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Original article

Involvement of glycative stress in diabetic nephropathy and effects of anti-glycation material Kuromoji (*Lindera umbellata* Thunb.)

Kiriko Akiyama [1], Akihiko Shimode [1], Shigeru Matsumi [1], Tetsuya Maruyama [1], Wakako Takabe [2], Masayuki Yagi [3], Yoshikazu Yonei [3]

1) Yomeishu Seizo Co. Ltd., Tokyo, Japan
2) Department of Materials and Life Science, Faculty of Science and Technology, Shizuoka Institute of Science and Technology, Shizuoka, Japan
3) Anti-Aging Medical Research Center/Glycative Stress Research Center, Graduate School of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Abstract

***Objective*:** The purpose of this study is to investigate the effects of anti-glycation activity of kuromoji (*Lindera umbellata* Thunb.) extract (KE) on diabetic nephropathy.

***Methods*:** Using streptozotocin-induced diabetic rats (STZ rats) and spontaneously diabetic mice (db/db mice), glycative stress-related indicators and biochemical changes were investigated during continuous administration of KE, and the effects of KE on inflammatory response in renal tissue was compared between two animal models. Then, mouse macrophage cells (RAW264.7 cells) were used to investigate the effects of glycated substances on the inflammatory response.

***Results*:** KE suppressed the amount of inflammatory cytokines (TNF-α, IL-6) in the renal tissue of STZ rats and db/db mice. In STZ rats, KE suppressed the increase in renal weight and the amount of autoantibodies against N^{ε}-(Carboxymethyl) lysine (CML). In db/db mice, the elevation of serum total cholesterol was suppressed. Treatment of RAW264.7 cells with CML-HSA (human serum albumin) increased TNF-α production, while CML alone did not.

***Conclusion*:** KE is thought to suppress the development of inflammatory response in renal tissue by reducing glycative stress during diabetic conditions.

***KEY WORDS*:** kuromoji (*Lindera umbellata* Thunb.), advanced glycation end products (AGEs), N^{ε}-(Carboxymethyl)lysine (CML), autoantibody, diabetic nephropathy, inflammatory cytokines

Introduction

Lauraceae Kuromoji (*Lindera umbellata* Thunb.) is an aromatic plant containing essential oils, *i.e.* linalool, geraniol [1, 2], and the dried branches are used in medicinal liquor as a crude drug called "usho (*Lindera umbellatae* Ramus)" [3]. Its non-volatile extract has various pharmacological actions including antioxidant, anti-glycation, anti-ulcer action, immunopotentiative action and antiviral action, and therefore it is expected to work as a functional material aimed at preventing lifestyle-related diseases [4-8].

We have used the human serum albumin (HSA) - glucose (GLU) glycation reaction system to target more than 500 types of food materials. As a result of evaluating the inhibitory activity of advanced glycation end products (AGEs) formation, it was clarified that kuromoji extract (KE) shows strong activity among these food materials [5]. KE had a strong inhibitory activity on AGEs formation, not only for HSA but also for collagen and elastin in the GLU glycation reaction system. Also, KE has, as an AGEs decomposition action, an activity enhancing cross-linking cleavage action and oxidized protein hydrolase (OPH) [6]. Furthermore, it

Corresponding author: Professor Masayuki Yagi, PhD
Anti-Aging Medical Research Center,
Graduate School of Life and Medical Sciences, Doshisha University
1-3, Tatara Miyakodani, Kyotanabe-shi, Kyoto, 610-0321 Japan
TEL & FAX: +81-774-65-6394 e-mail: myagi@mail.doshisha.ac.jp
Co-authors: Akiyama K, k-akiyama@yomeishu.co.jp; Shimode A, a-shimode@yomeishu.co.jp; Matsumi S, s-matsumi@yomeishu.co.jp; Maruyama T, te-maruyama@yomeishu.co.jp; Takabe W, takabe.wakako@sist.ac; Yonei Y, yyonei@mail.doshisha.ac.jp

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was observed that administration of KE to streptozotocin (STZ) -induced diabetic rats (STZ rats) suppressed increased levels of inflammatory cytokines, *i.e.* tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in renal tissue, resulting in improvement of renal function[5]. It is reported that infiltration of inflammatory cells, mainly macrophages, is observed in the renal tissue of diabetic nephropathy. Additionally, inflammatory cytokines are increased[9], and glycation and oxidative stress are involved as the mechanism of progression of nephropathy[10]. In consideration of the above, it is considered that the suppression of inflammatory reaction in renal tissue observed in STZ-induced diabetic rats may be related to the inhibitory action by KE on AGEs formation and accumulation.

In this study, we measured the amount of anti-CML (N^{ε}-(Carboxymethyl) lysine) autoantibodies in the serum of STZ rats and spontaneously diabetic mice (db/db mice), and investigated the effect of KE on the reduction of glycative stress and renal inflammation. Concurrently, the involvement of glycated substances in inflammation using macrophage cells (RAW264.7) was investigated.

Methods

1. Subjects

The preparation of KE follows the previously reported method[5]. Briefly, KE was prepared by adding 10 times the amount of water to the branch after finely chopping kuromoji (*Lindera umbellata* Thunb.), then heated at 95 °C for one hour to obtain extract and centrifuged to remove insoluble matter, followed by concentration by using a vacuum concentrator. Positive control drugs used were aminoguanidine (AMG) hydrochloride (Fuji Film Wako Pure Chemical Industries, Ltd., Osaka, Japan), a glycation reaction inhibitor, and metformin (MET) hydrochloride (Fuji Film Wako), an antidiabetic drug for reducing insulin resistance.

2. Effect of KE on diabetic model animals

1) Experimental animals

Six-week-old Sprague Dawley (SD) male rat (Nippon SLC Co., Ltd., Hamamatsu, Shizuoka, Japan), 7-week-old C57BLKS / J Iar-m +/+ Leprdb (db/+) male mouse, and C57BLKS / J Iar -+ Leprdb / + Leprdb (db/db) male mice (Nippon SLC Co., Ltd.) were bred for one week in a barrier facility set at temperature: 24 ± 2 °C, humidity: 50 ± 10%, and lighting time: 12 hours a day (7:00 to 19:00), and then used for the experiment.

2) Breeding management

Throughout the entire test period, rats were individually housed in polycarbonate cages (W26 x D42 x H18 cm) and mice were housed individually in polycarbonate cages (W11 x D18 x H11 cm). The cage used was equipped with a fir-made floor (floor for laboratory animals, soft tip: Nippon SLC Co., Ltd.).

For feed, Powder Lab MR Stock (Nihon Nosan Corporation, Yokohama, Kanagawa, Japan) was used, and for drinking water, well water was freely ingested using a water bottle. Body weight and food intake were measured once a week.

3) Preparation and group composition of model animals

a) Streptozotocin -induced diabetic rats (STZ rats)

STZ rats were prepared by intraperitoneally administering 60 mg/kg of STZ to 7-week-old rats after the completion of pre-breeding, followed by measuring the blood GLU level 3 days later to confirm diabetic state (300 mg/dL or more). The test subject was administered by gavage daily for 8 weeks using a disposable syringe and an oral sonde. The rats were grouped as follows so that there was no bias in blood GLU level and body weight using a grouping software (StatLight: Yukms Corp, Kawasaki, Kanagawa, Japan); No-treated group (Normal), group in which distilled water was administered to SD rats (n = 8); Control group (Vehicle), STZ rats treated with distilled water (n = 8); KE low-dose group (KE100), STZ rats treated with 100 mg/kg/day of KE (n = 8); KE high-dose group (KE300), STZ rats administered KE 300 mg/kg/day (n= 8); AMG-administered group (AMG100), STZ rats administered 100 mg/kg/day of AMG hydrochloride (n = 8).

b) Spontaneous diabetic (db/db) mice

After the completion of pre-breeding, the body weights of 8-week-old mice were individually measured and grouped using the above-mentioned software so that the average body weight and blood GLU level of each group became almost uniform. The test subject was administered by gavage daily for 12 weeks using a disposable syringe and an oral sonde. The group composition was as follows: db/+ group, db/+ mice treated with distilled water (n = 10); Control group (Vehicle), db/db mice treated with distilled water (n = 10); KE low-dose group (KE100), db/db mice treated with 100 mg/kg/day of KE (n = 10); KE high-dose group (KE300), db/db mice treated with 300 mg/kg/day KE (n = 10); MET-administered group (MET350), db/db mice were treated with 350 mg/kg/day of MET hydrochloride (n = 10).

4) Blood sampling and autopsy

Partial blood sampling was performed before and after administration every 4 weeks as follows: for blood GLU, specimen collected from the tail vein without anesthesia; for hemoglobin A1c (HbA1c), rat specimen collected from the jugular vein under isoflurane inhalation anesthesia and mouse specimen from the orbital vein under isoflurane anesthesia. Whole blood was collected after a 16-hour fast from the evening of the end of administration to the next day. Blood was collected from the abdominal aorta under isoflurane anesthesia for rats and from the heart under isoflurane anesthesia for mice. Serum was centrifuged, separated and cryopreserved. After total blood collection, the abdominal aorta and posterior vena cava were amputated and exsanguinated, followed by sampling the right kidney and weighing. Radio-immunoprecipitation (RIPA) buffer (Fuji Film Wako) was added in 4-fold doses in rats and 5-fold

dose in mice to the kidney, followed by homogenizing and centrifuge (14,000 g, 30 minutes) to obtain the supernatant, which was then stored frozen until measurement.

5) Measurement

The blood GLU level was measured using a simple blood GLU meter (One Touch Ultra: Johnson & Johnson Co., Ltd. or Precision Exceed: Abbott Japan Co., Ltd., Tokyo, Japan). HbA1c was measured using the DCA2000 system (Bayer Yakuhin, Ltd., Osaka, Japan). Rat serum total cholesterol (TC), triglyceride (TG), free fatty acids (NEFA), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), total bilirubin (T-BIL), GLU and glycoalbumin (GA) were measured by an automatic biochemical analyzer (Hitachi Co., Ltd., Tokyo, Japan).

For mouse serum, Cholesterol E-test Wako, Triglyceride E-test Wako, LDL-C/M E-test Wako and HDL-C E-test Wako (Fuji Film Wako) was used for measuring respectively TC, TG, LDL-C and HDL-C, and Levis high-density adiponectin-mouse/rat (Fuji Film Wako) was used for adiponectin measurement. Additionally, for the measurement of CML autoantibodies in serum, rats used the CircuLex Anti-CML rat autoantibody ELISA Kit (CycLex Co., Ltd., Ina, Nagano, Japan) and mice used CircuLex Anti-CML mouse autoantibody ELISA Kit (CycLex). For measurement of TNF-α and IL-6 in renal tissue supernatant in the homogenized renal-tissue supernatant, rats used Rat OptEIA ELISA Set (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and mice used Mouse OptEIA ELISA Set (Becton). The protein amount in the renal-tissue supernatant was measured using the BCA Protein Assay Kit (Takara Bio Inc., Kusatsu, Shiga, Japan) for both rats and mice.

6) Ethical standards for the animal study

In conducting this test, "Act on welfare and management of animals (Act No. 105 of October 1, 1973, revised on June 22, 2005)" and "Standards for breeding and storage of laboratory animals and reduction of pain" (April 28, 2006, Ministry of the Environment Notification No. 88) was respected, followed by inspectiom and approval (Approval No. 17117 and 17118) based on the Laboratory Animal Welfare Regulations (Nippon SLC Co., Ltd.,).

3. Effect of glycated substances on mouse macrophage cells (RAW264.7)

1) Culture cells

RAW264.7 cells derived from mouse macrophage were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells are cultured at 37°C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich Japan GK, Tokyo, Japan) supplemented with 10% deactivated fetal bovine serum (FBS, Equitech-Bio, Inc., Kerrville, TX, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Nacalai Tesque, Inc., Kyoto, Japan)

2) Solution of glycated materials

CML (Nippi Co., Ltd., Tokyo, Japan) and CML-HSA (CycLex) were diluted with phosphate-buffered saline (PBS) and used as glycated materials.

3) Measurement

After 4×10^5 cells/mL RAW 264.7 cells were seeded on a 48-well plate, the cells were cultured for 24 hours, washed, and then 50 μL of CML diluted to an arbitrary concentration was added so that the total volume became 500 μL, followed by culture for 24 hours. Additionally, 125 μL of CML-HSA or HSA diluted to an arbitrary concentration and added so that the total volume became 500 μL, followed by culture for 3 hours. Then, the medium supernatant was collected, diluted to an arbitrary concentration with Assay Diluent (Becton), and TNF-α was measured using Mouse OptEIA ELISA Set.

4. Data processing

The results are expressed as mean ± standard deviation (SD). For statistical processing, Wilcoxon rank sum test was used for comparison between two groups, and nonparametric Bonferroni multiple comparison test was used for comparison between multiple groups. When the p values are < 0.05, it is defined as having a significant difference.

Results

1. Effect of KE on diabetic model animals

1) Body weight, food intake and kidney weight

a) Body weight

The transition of body weight is shown in ***Fig. 1***. In rats, the transition of weight gain seen in Normal was not observed in the STZ-treated groups. The body weight after 8 weeks was significantly lower in Vehicle (255 ± 37.3 g) than in Normal (419 ± 29.1 g), while there was no significant difference in the body weights between Vehicle and the test groups (KE100, KE300, AMG100) (***Fig. 1-a***).

In mice, the body weight of Vehicle was higher than that of db/+ from the start of the test, while the body weight of Vehicle (38.0 ± 5.4 g) was higher than that in db/+ (26.7 ± 0.8 g) until 12 weeks. There was no difference in body weight between Vehicle and the test groups (KE100, KE300, MET350) (***Fig. 1-b***).

b) Food intake

The transition of food intake is shown in ***Fig. 2***. In rats, the mean daily food intake throughout the study period, compared to STZ Vehicle (37.4 ± 2.29 g/day), was significantly lower in Normal (24.9 ± 1.07 g/day) and KE100 (34.0 ± 3.62 g/day), while not different with AMG100 (***Fig. 2-a***).

In mice, the daily food intake of Vehicle (6.51 ± 0.62 g/day) was higher than that of db/+ (4.35 ± 0.53 g/day), while no difference noted between Vehicle and the test groups (KE100, KE300, MET350) (***Fig. 2-b***).

c) Kidney weight

Figure 3 shows the kidney weight at autopsy. The rat renal weight of STZ Vehicle (2.50 ± 0.24 g) was higher than that of Normal (2.24 ± 0.15 g). The renal weight of KE100 (2.25 ± 0.19 g) was significantly lower than that of Vehicle, while that in AMG100 was not different from Vehicle (***Fig. 3-a***).

The mouse renal weight of Vehicle (0.40 ± 0.03 g) was higher than that of db/+ (0.36 ± 0.02 g), while there was no difference between Vehicle and the test groups (KE100, KE300, MET350) (***Fig. 3-b***).

2) Biochemical test

Table 1-a shows the results of the STZ rat study after 8 weeks. For GLU metabolism-related indicators, GLU, GA and HbA1c were significantly higher in STZ Vehicle than in Normal, while there was no difference between Vehicle and the test groups (KE100, KE300, AMG100). For lipid metabolism-related indicators, TG and HDL-C were significantly higher in Vehicle than in Normal, and tended to be higher in the KE-treated groups than in Vehicle (TG in KE300, $p = 0.088$; HDL-C in KE100, $p = 0.095$). TC and NEFA did not differ in Vehicle compared to Normal. TC in KE-treated groups was significantly higher than in Vehicle. NEFA in KE300 and AMG100 was significantly higher than in Vehicle.

Table 1-b shows the results of the db/db mouse study after 12 weeks. GLU and HbA1c were significantly higher in Vehicle than in db/+. GLU tended to be lower in MET350 than in Vehicle ($p = 0.059$). HbA1c was significantly lower in MET350 than in Vehicle. TC, LDL-C and HDL-C showed significantly higher values in Vehicle than in db/+. In KE300, TC was significantly lower than in Vehicle, TG and LDL-C tended to show lower values than in Vehicle (TG, $p = 0.067$; LDL-C, $p = 0.084$). In MET350, TG and LDL-C were significantly lower than in Vehicle.

3) Anti-CML autoantibodies in serum

The results of anti-CML autoantibodies in serum after 8 weeks in the STZ rat study are shown in ***Fig. 4-a***. The amount of anti-CML autoantibodies in STZ Vehicle (321 ± 171.6 μg/mL) was significantly higher than Normal (108 ± 54.4 μg/mL). It was significantly lower in KE-treated groups (KE100, 71 ± 55.8 μg/mL; KE300, 113 ± 89.3 μg/mL) and AMG100 (122 ± 76.3 μg/mL) compared to Vehicle.

The results of anti-CML autoantibodies in serum after 12 weeks in the db/db mouse study are shown in ***Fig. 4-b***. The anti-CML autoantibody level in Vehicle (37.7 ± 20.9 μg/mL) was significantly higher than that in db/+ (22.6 ± 4.9 μg/mL). There were no significant difference between Vehicle and the test groups.

4) Inflammatory cytokines in kidney tissue

The results of TNF-α and IL-6 in the renal tissue after 8 weeks in the STZ rat study are shown in ***Fig. 5-a***, ***b***. The amount of TNF-α per protein in renal tissue was significantly higher in STZ Vehicle (1.72 ± 0.39 ng/mg protein) than in Normal (1.26 ± 0.22 ng/mg protein). KE100 (1.01 ± 0.30 ng/mg protein) and AMG100 (0.85 ± 0.12 ng/mg protein) showed significantly lower values than Vehicle. IL-6 was significantly lower in KE groups (KE100: 4.54 ± 1.63 ng/mg protein, KE300: 4.24 ± 1.75 ng/mg protein) and AMG100 (4.03 ± 0.51 ng/mg protein) compared with Vehicle (6.39 ± 1.27 ng/mg protein). There was no difference between STZ Vehicle and Normal.

The results of TNF-α and IL-6 in the renal tissue after 12 weeks in the db/db mouse study are shown in ***Fig. 5-c***, ***d***. TNF-α per protein in the renal tissue was significantly lower in KE300 (0.47 ± 0.12 ng/mg protein) than in Vehicle (0.70 ± 0.19 ng/mg protein). IL-6 was significantly lower in KE300 (0.32 ± 0.08 ng/mg protein) and MET350 (0.35 ± 0.10 ng/mg protein) compared with Vehicle (0.47 ± 0.12 ng/mg protein). There was no difference in TNF-α and IL-6 between db/+ and db/db Vehicle.

2. Effect of glycated substances on mouse macrophage cells (RAW264.7)

Figure 6 shows the amount of TNF-α produced by adding glycated substances to RAW264.7.

TNF-α produced in the culture solution 24 hours after the addition of the CML solution did not change as compared with that without the addition (***Fig. 6-a***). TNF-α produced in the culture medium 3 hours after the addition of the CML-HSA was observed to increase in a concentration-dependent manner, and was significantly increased by the treatments of 5 μg/mL and 10 μg/mL. In contrast, no increase in TNF-α was observed with the treatment of HSA alone (***Fig. 6-b***).

Discussion

1. Diabetes model animals

STZ rats are used as a model for type 1 diabetes mellitus (T1DM). The administration of STZ induces the production of reactive oxygen species (ROS) in pancreatic β-cells, causing cell death in β-cells and inhibiting insulin secretion, resulting in a rapid increase in blood GLU and maintenance of hyperglycemia[11,12]. On the other hand, db/db mice are used as a type 2 diabetes mellitus (T2DM) model. Since db/db mice have a deficiency in the receptor for the appetite-suppressing hormone "leptin", they become obese due to overeating and develop hyperglycemia at 6 to 10 weeks of age[13]. In these diabetic models, enlargement of the glomerular mesangium matrix and thickening of the glomerular basement membrane, which are characteristic of diabetic nephropathy, were observed in the kidney[13], and an elevation in inflammatory cytokines was recognized in the renal tissues[14,15]. Furthermore, AGEs are accumulated in the kidney, therefore, it is considered that the glycative stress generated by the glycation reaction of biological proteins is deeply involved in the onset of nephropathy[10,16,17]. In this study, renal weight was increased in both model animals, and elevation of TNF-α and IL-6 production was observed in the renal tissue of STZ rats. The increase in renal weight is considered to be due to renal hypertrophy, which is also observed in the initial

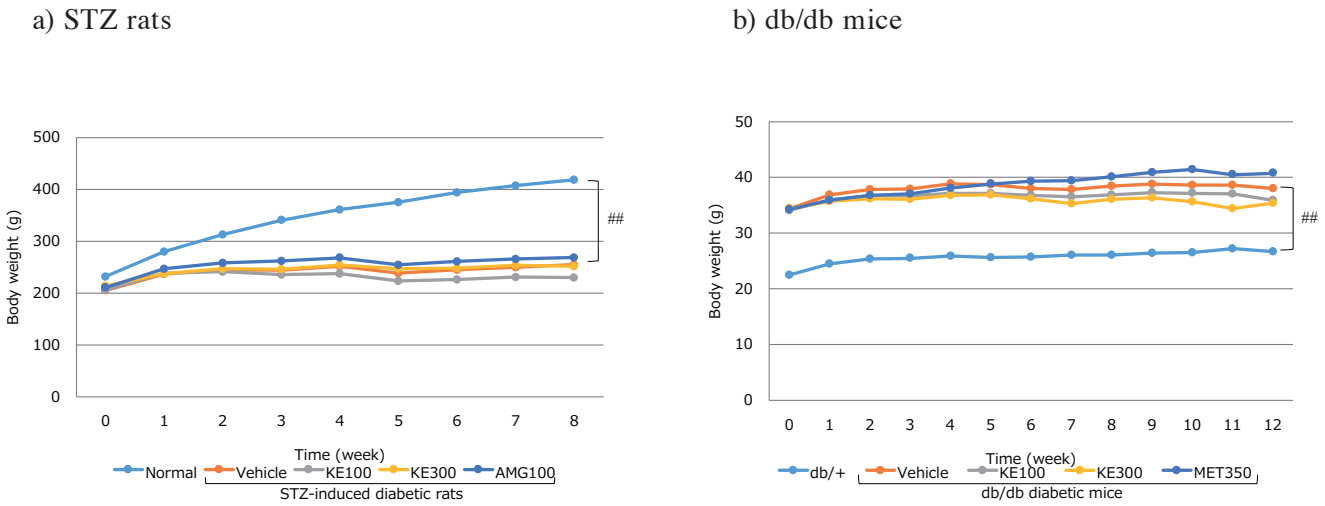

Fig. 1. **Change of weight in the diabetic model animals.**

a) STZ rats. Normal, no STZ-treat and vehicle treated group; Vehicle, STZ and vehicle treated group; KE100, STZ and 100 mg/kg/day KE treated group; KE300, STZ and 300 mg/kg/day KE treated group; AMG100, STZ and 100 mg/kg/day AMG treated group. Results are expressed as mean, n = 8, $^{\#\#}p < 0.01$ vs. Normal.

b) db/db mice. db/+, non-diabetic mice and vehicle treated group; Vehicle, db/db diabetic mice and vehicle treated group; KE100, db/db diabetic mice and 100 mg/kg/day KE treated group; KE300, db/db diabetic mice and 300 mg/kg/day KE treated group; MET350, db/db diabetic mice and 350 mg/kg/day MET treated group. Results are expressed as mean. n = 9 – 10, $^{\#\#}p < 0.01$ vs. db/+.

STZ, streptozotocin (i.p.); KE, kuromoji extract (p.o.); AMG, aminoguanidine (p.o.); MET, metformin (p.o.); i.p., intraperitoneal administration; p.o., oral administration.

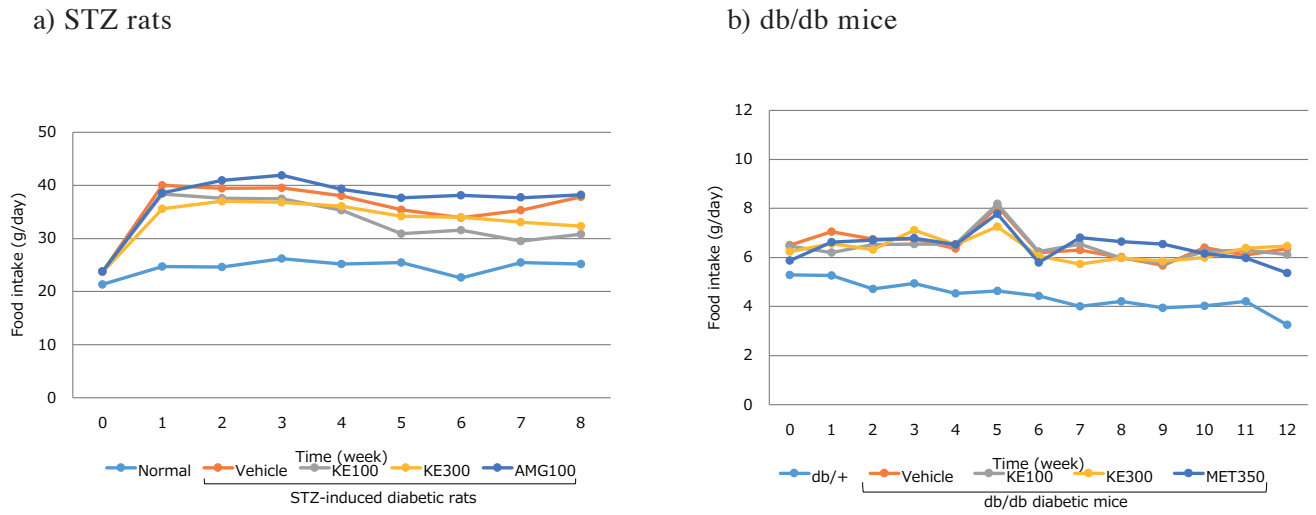

Fig. 2. **Change of food intake amount in the diabetic model animals.**

a) STZ rats. Normal, No STZ-treat and vehicle treated group; Vehicle, STZ and vehicle treated group; KE100, STZ and 100 mg/kg/day KE treated group; KE300, STZ and 300 mg/kg/day KE treated group; AMG100, STZ and 100 mg/kg/day AMG treated group. Results are expressed as mean, n = 8

b) db/db mice. db/+, non-diabetic mice and vehicle treated group; Vehicle, db/db diabetic mice and vehicle treated group; KE100, db/db diabetic mice and 100 mg/kg/day KE treated group; KE300, db/db diabetic mice and 300 mg/kg/day KE treated group; MET350, db/db diabetic mice and 350 mg/kg/day MET treated group. Results are expressed as mean, n = 9 - 10.

STZ, streptozotocin (i.p.); KE, kuromoji extract (p.o.); AMG, aminoguanidine (p.o.); MET, metformin (p.o.) ; i.p., intraperitoneal administration; p.o., oral administration.

Table 1. The values of blood markers after treatment in the diabetic model animals.

a) STZ rats

	STZ(-)	STZ(+)			
	Normal	Vehicle	KE100	KE300	AMG100
GLU (mg/dL)	243 ± 37.3	496 ± 66.3 $^{\#\#}$	480 ± 42.6	464 ± 63.5	530 ± 53.6
GA (%)	1.81 ± 0.22	8.41 ± 0.81 $^{\#\#}$	8.85 ± 2.71	7.94 ± 2.38	8.79 ± 0.68
HbA1c (%)	3.53 ± 0.14	8.06 ± 0.95 $^{\#\#}$	8.53 ± 1.95	7.98 ± 1.71	8.53 ± 1.14
TC (mg/dL)	77 ± 9.0	96 ± 37.8	171 ± 65.4 **	145 ± 40.1 *	109 ± 17.4
TG (mg/dL)	99 ± 21.7	481 ± 385.2 $^{\#}$	868 ± 529.2	1101 ± 881.0	544 ± 211.4
NEFA (μEq/L)	376 ± 63.3	457 ± 138.3	644 ± 123.3	847 ± 307.1 **	655 ± 129.8 *
LDL-C (mg/dL)	8.3 ± 1.2	16.6 ± 12.8	38.4 ± 29.7	34.6 ± 23.2	14.5 ± 3.4
HDL-C (mg/dL)	28.1 ± 2.0	35.0 ± 9.1 $^{\#}$	45.0 ± 11.6	38.8 ± 6.3	41.3 ± 6.6

b) db/db mice

	db/+	db/db			
		Vehicle	KE100	KE300	MET350
GLU (mg/dL)	102 ± 36.3	428 ± 64.4 $^{\#\#}$	419 ± 79.7	381 ± 101.3	330 ± 109.6
HbA1c(%)	3.87 ± 0.12	9.53 ± 1.18 $^{\#\#}$	9.61 ± 1.09	8.88 ± 1.62	7.99 ± 1.71 *
TC (mg/dL)	103 ± 12.0	132 ± 15.4 $^{\#\#}$	118 ± 12.6	117 ± 10.0 *	120 ± 16.8
TG (mg/dL)	109 ± 25.4	133 ± 27.1	111 ± 25.3	101 ± 26.4	77 ± 18.4 **
LDL-C (mg/dL)	39.2 ± 6.4	48.5 ± 12.5 $^{\#}$	42.2 ± 6.4	37.9 ± 4.3	27.6 ± 3.4 **
HDL-C (mg/dL)	62.6 ± 9.6	86.4 ± 13.2 $^{\#\#}$	84.6 ± 11.1	84.4 ± 6.9	88.8 ± 15.5
Adipo (μg/mL)	7.39 ± 3.94	7.78 ± 1.24	6.96 ± 1.95	6.60 ± 1.87	9.38 ± 7.35

a) STZ rats. Normal, no STZ-treat and vehicle treated group; Vehicle, STZ and vehicle treated group; KE100, STZ and 100 mg/kg/day KE treated group; KE300, STZ and 300 mg/kg/day KE treated group; AMG100, STZ and 100 mg/kg/day AMG treated group. Results are expressed as mean ± SD, n = 8, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. Normal; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Vehicle.

b) db/db mice. db/+, non-diabetic mice and vehicle treated group; Vehicle, db/db diabetic mice and vehicle treated group; KE100, db/db diabetic mice and 100 mg/kg/day KE treated group; KE300, db/db diabetic mice and 300 mg/kg/day KE treated group; MET350, db/db diabetic mice and 350 mg/kg/day MET treated group. Results are expressed as mean ± SD, n = 9 - 10), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. db/+; $^{**}p < 0.01$, $^{*}p < 0.05$ vs. Vehicle.
STZ, streptozotocin (i.p.); KE, kuromoji extract (p.o.); AMG, aminoguanidine (p.o.); MET, metformin (p.o.); GLU, glucose; GA, glycoalbumin; HbA1c, hemoglobin A1c; TC, total cholesterol; TG, triglycerides; NEFA, non-esterified fatty Acid; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; Adipo, adiponectin; SD, standard deviation; i.p., intraperitoneal administration; p.o., oral administration.

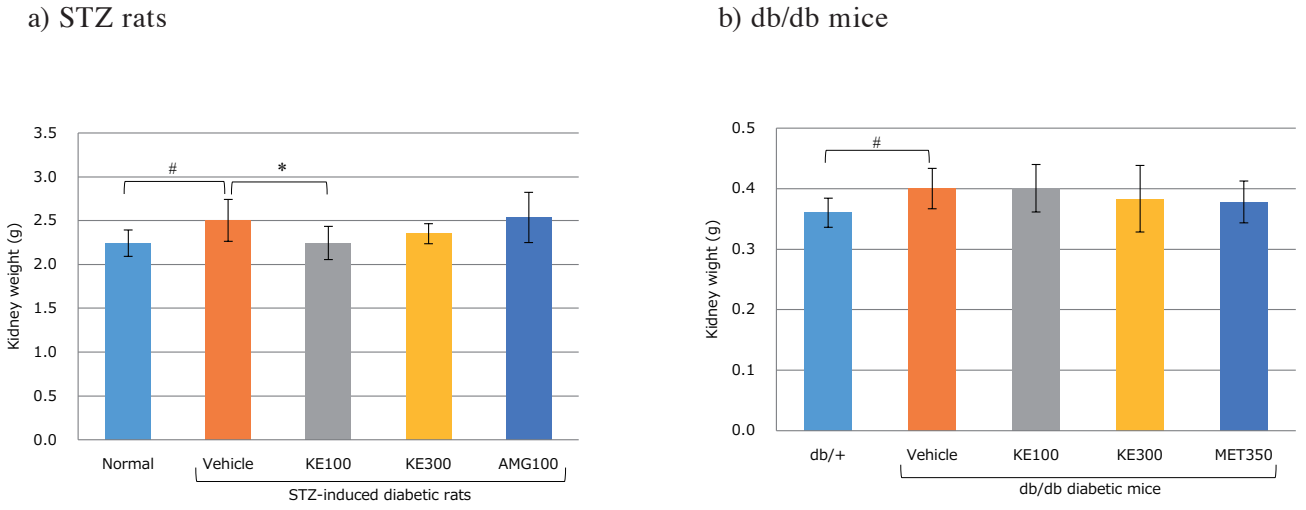

Fig. 3. **Kidney weight after treatment in the diabetic model animals.**

a) STZ rats. Normal, No STZ-treat and vehicle treated group; Vehicle, STZ and vehicle treated group; KE100, STZ and 100 mg/kg/day KE treated group; KE300, STZ and 300 mg/kg/day KE treated group; AMG100, STZ and 100 mg/kg/day AMG treated group. Results are expressed as mean ± SD, n = 8, $^{\#}p < 0.05$ vs. Normal; $^{*}p < 0.05$ vs. Vehicle.

b) db/db mice. db/+, non-diabetic mice and vehicle treated group; Vehicle, db/db diabetic mice and vehicle treated group; KE100, db/db diabetic mice and 100 mg/kg/day KE treated group; KE300, db/db diabetic mice and 300 mg/kg/day KE treated group; MET350, db/db diabetic mice and 350 mg/kg/day MET treated group. Results are expressed as mean ± SD, n = 9 – 10, $^{\#}p < 0.05$ vs. db/+.

STZ, streptozotocin (intraperitoneal administration, i.p.); KE, kuromoji extract (oral administration, p.o.); AMG, aminoguanidine (p.o.); MET, metformin (p.o.); SD, standard deviation; i.p., intraperitoneal administration; p.o., oral administration.

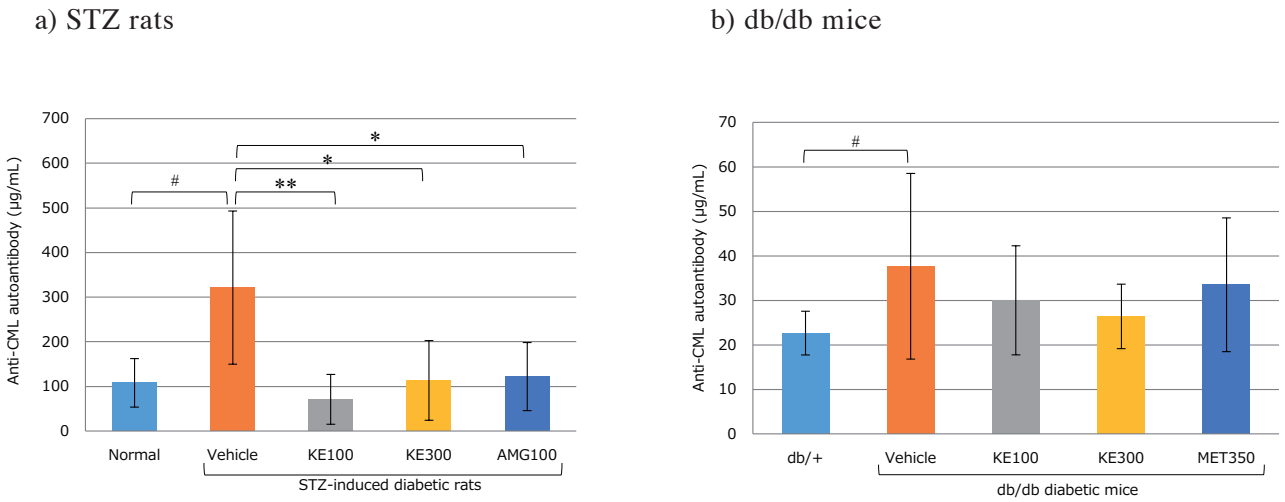

Fig. 4. **The values of blood anti-CML autoantibody after treatment in the diabetic model animals.**

a) STZ rats. Normal, No STZ-treat and vehicle treated group; Vehicle, STZ and vehicle treated group; KE100, STZ and 100 mg/kg/day KE treated group; KE300, STZ and 300 mg/kg/day KE treated group; AMG100, STZ and 100 mg/kg/day AMG treated group. Results are expressed as mean ± SD, n = 8, $^{\#}p < 0.05$ vs. Normal; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Vehicle.

b) db/db mice. db/+, non-diabetic mice and vehicle treated group; Vehicle, db/db diabetic mice and vehicle treated group; KE100, db/db diabetic mice and 100 mg/kg/day KE treated group; KE300, db/db diabetic mice and 300 mg/kg/day KE treated group; MET350, db/db diabetic mice and 350 mg/kg/day MET treated group. Results are expressed as mean ± SD, n = 9 – 10, $^{\#}p < 0.05$ vs. db/+.

STZ, streptozotocin (i.p.); KE, kuromoji extract (p.o.); AMG, aminoguanidine (p.o.); MET, metformin (p.o.); CML, N^{ε}-(Carboxymethyl) lysine; SD, standard deviation; i.p., intraperitoneal administration; p.o., oral administration.

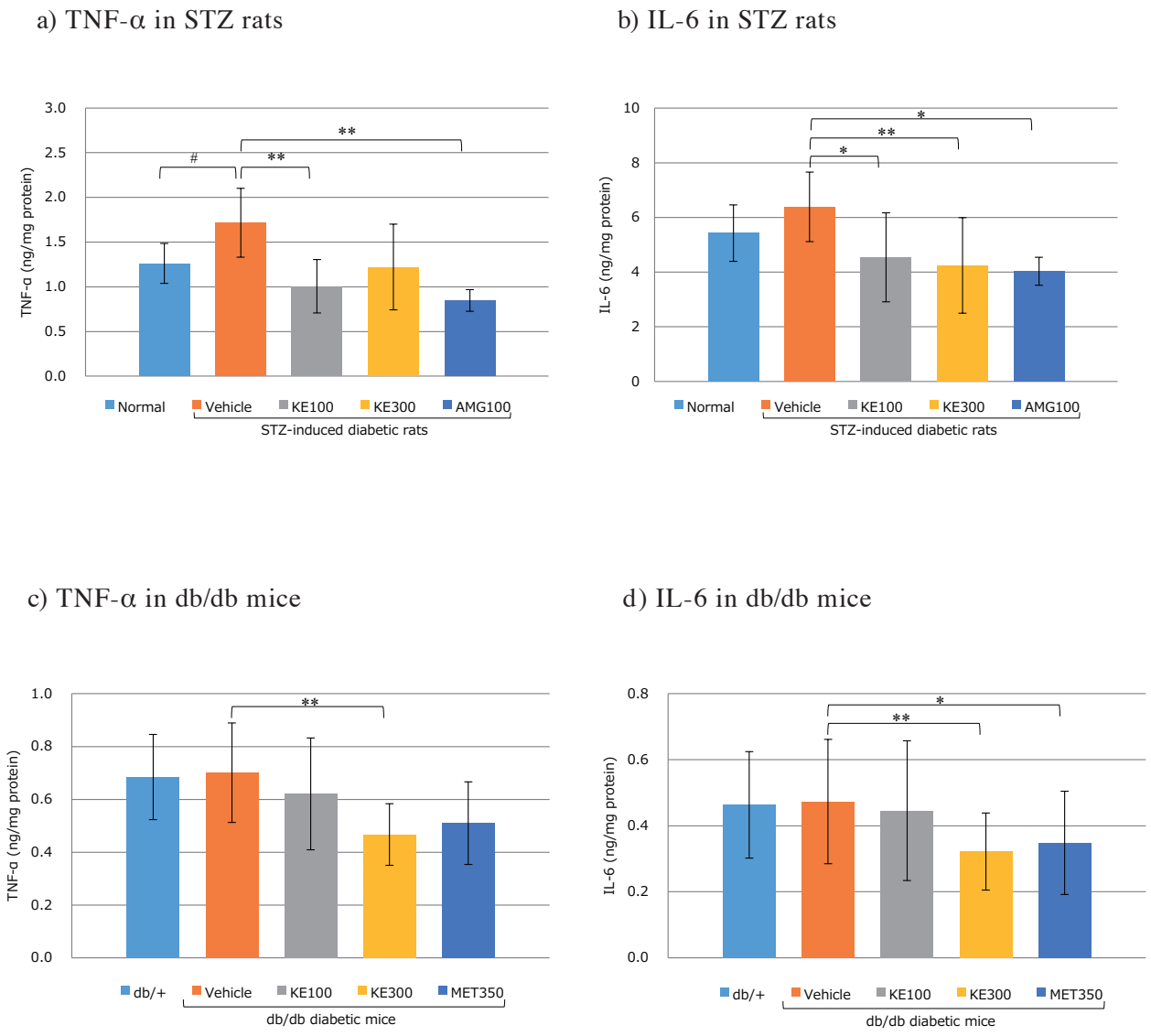

Fig. 5. **The values of kidney cytokine after treatment in the diabetic model animals.**

a) TNF-α, **b)** IL-6 in STZ rats. Normal, no STZ-treat and vehicle treated group; Vehicle, STZ and vehicle treated group; KE100, STZ and 100 mg/kg/day KE treated group; KE300, STZ and 300 mg/kg/day KE treated group; AMG100, STZ and 100 mg/kg/day AMG treated group. Results are expressed as mean ± SD, n = 8, $^{\#}p < 0.05$ vs. Normal; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Vehicle.

c) TNF-α, **d)** IL-6 in db/db mice. db/+, non-diabetic mice and vehicle treated group; Vehicle, db/db diabetic mice and vehicle treated group; KE100, db/db diabetic mice and 100 mg/kg/day KE treated group; KE300, db/db diabetic mice and 300 mg/kg/day KE treated group; MET350, db/db diabetic mice and 350 mg/kg/day MET treated group. Results are expressed as mean ± SD, n = 9 – 10, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. Vehicle.

STZ, streptozotocin (intraperitoneal administration, i.p.); KE, kuromoji extract (oral administration, p.o.); AMG, aminoguanidine (p.o.); MET, metformin (p.o.); TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; SD, standard deviation; i.p., intraperitoneal administration; p.o., oral administration.

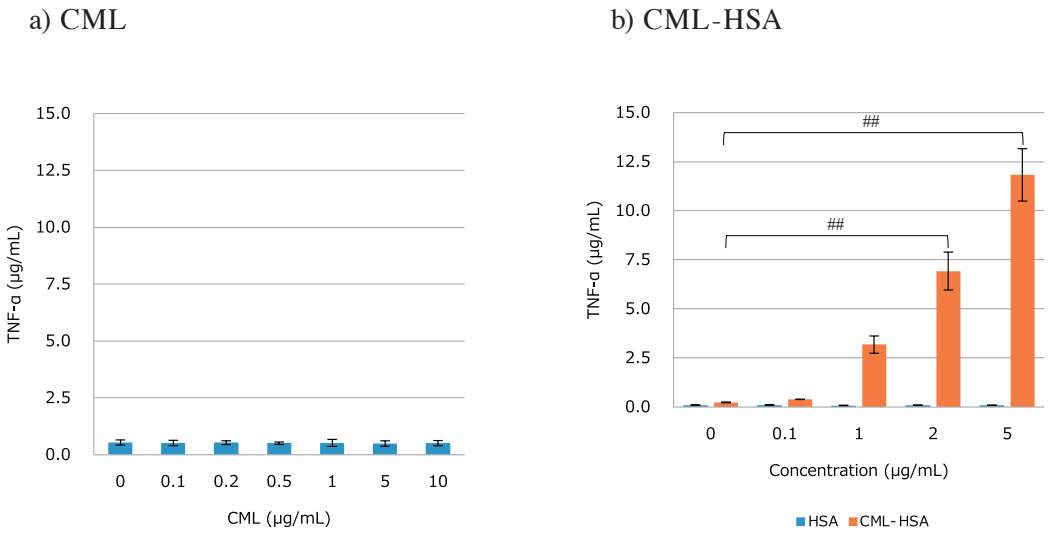

Fig. 6. **The values of TNF-α production by treatment with glycated materials in RAW264.7 cells.**
a) CML treatment. **b)** CML-HSA treatment. The culture supernatant was collected 24 hours (a) or 3 hours (b) after the application and measured for TNF-α content by ELISA. Results are expressed as mean ± SD, n = 3, $^{\#\#}p < 0.05$ vs. 0 μg/mL (control). CML, N^{ε}-(Carboxymethyl)lysine; TNF-α, tumor necrosis factor-α; HSA, human serum albumin; ELISA, enzyme-linked immuno sorbent assay: SD, standard deviation.

symptoms of diabetic nephropathy. Persistent hyperglycemia in both models may be involved in the development of the inflammatory response in the kidney.

AGEs are said to increase with age in human and animal tissues and be associated with diabetic nephropathy [18], chronic renal failure [19], atherosclerotic lesions of the arterial wall [20] and Alzheimer's disease [21]. In addition, AGEs are known to have antigenicity and produce autoantibodies. Autoantibodies against AGEs are detected in high concentrations in the sera of diabetic patients, and are thought to be associated with the various diseases mentioned above [22]. In particular, autoantibodies against AGEs are known to be associated with the severity of diabetic nephropathy, and higher concentrations are detected in patients with renal failure who require dialysis than in patients with mild microalbuminuria [23]. Furthermore, CML autoantibodies, which have an epitope common to various AGEs, increase about 5 times in T2DM patients with blood GLU levels of 200 mg/dL or higher compared with healthy subjects [22]. Taking these findings into consideration, the amount of autoantibodies to CML reflects the formation and accumulation of AGEs *in vivo*, and is considered to play an important role in the early detection of secondary complications of diabetes. In this study as well, a marked increase in anti-CML autoantibodies was observed in the sera of both model animals. The increase in antibody level was remarkable particularly in STZ rats, and this increase was suppressed by administration of AMG. This indicates that glycative stress with increased production of AGEs is strongly involved in the renal inflammatory response in both model animals, and is effect appears to be more pronounced in STZ rats that is the T1DM model.

As one of the mechanisms of elevation of inflammatory cytokines by AGEs, it has been reported that they are involved in the AGEs/RAGE inflammatory signal transduction system via RAGE (Receptor for AGEs) present in macrophages. In patients with nephropathy, there are marked infiltration of inflammatory cells mainly macrophages, in the glomerulus and interstitium [10, 24], and increased RAGE in the renal tissue of STZ rats and db/db mice [15, 25, 26]. However, contradictory discussions continue on each AGE regarding the induction of inflammatory reactions via RAGE, and some reports insist that CML does not have the necessary structure to bind to RAGE [27, 28]. On the contrary, it has been shown that modification of proteins to AGEs is important for binding to RAGE. Co-treatment of CML alone did not conflict with the binding of CML-BSA to RAGE [29]. Also, treatment with CML alone did not induce TNF-α production from human monocyte-derived cells, whereas it was produced by casein glycation metabolites, which were co-treated with an anti-CML antibody or a RAGE antagonist, resulting suppression of TNF-α production [27]. We also confirmed that TNF-α production was not observed with CML alone of RAW264.7 cells but was increased in a dose-dependent manner with CML-HSA treatment. This is a result supporting the importance of protein modification to AGEs for the induction of inflammation of macrophages.

2. *Actions of Kuromoji*

KE strongly suppressed increasing anti-CML autoantibody in STZ rat serum and decreased renal weight and inflammatory cytokines in renal tissue. Our previous study showed that KE suppresses the production of fluorescent AGEs, CML, 3-deoxyglucosone (3DG), glyoxal (GO), and methylglyoxal (MGO) in the GLU-glycation

reaction system for HSA, collagen and elastin, and especially for CML which was strongly inhibited [6]. Also, it has the cleaving effect of AGE cross-linking and the enhancing effect of the activity of oxidative proteolytic enzyme (OPH), thus exerting an action of degrading AGEs [6]. Furthermore, the kuromoji branches contain flavan-3-ol derivatives, *i.e.* procyanidin B1, and procyanidin B2, and flavonoid compounds, *i.e.* kaempferol, quercetin, hyperoside, and pinocembrin, which are reported to have an inhibitory effect on AGEs production, a trapping effect on MGO and GO, and an inhibitory effect on the AGEs-induced apoptosis [30-36].

The amount of autoantibodies to CML, which has an epitope common to various AGEs, may reflect the formation and accumulation of AGEs *in vivo*. It is considered that the strong anti-glycation activity of KE reduces the production and accumulation of AGEs, thereby suppressing the activation of inflammatory cells, *i.e.* macrophage, via the AGEs/RAGE inflammatory signal transduction system and reducing the progression of the inflammation in the kidneys. The influence of glycative stress was stronger in STZ rats than in db/db mice, however the effect of KE was more obvious in STZ rats. It is considered that the action of KE was more remarkable in the T1DM model in which the production and accumulation of AGEs progressed earlier.

On the other hand, in db/db mice, the increase in TC was suppressed by KE administration. Generally, the main sites of cholesterol synthesis are the liver and small intestine. It has been reported that, in STZ rats, cholesterol is mainly synthesized in the small intestine, whereas in db/db mice, cholesterol is highly synthesized in the liver [37]. There are differences in cholesterol synthesis between insulin-dependent diabetic animals, *i.e.* STZ rats and non-insulin-dependent diabetic animals, *i.e.* db/db mice. Concurrently, procyanidin B1 and procyanidin B2 have been reported to suppress the increase in TG and TC in db/db mice [25], but no reports have been found in STZ rats. The inhibitory effect of KE on cholesterol elevation, which was observed only in db/db mice this time, is considered to be influenced by the difference in the mechanism of lipid metabolism in each diabetes model.

Conclusion

In the diabetes model, it was considered that the increased AGEs *in vivo* caused renal inflammation by stimulating inflammatory cells such as macrophages. KE, which has an anti-glycation effect, suppressed renal inflammation in STZ rats and db/db mice, but the suppressive effect was more pronounced in STZ rats, a T1DM model in which the effect of glycative stress is large. It is possible that the strong inhibitory action of KE on glycative stress is involved in the suppression of the inflammatory response in renal tissues during diabetic conditions.

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Conflict of interest declaration

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