Title: Heterogeneity in liver histopathology is associated with GSK-3β activity and mitochondrial dysfunction in end-stage diabetic rats on differential diets

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Running Title: Liver histopathology in diabetic rats on differential diets.

Keywords: diabetes, GSK-3β, glycogenic hepatopathy, mitochondrial function, non-alcoholic hepatosteatosis

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Supplementary Materials and Methods

Animals and experimental design

Male Sprague-Dawley rats (11-weeks-old, specific pathogen-free) were purchased from Orient-Bio (Seongnam, South Korea). All animal protocols were approved by the Institutional Animal Care and Use Committee at Konkuk University. To mimic end-stage diabetes, we used subtotal-pancreatectomized diabetic rats. Briefly, after a 2-week acclimation period, surgery was performed as previously described (1): 11 sham operations were conducted as a control group (18S), and 33 operations were performed for subtotal pancreatectomy (Px) in the diabetes groups. All Px rats were fed an ad libitum diet for 7 weeks to induce diabetes (diabetes induction period), after which they were divided into three groups. During the subsequent 7 weeks of the differential diet period, the groups were fed the following diets: 11 rats were fed ad libitum (18AL), 11 rats were in the calorie-restriction group (18R), and the remaining 11 were in both the calorie- and protein-restriction group (6R). The 6R group was designed to simulate a low-protein diet that is frequently prescribed for patients with diabetic nephropathy.

Diet and basic measurements

All rats were fed a standard chow based on AIN-76A (GF 2005; Feed-Lab, Guri, South Korea; carbohydrate 62 %, protein 18 %, and lipid 20 %, by calories, Table S1), except for rats in the 6R group, which were fed a low-protein chow (modified GF 2005; Feed-Lab; carbohydrate 74 %, protein 6 %, and lipid 20 %, by calories, Table S1) during the differential diet period: for 7 weeks of the diabetes induction period after surgery, all rats were fed ad libitum the standard chow, and then we divided the diabetic Px rats into three groups. During the subsequent 7 weeks of the differential diet period, Px rats in the 18AL group were fed ad libitum the standard chow to maintain hyperglycemia, while the remaining Px rats were fed...
calorie-restricted diets with the standard chow (18R) or the low-protein chow (6R) to achieve euglycemia. Food intake (g) was individually measured every day, and the average rate of daily food intake (g/kg of body weight/day) was calculated weekly. Both the 18S and 18AL groups were fed ad libitum throughout the study, whereas both R groups were fed at the same rate of daily food intake as the 18S group, to achieve euglycemia during the differential diet period. Fasting blood glucose levels (mg/dL) were measured after overnight fasting at 9:00 am every other week. Body weight was measured every weekend, and before the rats were euthanized. Blood samples were collected immediately before euthanization. After death, liver weights were measured in all overnight fasted rats, before euthanization.

**Biochemical analyses**

Plasma insulin and C-peptide levels were determined using radioimmunoassay kits (Millipore, Billerica, MA, USA), and radioactivity was measured using a γ-counter (Beckman Coulter, Brea, CA, USA). Serum total cholesterol (TC) and triglycerides (TG) and hepatic TG levels were determined using enzymatic assay kits (Bio Clinical System, Anyang, South Korea). Serum high-density lipoprotein cholesterol (HDL-C) level was quantified using a polyethylene glycol precipitation kit (Young-dong, Seoul, South Korea). Hepatic glycogen was determined using anthrone reagent (2) and a glucose analyzer (YSI Life Science, Yellow Springs, OH, USA).

**Histology**

Overnight-fasted liver tissues were excised and pre-fixed in 4% paraformaldehyde, and then embedded in paraffin, and sliced into 4-μm-thick sections. The liver sections were stained with haematoxylin and eosin (H&E) or periodic acid-Schiff (PAS).
Transmission-electron microscopy (TEM) and counting of mitochondria
Liver tissues were fixed for 2 h at 4 °C in 0.1 M sodium phosphate buffer (pH 7.4) containing a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde. Fixed liver tissues were post-fixed for 2 h in the same buffer containing 1% OsO4. The liver tissue pieces were subsequently washed and dehydrated using graded acetone, and embedded in Epon-Araldite. Thin sections were prepared on an ultra-microtome (MT-X; RMC, Tucson, AZ, USA). The sectioned liver tissues were mounted on copper grids, and double-stained with uranyl acetate and lead citrate. The grids were visualized using a transmission electron microscope (H-7600; Hitachi, Tokyo, Japan). The number of mitochondria per hepatocyte was counted in the TEM images by using iSolution Lite (IMT i-solution, Vancouver, Canada).

Western blot analysis
Frozen-ground liver tissue was processed as described previously (1). The following primary antibodies were incubated with the tissues overnight at 4 °C: LC3B (Catalog No. 3868, Cell Signaling Technology, Danvers, MA, USA), total Akt (Catalog No. 4691, Cell Signaling Technology)/phosphorylated Akt (Catalog No. 4060, Cell Signaling Technology), total glycogen synthase kinase-3β (GSK-3β, Catalog No. 5558, Cell Signaling Technology)/phosphorylated GSK-3β (Catalog No. 12456, Cell Signaling Technology), total acetyl-CoA carboxylase (ACC, Catalog No. 3662, Cell Signaling Technology)/phosphorylated ACC (Catalog No. 3661, Cell Signaling Technology), total AMP-activated protein kinase (AMPK, Catalog No. 2532, Cell Signaling Technology)/phosphorylated AMPK (Catalog No. 4188, Cell Signaling Technology), glucose-6-phosphatase (G6Pase, Catalog No. 8866, Cell Signaling Technology), β-actin (Catalog No. 4967, Cell Signaling Technology; 1:5,000, except for G6Pase 1:1,000], mitochondrial respiratory chain complex I (COX I, Catalog No. PA5-79700, Invitrogen,
Carlsbad, CA, USA), COX IV (Catalog No. PA5-29992, Invitrogen), and sterol regulatory
element-binding protein-1c (SREBP-1c, Catalog No. sc-13551, Santa Cruz Biotechnology,
Dallas, TX, USA). The membranes were developed using horseradish peroxidase-conjugated
anti-rabbit IgG (Cell Signaling Technology; 1:5,000), followed by detection with ECL
reagent (GE Healthcare, Little Chalfont, UK). The immunoreactive protein bands were
quantified using Multi Gauge version 3.1 software (Fujifilm, Tokyo, Japan).

**Real-time RT-PCR**

Total RNA was isolated from liver tissues using TRI reagent (Sigma-Aldrich, St. Louis, MO,
USA). One microgram of total RNA was reverse-transcribed into cDNA using oligo-dT
primers (Thermo Fisher Scientific). Real-time PCR analysis was conducted using the
LightCycler FastStart DNA Master HybProbe kit (Roche, Basel, Switzerland) using TaqMan
primers (Applied Biosystems, Foster City, CA, USA) on a LightCycler 2.0 (Roche). Relative
mRNA levels were calculated using the comparative ΔCt values (3). The following
TaqManTM primers were used: ApoB forward 5′-TAC CTC CGG CAG CTC CAT TCC-3′;
ApoB reverse 5′-TGC GCT TCC TGC TCT TGC TGT T-3′; β-actin forward 5′-TCC TCC
TGA GCG CAA GTA CTC T-3′; β-actin reverse 5′-GCT CAG TAA CAG TCC GCC TAG
AA-3′.

**Statistical analysis**

Statistical analysis was performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA).
Statistical significance was evaluated by one-way analysis of variance (ANOVA) with
Tukey’s *post hoc* test. P < 0.05 was considered to indicate statistical significance.
Supplementary Table S1. The components of the diets.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Low Protein (6%)</th>
<th>Standard Protein (18%)</th>
<th>AIN-76A</th>
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<tr>
<td></td>
<td>gm.% kcal.%</td>
<td>gm.% kcal.%</td>
<td>gm.% kcal.%</td>
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<tr>
<td>Protein</td>
<td>6.2 6.0</td>
<td>18.6 18.0</td>
<td>20.0 20.5</td>
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<tr>
<td>Carbohydrate</td>
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<td>62.8 62.0</td>
<td>66.0 69.0</td>
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<tr>
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<td>9.7 20.0</td>
<td>5.0 10.5</td>
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<tr>
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<td><strong>g kcal</strong></td>
<td><strong>g kcal</strong></td>
<td><strong>g kcal</strong></td>
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<tr>
<td>Milk casein</td>
<td>62.0 248.0</td>
<td>186.0 744.0</td>
<td>200.0 800.0</td>
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<td>Corn starch</td>
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<td>117.5 470.0</td>
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<td>Sucrose</td>
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<td>50.0 0.0</td>
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<tr>
<td>Corn oil</td>
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<td>96.5 868.5</td>
<td>50.0 450.0</td>
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<td>3.0 12.0</td>
<td>3.0 12.0</td>
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<td>Choline Bitartrate</td>
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<td>2.0 0.0</td>
<td>2.0 0.0</td>
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<tr>
<td><strong>Total</strong></td>
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<td>1,000.0 4,134.50</td>
<td>1,000.0 3,902.00</td>
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References

