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ORIGINAL ARTICLE

Daily Coffee Intake Inhibits Pancreatic Beta Cell Damage and Nonalcoholic Steatohepatitis in a Mouse Model of Spontaneous Metabolic Syndrome, Tsumura-Suzuki Obese Diabetic Mice

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Abstract

Background: Metabolic syndrome is one of the most important health issues worldwide. Obesity causes insulin resistance, hyperlipidemia, diabetes, and various diseases throughout the body. The liver phenotype, which is called nonalcoholic steatohepatitis (NASH), frequently progresses to hepatocellular carcinoma. We recently established a new animal model, Tsumura-Suzuki obese diabetic (TSOD) mice, which spontaneously exhibit obesity, diabetes, hyperlipidemia, and NASH with liver nodules.
Methods: We examined the effects of coffee intake on various conditions of the metabolic syndrome using TSOD mice. The daily volume of coffee administered was limited so that it reflected the appropriate quantities consumed in humans. To clarify the effects of the specific components, animals were divided into two coffee-intake groups that included with and without caffeine.
Results: Coffee intake did not significantly affect obesity and hyperlipidemia in TSOD mice. In contrast, coffee intake caused various degrees of improvement in the pancreatic beta cell damage and steatohepatitis with liver carcinogenesis. Most of the effects were believed to be caused by a synergistic effect of caffeine with other components such as polyphenols. However, the antifibrotic effects of coffee appeared to be due to the polyphenols rather than the caffeine.
Conclusions: A daily habit of drinking coffee could possibly play a role in the prevention of metabolic syndrome.

Keywords: coffee intake, nonalcoholic steatohepatitis, beta cell failure, animal model, hepatocellular carcinoma

Introduction

Metabolic syndrome is one of the most important health issues worldwide. Obesity causes insulin resistance, hyperlipidemia, diabetes, and various diseases throughout the body.[1] This liver phenotype, which is referred to as nonalcoholic steatohepatitis (NASH), frequently progresses to hepatocellular carcinoma (HCC).[2–10] In Asian countries, HCC is the second most common cancer and remains an intractable tumor with a high mortality rate.[11] Although the main cause of HCC is a viral infection, such as hepatitis B virus (HBV) or hepatitis C virus (HCV) infections, the implementation of transfusion screening and the development of vaccines have the potential to lead to a marked decrease in the number of patients with viral hepatitis in the near future. In contrast,

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steatohepatitis caused by metabolic syndrome is rapidly increasing in developed countries, with HCC-associated steatohepatitis increasing in a dramatic manner.[3,9] Both epidemiological and experimental analyses have demonstrated that coffee drinking reduces the risk of metabolic syndrome, NASH, and HCC.[4,12–19] In addition, it has been reported that there was an enhanced effect of antiviral drugs (*e.g.*, peginterferon and ribavirin) in a group of individuals who exhibited a high consumption of coffee (three cups or more/day).[20] In contrast, the mechanism responsible for the effect of coffee on hepatitis and on the HCC associated with metabolic syndrome is yet to be fully elucidated. Within the context of translational research using animal models, previous studies have primarily used genetically modified animal models and/or special diet-induced animal models for metabolic syndrome with steatohepatitis and HCC. However, since these artificial animal models exhibit severe disease conditions, they have proven to be inadequate for analyzing the mild and gradual effects of coffee consumption.

We have recently reported that the Tsumura-Suzuki obese diabetic (TSOD) mice, which are an inbred strain of mice created in Japan from the *ddy* strain, sequentially develop obesity, type 2 diabetes, and hyperlipidemia after 3 months of age without any special treatment. Subsequently, they also develop NASH, including steatosis of the hepatocytes, ballooning degeneration, and lobular inflammation, at the age of 4 months. After 10 months of age, these mice develop liver tumors that exhibit two types of histological patterns.[21–26] Approximately 70% of the tumors exhibit cellular and structural atypia and glutamine synthetase (GS) positivity, thereby mimicking the human dysplastic nodule-HCC sequence. The remaining 30% of the tumors exhibit a less atypical change, with a foamy cytoplasm and liver fatty acid binding protein (LFABP) negativity, thereby mimicking the human hepatocellular adenoma. In contrast to the above characteristics, the TSOD mice are considered to be a unique animal model, as they exhibit clinical and pathological processes that are similar to human patients with metabolic syndrome.

In our previous studies, we used metabolic syndrome model animals to examine various types of natural products.[27–34] The aim of the present study was to analyze the advantageous effects of moderate coffee intake quantities on various types of metabolic symptoms through the use of a spontaneous metabolic syndrome/steatohepatitis/HCC mouse model, the TSOD mice. Appropriate doses of coffee (or its components) used in this model were determined by converting the coffee intake in humans according to body weight. To further clarify the effects caused by specific components, we divided the animals into coffee-intake groups with and without caffeine. This study histopathologically and serologically evaluated obesity, hyperlipidemia, type 2 diabetes, severity of steatohepatitis, and frequency of HCC.

Materials and Methods

Animals

This study used male TSOD mice, as the condition is more severe in males versus females. All TSOD mice were purchased from the Institute for Animal Reproduction, with the breeding/experiments entrusted to the same institute. Only for the comparison, we added the data of serum samples from previously stocked normal mice [Tsumura-Suzuki-nonobese (TSNO) mice].

Experimental design

This study was performed in accordance with the animal experiment guidelines specified in the Institute for Animal Reproduction (Ibaraki, Japan) (Permission number: IarAW No. H24-29), which strictly observed the animal research ethics guidance rules entitled, ''Consensus Author Guidelines on Animal Ethics and Welfare,'' and which were developed and published in 2010 by the International Association of Veterinary Editors. Four-month-old male TSOD mice were purchased and used in the experiments after 1 week of quarantine.

This study used three experimental groups that included a water control group (control: TSOD mice without coffee administration), a caffeine-free coffee group (caffeine free: TSOD mice given Nescafe Gold Blend Red Label; Nestle Japan, Tokyo), and a caffeine-containing coffee group (coffee: TSOD mice given Nescafe Gold Blend Gold Label; Nestle Japan). Coffee was administered by dissolving the test substance in self-pumped water containing sodium hypochlorite. The animals had free access to their respective drinks. No differences were noted in the volume of drinking among the three groups throughout the course of the study. The animals had free access to autoclave-sterilized usual solid feed (MF) for mice (Oriental Yeast Co., Ltd., Tokyo, Japan). Although we wanted to evaluate the progress of liver pathology in the same mouse without having to sacrifice it, this would have required a long time to document any significant effect associated with the coffee intake. Thus, we performed a liver biopsy when the mice were 6 months of age, with a small amount of liver tissue extracted under minimal laparotomy. No critical side effects, such as weight change, were observed after the liver biopsy. The experiment was continued after the liver biopsy. The endpoint of the study was 11 months of age, with each mouse sacrificed to collect blood samples and organs.

The coffee types used in the experiment were caffeine-free Nescafe Gold Blend Red Label, which contained polyphenol (chlorogenic acid; 14,000 mg/100 g), and caffeine (0.11/100 g) and Nescafe Gold Blend Gold Label, which contained polyphenol (chlorogenic acid; 15,000 mg/100 g) and caffeine (2.5/100 g). Even though the caffeine-free brand contained a very small amount of caffeine, for the purpose of this experiment, only the latter brand was considered to contain caffeine. One gram of each test substance was dissolved in 1000 mL of water and administered to the mice. All of the mice examined drank $\sim$10 mL of diluted coffee during each day. Equivalently, each mouse in the caffeine-free group ingested 1.5 mg of chlorogenic acid per day, while each animal in the coffee group ingested 0.25 mg of caffeine and 1.5 mg of chlorogenic acid per day. This amount is equivalent to 330 mg of caffeine in an adult human with a body weight of 60 kg. The maximum daily intake volume recommended by the European Food Safety Authority is 400 mg of caffeine. Thus, the coffee intake volume in this study was within the recommended volume.

Measurement items

Monthly. Body weight and urine sugar were measured.

Six months of age. Histopathological evaluation of the liver biopsy using hematoxylin and eosin (H&E) and Azan

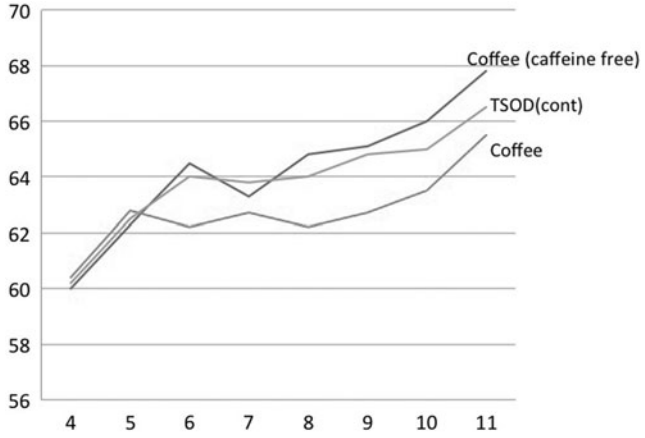

FIG. 1. Alteration over time of averaged body weight (g) in the three groups. *Top line*: caffeine-free coffee group, *bottom line*: caffeine-containing coffee group, *middle line*: control group.

staining was performed to evaluate the fibrosis. The histopathological evaluation of the NASH severity in humans was based on the commonly used nonalcoholic fatty liver disease (NAFLD) activity score (NAS) system, with a high NAS indicative of a suspected case of NASH. NAS is calculated as the sum of the score for the degree of hepatocellular steatosis spreading from around the central veins (0–3), hepatocellular ballooning (0–2), and lobular inflammation (0–3).

Eleven months of age. After sacrificing the animals, histopathological evaluations of the tissue samples using H&E and Azan staining were performed to evaluate the fibrosis. In addition, blood samples were tested (blood sugar and lipid analysis) and histopathological analyses of the visceral fat, pancreas, and liver were performed. As TSOD mice develop hyperlipidemia early in life, they are known to have elevated total cholesterol and triglyceride levels. In particular, these mice also have significant increases in the low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) fractions. Serum samples collected from each of the mice in the present study and samples from previously stocked normal mice (TSNO mice) specimens were analyzed by high-performance liquid chromatography (HPLC) (outsourced to LipoSEARCH, Akita, Japan), with the results used to compare the changes in each lipid fraction. For fat evaluation, the size of the adipose cells and the degree of crown-like structures, which represent macrophage accumulation, were evaluated. For pancreatic evaluation, we performed scoring of the degree of enlargement and degeneration/atrophy of the islets of Langerhans, as described below. Significant enlargement of the pancreatic islets of Langerhans has been demonstrated in TSOD mice due to the persistent hyperglycemia and insulin resistance that occur in these animals. These changes are followed by atrophy and degeneration of the beta cells, which are then replaced by exocrine cells, at $\sim$10 months of age. Analysis of the islets of Langerhans was performed with a Score of 1 indicating enlarged islets, a Score of 2 indicating mild degeneration and/or atrophy with substitution with exocrine cells in some enlarged islets, and a Score of 3 indicating degeneration and/or atrophy with substitution with exocrine glands in more than half of the islets. For the evaluation of the liver, the NAS, degree of fibrosis, and characteristics of liver tumors were evaluated.[35] There are two types of liver tumors that develop in TSOD mice. The first type is an atypical tumor exhibiting a malignant course of dysplastic nodule-HCC [glutamine synthetase (GS) positivity and liver fatty acid binding protein (LFABP) positivity]. The second type exhibits a less atypical tumor, which is equivalent to one of the subtypes of hepatocellular adenoma (GS negativity and LFABP negativity). Immunostaining for GS and LFABP was performed using the Envision-PO system (DAKO, Glostrup, Denmark) with the diaminobenzidine reaction.[36,37]

Statistical analysis

All continuous variables are expressed as the mean $\pm$ standard error of the measurement (SEM) and compared among groups using a one-way analysis of variance followed by Bonferroni correction for multiple testing. All analyses were two tailed, with $P < 0.05$ considered statistically significant. Statistical analysis was performed using Statcel version 3.0, which is an add-on of the Excel software (OMS Publ., Saitama, Japan).

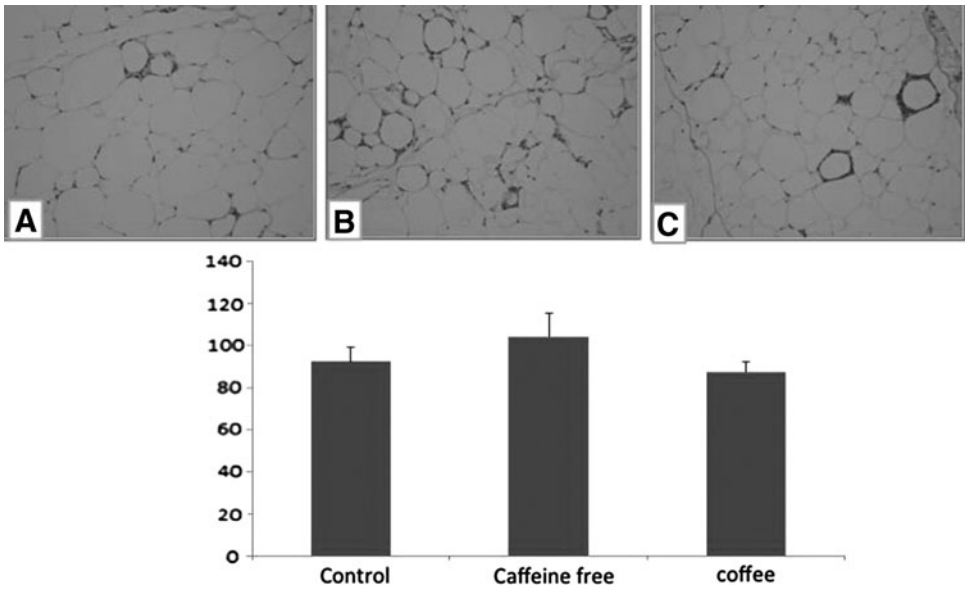

FIG. 2. Representative pictures of the control group **(A)**, caffeine-free group **(B)**, and coffee group **(C)**. The graph is the number of adipose cells in a visual field at 200× magnification.

Table 1. Measurement of Urinary Sugar Score

	Caffeine free						*Coffee*					
Urinary sugar	–	±	+	++	+++	++++	–	±	+	++	+++	++++
Months of age												
4	1	1	3	0	0	0	1	0	4	0	0	0
5	1	1	0	1	2	1	1	0	2	1	0	0
6	0	0	0	0	2	4	2	0	0	0	0	3
7	0	0	0	0	1	5	1	0	1	0	0	3
8	0	0	0	0	2	3	1	0	1	0	0	2
9	0	0	3	0	0	2	1	0	1	1	0	2
10	0	0	1	0	3	1	2	0	1	0	1	1
11	0	0	2	1	2	0	1	0	0	2	1	1

Urinary sugar score (–, negative; ±, false positive; +, weak positive; ++, positive; +++, strong positive; ++++, very strong positive.)

Results

Body weight change, size of visceral fat, and degree of crown-like structure (obesity-related findings)

All groups exhibited obesity from 4 weeks of age. No significant differences were noted in the body weight throughout the course of the study, and there was no observed weight change or serious conditions due to the coffee intake (Fig. 1). The size of the adipose cells in the visceral fat increased in all of the groups. No significant differences were noted in the number of fat cells per visual field at a magnification of 200× among the groups (Fig. 2). No significant differences were noted regarding the number of macrophages accumulated per unit area among the three groups (data not shown).

Urine sugar and the degree of beta cell damage in pancreatic islets of Langerhans (type 2 diabetes-related findings)

Urine sugar showed a tendency to gradually worsen in the control and caffeine-free groups. In contrast, the urine sugar decreased and increased during the course of the study in some mice in the coffee group, with the degree of urine sugar tending to decline compared with the other two groups (Table 1). In the pancreas, degeneration of the islets was milder in the caffeine-free and coffee groups compared with the control group, and was significantly milder in the coffee group (Fig. 3).

Serum lipoprotein fractions (hyperlipidemia-related findings)

Both the cholesterol and triglyceride levels tended to be higher in the caffeine-free and coffee groups compared with the control group. However, there was no significant difference observed among the three groups (Fig. 4). The LDL and VLDL fractions, which are believed to be involved in the aggravation of metabolic syndrome, also exhibited no trend toward improvement after the administration of coffee (data not shown).

Histopathological images of the liver (6 months of age: reflection of the condition of NASH)

At 6 months of age, TSOD mice present histopathological images that are equivalent to those for NASH when

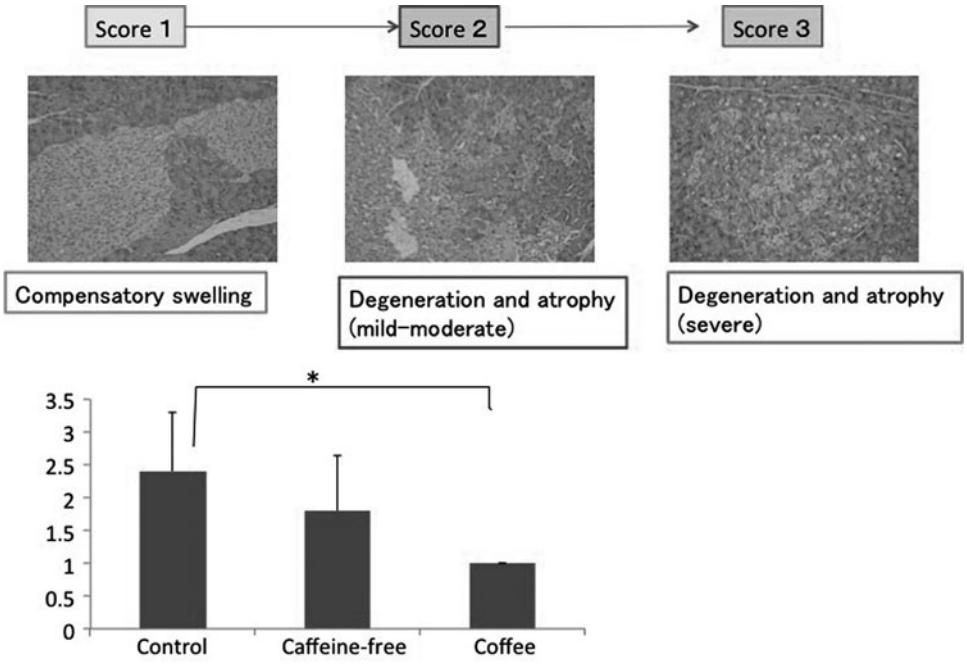

FIG. 3. Representative histology of score 1–3 islets of Langerhans in the pancreas, and graph of the score among the three groups. Degeneration of the islets was milder in the caffeine-free and coffee groups compared with the control group; in particular, it was significantly milder in the coffee group. $*p < 0.05$.

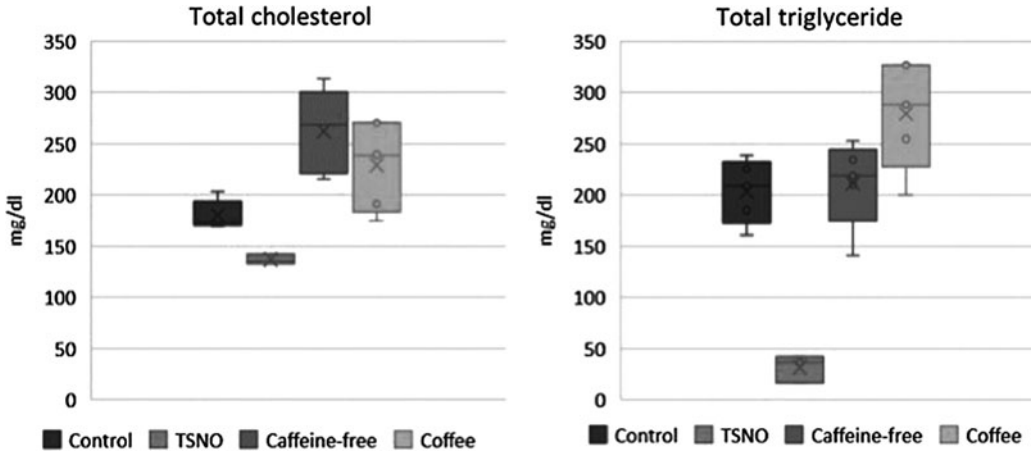

FIG. 4. Comparison of fractional serum cholesterol by HPLC among the three groups and normal mice (TSNO mice) (*left*: cholesterol; *right*: triglycerides). HPLC, high-performance liquid chromatography; TSNO, Tsumura-Suzuki-nonobese.

observed in humans, such as steatosis of hepatocytes around the central veins, inflammatory cell infiltration in the liver parenchyma, and hepatocellular ballooning degeneration. However, since our current specimens were collected through a needle biopsy at 6 months of age, there was no guarantee that the specimens included tissues around the central veins. Therefore, it was difficult to evaluate the degree of lobular inflammation that developed in the skin lesion. Accordingly, we compared the three groups using only the degree of hepatocellular ballooning as an index. This comparison showed that the degree of ballooning, which reflects the hepatocellular damage, was milder in the caffeine-free and coffee groups compared with the control group (Fig. 5).

Histopathological images of the liver (11 months of age: reflection of the degree of NASH, degree of fibrosis, and frequency of liver tumors)

Degree of NASH. We calculated the NAS for each animal and compared it among the three groups. The NAS was slightly lower in the caffeine-free and coffee groups, although this result was not significant (Table 2).

Degree of fibrosis. Fibrosis in NASH develops around the central veins and gradually progresses, weaving delicately into the surrounding hepatocytes. In the control group, mild fibrosis was observed around the central veins (1A) and there was slight progression to the surrounding hepatocytes (1B). A similar mild fibrosis was observed in the coffee group. In contrast, none of the animals in the caffeine-free group developed fibrosis, thereby indicating the presence of significant curative effects against the condition (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/met).

Frequency of liver tumors. Liver tumors were macroscopically identified, and tumor tissue samples were prepared for pathological evaluation. Liver tumors were observed in four of five (80%) mice in the control group. Two of these tumors (40%) were GS- and LFABP-positive atypical tumors, which are considered to be dysplastic nodule-HCC. The frequency of tumors was 80% (four out of five) in the caffeine-free group and 60% (three out of five) in the coffee group. The frequency of atypical tumors showing GS and LFABP positivity, however, was only 20% (one out of five) in both the caffeine-free and coffee groups (Table 2, Fig. 6, and Supplementary Table S2). Unfortunately, the frequency of tumors

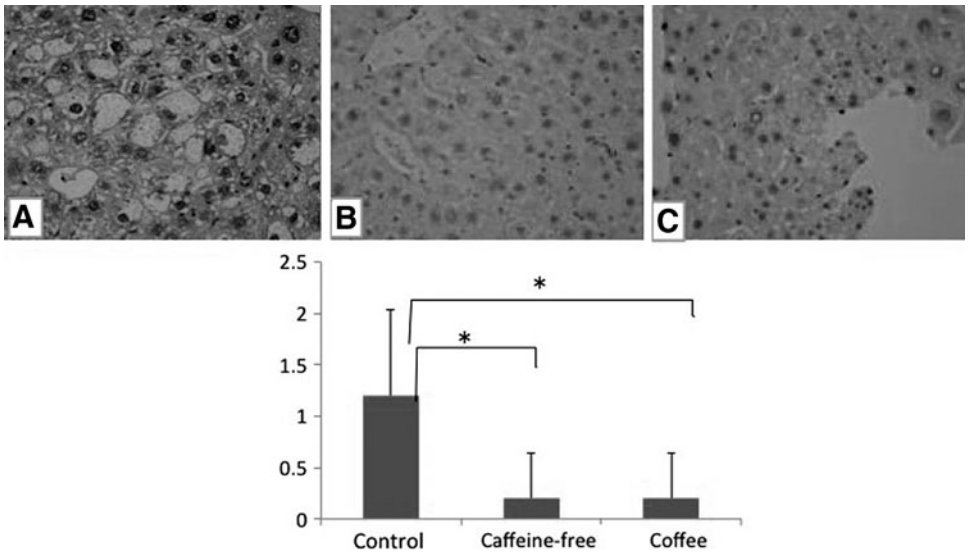

FIG. 5. The degree of ballooning, which reflects hepatocellular damage, in the liver of animals in the three groups at 6 months of age. Representative pictures of the control group **(A)**, caffeine-free group **(B)**, and coffee group **(C)**. The graph is the score of ballooning in the three groups. Ballooning was milder in the caffeine-free and coffee groups compared with the control group. $^*p < 0.05$.

Table 2. Frequency of Liver Tumor and GS(+) Tumor in Three Groups

	Frequency of tumor	*Frequency of GS(+) tumor*
Control	4/5 (80%)	2/5 (40%)
Caffeine free	4/5 (80%)	1/5 (20%)
Coffee	3/5 (60%)	1/5 (20%)

GS(+), glutamine synthetase positivity.

in the control group was lower than expected, and there was no significant difference among the three groups in the present study.

Discussion

We histopathologically examined the effects of coffee intake (with or without caffeine) in moderate quantities on obesity, type 2 diabetes, hyperlipidemia, and hepatic disorders (NASH and liver tumors) using a new mouse model (TSOD mice) that spontaneously develops metabolic syndrome and NASH with liver tumors. Our examinations did not find any significant improvement in either the findings associated with obesity, such as body weight, size of adipose cells in visceral fat, and frequency of crown-like structure, or with hyperlipidemia, such as changes in lipoprotein, even in the coffee-drinking groups. In contrast, the coffee-drinking groups did have some effects on the type 2 diabetes, such as a decrease in urine sugar, reduction of beta cell damage in pancreatic islets of Langerhans, and reduction of the hepatocellular ballooning at 6 months of age, as well as exhibiting a trend toward improvement in the NAS and a decrease in the frequency of malignant tumors at 12 months of age.

With regard to most of these items, the coffee group exhibited the same greater effects compared to that seen for the caffeine-free group, whereas an improvement effect on the liver fibrosis at the age of 12 months was only observed in the caffeine-free group. Previous reports have suggested that there may be an antifibrotic activity for caffeine.[38–48] In contrast, the present results indicated that substances other than caffeine that are present in coffee may inhibit fibrosis. Chlorogenic acid is a type of polyphenol with anti-inflammatory and antioxidant activities. Several studies have previously reported that chlorogenic acid reduces liver inflammation and fibrosis.[49,50] This suggests that chlorogenic acid may be a candidate inhibitor of fibrosis. Therefore, not only caffeine but also other components of coffee, such as chlorogenic acid, may work in a coordinated manner and act as antifibrotic agents.

In contrast to the previous reports, we did not observe any obvious improvement in the obesity or hyperlipidemia in the present study.[39,43,51,52] However, these changes may be dependent on the characteristics of the model animals (TSOD mice) and the amount of coffee ingested, which was ~1/2 to 1/5 of that reported in the previous studies. In contrast, the present results do suggest that the daily intake of several cups of coffee may inhibit the progression of liver fibrosis.

There were several limitations for our current study. First, this study only included a small number of samples (only five mice in each group) and only male mice were examined. Second, even TSOD mice are not completely representative of the human form of this disease, as the disease in humans is a more complex interplay of genetic and nutritional factors. Thus, an additional study that examines coffee intake using TSOD mice administered a high-fat and high-carbohydrate meal is currently being undertaken. Moreover, a further study with a larger sample size and

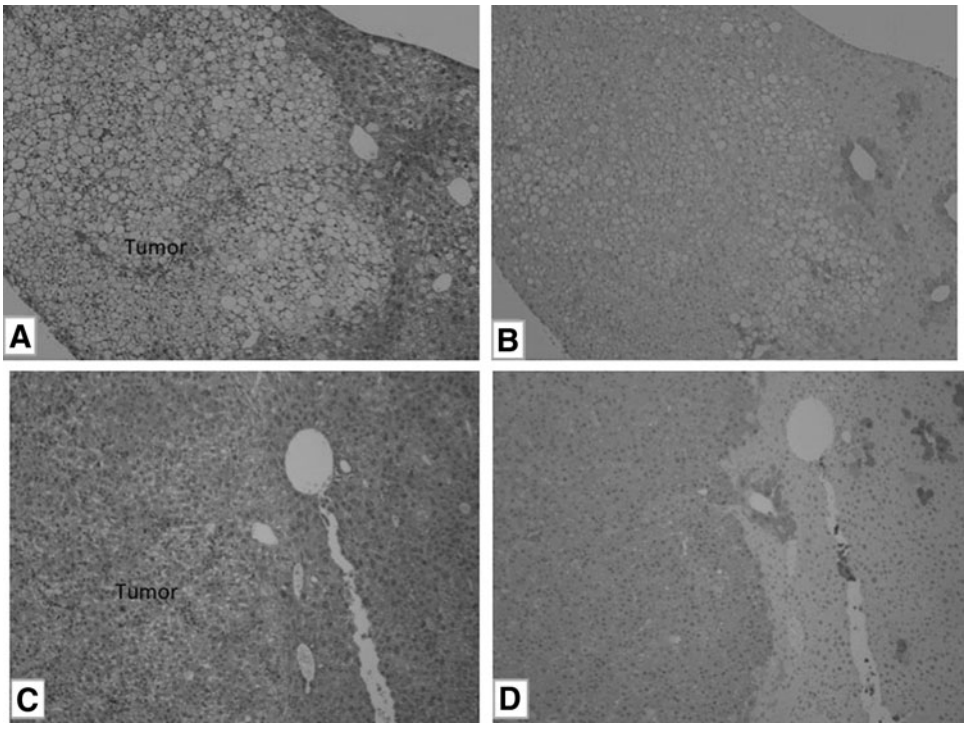

FIG. 6. Characters of liver nodules at 11 months of age. **(A, B)** Liver nodule showing less atypical changes, with steatosis showing negative staining against glutamine synthetase. **(C, D)** Liver nodule showing cellular and structural atypia, with positive staining against glutamine synthetase. **(A, C)** H&E staining at 100× magnification. **(B, D)** Immunostaining for glutamine synthetase. The Envision-PO system with diaminobenzidine reaction was used (100× magnification). H&E, hematoxylin and eosin.

longer observation period will need to be undertaken to confirm the mechanism of action of the components of coffee against the various symptoms of metabolic syndrome. Finally, future human studies will need to be performed to better understand the specific aspects of coffee that are beneficial to patients.

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