Influence of trans-fatty acids on non-alcoholic steatohepatitis with hepatic fibrosis induced by a choline-deficient, methionine-lowered, L-amino acid-defined diet in male Harlan Sprague Dawley rats

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ABSTRACT — Trans fatty acids (TFAs) are risk factors for cardiovascular diseases and have been suggested to play roles in various metabolic diseases, including non-alcoholic steatohepatitis (NASH). The present study aimed to assess the influence of TFAs on the development of NASH lesions. Male Harlan Sprague Dawley rats were fed a choline-deficient, methionine-lowered, L-amino acid-defined (CDAA) diet with or without TFAs for two, four, and 13 weeks. The CDAA diet caused hepatic steatosis, macrophage infiltration, and fibrosis. Further, it increased serum activities of aspartate and alanine aminotransferase, and upregulated inflammation- and fibrosis-related genes in the liver. TFAs enhanced or tended to enhance the serum ALT activity but did not affect other outcomes. In the present study using the CDAA rat model, NASH lesions were clearly induced; however, the effect of TFAs was minimal. In conclusion, TFAs may be a risk factor for NASH by enhancing hepatocellular injury. With the composition and amount used in the present study, TFAs did not affect hepatic steatosis, chronic inflammation, or fibrosis in rats fed with the CDAA diet. To assess the risk of TFAs for NASH, more comprehensive studies are warranted, using other compositions and/or amounts of TFAs.

Key words: Non-alcoholic steatohepatitis, Fibrosis, Trans fatty acids

INTRODUCTION

The Codex definition states that trans fatty acids (TFAs) are all the geometrical isomers of monounsaturated and polyunsaturated fatty acids that are non-conjugated, interrupted by at least one methylene group and carbon-carbon double bond in the trans-configuration (Wang and Proctor, 2013). TFAs are known to be a risk factor for cardiovascular diseases, as evidenced by epidemiological investigations in western countries (Ghahremanpour et al., 2008; Mozaffarian et al., 2004). Excessive TFA intake promotes inflammation and oxidative stress, which in turn facilitates atherosclerosis (Monguchi et al., 2017). The World Health Organization (WHO) issued a state-
ment in 2019 to eliminate industrially produced TFAs from the global food supply by 2023. The Food and Drug Administration (FDA) of the United States decided to remove artificial trans fats in processed foods, and in 2015, partially hydrogenated oils (PHOs), the major source of artificial trans fat in the food supply, were no longer “Generally Recognized as Safe” or GRAS. Since June 18, 2018, manufacturers could not add PHOs to foods. In Japan, because the intake of TFAs was estimated to be a substantially low, i.e., 0.3 to 0.9% (Hunter, 2006; Sugano, 2008), there has been no regulatory restriction, but both risk assessment and risk management bodies of the government encourage suppliers to reduce artificial TFAs, continue monitoring the intake of TFAs, and communicate about the risk of TFA use.

The number of people with non-alcoholic fatty liver disease (NAFLD), a liver phenotype associated with metabolic syndrome, are increasing worldwide. Its prevalence in Japan was estimated to be 18% by a medical health checkup program in a general hospital in 2001 (Hamaguchi et al., 2005), and 29.7% in a cross-sectional study conducted in three health centers from 2009 to 2010 (Eguchi et al., 2012). NAFLD is divided into two categories: non-alcoholic fatty liver (NAFL), a simple fatty liver, and non-alcoholic steatohepatitis (NASH), characterized by hepatic inflammation and fibrosis. As a pathogenic mechanism of NASH, the multiple parallel hit hypothesis has been proposed, in which various factors are thought to be implicated in parallel, such as insulin resistance, oxidative stress, adipocytokines, gut microbiome, and nutritional factors (Buzzetti et al., 2016). The NASH liver demonstrates hepatic fibrosis, and the risk of mortality is closely related to the progression of hepatic fibrosis (Dulai et al., 2017). NASH patients have a higher risk of cirrhosis and hepatocellular carcinoma than non-NASH NAFLD patients, and about 10% to 20% of NASH patients develop cirrhosis, a major factor of liver transplantation (von Roenn, 2018). Since the mechanisms underlying the development and progression of NASH are still largely obscure, their elucidation has attracted attention for developing preventive and therapeutic measures. Extensive efforts have been made, and a variety of NASH animal models are being used to provide pathophysiological situations extrapolating to their human counterparts. A rodent model featuring chronic feeding of a choline-deficient, methionine-lowered, L-amino-acid-defined (CDAA) diet is one of the most used NAFLD/NASH animal models because of the numerous phenotypical and mechanistic similarities to the human situation, especially oxidative stress, and hepatic fibrosis (Tølbøl et al., 2019). Among 344 male Fischer rats, the CDAA diet clearly induced hepatic phenotypes of hepatocellular fatty change, inflammatory cell infiltration, significant fibrosis ending in cirrhosis, and a high incidence of hepatocellular carcinomas. Oxidative stress and related signaling alterations have been shown to be involved in the underlying mechanisms.

TFAs have been reported to lower blood HDL cholesterol and increase LDL cholesterol, thus playing a role not only in cardiovascular diseases but also in various other metabolic diseases, including NAFLD/NASH (Oteng et al., 2019). However, it is unclear how TFAs are involved in NAFLD/NASH. In this context, the present study aimed to assess the influence of TFAs on the development of NASH lesions in rats induced by the CDAA diet, especially hepatic fibrosis.

MATERIALS AND METHODS

Animal and treatment

We purchased 36 male Harlan Sprague Dawley rats, aged five weeks, from Japan SLC, Inc. (Shizuoka, Japan) and housed them at an average temperature of 23°C under the air-controlled conditions in colony cages with a 12-hr light/12-hr dark cycle. The rats were allowed ad libitum access to food and water during both the acclimation and treatment periods. At six weeks of age, the rats were randomly assigned to three groups: a standard diet (CE-2; CLEA Japan Inc., Tokyo, Japan), the CDAA diet without TFAs (CDAA-T−) (A16032903; Research Diet Inc., New Brunswick, NJ, USA), and the CDAA with TFAs (CDAA+T) (A16032904; Research Diet Inc.) for up to 13 weeks. The CE-2 diet was composed of 58% carbohydrate, 13% fat, and 29% protein containing 0.21% choline and 0.44% methionine. CDAA diets were composed of 13% protein, 57% carbohydrate, and 30% fat. The components of the CDAA diets are shown in Table 1. Body weight and food consumption were monitored weekly.

The rats were sacrificed at the end of weeks 2, 4 (n = 3), and 13 (n = 6) after overnight fasting. At the scheduled sacrifice under isoflurane anesthesia, blood samples were collected from the abdominal aorta for serum biochemical examinations, and the rats were euthanized by exsanguination. The liver was carefully studied during autopsy, excised, and weighed. Portions of the livers were immediately fixed in 10% neutrally buffered formalin for histopathological and immunohistochemical examinations, and the remaining samples were stored at −80°C for molecular biological assessments.

Serum biochemical examinations

Serum activities of alanine aminotransferase (ALT)
and aspartate aminotransferase (AST) were measured using an automatic analyzer (DRI-CHEM NX500V, FUJIFILM Medical Co., Ltd., Tokyo, Japan). Histofine Simple Stain Rat MAX-PO (MULTI) (Nichirei Bioscience Inc., Tokyo, Japan) was used as a secondary antibody, and the signals were visualized using 3,3'-diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Using SR-stained specimens, the area of fibrosis was measured using CellSens Dimension software (Olympus Corp., Tokyo, Japan).

**Molecular biological examinations**

Hepatic total RNA was extracted using Sepasol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan) and was reverse transcribed to cDNA using a ReverTra Ace qPCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) and a Thermal Cycler Dice (Takara Bio Inc., Shiga, Japan). Quantitative real-time PCR (qPCR) was performed on a Thermal Cycler Dice Real Time System II (Takara Bio) using a Thunderbird SYBR qPCR Mix (Toyobo). All procedures were performed according to the manufactures’ protocols. The primer sequences used for qPCR are listed in Table 2.

**Statistical analysis**

Statistically significant differences between the control, CDAAT-, and CDAAT+ groups at each time point were determined using one-way ANOVA. Statistical significance was set at $p < 0.05$.

**Ethical consideration**

All animal husbandry and experiments were conducted in compliance with the guiding principle of the Tokyo University of Agriculture and approved by the Animal Experiment Committee of the university. Consequently, this study complied with all related domestic and international laws, regulations, and guidelines.

**RESULTS**

**Body and liver weights**

Changes in the body, absolute, and relative liver weights are shown in Fig. 1. While body weights were sequentially increased in all groups, those of the CDAAT diet groups were lower or tended to be lower than the control diet group value at each time point. Absolute and relative liver weights in the CDAAT diet groups were higher than those in the control diet group at the end of weeks two and four, but became unapparent at the end of week 13. TFAs exerted no apparent effect in the CDAAT diet groups.

**Serum biochemistry**

Changes in the serum biochemical parameters are
shown in Fig. 2. AST and ALT activities in the CDAA diet groups were higher or tended to be higher than those in the control diet group at each time point, especially at the end of week two. Within the CDAA diet groups, TFAs tended to increase the enzyme activities at each time point, and a significant effect was observed at the end of week 13, where ALT activity in the CDAAT+ group was higher than that of the CDAAT- group.

**Table 2. qPCR primers.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>TNF-alpha</td>
<td>GAACCTCAGCGAGGACACCAA</td>
<td>GCCAGTGTGTAGAGGGACG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CTTCCCTCACCCTATGCAGG</td>
<td>GATGCTACAGGCAGCAACTG</td>
</tr>
<tr>
<td>Collagen type 1</td>
<td>GTACATCGCACAACCCCA</td>
<td>CAGGATCGAAACCTTCGCTT</td>
</tr>
<tr>
<td>Collagen type 4</td>
<td>CTTGTTGGCCTCTTGTGCG</td>
<td>TGCACCTGGATTGCAAAAAGGC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTGCCAGCCTGTTCATA</td>
<td>AAGAAGGCGAGCCTGGTA</td>
</tr>
</tbody>
</table>

**Fig. 1.** Body and liver weights. Changes in body weight (A), absolute liver weight (B), and relative liver weight (C) during the experiment. Data are presented as mean ± standard deviation. *Significantly different from the control diet group value at the same time point.

**Fig. 2.** Serum AST and ALT activities. Data are presented as mean ± the standard deviation. *Significantly different from the control diet group value at the same time point.
**Histopathological and immunohistochemical outcomes in the liver**

Morphological changes in the liver are shown in Fig. 3. The CDAA diets induced NASH lesions, including hepatocellular fatty change, infiltration of inflammatory cells (ED-1-positive macrophages), and hepatic fibrosis. Fat-ty changes in hepatocytes and inflammatory cell infiltration were observed from the end of week two (data not shown) and were found to have been maintained until week 13. Hepatic fibrosis was slightly observed at the end of week four and progressed to week 13. TFAs exerted no apparent effect in the CDAA diet groups.
Hepatic gene expression profile

Changes in hepatic gene expression are shown in Fig. 4. The mRNA expression of inflammation-related, tumor necrosis factor alpha (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) were higher or tended to be higher in the CDAA diet groups than in the control group throughout the experimental period. The mRNA expression of fibrosis-related genes, collagen type 1 and collagen type 4, were also higher or tended to be higher in the CDAA diet groups than in the control group throughout the experimental period. TFAs exerted no apparent effect in the CDAA diet groups.

DISCUSSION

NAFLD is a hepatic phenotype of lifestyle-related diseases, and its prevalence is increasing worldwide and the ratio is higher in male and relatively low in female in Japan (Hamaguchi et al., 2012; Eguchi et al., 2012). Understanding the underlying mechanisms of disease progression is important for evidence-based research and development of molecular-targeted therapeutic and preventive measures. Chronic inflammation, hepatocellular damage, and fibrosis are the three major hepatic changes in NASH and must be primary targets in the control of the disease. Among them, hepatic fibrosis is the most centered target, because it has been shown that fibrosis increases mortality and is a factor determining the prognosis of NASH (Dulai et al., 2017; Heyens et al., 2021). Various risk factors, including nutrients, are known to affect the development and progression of the disease (Ullah et al., 2019), and TFAs may be one such risk factor. TFAs are known to be involved in cardiovascular events and have recently been shown to be associated with NAFLD with severe fatty changes (Tabuchi et al., 2014; Obara et al., 2010). However, it is largely unclear how TFAs are involved in NAFLD/NASH, especially in NASH-associated hepatic fibrosis, which is the rationale behind the present study.

In the present study’s CDAA diet groups, liver weights were elevated, hepatocellular fatty change and infiltration of macrophages were induced in the liver, and the hepatic inflammation-related genes, such as TNF-α and MCP-1, were upregulated, but the TFAs did not exert any apparent effect. In contrast, TFAs enhanced or tended to enhance the serum AST and ALT activities in the CDAA diet. Therefore, at least in the present conditions, TFAs may not be involved in the development of steatosis and inflammation, but may be involved in the hepatocellular injury in NASH, among the CDAA diet fed rats. Previous in vivo studies have reported an increase in ALT activity in mice fed a TFA-containing diet in mice (Tetri et al., 2008). In an epidemiological study of Japanese males, plasma levels of TFAs have been shown to be related to ALT activity (Tabuchi et al., 2014). The mechanism of cell injury by TFAs is attributed to apoptosis associated
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with DNA damage (Hirata et al., 2020). Because we have shown that hepatocellular injury in the CDAA model is a result of oxidative stress-derived apoptosis, it is suggested that TFAs enhance this process to cause greater hepatocellular injury.

The results of our study show that in the CDAA diet groups, hepatic fibrosis was induced from the end of week four, followed by substantial progression, and the hepatic fibrosis-related genes, such as collagen type 1 and collagen type 4, were upregulated, but TFAs did not exert any apparent effect. Although several studies have reported that TFAs play an important role in the development of hepatic fibrosis, by the proliferation of collagen fiber due to the upregulated fibrosis-related gene expression (Harris et al., 2020; Kohli et al., 2010; Jeyapal et al., 2018), TFAs may not be able to initiate fibrogenesis, but may only be able to promote fibrosis (Jia et al., 2021). Thus, TFAs can exert this promoting effect when fibrogenesis is initiated by certain other causes, and when the initiated fibrotic mechanisms are associated with the action of TFAs. It is therefore conceivable that this potential promoting effect of TFAs may not act on the fibrotic mechanisms of the CDAA diet model, or that the presently used composition and/or amount of TFAs may not be enough to exert effects on hepatic fibrosis.

In conclusion, TFAs may be a risk factor for NASH by enhancing hepatocellular injury. With the composition and amount used in the present study, TFAs did not affect hepatic steatosis, chronic inflammation, or fibrosis in rats fed with the CDAA diet. To assess the risk of TFAs for NASH, more comprehensive studies are warranted, using other compositions and/or amounts of TFAs.

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

REFERENCES


Obara, N., Fukushima, K., Ueno, Y., Wakui, Y., Kimura, O., Tamai, K., Kakazu, E., Inoue, J., Kondo, Y., Ogawa, N. and Sato, K.,
Liver Physiol., 295, G987-G995.