

Glycosaminoglycan derived from field cricket and its inhibition activity of diabetes based on anti-oxidative action

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Running title: Anti-oxidative effect of cricket glycan in db mice

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Abstract: Field cricket (*Gryllus bimaculatus*) is newly emerged as an edible insect in several countries. Anti-inflammatory effect of glycosaminoglycan derived this cricket was not fully investigated on chronic disease animal model such as diabetic mouse. For potential therapeutic agents, anti-diabetic activities of field cricket glycosaminoglycan (GbG) was evaluated in diabetic mice based on their abilities to reduce glucose, ALT, AST, LDL-cholesterol, and BUN levels, compared with dung beetle (*Catharsius molossus*) glycosaminoglycan (CaG) as a positive control glycosaminoglycan. Db mice were orally administered for one month according to their groups: Db Hetero (normal), Db Homo (type-2 diabetic), CaG (5 mg/kg), GbG (5 mg/kg), and metformin (10 mg/kg). Blood glucose level was decreased after 1st week treatment with GbG. It also inhibited LDL-cholesterol and alkaline phosphatase levels. Regarding oxidative damage of diabetic state, levels of hepatocellular biomarkers levels and protein carbonyl content were reduced in db mice treated with GbG. Especially anti-oxidative activities of catalase, superoxide dismutase, and glutathione peroxidase were significantly increased in GbG treated group compared to those in the control. GbG was composed of heparin disaccharides and main N-glycan was identified as Hex₉GlcNAc₂ (m/z 1905.7) of with neutral mono-sugar mainly comprising of hexose, L (+) rhamnose by mass spectroscopy. These results from sero-biochemical, hepatocellular anti-oxidant assay in db mice data suggest cricket (*G. bimaculatus*) glycosaminoglycan might play a role in its anti-diabetic action.

Keywords: cricket glycosaminoglycan, N-glycan, homo db mice, anti-oxidant enzyme.

1. Introduction

The custom of eating of insects has come to be spread worldwide. Edible crickets acquired from industrial rearing systems are an alternative food increasingly used in countries including the Netherlands and several others. *Gryllus bimaculatus* (field cricket, Gb) water extract has been used in Oriental medicine as a crude drug for an antifebrile and lowering agent for high blood pressure. Recently, this extracts from this cricket, have the lowering activity of blood ethanol metabolite concentrations by enhancing the liver mitochondrial alcohol and acetaldehyde dehydrogenases [1] and anti-obesity effect through the inhibition of adipose tissue accumulation in high fat diet rats [2]. Recently, we found a significant report on showing good result effects by consuming crickets: edible cricket consumption on gut microbiota in healthy adults [3]. We are concerned about the type-2 diabetes is related with oxidative stress and influenced vascular disease such as a chronic disease, atherosclerosis, hypertension and nephropathy [4].

The antioxidant and anti-inflammatory activities of enzymatic hydrolysates and peptide fractions from selected heat-treated edible insects including cricket have been ascertained [5] with exception of other carbohydrate components of field cricket. Indeed, all human cells were coated with an array of glycoproteins, glycolipids and polysaccharides named glycocalyx, the surface proteoglycan/glycoprotein layer [6]. Therefore, glycans can appeal distinct properties as biomarker targets [7]; especially N-glycans are also ubiquitous in nature

1 providing structural and functional stability to N-linked glycoprotein, with flexibility [8].

2 In the overview of a total insect component survey concept, as one of the functional
3 components, a mucin polysaccharide, glycosaminoglycan as an ingredient, is required to
4 standardize and to manufacture not from a versatile natural source but from the same
5 conditions of insect rearing. The distinctive role of Gb glycosaminoglycan has been reported
6 as an anti-inflammatory effect in arthritis induced rat model [9], anti-obesity [10] and
7 antilipidemic [11] effects in a high-fat diet. Nowadays, some glycosaminoglycans so retained
8 anti-oxidant activity robust enough for that they scavenge free radicals thus repaired cellular
9 oxidative damages [12].

10 We commonly use db/db mouse model to conduct research in this experiment on type 2
11 diabetes mellitus and its comorbidities including obesity and hypertension [13]. Hence, there
12 are some reports that a kind of glycosaminoglycan, heparin sulfate, plays a crucial role in
13 proliferation, development and maturation of beta-cells thereby contributing to normal
14 glucose hemostasis [14] and mouse β cell survival in the autoimmune type 1 diabetes [15].
15 Here, this *G. bimaculatus* glycosaminoglycan with anti-oxidant activities endeavored to
16 contribute to anti-diabetic activities thus to repair cellular oxidative damage in protein
17 carbonyl level and anti-oxidant enzyme induction ratios after treatment in db/db mice.

18 As a comparable insect glycosaminoglycan sample, following to a previous report, the
19 dung beetle (*Catharsius molossus*, Ca) GAG with marked anti-aging activity by reducing

oxidative damage in aged rats [16], was used as a positive control. In this work, we also characterized purified N-glycans throughout deglycosylation of GbG and analysis by MS and MS/MS (MALDI TOF MS). In this study, we found that field cricket revealed elements constituting glycosaminoglycan and displayed anti-oxidative activity for use as an anti-diabetic agent with reducing cellular oxidative damages.

2. Materials and Methods

2.1.Preparation of field cricket glycosaminoglycan

Field cricket (*Gryllus bimaculatus*) was supplied from a cricket farm located in Hwasung, South Korea. These insects were freeze-dried, in the Department of Agricultural Biology, National Academy of Agricultural Science (NAAS), South Korea. Dried dung beetle (*C. molossus*) was purchased at a local market in China by Chinese phamacognosist and posted to NAAS.

These each insect glycosaminoglycan was purified by previous reported method [17] using removal of fat by ethanol and acetone, protein enzymatic hydrolysis by putting Alcalase (Sigma Aldrich, St. Louis, Mo., USA), protein precipitation by trichloroacetic acid (5%), impure materials cleansing with detergent, cetylpyridinium chloride (5%), dissolving of non-glycosaminoglycan with 2.5 M NaCl. We added five volumes of ethanol followed by centrifugation at 8000 g for 30 min. Thus the precipitate was then dissolved in water and then dialyzed against 100 volumes of water and freeze-dried. We loaded crude field cricket or

1 dung beetle GAG onto a DEAE Sephadex A-25 gel chromatography column to be
2 equilibrated with 50 mM phosphate buffer (pH 7.4) using a linear sodium chloride gradient
3 from 0 to 2.5 M NaCl. Dialyzed these glycosaminoglycan was freeze-dried to obtain pure
4 GAG. Main peak of digested GbGAG by co-incubation with heparinase I, II and III was
5 pooled using strong anion exchange (SAX) column (Phenomenex, 5 m, 250 x 10.0 mm,
6 Torrance, CA, USA) by high performance liquid chromatography (HPLC), determined its
7 purity and compared with heparin disaccharides or carbohydrate standards by ESI (POS
8 polarity) Mass Spectrometer (SYNAPT G2, Waters, U.K.) chromatogram (Time of flight
9 analyzer 50 ~ 1,500 m/z).

10 **2.2. N-glycan preparation derived from insect glycosaminoglycan**

11 A purified cricket glycosaminoglycan DEAE Sephadex A-25 gel 0.5 M NaCl fraction (GbG
12 0.5M) was further purified in order to obtain a low molecular weight, N-glycan by enzymatic
13 release, and then enrichment of N-glycan via solid-phase extraction. This GbG was denatured
14 by handling of rapid treatment at 100 °C, for 10 minutes in an aqueous solution of 100 mM
15 ammonium bicarbonate with 5 mM dithiothreitol. After cooling, 2.0 µL (or 1000 U) of
16 peptide N-glycosidase F (PNGase F) (New England Biolabs, Ipswich, MA, USA) was added in
17 order to release N-glycans. The mixture was then incubated in a 37 °C water bath for 16
18 hours. Graphitized carbon cartridges (Extract clean™ carbo, carbograph, Grace Davison
19 discovery Sciences, Deerfield, IL, USA) were then washed with 80% acetonitrile/0.10%

trifluoroacetic acid (v/v) and conditioned with water. Released N-glycans were loaded onto these cartridges thus washed with pure water so as to remove any salt of buffer residues. Then, dried N-glycan were suspended in 25% acetonitrile/0.05% trifluoroacetic acid in water (v/v) (acidic fraction) and mixed well. N-glycan derived GbG were chromatographed at a condition of 50% acetonitrile eluent with 2,5-dihydroxybenzoic acid (DHB) as a Matrix to MS and MS/MS analysis (Matrix assisted laser ionization (MALDI) Time of flight (TOF) analyzer (AXIMA Resonance, Shimadzu) in positive polarity. A quadrupole ion trap was acquired in a positive ion mode over a mass range of m/z 100–2000 with an 800~5000 m for N-glycan detection from GbG.

2.3. Neutral monosugar composition of digested N-glycan on the basis of GC-MS analysis

To induce the trimethylsilylation of samples, 10 mg of isolated Gb N-glycan in 100 μ L was hydrolyzed by 1N HCl for 10 minute period, then dried with N_2 gas. Accordingly, the evaporated samples were added to 200 μ L pyridine, and 120 μ L *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (Sigma Co., USA), reacted for 30 min at 65°C, and then injected into the GC-MS. In order to confirm the target components, we made a TMS mono, di-carbohydrate standard mixture, and further blended the dried standard mixture with a derivative sample of the TMS standard mixture. GC-MS analysis was executed on the basis of Agilent 6890 Gas Chromatograph coupled to a 5973N Mass-Selective Detector (MSD) with a HP-5ms capillary column (Agilent, Santa Clara, USA). MS acquisition parameters included scanning from m/z 30 to 600 in electron impact (EI) mode

2.4. Animals

Two kinds of db mice, BKS.Cg-M^{+/+} Lepr^{db}, heterozygous (normal) and homozygous (diabetes) male db mice at 12-weeks of age, were purchased from Samtako Co. Ltd. (Osan, Korea). All procedures were accorded with NIH Guidelines for Care and Use of Laboratory Animals. All experiments were approved (approval number: NIAS 201605) by Laboratory Animals' Ethical Committee of the National Academy of Agricultural Science, Rural Development Administration, Republic of Korea. Mice were acclimated for 6 months under normal husbandry conditions. They were provided free access to normal diet (D10001, AIN-76A rodent diet, Research Diet Inc., New Brunswick, NJ, USA) and water *ad libitum*. These mice were allocated into two (negative and positive) control groups and two groups with each GAG treatment (11 mice per group). They were distributed according to similarity in weight (27.86 ± 1.14 g in DB-Hetro, 46.73 ± 4.73 g in DB-Homo). Treatments were offered in phosphate buffered saline daily. Each treatment was administrated intraperitoneally. The following treatment groups were used: 1) normal (DB-Hetero), 2) control (DB-Homo), 3) 5 mg/kg treatment of CaG (CaG5), 4) 5 mg/kg treatment of GbG (GbG5), 5) 10 mg/kg treatment of Metformin (Metformin10). Mice in each group were maintained with normal diet (AIN-76A rodent diet, Research Diet). Animal experimental design is shown in Figure.1.

2.5. Body weight and blood glucose detection

Body weights were measured per every week. Blood glucose levels were recorded weekly

with glucose stick by using a blood glucose Nocodingone detector (Theragenetex.Co., Sunngnam, Korea). Serum glucose levels were recorded by means of an auto-analyzer on the last day of the treatment schedule, after one-month of treatment was recorded using an auto-analyzer. Metformin was used as a positive control drug to retain an antidiabetic effect [18].

2.6. Organ and Adipose Tissue Weights

Absolute and relative weights (organ-to-body ratio) were measured for adrenal glands, kidneys, heart, liver, lung, spleen, stomach and pancreas. Abdominal fat to-body weight ratio was also determined. These measurements were made after sacrifice at the end of the one-month treatment period.

2.7. Blood sampling and serum assay

After treatment with CaG5, GbG5, or metformin treatment, for 1-month, all non-diabetic heterozygous control mice, db control mice, and db treated mice were sacrificed for serum assay. Then approximately 1 ml of blood was collected from the posterior vena cava under light CO₂ inhalation and used for serum chemistry measurements. The following parameters were examined: albumin, hyaluronic acid (HA), free fatty acid (FFA), alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (AST), glutamic pyruvic transaminase (ALT), lactic dehydrogenase (LDH), creatinine phosphokinase (CK), glucose, total cholesterol

triglyceride, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, creatinine, blood urea nitrogen (BUN), total protein, sodium (Na), chloride (Cl), c-reactive protein (CRP), calcium (Ca), potassium (K), total IgE, C-peptide and insulin. These parameters were evaluated by means of an auto analyzer (Hitachi 7060 Automatic Clinical Analyzer, Tokyo, Japan).

2.8. Oxidative protein damage

Liver homogenate and blood were centrifuged (8000 g for 30 min). Supernatants were used to determine of carbonyl content. Protein oxidative stress was evaluated by measuring protein carbonyl content in the blood. Carbonyl content was determined with an enzyme-linked immunosorbent assay (ELISA) by means of OxiSelect™ protein carbonyl ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's protocol. Catalase, CAT activity (U/mg protein) was measured on the basis of CAT-mediated decomposition of H₂O₂ [19].

2.9. Liver homogenate in preparation for oxidative enzyme detection

Five groups (DB-Hetero, DB-Homo, CaG5, GbG5, Metformin10) of liver tissues were homogenized on ice in a 10-fold volume lysis buffer PRO-PREP™ protein extraction solution (iNtRON, Busan, Korea). The supernatant of each liver homogenate after

centrifugation (800 g, 10 min) was assayed for catalase, glutathione peroxidase, glutathione s-transferase and superoxide dismutase activities [20] using OxiSelect™ ELISA kit (Cell BioLabs, Inc., San Diego, CA, USA) according to the assay manual.

2.10. Endothelial Nitric oxide synthase, laminin and VEGF on diabetic endothelial cells

Vasorelaxation and growth factor were also measured in adult diabetic type2 microvascular endothelial cells (D- HMVECs) were obtained from type 2 diabetics patients (Clonetics™, diabetic type II, Lonza CC-2928, Cambrex, Walkersville, MD, USA). Cells were grown in an endothelial cell basal medium (EBM)-2 with EGM-2 singlequots (Cambrex) at 37°C in an atmosphere containing 5% CO₂. Cells were pretreated with 0.2 mg/ml of either GAG or Pravastatin were incubated prior to the determination of endothelial nitric oxide synthase (eNOS) [21] and Vascular endothelial growth factor (VEGF) [22] (Quantikine, R&D Systems, Inc., Minneapolis, MN, USA) as according to the manufacturers' instructions. The laminin level in endothelial cells was measured using Quantimatrix™ human laminin ELISA kits (Millipore, Billerica, MA, USA) in HMVEC cells or in upper mentioned hepatocytes according to the manufacturer's instructions. Positive controls were Paravastatin (CJ Healthcare Co., Seoul Korea) and Chitosan (Sigma Co., USA).

2.11. Adipocyte density and pathological observation

The excised organs were included kidneys, heart, liver, lung, spleen, stomach, pancreas, testis and adipose tissue to be fixed 10% neutral formalin. After paraffin embedding, they were stained with hematoxylin and eosin, and Toluidine blue O, examined by light microscopy (Leica CTR6000, Hesse, Germany), and photographed. Adipocyte density (cells/mm²) was determined in treated and control tissue by toluidine blue O stain (original magnification, x400).

2.12. Statistical Analysis

Means and standard errors of parameters were determined for each group through the analysis of variance (ANOVA). Student's *t*-test was applied to determine significant differences between control and treated groups. A *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Yield of field cricket glycosaminoglycan

From 1 kg of each dried insect, the yield of freeze-dried GAG powder was about 1.52 g for CaG and 3.4 g for GbG

3.2. Body weight and abdominal fat detection

There were no significant differences in total mean body weight between the control and treatment groups at one week after the beginning of treatment: DB-Hetero, 27.9 ± 0.9 g; DB-Homo, 46.8 ± 7.3 g; CaG5, 46.7 ± 6.2 g; GbG5, 46.9 ± 4.8 g; and Metformin 10, 46.5 ± 4.0 g. The body weights at the end of five weeks (about one month period) of treatment were as follows: DB-Hetero, 31.3 ± 1.2 g; DB-Homo, 53.1 ± 5.5 g; CaG5, 45.9 ± 4.3 g; CaG5, 48.6 ± 4.2 g; and Metformin 10, 45.9 ± 3.0 (Metformin10 vs CON, $p < 0.05$) (Figure 2A). The abdominal fat weight (g) of the treated GAG group over an one month, showed significant differences from that of the DB-Hetero (normal db mice) group but was no significant difference from that of the DB-Homo group: DB-Hetero, 0.50 ± 0.23 ; DB-Homo, 2.72 ± 0.90 ; CaG5, 2.72 ± 0.59 ; GbG5, 2.79 ± 0.43 ; Metformin 10, 2.79 ± 0.37 .

3.3. Effects of CaG5 on blood glucose level

The blood glucose levels on heterozygous (normal) db mice were about 182.30 mg/dL. The glucose levels on homozygous db mice were shown to be increased with aging, proved from 486.5 mg/dL at the age of 12 weeks to 578.2 mg/dL at the age of 17 weeks. The second week glucose levels in the treatment groups were as follows: DB-Hetero (normal), 192.60 ± 25.34 mg/dL; DB-homo, 524.42 ± 35.75 mg/dL; CaG5, 539.25 ± 46.88 mg/dL; GbG5, 523.55 ± 43.46 mg/dL; and Metformin 10, 540.08 ± 48.16 mg/dL (Figure 2B). Early GbG treatment in the second week showed the lowering effect on the blood glucose level in db mice but long GbG

treatment until the fourth week did not turn out to have antidiabetic effect on blood glucose level.

3.4. Sero-biochemical finding of db mice after treatment with GbG

Serum albumin, alkaline phosphatase (ALP), ALT (GPT) and AST (GOT) levels in CaG- and GbG- treated groups were lower than those in the control group in one month after treatment yet, this trend was not statistically significant (Figure 2C). The BUN levels in GbG-treatment db mice (mg/dL) were also significantly lower than those in the control group: CON (DB-Homo), 34.45 ± 2.15 mg/dL; CaG5, 23.60 ± 1.47 mg/dL (CaG5 vs CON, $p < 0.05$); GbG5, 26.0 ± 0.9 mg/dL; Metformin10, 23.57 ± 2.31 mg/dL (Metformin10 vs CON, $p < 0.05$). Further creatinine levels were also slightly decreased thereby sample treatment: DB-Hetero, 0.23 ± 0 mg/dL; DB-Homo, 0.37 ± 0.01 mg/dL; CaG5, 0.32 ± 0.04 mg/dL; GbG5, 0.36 ± 0.01 mg/dL; and Metformin10, 0.32 ± 0.06 mg/dL (Figure 2D). Other detected sero-parameters in this study did not be shown any difference compared to CON.

3.5. Decrease in oxidative damages

Protein carbonyl concentrations in blood was shown quite decrease along with treatment with insect glycosaminoglycans. Decreased levels (nmol/mg protein) were found in DB-Hetero, 8.39 ± 0.24 nmol/mg protein; DB-Homo, 7.25 ± 0.17 nmol/mg protein; CaG5,

5.91±0.56 nmol/mg protein (CaG5 vs. CON, $p < 0.05$); GbG5, 5.91±0.58 mg/dL (GbG5 vs. CON, $p < 0.05$); and Metformin10, 6.74±0.71 mg/dL, respectively (Fig. 3A). However, we cannot find statically significant differences in the case of hepatocyte carbonyl contents between the control and treatment groups of db mice (Figure 3A and 3D). As for blood cellular oxidative damage, protein oxidative damage was also reduced (CaG5, 81.5; GbG5, 81.5% and Metformin10, 93.0%) by these GaGs based on blood neutrophil carbonyl content.

3.6. Oxidative enzyme (catalase, GPx, GST, SOD) quantitation

We also found Figure 3B showed the Catalase, GPx and GST increase levels of db mice treated with GbG for a month. Catalase activities (mg protein/min) in db mice blood after one month of GAG treatment were as follows: db mice (CON), 32.18±1.18; CaG5, 35.6±1.65 (CaG5/CON: 110.6%, CaG5 vs. CON, $p < 0.05$); GbG5, 36.97±1.95 (114.9%, GbG5 vs. CON, $p < 0.05$); and Metformin10, 32.92±1.46 (Met/CON: 102.2%). Catalase activities in all GAG-treated blood groups were increased when compared to those in the control group. Glutathione peroxidase activities (Unit/mg protein) in all GAG treated groups were also so increased: db mice (CON), 10.48±1.7; CaG5, 24.7±1.3; GbG5, 26±1.8 (GbG5/CON: 248.0%, GbG5 vs. CON, $p < 0.05$); Metformin10, 14.16±1.8 (Met/CON: 135.1%). As a detoxifying hepatic enzyme, glutathione-s-transferase activities (nmol/min/ml) were as

follows: CON, 106.42 ± 8.25 ; CaG5, 116.35 ± 4.65 ; GbG5, 125.12 ± 3.75 ; and Metformin10, 103.92 ± 6.45 .

As a strong free radical scavenger, superoxide dismutase activities (nmol/min/ml) were significantly increased in treatment groups: DB-Hetero (normal mice), 486.96 ± 58.94 ; db mice (CON), 558.47 ± 50.06 ; CaG5, 704.73 ± 41.52 (126.1%, CaG5 vs. CON, $p < 0.05$); GbG5, 701.95 ± 65.69 (125.7%, GbG5 vs. CON, $p < 0.05$); and Metformin10, 667.91 ± 21.72 (Fig. 3C).

3.7. The role of endothelial Nitric oxide synthase, laminin and VEGF on HUVEC

Endogenous nitric oxide (eNOS, pg/ml) levels in human cardiac microvascular endothelia cells (from diabetic type 2) were shown decrease in decreased in the treatment with 5 or 10 mg/ml of GbG5 or GbG10, but by the treatment of 10 mg/ml pravastatin demonstrated significant increase: CON 2268.3 ± 603.4 ; GbG5 1530.6 ± 395.8 ; GbG10 1428.3 ± 264 ; Pravastatin10, 3632.8 ± 723.6 (160.16%, Pravastatin10 vs. CON, $p < 0.05$) (Figure 4A).

Laminin levels (ng/ml) in the same cell type were not significantly changed with the treatment either GbG or positive control treatment: CON 105.1 ± 1.8 ; GbG5 108.2 ± 39.8 ; GbG10 102.6 ± 1.8 ; Pravastatin (10 mg/ml), 139.5 ± 16.8 ; Chitosan (10 mg/ml) 207 ± 36.2 .

Accordingly VEGF levels (μ g/ml) in HMVEC diabetic cells show increase in a dose dependent manner by GbG treatment: CON 99.39 ± 6.21 ; GbG5 114.024 ± 24.15 ; GbG10

174.02±35.87 (175.08%, GbG10 vs. CON, $p < 0.05$); Pravastatin10 112.07±4.83; Chitosan10
184.76±24.84 (Figure 4B).

3.8. The manufacture of the insect N- glycan fraction

The N- glycan (N-glycan) is to be refined from the cricket (*G. bimaculatus*) origin insect
glycosaminoglycan and the concentration of the sodium chloride used in the ion exchange
chromatography process in the elution is 0 M (Figure 5A). By confirming whether it was the
low molecular weight or the degree of purity to confirm performed SAX (strong aion
exchange process, Phenomex, 250x 10mm) - HPLC, and 232 nm after glycan lyase (the
heparinase I,II,III, and the chondroitin ABCase) processing amid the single Peak was
detected or purity was confirmed in Figure 5A. As this figure showed SAX-HPLC result,
final low molecular weight chromatogram of cricket glycosaminoglycan noted from, the
major SAX-HPLC peak glycosaminoglycan of insect, exists as one. After pooling this single
peak, by heparinase II treatment, heparin disaccharide IV-H and I-S standard fragments were
detected throughout MADI-TOF mass data program in this peak.

3.9. Identification of N-glycan derived from GbG

We have already noted for the monosaccharide composition of GbG [11]. N-glycan of CbG
was identified as Hex₆, Hex₅GlcNAc₂, Hex₆GlcNAc₂, Hex_{7~10}GlcNAc₂ and m/z 1905.7 of
Hex₉GlcNAc₂ by mass/mass spectroscopy (Fig. 5B and Fig. 5C). We found that the neutral
mono-sugar of N-glycan derived from GbG, to be mainly hexose: L(+) rhamnose 81.10%,

D(-) ribose 7.16%, D(+)arabinose 5.91% and D(-)fructose 1.91% by TMS Gas chromatography-Mass data base (Table1).

3.10. Adipocyte density amid pathological observations

Pancreas tissues were repaired by treated GAG or metformin compared non-treated Homo-db mice at hematoxylin and eosin stain samples. Adipocyte density (cells/mm²) was decreased in treated cardiac tissues compared to control by toluidine blue O stain, but in other organ tissues, toluidine stain deposit number was not shown to decreasing discrepancy as shown in Figure 6.

4. Discussion

Glycosaminoglycan can play a role in the curing increased blood glucose, hyperglycemia, and other complicated diseases. As an insect source, glycosaminoglycan extracted from the mucous membrane in the cricket inner cortex has been reported to have an antilipidemic effect on high fat diet rat [11], and blood-pressure-lowering [23] and anti-inflammatory effects on adjuvant-induced edema rats [9]. The results of the present study displayed that, after, treatment with each glycosaminoglycan, sera BUN levels were decreased (CaG5, 68.5%; GbG5, 75.5% and Metformin10, 68.4%) compared to those in the DB-Homo group. Furthermore, the levels of total albumin, alkaline phosphatase, ALT, AST, creatinine, glucose,

1 and HDL-cholesterol were also decreased by these GAGs, demonstrating their anti-diabetic
2 thus thereby complicating disease amelioration effects.

3 In fact, some oxygenic functional groups, including epoxy, hydroxyl, and carbonyl groups
4 enable a chemical structure constituent to exert a potent free radical scavenging activities [24].

5 In addition to antioxidant activities, GbG displayed antidiabetic activities accompanied by no
6 adverse effects found in *in vitro* and *in vivo* models.

7 We also found some instances of the anti-oxidant action of glycosaminoglycan with
8 scavenging activities through free radical causing cellular oxidative damages [25]. On the
9 other hand, scavenger enzymes like SOD with cofactors, Cu or Zn in cardiovascular target
10 sites promote increases of antioxidant defenses [26]. Levels of anti-oxidative enzymes and
11 the following activities of catalase, glutathione peroxidase, glutathione-s-transferase and
12 SOD were also increased by this GbGs. Henceforth; hepatocellular oxidative stress by free
13 radical damage could be scavenged with the help of these antioxidant enzymes. In case of db
14 mice experiment, CbG5 appeared to have anti-oxidant activities that are increasing ones of
15 catalase by 114.9%, GPX by 248.1%, GST by 117.6% as well as SOD by 125.7%. We found
16 as for cellular oxidative damage, protein oxidative damage was also to be reduced (CaG5,
17 81.5; GbG5, 81.5% and Metformin10, 93.0%) by these GaGs on the basis of blood neutrophil
18 carbonyl content.

However, we recognize some down laying elements such as glycosaminoglycan and mucopolysaccharide: purified human GAGs have shown to reduce cell death, limit DNA fragmentation and thus protein oxidation, decreased $\text{OH}\cdot$ generation and lactate dehydrogenase activity, inhibited lipid peroxidation and improved endogenous antioxidant defenses [27]. On the other hand, the mucopolysaccharidosis type II (MPS II), a lysosomal storage disorder could be caused by deficient enzyme iduronate-2-sulfatase that is responsible for the degradation of glycosaminoglycans dermatan and heparin sulfate, could be protected against lipid peroxidation while protein damage in these patients by enzyme replacement therapy [28]. Furthermore, the proposed mechanism of action on cricket glycosaminoglycan in db mice can be summarized as figure 6B. This anti-oxidative action of cricket glycosaminoglycan in type 2 diabetic mice could be supported in the following role that GAG lowers molecular weight of heparin, increases SOD levels, and inhibits oxidative stress [29] and glycosaminoglycan (from *Urechis unicinctus*) significantly enhances liver SOD and GSH-Px activity [30]. We leave those concerns for further human clinical researches to come.

Conclusions

These results from sero-biochemical, hepatocellular anti-oxidant assay in db mice data suggest cricket (*G. bimaculatus*) could be used as natural anti-diabetic agents and functional food.

Abbreviation

CON: control group; GAG: glycosaminoglycan; CaG: dung beetle (*C. molossus*) glycosaminoglycan; GbG: *G. bimaculatus* (a type of cricket) glycosaminoglycan; CaG5: *C. molossus* glycosaminoglycan 5 mg/kg; GbG5: *G. bimaculatus* glycosaminoglycan 5 mg/kg; Metformin10: Metformin 10 mg/kg; eNOS: endothelial nitric oxide synthase; VEGF: Vascular endothelial growth factor; AST (GOT), glutamate oxaloacetate transaminase; ALT (GPT), glutamate pyruvate transaminase; BUN, blood urea nitrogen;

Declarations

Ethics approval and consent to participate

Studies involving animals were approved by the Laboratory Animals' Ethical Committee of the National Academy of Agricultural Science, RDA, South Korea (NIAS201605).

Consent to publish: Not applicable (not contain any individual person's data)

Availability of data and materials

All data generated or analyzed during this study are indicated in this article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MYA performed most of the experiments and prepared the manuscript: conceived of the

study, participated in its design and coordination, collected and analyzed data, and prepared the manuscript. BJK carried out the animal studies, participated in oxidative relating enzyme assay. HJK carried out HMVEC cell assays. MJJ carried out N-glycan sequence analysis. HJY reared and supplied of versatile insects. JSH participated in the enzymatic function. BML edited related references. All authors read and approved the final manuscript.

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Legends

Figure 1. Animal experimental design: Scheme of Db mice treated with CaG or GbG over one month.

CaG5: dung beetle (*C. molossus*) glycosaminoglycan 5 mg/kg; GbG5: field cricket (*G. bimaculatus*) glycosaminoglycan 5 mg/kg, and Metformin10: Metformin 10 mg/kg

Figure 2. Effect of (A) Body weight, (B) blood glucose level, (C) Serological level of ALT, AST, and BUN level, (D) HDL-cholesterol and LDL-cholesterol levels in db mice treated with *G. bimaculatus* glycosaminoglycan for a one month.

* $p < 0.05$, compared with CON (DB-Homo, diabetic) group

Figure 3. (A) Anti-oxidative effect of CaG or GbG on (A) carbonyl content, (B) catalase content, glutathione peroxidase (GPX), or glutathione-s transferase (GST) and (C) superoxide (SOD) content. Each value represents mean \pm S.D. statistically significant from DB-Homo group (* $p < 0.05$).

Figure 4. Effect of (A) endothelial nitric oxide synthase (eNOS), (B) laminin and vascular endothelial growth factor (VEGF) on human microvascular endothelial cells from type 2 diabetics.

As a D- HMVEC cell study, GbG5: *G. bimaculatus* glycosaminoglycan 5 mg/ml; GbG10: *G.*

bimaculatus glycosaminoglycan 10 mg/ml; Pravastatin: Pravastatin 10 mg/ml and Chitosan: Chitosan 10mg/ml. Each value represents mean \pm S.D. statistically significant from DB-Homo (* $p < 0.05$).

Figure 5. Identification of cricket glycosaminoglycan

A) HPLC chromatogram of GbG that digested by heparinase II and ESI Mass Spectrometer chromatogram (Time of flight analyzer).

B) N-glycan from *G. bimaculatus* glycosaminoglycan using MALDI MS/MS TOF analyzer (with quadrupole ion trap) at m/z 1905.7, C) GbG N-glycan chromatogram of MALDI (Matrix-assisted laser desorption/ionization) MS from m/z from 800 to 2200.

Figure 6. (A) Microscopy observation of pancreas, (A) hematoxylin & eosin stained cells and toluidine O-Blue stained depots: adipocyte in db mice treated with some GAG, (n = 10 per group, x 400).

(B) The proposed mechanism of action on cricket (*G. bimaculatus*) glycosaminoglycan in db mice.

Figure 1

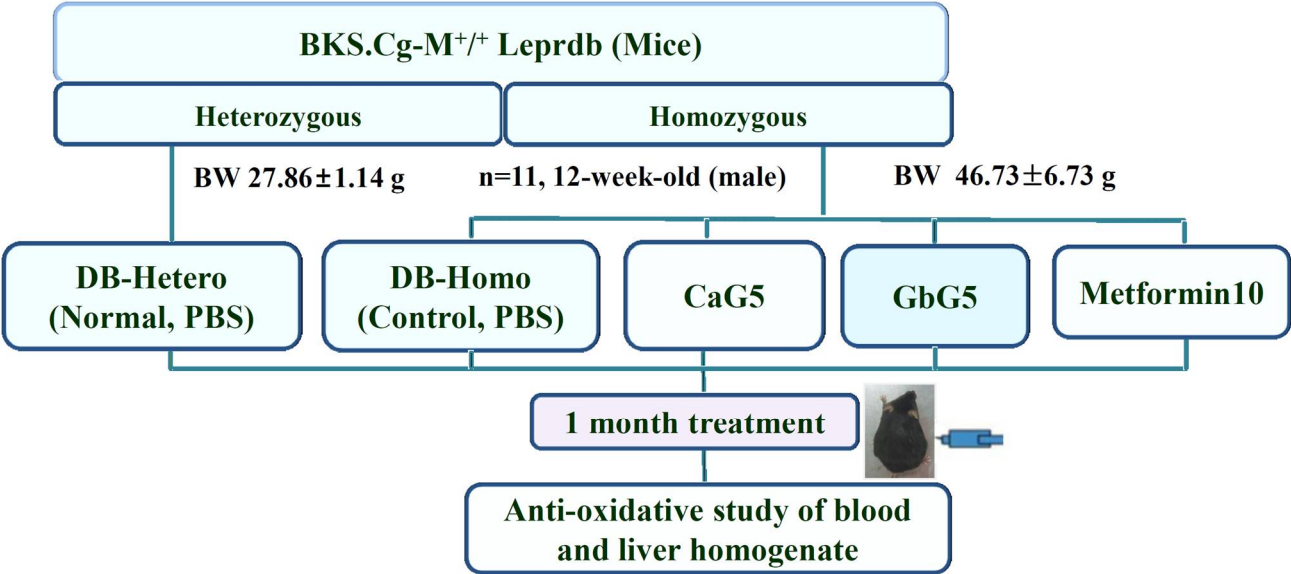
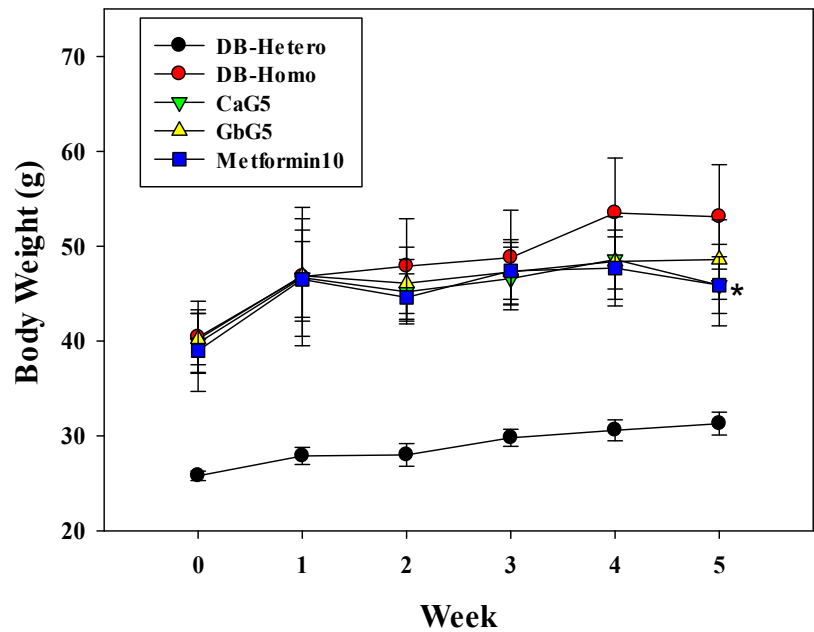
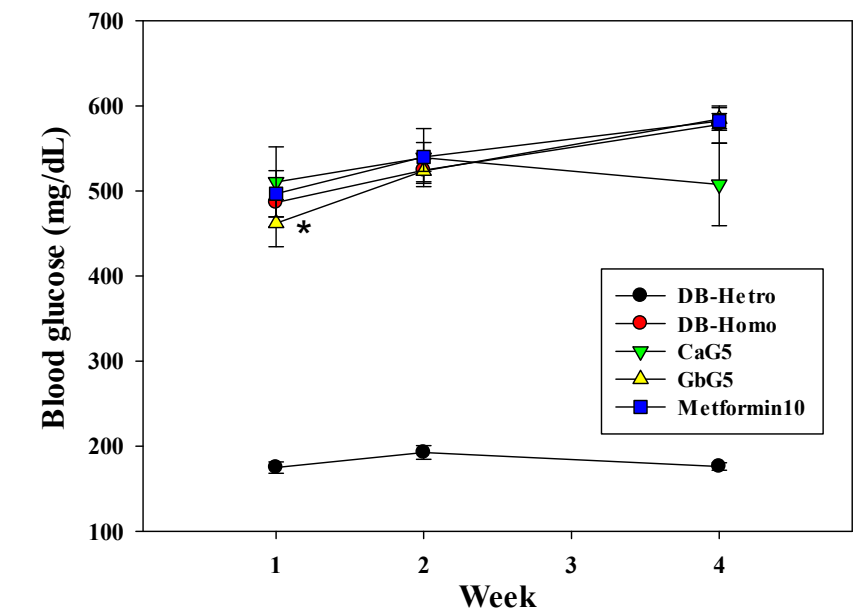


Figure 2

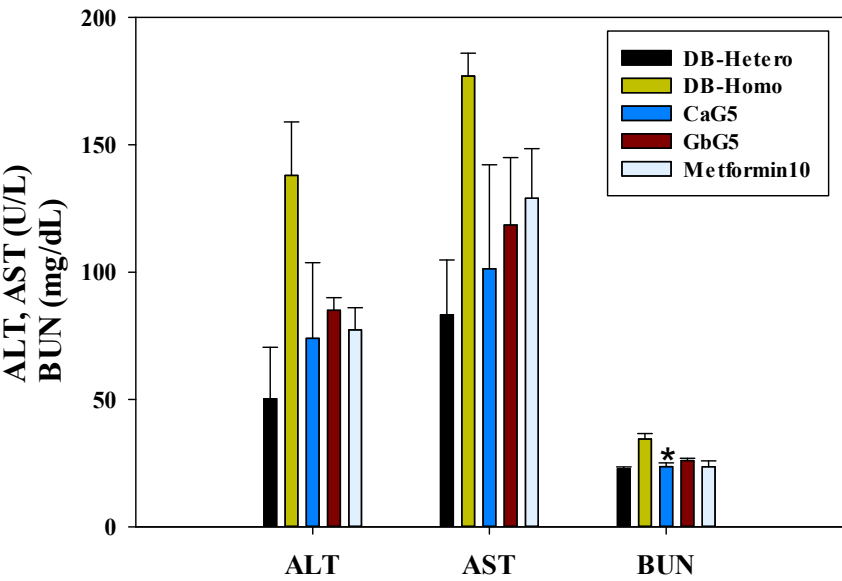
2A



2B



2C



2D

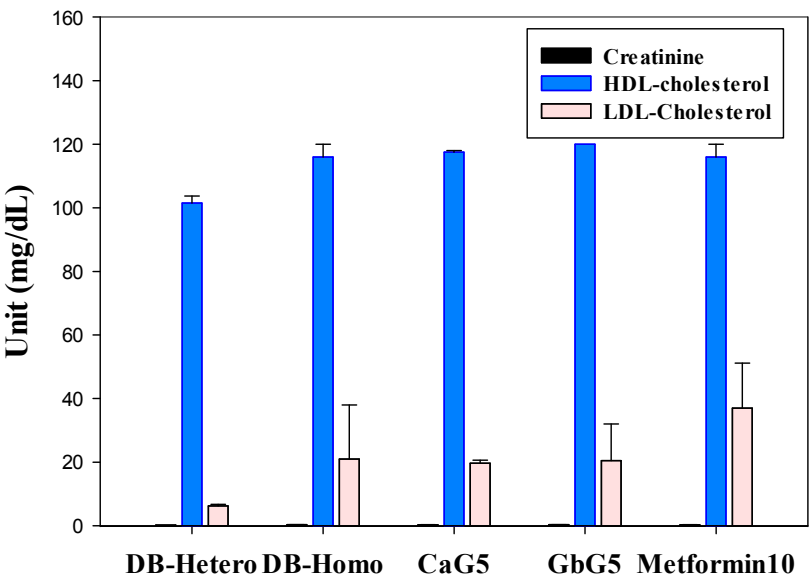
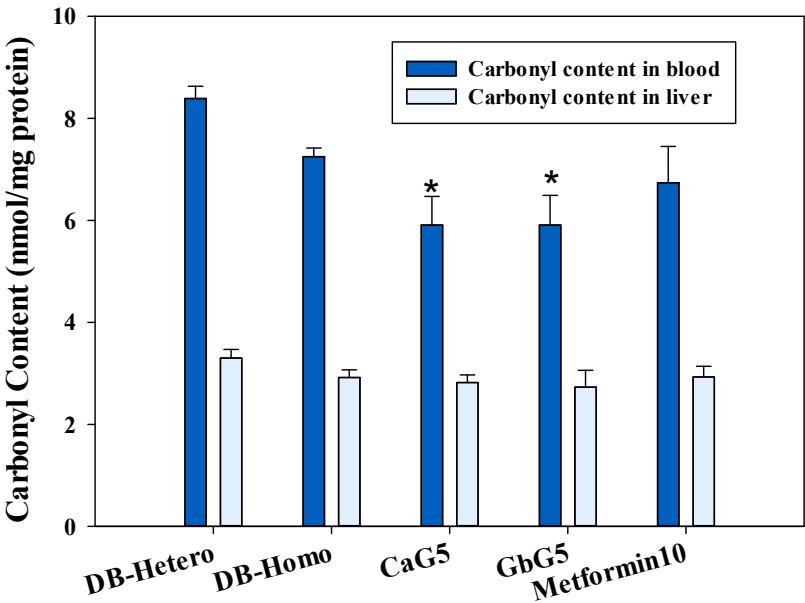
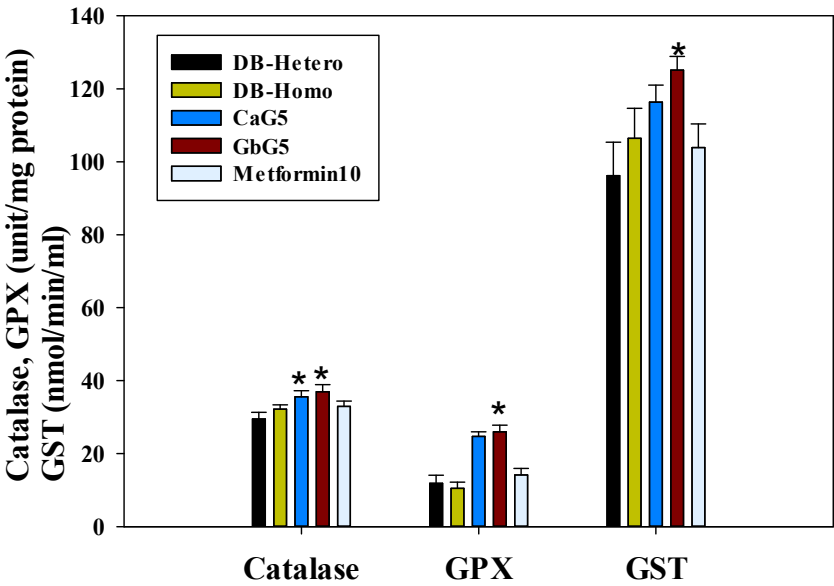


Figure 3
3A



3B



3C

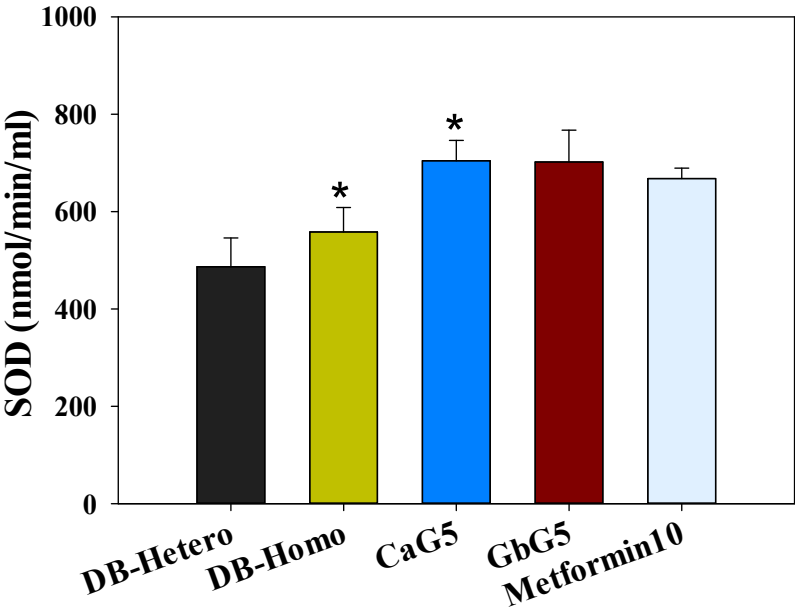
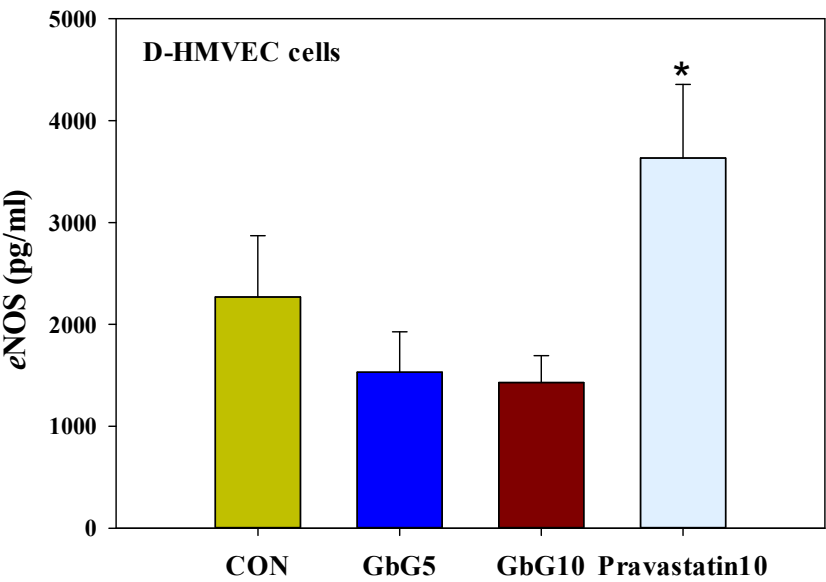


Figure 4

4A



4B

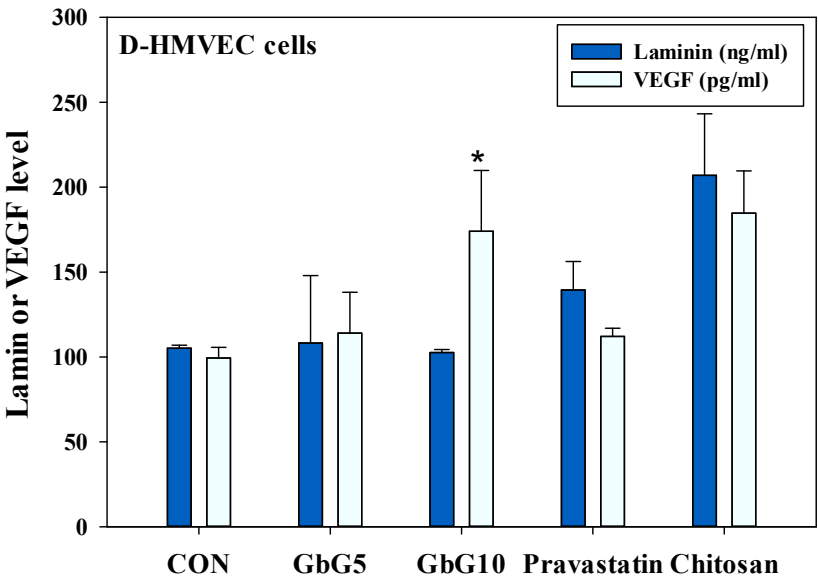
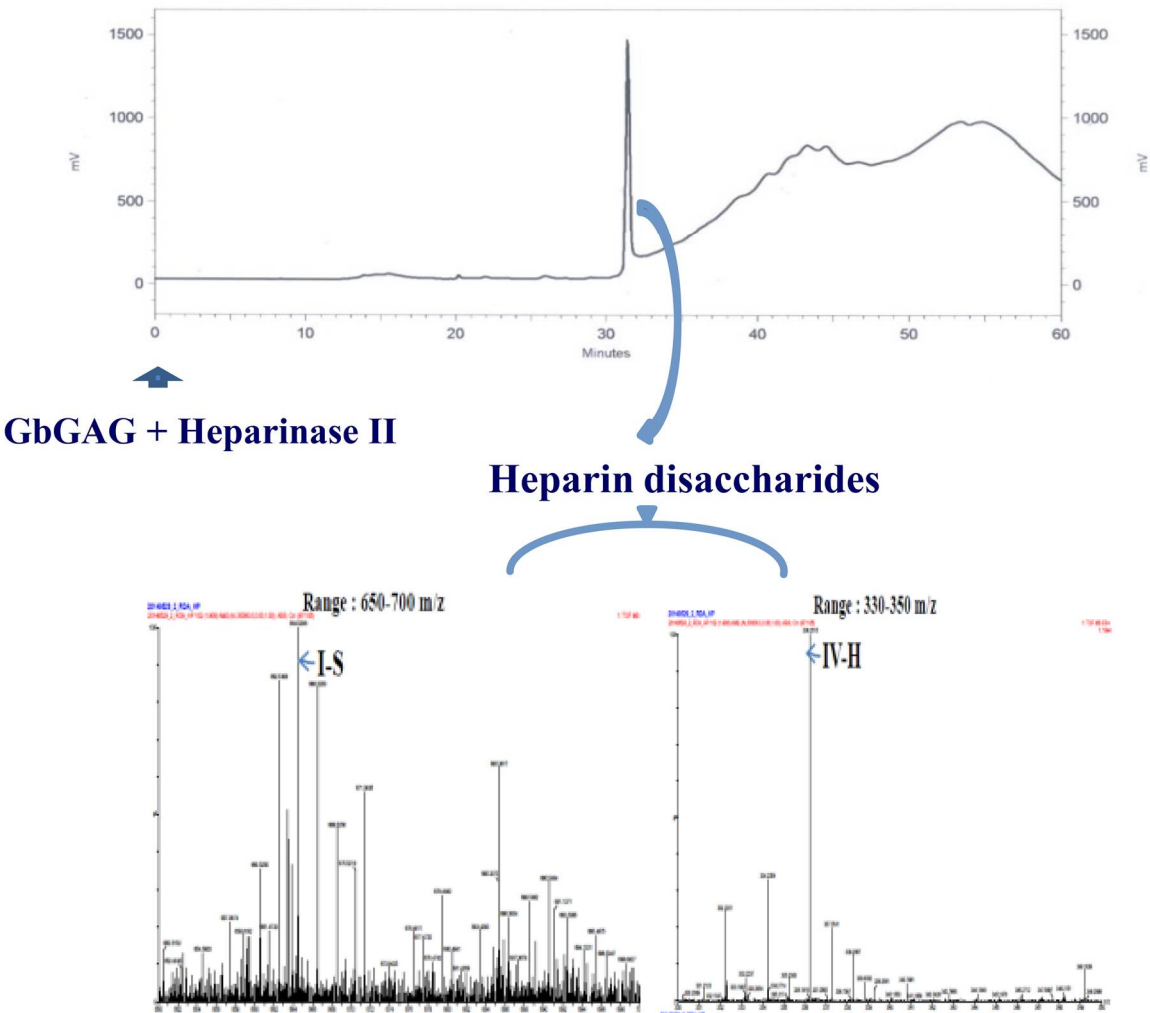


Figure 5

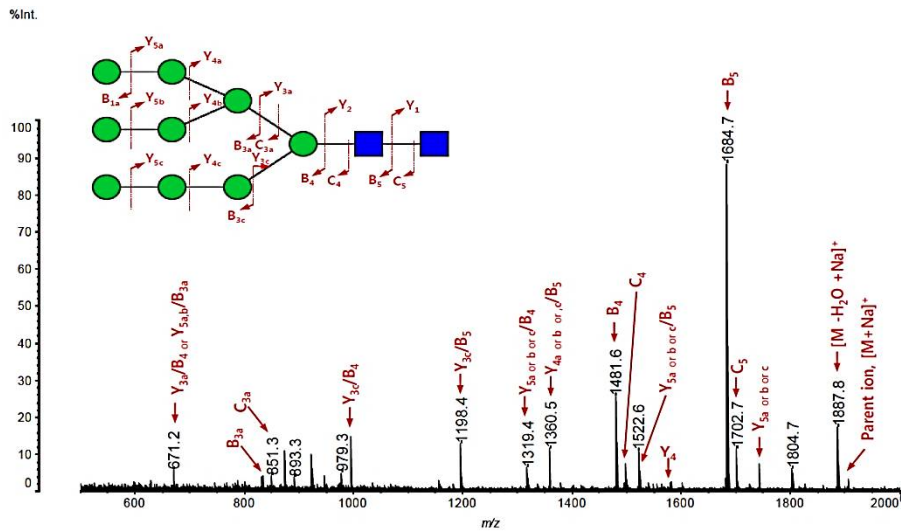
5A



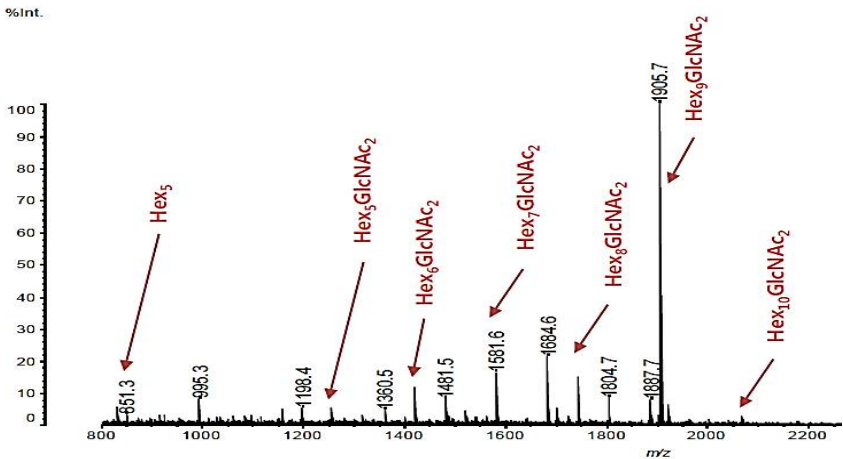
5B

Hex₉GlcNAc₂ (m/z 1905.7)

GlcNAc Hexose

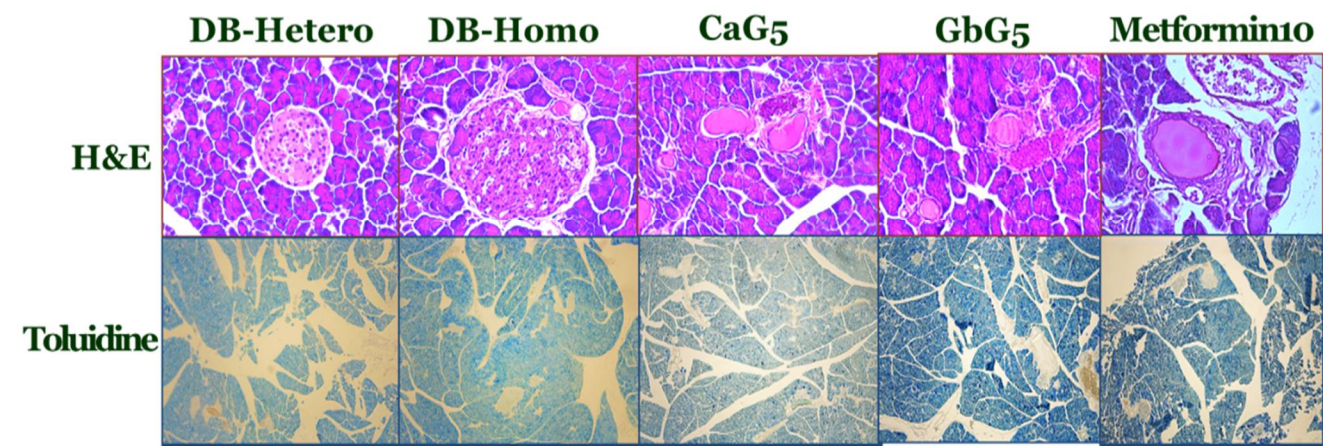


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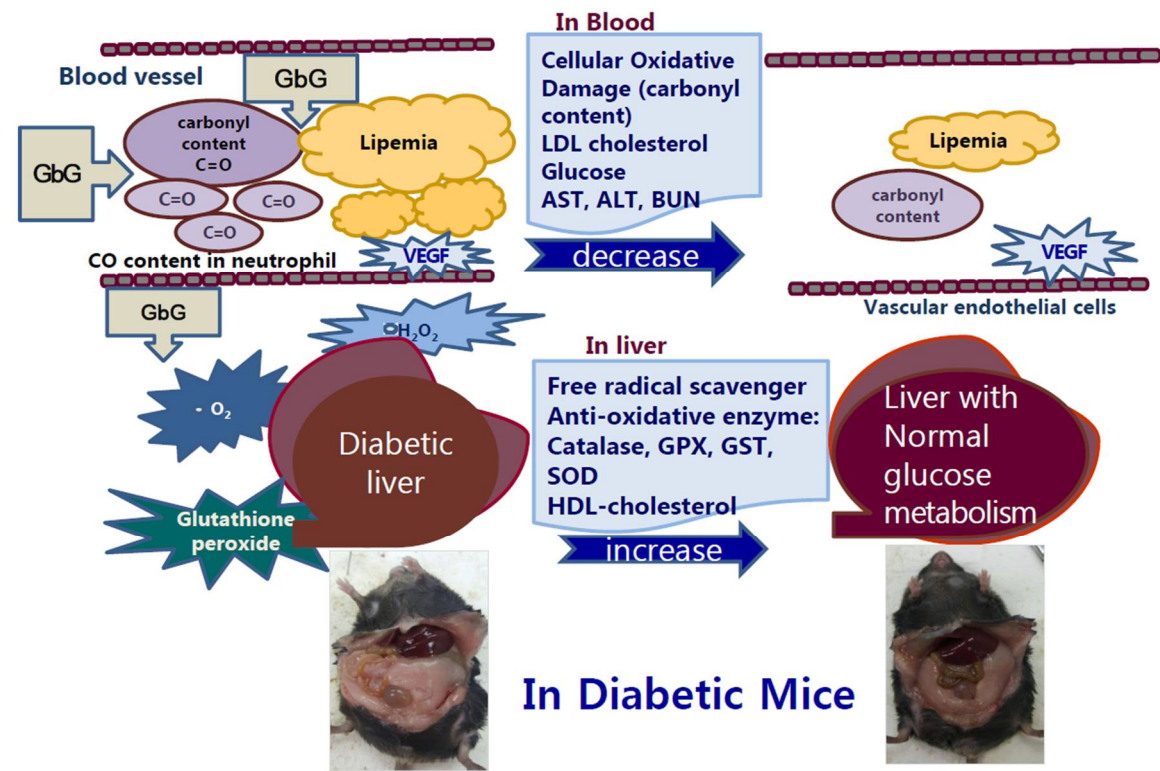


(logic value)	(detection value)	Intensity (Apex, mV)	Hexose (Hex)	GlcAc	Charges
851.3	851.3	4.21	5	0	+1 (Na)
1257.4	1257.5	6.46	5	2	+1 (Na)
1419.5	1419.5	11.49	6	2	+1 (Na)
1581.5	1581.6	20.11	7	2	+1 (Na)
1743.6	1743.7	16.52	8	2	+1 (Na)
1905.6	1905.7	122.75	9	2	+1 (Na)
2067.7	2067.8	3.34	10	2	+1 (Na)

Figure 6
(A)



(B)



1 Table 1. Antioxidant enzyme of hepatocyte in db (+leptin) mice treated with
2 field cricket glycosaminoglycan for one month

Antioxidant enzyme	Unit	DB-Hetero	DB-Homo	CaG5	GbG5	Metformin10
Catalase	Unit/mg protein	29.5±1.81	32.18±1.18	35.6±1.65*	36.97±1.95*	32.92±1.46
Glutathione peroxidase	Unit/mg protein	11.87±2.20	10.48±1.70	24.70±1.30	26.00±1.80*	14.16±1.80
Glutathione-s-transeferase	nmol/min/ml	96.20±9.15	106.42±8.25	116.35±4.65	125.12±3.75	103.92±6.45
Superoxide dismutase	nmol/min/ml	486.96±58.94	558.47±50.06	704.73±41.52*	701.95±65.69*	667.91±21.72
Carbonyl content in blood	nmol/mg protein	8.39±0.24	7.25±0.17	5.91±0.56*	5.91±0.58*	6.74±0.71
Carbonyl content in liver	nmol/mg protein	3.30±0.17	2.92±0.15	2.82±0.15	2.73±0.33	2.93±0.21

18
19 Each values represents mean±SE statistically significant from DB-Homo group
20 (*p<0.05)

21
22
23

Table 2. N-glycan composition and structure analysis of GbG: monosaccharide (neutral sugar) composition of N-glycan from GbG by GC-ESI MS (TMS derivertized)

Neutral monosaccharide (hexose)	Component ratio (%)
D(-) ribose	7.16
L(+) rhamnose	81.10
D(+) mannose	1.13
D(+) xylose	1.05
D(+) galactose	0.12
L(+) arabinose	5.91
D(-) fructose	1.91
α -D(+)-glucose	0.27
β -D(+)-glucose	1.35
Total percent	100

Mass Accuracy: <40 ppm, Hex: hexose, GlcNAc: N-acetylglucosamine, Source: GbG 0.5M