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Supplementation of concentrated Kurozu, a Japanese black vinegar, reduces the onset of hepatic steatosis in mice fed with a high-fat diet

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Submission Date: February 4th, 2019**, Acceptance Date:** April 28th, 2019, **Publication Date:** April 31st, 2019

Citation: Shibayama Y., Nagano M, Hashiguchi K., Fujii A., Iseki K. Supplementation of concentrated Kurozu, a Japanese black vinegar, reduces the onset of hepatic steatosis in mice fed with a high-fat diet. *Functional Foods in Health and Disease* 2019; 9(4): 276-296. DOI: https://doi.org/10.31989/ffhd.v9i4.596

ABSTRACT

Background: Hepatic steatosis is among the most common causes of chronic liver diseases, although established effective treatments are not evident. Previous studies reported that Kurozu improved hypercholesterolemia and carbohydrate metabolism. However, the effect of Kurozu on the incidence of hepatic steatosis is not clear.

Objective: The effect of Kurozu on the onset of hepatic steatosis by administering a high-fat diet (HFD) for 110 weeks was evaluated in C57BL/6J mice.

Methods: HFD treatment for 110 weeks accelerated the onset of hepatic steatosis more than a standard diet, whereas concentrated Kurozu (CK) supplementation ameliorated the effect of an HFD feeding. The effect of supplementation with resveratrol in an HFD on the onset of hepatic steatosis was also evaluated. To elucidate the mechanism of the effect of Kurozu on the expression of lipid metabolism genes, acute treatment for 10 days with Kurozu was also examined.

Results: Supplementation with resveratrol in HFD-fed mice did not ameliorate hepatic steatosis. Body weights were significantly lower in the CK + HFD and Resveratrol + HFD groups than in the control HFD group in middle age. No significant differences in all-cause mortality were observed following supplementation with CK or resveratrol. CK and resveratrol supplements significantly inhibited decreases in dehydroepiandrosterone sulphate serum levels at postnatal week 120. CK and resveratrol supplements did not affect the survival of mice. The ingestion of Kurozu for 10 days significantly elevated the expression levels of *Sirt1*, *Pgc-1α*, *Lpin1*, and *Igfbp1* in the liver.

Conclusion: These results suggest that ingesting CK may delay the onset of hepatic steatosis HFD feeding causes.

Keywords: Kurozu, steatosis, Sirt1, Igfbp1, Lpin1, Pgc-1α, resveratrol

BACKGROUND

Hepatic steatosis is one of the most common chronic liver diseases. An increase in the prevalence of obesity has heralded a rise in associated non-alcoholic fatty liver disease (NAFLD). Hepatic steatosis is an early stage on the spectrum of NAFLD. Treatments for NAFLD include weight loss, exercise, and drug treatments; however, there are no established management options for patients with NAFLD [1, 2] The accumulation of excess liver fat, obesity/insulin resistance, and disorders of lipid metabolism are major risk factors for hepatic steatosis [3].

Some of the molecular mechanisms of lipid and glucose metabolism have recently been elucidated. NAD^+-dependent deacetylase sirtuin 1 (SIRT1) has been identified as one of the key regulators of lipid metabolism, lifespan, gluconeogenesis, and carcinogenesis [4]. Peroxisome proliferator-activated receptor gammacoactivator-1α (PGC-1α) is a known target of SIRT1-dependent deacetylation [5]. PGC-1α is a member of the nuclear receptor PGC-1 superfamily, which comprises coactivators that have key functions in the dynamic

transcriptional control of energy metabolic pathways in various mammalian tissues [6]. Phosphatidate phosphatase LPIN1 (LPIN1) plays a key role in lipid metabolism and enhances peroxisome proliferator-activated receptor alpha (PPARα)–PGC-1α by forming a complex with PGC-1α and PPARα. LPIN1 gene expression is induced by an activating ligand of the liver X receptor (LXR). LXR agonists trigger deacetylation as SIRT1 interacts with LXR in a ligand-dependent manner. SIRT1 has been shown to deacetylate and positively regulate LXR, which affects LPIN1 gene expression [7]. Insulin-like growth factor (IGF-1) has an important role in lipid and glucose metabolism. IGF-binding proteins (IGFBP) are serum proteins that bind IGF-1 and regulate its turnover, transport, and tissue availability, and are the major determinant of free IGF-1 concentrations [8]. In summary, SIRT1 plays key roles in energy metabolism and aging, and it regulates the gene expression of PGC-1α, IGFBP1, and LPIN1. Upregulation of SIRT1, PGC-1α, IGFBP1, and LPIN1 are assumed to improve metabolic- and age-related diseases.

The activity of IGF-1 is regulated by growth hormone (GH). GH functions as a major metabolic hormone in adults by regulating energy and substrate metabolism. GH also enhances the clearance of low-density lipoprotein (LDL) by activating the expression of hepatic LDL receptors [9]. The adrenal prohormone dehydroepiandrosterone sulphate (DHEAS) is a biomarker of adrenal gland function, and the adrenal prohormone DHEAS conjugate plays an important role by regulating responses to stress, and it is generally regarded as a reliable endocrine marker of aging [10].

Vinegar, a common food flavoring, has potential health benefits and has been used for medical purposes. Functional and therapeutic properties of vinegar include an antibacterial action, reducing blood pressure, antioxidant effects, the amelioration of diabetes, prevention of cardiovascular diseases, and increased vigor following exercise [11]. Black vinegar (also called “Kurozu”) is a Japanese traditional vinegar made from rice, and it is commonly used as a healthcare supplement [12]. Previous studies reported that Kurozu improved the symptoms of hypertension, allergies, and hypercholesterolemia [13], enhanced carbohydrate metabolism [12], and inhibited tumor growth [14-16]. Previous reports suggested that Kurozu improved hypercholesterolemia; however, the details of its molecular function on lipid and glucose metabolism and effect on a high-fat diet on the development of hepatic steatosis remain unclear. To elucidate the effect of Kurozu on lifespan, we examined chronic treatment for 110 weeks in accordance with Baur JA. et al. [17].

In the present study, the incidence of hepatic steatosis was evaluated in mice fed a high-fat diet combined with concentrated Kurozu (CK). To elucidate the details of the molecular function of Kurozu on lipid and glucose metabolism, the effect of acute treatment

with Kurozu on the expression levels of metabolic- and aging-related genes: Sirt1, Pgc-1α, Igfbp1, and Lpin1 in the liver tissues of C57BL/6J mice were also evaluated.

METHODS

Reagents

Native Kurozu (containing 4% acetic acid) and CK from a jar (10-fold concentrated, pH 4.41, acetic acid distilled) were obtained from Sakamoto Kurozu, Inc. (Kagoshima, Japan). CK contained 3.23% lactic acid and 1.99% ash, and all acetic acid had been removed. Purified resveratrol made from *Polygonum cuspidatum* root was purchased from PureBulk, Inc (Roseburg, OR). TaqMan Gene Expression Assays, RNA*later*®, and TRIzol reagent were obtained from Applied Biosystems (Carlsbad, CA, USA). Real-time PCR master mix, THUNDERBIRD qPCR Mix, and reverse transcriptase, ReverTra Ace were purchased from TOYOBO Co., Ltd. (Osaka, Japan). A RNeasy Plus Mini Kit was purchased from Qiagen (Valencia, CA, USA). Protease inhibitor cocktail tablets (Complete®, EDTA-free) were purchased from Roche Diagnostics GmbH (Buckinghamshire, UK). Mouse monoclonal antibodies specific for B2m (B2M-01, Sigma-Aldrich, St. Louis, USA) and Sirt1 (7c2, Abnova, Taipei, Taiwan) were used. Rabbit polyclonal antibodies specific for Pgc-1α (101707, Cayman Chemical, MI, USA), Igfbp1 (13981-1-AP, ProteinTech Group, Inc., Chicago, USA), and Lpin1 (PAB12400, Abnova, Taipei, Taiwan) were also used. Secondary antibodies, anti-rabbit and anti-mouse IgG antibodies conjugated with horseradish peroxidase (HRP), were purchased from Nacalai Tesque (Kyoto, Japan). Nitrocellulose membranes, ECL Western Blotting detection system, and Hyperfilm ECL were purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK). Mouse dehydroepiandrosterone sulfate, DHEA-S ELISA kit, Mouse/Rat IGF-1 Quantikine ELISA kit, and Rat/Mouse Growth Hormone ELISA kit were purchased from TSZ Scientific (MA, USA), R & D System, Inc. (MN, USA), and Millipore (MA, USA), respectively. Bio-Gel P4 and the DC protein assay were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Forage

A standard-diet (STD) feed, CE-2, and high-fat diet (HFD) feed (QuickFat®) were purchased from CLEA Japan, Inc. (Tokyo, Japan). CE-2 contains 4.8% fat made from soybeans and wheat germ. QuickFat contains 13.9% fat made from cattle suet and wheat germ. Kurozu forage contained 0.6% (V/W) CK in QuickFat. The concentrate composition of Kurozu

except for acetic acid was added to QuickFat® to exclude the influence of acetic acid on the feed. The concentration of Kurozu in feed was configured to a 1-day average amount of food ingested by a mouse and the standard intake volume of Kurozu for humans as 30 mL per day. The ingested amount of Kurozu forage per mouse per day corresponded to the average intake in humans per body surface area. Resveratrol forage contained a concentration of 0.04% (W/W) resveratrol [17] in QuickFat. Resveratrol was added as a supplement to the HFD as a positive control for the prevention of liver steatosis. It has been reported that supplemental treatment with resveratrol reduced hepatic steatosis in rats [18] and mice [19] fed an HFD.

Animals

Male C57BL/6J mice (7 or 10 weeks old, Hokudo, Sapporo, Japan) were housed in standard conditions (21 ± 2ºC, ventilated rooms, 12-h light/dark cycle). The animals were allowed free access to water and feed during the experimental period. Acetic acid solution for the experiments was prepared using acetic acid diluted with water to a concentration of 4%. Repeated oral administration tests were performed for 10 days as follows: 7-week-old mice were treated by oral gavage once a day; vehicle group, distilled water was given at a dose of 7.5 mL/kg bodyweight; acetic acid group, acetic acid solution neutralized with sodium bicarbonate was given at a dose of 7.5 mL/kg bodyweight; Kurozu group, a stock solution of Kurozu neutralized with sodium bicarbonate was given at a dose of 7.5 mL/kg bodyweight; CK group, CK diluted 10-fold with water was given at a dose of 7.5 mL/kg bodyweight. CE-2 was given to mice as forage. As a positive control, mice fasted for 24-h before tissue collection were used [20].

Chronic treatment for 110 weeks was performed as follows: animals were given free access to forage and water during the experimental period; the STD group was given CE-2, the HFD group was given QuickFat, the CK + HFD group was given CK-containing QuickFat, and the Resveratrol + HFD group was given 0.04% resveratrol-containing QuickFat. Mice were sacrificed by exsanguination after blood collection. The target tissues were promptly removed and stored at –30°C until western blotting analysis. Target tissues for RNA analysis were stored in RNA*later* at –30°C to stabilize and protect cellular RNA until RT-PCR analysis. Serum prepared from each mouse was stored at 4°C until analysis. Hemolysate samples were excluded from samples for analysis. The present study was approved (approved number, 10-0122) by the Hokkaido University committee for the use and care of experimental animals.

Assays, microarray, and histological analysis

Protein concentrations were determined using the DC protein assay (Bio-Rad Laboratories Inc. Hercules, CA, USA). The optical densities of bands in western blotting analyses were measured using the public domain program Image J (developed by the US National Institutes of Health and available at http://rsb.info.nih.gov/ij/download.html). The concentrations of aging-related hormones were determined in accordance with the manufacturer's instructions. Messenger RNA for microarray analysis was purified using an RNeasy Plus Mini kit, and microarray analysis was performed by TAKARA Bio Inc. (Otsu, Japan). The target genes were screened based on the results of the microarray analysis. The microarray analysis of genetic alteration in the liver was performed using Kurozu treatment for 10 consecutive days. Target tissues were fixed in 10% formalin neutral buffer solution. Paraffin-embedded sections were stained with hematoxylin-eosin staining by conventional methods.

Gel filtration chromatography

CK was separated using the size exclusion chromatography system. CK (10 mL) was injected onto a porous polyacrylamide bead column (Bio-Gel P4, φ5.0×70 cm). Operation conditions were as follows: detector, ultraviolet absorption photometer wavelength of 280 nm; mobile phases, pure water; flow rate of 3 mL/min; fraction size, 30 mL. The fractions were obtained from 100 ml of Kurozu: Fraction 1, 0.34 g; Fraction 2, 1.38 g; Fraction 3, 30 mg Fraction 4, 25 mg. Fractions 3 and 4 were insoluble in water.

Real-time quantitative RT-PCR analysis

Total RNA was isolated from target tissues using TRIzol reagent in accordance with the manufacturer's instructions. Single-stranded cDNA was synthesized using reverse-transcriptase (RT) using ReverTra Ace based on the manufacturer's instructions. Real-time PCR was performed using a LightCycler 480 II System (Version 1.5, Roche Diagnostics GmbH, Mannheim, Germany) with TaqMan gene expression assays in accordance with the manufacturer's instructions. The comparative quantification cycle threshold (Cq) method was used to determine the relative expression levels of target genes. Cq values were calculated using the 2nd derivative maximum method. Beta-2-microglobulin (*B2m*) was analyzed as a reference gene for mRNA. The cycle number difference (ΔC_q = reference genes – target genes) was calculated for each replicate. Relative target gene expression values were calculated using the mean of ΔCq from the duplicates, $\mu(\Delta Cq) = (\Sigma\Delta Cq)/2$, and expressed as $2^{\mu(\Delta Cq)}$. The primers sets for real-time PCR are indicated in Table 1.

Table 1. The sequences of primers

Gene	Sense (5' – 3')	Reverse (5' – 3')
B2m	GACCGGCCTGTATGCTATCC	TCAGTCTCAGTGGGGGTGAA
Sirt1	CCTTTCAGAACCACCAAAGCG	GGCGAGCATAGATACCGTCT
Pgc-1α	AGCGGTTCTCACAGAGACAC	GGTTTGGTGTGAGGAGGGTC
Lpin1	GGAAAAGGAGGAGGAAGGCC	TACGATGCTGACTGGGGGTA
Igfbp1	TCTGAGAGCCCAGAGATGACA	GTAGACACACCAGCAGAGTCC

Electrophoresis and western blotting

Whole cell homogenates (B2m, Pgc1-α, Sirt1: 4 μg; other proteins: 8 μg) were separated using 10% SDS polyacrylamide gels, and proteins were then transferred to a nitrocellulose membrane. The membranes were incubated with either the AC-74 (100,000-fold dilution), 101707 (500-fold dilution), 7c2 (500-fold dilution), PAB12400 (1,000-fold dilution), B01P (1,000-fold dilution), 13981-1-AP (500-fold dilution), or F4 (1,000-fold dilution) primary antibodies and with a rabbit polyclonal antibody (1,000-fold dilution) in blocking solution. The detection method was described in detail in a previous study [23].

Statistical analysis

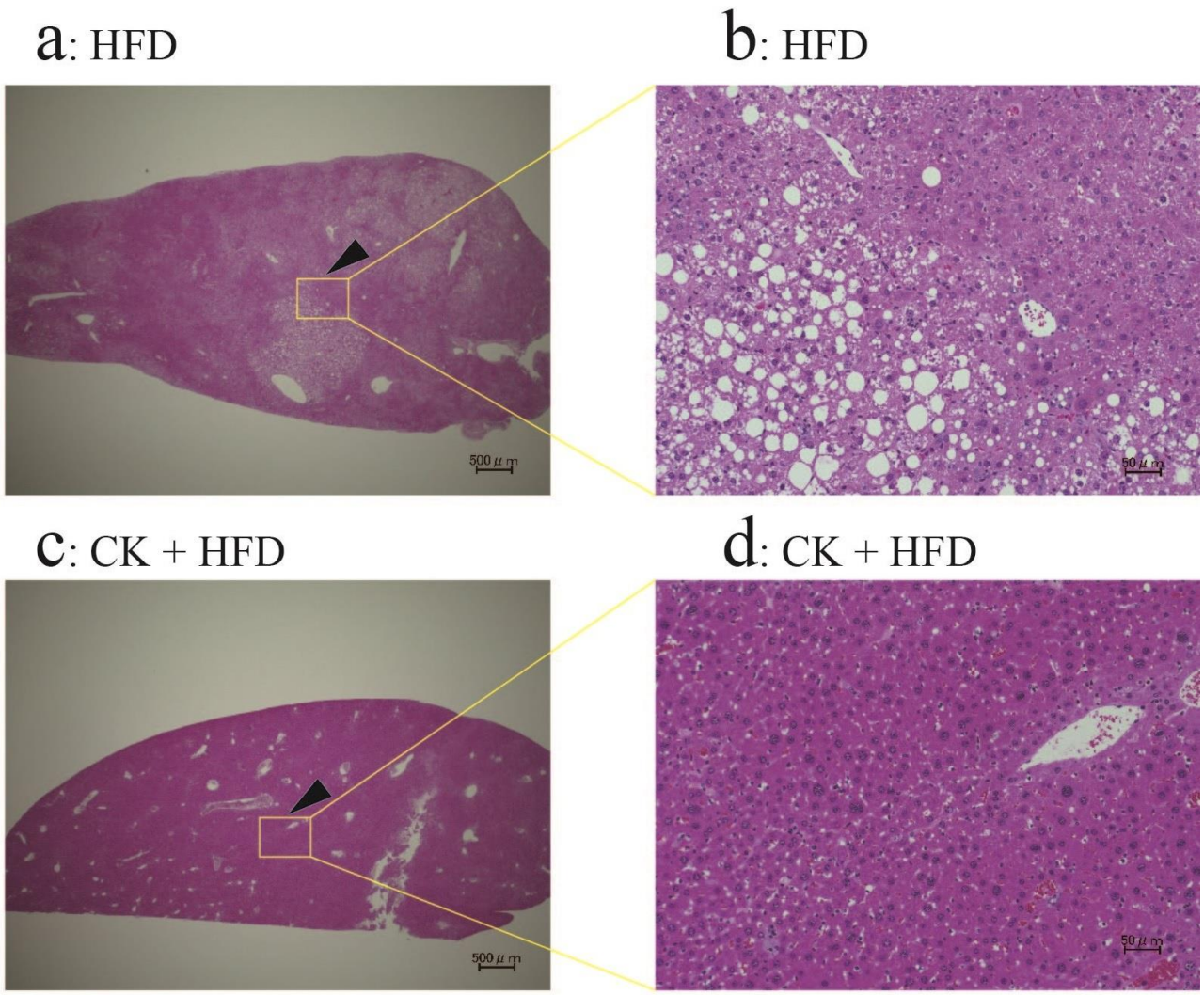
Statistical analyses were performed using the Tukey-Kramer test and Fisher's exact test. Survival was plotted using Kaplan-Meier curves, and significant differences in mortality were evaluated using the Log-rank test. Hazard ratios of overall survival were evaluated using a Cox proportional hazards model. All indicated *p*-values were two-sided.

RESULTS

Chronic treatment with CK reduced the incidence of hepatic steatosis with a high-fat diet

Ten-week-old C57BL/6J mice were fed STD, HFD, CK + HFD, or Resveratrol + HFD forage for 110 weeks. Livers and kidneys were collected at 120 postnatal weeks. The incidences of hepatic steatosis and neoplasms in the abdomen were evaluated visually when collecting the tissues. HFD treatment generated hepatic steatosis (Table 2), and this was confirmed in the liver. Histological examinations also confirmed steatosis in the liver (Figure 1a and 1b). Figure 1b is a magnified image of the region in Figure 1a. On the other hand, the incidence of hepatic steatosis was significantly lower in the CK + HFD group than in the HFD group

(Table 2). A typical histological image of a normal liver obtained from a CK-treated mouse is shown in Figure 1c and 1d. Figure 1d is a magnified image of the region in Figure 1c. The incidence of hepatic steatosis was lower in the Resveratrol + HFD group than in the HFD group. Visual and histological changes in the kidney were not observed in the Resveratrol + HFD group. Hepatic neoplasms were detected in the most mice; a typical neoplasm is shown in Figure 1f. Hepatic neoplasms with strong nuclear staining were detected in liver cells. Figure 1f and 1g shows a histological image of a tumor mass in the liver. Figure 1h and 1i show histological images of an intestinal metastatic lesion site in the same mouse as Figure 1f. No significant differences were observed in the incidence of neoplasms between the CK + HFD group and other groups (Table 2).



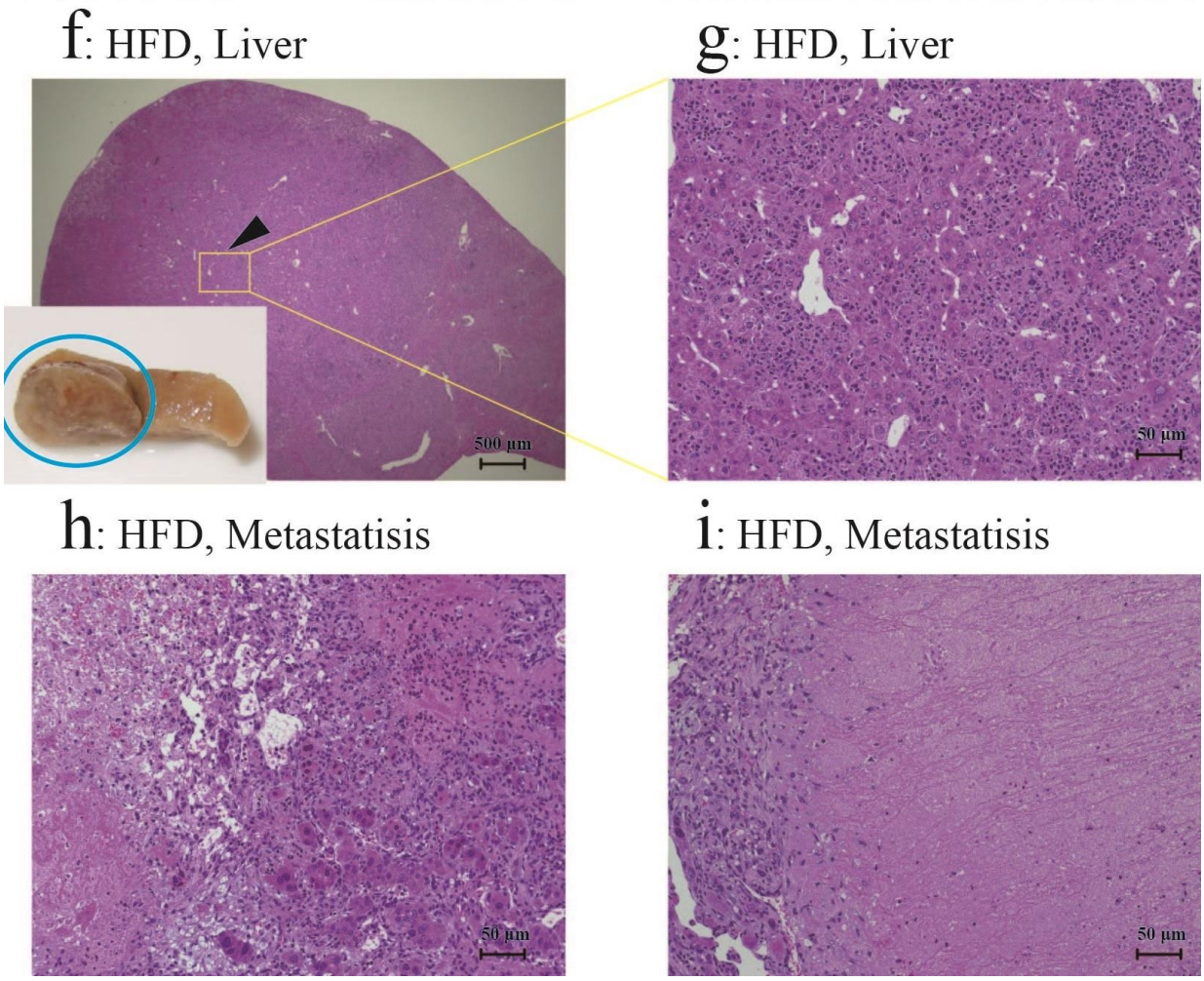


Figure 1. Microscopic images and gross morphology of the liver and metastatic region. The liver was fixed with 10% formalin neutral buffer solution. Paraffin sections were stained with hematoxylin and eosin. a: Macro image of hepatic steatosis generated in the HFD group. b: High-magnification histology of macro image "a." The arrow domain in the image was expanded. Cytoplasmic lipid droplets were observed as vacuoles. c: Macro image of a normal liver in the CK + HFD group. d: High-magnification histology of macro image "c". e: Macro images of neoplasms and hepatic steatosis in the HFD group. The image of the two upper parts shows a neoplasm, and the image of the two lower parts shows hepatic steatosis due to HFD. Hepatic steatosis was observed as cysts. f: A macro image of a tumor mass in a liver of the HFD group. g: High magnification histology of macro image "f." The arrow domain in the image was expanded. Hepatic neoplasms with strong nuclear staining were observed in the liver cells. h: The histology of the hepatic neoplasm in the intestinal metastatic region of the same mouse. Similar hepatic neoplasm cells to those in image "g" were observed. i: A macro image of a tumor mass in the liver. Hepatic carcinoma cells were observed on the left side, and hypertrophic cell changes were observed on the right side.

Table 2. Incidence rate of hepatic steatosis and neoplasms

	Number	Steatosis	Neoplasms	Metastasis
HFD	10	9	3	2
CK + HFD	5	1*	1	1
Resveratrol + HFD	6	3‡	1	0
STD	8	1†	1	1

Hepatic steatosis and neoplasms in the abdomen were identified by visual and microscopic examination. Statistical analyses were performed using Fisher's exact test. *: p=0.017; †: p=0.003; ‡: p=0.11; versus the HFD group.

Effects of chronic treatment with CK on body weight changes, survival, and DHEAS serum levels

Body weights were significantly higher in the HFD group than in the STD group. The average weekly values for each group are shown in Figure. 2a. Body weights were significantly lower in the CK + HFD and Resveratrol + HFD groups than in the control HFD group during postnatal weeks 21-33. Body weights were significantly higher in the CK + HFD group than in the HFD group during postnatal weeks 86-106.

Regarding all-cause mortality, the Kaplan-Meier survival curve is shown in Figure. 2b. No significant differences were observed in survival among the groups examined during the 110-week observation period (CK + HFD-HFD, p = 0.12; CK + HFD -Resveratrol, p = 0.60; CK + HFD-STD, p = 0.47; Log-rank test). When observed for 100 weeks, STD treatment significantly reduced survival compared with HFD treatment (Log-rank test, p = 0.014; Cox proportional hazards model, HR = 8.8, 95% CI: 1.08-71.6, p = 0.042), but no effect was observed with CK + HFD and Resveratrol + HFD treatment (CK + HFD-HFD: Log-rank test, p = 0.087, Cox proportional hazards model, HR = 5.3, 95% CI, 0.62-45.7, p = 0.127; Resveratrol + HFD-HFD: Log-rank test, p = 0.146, Cox proportional hazards model, HR = 4.4, 95% CI, 0.49-39.7, p = 0.183).

Lipid metabolism-related hormone levels in the serum were evaluated. Mice were fed for 110 weeks and sacrificed at 120 postnatal weeks. The liver, kidney, and blood were collected for analysis. DHEAS, IGF-1, and GH levels were evaluated in the serum. DHEAS levels in the serum were significantly higher in the CK group than in the HFD group (Table 3). DHEAS levels were also significantly higher in the Resveratrol + HFD group than in the HFD group. No significant differences were observed in IGF-1 and GH levels in the serum among the groups examined.

Table 3. Concentrations of aging-related hormones in serum

	HFD	CK + HFD	Resveratrol + HFD	STD
DHEAS (pg/ml)	7.3 ± 3.8	15.2 ± 3.9*	13.5 ± 5.9*	9.7 ± 5.8
IGF-1 (pg/ml)	682 ± 251	922 ± 231	862 ± 262	844 ± 214
GH (ng/ml)	30.1 ± 19.5	24.1 ± 4.2	34.0 ± 23.6	34.5 ± 21.5

Each value is the mean ± standard error. *: $p<0.05$, significantly different from the HFD group, Tukey-Kramer test. DHEAS: dehydroepiandrosterone sulfate, IGF-1: insulin-like growth factor-1, GH: growth hormone. Administered forages were as follows: HFD, QuickFat; CK + HFD, 0.6% (V/W) CK in QuickFat; Resveratrol + HFD, 0.04% (w/w) resveratrol in QuickFat; STD: CE-2.

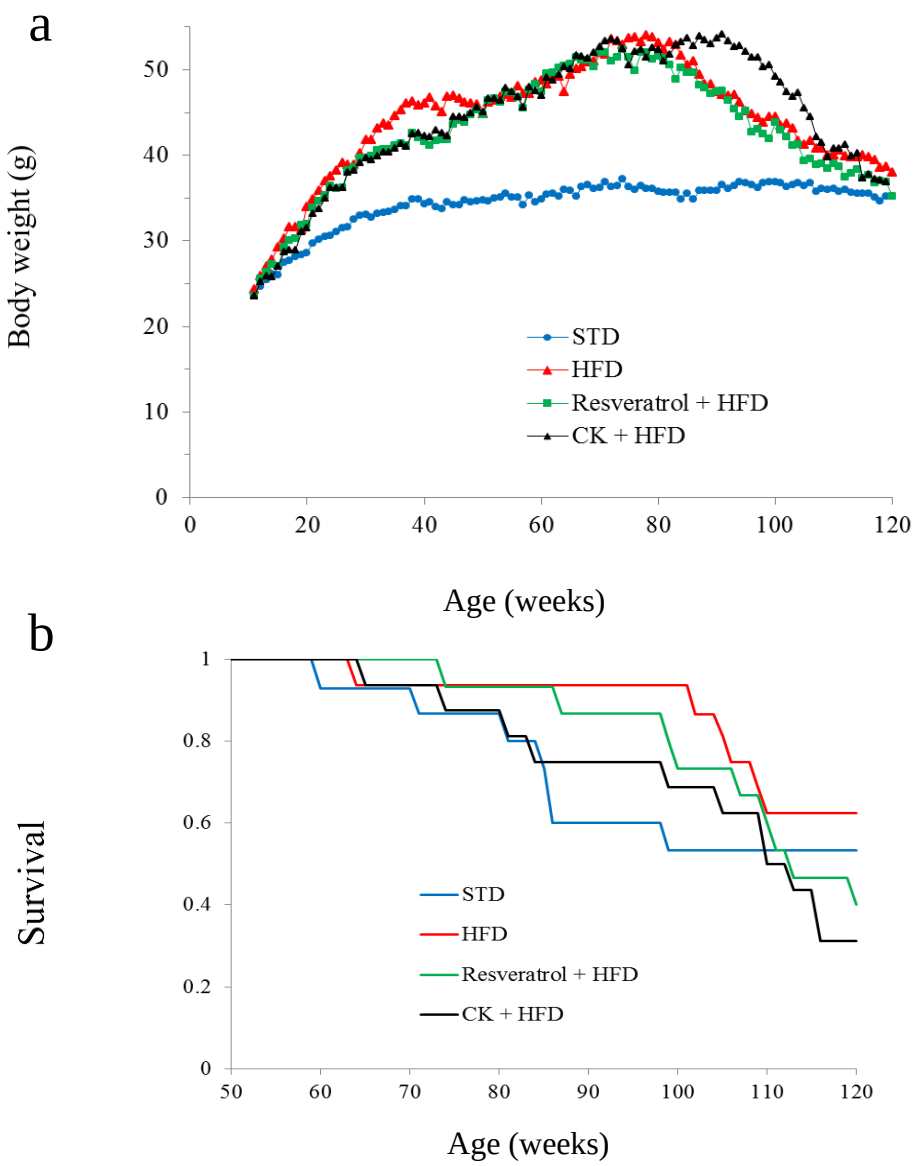


Figure 2. Survival curve and body weight changes. a, Body weight changes. The average weekly values of each group are shown. Body weights were significantly lower in the CK+ HFD and Resveratrol + HFD groups than in the control HFD group during postnatal weeks 21-33. Body weights

were significantly higher in the CK group than in the HFD group during postnatal weeks 86-106. Statistical analysis was performed using the Tukey-Kramer test. STD: standard diet; HFD: high-fat diet; Resveratrol + HFD: high-fat diet with resveratrol; CK + HFD: high-fat diet with 0.6% CK. b, Kaplan–Meier survival curves for all-cause mortality in old-onset mice. All-cause mortality was analyzed using the Log-rank test. No significant differences were observed between the CK + HFD group and other groups during the 110-week observation period. When observed for 100 weeks, STD treatment significantly reduced survival compared with HFD treatment, but no effect was observed on CK + HFD and Resveratrol + HFD treatment. Statistical values are described in the Results.

Kurozu treatment elevated the expression levels of metabolism-related genes in the liver

To estimate effects of Kurozu treatment on the expression levels of metabolism-related genes, a preliminary experiment with microarray analyses of genetic alterations in the liver following Kurozu treatment was performed. The results suggested that Kurozu treatment significantly upregulated the expression levels of *Sirt1*, *Pgc-1α*, *Lpin1*, and *Igfbp1* in the liver. Expression levels were also evaluated in detail by real-time PCR analysis. Mice were treated by oral gavage once a day for 10 days. Kurozu and CK increased the mRNA expression levels of *Sirt1*, *Pgc-1α*, *Lpin1*, and *Igfbp1* in the liver (Table 4). The treatment also increased the protein expression levels of Sirt1, Pgc-1α, Lpin1, and Igfbp1 in the liver (Table 5). The ingestion of Fractions 1 and 2 also increased the mRNA expression levels of *Sirt1* and *Pgc-1α* (Figure 3, Table 6). In the positive control for the up-regulated expression of *Sirt1* [5], fasting treatment elevated the expression levels of *Sirt1* and *Pgc-1α* in the liver (Table 4).

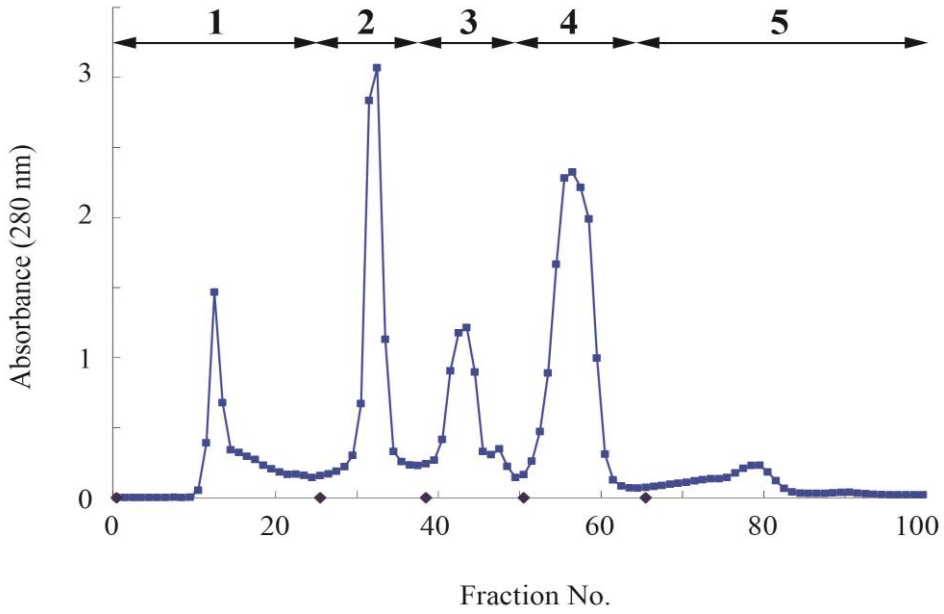


Figure 3. Concentrated Kurozu purified by gel filtration chromatography CK was separated by gel filtration chromatography. The eluate of CK was divided into five fractions. The fractions were detected at 280 nm. The collected fractions were powdered by lyophilization. The major fractions

(Fractions 1 and 2) were evaluated regarding their effect of altering mRNA expressions of *Sirt1* and *Pgc-1α*.

Table 4. Alterations in mRNA expression levels in the liver relating to lipid metabolism following ingestion of Kurozu, CK, or acetic acid in mice

	Water	Acetic acid	Kurozu	CK	Fasted
Sirt1	1.00 ± 0.08	1.43 ± 0.26	2.18 ± 0.38**	2.91 ± 0.60**	2.61 ± 0.89
Pgc-1α	1.00 ± 0.13	1.32 ± 0.16	2.96 ± 0.66***	1.98 ± 0.34*	4.56 ± 1.70
Lpin1	1.00 ± 0.20	1.54 ± 0.37	8.02 ± 0.76***	7.01 ± 0.96***	ND
Igfbp1	1.00 ± 0.10	1.47 ± 0.34	2.76 ± 0.39**	1.94 ± 0.21*	ND

C57BL/6J mice received CK, Kurozu, 4% acetic acid, or water for 10 consecutive days. The mice were fed Kurozu solution and 4% acetic acid neutralized with sodium bicarbonate. Mice were treated by oral gavage once a day at a dose of 7.5 mL/kg bodyweight. The fasted mice underwent 24-h fasting. Relative expression levels of mRNAs compared with the water-treated group. The values were calculated from the ratio of target gene / *B2m* expression levels. The experimental method is described in the Methods in detail. Each value is the mean ± standard error. Statistical analyses were performed using the Tukey-Kramer test. Significantly different from the water-treated group: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. ND: no data.

Table 5. Alterations in protein expression in the liver relating to lipid metabolism following ingestion of Kurozu, CK, or acetic acid in mice

	Water	Acetic acid	Kurozu	CK
Sirt1	1.00 ± 0.07	1.02 ± 0.15	1.55 ± 0.17*	1.97 ± 0.18**
Pgc-1α	1.00 ± 0.31	1.39 ± 0.39	2.95 ± 0.41*	2.18 ± 0.21*
Lpin1	1.00 ± 0.21	1.29 ± 0.23	2.99 ± 0.87*	2.74 ± 0.36**
Igfbp1	1.00 ± 0.12	1.51 ± 0.16	2.00 ± 0.30*	1.87 ± 0.21*

Relative expression levels of proteins compared with the water-treated group. The values were calculated from the ratio of target gene / *B2m* expression levels. The experimental method is described in the Method in detail. Each value is the mean ± standard error. Statistical analyses were performed using the Tukey-Kramer test. Significantly different from the water-treated group: *: $p<0.05$, **: $p<0.01$.

Table 6. Alterations in mRNA expression levels in the liver following ingestion of the purified fraction of CK for 10 consecutive days in mice

	Water	Fraction 1	Fraction 2
Sirt1	1.00 ± 0.08	4.03 ± 0.45***	4.82 ± 0.51***
Pgc-1α	1.00 ± 0.13	1.43 ± 0.11*	2.69 ± 0.37***

C57BL/6J mice were fed the purified fractions of CK or water. Mice received a dose of 7.5 mL/kg (water), 1.7 g/kg (Fractions 1 and 2, diluted with water at a concentration of 100 mg/mL, which corresponded to the CK-treated dose). The fasted group underwent 24-h fasting. Relative expression levels of mRNAs compared with the water-treated group. The values were calculated from the ratio of target gene / *B2m* expression levels. Each value indicated the mean ± standard error. Statistical analyses were performed using the Tukey-Kramer test. Significantly different from the water-treated group: *: $p<0.05$, ***: $p<0.001$.

DISCUSSION

The effect of CK supplementation on the onset of hepatic steatosis on administering an HFD for 110 weeks was evaluated in the present study. The lifelong ingestion of CK significantly reduced the incidence of hepatic steatosis in mice. Kurozu was previously shown to contain organic and amino acids. Shimoji Y. et al. reported that Kurozu contained polyphenolic compounds, for example, dihydroferulic and dihydrosinapic acids, which exhibit anti-oxidative activities [22]. Polyphenolic plant metabolites, such as butein, quercetin, and resveratrol, exhibit anti-oxidative activities. These polyphenolic compounds were identified as small-molecule activators of SIRT1 [23]. The positive control for the prevention of liver steatosis, 0.04% resveratrol-containing forage, was fed to mice [24]. The addition of resveratrol reduced the onset of hepatic steatosis with an HFD (Table 2). Anti-oxidative actions of Kurozu-containing organic acids might reduce the onset of hepatic steatosis on administering an HFD; however, the effects of organic acid treatments on the prevention effect of hepatic steatosis.

Baur JA, et al. reported that resveratrol supplement improved the survival of mice on an HFD [17]. However, significant difference was not observed in survival between the HFD and STD groups during the 110-week observation period (Figure 2b). When observed for 100 weeks, STD ingestion significantly decreased survival compared with HFD ingestion (Log-rank test, $p = 0.014$; Cox proportional hazards model, HR = 8.8, 95% CI: 1.08-71.6, $p = 0.042$), but no effect was observed with CK and resveratrol supplementary treatment (Figure

2). The STD forage, CE-2 contains 4.8% fat from soybean and germ. The HFD forage, QuickFat, had 13.9% fat from cattle suet and germ. Baur JA, et al. fed animals high-fat AIN-93G, which was modified by the addition of hydrogenated coconut oil to provide 60% of the total calories from fat [17]. Mattison JA, et al. reported that calorie restriction did not affect survival in rhesus macaques; nutritional factors play an important role in mortality [25]. Mortality differences were not observed between the HFD and Resveratrol + HFD groups, indicating that nutritional factors may influence the effects of prolonging mortality with resveratrol supplementation.

Baur JA, et al. also demonstrated that resveratrol supplementation suppressed body weight increases [17]. Resveratrol supplementation also significantly suppressed body weight increases during postnatal weeks 21-33 in the present study. CK supplementation also had suppressive effects. Hamadate N, et al. reported that powdery CK treatment for 12 weeks decreased body weight in humans [26].

In old age, the body weights were significantly higher in the CK+ HFD group than in the HFD group during postnatal weeks 86-106. Tong LT, et al. reported that CK treatment decreased adipocyte sizes, and the number of cells in subcutaneous adipose tissues of the CK-treated group was significantly increased compared with the control group [27]. It was estimated that increased adipocyte cells inhibited weight loss in old age. The results of the present study suggested that CK may prevent weight gain in middle age, thereby inhibiting weight loss in old age.

A previous study reported that obesity induced hepatocellular carcinoma [28]. Kurozu has been shown to exhibit antitumor effects. Shimoji Y, et al. reported that the administration of Kurozu significantly inhibited the incidence and multiplicity of colon adenocarcinoma in rats [16]. Kurozu also inhibited the proliferation of all tested cell lines *in vitro* [29]. Neoplasms in the liver were observed in mice in this study; however, there were no significant differences in the onset of hepatic neoplasms in the four groups. Further investigations are needed to confirm the effect of carcinogenicity.

Body weights were significantly higher in the CK+ HFD group than in the HFD group during postnatal weeks 86-106. Auyeung TW, et al. reported that older men were resistant to the risks associated with being overweight and adiposity; being mildly overweight, obese,

and even having central obesity may be protective [30]. Previous studies suggested that reductions in body weight with old age may reduce survival rates. CK supplementation significantly inhibited body weight loss in old age. CK and resveratrol supplementation significantly inhibited decreases in DHEAS serum levels (Table 3). These results suggested that CK and resveratrol supplementation delayed senescence.

Native Kurozu and CK upregulated the expression levels of *Sirt1*, *Pgc-1α*, *Lpin1*, and *Igfbp1* in the liver. Fractions 1 and 2 of CK also upregulated the expression levels of the genes. The results suggested that Kurozu contains constituents that upregulate gene expression. Li Y, et al. reported that the hepatic overexpression of Sirt1 ameliorated hepatic steatosis and glucose intolerance in obese mice [31], whereas defects in hepatic Sirt1 exacerbated hepatic steatosis and ultimately increased weight gain [32]. Jeon BT, et al. demonstrated that the myeloid-specific deletion of SIRT1 also exacerbated hepatic steatosis [33], and Kurozu treatment upregulated the expression levels of Sirt1 in the liver, which may, in turn, have exacerbated hepatic steatosis. Finck BN, et al. identified Lpin1 as a selective physiological amplifier of the PGC-1α/PPARα-mediated control of hepatic lipid metabolism [34]. Fatty acid oxidative metabolism by inducing Lpin1 expression may have exacerbated hepatic steatosis in this study. The upregulated expression of *Sirt1*, *Pgc-1α*, *Lpin1*, and *Igfbp1* was observed in mice (Table 4, 5).

Ceddia et al. reported that acetic acid upregulated uncoupling protein-2 (UCP2) mRNA level [35]. Elevated UCP2 in turn reduces oxidative stress, which provides a growth advantage for cancers [36]. Previous studies reported that Kurozu inhibited tumor growth [14-16]. Previous reports suggest that acetic acid as a constituent of Kurozu, in itself, inhibited tumor growth, but acetic acid free CK reduced the onset of hepatic carcinoma in this study. Further investigation is needed to confirm the effect of Kurozu and CK on tumorigenicity.

Sirt1 is one of the key regulators of lipid metabolism and carcinogenesis, upregulation effects of *Lpin1* and *Igfbp1* resulted from increased expression of *Sirt1* by Kurozu treatment. Taken together, our data demonstrate that upregulated expression of *Sirt1*, *Pgc-1α*, *Lpin1*, and *Igfbp1* may reduce the onset of hepatic steatosis. To illustrate the effects of Kurozu on hepatic steatosis and carcinogenesis, a possible signaling pathway is indicated in Figure 4.

CONCLUSION

These results suggest that Kurozu feeding increases the expression levels of Sirt1, Pgc-1α, Lpin1, and Igfbp1 in the liver. Lifelong ingestion with CK supplementation may delay the onset of hepatic steatosis caused by HFD feeding. CK supplementation may prevent elevations in weight in middle age, as well as the loss of weight in old age.

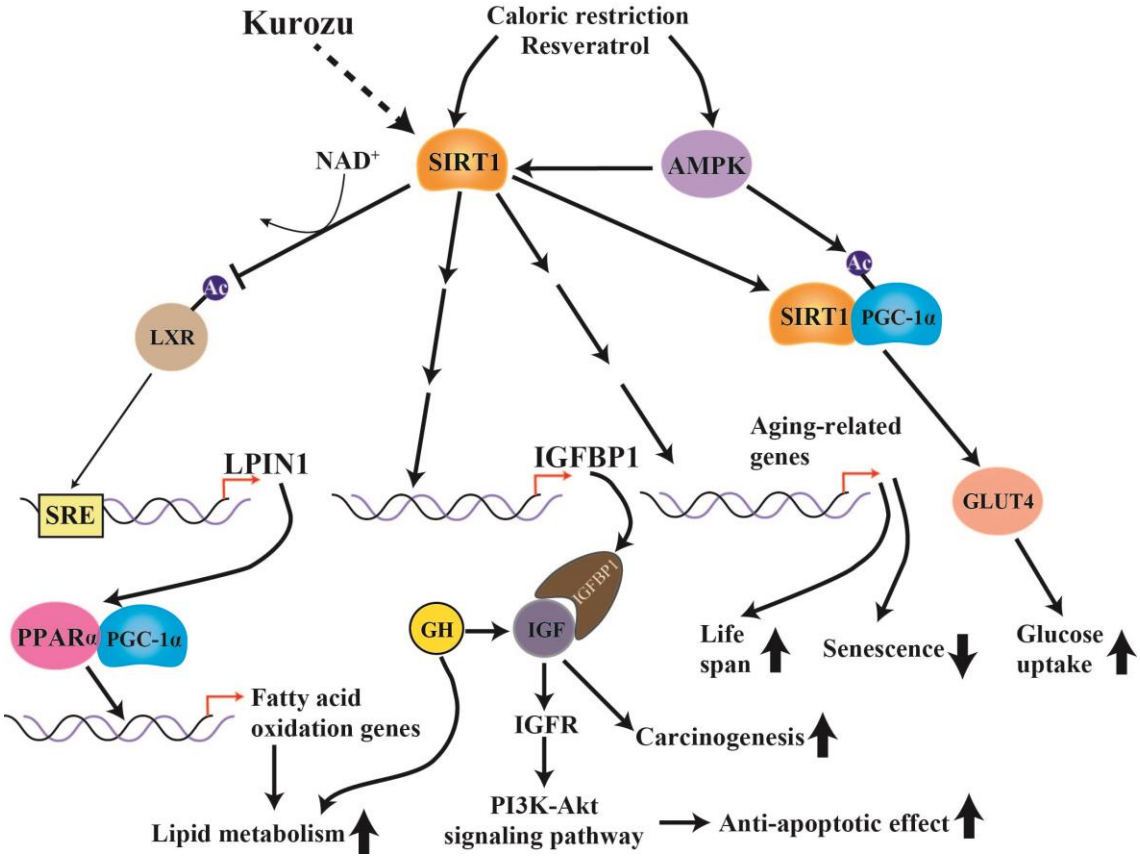


Figure 4. The SIRT1 signaling pathway regulates metabolism, cancer and aging-related genes. Both SIRT1 and AMPK are activated by calorie restriction or supplementation of resveratrol. SIRT1 deacetylates LXR, which leads to ubiquitination and degradation of LXR. This mechanism results in enhancement of its transcriptional activity and target genes. SIRT1 controls the expression of IGFBP1. IGFBP1 regulates the half-life of IGF, which gives rise to carcinogenesis and anti-apoptotic effects. GH is implicated in lipid metabolism. SIRT1 has a role in regulation of the transcription leading to metabolic and aging-related genes. AMPK: AMP-activated protein kinase, GH: growth hormone, GULT4: glucose transporter type 4, IGFR: insulin-like growth factor receptor, LXR: liver X receptor SRE: sterol-response element.

List of Abbreviations: CK, concentrated Kurozu; DHEAS, dehydroepiandrosterone sulfate; GH, growth hormone; HFD, high-fat diet; HR, hazard ratio; IGF, insulin-like growth factor;

Igfbp1, IGF-binding protein1, STD, standard diet.

Competing Interests: MN, KH, NH, and AF are employees of Sakamoto Kurozu, Inc.

Authors' Contributions: YS and KI conducted the study. YS performed animal experiments and analyses. MN, KH, NH, and SF generated Kurozu and CK. YS prepared the manuscript and all authors contributed to proofreading and revisions.

Acknowledgements and Funding: This work was supported by grants from Sakamoto Kurozu, Inc.

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