



Original Article

High-dose β -carotene exacerbates high-fat/high-sucrose diet-inducible fibrosis in the liver of Mongolian gerbils

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ABSTRACT — β -Carotene, a natural additive that is often used for food coloring, is converted into vitamin A in the body and it removes reactive oxygen species. β -Carotene accumulates in the liver and adipose tissue, where it can induce adverse effects if present in excess. In this study, we determined whether β -carotene supplementation of a high-fat/high-sucrose diet enhances liver inflammation and fibrosis in Mongolian gerbils, which accumulate β -carotene in the liver. Ten-week-old male Mongolian gerbils were divided into four groups and fed either a normal diet, a high-fat/high-sucrose diet, a high-fat/high-sucrose diet supplemented with a low dose of β -carotene (0.001%), or a high-fat/high-sucrose diet supplemented with a high dose of β -carotene (0.004%). An oral glucose tolerance testing was performed after 12 weeks, and non-fasted serum and tissue samples were collected after 13 weeks of diet feeding. The high dose of β -carotene increased triglycerides and total cholesterol serum concentrations. Intake of a high-fat/high-sucrose diet with a high dose of β -carotene increased the β -carotene content, volume occupied by fat droplets, degree of fibrosis, and protein levels of matrix metalloproteinase-9 (a marker of inflammation in steatohepatitis) in the liver. These data suggest that the intake of excessive amounts of β -carotene for 13 weeks exacerbates high-fat/high-sucrose diet-inducible inflammation and fibrosis in the liver in Mongolian gerbils. This model is suitable for studying the mechanism of β -carotene-induced liver injury, including liver fibrosis.

Key words: β -Carotene, Mongolian gerbil, Fibrosis, Liver, Supplement

INTRODUCTION

Carotenoids, natural additives that are often used for food coloring, are thought to play important roles in maintaining health by acting as vitamin A and antioxidants in the body. Among the carotenoids, α -, β -, and γ -carotene and β -cryptoxanthin are called provitamin A, because they can be converted into vitamin A in the body. β -Carotene is easily converted into vitamin A, and this is thought to occur only when insufficient vitamin A is present in the body. However, carotenoids such as lycopene, lutein, and

astaxanthin are not converted into vitamin A. Therefore, the risk of developing a vitamin A overload disorder is low. The carotenoids, including β -carotene, can remove reactive oxygen species, such as singlet oxygen (Black and Gerguis, 2003), and accumulate in the liver and adipose tissue, and can therefore efficiently prevent oxidative damage in fat-rich tissues (Hanachi and Naghavi, 2016).

β -Carotene can be consumed in large amounts in supplement form. Because it accumulates in the body in fat, β -carotene has the potential, as do other fat-soluble food ingredients, to cause disorders if they are present in

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excess. β -Carotene can induce a mutagenic adduct 1,N(2)-etheno-2'-deoxyguanosine in DNA *in vitro* (Marques *et al.*, 2004). A cell culture study showed that oxidized β -carotene induced DNA damage in human foreskin fibroblast Hs68 cells (Yeh and Hu, 2001).

Many epidemiologic studies on the efficacy and safety of carotenoids containing β -carotene have been conducted. A cross-sectional study in adults over 25 years of age in Australia showed that serum carotenoid concentrations (α -, β -, and γ -carotenes, β -cryptoxanthin, lycopene, and lutein) were inversely associated with the presence of glucose intolerance or type 2 diabetes (Coyne *et al.*, 2005). Additionally, a meta-analysis of 13 studies that evaluated the relationship between the risk of gastric cancer and carotene intake (Zhou *et al.*, 2016) showed that the ingestion of α - and β -carotenes reduced the incidence of this type of cancer. However, a meta-analysis summarizing 50 randomized controlled trials reported that supplemental intake of antioxidants, including vitamins and β -carotene, in subjects with cardiovascular disease (myocardial infarction, angina pectoris, stroke, and/or transient ischemia) did not affect the subsequent onset and the risk of vascular disease-related death (Myung *et al.*, 2013). A systematic review and meta-analysis of 13 studies, including nine randomized controlled trials, also showed that the intake of β -carotene supplements (20-30 mg/day) increased the incidence of lung or gastric cancer in smokers and people with a history of working with asbestos (Druesne-Pecollo *et al.*, 2010). These data indicate that β -carotene supplementation is not always effective for preventing metabolic disease or cancer. Thus, the intake of carotenoids, including β -carotene, may have differing effects, depending on the patient's life or disease stage, and the optimal dose and tolerable upper intake level may differ accordingly. Therefore, β -carotene's effectiveness and safety and its optimal doses at each life stage and each disease stage need to be examined using animal models.

Few studies on the efficacy and safety of β -carotene supplementation in the diet have been conducted to date in animal models of lifestyle-related diseases. This is because most β -carotene is converted into vitamin A in mice and rats (Takeda *et al.*, 2011). The β -carotene serum concentration was reported to be $7.9 \pm 0.9 \mu\text{g/L}$ when 2 g/kg β -carotene was administered to rats for 14 days. In adult men, however, the serum β -carotene concentration was $219 \pm 4.2 \mu\text{g/L}$ after a single dose of 2.65 mg β -carotene, meaning that the resulting concentration in rats is $< 5\%$ of that in humans (Takeda *et al.*, 2011). Thus, the β -carotene dose given to rats was approximately 5,400 times higher than that used in humans, when the

two doses are compared on an equivalent body mass basis.

Previous studies have been conducted in Mongolian gerbils, which, like humans, efficiently accumulate β -carotene in the liver. β -Carotene was reported to accumulate in the liver to $24.2 \pm 9.4 \mu\text{g}$ ($45.1 \pm 17.5 \text{ nmol}$)/liver following administration of 0.36 g/kg diet β -carotene diet for 31 days (Lee *et al.*, 1998). Thus, if a Mongolian gerbil (100 g body mass) consumes 5 g of food including β -carotene at 0.36 g/kg food per day, the amount of β -carotene intake corresponds to 0.018 g/kg body mass, which is less than one-hundredth of the amount that was previously administered to rats (2 g/kg body mass) (Takeda *et al.*, 2011). However, the β -carotene doses used in these studies were considerably higher than the highest amount of β -carotene used in human supplements, and the duration of its administration was relatively short. Additionally, Mongolian gerbils developed fatty liver following administration of a high-fat/high-sucrose diet for 8 months (Liu *et al.*, 2014). However, no studies have evaluated the efficacy and safety of β -carotene administration in Mongolian gerbils during the development of fatty liver.

Therefore, in this study, we evaluated the efficacy and safety of β -carotene with regard to the onset of fatty liver in Mongolian gerbils consuming a high-fat/high-sucrose diet administered at two different concentrations: 0.001% (0.01 g/kg diet), which is approximately equivalent to typical supplemental intake, or 0.004% (0.04 g/kg diet), which is approximately equivalent to the upper supplementation level. Furthermore, fibrosis and inflammatory protein (interleukin (IL)-18 and matrix metalloproteinase (MMP)-9) levels were measured in the liver.

MATERIALS AND METHODS

Animals

Five-week-old male Mongolian gerbils purchased from SLC Co., Ltd. (Shizuoka, Japan) were fed a standard laboratory diet (MF, Oriental Yeast, Tokyo, Japan), and subsequently fed an AIN-93M diet (MF, Oriental Yeast) beginning at 9 weeks of age and continuing for 1 week. They were then divided into four groups that were fed their normal diet (N, n=7), a high-fat/high-sucrose diet (control diet, C, n=8), a high-fat/high-sucrose diet containing β -carotene at a low dose (0.001% by mass; Low β -carotene diet, LC, n=8), or a high-fat/high-sucrose diet containing β -carotene at a high dose (0.004% by mass; High β -carotene diet, HC, n=8; Supplemental Table 1), which were manufactured by a company (Oriental Yeast). In this study, we defined 0.001% as the low dose and

0.004% as the high dose for the experimental groups. There were no significant differences in 12-hr fasting glucose concentration or body mass among the four groups at 10 weeks of age. The diets and water were provided *ad libitum* for 13 weeks. Food intake and body weights were measured once every week. The gerbils were housed in cages and maintained at a stable temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 10\%$), and on a 12-hr light/dark cycle (lights on 06:00-18:00 hr). An oral glucose tolerance test (OGTT) was performed after 12 weeks of feeding the test diets. The Mongolian gerbils were orally administered a glucose solution (2 g/kg body weight; 10 mL/kg body weight) after a 14-hr fast. In this test, blood samples (150 μL) were collected from the tail vein of the gerbils at 0, 15, 30, 60, 90, and 120 min after a glucose load (2 g/kg) using a capillary without coagulant agents. Blood samples were also collected from the carotid artery when the animals were sacrificed, and serum was obtained. The fasting blood glucose concentration was measured every 4 weeks during test-diet feeding, and the blood glucose concentration during OGTT and non-fasting blood glucose concentration were measured at the end of the study (23 weeks old) using a hand-held glucose monitor (Glutest Mint, Sanwa Kagaku Kenkyusho, Co. Ltd., Aichi, Japan). At that time, body and tissues (mesenteric and epididymal adipose tissue and liver) are weighted and these tissues samples were collected. The experimental procedures conformed to the Animal Use Committee of the University of Yamanashi guidelines (assigned number: A27-21), which are based on the guidelines issued by the Ministry of Education, Culture, Sports, Science and Technology in Japan.

Biochemical parameters

Serum insulin concentrations were measured using commercial kits (Rebisu Insulin Rat ELISA Kit; Shibayagi Co., Ltd., Gunma, Japan). Serum triglyceride, total cholesterol, and high-density lipoprotein (HDL)-cholesterol were measured using assay kits (triacylglycerol E-test, cholesterol E-test; HDL-cholesterol E-test; Wako Pure Chemical Industries, Osaka, Japan). Glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were measured using a transaminase C II test kit (Wako Pure Chemical Industries).

β -Carotene concentrations were measured using high performance liquid chromatography (HPLC) at a contract research organization (Nikken Seil Co. Ltd., Tokyo, Japan). Liver samples were homogenized 30 times in 100% methanol containing 0.1% butylated hydroxytoluene and the homogenates were then centrifuged at 21,900

g for 20 min at 4°C . The supernatants were collected and stored at -80°C . Two hundred microliters of each sample were vortexed with 200 μL of α -tocopherol acetate and 800 μL of n-hexane was then added; the mixture was then revortexed and centrifuged at 2,300 g for 2.5 min. The n-hexane layer was then collected and dried in an evaporator. To the residue, 20 μL of ethyl acetate and 105 μL of ethanol were added, and the dissolved samples were analyzed using HPLC (Wakosil-II 5C18), with signal detection using diode array (0 min, 325 nm; 6.3 min, 455 nm; 30.0 min, 455 nm) and fluorescence (excitation wavelength 295 nm/emission wavelength 335 nm) detectors.

Histological staining of liver sections

Histological staining was performed at a contract research organization (New Histro. Science Laboratory Co., Ltd., Kanagawa, Japan). Liver samples from gerbils fed each diet were fixed in 10% formalin phosphate buffer and processed to generate paraffin sections. The sections were then subjected to oil red O and Azan staining and examined using a phase-contrast microscope (Olympus CX41, Olympus, Tokyo, Japan). We assessed the positive areas of Azan staining and oil red O staining using the Image J program that was developed at the National Institutes of Health (Bethesda, MD, USA). Blue areas stained by the Azan and red areas stained by the oil-Red O were extracted and measured in all samples of images obtained under the same conditions (diaphragm, optical source, and magnification) and these signals were averaged.

Protein expression analysis by western blotting

For a western blot, we randomly selected three samples in the N group, four samples in the C group, four samples in the LC group, and four samples in LH group. Liver lysates were homogenized 20 times using a homogenizer (Digital Homogenizer, As One, Osaka, Japan) in radioimmunoprecipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 mM tris-HCl pH 8.0, 5 mM EDTA, and 150 mM NaCl), which is regularly used for tissue homogenization (Elinger *et al.*, 2019), containing protease inhibitor tablets (Complete Mini; Roche, Diagnostics, Mannheim, Germany) and centrifuged at 17,800 g for 30 min at 4°C . The supernatants were collected, their total protein content measured using the Lowry method, and the concentration of each was equalized to 8.5 μg . Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to Immobilon membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 1 hr in 10% skimmed milk in phos-

phate-buffered saline containing 0.05% Tween-20 and 0.5 M NaCl pH 7.4 (PBS-Tween-NaCl). The membranes were then incubated with anti-MMP-9 or anti-interleukin (IL)-18 (Medical and Biological Laboratories Co., Ltd., Aichi, Japan) primary antibodies in PBS-Tween-NaCl containing 10% skimmed milk at 4°C for >12 hr. After washing in PBS-Tween-NaCl, the membranes were incubated with anti-rabbit or anti-mouse immunoglobulin G conjugated to biotin (GE Healthcare, Chicago, IL, USA), as secondary antibodies, in PBS-Tween-NaCl containing 3% skimmed milk. After further washing in PBS-Tween-NaCl and incubation with a horseradish peroxidase-conjugated anti-biotin tertiary antibody (Cell Signaling Technology, Beverly, MA, USA), positive signals were detected by chemiluminescence (ECL Plus; GE Healthcare), using a luminescent image analyzer (ChemiDog™, Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Data are expressed as the mean \pm standard error of the mean (SEM). Differences between two groups (N vs. C) were analyzed using the Student's *t*-test and differences among the three groups (C, LC, and HC) were analyzed using the Tukey-Kramer test. Values of $P < 0.05$ were considered to demonstrate significance.

RESULTS

Effects of β -carotene supplementation on body and tissue mass and blood biochemical parameters

Basic parameters are shown in Table 1. The fasting blood glucose concentrations after 4 and 8 weeks of feeding the diet were greater in the C group compared with the N group. The body mass, liver mass, total energy intake, non-fasted blood glucose and total cholesterol, were greater in the C group compared with the N group at

Table 1. Body mass, tissue mass, and blood biochemical parameters in Mongolian gerbils fed diets containing β -carotene.

Groups	N	C	LC	HC
Total food intake (g)	363.1 \pm 43.7	373 \pm 28.6	367.4 \pm 31.8	404.8 \pm 37.4
Total energy intake (kcal)	1383 \pm 166	1736 \pm 133 *	1710 \pm 148	1884 \pm 174
Food intake (g/day)	3.99 \pm 0.48	4.10 \pm 0.31	4.04 \pm 0.35	4.45 \pm 0.41
Fasting blood glucose after 4 weeks of diet (mg/dL)	84.4 \pm 10.7	100.0 \pm 16.3 *	98.8 \pm 12.5	103.3 \pm 12.1
Fasting blood glucose after 8 weeks of diet (mg/dL)	82.8 \pm 6.5	99.5 \pm 6.8 *	91.8 \pm 7.2	91.5 \pm 20.4
Autopsy after 13 weeks of consuming test diets				
Body mass (g)	75.5 \pm 13.7	90.6 \pm 14.5 *	89.3 \pm 12.9	99.7 \pm 18.0
Liver mass (g/10g body mass)	0.34 \pm 0.03	0.40 \pm 0.05 *	0.43 \pm 0.09	0.48 \pm 0.14
Epididymal fat mass (/10g body mass)	0.15 \pm 0.08	0.21 \pm 0.09	0.23 \pm 0.09	0.29 \pm 0.09
Mesenteric fat mass (/10g body mass)	0.04 \pm 0.03	0.07 \pm 0.04	0.07 \pm 0.04	0.10 \pm 0.04
Non-fasted blood glucose (mg/dL)	117.7 \pm 13.2	123.3 \pm 10.6	128.1 \pm 30.5	122.6 \pm 23.0
Total cholesterol (mg/dL)	55.4 \pm 12.1	110.2 \pm 7.7 * a	211.5 \pm 30.5 ab	243.1 \pm 37.9 b
HDL-cholesterol (mg/dL)	60.8 \pm 11.1	60.1 \pm 3.7	77.6 \pm 4.8	60.3 \pm 4.6
Serum triglyceride (mg/dL)	196.1 \pm 51.2	201.9 \pm 24.2 a	364.6 \pm 67.9 ab	420.9 \pm 66.0 b
Serum insulin (mg/dL)	705.4 \pm 120.7	938.7 \pm 107.0	1834 \pm 699.2	2611 \pm 670.9
GOT (IU/L, 25°C)	153.3 \pm 13.9	140.1 \pm 6.3	158.9 \pm 9.3	168.4 \pm 11.1
GPT (IU/L, 25°C)	49.5 \pm 3.3	52.6 \pm 3.9 a	124.7 \pm 27.6 b	85.3 \pm 18.0 a
Liver content of β -carotene (μ g/g liver mass)	undetected	0.25 \pm 0.05 a	4.15 \pm 0.85 a	25.4 \pm 8.99 b
Oral glucose tolerance test after 12 weeks of consuming test diets				
Blood glucose concentration				
fasting	79.0 \pm 5.8	111.9 \pm 8.9 *	105.0 \pm 8.0	106.8 \pm 8.1
15 min	245.9 \pm 16.7	251.5 \pm 24.4	262.6 \pm 10.3	275.8 \pm 15.3
30 min	196.8 \pm 21.8	242.8 \pm 19.1	235.6 \pm 15.8	265.1 \pm 25.0
60 min	162.1 \pm 13.4	183.8 \pm 15.1	182.4 \pm 21.8	196.5 \pm 24.5
120 min	126.5 \pm 8.1	128.8 \pm 6.6	132.4 \pm 10.0	156.1 \pm 20.8

Values are presented as the mean \pm SEM of 7-8 animals.

Abbreviations: N, group fed a normal diet (AIN93G diet); C, group fed a high-fat, high-sucrose diet; LC, group fed a low β -carotene diet; HC, group fed a high β -carotene diet; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase

* $p < 0.05$, significant difference N vs. C (Student's *t*-test)

Different letters indicate groups showing significant differences ($p < 0.05$; Tukey-Kramer test).

autopsy. Epididymal fat mass, mesenteric fat mass, total food intake, food intake per day, HDL-cholesterol, triglyceride, and insulin, and serum activities of GOT and GPT at autopsy did not differ between the N and C groups at the end of the study. Among groups fed the high-fat/high-sucrose diet with or without β -carotene, the total cholesterol and serum triglyceride concentrations were greater in the HC group compared with the C group. The HDL-cholesterol concentration was not different among groups. GPT activity was significantly higher in the LC group compared with the C and HC groups. The hepatic β -carotene content was $0.25 \pm 0.05 \mu\text{g/g}$ in the C group, $4.15 \pm 0.85 \mu\text{g/g}$ in the LC group, and $25.4 \pm 8.9 \mu\text{g/g}$ in the HC group, the hepatic β -carotene content was higher in HC-group than in C and HC groups.

OGTTs were performed after 12 weeks of feeding the test diets. The fasted blood glucose concentrations were greater in the C group compared with the N group. The blood glucose concentrations after the glucose load were not different among the C, LC, and HC groups.

Effects of β -carotene supplementation on fat accumulation and fibrosis in the liver

Results of oil red O and Azan staining are shown in Fig. 1. Oil red O staining showed that the volume occupied by lipid droplets was greater in the C group compared with the N group. Fat droplets were not significantly different among the C-, LC-, and HC-groups ($p=0.069$; Fig. 1A). Azan staining showed no significant difference in the area occupied by fibers around blood vessels between the C and N groups. However, the fibrous areas around blood vessels were larger in the HC group compared with the LC group (Fig. 1B)

Effects of β -carotene supplementation on hepatic inflammatory protein expression

MMP-9 and IL-18 protein levels are shown in Fig. 2. Hepatic MMP-9 protein expression was higher in the HC group compared with the C and LC groups, but IL-18 expression did not differ among the groups fed a high-fat/high-sucrose diet with or without β -carotene.

DISCUSSION

In this study, we evaluated the efficacy and safety of relatively long-term β -carotene administration to Mongolian gerbils supplementing their high-fat/high-sucrose diet with β -carotene at 0.001% or 0.004% by mass. Previous intervention studies targeted to metabolic diseases such as macrovascular diseases showed that β -carotene was supplemented at 20-60 mg/day in humans (Kataja-Tuomola

et al., 2010; Shaish *et al.*, 2006; Zhou *et al.*, 2016). Therefore, we chose 30 mg β -carotene/day as a reference value. If a Mongolian gerbil that was 100 g body mass ate 5 g of food containing 0.001% or 0.004% β -carotene per day, the β -carotene intake was 0.05 mg or 0.2 mg/100 g body weight. This corresponds to 30 mg/60 kg body weight and 120 mg/60 kg body weight in humans. In this study, we demonstrated substantial accumulation of β -carotene in the liver of the gerbils consuming supplemental β -carotene, and this accumulation was dose-dependent. The hepatic β -carotene content of Mongolian gerbils fed a diet including β -carotene at 0.036 g/kg diet for 4 weeks was $7.91 \pm 2.48 \mu\text{g}$ (Sulaeman *et al.*, 2002). In this study, we have shown that β -carotene can also accumulate in the liver of gerbils when it is administered at a dose of 0.04 g/kg in the diet for 10 weeks, resulting in levels of $25.8 \pm 2.48 \mu\text{g}$. The reason the amount of β -carotene content in the liver was higher in this study compared with the previous study, even with almost the same amount administered, may be because the duration of feeding the β -carotene diet was longer in this study than in the previous study, and the diet composition in this study included more fat than that in the previous study. Thus, we consider that Mongolian gerbils are suitable to evaluate the efficacy and safety of β -carotene accumulation obtained from the diet.

In this study, consumption of a diet high in β -carotene enhanced the non-fasted serum triglyceride and total cholesterol concentrations, but not non-fasted HDL-cholesterol or fasting- and postprandial-blood glucose concentration during an OGTT. These findings suggest that β -carotene supplementation, particularly at higher doses, impairs lipid abnormalities when consuming a high fat/sugar diet. Additionally, there was an increase in fibrosis with the intake of a high-fat/high-sucrose diet containing a high dose of β -carotene. These findings suggest that excessive dietary β -carotene supplementation in Mongolian gerbils promotes the development of fibrosis in the liver.

Criteria for diagnosing fibrosis as steatohepatitis in animals have not been determined. Therefore, many previous studies that have shown fibrosis in the liver in rodents as determined by Azan staining was assessed by comparison to control animals (Arima *et al.*, 2014; Imaeda *et al.*, 2014; Inami *et al.*, 2015; Kolodziejczyk *et al.*, 2015), which is consistent with this study. Therefore, we assessed the fibrosis in Mongolian gerbils fed a high-fat/high-sucrose diet with β -carotene compared to those without β -carotene, as well as those fed a normal diet. The degree to which fibrosis in Mongolian gerbils corresponds to steatohepatitis in humans should be investigat-

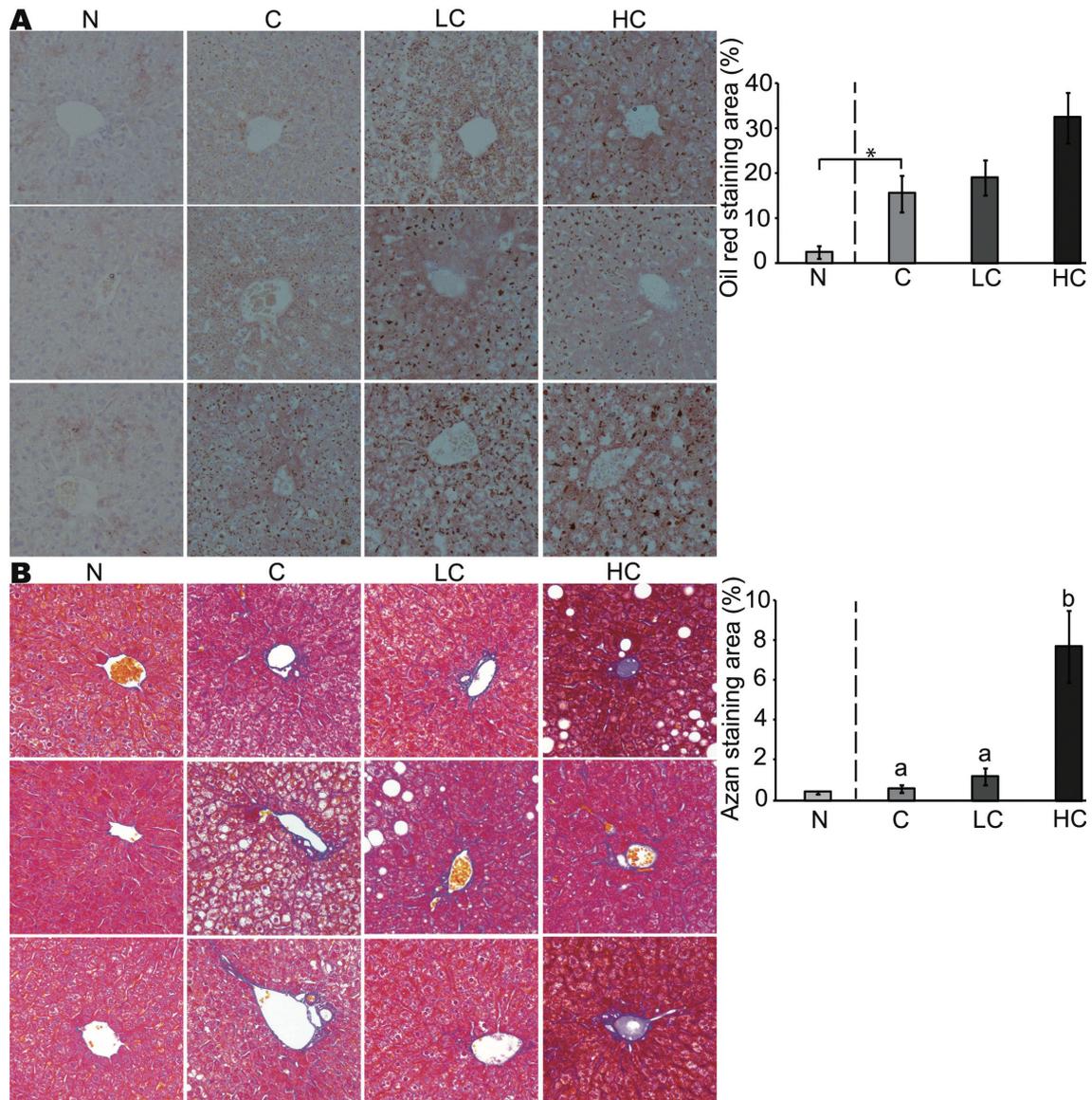


Fig. 1. Lipid deposition and fibrosis in the liver of Mongolian gerbils fed a high-fat/high-sucrose diet with or without β -carotene. Normal diet (N), high-fat/high-sucrose diet (C), high-fat/high-sucrose diet containing carotene at a low dose (0.001% by mass) (LC), or high-fat/high-sucrose diet containing carotene at a high dose (0.004% by mass) (HC). A) Oil red O staining. B) Azan staining. Original magnification $\times 400$. Oil red O and Azan staining were quantified using Image J software. Values are presented as the mean \pm SEM (n=7-8). Different letters indicate significant differences among groups (one-way ANOVA and post-hoc Tukey-Kramer test ($P < 0.05$)). * $p < 0.05$, significant difference shown by N vs. C using the Student's *t*-test ($P < 0.05$).

ed.

Recent studies have demonstrated that proinflammatory cytokines can contribute to the development of liver injury. Among pro-inflammatory cytokines, IL-18 is related to development of hepatic fibrosis. For example, a high-fat diet increased IL-18 levels in rat liver (Wang *et*

al., 2008). Primary hepatocytes with steatosis were reported to secrete IL-18 (Pan *et al.*, 2015), and a genetic deficiency in IL-18 reduced fat accumulation and liver damage in mice (Lana *et al.*, 2016). Therefore, we focused on IL-18. Additionally, MMPs have protective effects in liver injury. Amelioration of fibrosis by placental extract

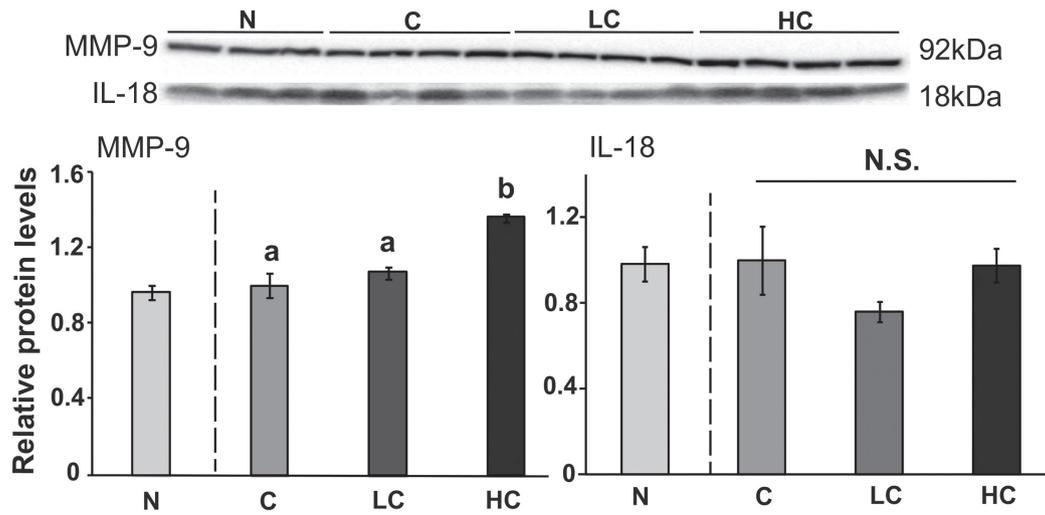
Effects of excessive β -carotene on liver fibrosis

Fig. 2. Western blotting analysis of MMP-9 and IL-18 protein expression in the liver of Mongolian gerbils fed a high-fat/high-sucrose diet with or without β -carotene. Normal diet (N), high-fat/high-sucrose diet (C), high-fat/high-sucrose diet containing carotene at a low dose (0.001% by mass) (LC), or high-fat/high-sucrose diet containing carotene at a high dose (0.004% by mass) (HC). Values are presented as the mean \pm SEM ($n=3-4$). Different letters indicate significant differences among groups (one-way ANOVA and post-hoc Tukey-Kramer test; $P < 0.05$).

in mice was closely associated with reduced MMP-9 expression (Yamauchi *et al.*, 2017). However, other pro-inflammatory cytokines may be related to liver fibrosis. Additionally, these genes are not cloned. Therefore, we focused on IL-18 and MMP-9 expression in the liver of Mongolian gerbils that received β -carotene supplementation. In this study, IL-18 protein expression in the liver was not changed by feeding gerbils a high-fat/high sucrose diet with or without β -carotene. However, hepatic MMP-9 protein expression was higher in gerbils fed a high-fat/high-sucrose diet with high β -carotene compared with that without β -carotene, but there were no differences between the N and C groups. MMP-9 is a collagen-degrading enzyme that is involved in degrading collagen that accumulates in the liver as part of the inflammatory response (Grobewska *et al.*, 2011). MMP-9 levels in plasma were shown to be higher in NASH patients than in healthy subjects (D'Amico *et al.*, 2010), and rats consuming a diet containing 71% fat (w/w) showed higher hepatic MMP-9 expression (Esposito *et al.*, 2009). The current study showed that high-fat/high-sucrose diet intake for 13 weeks did not induce hepatic inflammation and fibrosis in the liver, but the addition of β -carotene does cause development of these pathologies. This may be because of oxidation of other molecules after oxidation of β -carotene (Viskin, 2017), although it must be determined whether this mechanism exists and whether oxidized β -carotene can induce NASH *in vivo*. Patients with NASH frequent-

ly develop liver cirrhosis and hepatocellular carcinoma (Bellentani, 2017). Additionally, serum MMP-9 concentrations were higher in patients with liver cirrhosis compared with healthy subjects (Kozłowska *et al.*, 2016). The serum MMP-9-to-MMP-2 ratio was higher in chronic hepatitis B patients with hepatocellular carcinoma than in healthy carriers (Yeh *et al.*, 2010). These results suggest that increased MMP-9 protein expression in the liver in Mongolian gerbils and excessive β -carotene intake lead to an increased risk of developing liver cirrhosis and hepatocellular carcinoma. Further research on continuous feeding of the high-fat/high-sucrose diet with excessive β -carotene is required to determine if it induces cirrhosis and hepatocellular carcinoma. Additional investigation of the association between MMP-9 in the liver or serum and liver cirrhosis or hepatocellular carcinoma is also required. Future studies should investigate whether other pro-inflammatory cytokines are related to hepatic liver fibrosis development.

It is unknown whether the mechanism of hepatic fibrosis development in the liver induced by excessive β -carotene intake in Mongolian gerbils is similar to that in humans. We also did not assess the effect of excessive β -carotene supplementation in a normal diet in the gerbils. Moreover, there are limitations to using gerbils for analyzing mRNA expression patterns because the genome of this species has not been fully mapped. However, in this study, we demonstrated that long-term excessive intake of

β -carotene, even at doses equivalent to those consumed in supplements by people, induces inflammation and fibrosis in the liver. To further characterize the effect of consuming excessive β -carotene by Mongolian gerbils, the effect of a range of doses should be tested using a range of time periods.

In conclusion, we have demonstrated that excessive β -carotene intake for 13 weeks exacerbates high-fat/high-sucrose diet-inducible hepatic fibrosis in Mongolian gerbils. Our results suggest that this species may represent a suitable model in which to determine the mechanism of liver injury, including that associated with hepatic fibrosis.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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