Oral Administration of Spa-derived Green Alga Improves Insulin Resistance in Fructose-fed Rats and Slightly Overweight Subjects

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Abstract

Advanced glycation end products (AGEs) and their receptor (RAGE) system evoke inflammatory reactions and insulin resistance in adipocytes. Spa-derived green alga *Mucidosphaerium* sp. (MS) had anti-inflammatory properties *in vitro*. We examined here whether and how MS could ameliorate insulin resistance in fructose-rich diet-fed rats, and conducted a randomized, double blind, placebo-controlled trial to investigate the effects of MS on insulin resistance in overweight subjects. Oral administration of MS for 8 weeks significantly decreased random blood glucose, and fasting insulin, oxidative stress levels, and improved homeostasis model assessment of insulin resistance (HOMA-IR) values in fructose-fed rats, which were associated with the reduction of AGEs, RAGE, 8-hydroxy-2'-deoxy-guanosine, NADPH oxidase activity, macrophage and lymphocyte infiltration, monocyte chemoattractant protein-1 (MCP-1) expression, and adipocyte size in the adipose tissues as well as restoration of adiponectin levels. MS decreased the AGE-induced NADPH oxidase activity, ROS generation, *MCP-1* and *RAGE* gene expression, and lipid accumulation in differentiated adipocytes, while it restored the decrease in adiponectin mRNA levels. An anti-oxidant, *N*-acetylcysteine mimicked the effects of MS on ROS generation, *RAGE* gene expression, and lipid accumulation. Oral intake of MS for 12 weeks significantly decreased systolic and diastolic blood pressure, fasting plasma glucose, fasting insulin, HOMA-IR, HDL-cholesterol and creatinine in overweight subjects. Baseline-adjusted diastolic blood pressure, fasting plasma glucose, fasting insulin, and HOMA-IR values were significantly lower in MS treatment group than in placebo. Our present findings suggest that MS may improve insulin resistance by blocking the AGE-RAGE-oxidative stress axis in the adipose tissues.

Keywords; Advanced glycation end products (AGEs), Adipose tissue, Inflammation, Insulin resistance, RAGE

Abbreviations

8-iso-PGF2α, 8-iso prostaglandin F2α

AGEs, advanced glycation end-products

CML, carboxymethyllysine

CV, dry powder of Chlorella vulgaris CK22

DGDG, digalactosyl diacylglycerol

HOMA-IR, homeostasis model assessment of insulin resistance

NAC, N-acetylcysteine

MCP-1, monocyte chemoattractant protein-1

MS, *Mucidosphaerium* sp.

MSGL, MS-derived glycolipids

RAGE, receptor for AGEs

ROS, reactive oxygen species

Introduction

Monosaccharides, such as glucose, glyceraldehyde, and frucotse react non-enzymatically with amino gropus of proteins, lipids, and nucleic acids to form Amadori compounds, which could be subsequently converted to advanced glycation end products (AGEs) over a course of days to weeks via a complex series of reactions.[1-9] Modification of amino groups of molecules by AGEs alter the structural integrity and functional property of numerous types of proteins and lipids, including collagen and low-density lipoprotein, thereby being involved in atherosclerotic cardiovascular disease and osteoporosis.[1-9] Furthermore, AGE-modified molecules can interact with a cell surface receptor, RAGE, which evokes oxidative stress and inflammatory reactions, thereby contributing to various aging and/or diabetes-related complications.[1-9] Since the formation and accumulation of AGEs have progressed under hyperglycemic, oxidative stress, and/or inflammatory conditions and that RAGE expression is enhanced by its ligand AGEs, AGE-RAGE interaction may form a positive feedback loop that further promotes the development of aging-related disorders.[1-9]

Insulin resistance and obesity are associated with inflammatory conditions, which also play a role in the development and progression of aging-related disorders, such as diabetes, cancer, and Alzheimer's disease.[10-12] We have previously found that

interaction of AGEs with RAGE evokes oxidative stress and inflammatory reactions in adipocytes, being involved in insulin resistance of obese type 2 diabetic mice.[13,14] Furthermore, we have found that serum levels of AGEs are independently correlated with inflammatory activity in visceral adipose tissues and homeostasis model assessment of insulin resistance (HOMA-IR), a marker of insulin resistance in humans.[15,16] These observations suggest that activation of the AGE-RAGE axis could contribute to insulin resistance, which may serve as "common soil" for promoting various aging-related diseases and also be a therapeutic target for these devastating disorders.

We have isolated a novel *Mucidosphaerium* sp. strain (MS) from hot springs in Beppu city, one of the most famous resorts in Japan with the world's second-largest number of hot springs and recently found that extract of the green alga exhibits anti-inflammatory and anti-oxidative properties in cultured human dermal fibroblasts, synoviocytes, and papilla cells.[17] Several types of algae have been reported to inhibit the formation of AGEs *in vitro*.[18-20] However, effects of MS on AGE-RAGE axis, inflammation, and insulin resistance remain to be elucidated. Therefore, in this study, we examined whether and how MS could ameliorate insulin resistance in high fructose diet-fed rats, studied the effects of MS on AGE-exposed human differentiated

adipocytes, and then conducted a randomized, double blind, placebo-controlled trial to investigate the effects of oral intake of MS on anthropometric and metabolic parameters, including HOMA-IR in apparent healthy overweight Japanese adults.

Research Design and Methods

Materials

Bovine serum albumin (BSA), D-glyceraldehyde, and *N*-acetylcysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). Digalactosyl diacylglycerol (DGDG) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Fructose-enriched diet consists of 60% fructose, 20.7% casein, 0.3% methionine, 5% lard, and 9.25% cellulose with vitamin and mineral mixture (350 kcal/100g) (Oriental Yeast Co., Ltd., Shiga, Japan). Dietary fructose is replaced by cornstarch in control diet. Dry powder of Chlorella vulgaris CK22 (CV) which contained 10mg DGDG/g CV was provided from Chlorella Industry Co., LTD. (Tokyo, Japan).

Preparation of MS powder

MS was isolated from hot springs in Beppu city and cultured as described previously.[17] Dry powder of MS was obtained by a spray dryer.

Preparation of AGEs

AGEs were prepared by incubating BSA (25 mg/ml) with 0.1 M D-glyceraldehyde at 37 °C for 7 days as described previously.[21] Control non-glycated BSA was incubated in the same conditions except for the absence of D-glyceraldehyde.

Animals

Six-week-old male Wistar rats (Charles River Breeding Laboratories, Yokohama, Japan) were fed a fructose-rich diet (Fructose) or control diet (Control) with or without 0.02% MS (0.2mg/g diet). Number of Control rats, Fructose-fed rats, and Fructose+MS-fed rats were 6, 6, and 8, respectively. Systolic and diastolic blood pressure (BP) and heart rate were monitored by a tail-cuff sphygmomanometer (BP-98A; Softron, Tokyo, Japan) at 8 weeks after treatment. Body weight was measured at 9 weeks after treatment, and then rats were sacrificed. Visceral adipose tissues were excised for immunohistochemical and morphological analyses, and blood biochemistry was determined as described previously.[22] Serum levels of AGEs were measured with an enzyme-linked immunosorbent assay (ELISA); one unit (U)/ml corresponded to half maximal (50%) inhibitory concentration of AGEs.[23] Serum and urinary levels of 8-iso

prostaglandin F2α (8-iso-PGF2α), a marker of oxidative stress were determined with ELISA kits derived from Enzo Life Science Inc. (Farmingdale, NY). All experimental procedures were conducted in accordance with the National Institutes Health Guide for Care and Use of Laboratory Animals and were approved by the ethnical committee of Kurume University School of Medicine.

Immunostaining and morphological analysis

Adipose tissue sections derived from visceral fats were incubated overnight at 4°C with antibodies, and the reactions were visualized with a Histofine Simple Stain Rat MAX-POMULTI kit (Nichirei Co., Japan).[22,24] Antibodies raised against AGEs, carboxymethyllysine (CML) (TransGenic Inc., Japan, cat #KH011-02, lot #TG240914), one of the well characterized AGEs, RAGE (Santa Cruz Biotechnology, Dallas, TX, cat #SC-5563, Lot #I2515), 8-hydroxy-2'-deoxy-guanosine (8-OHdG) (Nikken Seil Co., Shizuoka, Japan, cat #MOG-100P, lot #008MOG-100P), F4/80 (Abcam, Cambridge, MA, cat #ab111101, lot #GR201096-5), CCR7 (Epitomics Inc., Burlingame, CA, cat #2059-1, lot #Y105952SD), monocyte chemoattractant protein-1 (MCP-1) (Abcam, cat #ab7202, lot #GR47125-26), and adiponectin (Santa Cruz Biotechnology, cat #SC-26496, lot #F2204) were used for immunohistochemical analyses.

Immunohistoreactivity in 5 different fields in each sample was measured by cellSens software version 1.14 (Olympus Co., Japan). For morphological analysis, the sections were stained with hematoxylin and eosin, and adipocyte size in 6 different field of each sample was analyzed by cellSens software.

Measurement of NADPH oxidase activity

Adipose tissue NADPH oxidase activity was measured by a luminescence assay as described previously.[24]

Preparation of MS-derived glycolipids (MSGL)

Crude lipids were extracted from MS powder as described before.[25] Glycolipids were further fractionated with n-hexane and 10% water/methanol according to the method of Marcolongo et al.[26]

Cell experiments

3T3-L1 cells (American Type Culture Collection, Manassas, VA) were maintained in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) and

differentiated to mature adipocytes using insulin, dexamethasone, and 3-isobutyl-1-methyl-xanthine as described previously.[13] After the medium was changed back to original DMEM containing 10 % fetal bovine serum, differentiated adipocytes were treated with 100 μg/ml AGE-modified BSA or non-glycated BSA in the presence or absence of 0.8~3.2 μg/ml MS, 0.8~3.2 μg/ml CV, 5~20 mM NAC, 0.2~1 μg/ml MSGL, or 0.001~10 μg/ml DGDG for the indicated time periods. Then real-time reverse transcription-polymerase chain reactions (RT-PCR) analysis and Oil Red O staining were performed, and reactive oxygen species (ROS) and NADPH oxidase activity were measured. 3T3-L1 cells in passage numbers between 5 and 20 were used.

ROS measurement

Superoxide generation was measured with carboxy-H_2DFFDA (Life Technologies Japan) as described previously.[24] ROS in 9 different fields in each sample was measured.

RT-PCR

RT-PCR was performed as described previously.[24] Identifications of primers for mouse *RAGE, MCP-1, adiponectin, 18S ribosomal RNA, 28S ribosomal RNA,* and *TATA-box*

binding protein genes were Mm01134790_g1, Mm004441242_m1 Mm00456425_m1, Hs99999901_s1, Mm03682676_s1, and Mm00446973_m1, respectively.

Oil Red O staining

Intracytoplasmic lipids were quantitated by staining the cells with Oil Red O (Sigma) as described previously.[13]

Test capsules

Composition of MS-containing brown opaque hard gelatin capsule was 100 mg MS powder, 257 mg starch, 7.4 mg calcium stearate, and 5.6 mg micronized silica. Same shape and color placebo capsule included the same quantity of additives but MS powder was replaced with 100 mg starch.

Subjects and clinical study design

Fifty-three apparent healthy, non-diabetic Japanese adults with glycated hemoglobin (HbA1c) values less than 6.5 % (20~64 years old) whose body-mass index was ≥25 but <30 kg/m^2 were recruited into the study. We excluded any subjects with hypertension, hypercholesterolemia (total cholesterol ≥240 mg/dl), liver diseases with aspartate

aminotransferase, alanine aminotransferase (ALT) or γ-glutamyl transpeptidase ≥2.5x upper limit of normal, acute infections, active inflammatory diseases, anemia, pregnancy, lactation, a history of hypersensitivity reactions to test capsules, heart disease or cancer, or who had taken drugs or supplements that could affect blood glucose or insulin levels. During the study period, participants were instructed not to change their life habits. This study was a single center, randomized, double-blind, placebo-controlled clinical trial to examine the effects of oral intake of 500 mg MS (5 capsules) once a day for 12 weeks on insulin resistance and metabolic parameters in overweight adults. Based on the findings in animal experiments, clinical study was designed. The study was conducted in accordance with the Declaration of Helsinki and all procedures were approved by the Institutional Review Board of the Shiba Palace Clinic (Tokyo, Japan). Written informed consent was obtained from all the participants prior to their participation in the study. The trial was registered with the University Hospital Medical Information Network clinical trials database (number UMIN 000031585).

Data collection

Weight and height were measured to calculate body mass index (kg/m^2). Waist circumference was measured at the umbilical level in the late exhalation phase. After at least 5-min rest, BP was measured in supine position using a standard sphygmomanometer.

After overnight fast, peripheral blood was drawn from the antecubital vein. Blood chemistry was analyzed with standard methods by BML, Inc., Tokyo, Japan. Insulin resistance was estimated using the HOMA-IR from fasting plasma glucose and fasting insulin concentrations using the following formula: HOMA-IR=(fasting insulin [μU/ml] × fasting plasma glucose [mg/dl]) /405.

Statistical analysis

All data are presented as mean ± standard deviation. Student's t-test and analysis of variance (ANOVA) followed by Steel-Dwass or Turkey HSD were performed for statistical comparisons among groups in cell culture and animal model experiments, respectively. Treatment groups were compared at baseline by using an unpaired *t*-test. Paired *t*-test was performed for comparisons between baseline and post-treatment. Post-treatment clinical variables were adjusted for baseline values, and statistical differences

between the two groups were analyzed by one-way analysis of covariance, ANCOVA. $p<0.05$ was considered to be statistically significant.

Results

Safety of MS

Acute and chronic oral toxicity studies of MS were performed according to the guidelines of Organization for Economic Co-operation and Development.[27,28] No treatment-related mortality or adverse effects were observed in 5 male ICR mice at 2,000 mg/kg body weight of MS during the 14 day-observation period. Furthermore, there were no signs of toxicity in 5 male and 5 female SD rats received 1,000 mg/kg body weight of MS for 90 days. Ames test also revealed no mutagenic properties of MS. Heavy metals were not detectable in MS powder, while content of arsenic was within the acceptable range (0.6 ppm).

Effects of MS on clinical variables in Fructose-fed rats

We first examined the effects of MS on clinical variables in Fructose-fed rats. There were no differences of spontaneous food intake among three groups (*ca.* 22-23 g/day). As shown in Table 1, random blood glucose, fasting insulin, HOMA-IR, ALT, ratio of

liver weight to body weight in Fructose-fed rats were significantly higher than those of Control rats. Oral administration of MS for 8 weeks significantly decreased random blood glucose, fasting insulin, and HOMA-IR values, but increases in ALT and ratio of liver weight to body weight of Fructose-fed rats were not ameliorated by the treatment with MS.

Effects of MS on AGE-RAGE-oxidative stress system in the adipose tissues of Fructose-fed rats

As shown in Fig. 1*A-C*, levels of AGEs, CML, and RAGE in the adipose tissues of Fructose-fed rats were significantly increased compared with Control rats, all of which were prevented by the treatment with MS. Furthermore, NADPH oxidase activity and 8-OHdG levels in the adipose tissues, and serum and urinary excretion values of 8-iso-PGF2α were significantly higher in Fructose-fed rats than in Control rats (Fig. 1*D-G*). Oral administration of MS for 8 weeks completely inhibited the increases in these oxidative stress markers of Fructose-fed rats.

Effects of MS on macrophage and lymphocyte infiltration, MCP-1 and adiponectin Expression, and remodeling in the adipose tissues of Fructose-fed rats

As shown in Fig. 2*A* and *B*, macrophage and lymphocyte infiltration into the adipose tissues of Fructose-fed rats, evaluated by F4/80 and CCR7 immunostainings,[24,29] were significantly increased compared with Control rats, which were completely blocked by the treatment with MS. Moreover, compared with Control rats, MCP-1 levels in the adipose tissues were significantly higher and adipocyte size grew larger in Fructose-fed rats, while adipose tissue adiponectin expression levels were decreased (Fig. 2*C-E*). Eight-week oral administration of MS completely prevented the MCP-1 overexpression in the adipose tissues as well as adipocyte remodeling and restored the decrease in adiponectin expression of Fructose-fed rats (Fig. 2*C-E*).

Effects of MS on NADPH oxidase activity, ROS generation, *RAGE*, *MCP-1*, and *adiponectin* gene expression, and intracytoplasmic lipid accumulation in AGE-exposed adipocytes

AGEs significantly increased NADPH oxidase activity and ROS generation in differentiated adipocytes, which were inhibited by 0.8, 1.6, and 3.2 μg/ml MS (Fig. 3*A* and *B*). MS at 0.8, 1.6, and 3.2 μg/ml significantly reduced ROS generation in non-glycated control BSA-exposed adipocytes as well (Fig. 3*B*). MS at 0.8 and 1.6 μg/ml, but not at 3.2 μg/ml significantly inhibited basal and AGE-induced *RAGE* gene

expression in adipocytes (Fig. 3*C*). Although AGEs did not affect *MCP-1* gene expression, they significantly decreased adiponectin mRNA levels in AGE-exposed differentiated adipocytes (Fig. 3*D* and *E*). MS at 3.2 μg/ml significantly decreased MCP-1 mRNA levels and restored *adiponectin* gene expression in adipocytes (Fig. 3*D* and *E*). AGEs increased intracytoplasmic lipid accumulation in differentiated adipocytes, which was significantly suppressed by 0.8, 1.6, and 3.2 μg/ml MS (Fig. 3*F*). MS at 3.2 μg/ml also reduced lipid accumulation in BSA-exposed adipocytes (Fig. 3*F*).

An anti-oxidant NAC dose-dependently inhibited the AGE-induced ROS generation, *RAGE* gene expression, and intracytoplasmic lipid accumulation in differentiated adipocytes (Fig. 3*G-I*). Regardless of the presence or absence of AGEs, NAC at 10 or 20 mM significantly reduced ROS generation in adipocytes, while 5~20 mM NAC decreased lipid accumulation. NAC at 20 mM completely inhibited up-regulation of RAGE mRNA levels in AGE-exposed adipocytes. CV had a tendency to inhibit the AGE-induced ROS generation and intracytoplasmic lipid accumulation in differentiated adipocytes in a dose-dependent manner, but the effects were modest and not significant (Fig. 3*J* and *K*).

Effects of MSGL and DGDG on ROS generation and intracytoplasmic lipid accumulation in AGE-exposed adipocytes

Both MSGL at 0.2~1 μg/ml and DGDG at 0.001~10 μg/ml significantly decreased the AGE-induced ROS generation in adipocytes (Fig. 4*A* and *B*). MSGL at 1 μg/ml reduced basal levels of ROS generation in adipocytes (Fig. 4*A*). MSGL at 0.2, 0.4 and 1 μg/ml and DGDG at 0.01~10 μg/ml also significantly suppressed intracytoplasmic lipid accumulation in AGE-exposed adipocytes (Fig. 4*C* and *D*).

Effects of oral intake of MS on metabolic parameters in overweight humans

A clinical study outline is shown in Fig. 5. We screened 53 apparently healthy overweight Japanese adults (25≤ body-mass index <30 kg/m^2) aged 20~64 years old whose HbA1c values were less than 6.5 %. Twenty-three participants met exclusion criteria and were not included in the present study. Thirty subjects were equally randomized to either active (MS-containing capsules) (N=15) or placebo treatment group (N=15). One subject in the placebo group declined to continue to participate in the study. Finally, 29 participants (N=15 in active treatment group and N=14 in placebo treatment group) completed the study. Adherence rates in taking 5 capsules per day were 99.4 % and 99.9 % in active treatment group and placebo group, respectively.

Clinical variables are shown in Table 2. There were no significant differences in clinical variables at baseline except for HDL-cholesterol between active and placebo treatment groups. HDL-cholesterol levels were significantly higher in active treatment group than in placebo group, but individual values of both groups were within the normal range. Oral intake of MS for 12 weeks significantly decreased systolic and diastolic BP, heart rate, fasting plasma glucose, fasting insulin, HOMA-IR, HDL-cholesterol and creatinine values, while blood urea nitrogen and ALT were elevated after placebo capsule treatment (Table 2). There were significant differences in baseline value-adjusted diastolic BP, fasting plasma glucose, fasting insulin, and HOMA-IR at 12 weeks between the two groups; all the parameters were significantly lower in active treatment group than in placebo group. No MS treatment-related adverse effects were observed during the study period.

Discussion

In this study, we showed for the first time that oral administration of spa-derived novel green alga, *Mucidosphaerium* sp., MS for 8 weeks significantly decreased random blood glucose, fasting insulin, and HOMA-IR values in Fructose-fed rats. Treatment with MS for 8 weeks also completely suppressed the increased levels of AGEs, CML, NADPH oxidase activity, and 8-OHdG, a marker of oxidative stress in the adipose

tissues of Fructose-fed rats in association with the reduction of serum and urinary excretion values of 8-iso-PGF2α, another marker of oxidative stress. Moreover, MS inhibited the increase of macrophage and lymphocyte infiltration evaluated by F4/80 and CCR7 immunostainings, MCP-1 expression, and adipocyte size in the adipose tissues of Fructose-fed rats, while it restored the decreased expression levels of adiponectin. High-fat diet has been shown to increase urinary excretion levels of 8-OHdG, gene expression of components of *NADPH oxidase* and *MCP-1*, formation of α-dicarbonyls, precursors of AGEs, and adipocyte cell size in the visceral adipose tissues of mice and reduce adipose tissue adiponectin mRNA levels as well as insulin sensitivity, all of which are inhibited by the treatment of pyridoxamine, a blocker of AGE formation.[30,31] Furthermore, we previously found that serum levels of AGEs were associated with insulin resistance in KK-A^y mice, and pyridoxamine dose-dependently reduced serum AGEs levels and ameliorated insulin sensitivity in these obese and type 2 diabetic animals.[14] In addition, RAGE-deficient or soluble RAGE-treated mice were resistant to high-fat diet-induced metabolic derangements; they were more insulin sensitive, and displayed less macrophage infiltration, smaller adipocyte cell size, lower *MCP-1* and higher *adiponectin* gene expression in the adipose tissues, compared with wild-type non-treated littermates.[32] There is accumulating evidence that macrophage

infiltration and inflammatory reactions in the adipose tissues play a central role in insulin resistance.[30-33] AGE-RAGE interaction evokes inflammatory reactions in various types of cells, including visceral adipocytes via NADPH oxidase-derived ROS generation.[34-36] Moreover, inhibition of NADPH oxidase-derived oxidative stress by pigment epithelium-derived factor has been shown to inhibit macrophage infiltration, adipocyte hypertrophy, and inflammatory reactions and ameliorate dysregulation of adipocytokines and insulin resistance in type 2 diabetic rats with obesity.[37] These observations suggest that MS may improve insulin resistance in Fructose-fed rats partly by inhibiting the AGE-RAGE-induced inflammatory reactions in the adipose tissues via suppression of NADPH oxidase-mediated ROS generation. Since the AGE-RAGE-evoked ROS generation further enhances RAGE expression and AGE accumulation, which could make a vicious cycle,[34,38] MS may break the crosstalk between AGE-RAGE axis and ROS in the adipose tissues.

To further elucidate the underlying mechanism by which MS ameliorated adipose tissue remodeling and dysregulation of adipocytokines in Fructose-fed rats, we performed *in vitro*-experiments using differentiated adipocytes. As was the case for animal experiments, we found here that MS significantly reduced the NADPH oxidase-driven ROS generation, *MCP-1* and *RAGE* gene expression, and lipid accumulation as

detected by Oil Red O staining and simultaneously restored the decreased adiponectin mRNA levels in AGE-exposed adipocytes. We have previously found that anti-oxidants, such as NAC and pigment epithelium-derived factor, not only attenuate the AGE-induced insulin resistance in adipocytes, but also inhibit the effects of AGEs on *MCP*-1 and *adiponectin* gene expression *in vitro*.[13,36] Moreover, in this study, NAC mimicked the effects of MS on ROS generation, *RAGE* gene expression, and lipid accumulation in AGE-exposed adipocytes. Engagement of RAGE with CML has been shown to stimulate adipocyte inflammatory reactions, *RAGE* gene expression, and lipid accumulation in association with the suppression of *adiponectin* gene expression.[39] In addition, obesity is associated with the activation of CML-RAGE axis in human adipose tissues, especially visceral adipose tissues.[38] These findings further support the concept that MS may improve insulin resistance in Fructose-fed rats by blocking the harmful effects of AGEs on adipocytes via inhibition of ROS generation.

In the present study, we also investigated the effects of glycolipids and DGDG on cultured adipocytes because DGDG is one of the most abundant glycolipids in algal membranes and that it has anti-inflammatory properties *in vitro*.[40,41] Since spontaneous food intake was *ca.* 22 g/day in MS-treated rats and that MS contained DGDG (*ca.* 19mg/g MS), if we assume that absorption rate of DGDG is 1 % and that DGDG is

distributed to extracellular fluid volume, plasma concentration of DGDG is estimated to be 0.01 μg/ml. So, we chose the concentration of 0.001~10 μg/ml DGDG in the present experiments. Both MSGL and DGDG mimicked the effects of MS on ROS generation and lipid accumulation in AGE-exposed adipocytes, thus suggesting the pathological role of DGDG in insulin resistance.

In accordance with the results in cell culture and animal model experiments, we found here that compared with placebo, oral intake of MS for 12 weeks significantly decreased diastolic BP, fasting plasma glucose, fasting insulin, and HOMA-IR values in apparent healthy overweight subjects. Since no MS-related side effects were observed in this study, oral intake of MS may be a novel therapeutic strategy for preventing obesity-related insulin resistance in humans.

Limitations

The present study had several limitations. First, in this study, we could not identify active components in MS that might be responsible to the observed effects. DGDG mimicked the effects of MS on adipocytes; it inhibited the AGE-induced ROS generation and lipid accumulation in adipocytes. However, the exact absorption rates of DGDG and effects of DGDG on insulin resistance in animals and humans remain

unclear. Moreover, since the effects of CV that contained 10 mg DGDG/g dry powder on ROS generation and lipid accumulation in adipocytes were modest and not significant, other components than DGDG may contribute to the beneficial effects of MS. Second, we did not know the reason why MS at 3.2 μg/ml did not inhibit basal or AGE-induced *RAGE* gene expression in adipocytes, while lower concentration did (Fig. 3*C*). Alternative techniques, such as western blot analysis and enzyme-linked immunosorbent assay would be helpful to confirm the present findings. Third, in this study, rats were kept in metabolic cages individually for measurement of metabolites in the urine. It could cause depression that affected the present findings. Fourth, orally ingested glycolipids, such as DGDG are rapidly hydrolyzed in the rat small intestine, and released galactosylglycerols are fermented by the colon microbiota to generate short-chain fatty acids,[42] which could also be derived via fermentation of indigestible fibers contained in MS.[43] Therefore, we did not know how much microbiota-generated short-chain fatty acids may contribute to the improvement of insulin resistance in both Fructose-fed rats and overweight subjects. Fifth, in the present study, we examined the effects of MS on insulin resistance in Fructose-fed rats, especially focusing on adipose tissues because the elevations in ALT and ratio of liver weight to body weight in Fructose-fed rats were not ameliorated by the treatment with MS. However, it would be

interesting to examine the effects of MS on insulin sensitivity in the liver and skeletal muscles of Fructose-fed rats. Moreover, due to ethical considerations, it was not possible to obtain adipose tissue samples from study participants. So, we could not assess the effects of MS on AGE-RAGE-oxidative stress system in the adipose tissues of overweight subjects. Although we, along with others, have previously shown the active involvement of AGE-RAGE axis in insulin resistance in animal models and humans,[14,30-32] further basic study using RAGE-aptamer that could block the interaction of AGEs and RAGE is need to clarify the pathological role of AGE-RAGE axis in metabolic derangements.[44,45] Finally, the study population consists of apparent healthy overweight Japanese adults. Accordingly, our clinical results might not be generalized to other populations.

Conclusions

Our present findings suggest that MS may improve insulin resistance by blocking the AGE-RAGE-oxidative stress axis in the adipose tissues.

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Authors' Contributions

S.Y. conceptualized and designed the study, acquired, analyzed, and interpreted data, and drafted the manuscript, and took responsibility for the integrity of the data and the accuracy of the data analysis. K.K. organized and supervised the study. H.K. supervised the clinical study. M.T. designed the clinical study and acquired the data. Y.K., M.S., S.I., M.M., C.K.M., N.Y., W.S., M.F., A.S., and T.M. acquired, analyzed, and interpreted data.

Author Disclosure Statement

Y.K., M.S., S.I., M.M., C.K.M., N.Y., W.S., M.F., M.T., and K.K. were employees of Saravio Cosmetics Ltd.

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FIGURE LEGENDS

Fig. 1. Effects of MS on AGEs, RAGE, and oxidative stress in the adipose tissues of Fructose-fed rats and serum and urinary excretion levels of 8-iso-PGF2α. *A-E*: Adipose tissue AGEs (*A*), CML (*B*), RAGE (*C*), NADPH oxidase activity (*D*), and 8-OHdG levels (*E*). Each upper panel shows representative immunostainings of AGEs (*A*), CML (*B*), RAGE (*C*), and 8-OHdG (*D*) in the adipose tissues. Each lower panel shows the quantitative data. *F*: Serum 8-iso-PGF2α levels. *G*: Urinary excretion levels of 8-iso-PGF2α.

Fig. 2. Effects of MS on inflammatory reactions, MCP-1 and adiponectin expression in the adipose tissues and adipocyte size of Fructose-fed rats. *A-D*: Each upper panel shows representative immunostainings of F4/80 (*A*), CCR7 (*B*), MCP-1 (*C*), and adiponectin (*D*) in the adipose tissues. Each lower panel shows the quantitative data. *E*: Adipose tissue sections were stained with hematoxylin and eosin, and adipocyte size was analyzed. Left panels show representative hematoxylin and eosin-stained adipose tissue sections. Right panel shows the quantitative data.

Fig. 3. Effect of MS or NAC on NADPH oxidase activity (*A*), ROS generation (*B*, *G*, and *J*), RAGE (*C* and *H*), MCP-1 (*D*), and adiponectin mRNA levels (*E*), and intracytoplasmic lipid accumulation (*F*, *I*, and *K*) in AGE-exposed differentiated adipocytes. Differentiated adipocytes were treated with 100 μg/ml AGE-BSA or non-glycated BSA for 1 hours (*A*, *B*, *D*, *G*, and *J*), 2 hours (*E*), 8 hours (*C* and *H*), and 2 days (*F*, *I*, and *K*) in the presence or absence of the indicated concentrations of MS, CV, or NAC. *C-E*, and *H*: Total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of 18S ribosomal RNA (*C* and *H*, 28S ribosomal RNA (*D*)-, or TATA-box binding protein mRNA (*E*)-derived signals and then related to the values obtained with non-glycated BSA alone. *F*, *I*, and *K*: Intracytoplasmic lipid accumulation was quantitated by staining the cells with Oil Red O. Each upper panel shows representative Oil Red O stainings. Each lower panel shows the quantitative data. * and **, $p<0.05$ and $p<0.01$ compared with AGEs alone, respectively. # and ##, $p<0.05$ and $p<0.01$ compared with non-glycated BSA alone, respectively. (*A*) *N*=6 per group. (*B*) *N*=18 per group. (*C*) *N*=8 per group. (*D*, *E*, and *H*) *N*=4 per group. (*F*, *I*, and *K*) *N*=15 per group. (*G*) *N*=27 per group. (*J*) *N*=18 per group.

Fig.4. Effect of MSGL or DGDG on ROS generation (*A* and *C*) and intracytoplasmic lipid accumulation (*B* and *D*) in AGE-exposed differentiated adipocytes. Differentiated adipocytes were treated with 100 μg/ml AGE-BSA or non-glycated BSA for 1 hours (*A*), 4 hours (*C*), and 2 days (*B* and *D*) in the presence or absence of the indicated concentrations of MSGL or DGDG. *A* and *C*: ROS generation was measured with carboxy-H_2DFFDA. *B* and *D*: Intracytoplasmic lipid accumulation was quantitated by staining the cells with Oil Red O. Each upper panel shows representative Oil Red O stainings. Each lower panel shows the quantitative data. * and **, $p<0.05$ and $p<0.01$ compared with AGEs alone, respectively. #, $p<0.05$ compared with non-glycated BSA alone. (*A*) *N*=27 per group. (*B*) *N*=10 per group. (*C*) *N*=18 per group. (*D*) *N*=10 per group.

Fig. 5. A clinical study outline.

Table 1. Characteristics of animals

	Control rats	Fructose-fed rats	Fructose- and MS-fed rats
Number	6	6	8
Body weight (g)	433 ± 11	422 ± 37	413 ± 33
Heart rate (beats/min)	408 ± 43	394 ± 35	386 ± 48
Mean blood pressure (mmHg)	109 ± 10	107 ± 10	101 ± 7
Systolic blood pressure (mmHg)	127 ± 9	124 ± 12	119 ± 10
Diastolic blood pressure (mmHg)	100 ± 11	98 ± 10	93 ± 5
Fasting blood glucose (mg/dl)	157 ± 44	163 ± 17	150 ± 12
Random blood glucose (mg/dl)	131 ± 22	173 ± 21*	136 ± 18††
Fast insulin (μU/ml)	27.1 ± 7.5	40.8 ± 23.7**	23.5 ± 16.6††
HOMA-IR	10.4 ± 4.0	16.7 ± 11.1**	8.5 ± 5.7††
HbA1c (%)	4.9 ± 0.1	4.9 ± 0.1	5.0 ± 0.1
Total cholesterol (mg/dl)	58 ± 4	69 ± 16	66 ± 15
LDL-cholesterol (mg/dl)	20 ± 6	20 ± 4	19 ± 4
Triglycerides (mg/dl)	102 ± 60	140 ± 36	127 ± 62
HDL-cholesterol (mg/dl)	44 ± 8	52 ± 13	49 ± 12
Non-esterified fatty acid (mEq/l)	0.64 ± 0.08	0.63 ± 0.09	0.67 ± 0.07
Blood urea nitrogen (mg/dl)	14.5 ± 1.7	14.2 ± 2.0	13.4 ± 1.5
Creatinine (mg/dl)	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Aspartate aminotransferase (U/l)	60 ± 14	60 ± 18	68 ± 12
Alanine aminotransferase (U/l)	21 ± 4	30 ± 6**	32 ± 14
Kidney weight/body weight (%)	0.45 ± 0.07	0.49 ± 0.03	0.50 ± 0.05
Heart weight/body weight (%)	0.45 ± 0.08	0.48 ± 0.06	0.50 ± 0.04
Liver weight/body weight (%)	2.63 ± 0.22	3.36 ± 0.29**	3.07 ± 0.23
AGEs (U/ml)	6.6 ± 3.1	8.9 ± 4.1	7.8 ± 2.8

Data are presented as mean ± standard deviation.

* and **, $p<0.05$ and $p<0.01$ compared with Control rats, respectively. ††, $p<0.01$ compared with Fructose-fed rats. HOMA-IR; homeostasis model assessment of insulin resistance, HbA1c; glycated hemoglobin, LDL-cholesterol; low-density lipoprotein cholesterol, HDL-cholesterol; high-density lipoprotein cholesterol, AGEs; advanced glycation end products.

Table 2. Clinical variables of active and placebo treatment groups

	Active treatment group (n=15; 7 males and 8 females)		Placebo treatment group (n=14; 8 males and 6 females)	
	baseline	post-treatment	baseline	post-treatment
Age (years old)	45.7 ± 9.6	46.0 ± 9.6	46.1 ± 10.6	46.4 ± 10.6
Body weight (kg)	75.8 ± 9.5	76.2 ± 9.5	73.5 ± 7.6	74.4 ± 7.9
Body mass index (kg/m^2)	27.1 ± 1.3	27.3 ± 1.8	27.1 ± 1.3	27.4 ± 1.7
Waist circumference (cm)	90.5 ± 7.7	89.0 ± 7.7	86.9 ± 3.6	87.4 ± 4.7
Systolic blood pressure (mmHg)	121 ± 18	116 ± 11*	121 ± 9	119 ± 11
Diastolic blood pressure (mmHg)	75.4 ± 11.7	69.7 ± 8.6**§	73.2 ± 9.8	74.1 ± 9.7
Heart rate (bpm)	77.5 ±15.6	71.8 ± 11.1*	76.9 ± 12.9	75.2 ± 12.7
Fasting plasma glucose (mg/dl)	94.5 ± 12.3	89.3 ± 8.9*§	92.6 ± 6.6	92.4 ± 8.5
Fasting insulin (μU/ml)	7.28 ± 3.44	5.91 ± 2.41*§	7.71 ± 4.67	8.01 ± 3.27
HOMA-IR	1.75 ± 1.07	1.32 ± 0.65*§	1.79 ± 1.12	1.85 ± 0.82
HbA1c (%)	5.5 ± 0.4	5.5 ± 0.4	5.5 ± 0.4	5. 6 ± 0.4
Total cholesterol (mg/dl)	203 ± 21	199 ± 22	199 ± 21	206 ± 26
LDL-cholesterol (mg/dl)	127 ± 26	124 ± 24	135 ± 24	138 ± 30
Triglycerides (mg/dl)	109.9 ± 31.1	110.3 ± 37.5	91.4 ± 43.2	99.9 ± 43.4
HDL-cholesterol (mg/dl)	63.9 ± 10.3†	60.4 ± 8.4*	55.6 ± 11.4	54.4 ± 10.1
Blood urea nitrogen (mg/dl)	11.7 ± 3.1	11.9 ± 2.8	12.7 ± 3.4	14.0 ± 3.5#
Creatinine (mg/dl)	0.73 ±0.13	0.71 ± 0.13*	0.77±0.23	0.76 ±0.24
Uric acid (mg/dl)	5.8 ± 1.0	5.6 ± 0.8	6.0 ± 1.2	5.9 ± 1.2
Aspartate aminotransferase (U/l)	20.7 ± 6.0	22.5 ± 11.0	19.9 ± 4.1	21.6 ± 5.2
Alanine aminotransferase (U/l)	18.8 ± 6.7	22.8 ± 13.4	21.1 ± 12.1	25.0 ± 11.0#
γ-glutamyl transferase (U/l)	29.1 ± 13.5	37.4 ± 40.4	30.1 ± 20.3	29.7 ± 15.9

Values are shown as means ± standard deviation. Treatment groups were compared at baseline by using an unpaired *t*-test. Paired *t*-test was performed for comparisons between baseline and post-treatment. Post-treatment clinical variables were adjusted for baseline values, and statistical differences between the two groups were analyzed by one-way analysis of covariance. * and **, $p<0.05$ and $p<0.01$ compared with baseline values of active treatment group, respectively. #, $p<0.05$ compared with baseline values of placebo treatment group. † and §, $p<0.05$ compared with baseline and post-treatment values of placebo group, respectively. HOMA-IR; homeostasis model assessment of insulin resistance, HbA1c; glycated hemoglobin, LDL-cholesterol; low-density lipoprotein cholesterol, HDL-cholesterol; high-density lipoprotein cholesterol.

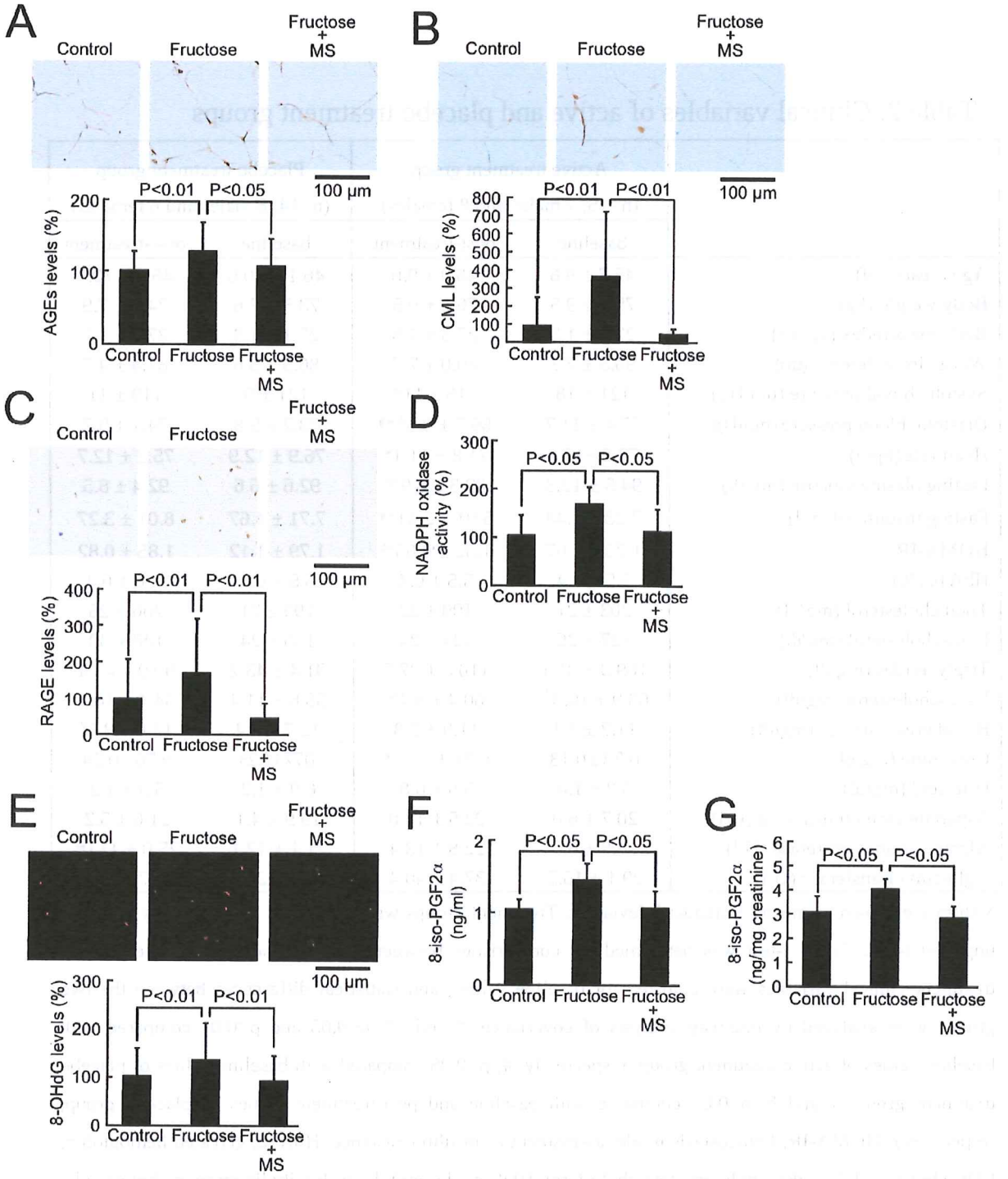

A
Control
Fructose
Fructose + MS
100 μm
AGEs levels (%)
P<0.01
P<0.05
B
CML levels (%)
P<0.01
P<0.01
C
RAGE levels (%)
P<0.01
P<0.01
D
NADPH oxidase activity (%)
P<0.05
P<0.05
E
8-OHdG levels (%)
P<0.01
P<0.01
F
8-iso-PGF2α (ng/ml)
P<0.05
P<0.05
G
8-iso-PGF2α (ng/mg creatinine)
P<0.05
P<0.05

Fig.1

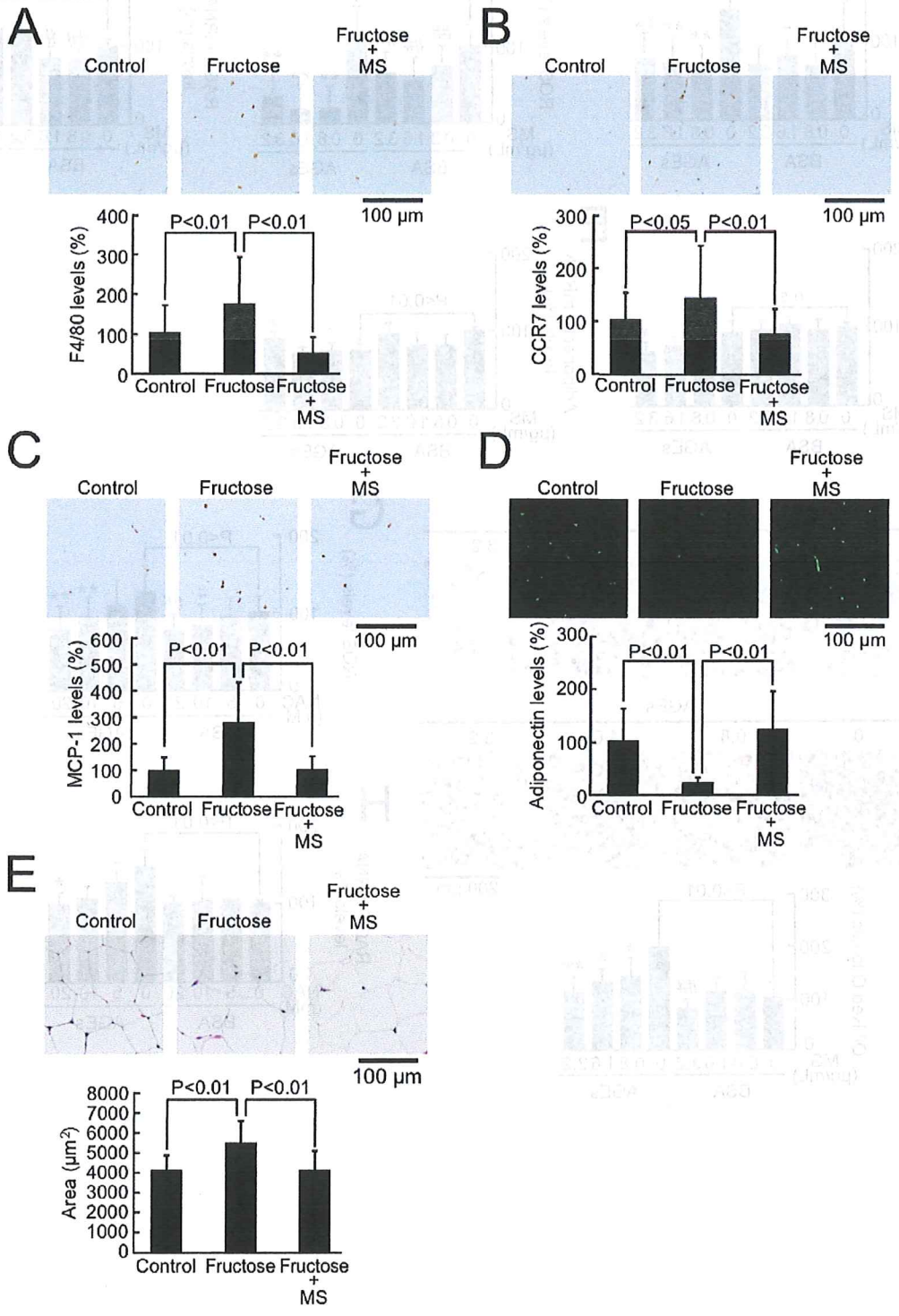
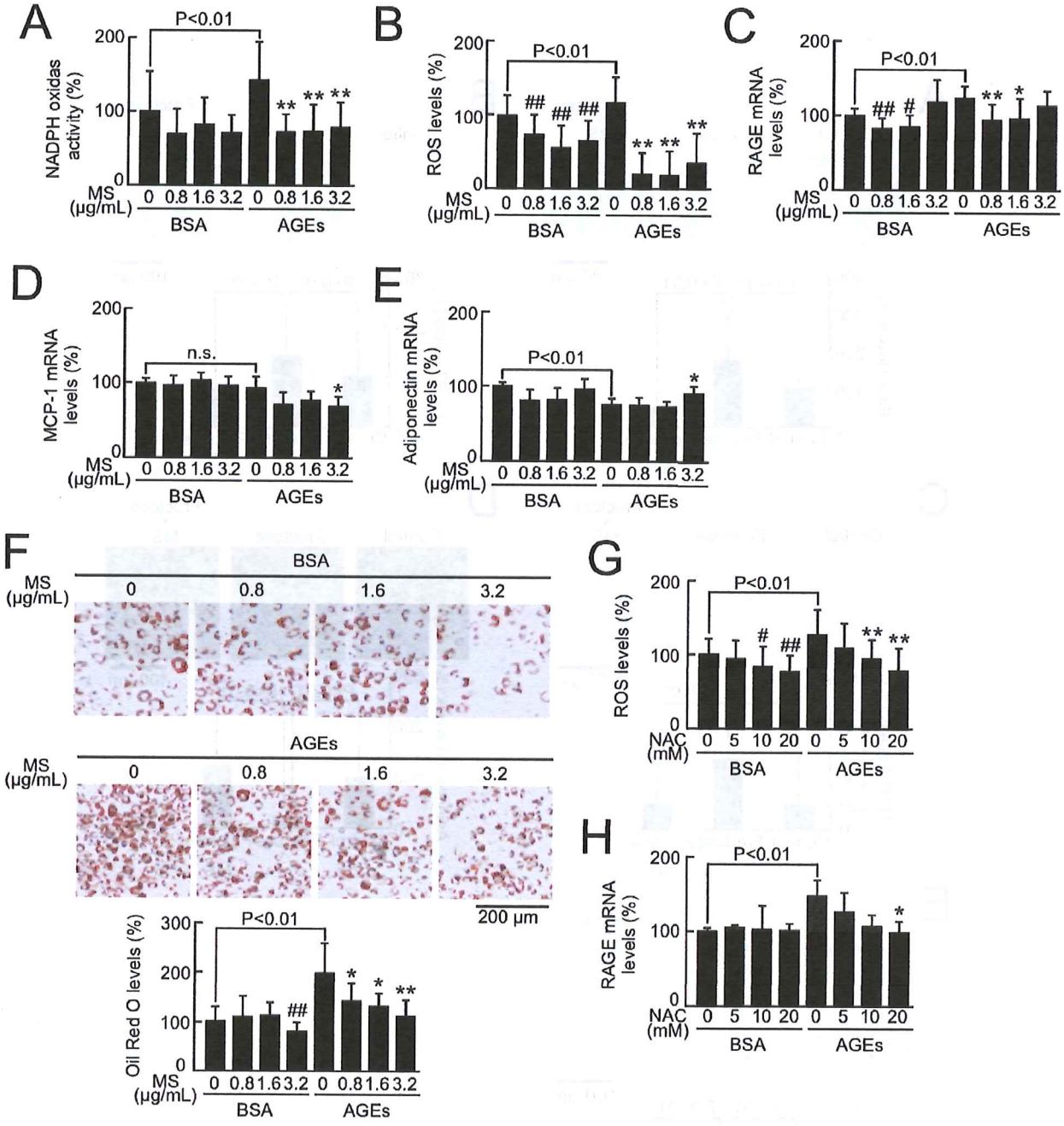

A
Control
Fructose
Fructose + MS
100 μm
F4/80 levels (%)
400
300
200
100
0
P<0.01
P<0.01
Control
Fructose
Fructose + MS
B
Control
Fructose
Fructose + MS
100 μm
CCR7 levels (%)
300
200
100
0
P<0.05
P<0.01
Control
Fructose
Fructose + MS
C
Control
Fructose
Fructose + MS
100 μm
MCP-1 levels (%)
600
500
400
300
200
100
0
P<0.01
P<0.01
Control
Fructose
Fructose + MS
D
Control
Fructose
Fructose + MS
100 μm
Adiponectin levels (%)
300
200
100
0
P<0.01
P<0.01
Control
Fructose
Fructose + MS
E
Control
Fructose
Fructose + MS
100 μm
Area (μm²)
8000
7000
6000
5000
4000
3000
2000
1000
0
P<0.01
P<0.01
Control
Fructose
Fructose + MS

Fig.2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NADPH oxidas activity (%)
P<0.01
MS (μg/mL)
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BSA
AGEs
B
ROS levels (%)
P<0.01
C
RAGE mRNA levels (%)
P<0.01
D
MCP-1 mRNA levels (%)
n.s.
E
Adiponectin mRNA levels (%)
P<0.01
F
BSA
AGEs
200 μm
Oil Red O levels (%)
P<0.01
G
ROS levels (%)
P<0.01
NAC (mM)
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H
RAGE mRNA levels (%)
P<0.01

Fig.3

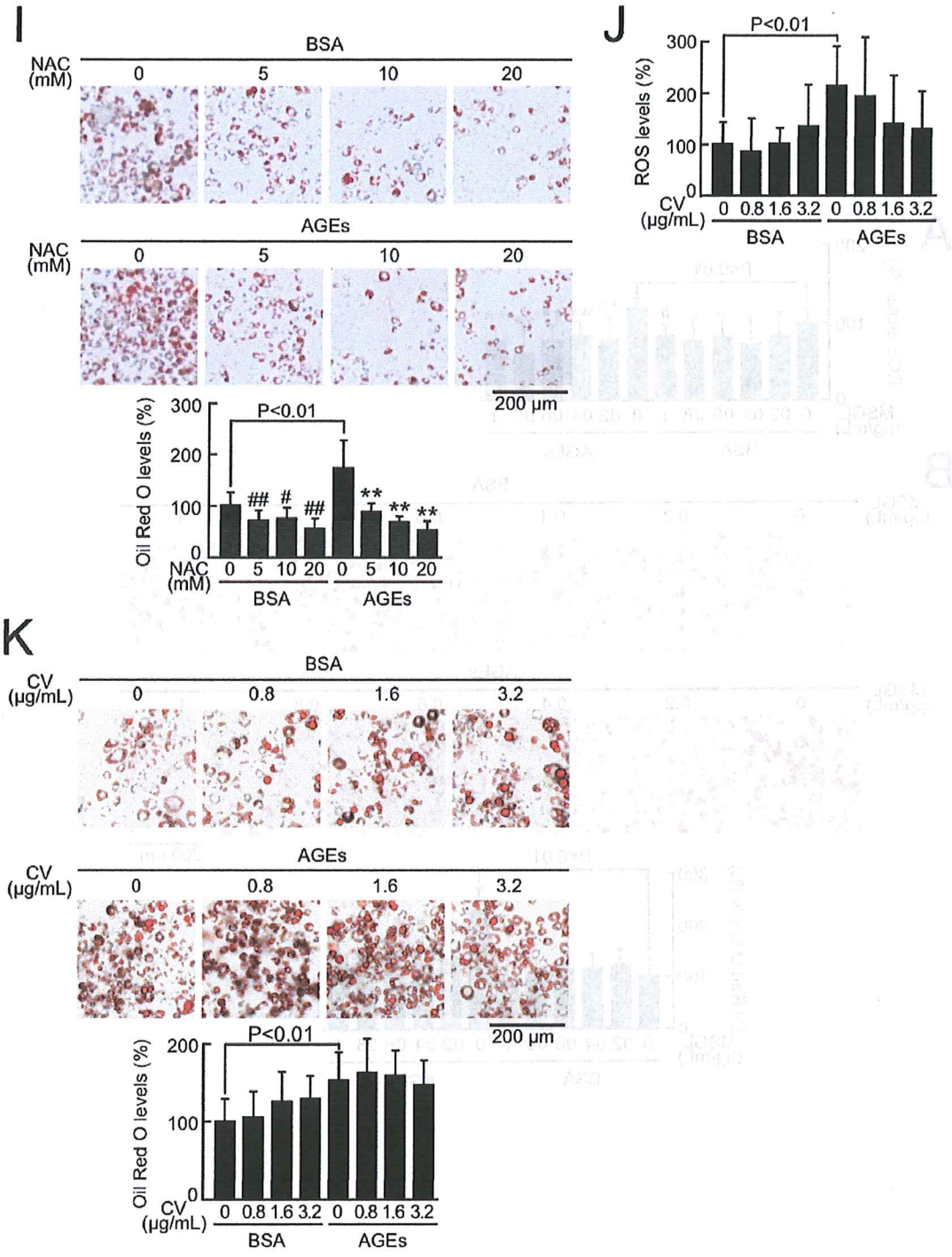

Fig.3 (continued)

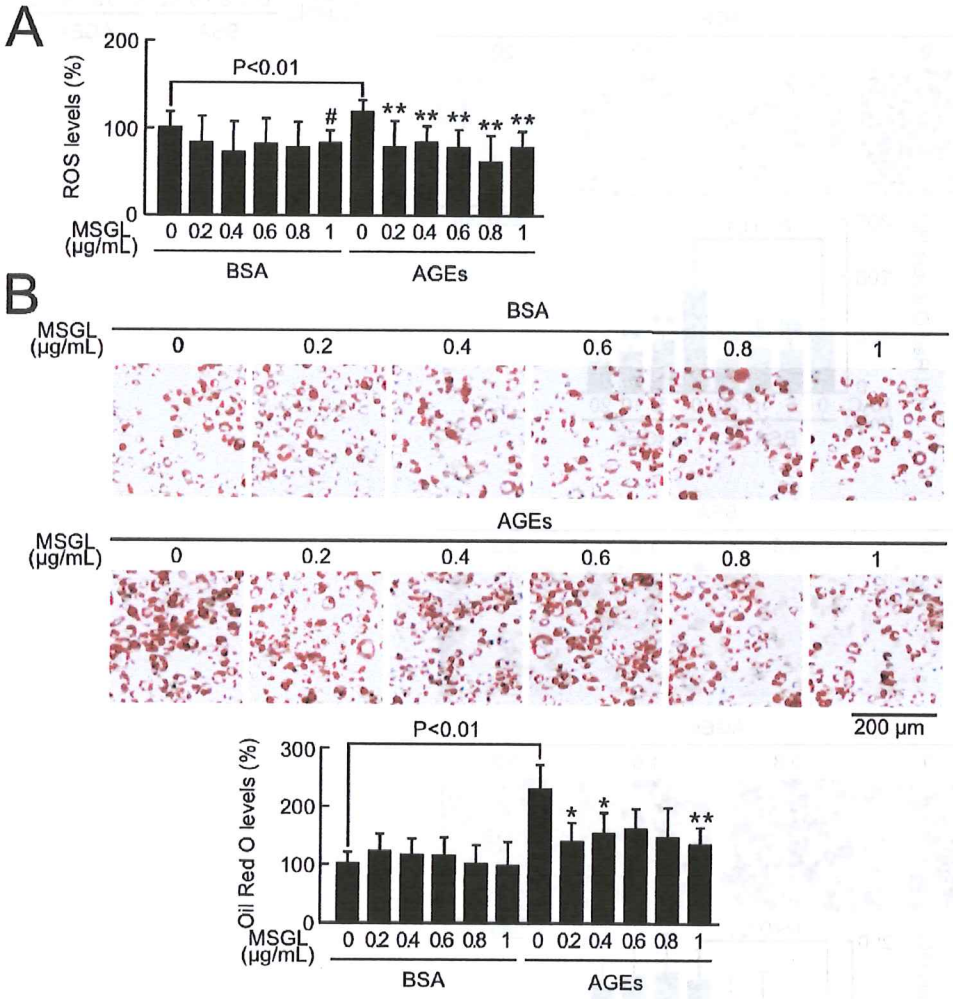
A
ROS levels (%)
200
100
0
P<0.01
#
**
MSGL (μg/mL)
0 0.2 0.4 0.6 0.8 1 0 0.2 0.4 0.6 0.8 1
BSA
AGEs
B
BSA
MSGL (μg/mL)
0
0.2
0.4
0.6
0.8
1
AGEs
200 μm
Oil Red O levels (%)
300
200
100
0
P<0.01
*
**

Fig.4

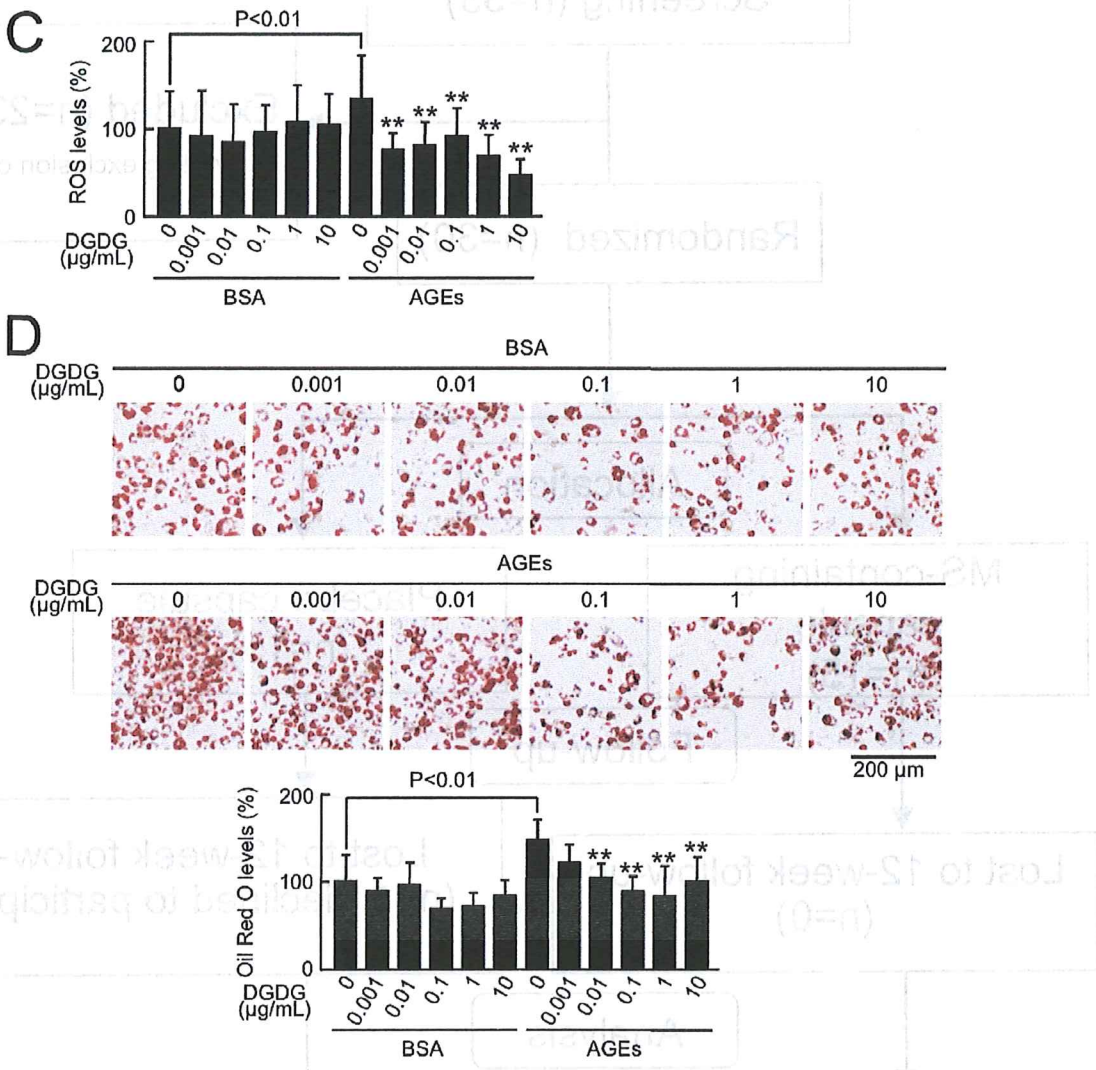

Fig.4 (continued)

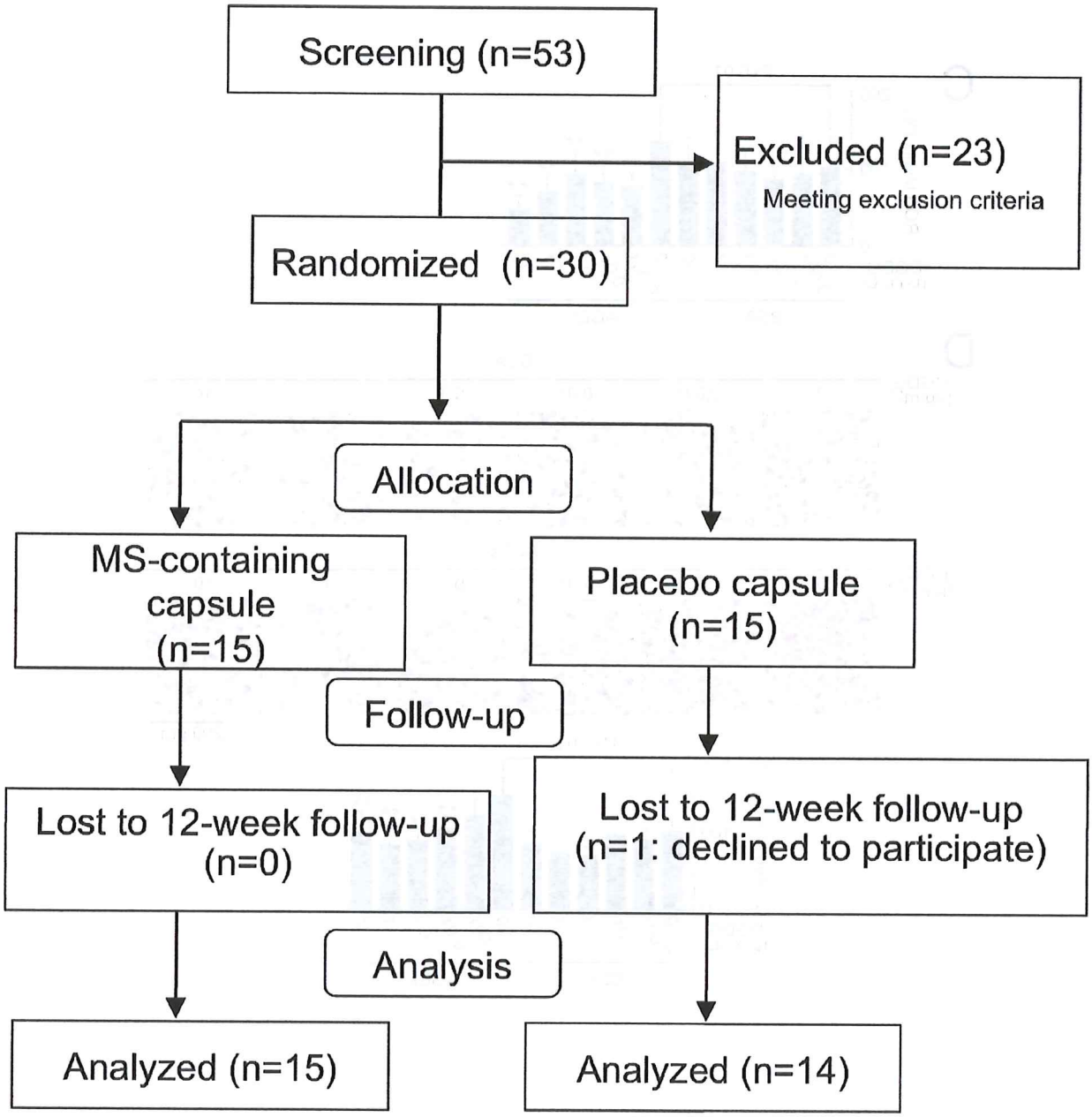
Screening (n=53)
Excluded (n=23)
Meeting exclusion criteria
Randomized (n=30)
Allocation
MS-containing capsule (n=15)
Placebo capsule (n=15)
Follow-up
Lost to 12-week follow-up (n=0)
Lost to 12-week follow-up (n=1: declined to participate)
Analysis
Analyzed (n=15)
Analyzed (n=14)

Fig.5

Author Disclosure Statement

Y.K., M.S., S.I., M.M., C.K.M., N.Y., W.S., M.F., M.T., and K.K. were employees of Saravio Cosmetics Ltd.