Original Article

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Bovine lactoferrin intake prevents hepatic injury in a mouse model of non-alcoholic steatohepatitis induced by choline and methionine deficiency

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SUMMARY: Lactoferrin, a multifunctional protein found in breast milk, is important for the regulation of immune function. Non-alcoholic steatohepatitis (NASH), which is characterized by hepatitis and fibrosis, has no established drug treatment. In this study, we aimed to investigate the effects of lactoferrin on hepatocyte inflammation in a mouse model of NASH induced with a choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD). As a method, C57BL/6JJmsSlc mice were fed CDAHFD for 14 days and simultaneously intake lactoferrin (3.3 g/kg or 6.6 g/kg) of water. Then, plasma levels aspartate aminotransferase (ALT) and alanine aminotransferase (AST) were measured and gene expression levels of inflammatory cytokines in the liver were examined. Plasma levels of ALT and AST significantly increased in the NASH model, indicating hepatocyte inflammation, and lactoferrin intake suppressed their elevation in a dose-dependent manner. Histological analysis revealed that lactoferrin alleviated the fatty liver-associated tissue damage. Additionally, lactoferrin suppressed the gene expression of the pro-inflammatory cytokines tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-6 and the macrophage migration factor (MCP)-1, suggesting inhibition of macrophage activation. Lactoferrin also significantly reduced the expression of apoptosis-related genes (caspase 3 and p53), indicating its anti-apoptotic effects. Furthermore, lactoferrin alleviated oxidative stress by suppressing inducible nitric oxide synthase expression. These findings suggest that lactoferrin prevented liver injury in the mouse model of NASH induced by CDAHFD feeding by inhibiting macrophage-mediated inflammation and alleviating oxidative stress caused by fat accumulation.

Keywords: Fat accumulation, macrophage, TNF-α, MCP-1, iNOS

1. Introduction

The incidence of non-alcoholic fatty liver disease (NAFLD) is increasing with the prevalence of metabolic syndrome, diabetes, and dyslipidemia (*1*). NAFLD development includes stages of non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), chronic inflammation, and fibrosis. Cirrhosis or hepatocellular carcinoma is the end stage of liver disease caused by NASH (*2,3*). The pathogenesis of NAFLD to NASH is considered to involve a variety of factors but is most likely due to the deposition of triglycerides and other lipids in hepatocytes as a result of long-term disordered eating habits. Consequently, increased inflammation is considered to be caused by increased oxidative stress and abnormal immune function following endotoxin exposure (*4*). This progression induces irreversible liver damage in patients with cirrhosis and hepatocellular carcinoma, which are also the major causes of liver transplantation and social problems (*5*). However, there is no treatment for NASH, and lifestyle modifications, such as diet and exercise therapy, are the mainstay of treatment.

Several mouse models have been used in NASH research, including a model induced with a choline-methionine-deficient diet (CDAHFD) (*6*). The liver synthesizes the lipoprotein very low-density lipoprotein (VLDL) and excretes lipid components into the blood.

Phosphatidylcholine (PC) is required for VLDL formation (*7*). The formation of PC, which imposes a burden on the liver, occurs *via* the methylation of phosphatidylethanolamine from active methionine. As 40% of active methionine is used to synthesize PC, reduced PC synthesis under methionine deficiency causes fatty liver because VLDL is not synthesized. Choline is also the starting substrate for PC synthesis in the CDP-choline pathway. In other words, PC is synthesized from choline and methionine *via* different synthesis pathways, and their loss causes substantial damage to hepatocytes. Decreased synthesis of VLDL and increased synthesis of triglycerides lead to the accumulation of fat in the liver and cause fatty degeneration. As a result, lipid peroxidation occurs, damaging the cell membranes and inducing inflammation.

As an environmental factor, high-fat diet intake disturbs the balance of the intestinal microbiota and increases lipopolysaccharide (LPS) concentration, which induces inflammation by exposing hepatocytes to plasma endotoxins. LPS intake induces damage to hepatocytes (*8*). LPS administration induces inflammation in macrophage-like cells (unpublished data).

Lactoferrin, a protein found in breast milk, enhances the immune function in infants, mostly through the action of neutrophils containing lactoferrin (*9,10*). However, in addition to neutrophils, it has been shown to affect macrophages and T cells, and enhance immune function (*11*). In an ovariectomy-induced osteopenia model, lactoferrin also inhibited the loss of bone mineral density by acting on macrophage-derived osteoclasts (*12*). Furthermore, lactoferrin has been shown to have anti-inflammatory effects in models of liver injury induced with acetaminophen and carbon tetrachloride (*13,14*). In hepatocytes, lactoferrin exerts effects on immune abnormalities and inflammation by acting on macrophage-derived Kupffer cells. Previously, we have shown that lactoferrin has an anti-inflammatory effect on hepatocytes in mice fed a high-fat diet (*15*). However, lactoferrin has also been reported to improve the intestinal microbiota (*16*), limiting the ability to test its direct effect on macrophages in high-fat diet models. Therefore, in this study, we aimed to investigate the anti-inflammatory effects of lactoferrin on hepatocyte inflammation and its preventive effects on liver injury in an NASH model with increased hepatocellular damage using a CDAHFD.

2. Materials and Methods

2.1. Animals

Sixty 5-week-old male C57BL/6JJmsSlc mice (Japan SLC, Shizuoka, Japan) were used in this study after 1 week of preliminary rearing. The rearing environmental conditions were as follows: room temperature of 24°C ± 1°C, 12 h of day light (light period 7:00–19:00 h, dark period 19:00–7:00 h), and humidity of 55%. During the pre-rearing period, the mice were fed CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan) and provided with tap water ad libitum. The experimental design of this study was approved by the Experimental Animal Committee of Yokohama University of Pharmacy (2020-009).

2.2. Model mice and lactoferrin intake

After 1 week of pre-rearing, the mice were divided into four groups (15 animals per group): control, NASH, NASH+lactoferrin-low (3.3 g/kg), and NASH+lactoferrin-high (6.6 g/kg) groups to ensure that there was no difference in body weight. The control group mice were fed a normal diet (CRF-1), and NASH model mice were fed CDAHFD 60 (choline-deficient methionine reduced 60 kcal% fat diet; EP Trading Co., Ltd., Kanagawa, Japan), as described by Matsumoto *et al*. (*6*). Mice from two of the CDAHFD 60-intake groups were provided lactoferrin at different concentrations 3.3 and 6.6 g/kg ad libitum for 14 days. Bovine lactoferrin was provided by NRL Pharma (NRL Pharma, Inc., Kanagawa, Japan). The group administered 3.3 g/kg lactoferrin was the lactoferrin-low group and the group given 6.6 g/kg was the lactoferrin-high group. The lactoferrin ingested was converted from the daily intake, with 1.65 g/100 mL of dissolved solution for the lactoferrin-low group and 3.3 g/100 mL for the lactoferrin-high group. Food and water intake was measured every 5 days and divided by the number of days and number of mice to obtain the daily food and water intake per mouse. Body weight was measured after pre-rearing and every 5 days from the start of intake.

2.3. Aspartate aminotransferase and alanine aminotransferase levels in plasma

Fifteen days after lactoferrin administration, blood samples were collected with the mice under isoflurane anesthesia, placed in a MiniCollect® Tube (Funakoshi Co., Ltd., Tokyo, Japan) and centrifuged; the supernatant was then collected and stored at -80°C until measurement. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using an aspartate aminotransferase kit, alanine aminotransferase kit, and transaminase CII-test Wako (Fujifilm Wako Pure Chemicals Corporation, Osaka, Japan).

2.4. Histological analysis

Fifteen days after lactoferrin administration, the mice were anesthetized with isoflurane and perfusion-fixed with saline and 10% Mildform® reagent (Fujifilm Wako Pure Chemicals Corporation). The livers were then removed and fixed in 10% Mildform® reagent at 4°C for 24 h. After 24 h, the tissues were immersed in 10%, 20%, and 30% sucrose solution dissolved in phosphate-

buffered saline (PBS) (pH 7.4) sequentially for 24 h at 4°C, embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Inc. Tokyo, Japan), and frozen. The samples were sectioned to 16-µm thickness using a cryostat HM550 (Thermo Fisher Scientific K.K., Tokyo, Japan). The frozen sections were washed with PBS and stained with hematoxylin–eosin (HE) to evaluate their morphology. The sections were photographed using an all-in-one fluorescence microscope BZ-X710 (KEYENCE, Osaka, Japan).

2.5. Real-time reverse transcription-polymerase chain reaction analysis of gene expression levels

After blood collection, the mice were sacrificed and their livers were removed. Total RNA was extracted by homogenizing the liver with 400 µL of Isogen (Nippon Gene Co., Ltd., Tokyo Japan), followed by centrifugation (16,900× *g*, 15 min) with 80 µL of chloroform. Thereafter, the supernatant was collected, 200 µL of isopropanol was added, and the precipitate was collected using centrifugation (16,900× *g*, 5 min). The precipitate was dissolved in sterile water and reverse-transcribed into cDNA using the SuperScript VIRO cDNA Synthesis kit (*Invitrogen*, Thermo Fisher Scientific Inc., Waltham, MA, USA.). The synthesized cDNA was used as a template with primers for each marker (Table 1) and the expression levels of genes related to inflammatory cytokines, oxidative stress, and apoptosis were detected using the TaqMan probe method on a LightCycler®480II (F. Hoffmann-La Roche, Ltd., Basel, Switzerland.). The expression levels of each gene was corrected using that of *GAPDH*.

2.6. Data analysis

Data are shown as mean ± standard error. Between-group comparisons for each dataset were performed using Dunnett's test after testing with a one-way analysis of variance. The statistical software Stat View 5.0 (SAS Institute Inc., version 5.0) was used for analyses. The significance level was set at $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Influence of CDAHFD on food and water intake and body weight of mice

To confirm the influence of CDAHFD, the diet used to generate NASH model mice, on body weight, we measured food and water intake and body weight of mice every 5 days. Food and water intake were measured in group-housed mice. Although statistical comparison was not performed, intake appeared similar across the groups (Table 2); however, the NASH group showed a significant decrease in body weight compared to the control group after 5 days of CDAHFD feeding (Table 3). This weight loss continued until day 15. Lactoferrin intake significantly reduced weight loss induced by CDAHFD feeding from day 10.

Table 1. Primers for each marker used in real-time RT-PCR

Protein/Gene	Universal Probe Library Probe No.	Forward primer (5'→3')	Reverse primer
GAPDH (*GAPDH*)	#9	agcttgtcatcaacgggaag	tttgatgttagtggggtctcg
TNF-α (*TNF*)	#103	tgctgggaagcctaaagg	cgaattttgagaagatgatcctg
IL-6 (*IL6*)	#6	gctaccaaactggatataatcagga	ccaggtagctatggtactccagaa
IL-1β (*IL1B*)	#60	tcttcctaaagtatgggctgga	aaagggagctccttaacatgc
MCP-1(*CCL2*)	#2	gggacactggctgcttgt	gttgttaagcagaagattcacgtc
iNOS (*NOS2*)	#13	ctttgccacggacgagac	tcattgtactctgagggctgac
p53 (*TP53*)	#9	gacggaggtcgtgagacg	atttccttccacccggatac
Caspase 3 (*CASP3*)	#80	gaggctgacttcctgtatgctt	aaccacgacccgtccttt

GAPDH: glyceraldehyde-3-phosphate dehydrogenase, TNF-α: tumor necrosis factor-α, IL-6: interleukin-6, IL-1β: interleukin-1β, MCP-1: monocyte chemotactic protein-1, iNOS: inducible nitric oxide synthase.

Table 2. Food and water intake

Group	Food intake (g)			Water intake (mL)		
	Day 5	Day 10	Day 15	Day 5	Day 10	Day 15
Control	3.2	3.0	3.3	4.6	4.3	4.2
NASH	2.4	2.6	3.0	4.1	5.9	5.4
Lactoferrin low	3.8	5.2	5.2	4.3	4.5	4.6
Lactoferrin high	2.3	2.0	2.4	4.0	4.4	4.8

Food and water intake were measured in group-housed mice, and no obvious differences were observed between the groups. NASH: choline-deficient, L-amino acid-defined, high-fat diet fed group, Lactoferrin low: NASH + lactoferrin-low (3.3 g/kg) group, Lactoferrin high: NASH + lactoferrin-low (6.6 g/kg) group.

3.2. Effect of lactoferrin on AST and ALT levels in CDAHFD-fed mice

To assess liver damage caused by CDAHFD feeding, we measured plasma AST and ALT levels. Both AST and ALT levels significantly increased on day 15 of CDAHFD feeding compared with those in the control group (Figure 1). Both low and high doses of lactoferrin significantly suppressed the increase in the AST and ALT levels induced by CDAHFD feeding (Figure 1).

3.3. Effect of lactoferrin on the morphology of the liver

We performed HE staining to morphologically assess the liver damage caused by CDAHFD60 feeding (Figure 2). We observed large fat droplet deposits and vacuoles in hepatocytes after CDAHFD feeding. In mice receiving lactoferrin, the number and size of vacuoles appeared reduced, and hepatocytes showed more regular morphology with less swelling, suggesting partial alleviation of hepatocyte damage. These qualitative observations provide meaningful insights, although the distribution of vacuoles and lipid droplets was highly heterogeneous within and among liver sections.

3.4. Effect of lactoferrin on hepatotoxicity-related gene expression levels in CDAHFD-fed mice

To examine the effect of lactoferrin on liver inflammation, we measured gene expression levels of the inflammatory cytokines tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-6 using real-time reverse

Table 3. Effect of lactoferrin on body weight

Group	Body weight (g)			
	Day 1	Day 5	Day 10	Day 15
Control	19.6 ± 0.2	20.9 ± 0.2	21.8 ± 0.2	22.0 ± 0.3
NASH	19.4 ± 0.2	19.7 ± 0.2*	19.7 ± 0.2**	19.9 ± 0.2**
Lactoferrin low	20.0 ± 0.3	20.9 ± 0.3	21.0 ± 0.4$^{\#}$	21.6 ± 0.4$^{\#}$
Lactoferrin high	19.8 ± 0.2	20.7 ± 0.3	20.7 ± 0.3$^{\#}$	21.3 ± 0.3$^{\#}$

Lactoferrin intake significantly reduced body weight loss due to CDAHFD feeding from day 10 (**$p < 0.01$, *$p < 0.05$ compared to the control, #$p < 0.05$ compared to NASH, Tukey's test). The results are presented as mean ± standard error. NASH: choline-deficient, L-amino acid-defined, high-fat diet fed group, Lactoferrin low: NASH + lactoferrin-low (3.3 g/kg) group, Lactoferrin high: NASH + lactoferrin-low (6.6 g/kg) group.

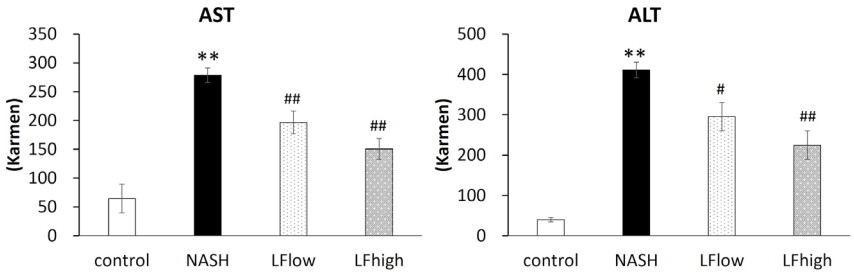

Figure 1. Effects of lactoferrin on aspartate aminotransferase (AST) and alanine aminotransferase (ALT) level in NASH model mice. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. CDAHFD feeding significantly increased the AST and ALT levels. Lactoferrin intake significantly suppressed this increase (**$p < 0.01$, *$p < 0.05$, Dunnett's test). Bars indicate mean ± standard error. NASH: choline-deficient, L-amino acid-defined, high-fat diet fed group, LF low: NASH + lactoferrin-low (3.3 g/kg) group, LF high: NASH + lactoferrin-low (6.6 g/kg) group.

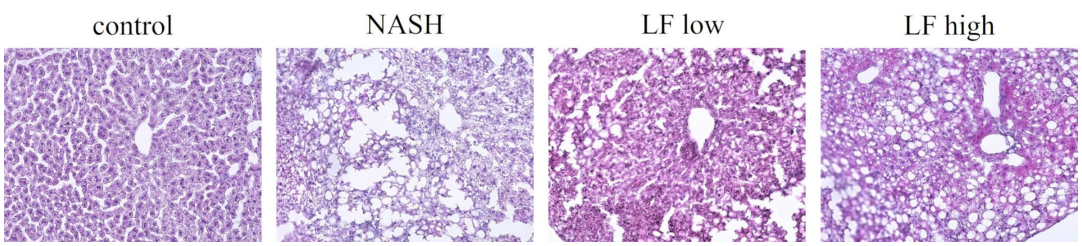

Figure 2. Effects of lactoferrin on the morphology of liver in NASH model mice, as determined using hematoxylin–eosin (HE) staining. The control group showed normal lobular cells. The NASH group showed large fat droplet deposits and vacuoles in hepatocytes. Lactoferrin intake decreased the number of vacuoles. NASH: choline-deficient, L-amino acid-defined, high-fat diet fed group, LF low: NASH + lactoferrin-low (3.3 g/kg) group, LF high: NASH + lactoferrin-low (6.6 g/kg) group.

transcription-polymerase chain reaction (RT-PCR). CDAHFD feeding induced a significant increase in the expression of TNF-α, IL-1β, and IL-6 (Figures 3A, 3B, and 3C). High-dose intake of lactoferrin significantly suppressed this increase (Figures 3A and 3B), and the gene expression of IL-6 was significantly suppressed by both low- and high-dose lactoferrin intake (Figure 3C). Next, we measured the gene expression level of the macrophage migration factor (MCP)-1, a macrophage chemotaxis factor. Its expression was significantly increased by CDAHFD feeding and significantly suppressed by high-dose lactoferrin intake (Figure 3D). We measured the gene expression of p53 and caspase 3 to assess hepatocyte apoptosis. The gene expression of p53 and caspase 3 was significantly increased by CDAHFD feeding (Figures 4A and 4B), and high-dose lactoferrin intake significantly suppressed the increase in the gene expression of caspase 3 (Figure 4B). We also measured the gene expression levels of inducible nitric oxide synthase (iNOS) to examine the involvement of oxidative stress; its level was significantly elevated by CDAHFD feeding and significantly suppressed by high-dose lactoferrin intake (Figure 4C).

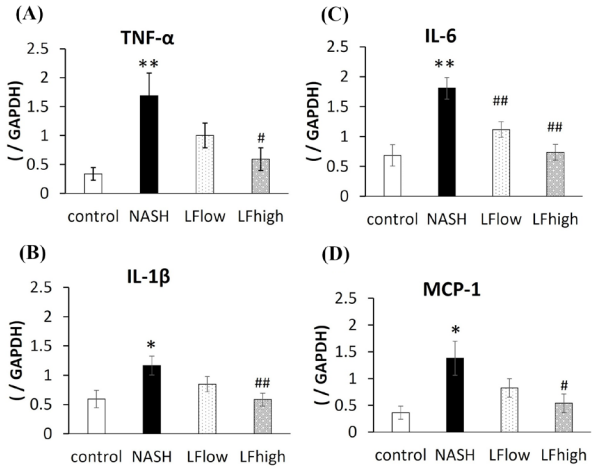

Figure 3. Effects of lactoferrin on mRNA expression levels of the cytokines tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-6 and chemokine monocyte chemoattractant protein 1 (MCP-1) in the liver of NASH model mice. Expression levels of hepatotoxicity-related genes as determined using real-time RT-PCR. CDAHFD feeding induced a significant increase in the gene expression of TNF-α (A), IL-1β (B), IL-6 (C), and MCP-1 (D). High-dose lactoferrin intake significantly suppressed this increase (**$p < 0.01$, *$p < 0.05$, Dunnett's test). Bars indicate mean ± standard error. NASH: choline-deficient, L-amino acid-defined, high-fat diet fed group, LF low: NASH + lactoferrin-low (3.3 g/kg) group, LF high: NASH + lactoferrin-low (6.6 g/kg) group.

4. Discussion

In this study, we investigated the effects of lactoferrin intake in a mouse model of NASH induced with a CDAHFD. We observed a significant decrease in body weight from day 5 of CDAHFD feeding compared with that of the control group, and this decrease continued until day 15 of feeding. Both the low- and high-dose lactoferrin groups showed no weight decrease on day 5 of treatment and significant weight gain compared with that of the NASH group from day 10 of treatment. The amount of food and water intake of mice in each group was measured, and no significant differences were found among the groups. These results suggest that lactoferrin improves weight loss with abnormal lipid metabolism in hepatocytes caused by CDAHFD feeding. We have previously shown that lactoferrin reduced liver inflammation induced by a high-fat diet (*15*). Therefore, we examined the plasma AST and ALT levels to determine whether lactoferrin is involved in the suppression of hepatocyte inflammation. A significant increase in the AST and ALT levels was observed after 16 days of CDAHFD feeding, indicating that CDAHFD feeding induced hepatocyte inflammation. Similar results have been reported by Matsumoto *et al.* (*6*). We also found that lactoferrin intake had a significant concentration-dependent inhibitory effect on the elevation in AST and ALT levels caused by hepatocyte inflammation. It has been reported that lactoferrin exhibited anti-inflammatory effects in a liver injury model developed using acetaminophen and carbon tetrachloride (*13,14*). This finding suggests that lactoferrin exerted an anti-inflammatory effect in our mouse model of NASH

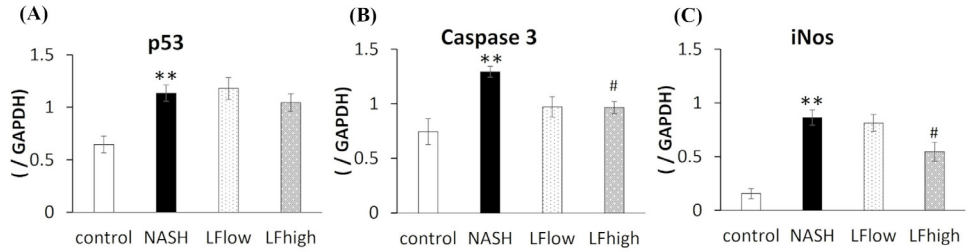

Figure 4. Effects of lactoferrin on the mRNA expression levels of p53, caspase 3, and inducible nitric oxide synthase (iNOS) in the liver of NASH model mice. Expression levels of hepatotoxicity-related genes as determined using real-time RT-PCR. CDAHFD feeding significantly increased the gene expression of p53 (A), caspase 3 (B), and iNOS (C). High-dose lactoferrin intake significantly suppressed p53 and iNOS expression (**$p < 0.01$, *$p < 0.05$, Dunnett's test). Bars indicate mean ± standard error. NASH: choline-deficient, L-amino acid-defined, high-fat diet fed group, LF low: NASH + lactoferrin-low (3.3 g/kg) group, LF high: NASH + lactoferrin-low (6.6 g/kg) group.

induced with a CDAHFD.

In this study, lactoferrin suppressed the elevation in plasma AST and ALT levels; therefore, sections of liver tissue were prepared and compared histologically using HE staining. The CDAHFD-fed group had more vacuoles in liver tissue sections than the control group, indicating fatty liver and tissue damage. Therefore, it was considered that lactoferrin intake reduced liver tissue damage; the histological studies also suggested that lactoferrin reduced liver tissue damage caused by CDAHFD feeding.

To further investigate the inhibitory effect of lactoferrin on hepatocyte inflammation, its effect on gene expression was examined using real-time RT-PCR. The gene expression of TNF-α, IL-1β, and IL-6 significantly increased in the CDAHFD group compared with that in the control group. As TNF-α, IL-1β, and IL-6 are inflammatory cytokines released by macrophages, it was suggested that activation of Kupffer cells is involved in hepatocyte inflammation. Ganguly *et al*. reported that different mouse models of NASH exhibit immune changes in hepatocytes, and it is likely that similar changes occurred in the model used in this study (*17*). Lactoferrin intake inhibited the elevation in the levels of these inflammatory cytokines, and significantly suppressed their expression at higher doses. Lactoferrin has been shown to be closely related to immune function in infants and likely involved in the activation of neutrophils (*10*). On the contrary, Hwang *et al*. reported that in *Bacillus* Calmette Guerin and human lactoferrin-treated $CD16^{+}$ macrophages, lactoferrin decreased TNF-α level (*18*). As lactoferrin significantly suppressed the release of inflammatory cytokines from macrophages, we investigated the gene expression of MCP-1, which is involved in macrophage chemotaxis. Lactoferrin intake significantly suppressed the increase in the gene expression of MCP-1 caused by CDAHFD feeding. MCP-1 has been shown to be a migratory factor for macrophages (*19*), suggesting that lactoferrin inhibits macrophage migration. It is also possible that CDAHFD intake causes an imbalance in the intestinal microflora, resulting in increased LPS level and inflammation induced by the activation of Kupffer cells in the liver. Lactoferrin has been reported to improve the imbalance of intestinal microflora (*20*). Although not clarified in the present study, we cannot rule out the possibility that lactoferrin positively affects the intestinal microflora and suppresses the induction of LPS.

In this study, lactoferrin suppressed the increase in plasma AST and ALT levels and histological cell damage caused by CDAHFD feeding, suggesting that lactoferrin also has an effect on hepatocyte cell death. Therefore, we examined the gene expression levels of caspase 3 and p53, which are markers of apoptosis. The results indicated that CDAHFD feeding significantly increased the gene expression of caspase 3 and p53, suggesting the induction of apoptosis in hepatocytes. Lactoferrin intake decreased the gene expression of p53 and significantly suppressed the gene expression of caspase 3. This finding suggests that lactoferrin inhibited the induction of apoptosis in the NASH mouse model used in this study. It has been shown that inflammatory and oxidative stresses are associated with the induction of apoptosis (*21*). NO release is also closely associated with these stresses (*22*). The enzymes neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS are known to be involved in NO synthesis. Mgbemena *et al*. demonstrated the importance of iNOS in inducing apoptosis *via* oxidative stress in a mouse mode (*23*). Therefore, the gene expression level of iNOS was also examined in this study. The expression of iNOS was significantly higher in the CDSHFD group than in the control group, and this increase was significantly suppressed by lactoferrin intake. These results suggest that lactoferrin suppresses oxidative stress-induced apoptosis induced by CDAHFD feeding. In a study on hepatocellular carcinoma-bearing mice, Abdelmoneem *et al*. showed that lactoferrin inhibited cancer cell growth by suppressing the expression of the transcription factor NF-κB (*24*). As NF-κB expression is known to induce the gene expression of iNOS (*25*), lactoferrin may have suppressed the gene expression of iNOS by suppressing the expression level of NF-κB.

Our results showed that lactoferrin prevented liver injury in the model mice with NASH induced by CDAHFD feeding through anti-inflammatory effect by inhibiting the migration to macrophages and *via* antioxidant stress effects on oxidative stress caused by fat accumulation. However, lactoferrin is known to have multiple functions, and its effects may also involve other immune cells or actions on adipose tissue, which cannot be excluded.

Our study opens up the possibility that lactoferrin could be a new treatment for NASH by reducing the development of lifestyle-related hepatocyte inflammation. Nevertheless, some limitations should be acknowledged. In this study, we focused on the preventive effects of lactoferrin during the early stage of NASH using the CDAHFD model. Previous studies have shown that significant hepatic fibrosis develops after approximately 6 weeks of CDAHFD intake (*6*). Thus, our 14-day experimental period was intended to capture the initial stage of disease progression. However, the pathogenesis of human NASH is more complex and chronic, and whether lactoferrin can suppress long-term progression such as advanced fibrosis or cirrhosis remains to be clarified. This limitation should be addressed in future studies with extended intake periods. Although there are some limitations, our findings suggest that lactoferrin may help prevent the development of NASH, providing a basis for future studies to explore its potential in NASH prevention.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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