Identification of entero-endocrine mechanisms in the improvement of glucose tolerance after foregut bypass surgery for type 2 diabetes in Zucker diabetic fatty rats

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Directed by Professor Woo Jin Hyung

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ABSTRACT

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Background and objective: It is not clear whether the anti-diabetic effect of metabolic surgery is due to the expedited delivery of nutrients to the distal intestine and consequent enhancement of glucagon-like peptide-1 (GLP-1) (incretins) secretion (hindgut hypothesis) or the exclusion of the duodenum and proximal jejunum and consequent reduction of "putative diabetogenic factors" (foregut hypothesis). Understanding how the bypassed foregut contributes to glycemic regulation could help optimize the metabolic surgical procedure and identify critical targets for novel therapeutic interventions. We aimed to characterize how foregut bypass surgery alone (duodenojejunal bypass [DJB] surgery) affects glucose tolerance and beta-cell functions and investigate the distinct effect of foregut bypass surgery on entero-insular hormonal regulation of glucose homeostasis. We also aimed to draw a rationale for further research to discover a diabetogenic anti-incretin factor in the proximal intestine. We hypothesize that (1) the long-term beneficial effect of foregut bypass surgery on glycemic control is primarily associated with beta-cell function and (2) the post-bypass glucose tolerance improvement is induced by eliminating the anti-incretin activity contributing to the pathophysiology of diabetes.

Materials and Methods: In the first set of experiments, Zucker Diabetic Fatty (ZDF) rats were randomly assigned to the DJB, sham-operated DJB pair-fed (sham-DJBPF), and sham-operated ad-libitum fed (sham-AL) groups. Glucose tolerance (GT) and plasma insulin levels were measured during periodically performed oral glucose tolerance test up to 16 weeks postoperatively. Histomorphometric analyses were performed to evaluate the modification of islet architectures. Intracellular insulin signaling in visceral adipocytes was measured by the phosphorylated Akt/Akt ratio using Western blot. In the second set of experiments, the effects of DJB surgery in ZDF rats based on entero-insular hormonal changes were compared to those of gastric resection alone (sleeve gastrectomy [SG]). Other animals underwent a combination procedure (SG + DJB surgery). Outcome measures were weight, food intake (FI), GT, and gut hormones.

Results: DJB surgery did not substantially affect weight and FI. GT in DJB animals more significantly improved compared to that in sham-DJBPF and sham-AL animals ($p < 0.01$), and the value of GT 2 weeks after surgery were significantly better compared to its preoperative value ($p < 0.05$). Postoperatively, in contrast to the progressive deterioration of GT and the decrease in plasma insulin levels in sham-operated animals ($p < 0.01$), DJB animals prevented GT aggravation and plasma insulin level declining. The proportion of the islets having expanding projections was significantly higher in DJB animals than in sham-operated animals ($p < 0.01$). DJB had larger fractional area of beta cells ($p < 0.01$) and lesser incidence of fibrosis in the islet than sham-operated animals ($p = 0.083$). DJB animals had significantly higher levels of Akt phosphorylation in the visceral fat tissues than sham-operated animals ($0p < 0.05$).

SG significantly reduced weight gain and food consumption. DJB rats showed weight-independent improvement in GT, which improved less after SG. Furthermore, SG significantly increased insulin, GLP-1, glucose-dependent insulinotropic peptide, and peptide YY responses to oral glucose. In contrast, DJB

surgery had no effects on the postprandial levels of these entero-endocrine hormones. DJB surgery restored postprandial glucagon suppression in diabetic rats, whereas SG had no effect in ZDF rats' glucagon response. The combination procedure had higher GT than SG alone with modestly compromised GLP-1 levels.

Conclusion: Foregut bypass surgery improved GT immediately after surgery and prevented the further decrease of plasma insulin levels, which may be attributable to preserving the morphological compensation of islets to insulin resistance. Moreover, this surgery also facilitated insulin signaling in its peripheral target tissues.

Foregut bypass surgery did not improve diabetes by increasing incretin levels or enhancing factors that reduce glycemia. It may work by reducing the mechanism promoting hyperglycemia, which would be consistent with the anti-incretin theory. Considering abovementioned results, further elucidation of the molecular mechanism, which may be mediated by putative anti-incretin, is required.

Key words: diabetes, foregut, bypass, surgery, beta-cell, incretin, anti-incretin

Identification of enteroendocrine mechanisms of foregut bypass surgery improving glucose tolerance in type 2 diabetes animal in Zucker diabetic fatty rats

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I. INTRODUCTION

According to the International Diabetes Federation (IDF), the worldwide estimated diabetes prevalence for adults aged between 20 and 79 years was 415 million in 2015. Diabetes is expected to affect 642 million people by 2040. It is estimated that 193 million individuals globally have undiagnosed diabetes. Most of these individuals have type 2 diabetes.[1] Considering the epidemic growth of such conditions, determining treatments of curative intent has been considered significantly important over time. The development of alterations in glucose metabolism results from the gradual decrease in beta-cell function within a background of insulin resistance.[2,3] However, determining the effective treatment for diabetes is hampered by an incomplete understanding of the etiology and pathophysiology of these conditions.

Roux-en-Y gastric bypass (RYGB) surgery dramatically improves or remits type 2 diabetes mellitus (T2DM)[4], and the surgical procedures, commonly involving proximal intestinal bypass, reduce peripheral insulin resistance or directly enhance pancreatic beta-cell function.[4–7] The exclusion of nutrient passage from

the duodenum and proximal jejunum is considered the main contributing mechanism, by which these procedures confer anti-diabetic effect.[8,9] In reality, the duodenojejunal bypass (DJB) (foregut bypass surgery alone) surgery ameliorates T2DM in various strains of rodent models.[8–12] Moreover, short-term follow-up data on patients with T2DM who underwent DJB surgery have shown favorable results.[13,14] However, characterizing the long-term effects of foregut bypass surgery on T2DM in both human and animal studies has a paucity of data. Moreover, the primary effects of DJB surgery on the pancreatic beta cells and its histopathological changes in islets remain to be clarified. However, inadequate knowledge about the effect of surgical procedures, specifically DJB surgery commonly involved in bariatric procedures, on the treatment of T2DM hampers the interpretation of the primary role of the surgery in islet per se. It results in formulating several hypotheses rather than drawing conclusions. As is often observed in medicine, finding effective clinical remedies may provide essential insights for understanding the disease. Bariatric metabolic surgery causes durable weight loss; results in the remission or improvement of type 2 diabetes, hypertension, dyslipidemia, and sleep apnea; and reduces the incidence of cardiovascular diseases and death.[15-17] Although weight reduction might in theory results in the improvement of insulin resistance and associated conditions, studies in rodents[8,18,19] and humans[20-22] showed that the anti-diabetic effect of certain bariatric procedures, particularly RYGB surgery, results from several mechanisms apart from weight loss.[23,24] Understanding the mechanisms of action of surgery provides an opportunity to elucidate the pathophysiology of diabetes and may lead to the development of new treatments of curative intent.

However, investigating the mechanisms behind the surgical control of diabetes is particularly challenging. In fact, bariatric metabolic surgery includes a variety of procedures that impose numerous and distinct changes to gastrointestinal (GI) anatomy. In addition, the physiology of the digestive system and its role in glucose and energy homeostasis are complex and incompletely understood. Over

the last decades, the study of GI physiology has advanced at cellular and molecular levels, revealing that several GI factors have played important roles in the regulation of energy and glucose homeostasis including bile acid pertubations, gut microbiota alterations, neural signal changes, and intestinal nutrient sensing.[18,25-30] Paradoxically, however, the understanding of how the bypassed anatomical segment of the GI tract contributes to metabolic regulation remains unclear.

Originally, bariatric procedures were designed with the assumption that gastric restriction could mechanically reduce food intake (FI), whereas intestinal bypass would reduce nutrient absorption, inducing weight loss. Considering the several metabolic and endocrine functions of the GI tract, it is now recognized that changing the anatomy of the stomach and small intestine exerts far more complex effects on energy homeostasis rather than just mechanical decrease in energy intake. The exact contribution of the stomach and intestine to the improvement of diabetes after these procedures, however, remains unclear.

It has been hypothesized that the beneficial metabolic effects of RYGBR for the patients with T2DM may be secondary to the re-routing of the small bowel. However, it is not clear whether this is due to the expedited delivery of nutrients to the distal intestine and consequent enhancement of GLP-1 (incretins) secretion (hindgut hypothesis)[23,30] or results from the exclusion of the duodenum and proximal jejunum and consequent reduction of “putative diabetogenic factors” (foregut hypothesis).[31,32]. Understanding how the bypassed duodenum and proximal jejunum contribute to metabolic regulation could help optimize metabolic surgical procedures, identify important targets for novel therapeutic interventions, and help interpret the biological and clinical relevance of molecular aspects of GI physiology.

We hypothesized that the anti-diabetic effect of foregut bypass surgery is implicated in the improvement of pancreatic beta-cell function, which is responsible for the plasma insulin concentration, apart from the changes in insulin

resistance. In addition, we hypothesized that post-bypass improvement of T2DM might primarily result from a reversal of imbalanced putative anti-incretin activity, which may play a critical role in the entero-insular hormonal axis. We aimed to characterize how DJB surgery (proximal intestinal bypass alone) affects glucose tolerance (GT) and pancreatic islets and insulin signaling pathway in a T2DM rat model. We also aimed to demonstrate the distinct entero-insular hormonal mechanism of glucose homeostasis regulation primarily mediated by foregut bypass surgery.

II. MATERIALS AND METHODS

1. Animal model

Ten-week-old male Zucker diabetic fatty (ZDF; fa/fa) rats (Charles River, Tokyo, Japan) were individually housed and maintained at 12/12-h light/dark cycle at constant ambient temperature and humidity (25°C, 50%-60%) (Figure 1A). Rats were fed Purina Lab diet #5008 (WF Fisher & Son, Bound Brook, NJ) for the entire duration of the study. After 1 week of acclimatization, rats underwent oral glucose tolerance test (OGTT) to examine their diabetes status. Only ZDF rats with overt diabetic phenotype (2-h glucose > 200 mg/dL) were included in the study. The Hallym University Medical College Institutional Animal Care and Use Committee approved this study (HMC2012-1-0706-6). All animal procedures were carried out according to the standard guidelines of IACUC.

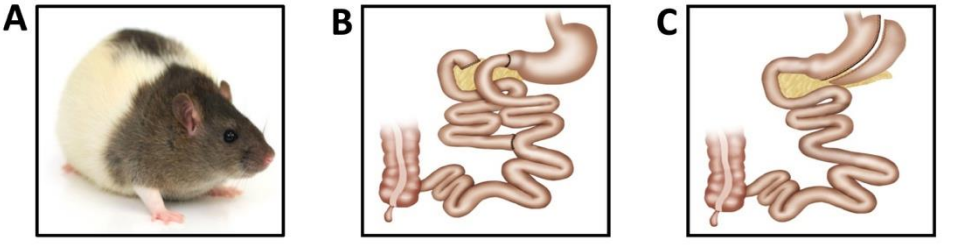

Figure 1. The animal model and surgical procedures. Zucker diabetic fatty rat

(A). Duodenojejunal bypass surgery (B). Sleeve gastrectomy (C).

2. Study design

In the first set of experiments, 12-week-old ZDF rats with overt diabetes were randomly assigned to the DJB, sham-operated DJB pair-fed (sham-DJBPF), and sham-operated ad-libitum fed (sham-AL) groups. To investigate acute weight independent mechanism of surgical interventions, the pair-fed group was included for 1 week postoperatively. The remaining groups were followed up 16 weeks postoperatively. The pancreas was obtained from the subjects of each group at the terminal tissue harvest. Additionally, to investigate the natural course of the changes in beta-cell function in this animal model, plasma insulin levels were measured in a separate set of unoperated control ZDF rats and normal Sprague-Dawley rats at several time points from age 7 to 29 weeks. In the second set of experiments, 12-week-old ZDF rats with a full-blown diabetic phenotype were randomly assigned to the DJB, sleeve gastrectomy (SG), sham-DJBPF, sham-operated SG pair-fed (sham-SGPF), and sham-ALgroups. An additional subset of 12-week-old ZDF rats with a full-blown diabetic phenotype was selected to undergo either SG alone or SG in combination with DJB (SG+DJB) to investigate if the effects of gastric and intestinal alterations are additive. Twelve-week-old Sprague-Dawley rats were included as healthy controls to investigate the hormonal effects of operations on glucose metabolism in non-diabetic rats.

3. Surgical procedures

DJB surgery was designed complying with the concept of the stomach-sparing bypass that excludes approximately the same length of the proximal intestine as in human standard RYGB (Figure 1B). DJB surgery was performed as previously

described.[9] Briefly, the duodenum was divided just distal to the pylorus, and the distal segment was closed with number 5-0 Prolene sutures (Ethicon, Piscataway, NJ). The jejunum was divided 10 cm distal to the ligament of Treitz, and the distal limb was anastomosed using number 5-0 Vicryl suture (Ethicon, Piscataway, NJ) to the proximal segment of the duodenum. The continuity of the biliopancreatic limb was restored by end-to-side anastomosis with the alimentary limb 15 cm distal to the gastrojejunostomy site in a Roux-en-Y manner. The abdominal cavity was closed in layers.

Sleeve gastrectomy (SG) was performed according to the same concept used for human surgery where the stomach is subjected to vertical partial gastric resection (Figure 1C). Different from humans, where an intra-gastric tube is used to calibrate the final volume, our procedure was standardized according to the rats' anatomy. DeBakey curved forceps was precisely positioned 1 cm from the pylorus, engaging the full extent of the stomach up to the left side of the esophageal–gastric junction. Scissors were used to divide the stomach along the DeBakey forceps removing 90% of the forestomach and 70% of the glandular stomach. The divided stomach was subsequently closed with number 6-0 PDS II suture (Ethicon, Piscataway, NJ) in two layers along the DeBakey forceps, creating the vertical gastric sleeve. The abdominal cavity was closed in layers.

For SG + DJB, the SG was performed first, followed by DJB surgery as described above. For the sham-operated controls (DJBPF, SGPF, and AL), specifically for the sham-DJB operation, transverse incisions and primary closures were performed on the antimesenteric intestinal walls at all the sites where enterotomies were performed for DJB surgery. The sham-SG operation was performed with a longitudinal incision at the same level where the resection was done in the SG followed by primary suture. For the sham-AL group, 50% of animals underwent sham-SG, and 50% of animals underwent sham-DJB surgery. The operation time was prolonged in sham-operated rats to produce a similar degree of anesthetic stress as in the respective surgical group.

4. Postoperative care

Subcutaneous injections of buprenorphine (0.01 mg/kg body weight (BW) twice daily 2 days) were administered to all postoperative rats. Antibiotics were not administered to rats after surgery.

5. Outcome measures

Body weight and FI were measured daily between 9:00 and 10:00 AM throughout the study period. Sham operated pair-fed rats were given access to the exact amount of food eaten by the surgical rats on the previous day.

6. Oral glucose tolerance test

After an overnight fast (12 h), rats received an oral load of glucose (2 g/kg body weight) via oral gavage. Blood samples were collected from the rats' tail vein before (t = 0 min) and 15, 30, 60, 120, and 180 min after glucose administration using Microvette CB 300 K2E tubes (Sarstedt, Nuernbrecht, Germany) containing dipeptidyl peptidase IV inhibitor (Millipore, MA). Blood glucose levels were determined using an automated blood glucose reader (YSI2700 SELECT^{TM} Bio- chemistry Analyzer, Biocompare, SanFrancisco, CA). Tubes were immediately placed on ice and, within 1 h, the plasma was prepared by centrifugation (5000 rpm at 4°C) using an Eppendorf bench top cen trifuge and stored at -80°C until assay. Glucose tolerance (GT) was calculated as area under the curve (AUC) with t_0 starting and t_{180} ending points for each experiment. Results are expressed as AUC glucose and delta AUC glucose. OGTT was performed preoperatively (pre-op) and repeated at weeks 1 (short term), weeks 4-8 (mid- term) and week 16 (long-term) postoperatively.

7. Histomorphometric study for the pancreatic islets and beta cells.

A. Hematoxylin and eosin (H&E), anti-insulin immunohistochemistry (IHC), anti-pancreatic duodenal homeobox-1 (PDX-1), and Masson's trichrome stain

Bodies of the pancreas were obtained 16 weeks postoperatively from the subjects of each group at terminal tissue harvest in the first set of studies. The pancreas was removed and fixed by immersion in 10% neutral buffered formalin 24 hours before standard processing and embedding in paraffin wax. The pancreas was embedded to demonstrate the head/tail orientation and maximize the area of tissue sections, which were stained with hematoxylin and eosin (H&E) followed by subsequent histopathological examination. Anti-insulin immunohistochemistry (IHC) was performed to identify beta-cells containing cytoplasmic insulin granules by using a mouse anti-insulin monoclonal primary antibody (Santa Cruz biotechnology, Santa Cruz, CA) and a biotinylated mouse secondary antibody (Vector Labs, Burlingame, CA) with strept-ABC/HRP kit (Vector Labs, Burlingame, CA). Insulin immunopositivity was visualized as brown staining of beta cell cytoplasm. For pancreatic duodenal homeobox-1 (PDX-1) activity in the beta-cell nucleus, the sections were immunostained for immunoperoxidase using the ABC kit (Vector Laboratories, Burlingame, CA) or immunofluorescence. Histochemical demonstration of the infiltration of fibrous tissue in the islets was performed using Masson's trichrome stain.

B. Histomorphometric analysis of islets

To evaluate the time-course changes in islet morphology, the pancreas was harvested from ZDF control rats (12- and 20-week-old ZDF rats; n = 6 in each age group). In H&E stained sections, islets were defined as clusters of eight or

more beta cells associated with other morphologically identifiable endocrine cells, and all visualized islets were counted in every section. The proportion of the number of islets showing expanding features (asteroid shape) to the total islets was calculated in each section from control ZDF rats and every group. Two pathologists counted the total number of islets from each section and counted the number of islets that have expanding features in each section. The proportions of expanding islet in every section generated by each pathologist were averaged (< 5 % variability in the proportions between the pathologist). The fractional area of beta cells per unit pancreas sectional area was measured. For each pancreas, two sections separated by 250 μm were analyzed. Islet images were captured using a MIRAX SCAN® (Carl Zeiss, Thornwood, NY) with a 200 x objective lens. Light source intensity and image hue, brightness and saturation were standardized in every section. The beta-cell area was automatically determined by using a preset positive pixel count algorithm with MetaMorph software version 7.7.4.0 (Molecular Devices, Inc., Sunnyvale, CA). The fractional area of β-cell was expressed by a ratio of the positive green pixel counts to the total pixel counts corresponding to the total pancreas area in a section. The proportions of the number of anti-insulin positive beta cells per total number of the cells in the islet and the proportion of nuclear PDX-1 positive beta cells per total number of the anti-insulin positive beta cells in the islet were also calculated. In the Masson's trichrome-stained sections, the degree of fibrosis of islet in each group was assessed by two pathologists blindly using the semi-quantifying fibrosis score (0 = none, 1 = mild, 2 = moderate, 3 = marked, 4 = complete effacement). The fibrosis scores in every section that were estimated by each observer were averaged.

8. Western blot for Akt phosphorylation in visceral fat

Visceral fat tissues were harvested 16 weeks postoperatively from ZDF rats in the

DJB, DJBPF, and AL groups. Tissues were homogenized in 1 mL of NP40 containing lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0; 5 mM EDTA, 1% NP40) containing protease and phosphatase inhibitors (2 lg/mL leupeptin, 2 lg/mL aprotinin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). Tissue extract was cleared by centrifugation (12000 g for 15 min). Protein samples were boiled with 4x lithium dodecyl sulfate Nupage sample buffer from Invitrogen (NP0007) for 2 min before loading. Electrophoresis was done at 80 mA using MES-SDS running buffer from Invitrogen. Transfer was performed at 4°C, 100 V for 90 min. Polyvinylidene difluoride membranes were blocked in 5% milk-Tris-buffered Saline-T. Anti-pAkt, anti-Akt, and anti-actin antibodies were probed overnight at 4°C.

9. Insulin and enteroendocrine hormones

Plasma insulin was determined using Ultra-Sensitive Rat Insulin enzyme-linked immunosorbent assay (ELISA) kit (Crystal Chem, Downers Grove, IL). Plasma active GLP-1 (GLP-17-36 and GLP-17-37), glucose-dependent insulinotropic peptide (GIP) (total), and ghrelin (total) were determined using rat specific ELISA kits (Millipore, St. Charles, MO). The total peptide YY (PYY) (PYY1-36 and PYY3-36) and pancreatic glucagon levels were measured using rat-specific enzyme immunoassay kits (ALPCO Diagnostics, Salem, NH). Glucagon level was measured using the antibody against the C-terminal fragment[28] of glucagon, which has high specificity to pancreatic glucagon and shows no cross-reactivity with intestinal glucagon, GLP-1, and GLP-2. Level of insulin, GLP-1, GIP, PYY, and glucagon were measured pre-operatively and at week 1 and 16 post-operatively.

10. Acetaminophen and D-xylose absorption test

The acetaminophen absorption test measures the rate of gastric emptying by calculating the plasma concentration of acetaminophen after the ingestion of a known amount of acetaminophen. Overnight (12-h) fasted animals were administered with 75 mg/kg acetaminophen solution in normal saline (0.9%) by oral gavage. Blood samples were drawn from the rats' tail at baseline and at 15 and 30 min after acetaminophen administration. Plasma acetaminophen concentrations were measured using an enzymatic method (Stanbio Laboratory). D-xylose absorption test measures intestinal glucose absorption by calculating the plasma concentration of D-xylose after the ingestion of a known amount of D-xylose. After 12-h overnight fasting, D-xylose (2 g/kg) was administered by oral gavage. Blood samples were drawn from the rats' tail at baseline and at 15 and 30 min after D-xylose administration. Plasma xylose concentrations were measured using the chemical method. Tests for gastric emptying and intestinal glucose absorption (D- xylose) were measured at weeks 4-8 postoperatively.

11. Statistical analyses

Data were analyzed using Student's t-test and one-way and two-way analysis of variance (ANOVA) where appropriate. Significant differences between treatment groups were followed up with post hoc Bonferroni multiple comparison tests ($p < 0.05$). The data are expressed as mean ± standard error of the mean (SEM). Sequential variations were analyzed by repeated measures of analysis of variances (RMANOVA) with post-hoc differentiation. Non-parametric tests (e.q., the Mann-Whitney U test or the Kruskal-Wallis test) were performed if appropriate. P value < 0.05 was considered statistically significant.

III. RESULTS

In the first set of experiments, we compared the effects of DJB (bypass alone without gastric resection) versus DJBPF in ZDF rats in terms of GT, plasma

insulin level, changes in pancreatic beta cells and insulin signaling in visceral fat tissues.

1. Effect of foregut bypass surgery on glucose tolerance and pancreatic beta cells

A. Effect of body weight and calorie intake on glucose tolerance

Before surgery, there was no difference in BW, FI, fasting glycemia, and GT between the DJB and sham-operated groups. Food intake in the DJB group was significantly lower compared to that of the sham-AL group until 1 week after surgery ($p < 0.01$). However, there was no significant difference in average daily FI during the study period between DJB and DJBPF (Figure 2A). BW in DJB and PF animals was significantly lower than that in AL animals 1 week after surgery ($p < 0.05$). Subsequently, all three groups did not show any difference in weight gain (weight gain %) 16 weeks postoperatively (Figure 2B).

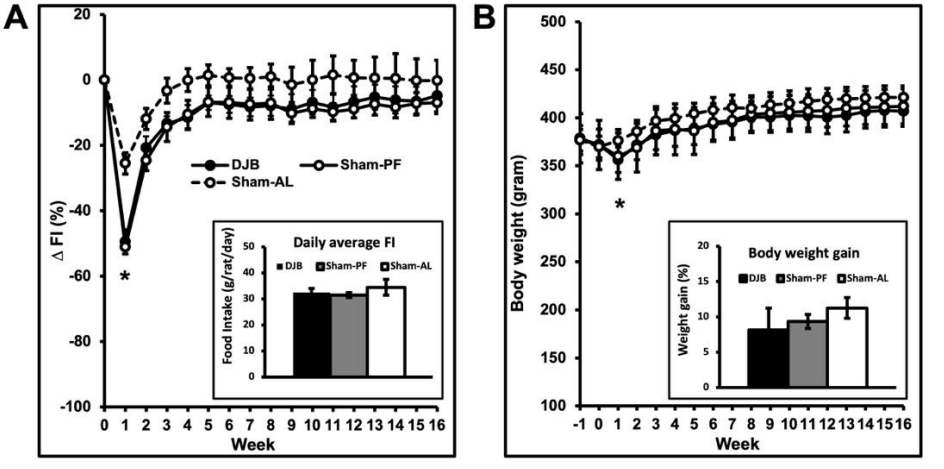

Figure 2. Postoperative changes in food intake (A) and body weight (B). Δ food intake (Δ FI) in duodenojejunal bypass (DJB) animals was significantly lower

than that in ad-libitum (AL) rats until 1 week after surgery (*$p < 0.01$), and the difference weakened over time. The control for paired-feeding resulted in equivalent FI between DJB and paired-fed (PF) rats (A). There was no significant difference in average daily FI during the study period (the bar chart in pane A). There was no significant difference in body weight between DJB and PF rats throughout the study period except for 1 week after surgery. At 1 week after surgery, DJB and PF rats transiently showed lower body weight than AL rats (*$p < 0.05$) (B). All three groups did not show a significant difference in weight gain (weight gain %) at 16 weeks after surgery (the bar chart in pane B). Δ FI (%) = FI (postop – preop)/preop x 100. FI (g/rat/day) is expressed as average daily food intake during the 16 weeks of the study period. Weight gain (%) = BW (16 weeks – a week before surgery)/a week before surgery x 100. Data are shown as mean ± standard error of the mean. DJB (n = 16), PF (n = 10), and AL (n = 10) rats.

DJB animals showed higher GT than DJBPF and AL animals 2 weeks after surgery ($AUC\text{-}OGTT_{2wk}$: 33188 ± 2919, 44555 ± 1977, 49161 ± 823 mg/dl x min; $p < 0.01$, Figure 3A). The effect on GT improvement attenuated 4 and 8 weeks after surgery. However, DJB animals showed significantly lower GT again 16 weeks after surgery than DJBPF and AL animals (Figure 3A). GT of DJB animals was significantly better 2 weeks postoperatively than their preoperative values ($\Delta AUC\text{-}OGTT_{preop-2wks}$ = - 13.1 ± 6.5 %; $p < 0.05$, Figure 3B). Over time, GT progressively worsened in PF and AL animals ($p < 0.01$ by RMANOVA), while the time-course change in GT in DJB animals was significantly different from the sham-operated animal groups ($p = 0.01$; RMANOVA with Turkey's post hoc test) (Figure 3B) despite similar FI and weight gain to PF and AL animals.

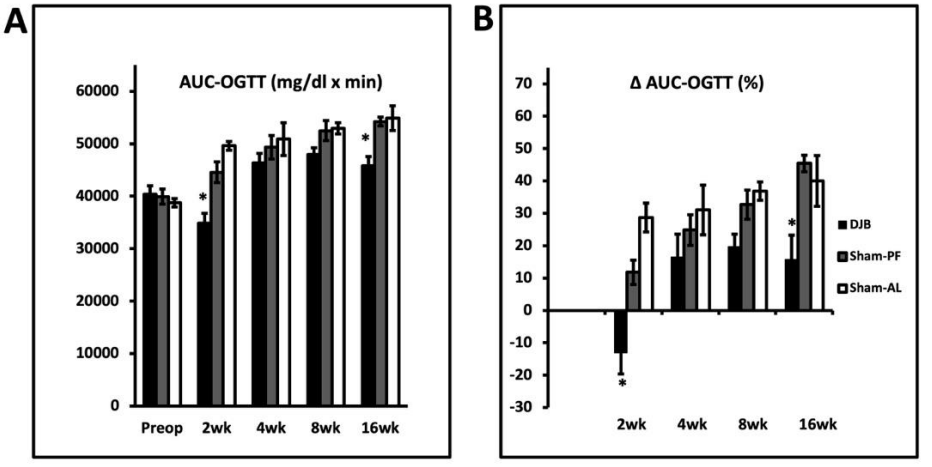

Figure 3. Effect of duodenojejunal bypass (DJB) surgery on postoperative glucose tolerance. DJB rats showed significantly better glucose tolerance 2 weeks postoperatively than sham-operated rats. The statistical significance was no longer observed 4 and 8 weeks after surgery; however, 16 weeks after surgery, DJB rats showed significantly lower AUC-OGTT again than sham-operated rats ($^{*}p < 0.01$ by analysis of variance [ANOVA]) (A). DJB rats had significantly more improved glucose tolerance 2 weeks after surgery compared to preoperative levels ($^{**}p < 0.05$) (B). Glucose tolerance progressively worsened in pair-fed (PF) and ad-libitum (AL) rats over time ($^{*}p < 0.01$ by repeated measures ANOVA), while time-course changes in glucose tolerance in DJB rats was significantly different from those of the sham-operated rat groups ($^{\dagger}p < 0.05$; Tukey's post-hoc test). Δ AUC-OGTT (%) = AUC-OGTT (post-op – pre-op)/pre-op x 100. AUC-OGTT: area under the curve of oral glucose tolerance test. Data are expressed as mean ± standard error of the mean. DJB (n = 16), PF (n = 10), and AL (n = 10) animals.

B. Characteristics of the development and progression of diabetes in Zucker diabetic fatty rats

Naturally, ZDF rats developed fasting hyperglycemia between 7 and 10 weeks of age (Figure 4A), while plasma insulin levels display a biphasic pattern (Figure 4B), increasing until 8 weeks during the pre-diabetic state and decreasing afterward ($\Delta Insulin_{8-29wk}$ = - 84.8 ± 8.9 %; $p < 0.0$) (Figure 4B). Overt hyperglycemia is accompanied by decreased plasma insulin levels, confirming the decompensation of beta-cell function and progressive nature of insulin deficiency in this model.

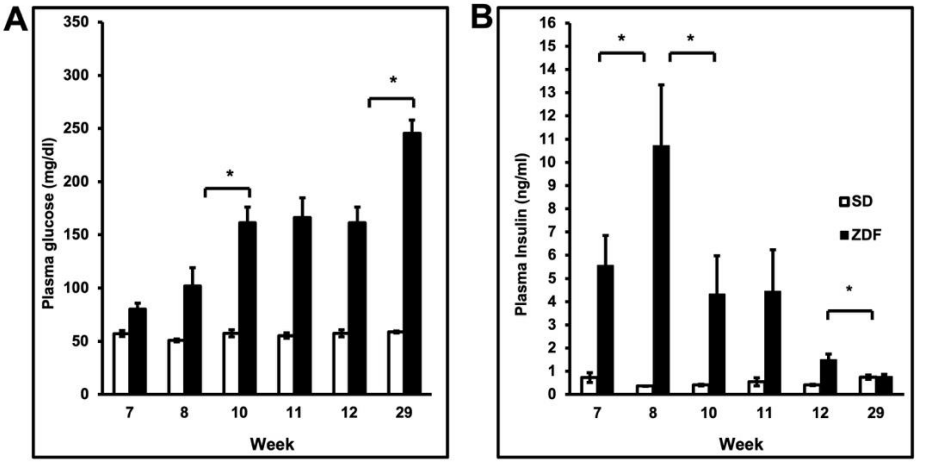

Figure 4. Characterizing the development of overt diabetes and its progression in Zucker diabetic fatty (ZDF) rats. Fasting plasma glucose levels were aggravated over time during the observational period in ZDF rats (from age 7 to 29 weeks; $^{*}p < 0.05$ by paired t-test), while these levels constantly remained in normal Sprague-Dawley (SD) rats (A). Plasma insulin levels increased from age 7 to 8 weeks ($^{*}p < 0.05$ by one-way analysis of variance [ANOVA]test). However, subsequently, plasma insulin concentrations progressively decreased during the observational period ($^{*}p < 0.05$ by one-way ANOVA test) and became similar to those of SD rats (B). Data are expressed as mean ± standard error of the mean. ZDF (n = 6) and SD (n = 6) rats.

C. Preservation of plasma insulin after bypass surgery

DJB (n = 16) animals maintained significantly higher fasting and postprandial insulin levels until 16 weeks after surgery than sham-operated animals (*$p < 0.05$ by RMANOVA with Tukey's post-hoc test) (Figure 5A). Fasting insulin levels in DJB animals were maintained similar to the preoperative value ($p = 0.51$). However, fasting insulin levels in DJB animals were significantly higher than those in sham-operated animals ($\Delta\ Insulin_{preop-16wk}$ = - 11.2 ± 1.3 vs. - 58.4 ± 9.5 and -69.9 ± 12.5 %, DJB vs. PF and AL, respectively; $p < 0.01$). Fasting and glucose-challenged plasma insulin levels of sham-operated animals significantly decreased 16 weeks postoperatively over time ($p < 0.05$) (Figure 5 A, B), and the patterns were exactly the same as in the un-operated ZDF rats (Figure 4B).

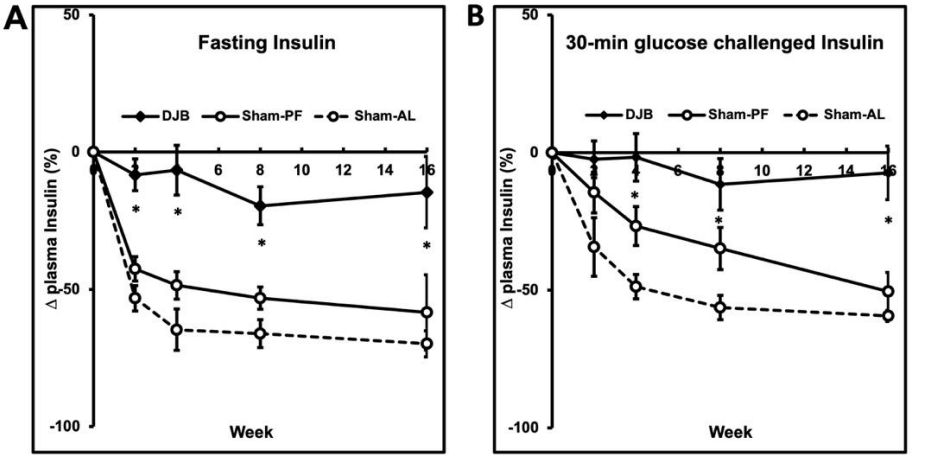

Figure 5. Effect of duodenojejunal bypass (DJB) surgery on the preservation of the postoperative plasma insulin levels. DJB (n = 16) animals maintained significantly higher fasting and postprandial insulin levels 16 weeks after surgery than sham-operated rats (*$p < 0.05$ by RMANOVA with Tukey's post-hoc test). Fasting insulin level in DJB animals was maintained similar to the preoperative value ($p = 0.51$). Δ Plasma insulin = insulin (postop – preop)/preop x 100 (%).

Data are expressed as mean ± standard error of the mean. DJB (n = 16). Sham PF: sham-operated paired-fed (n = 10), Sham-AL = and ad-libitum (n = 10) rats.

D. Histomorphometry of islets

(A) The effect of duodenojejunal bypass (DJB) on the preservation of the morphological modification of the islets

The islets of twelve 12-week-old control ZDF rats were distinguishable from those of 20-week-old rats because of the former's predominant features of asteroid-like expansions (Figure 6 A, B). However, these findings were not frequently observed in 20-week-old rats. Similarly, the islets in DJB animals (C) were also more distinguishable compared to those in sham-operated animals (D, E). DJB animals showed a significantly higher proportion of the asteroid-like islets than sham-operated animals ($p < 0.01$, Figure 6F), which was similar in 12-week-old ZDF rats ($p=0.98$).

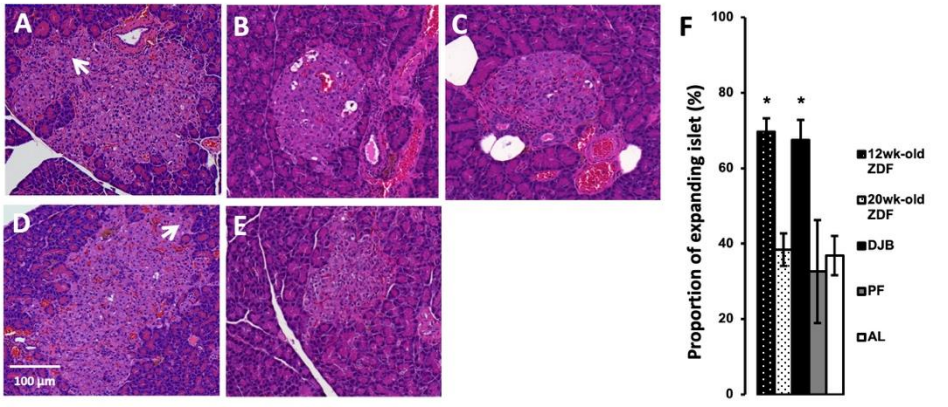

Figure 6. Effect of duodenojejunal bypass (DJB) surgery on the preservation of the morphological modification of the islets. The majority of islets in DJB (A) rats showed the asteroid-like projections similar to 12-week-old ZDF controls (D).

These projections of the islets were only occasionally noted in the pair-fed (PF) (B), ad-libitum fed (AL) (C) rats, and 20-week-old ZDF controls (E). Twelve-week-old Zucker diabetic fatty (ZDF) controls (A) presented vague asteroid-like expansions of the islets beyond the usual boundaries (arrow) (D). The proportions of the islets presenting the asteroid-like expansions were significantly higher in 12-week-old ZDF controls and DJB rats than those in 20-week-old ZDF controls and PF and AL rats (*$p < 0.01$ by analysis of variance with Tukey's post-hoc test) (F). (Hematoxylin and eosin stain, pancreas, × 200). DJB (n = 9), PF (n = 7), and AL (n = 7) rats.

(B) Changes in the islet beta cells after DJB surgery

Areas of asteroid-like expansions (Figure 6C) were positively stained by anti-insulin IHC in matched pancreatic sections (Figure 7A).

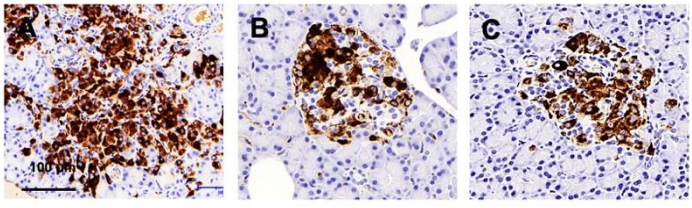

Figure 7. Changes in the beta cells outside of the islet boundaries after duodenojejujal bypass (DJB) surgery. In DJB animals (A), anti-insulin IHC-positive cells were present beyond the boundaries of a typical discrete islets (i.e. consistent with the area of islet expansion as shown in hematoxylin and eosin stain) in the majority of islets, in contrast to paired-fed (PF) (B) and ad-libitum fed (AL) (C) rats. IHC stain (pancreas, x 200). DJB (n = 9), PF (n = 7), and AL (n = 7) rats.

Moreover, fractional area of anti-insulin IHC-positive beta-cell per a unit

microscopic field was threefold wider in DJB animals than in PF and AL animals (0.029 ± 0.08, 0.008 ± 0.003, and 0.007 ± 0.002, respectively; p <0.01) (Figure 8D).

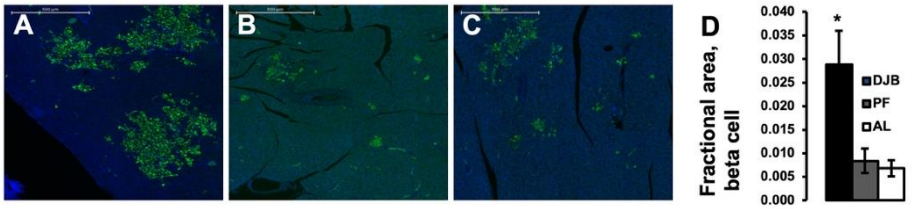

Figure 8. Fractional area of beta cells in the islet after the duodenojejunal bypass surgery (DJB). The anti-insulin IHC-positive beta-cell area in the pancreas was prominent in DJB animals (A) than that in the paired-fed (PF) (B) and ad-libitum fed (AL) (C) rats (fluorescence in green). The fractional area of beta cells per unit pancreas area was wider in DJB animals than those in sham-operated rats (*p < 0.01) (D). Immunohistochemical stain, x 100). DJB (n = 9), PF (n = 7), and AL (n = 7) rats.

(C) Beta-cell maturation after DJB

The proportion of the nuclear PDX-1-positive beta cells was threefold higher in DJB animals than in sham-operated animals (p < 0.01) (Figure 9).

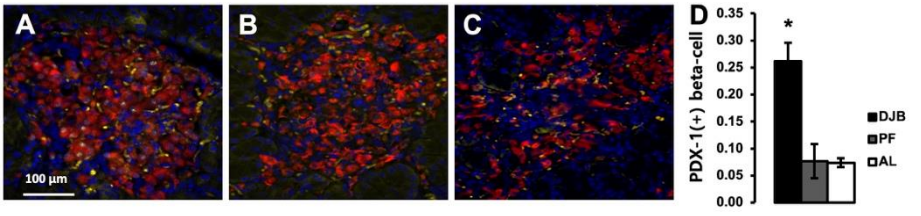

Figure 9. Beta-cell maturation after the procedures. The nuclear (fluorescence in blue) anti-pancreatic duodenal homeobox-1 (PDX-1) immunohistochemistry (IHC) positivity (in white) in the beta cells (in red) was predominant in duodenojejunal bypass (DJB) rats (A) than in pair-fed (PF) (B) and ad-libitum

fed (AL) rats (C). The proportion of the number of PDX-1 IHC-positive beta cells per total number of beta cells in a unit islet was higher in DJB rats than those in sham-operated animals (*$p < 0.01$) (D). (Immunohistochemical stain, pancreas, × 200). DJB (n = 9), PF (n = 7), and AL (n = 7) rats.

In addition, AL animals presented larger areas of fibrous tissue infiltrations (no significance) in the islets than DJB and PF animals (fibrosis score: DJB, PF, and AL, 1.7 ± 0.4, 1.9 ± 0.5, and 2.4 ± 0.5, respectively; p=0.083) (Figure 10).

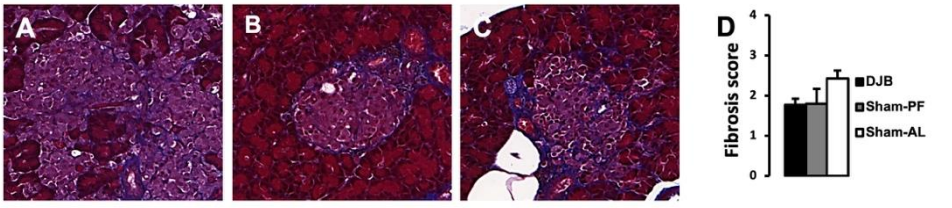

Figure 10. The fibrous tissue infiltration in the islets after the procedures. Blue-stained islet areas present collagen and fibrous infiltration in duodenojejunal bypass (DJB) (A), pair-fed (PF) (B), and ad-libitum fed (AL) (C) rats. The islets of DJB animals showed a lower marginal fibrosis score than those in AL rats. (p = 0.083) (D). (Masson's trichrome stain, pancreas, × 200). DJB (n = 9), PF (n = 7), and AL (n = 7) rats.

2. Insulin signaling in visceral fat

The phosphorylated Akt level in visceral adipose tissue was significantly higher in DJB animals than in PF and AL animals ($p < 0.05$) (Figure 11).

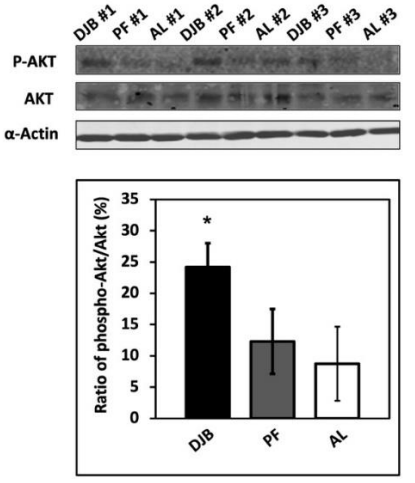

Figure 11. The effect of bypass surgery on insulin signaling in visceral fat. Akt phosphorylation in visceral fat was evaluated by Western blotting. Western blots were probed with anti-phospho-Akt and anti-Akt antibodies. The blot represents at least three independent experiments. The phosphorylated Akt/Akt ratio was significantly higher in duodenojejunal bypass (DJB) animals than those in sham-operated paired-fed (PF) and sham-operated ad-libitum fed (AL) rats (*$p < 0.05$). The results are expressed as the percentage of the phosphorylated Akt/Akt ratio. Data are expressed as means ± standard error of the mean. DJB (n = 9); PF = pair-fed (n = 7); AL = ad-libitum (n = 7) rats.

In the second set of experiments, to understand the foregut mechanism, we compared the effects of DJB (bypass alone without gastric resection) versus SG (gastric resection without bypass) on changes of entero-insular hormones in ZDF rats.

3. Evidence of the putative foregut mechanism

A. Effects of DJB and sleeve gastrectomy (SG) on body weight, calorie intake, and glucose tolerance

There were no statistical differences in BW and FI between the groups preoperatively. DJB surgery had no significant effect on BW. Furthermore, there was no difference in BW between DJB and DJBPF animals 1 week after surgery, thus providing the right experimental conditions for investigating weight-independent effects of DJB surgery (Figure 12A, B). In contrast, SG had substantial effects on weight loss, that is, SG animals had significantly lower BW than DJB and AL animals 4 weeks after surgery (Figure 12A, B). SG animals had significantly lower FI than DJB and AL animals 8 weeks after surgery, while DJB animals had reduced FI only 1 week postoperatively (Figure 12C, D). At long-term follow-up, there was no difference in BW and FI between the study groups. DJB animals had significantly better GT early after surgery, as shown by lower AUC for glucose (Δ AUC-OGTT), compared to AL and DJBPF animals (Figure 12E). This effect was independent of weight changes because similar weight gain profiles to those of DJB rats were observed in PF and AD animals (Figure 12A, C). SG animals had marginally better GT, as shown by the nonsignificant improvement in Δ AUC-OGTT, compared to AL animals (Figure 12E). Analysis of glucose excursions following OGTT showed that DJB rats had improved glucose levels at all time points. In contrast, SG rats showed a biphasic response with a sharp increase in glucose levels at 15 and 30 min and a significant decrease of glucose levels at 120 min similar to DJB rats (Figure 12F). However, GT worsened in all groups over time, similar to the first set of experiments. There was no difference at mid-term (4–8 weeks) and long-term (16 weeks), consistent with the progressive nature of diabetes in this genetically programed diabetes model.

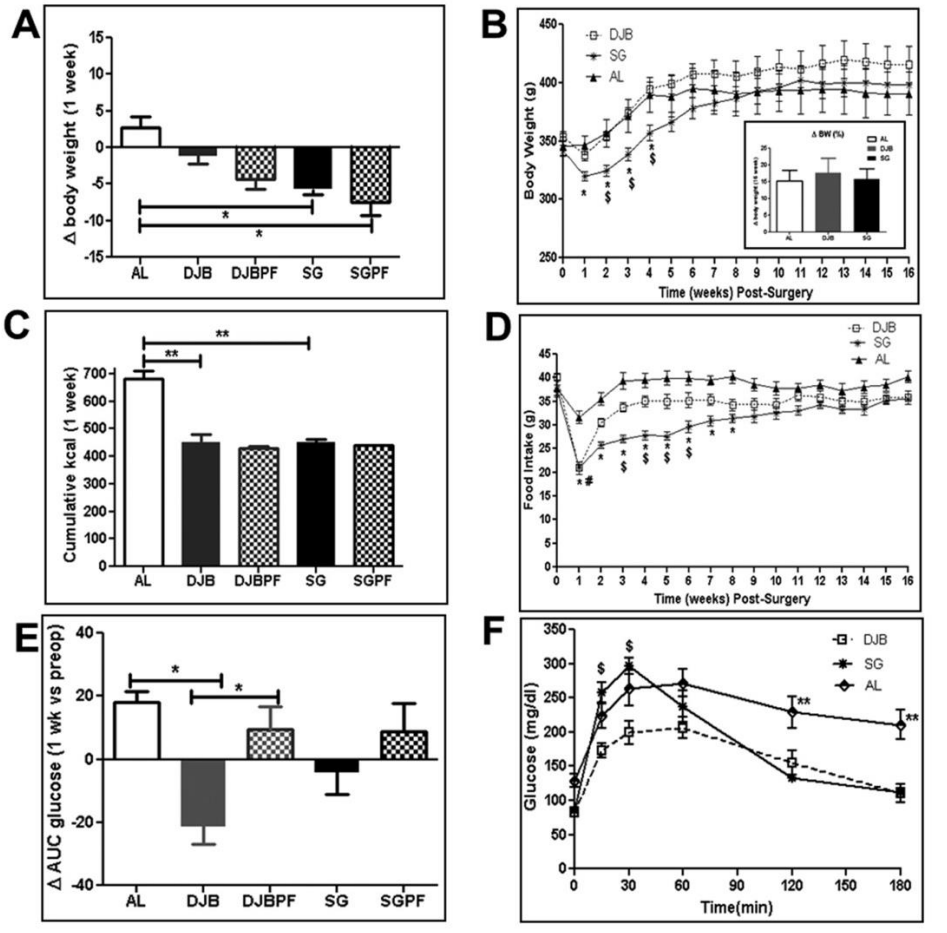

Figure 12. Postoperative changes in body weight, food intake, and glucose tolerance after surgery. Duodenojejunal bypass (DJB) surgery did not affect weight loss in the short- (A) and long-term follow-up period (B), whereas sleeve gastrectomy (SG) substantially reduced body weight up to 4 weeks postoperatively ($^{*}p < 0.05$) (B). At 1 week postoperatively, food intake (C) was significantly lower in all operated animals than that in ad-libitum fed (AL) controls ($^{**}p < 0.01$). In the long-term follow-up, DJB surgery did not affect food intake (D), while SG reduced food intake for at least 8 weeks after surgery. Changes in AUC-OGTT (E) from baseline and glucose excursions (F) 1 week postoperatively showed markedly higher glucose tolerance in DJB rats than DJB

paired-fed (DJBPF) rats and AL controls. SG only showed a trend toward improvement by AUC-OGTT, while glucose excursions were characterized by a sharp increase in glucose levels at 15 and 30 min and a decrease in glucose levels at 120 min. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\$}p < 0.05$ SG vs. DJB; $^{\#}p < 0.05$ DJB vs. AL. AUC-OGTT: area under the curve of oral glucose tolerance test. Data are expressed as means ± standard error of the mean. DJB (n = 12), SG (n = 12), DJBPF: DJB paired-fed (n = 8), SGPF: SG paired-fed (n = 8), and AL (n = 12) rats.

B. Effect of DJB on entero-insular hormonal axis

Both DJB and SG resulted in significantly lower fasting insulin levels in the short-term after surgery compared to controls. In the long-term after surgery, both DJB and SG had fasting insulin levels similar to preoperative values. In contrast, insulin levels significantly decreased in AL controls (Figure 13A) following the typical progressive decrease in insulin levels in this ZDF model (Figure 4B and Figure 5).[33] Postprandial insulin levels significantly increased in SG rats at weeks 1 and 16, while DJB rats had higher postprandial insulin levels, which only showed a nonsignificant trend, than sham-operated animals (Figure 13B), suggesting that improved GT after DJB surgery is, at least in part, independent on insulin secretion.

Preoperatively, oral glucose gavage resulted in a physiologic increase in GLP-1 levels in normal nondiabetic (SD) rats, an effect not observed in diabetic (ZDF) rats (Figure 13C), consistent with blunted GLP-1 response to glucose in diabetes. DJB surgery had no effect on postprandial GLP-1 levels at any time point (Figure 13D, E). In contrast, SG resulted in a supra-physiologic, fourfold increase in postprandial GLP-1 levels 15 min after oral glucose challenge, and the effect persisted for the entire study duration (16 weeks) (Figure 13D, E).

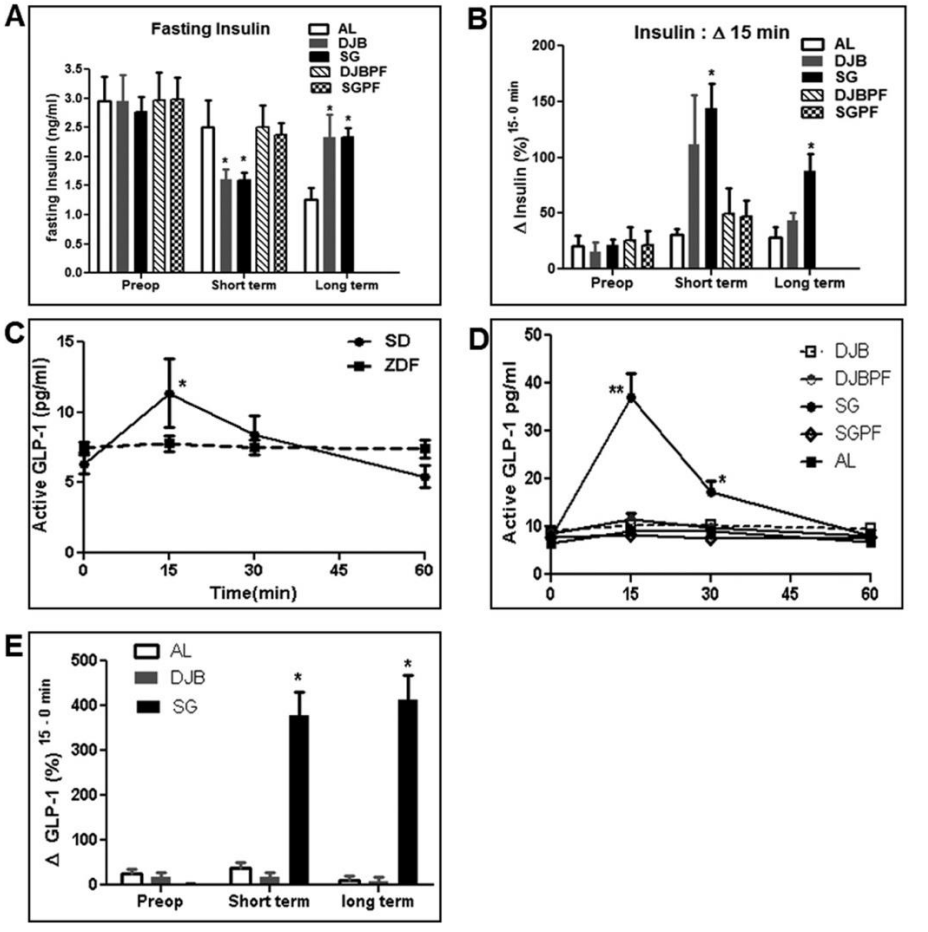

Figure 13. Postoperative changes in plasma insulin and active glucagon-like peptide-1 (GLP-1) levels. Duodenojejunal bypass (DJB) surgery and sleeve gastrectomy (SG) resulted in a significantly decreased fasting insulin level 1 week after surgery possibly due to the improvement of insulin resistance (A). At 16 weeks after surgery, both DJB and SG rats maintained insulin levels similar to preoperative values, whereas a sharp decrease in insulin level was observed in ad-libitum (AL) controls ($^{*}p < 0.05$) (A). Insulin response to oral glucose (Δinsulin, 15 min from baseline) at 1 and 16 weeks after surgery increased only by SG ($^{*}p < 0.05$) (B). GLP-1 excursion decreased in diabetic (Zucker diabetic fatty [ZDF]) rats but increased in nondiabetic (Sprague-Dawley) rats (C). SG

dramatically increased 15-min GLP-1 level response to glucose from baseline during the oral glucose tolerance test ($^{*}p < 0.05$) (D, E). Data are expressed as means ± standard error of the mean. DJB (n = 8), SG (n = 12), AL (n = 8), ZDF rats (n = 9), and Sprague-Dawley rats (n = 9).

The levels of other incretin hormones, such as GIP and PYY, increased fivefold after SG (Figure 14A, B). In contrast, there was no significant change in the levels of GIP and PYY after DJB surgery (Figure 14A, B). SG resulted in a dramatic decrease in fasting ghrelin levels. Other surgical interventions had no effect on postprandial ghrelin levels (Figure 14C).

Glucagon level decreased with the oral glucose challenge test in SD rats consistent with normal physiology. This normal glucagon suppression effect of the oral glucose challenge test was not observed in ZDF rats preoperatively (Figure 14 D). However, only DJB surgery restored glucagon suppression in ZDF rats, whereas the dysregulation of postprandial glucagon response persisted after SG (Figure 14E).

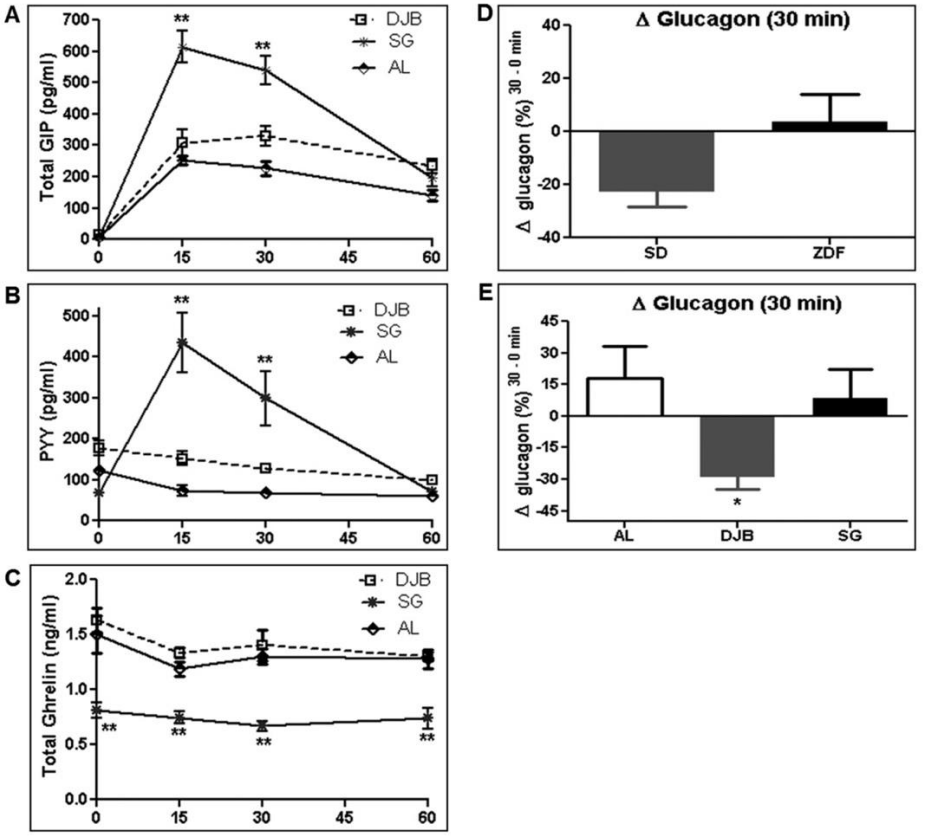

Figure 14. Postoperative plasma levels of enteroendocrine hormones and glucagon. Sleeve gastrectomy (SG) dramatically increased the levels of glucose-stimulated glucose-dependent insulinotropic peptide (GIP) and peptide YY (PYY) ($^{**}p < 0.01$) and decreased fasting ghrelin levels (C) ($^{**}p < 0.01$) (A, B, C). Thirty-min glucagon excursions expressed as a change from baseline (fasting) during the oral glucose tolerance test in nondiabetic (Sprague-Dawley), diabetic (Zucker diabetic fatty [ZDF]), duodenojejunal bypass (DJB), SG, and ad-libitum rats (D, E). Nondiabetic rats showed significant decrease in glucagon level ($^{*}p < 0.05$), whereas oral glucose failed to decrease glucagon level in ZDF rats. DJB surgery restored glucagon suppression in diabetic (ZDF) rats ($^{*}p < 0.05$). Data are expressed as means ± standard error of the mean.

C. Change in gastric emptying and glucose absorption after DJB and SG

SG rats showed significantly higher plasma levels of acetaminophen and D-xylose 15 min after oral gavage, suggesting an accelerated gastric emptying and glucose absorption, than DJB and AL rats (Figure 15).

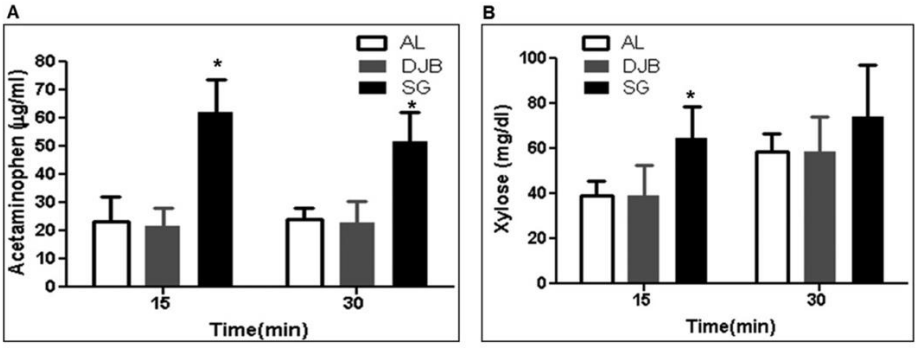

Figure 15. Gastric emptying and intestinal absorption. Sleeve gastrectomy (SG) rats showed significantly higher plasma levels of acetaminophen at 15 and 30 min, indicating accelerated gastric emptying (A). Fifteen-min D-xylose levels were higher in SG animals than those in duodenojejunal bypass (DJB) rats and controls, indicating faster glucose absorption (B). Data are expressed as means ± standard error of the mean. SG: sleeve gastrectomy, AL: ad-libitum fed.

D. Combination of SG with DJB surgery

Compared to SG alone, the combination of SG with DJB surgery resulted in greater weight loss (Figure 16A) without substantial change in FI (Figure 16B).

Furthermore, the addition of intestinal bypass to SG improved GT (Figure 16C), while the hormonal effects were slightly weakened but remained similar in pattern to that observed after SG alone (Figure 16D-F).

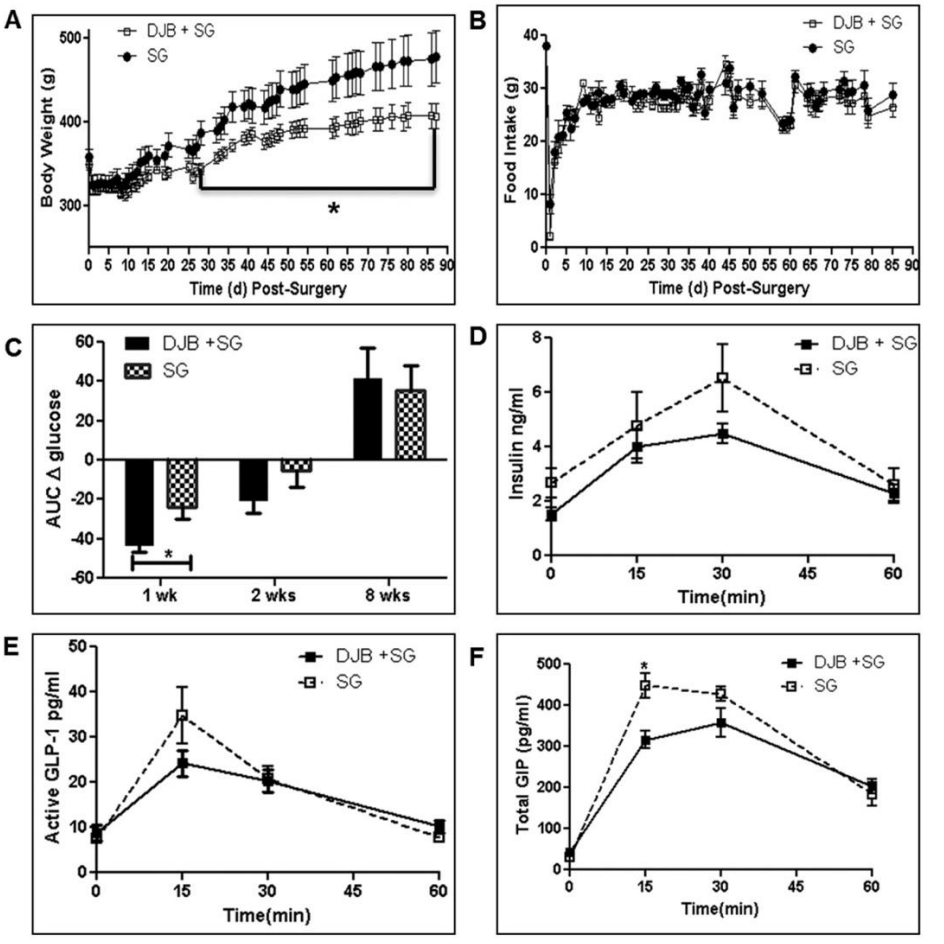

Figure 16. The combination of sleeve gastrectomy (SG) with duodenojejunal bypass (DJB). The combination of SG with DJB (SG + DJB) resulted in greater weight loss (A) without significant change in food intake (B). Compared to SG alone, the combination procedure improved oral glucose tolerance (Δ AUC-OGTT) (C) at 1 week after surgery without significant changes in insulin (D),

glucagon-like peptide-1 (E,) and glucose-dependent insulinotropic peptide (F) levels ($^{*}p < 0.05$). Data are expressed as means ± standard error of the mean. SG + DJB (n = 7) and SG (n = 7). Δ AUC-OGTT (%) = AUC-OGTT (post-op – pre-op)/pre-op x 100. AUC-OGTT: area under the curve of oral glucose tolerance test

IV. DISCUSSION

1. The first set of experiments

Given the early improvement in T2DM within a few days after RYGB or biliopancreatic diversion, it is suggested that there is a mechanism beyond weight loss to explain these beneficial effects on glucose homeostasis.[23,34,35,36] DJB surgery is considered an important surgery performed to investigate the effects of the exclusion of a nutrient passage from the duodenum on glucose homeostasis without food restriction.[8,9] In this study, we preserved the pylorus intact to investigate the sole effect of DJB surgery without any dumping-like symptoms that may reduce the FI and induce a concomitant weight loss. As a result, DJB animals quickly recovered from the immediate postoperative reduction in FI and BW, which became similar to the preoperative levels and those of AL animals as early as 2 weeks after surgery. Despite the absence of difference in FI and weight gain profile, GT was greater in DJB animals than in PF animals, which were designed to control the same amount of food consumed by DJB animals. Strikingly, only DJB animals showed a more significant improvement in GT compared to preoperative values at this time point. We could confirm that DJB surgery conferred the effect on GT improvement by the mechanism independent of calorie restriction and weight loss in ZDF rats.

The phenotype of ZDF rats is well recognized, which is characterized by rapid and severe diabetes progression with age.[33] In a previous study, GT was significantly greater in RYGB animals than in sham-operated animals.[37,38] However, impaired GT never reverted to the normal range after RYGB. In this study, the long-term follow-up results clearly showed distinguishable courses in T2DM progression between DJB and sham-operated animals, although the effect was transiently attenuated. In contrast to the rapid aggravation of GT in both PF and AL animals over time, only DJB animals maintained GT similar to the preoperative levels. This was observed throughout the study period. Interestingly, the previous study on Goto-Kakizaki rats showed a similar transient weakening of the effect on fasting glycemic control 4 weeks after DJB surgery and temporal variations for 9 months.[9] However, considering the genetically programmed diabetes progression in ZDF rats, it is surprising that DJB arrests diabetes phenotype progression throughout the long-term follow-up period in the first set of experiments. The early improvement of GT and transient attenuation followed by recovery of the long-term effect on glucose homeostasis may indicate variations of the combined mechanisms that play an alternative or cooperative role in improving T2DM after DJB surgery during the follow-up period. Even this longer-term recovery of the effect of DJB was not significant in the second set of experiments. Further investigation needs to define the longer-term time-course longitudinal variations of dominant mechanisms involving glucose homeostasis.

The exclusive role of the bypassed foregut in beta-cell insulin secretory function may not only be a key to treat T2DM but also an interesting piece of research in the pathophysiology of diabetes. Despite the current investigations regarding the effect of DJB on changes in plasma insulin concentrations, the paucity of data showing consistent results of beta-cell function after surgery makes firm conclusion difficult.[10,12,14,39] Moreover, previous studies have the following limitation: almost all the plasma insulin levels were measured at a single time

point postoperatively. For example, in our earlier study of ZDF rats, there were no significant differences in plasma insulin levels between DJB and sham-operated animals, measured 3 weeks after surgery.[10] A similar study performed using Goto-Kakizaki rats showed higher plasma insulin levels than sham-operated control 40 days after surgery[12] or the tendency to increase C-peptide levels measured 3 months after surgery.[14] Before these reports, Ramos et al. described the considerable improvement in plasma C-peptide concentrations by 25% between the third and the sixth months after DJB. However, it did not show any significant alteration until 3 months after surgery.[6] In the first set of this study, we aimed to investigate the time-course variations in plasma insulin concentrations after surgery to determine the sustained effect of DJB surgery on the amelioration of beta-cell deterioration. Plasma C-peptide concentrations were measured simultaneously to eliminate the probability of changes in hepatic clearance of insulin and reflect beta-cell function (data not shown). With DJB surgery, the plasma insulin level of ZDF rats did not further decrease, with the eventual preservation of the plasma insulin levels throughout the postoperative period. Given the data on greater plasma insulin concentrations coinciding with better GT throughout the study period, we suggested that the long-term effect of DJB surgery on GT is partly attributable to the preservation of the plasma insulin levels in ZDF rats.

We applied a morphometric investigation for the pancreatic islets that may primarily be responsible for the increase in plasma insulin levels to understand how DJB surgery maintained these levels during the entire study period. We found out that the predominant presentation of the asteroid-like expansions distinguished DJB animals from PF and AL animals postoperatively. Interestingly, however, these particular projections appeared only scarcely and heterogeneously in the islets of sham-operated animals. In DJB animals, over two-third of the islets expressed the projections that were twofold more frequent than in both PF and AL animals. The higher plasma insulin levels are attributable to a higher

proportion of these asteroid-like islet expansions after DJB surgery. A tendency for a larger fractional area of beta-cells seemed to be attributable to these striking features of projections of the islets beyond the boundaries and lesser degenerative changes in islets.

Therefore, to determine why only DJB animals presented predominant projections of the islets after surgery, we focused on the morphological modifications of islets that might occur during the development of diabetes in ZDF rats. Earlier reports demonstrated the hypertrophic islets with multiple irregular projections into the surrounding exocrine tissues in 12-week-old ZDF rats.[40,41] Tokuyama et al. reported these findings in 12-week-old ZDF rats with similar plasma insulin levels compared to those in 7-week-old rats.[40] Pick et al.[41] also demonstrated projections of hypertrophied islets in 12-week-old ZDF rats with a twofold increase in beta-cell masses than 7-week-old ZDF rats. However, it was not adequate to maintain the same level of fasting glycemia as in 7-week-old rats. The current report showed that hypertrophied expanding islets compressed adjacent exocrine as early as age 6 weeks.[42] Farilla et al.[43] reported these budding appearances in a 12-week-old ZDF rat. Interestingly, GLP-1 treated ZDF rats presented enhanced irregularity of margins of the islets and extensive branching out of cells from the periphery of the islets with 3.4-fold higher plasma insulin levels in 12-week-old ZDF rats compared to those in age-matched controls. In a recent report on the morphological decompensation and the natural deterioration of beta-cell mass in ZDF rats, islet degeneration progresses severely and widely spreads at age 14-16 weeks. The rat becomes severely hyperglycemic and hypoinsulinemic.[33] In this study, as shown in the age-matched ZDF controls, we confirmed that the islets naturally lost their expanding characteristics according to ages. Moreover, both sham-operated animals lost projections of the islets by the same degrees as in 20-week-old ZDF controls. DJB animals maintained higher proportions of projections similar to the preoperative levels. It seems that morphological modifications are not a de novo

phenomenon after DJB surgery. Collectively, it is clear that DJB surgery contributes to preserving the morphological modifications in this study and was initiated during a critical period of compensation to insulin resistance as an adaptive mechanism to insulin demand in ZDF rats. Conclusively, beta-cells appear to be protected from the natural deteriorations and maintain plasma insulin levels. DJB surgery partly plays a role in preventing further deterioration of GT in this genetically programmed progressive T2DM model via a yet to be elucidated mechanism(s).

Moreover, the nuclear PDX-1, which plays an essential role in the maturation of pancreatic beta cells, is dominantly expressed by the foregut bypass surgery. Further, the degenerative changes of the islet in DJB animals tended to be prohibited by the foregut bypass surgery, confirmed by lower fibrous tissue deposits. However, the exact mechanism of increased beta-cell maturation by DJB needs to be explained. Thus, in the second set of experiments, we aimed to investigate if these results were associated with the conventional incretins (GLP-1, GIP, and PYY) or putative anti-incretins.

Furthermore, we investigated how DJB surgery affected peripheral insulin-target tissues. DJB surgery increased insulin signaling in peripheral tissues. Akt, also known as protein kinase B, is a protein kinase that plays a pivotal role in various cellular processes, including cell proliferation, migration, apoptosis, and glucose metabolism. Dysregulation of AKT signaling leads to the development of obesity and type 2 diabetes due to defective glucose transport and glycogen synthesis. Hence, we examined whether the DJB surgery affects the activation status of Akt. We were able to show the induction of Akt activation, specifically in the adipose tissues, after foregut bypass surgery. Compared to PF and AL, DJB surgery exhibited more than twofold increase in phosphorylated Akt level. This result demonstrates that DJB surgery improves glucose metabolism by modulating Akt signaling efficiency in the peripheral tissues.

Considering the above results, foregut bypass surgery ameliorates glucose

homeostasis partly through its substantial role in preserving the pancreatic beta-cell viability and the upregulation of the insulin signaling pathway in peripheral tissues. We need to determine what would mediate those salutary effects of foregut bypass surgery in the entero-insular hormonal axis.

2. The second set of experiments

To understand the mechanisms of antidiabetic effects of the foregut bypass surgery that were clearly demonstrated in the first set of experiments, we aimed to investigate the evidence of putative diabetogenic factor (anti-incretin) secreted by enteroendocrine cells in the foregut that is no longer stimulated after bypass surgery. We compared the perioperative changes of the representative entero-insular hormones and the physiological alterations after bypass surgery alone with gastric resection alone. It was shown that SG resulted in modest improvement of GT with a marked increase in GLP-1 levels and insulin response. In contrast, simple proximal bypass dramatically improved GT via an incretin-independent mechanism, suggesting that the rerouting of the intestine may have a distinct effect on glucose homeostasis via a distinguishable mechanism.

It has been commonly believed that the increase in postprandial GLP-1 level after RYGB surgery as a result of the characteristic intestinal rerouting results in the expedited delivery of nutrients to the distal gut where they stimulate GLP-1-producing L-cells (hindgut hypothesis).[22,44] However, a similar increase in GLP-1 level is only observed after SG, which does not involve an expedited delivery of nutrients. On the contrary, we found that intestinal rerouting per se did not increase GLP-1 level. Therefore, these findings challenge the conventional "hindgut" hypothesis. Consistent with previous reports,[19,32,45] SG resulted in a para-physiologic increase in postprandial GLP-1 levels. These findings support the role of gastric manipulations (resection or reduction) in the enhancement of postprandial GLP-1 secretion. These suggest that the augmentation of GLP-1

response observed after RYGB surgery was induced by gastric mechanisms rather than by intestinal bypass mechanisms. This finding explains the similar effects of SG and RYGB on GLP-1 in previous human and animal studies because both procedures include gastric resection, different from DJB.

We might explain these results through the gastric inhibitory factor to GLP-1 secretion from intestinal L-cells. After SG, the lack of gastric inhibitory signals may lower the threshold for nutrient stimulation of L-cells, plausibly also after standard RYGB. The other hypothesis explaining these results would be that accelerated stimulation of the small intestine by chyme, which rapidly empties the altered stomach, may facilitate an increase in GLP-1 secretion by L-cells located in the proximal small intestine (in SG) and distal intestines (in RYGB). The proximal intestine also hosts L-cells, although GLP-1 is mainly secreted by L-cells located in the distal small bowel. After SG, the rapid increase in GLP-1 level only 15 min after a meal suggests that proximal rather than distal intestinal nutrient stimulation may be involved in the augmented GLP-1 response. The fact that the combination of SG with DJB also increases GLP-1 suggests that proximal intestinal nutrient stimulation is sufficient to make augmentation of GLP-1 response in the presence of accelerated gastric emptying from SG.

SG induced a biphasic pattern of glucose excursions and a rapid increase of GLP-1 and insulin levels and a subsequent reduction in glycemia. The data also showed that SG increases gastric emptying and accelerates glucose absorption. Accelerated gastric emptying and the rate of glucose absorption could potentially explain the early peak in glycemia. Similar observations of a sharp increase and subsequent decrease of glucose level have also been reported after RYGB in humans.[37] Intriguingly, since RYGB surgery involves a stomach resection, it plausibly accelerates gastric emptying. The early peak in glucose levels after RYGB may result from the accelerated gastric emptying and intestinal absorption of glucose.

SG only showed marginal improvement of GT despite the supra-physiologic

increase in incretin (GLP-1) levels. In contrast, DJB dramatically improved GT without GLP-1 changes. These results question the role of GLP-1 in the early and marked improvement of hyperglycemia after RYGB. The role of GLP-1 in improving diabetes after bariatric surgery has also been called into question by other recent studies.[18,36] In this regard, we need to determine the role of the proximal intestinal bypass from nutrient passage (foregut gut hypothesis).

Previous studies regarding the effects of DJB on GLP-1 showed conflicting results. Pacheco et al.[11] reported a modest decrease in postprandial plasma GLP-1 level at 1 week after DJB in GK rats. In contrast, Breen et al.[18] (7) reported a modest increase in GLP-1 levels in STZ-induced uncontrolled diabetic rats, with no changes in type 1 diabetic rats after DJB.[18] Other studies have reported modestly increased post-meal plasma GLP-1 levels after DJB surgery.[46,47] However, in general, post-meal plasma GLP-1 levels after DJB surgery is of far less magnitude compared to the effect reported after SG and RYGB.

Currently, possible mechanisms for the improvement of glucose homeostasis after procedures that involve a proximal intestinal bypass, such as DJB surgery, include gut microbiota alterations,[28,29] bile acid perturbations,[26,27] neuroendocrine signaling changes,[30] and putative gut factor alterations regulated by the duodenum, which may be involved in the diabetic alterations of glucose homeostasis (anti-incretin theory).[8,17]Lam et al.[18] demonstrated that DJB surgery activates nutrient-sensing signals in the jejunum. Furthermore, they reported that DJB surgery rapidly lowers endogenous hepatic glucose production in nonobese rats with uncontrolled diabetes via an unknown mechanism, suggesting a possible contribution of reduced hepatic glucose production to the glucose-lowering effects of bypass procedures.

Moreover, the data corroborate earlier reports of the weight-independent effects of DJB surgery on GT.[9,18,20,21] Foregut bypass shows that improvement in GT after DJB surgery is independent of changes in the conventional enteroendocrine incretin (GLP-1, GIP, and PYY) responses. The early and dramatic improvement

of GT is not induced by insulin secretion. In contrast, foregut bypass surgery is basically associated with the long-term viability of pancreatic beta cells and may be at least in part associated with enhanced intracellular insulin-signaling pathway in peripheral tissues. Most importantly, the novel finding of the second set of experiment is that DJB surgery also restores normal glucagon suppression in ZDF rats. We suggest that restoring the normal glucagon suppression may partly contribute to the outstanding improvement of GT that was observed after a simple foregut bypass surgery. In SG, an increase in glycemia was concomitant with a dramatic increase in insulin and GLP-1 levels. However, given the well-known suppressive effect of glucose, insulin, and GLP-1 on glucagon, it is intriguing that SG fails to suppress postprandial glucagon secretion, which was shown by this experiment and other studies using the combination procedure (RYGB).[25,38,48] The mechanism by which SG disrupts the regulation of glucagon by glucose, insulin, and GLP-1 remains unclear. Theoretically, it also critical to determine what the potential factor would be, which inhibits the role of glucose, insulin, and GLP-1 on glucagon suppression under the pathophysiologic state of type 2 diabetes.[48,49]

Although intestinal bypass alone had no significant effect on BW and FI, the combination of gastric resection with intestinal bypass further improved GT. Furthermore, the additive effect of simple foregut bypass surgery on GT is induced in the absence of an additional increase in incretin (GLP-1 and GIP) response and augmented insulin response. By contrast, the incretin effects were modestly weakened, while the typical incretin response pattern that was previously shown after gastric resection was maintained. We suggest that simple foregut bypass surgery could significantly improve GT by eliminating the pathologic diabetogenic factor (anti-incretin) from the proximal intestine rather than further activating the incretin pathway. Thus, further studies regarding the direct effect of anti-incretin are required.

In summary, the improvement in glucose homeostasis after DJB surgery is

independent of insulin changes and GLP-1 response and is associated with suppression of postprandial glucagon levels. Overall, this suggests that duodenal exclusion does not improve diabetes by increasing incretins or glycemia-reducing factors and might instead work by reducing mechanisms that promote hyperglycemia. This would be consistent with the anti-incretin theory, which postulates the existence of factors physiologically produced by the small intestine to prevent postprandial hypoglycemia from incretin-mediated insulin secretion. According to this theory, an average physiologic balance between incretins and "anti-incretins" would ensure standard excursions of blood glucose and proper beta-cell function, whereas an enhancement of anti-incretin mechanisms would characterize the diabetic state. Exclusion of the upper intestine from digestive continuity might restore a proper balance between incretins and anti-incretins, resulting in improved glycemia. This could potentially explain the improvement of diabetes after DJB surgery in the absence of substantial effects on plasma insulin levels, as shown by this study and by previous investigations. The experiments were designed to determine the firm evidence supporting anti-incretin mechanisms by analyzing representative entero-insular hormones characterizing the role of foregut bypass surgery rather than elucidating direct evidence of diabetogenic factors in the foregut. The data could offer a better understanding of the pathophysiologic background of the development and progression of diabetes. In this context, further studies are warranted to elucidate the direct evidence of potential enteroendocrine peptide responsible for the pathophysiology of diabetes.

This study has certain limitations. Regarding the ZDF rodent model, the phenotype of the ZDF rat includes severe insulin resistance and enhanced apoptosis of beta cells. As a result, the rats become progressively insulinopenic from approximately 14 weeks and experience further deterioration of glycemia observed in this study in the long-term, which is independent of the early effectiveness of interventions. Moreover, the pattern of mid-term attenuation of

GT and recovery of longer-term GT in the different batches of animals was not consistent in each group 16 weeks after DJB surgery. This model does not precisely mimic the pathophysiology of diabetes in most humans with type 2 diabetes who have the intact leptin receptor gene.

V. CONCLUSION

It was proven that foregut exclusion from the passage of food stimulation had a vital role in recovery from a pathophysiologic state of diabetes. The proximal intestinal bypass prevents the pancreatic beta cells from rapid deterioration and preserves their survival in the long run in ZDF rats. Foregut bypass also improves insulin signaling in the peripheral tissue in ZDF rats. The beneficial effects of the proximal intestinal bypass on both pancreatic beta cells and peripheral insulin target cells imply lowering hyperglycemia in ZDF rats. The underlying enteroendocrine antidiabetic mechanism of the foregut bypass surgery in the amelioration of GT is independent of well-known mediators such as incretin (GLP-1, GIP, and PYY) and insulin response.

Gastric and intestinal manipulations exert distinct effects on BW and glucose homeostasis. Intestinal bypass alone has a dominant effect on glucose homeostasis. The result of this study also contradicts the conventional hypothesis that the increase in incretin hormones observed after RYGB (particularly GLP-1) is due to proximal intestinal bypass and suggests a role of the stomach in regulating endogenous GLP-1. The effect of foregut bypass surgery on glucose homeostasis may be at least in part independent of incretin or insulin secretion.

The mechanism of the glucose-lowering effect of the foregut bypass surgery was associated with restoring the postprandial glucagon suppression via an unknown mechanism. We suggest that a potential diabetogenic factor released from proximal intestinal enteroendocrine cells may play a role in disturbing normal glucagon signaling pathways in the pancreas islet and insulin-target cells, such as

adipocytes and hepatocytes. This putative anti-incretin(s) could be eliminated by simple exclusion of the foregut from nutrient stimulation. Eventually, further elucidation of the molecular mechanism, which may be mediated by putative anti-incretin, is required.

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ABSTRACT (IN KOREAN)

Zucker Diabetes Fatty rat에서 전장우회술 후 제2형당뇨병의 내당능 개선에 대한 장 내분비 기전의 증명

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배경 및 목적: 대사수술의 항당뇨 효과가, 영양분의 빠른 소장 원위부 자극에 의한 인크레틴(incretin) 분비 증가(후장 가설) 때문인지, 또는 영양분의 소장 근위부 우회에 의한 가상의 '당뇨병 유발 인자(항인크레틴, anti-incretin)' 분비 회피(전장 가설)로 인한 것인지 명확치 않다. 전장우회술의 혈당 조절기전을 이해하는 것은 대사수술을 최적화하고 새로운 치료 방향 설정에 기여할 수 있을 것이다. 이 연구는 전장우회술 (foregut bypass; duodenojejunal bypass; DJB)의 1) 췌장 베타세포 및 인슐린 신호 전달에 대한 영향과, 2) 내당능 개선을 유도하는 장 내분비-췌도 호르몬 기전을 밝히려 하였다.

대상 및 방법: 실험 1) 당뇨비만 쥐(Zucker diabetic fatty rat; ZDF)을 DJB, 짝지워 먹이기(pair-fed; DJBPF) 및 임의 섭식(ad libitum fed; AL) 군으로 무작위 배정하였다. 경구혈당부하검사와 혈장 인슐린 농도 측정을 수술 후 16주까지 정기적으로 시행하였다. 세 군간의 췌도 구조 변화를 조직형태학분석을 통해 비교하였다. 내장지방 세포 내 인산화-Akt/Akt 비를 측정하여 인슐린 신호 전달을 비교하였다. 실험 2) ZDF rat에서 DJB 와 위절제술(sleeve gastrectomy; SG) 및 조합 수술(SG + DJB) 후 내

당능 개선과 호르몬 변화를 비교하였다.

결과: DJB 후 체중과 음식 섭취량 변화는 없었다. 수술 후 내당능은 비교군과 대조적으로 DJB 2주 후 현저히 개선되었($p<0.05$). 혈장 인슐린 농도는 DJB 군에서만 16주간 수술전과 차이없이 유지되었고, 비교군에서는 지속적으로 저하되었다($p<0.01$). 췌도는 DJB군에서 비교군보다 높은 비율의 확장투사형태(islets with expanding projections)를 보였고($P<0.01$), 수술 전 동일 연령 ZDF rat에서의 비율과 같았으며, 베타세포 분획은 비교 군들보다 3배 이상 넓었다($p<0.01$). DJB군은 세포핵내 PDX1발현도가 3배 이상 높았고($p<0.01$), 섬유화도가 낮은 경향을 보였다. 내장지방 세포 내의 Akt 인산화비는 DJB 군에서 유의하게 높았다 ($p < 0.05$).

SG 군의 체중과 섭취량은 유의하게 감소하였다. 내당능 개선 정도는 DJB 군에서 가장 현저하였고($p < 0.05$), SG 군은 AL 군과 유의한 차이를 보이지 않았다. 인크레틴(glucagon-like peptide-1, glucose-dependent insulinotropic peptide, peptide YY) 분비 반응은 SG 군에서 만 유의하게 증가하였고, DJB 군에서는 변화되지 않았다. 경구 당부하 글루카곤 억제 반응은 DJB 군에서만 정상 수준으로 회복되었다.

결론: 전장우회술은 당뇨쥐에서 체중 감소와 무관하게 내당능을 개선하였고, 혈장 인슐린 농도 감소를 장기간 방지였으며, 베타세포의 형태학적 적응 결과를 유지하였고, 인슐린 신호 전달을 촉진하였다.

전장우회술의 내당능 개선은 인크레틴 분비 반응과 무관하며, 고혈당 유발의 병리적 기전 일부를 정상화 시켰고, 이는 전장 가설에 합치한다. 향후 전장의 항인크레틴 증명 연구가 필요하다.

핵심되는 말: 당뇨, 전장, 우회, 수술, 베타 세포, 인크레틴, 항인크레틴