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Long-term administration of Kansui, a food additive that is commonly used in Chinese noodles, modulates gene expression in kidneys and spleens of mice.

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Abstract

The aim of this study was to investigate the effects of long-term administration of Kansui, a food additive that is commonly used as colorant and thickener in Chinese noodles, on gene expression patterns in mouse tissues. Mice were fed on a standard diet containing Kansui (0.33% w/w) for 32 weeks. Kansui administration caused decreases in food consumption and body weight gain. Kansui treatment modestly but significantly increased renal expression of proto-oncogenes (Fos, Jun, Junb, Myc and Fosb), stress response genes (Hspa1a and Mt2) and genes related to renal function (Adipoq, Fabp1, Vnn1, Retn, Il11, Tnfrsf12a, B2m, Il1rn and Plau). Kansui administration also resulted in the increased splenic expression of proto-oncogenes (Fos, Junb, Jun and Fosb) and inflammatory genes (Il6, Il1 β, Il10, Mcp-1 and KC). Increased gene expression of Mt1, Mt2, Fos, Junb and Fosb in the liver and elevated gene expression of Junb in the muscle were also noted. These results suggest that dietary Kansui intake can cause adverse health effects by modulating gene expression.

Key Words：Chinese noodle, food additive, gene expression, Kansui, kidney, spleen

Introduction

Chinese noodles, including instant ramen, are very popular in East Asian countries, because they are convenient with low-price [1),2)]. In 2018, 57.8 billion of instant ramen were consumed in Japan [3)]. In addition, the Japanese often eat Chinese noodles at the restaurants. Overeating of Chinese noodles causes excessive intakes of salt and fat. Excessive dietary salt intake is associated with an increased risk for hypertension, and high consumption of fat rich in saturated and monounsaturated fatty acids leads to obesity, a risk factor for cardiovascular disease. Habitual consumption of Chinese noodles also results in a high intake of Kansui, a food additive that is commonly added to these noodles. In Japan, more than 1,500 food additives have been allowed to be used as food colorant, flavor, thickener, sweetener, preservative and antioxidant [4)]. Kansui is usually

used as food colorant and thickener. Safety evaluations of these food additives are performed by Food Sanitation Act in Japan. However, it remains unclear whether Kansui have undesirable effects on health. The objective of this study was therefore to investigate the effects of long-term administration of Kansui on gene expression patterns in mouse tissues such as kidney, spleen, liver and muscle.

Materials and methods

Diets

Casein, α-corn starch, sucrose, cellulose powder, AIN-76 mineral mixture [5], AIN-76 vitamin mixture [5] and choline bitartrate were purchased from Oriental Yeast (Tokyo, Japan). DL-methionine and soybean oil were obtained from Wako Pure Chemical Industries (Osaka, Japan). Kansui was purchased from Happou (Chiba, Japan). Kansui consisted of 30% K_2CO_3, 57% Na_2CO_3, 7% Na_2HPO_4, 4% $Na_4P_2O_7$, and 2% $Na_nH_2PnO_{3n+1}$ (w/w). Purified powder standard diet was prepared in our laboratory using food-grade ingredients. This standard diet consisted of 60.075% α-corn starch, 10% sucrose, 15% casein, 0.225% DL-methionine, 5% soybean oil, 1% AIN-76 vitamin mixture, 3.5% AIN-76 mineral mixture, 0.2% choline bitartate, and 5% cellulose (w/w). A diet supplemented with Kansui were prepared by mixing 50g of the powder standard diet and 100g boiling water containing 0.5g Kansui. Diets without Kansui (control diet) were prepared by mixing 50g of the powder standard diet and 100g boiling water. The test diet with and without Kansui were kept at 4℃ until required.

Animals and experimental design

Specific-pathogen-free, 5-week-old female BALB/c mice were obtained from Charles River Japan (Atsugi, Japan). The animals were maintained on a commercial laboratory chow (Oriental Yeast) and were given water ad libitum. The non-purified diet comprised approximately 23.6% protein, 5.3% fat, 6.1% ash, 2.9% fiber and 54.4% nitrogen-free extracts. After an acclimatization period (5 days), the mice were separated into two different groups (n=16 per group) that were fed the test diets containing 1) the powder standard diet or 2) the powder standard diet and Kansui. Before starting the treatment with the test diets, body weights were comparable among the two groups. In both groups, a 12-h/12-h light/dark cycle was maintained and the room temperature was kept at 23 ± 1℃. Food intake and body weight were monitored every other day and weekly, respectively. At the end of the feeding experiment of the two dietary groups for 32 weeks, the animals were sacrificed by decapitation between 8:30 and 10:30 a.m. Blood was collected and allowed to clot for 1 hour at room temperature. Serum was then separated by centrifugation at 1200g for 20 min at 4℃ and stored at -80℃ until analysis. Tissue samples were weighed and stored at -80℃ or in RNAlater (Thermo Fisher Scientific, Tokyo, Japan) at -20℃. The experimental procedures used in the present study confirmed to the guidelines of the Animal Usage Committee of Chiba University and Sagami Women's University.

Real-time quantitative reverse transcriptase polymerase chain reaction

Total RNA was isolated from mouse kidneys, spleens, livers and muscles with an Isogen according to the manufacturer's instructions (Nippon gene co., Tokyo, Japan). The quantity and the quality of RNA samples were measured with a Nano Drop 1000 (Thermo Fisher Scientific), and 4 ug of RNA was taken for the synthesis of complementary DNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). The reaction product was amplified using the KAPA SYBR FAST qPCR Kit (Nippon Genetics Co, Ltd, Tokyo, Japan) by real-time quantitative polymerase chain reaction (PCR: Thermal Cycler Dice Real Time System TP870, Takara Bio Inc.,

Tokyo, Japan). The gene-specific primer sequences are listed in Table 1. The relative expression levels of the target gene products were calculated with the comparative threshold cycle method

Table 1. Primer sequences (5'→3')

Gene	Acc. number	Forward	Reverse
Adipoq	U49915	CTCTAAAGATTGTCAGTGGATCTG	AGTAACGTCATCTTCGGCAT
B2m	BC085164	TGGTGCTTGTCTCACTGACC	TTCAGTATGTTCGGCTTCCC
Cd14	BC057889	GCTTGTTGCTGTTGCTTCTG	CGTGTCCACACGCTTTAGAA
Cox2	AF378830	CAAGACAGATCATAAGCGAGGA	GGCGCAGTTTATGTTGTCTGT
Emr1	U66888	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Fabp1	BC009812	TGCAGAGCCAGGAGAACTTT	GATTTCTGAACACCCCTTGA
Fos	BC029814	CCTGTCCGGTTCCTTCTATG	AAGTAGTGCAGCCCGGAGTA
Fosb	BC132064	GAGTAACATTAAATACCCTGAAGGA	ACTGCATGATTTCCAAGTTGC
Hprt1	BC004686	GGAGCGGTAGCACCTCCT	CATAACCTGGTTCATCATCGC
Hspa1a	NM_010479	CGAGGCTGACAAGAAGAAGG	CTGGTACAGCCCACTGATGA
Il1b	NM_008361	TCGCAGCAGCACATCAACAA	GGTGCTCATGTCCTCATCCT
Il1rn	BC042532	GTGTGTTCTTGGGCATCCAC	CAGGACGGTCAGCCTCTAGT
Il6	BC138766	TGATGCACTTGCAGAAAACA	ACCAGAGGAAATTTTCAATAGGC
Il10	M84340	ATCGATTTCTCCCCTGTGAA	TGTCAAATTCATTCATGGCCT
Il11	BC134354	TGCTGACAAGGCTTCGAGTA	GAGCTGTAAACGGCGGAGTA
Jun	BC021888	ATGGGCACATCACCACTACA	GACACTGGGAAGCGTGTTCT
Junb	BC092302	CCCGTCTACACCAACCTCAG	GCATGTGGGAGGTAGCTGAT
KC	BC132502	GCCTATCGCCAATGAGCTG	GAACCAAGGGAGCTTCAGG
Mcp-1	BC145869	GGTCCCTGTCATGCTTCTGG	CCTTCTTGGGGTCAGCACAG
Mpo	BC053912	CCGCCTGAACAATCAGTACC	ATTCAGTTTGGCTGGAGTGG
Msr1	BC003814	TCAAACTCAAAAGCCGACCT	ACGTGCGCTTGTTCTTCTTT
Mt1	J00605	CCAACTGCTCCTGCTCCAC	ACAGCCCTGGGCACATTT
Mt2	BC031758	CTCCTGTGCCTCCGATGG	CGGAAGCCTCTTTGCAGAT
Myc	BC138931	CTGTGGAGAAGAGGCAAACC	GTTGTGCTGGTGAGTGGAGA
OPN	AF515708	GGACCTCACCTCTCACATGAA	CCGACTGATCGGCACTCT
Plau	BC120713	GAGGAAAGGCCAACACTGATA	CCAATCTGCACATAGCACCA
Rantes	BC033508	GTGCCCACGTCAAGGAGTAT	CCACTTCTTCTCTGGGTTGG
Retn	AF480491	TGAAGCCATCGACAAGAAGA	CTTCCCTCTGGAGGAGACTG
S100a8	BC078629	AGAAGGCCTTGAGCAACCTC	CCTTGTGGCTGTCTTTGTGA
S100a9	BC027635	GGAGGACCTGGACACAAACC	ACTTCCCACAGCCTTTGC
Tlr4	NM_021297	GGTTGAGAAGTCCCTGCTGA	CCAAGTTGCCGTTTCTTGTT
Tnfrsf12a	BC025860	CAGATCCTCGTGTTGGGATT	CAGTCCATGCACTTGTCGAG
Vnn1	BC019203	TTATGCCTTTGGAGCCTTTG	ACCACAGGTGCGTAAATTGG

using Hprt1 as the normalization control.

Biochemical analyses of serum parameters

The serum total protein, albumin, non-esterified fatty acid, glucose, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using commercially available kits as follows: A/G B-test, NEFA C-test, glucose CII-test and transaminase CII-test (Wako Pure Chemical Ind.). All assays were performed in duplicate and the data averages were statistically analyzed.

Statistical analysis

All values are expressed as means ± S.D. Statistical analysis was performed using SPSS version 25. Student t test was used for statistical analysis, with $P<0.05$ considered to indicate a significant difference.

Results

Food intake and result on body, liver, kidney and spleen weights

During a 32-week experimental period, Kansui-treated mice showed a decreased food intake at weeks 5, 7, 22, 24, 29 and 32 in comparison with control mice (Fig.1A). The body weight gain was significantly lower in Kansui-treated mice than in control mice at weeks 7-13, 15, 18 and 25-32 (Fig.1B). There was no significant difference in the weights of liver (mean ± SD 1.25 ± 0.12 g vs 1.21 ± 0.14 g; p=0.382), kidney (308 ± 26 mg vs 291 ± 21 mg: p=0.061) and spleen (129 ± 17 mg vs 132 ± 26 mg; p=0.736) between control and Kansui-treated mice.

Serum albumin, non-esterified fatty acid, glucose, total protein, ALT and AST levels

Kansui-treated mice showed slightly increased levels of serum albumin (3.06 ± 0.11 g/dL serum vs 3.15 ± 0.08 g/dL serum; p = 0.023) and non-esterified fatty acid (0.61 ± 0.12 mEq/L serum vs 0.70 ± 0.11 mEq/L serum; p = 0.049) compared with control mice. There was no significant difference in levels of serum glucose (169 ± 19 mg/dL serum vs 175 ± 15 mg/dL serum; p = 0.314), total protein (5.15 ± 0.19 g/dL serum vs 5.12 ± 0.23 g/dL serum; p = 0.684), ALT (4.91 ± 1.48 IU/L serum vs 4.58 ± 1.55 IU/L serum; p = 0.559) and AST (55.89 ± 18.20 IU/L serum vs 49.42 ± 10.20 IU/L serum; p = 0.228) between control and Kansui-treated mice.

Expression of specific genes in the kidney

The renal mRNA levels for oncogenic genes

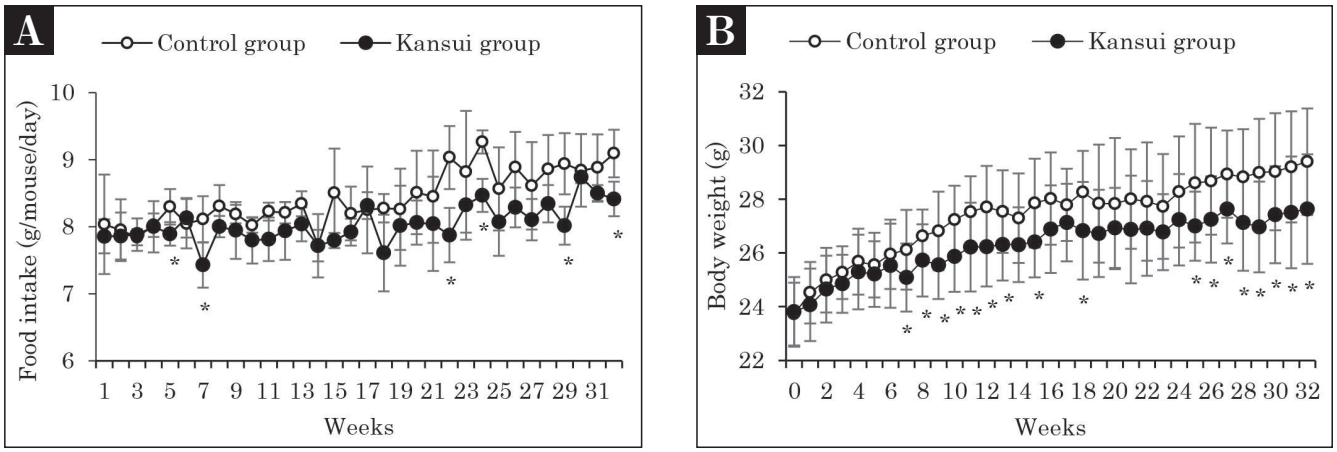

Fig. 1. Food intake (A) and body weight (B) in Kansui-treated mice. Results are expressed as mean ± S.D. (n=16 per test group). Values highlighted with asterisks are significantly different from control groups for the same day ($P<0.05$).

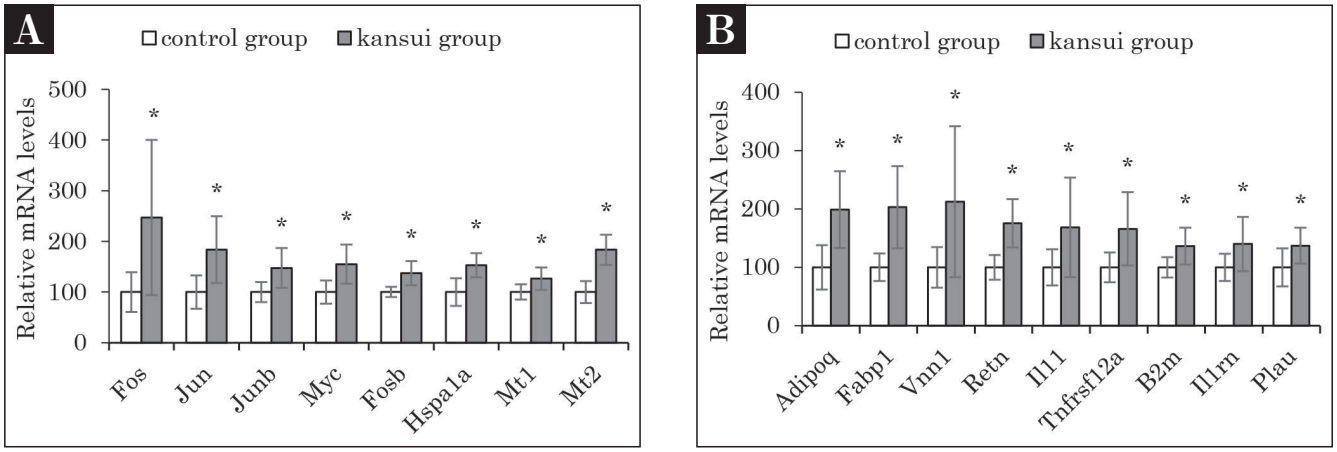

Fig. 2. Expression of proto-oncogenes (A), stress response genes (A) and genes related to renal function (B) in kidneys of Kansui-treated mice. The transcript levels for each gene are expressed as relative mRNA levels normalized to Hprt1. The values represent the mean ± S.D. (n=16 per test group). Values highlighted with asterisks are significantly different from control mice ($P<0.05$).

(Fos, Jun、Junb、Myc、Fosb) and stress response gene (Hspa1a) were higher in Kansui-treated mice compared with control mice (Fig.2A). Metallothioneins (MTs), a group of intracellular metal-binding proteins with high cysteine contents, are able to effectively protect cells or tissues from oxidative damage [6),7)]. MT1 and MT2 are the major isoforms found in mouse tissues [6),8)]. Kansui treatment resulted in the increased renal expression of Mt2, a gene encoding MT2. Adiponectin is a multifunctional cytokine that has a role in regulating inflammation [9)]. Fatty acid-binding protein 1 (FABP1) is expressed in various tissues, including the kidney, and is involved in the regulation of fatty acids uptake and the intracellular transport [10),11)]. FABP1 has also been demonstrated to protect cells from oxidative stress [12)]. Vanin 1 is an ectoenzyme with pantetheinase activity [13)]. The mRNA levels of Adipoq, Fabp1 and Vnn1 (the genes encoding adiponectin, Fabp1 and vanin1, respectively), in the kidneys of the Kansui-treated mice were 2-fold higher than those of control mice (Fig.2B). Kansui administration also caused the increased renal expression of genes related to renal function (Retn、Il11、Tnfrsf12a、B2m、Il1rn and Plau).

Expression of specific genes in the spleen

Kansui-treated mice showed the increased splenic expression of oncogenic genes (Fos, Jun, Junb and Fosb) when compared with control mice (Fig.3A). The mRNA levels of Mt2 in the spleens of the Kansui-given mice were 1.5 fold higher than those of control mice. Kansui treatment had no significant effects on the Hspa1a mRNA in the spleen. IL11, a member of the IL6 family of proinflammatory cytokines, has been produced by cells in an oxidative stress-dependent manner [14)]. Kansui treatment resulted in the elevated splenic expression of pro- and anti-inflammatory cytokines, including Il6, Il10, Il1β and Il11 (Fig.3B). In addition, Kansui also caused the increased splenic expression of migration factors for macrophages and neutrophils (Mcp-1, Rantes and KC), macrophage markers (Emr1 and CD14), neutrophil marker (Mpo) and other inflammatory genes (Cox2, Tlr4, Opn, Msr1, S100a8 and S100a9) (Fig.3C).

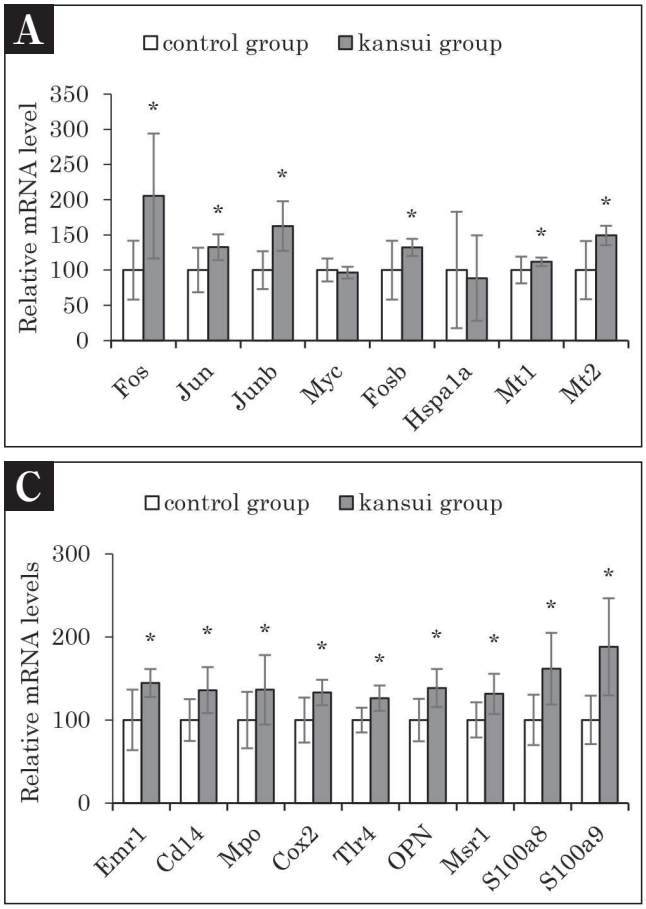

Fig. 3. Expression of proto-oncogenes (A), stress response genes (A) and inflammatory genes (B and C) in spleens of Kansui-treated mice. The transcript levels for each gene are expressed as relative mRNA levels normalized to Hprt1. Results are expressed as mean ± S.D. (n=16 per test group). Values highlighted with asterisks are significantly different from control mice ($P<0.05$).

Expression of specific genes in the liver

The mRNA levels of Mt1 and Mt2 in the livers of Kansui-treated mice were 2.9- and 4.4-fold higher than those of control mice, respectively (Fig.4). Kansui administration also resulted in the increased hepatic expression of oncogenic genes (Fos, Junb and Fosb), but had no significant effects on the hepatic mRNA levels for Jun, Myc and Hspa1a. IL-1 receptor antagonist (IL-1RA) is an agent that binds non-productively to the cell surface interleukin-1 receptor (IL-1R), the same receptor that binds IL-1, preventing IL-1 from sending a signal to that cell [15]. The mRNA levels of Il1rn, encoding IL-1RA, in the livers of Kansui-treated mice were 1.6-fold higher than those of control mice.

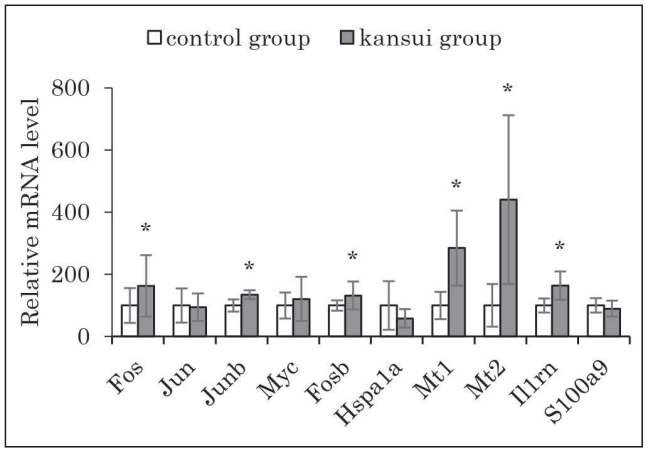

Fig. 4. Expression of proto-oncogenes and stress response genes in livers of Kansui-treated mice. The transcript levels for each gene are expressed as relative mRNA levels normalized to Hprt1. Results are expressed as mean ± S.D. (n=16 per test group). Values highlighted with asterisks are significantly different from control mice ($P<0.05$).

Expression of specific genes in the muscle

The mRNA levels of Junb in the muscles of Kansui-treated mice was 1.5-fold higher than those in control mice. However, Kansui administration had no significant effects on the mRNA of other oncogenic genes (Fos, Jun, Myc, Fosb) and stress response genes (Hspa1a, Mt1, Mt2) in the muscle (data not shown).

Discussion

We observed in our present study in mice that long-term administration of Kansui caused modulation of expression of genes associated with oncogenesis, stress response and inflammation in several tissues, particularly in the kidney and spleen. Kansui powder is generally added to wheat flower at a rate of 1-1.5 % for making Chinese noodles. Therefore, in our present study, Kansui was added to the powder standard diet at a rate of 1%. Kansui-treated mice showed reduced food intake and body weight gain when compared with control mice (Fig.1A and B). Kansui-supplemented diet used in this study was not alkaline, and it was neural. Thus, it is necessary to clarify the cause of decreased apatite by Kansui administration.

It has been demonstrated that adiponectin prevents glomerular and tubulointerstitial injury through modulating inflammation and oxidative stress [16]. Plasma adiponectin levels are dependent on kidney function, and being markedly increased among patients with kidney impairment [17]. Several studies have shown that increased urinary FABP1 is associated with the severity and clinical prognosis of diabetic kidney disease [18]. The Vnn1 gene was one of nine genes found to be upregulated in the early phase of acute kidney injury [19]. For example, increased urinary vanin 1 levels were observed in rats intraperitoneally injected with cisplatin or gentamicin sulfate to induce nephrotoxicity [20]. Taken together, these observations suggest a potential role for adiponection, FABP1 and vanin1 as biomarkers of renal injury. We found that Kansui administration resulted in the increased renal expression of Adipoq, Fabp1 and Vnn1 (Fig.2B). The contribution of these increases to renal protection in the Kansui-given mice should be further clarified. We observed the increased expression of markers of macrophage, neutrophil and their migration factors in the spleens of Kansui-supplemented mice (Fig.3B and C), indicating that long-term supplementation with Kansui leads to enhanced infiltration of macrophages and neutrophils into the spleen. This may be, at least in part, responsible for the elevated splenic expression of inflammation-related genes such as Il6, Il10, Il1β and Il11 in the Kansui-treated mice. Persistent inflammation and immune activation plays an important role in the organ dysfunction and the onset and progression of diabetes and arteriosclerosis[21,22]. The long-term administration of Kansui may contribute to the development of these diseases.

Kansui-induced modulation of gene expression was smaller in the liver than in the kidney and spleen, and Kansui administration had no significant effects on serum ALT and AST levels. Metallothionein is inducible to high levels by various oxidative or pathogenic stresses [8,23]. Under acute and chronic oxidative stress conditions such as treatment with doxorubicin, ischemia-reperfusion and dietary copper restriction, MT-overexpressing transgenic mouse hearts displayed a marked resistance to the injurious consequences, including biochemical, pathological and functional alterations [6]. In our present study, Kansui administration resulted in the increased expression of Mt1 and Mt2 in the tissues, but these increase were greater in the liver compared to the kidney and spleen. Thus, we speculate that the increased hepatic expression of Mt1 mRNA may at least in part contribute to reduced hepatic disorders caused by Kansui administration.

The results of the present study suggest that we need to pay attention to adverse health effects of long-term intake of Kansui. To clarify the health effects of Chinese noodles, the combined

effect of Kansui, salt and oil on biochemical and genetic conditions should be further investigated.

Abbreviation

Adipoq, Adiponectin; B2m, beta-2-microglobulin; CD14, CD14 antigen; Cox2, cytochrome c oxidase II; KC, chemokine (C-X-C motif) ligand 1; Emr1, adhesion G protein-coupled receptor E1; Fabp1, fatty acid binding protein 1; Fos, FBJ osteosarcoma oncogene; Fosb, FosB proto-oncogene; Hspa1a, heat shock protein family A (Hsp70) member 1A; Il1rn, interleukin 1 receptor antagonist; Jun, Jun proto-oncogene; Junb, JunB proto-oncogene; Mcp-1, C-C motif chemokine ligand 2; Mpo, myeloperoxidase; Msr1, macrophage scavenger receptor 1; Mt1, metallothionein 1; Mt2, metallothionein 2; Myc, myc proto-oncogene protein; OPN, osteopontin; Plau, plasminogen activator, urokinase; Rantes, C-C motif chemokine ligand 5; Retn, resistin; S100a8, S100 calcium binding protein A8; S100a9, S100 calcium binding protein A9; Spp1, secreted phosphoprotein 1; Tlr4, toll-like receptor 4; Tnfrsf12a, TNF receptor superfamily member 12A; Vanin 1, vascular non-inflammatory molecule-1.

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