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ORIGINAL ARTICLE

The novel sodium glucose transporter 2 inhibitor dapagliflozin sustains pancreatic function and preserves islet morphology in obese, diabetic rats

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Aims: To investigate whether glucose lowering with the selective sodium glucose transporter 2 (SGLT2) inhibitor dapagliflozin would prevent or reduce the decline of pancreatic function and disruption of normal islet morphology.

Methods: Female Zucker diabetic fatty (ZDF) rats, 7–8 weeks old, were placed on high-fat diet. Dapagliflozin (1 mg/kg/day, p.o.) was administered for ~33 days either from initiation of high-fat diet or when rats were moderately hyperglycaemic. Insulin sensitivity and pancreatic function were evaluated using a hyperglycaemic clamp in anaesthetized animals (n = 5–6); β-cell function was quantified using the disposition index (DI) to account for insulin resistance compensation. Pancreata from a matched subgroup (n = 7–8) were fixed and β-cell mass and islet morphology investigated using immunohistochemical methods.

Results: Dapagliflozin, administered from initiation of high-fat feeding, reduced the development of hyperglycaemia; after 24 days, blood glucose was 8.6 ± 0.5 vs. 13.3 ± 1.3 mmol/l ($p < 0.005$ vs. vehicle) and glycated haemoglobin 3.6 ± 0.1 vs. 4.8 ± 0.26% ($p < 0.003$ vs. vehicle). Dapagliflozin improved insulin sensitivity index: 0.08 ± 0.01 vs. 0.02 ± 0.01 in obese controls ($p < 0.03$). DI was improved to the level of lean control rats (dapagliflozin 0.29 ± 0.04; obese control 0.15 ± 0.01; lean 0.28 ± 0.01). In dapagliflozin-treated rats, β-cell mass was less variable and significant improvement in islet morphology was observed compared to vehicle-treated rats, although there was no change in mean β-cell mass with dapagliflozin. Results were similar when dapagliflozin treatment was initiated when animals were already moderately hyperglycaemic.

Conclusion: Sustained glucose lowering with dapagliflozin in this model of type 2 diabetes prevented the continued decline in functional adaptation of pancreatic β-cells.

Keywords: β-cell, diabetes mellitus, SGLT2 inhibitor, Zucker diabetic fatty rat

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Introduction

The kidney is an important contributor to blood glucose regulation [1]. In normal individuals, most of the glucose filtered by the kidneys is reabsorbed such that <1% of glucose is excreted via the urine. The majority of this reabsorption occurs in the S1 segment of the proximal tubule where the high-capacity, low-affinity sodium glucose co-transporter (SGLT)2 is located [2], and the remainder is removed via the high-affinity transporter SGLT1 in the S3 segment of the proximal tubule. Inhibition of SGLT2 is an insulin-independent mechanism for lowering blood glucose and is, therefore, not expected to cause weight gain, an inherent side effect of mechanisms that potentiate insulin secretion or insulin action [3]. SGLT2 inhibitors, which reduce renal glucose reabsorption, are now being developed as potential novel therapies for type 2 diabetes. Studies with phlorizin, a naturally occurring but non-selective SGLT inhibitor, and with several novel, synthetic inhibitors in diabetic animal models have provided non-clinical proof of concept [4–10]. Moreover, the highly selective SGLT2 inhibitor dapagliflozin was recently reported to improve glycaemic control in type 2 diabetic patients [11].

Type 2 diabetes develops when pancreatic β-cells fail to compensate for insulin resistance by appropriately increasing insulin secretion. This insulin secretion failure could result from diminished 'sensing' of glucose, inadequate insulin synthesis, incorrect proinsulin processing, failure to maintain or appropriately increase β-cell mass or a combination of these factors. Changes in β-cell mass may occur several years before hyperglycaemia is evident, as suggested by United Kingdom Prospective Diabetes Study (UKPDS) [12]. Indeed, prediabetic as well as diabetic subjects have a reduced β-cell mass [13]. Reduction in β-cell mass may occur following β-cell death (apoptosis) and/or reduced generation of new cells (replication or neogenesis). An ideal diabetes therapy should reduce the

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pancreatic defect in addition to directly lowering blood glucose concentration.

Several studies have explored the hypothesis that gluco-or lipotoxicity contributes to the processes of declining β-cell function and/or β-cell mass. SGLT2 inhibition provides the opportunity to investigate the effects of hyperglycaemia *per se*. Chronic phlorizin administration restored insulin secretion of islets from db/db mice [14]. In 85–95% pancreatectomized rats, phlorizin normalized β-cell volume and gene expression [15] and restored the *in vivo* insulin response to glucose [16]. In male Zucker diabetic fatty (ZDF) rats, the decline in insulin concentration was prevented [17]. The SGLT2 inhibitor T-1095 prevented the decline in plasma insulin and preserved pancreas insulin content in db/db mice [4].

The majority of non-clinical studies with SGLT2 inhibitors have been conducted in insulin-deficient animals [7–9,18–20]. Dapagliflozin markedly decreased plasma glucose in male ZDF rats [9] when treatment was initiated in animals at 15 or more weeks of age when β-cell mass and plasma insulin concentrations are already reduced [21]. Exploration of the potential of dapagliflozin to slow or even reverse the rate of pancreatic failure requires an animal model in which the functional and morphological condition of the pancreas has not irreversibly declined. In contrast to its male counterpart, the female ZDF rat, although insulin resistant, does not develop overt hyperglycaemia when fed on diets with a normal carbohydrate and fat content. However, when fed a specific high-fat diet, hyperglycaemia occurs almost immediately, as a result of decreased plasma insulin concentration [21]. Hyperglycaemia may be reversed by restoring the animals to a normal diet, provided the reversal occurs before hyperglycaemia has become excessively severe [22]. A study with a matrix metalloproteinase inhibitor PD166793 confirmed the utility of this model for exploring preservation of β-cell mass and morphology [23].

The current study evaluated the effect of insulin-independent reduction of hyperglycaemia with the selective SGLT2 inhibitor, dapagliflozin, upon the decline in β-cell function and islet morphology in female obese ZDF rats on high-fat diet.

Materials and Methods

Animals

Female obese ZDF rats (Gmi-*fa/fa*) and lean ZDF rats (Gmi-*fa*/+) were obtained from Charles River Laboratories, Belgium. Animals were housed in pairs on a 12-h light : dark cycle (lights on at 06:00 h) with free access to food and water. They acclimatized on standard rodent chow (RM1; Special Diet Services, Witham, Essex, UK) for 10–12 days before starting the experiment. Five days before the study, obese rats were randomized into control or treatment groups based on blood glucose and body weight. At 7 weeks of age, obese rats were transferred to high-fat diet (48% fat by calories, C13004; Research Diets, New Brunswick, NJ, USA). Lean rats were maintained on RM1 diet throughout.

All work was carried out in accordance with the Animals (Scientific Procedures) Act 1986.

Experimental Design

In a preliminary study, four obese female ZDF rats were placed on C13004 high-fat diet for 12–15 days, while four matched obese and four lean animals remained on RM1 chow diet. A hyperglycaemic clamp study was then carried out as described in subsequent text.

For the evaluation of dapagliflozin, two separate studies were performed in parallel in two separate batches of rats. In the first study ('prevention'), dapagliflozin (n = 14) was administered from the initiation of high-fat feeding. In the second study ('intervention'), dapagliflozin treatment (n = 14) was initiated 10 days after the start of high-fat diet when animals had become moderately hyperglycaemic. Dapagliflozin (1 mg/kg, p.o. in water) or vehicle was administered once daily at 08:00 h for 33 days. In both studies, 48 h after the final dose, following an overnight fast, all groups were subdivided into two matched subgroups based on day 24 glucose and glycated haemoglobin (gHb) levels. One subgroup (5–6 animals) was used for evaluation of pancreatic function by hyperglycaemic clamp. The remaining animals were rendered insentient by inhalation of a 5 : 1 mixture of CO_2: O_2 to minimize changes of glucose and insulin levels in blood samples taken by cardiac puncture into ethylenediaminetetraacetic acid (EDTA) blood syringes. The pancreas was removed and fixed in 10% neutral buffered formalin for 24–48 h, followed by conventional processing and embedding in paraffin wax.

Blood Sampling and Plasma Assays

Disease progression was monitored prestudy, and on days 14 and 24, by measurement of gHb and blood glucose from small samples taken from the tail vein in conscious animals [22]. Samples were taken at 08:00 h prior to the dose of dapagliflozin. Plasma insulin was measured by enzyme-linked immunosorbent assay (ELISA) (Ultrasensitive Rat Insulin ELISA kit; Mercodia, Uppsala, Sweden). Plasma C-peptide was measured by radioimmunoassay (Linco Research, St Charles, MO, USA). The plasma triglyceride assay was carried out using a Roche modular system by the Glycero-3-phosphate oxidase - para aminophenazone method.

Hyperglycaemic Clamp

Animals were terminally anaesthetized with sodium thiobutabarbitol (lean 120 mg/kg; obese 180 mg/kg both i.p.). Three catheters were placed into the right jugular vein for infusions of glucose, Haemacel and anaesthetic, respectively, and one placed into the left carotid artery for blood sampling and blood pressure recording. Catheter patency was maintained by continuous infusion of 20.6 mmol/l sodium citrate. Following an initial stabilization period (60 min), Haemacel infusion (10 µl/ min) was initiated to maintain plasma volume. Hyperglycaemic clamp was initiated with a priming dose of glucose (375 mg/kg; 1 min) administered into the jugular vein, then the infusion rate immediately reduced to 4–6 µl/min; and blood glucose concentration determined every 3–5 min. Blood/plasma glucose concentrations were maintained, at 5.4 mmol/l above mean basal concentrations for each individual animal, by adjustment

of the glucose infusion rate. Arterial blood samples (~300 µl) were taken at −20 and 0 min before the clamp and at 5, 15, 40, 70, 80 and 90 min of the clamp to determine blood (Roche Accuchek®, Burgess Hill, UK) and plasma (YSI 2700 Biochemistry Analyser, Yellow Springs, OH, USA) glucose concentrations, plasma insulin and C-peptide concentrations. These blood samples were collected into potassium EDTA-coated tubes (Microvette®, Sarstedt, UK) containing aprotinin (6.5 µl; 75 KIU). Each sample was centrifuged (15 700 × g, 3–5 min, 4 °C) immediately for plasma separation. Pancreatic function was assessed using the disposition index (DI) as the β-cell insulin secretion responses have to be evaluated in the context of compensation for insulin resistance [24].

Histology and Immunohistochemistry

For histopathological assessment, pancreatic sections were stained for haematoxylin and eosin. Immunohistochemical analysis on 4-µm lengthwise (head-to-tail) sections of the whole pancreas was undertaken as described previously [22]. Sections were mounted using Vectashield® hardest mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI).

Quantitation of β-Cell Morphology

β-Cells were assessed from insulin-immunostained sections using automated imaging and analysis systems. At least 100 images per animal were captured using ImageXpress 5000 (Molecular Devices, Sunnyvale, CA, USA). β-Cell mass and islet morphology were analysed using DEFINIENS image analysis software (Munich, Germany).

The relative cross-sectional area of β-cells was quantified as the area occupied by β-cells (where insulin staining was greater than background intensity, defined as 40 arbitrary units) in relation to the total pancreatic area. Individual β-cells were counted as nuclei (DAPI stained) associated with insulin staining.

Insulin staining intensity of greater than 120 was defined as a 'bright' β-cell. The degree of β-cell scattering within an islet was determined by defining β-cells that shared a common border as one object ('β-cell clump'). The islet morphology index was calculated by dividing the β-cell area by the number of β-cell clumps. All islets within the whole longitudinal pancreas section were analysed.

Data Analysis

An analysis of variance was carried out, assuming non-homogeneous variance across the treatment groups. Where available, this included a baseline value as covariate. Least-squares means and standard errors were estimated for each treatment group. Pair-wise contrasts were then used to test for differences between treatment groups.

Glucose infusion rates (GIRs), corrected for body weight, are expressed as µmol/kg/min.

Insulin sensitivity (M/I index) and DI were calculated during the final 30 min of hyperglycaemic clamp.

M/I index = GIR (µmol/kg/min)/[insulin] (pmol/l).

DI = insulin secretion [C-peptide] (nmol/l) × sensitivity index (M/I).

Results

Effect of Dapagliflozin on Hyperglycaemia

High-fat diet induced hyperglycaemia in obese female ZDF rats, which was evident within 10 days (Table 1). In pilot pharmacokinetic studies, a single dose of dapagliflozin lowered glucose within 4 h (from 7.3 ± 0.6 to 4.9 ± 0.2 mmol/l, n = 3, $p < 0.05$). After 14 days treatment, dapagliflozin significantly reduced the development of hyperglycaemia, with blood glucose being significantly lower than vehicle controls (Table 1). gHb levels were indistinguishable between groups at the start of high-fat feeding (lean 2.9 ± 0.1%; obese control 3.0 ± 0.1%; dapagliflozin 3.2 ± 0.0%, n = 7); however, after 24 days, the same obese vehicle-treated rats' gHb levels increased to 4.8 ± 0.1%, whereas treated animals were unchanged (3.6 ± 0.1%).

When dapagliflozin was administered to moderately hyperglycaemic animals, further development of hyperglycaemia was prevented during the following 14 days (Table 1). gHb was also lower in dapagliflozin-treated rats (4.0 ± 0.1% vs. 5.1 ± 0.2%, $p < 0.001$).

Fasted plasma glucose and insulin concentrations were determined 48 h following the last dose of dapagliflozin before the

Table 1. Dapagliflozin treatment of female ZDF rats for 24 days reduces the development of impaired glycaemic control.

Treatment group	n	Days on high-fat diet before start of treatment	Blood glucose (mmol/l)			Glycated haemoglobin (%)
			Day 0	Day 14	Day 24	Day 24
Prevention study						
Lean vehicle	14	0	5.4 ± 0.12*	5.4 ± 0.1‡	5.6 ± 0.1‡	2.9 ± 0.1‡
Obese vehicle	14	0	6.0 ± 0.23	10.0 ± 1.0	13.3 ± 1.3	4.8 ± 0.1
Obese dapagliflozin	14	0	6.0 ± 0.28	7.6 ± 0.4*	8.6 ± 0.5†	3.6 ± 0.1†
Intervention study						
Lean vehicle	14	10	5.6 ± 0.11‡	5.3 ± 0.2‡	5.6 ± 0.1	3.3 ± 0.1‡
Obese vehicle	14	10	7.5 ± 0.37	11.5 ± 0.8	13.4 ± 1.6	5.1 ± 0.2
Obese dapagliflozin	14	10	7.4 ± 0.39	7.5 ± 0.2‡	8.3 ± 0.5†	4.0 ± 0.1‡

ZDF, Zucker diabetic fatty.

*$p \leq 0.05$ vs. corresponding obese vehicle.

†$p \leq 0.01$ vs. corresponding obese vehicle.

‡$p \leq 0.001$ vs. corresponding obese vehicle.

hyperglycaemic clamp. Dapagliflozin had no residual effect on plasma glucose but there was a tendency to lower insulin concentration in treated animals whether administered in 'prevention' or 'intervention' mode (1367 ± 414 vs. 1873 ± 142 pmol/l; 1150 ± 149 vs. 1642 ± 184 pmol/l, respectively, p = 0.06).

Over the period of administration, dapagliflozin had no significant effect on the body weight gain of obese female high fat-fed ZDF rats. Dapagliflozin-treated obese rats had significantly lower plasma insulin and triglyceride concentrations after 35 days in the subgroup of animals used for pancreatic analysis (Table 3).

Effect of Dapagliflozin on Insulin Sensitivity

The insulin sensitivity index is derived during steady state of the hyperglycaemic clamp. In the preliminary study, female obese ZDF rats had an insulin sensitivity index of 0.06 ± 0.01 when fed on a standard chow diet, compared to 0.45 ± 0.02 in lean animals; 12–15 days of high-fat feeding reduced insulin sensitivity even further (M/I index 0.03 ± 0.00, $p < 0.01$) (figure 1). In the prevention study, dapagliflozin increased the glucose infusion rate required to maintain hyperglycaemia to that of lean controls, such that insulin sensitivity was improved compared with vehicle-treated animals (0.08 ± 0.01 vs. 0.02 ± 0.01 μmol/kg/min; Table 2), but not to the level of the insulin-sensitive lean rats (M/I index 0.23 ± 0.04 μmol/kg/min). Similarly, in the intervention study dapagliflozin also increased the insulin sensitivity index (0.05 ± 0.01 vs. 0.02 ± 0.01 μmol/kg/min, $p < 0.05$) (Table 2).

Effect of Dapagliflozin on Pancreatic Function

In the preliminary study, high-fat feeding of obese rats reduced the DI from 0.30 ± 0.02 to 0.15 ± 0.01 μmol/kg/min.pmol/l ($p < 0.001$). In the prevention study, dapagliflozin maintained the DI to the level of lean controls (Table 2). When dapagliflozin treatment was initiated after the onset of hyperglycaemia, β-cell function was improved to a similar level compared with the lean control group (Figure 2).

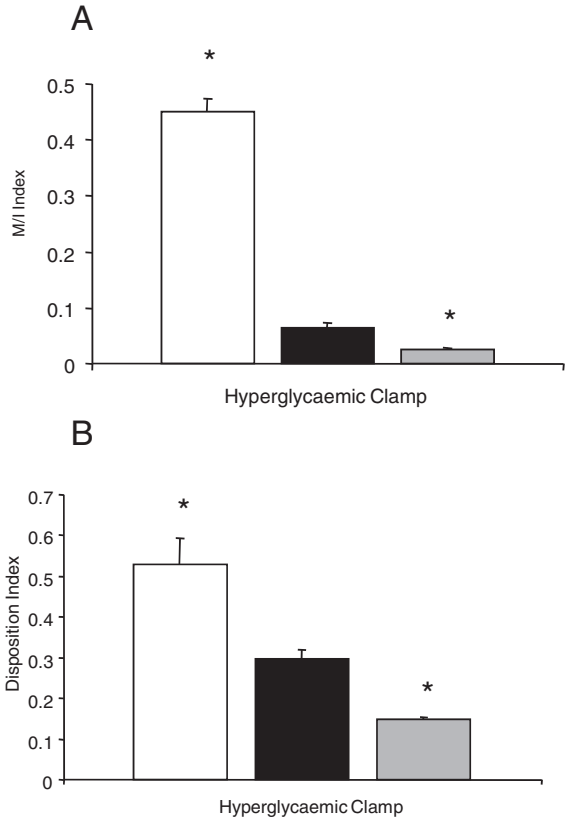

Figure 1. High-fat feeding reduces insulin sensitivity (A) and pancreatic function (B) in female obese Zucker diabetic fatty (ZDF) rats after 2 weeks. Open bars, lean; black bars, obese chow-fed; grey bars, obese high fat-fed. *$p < 0.05$ vs. obese chow-fed group.

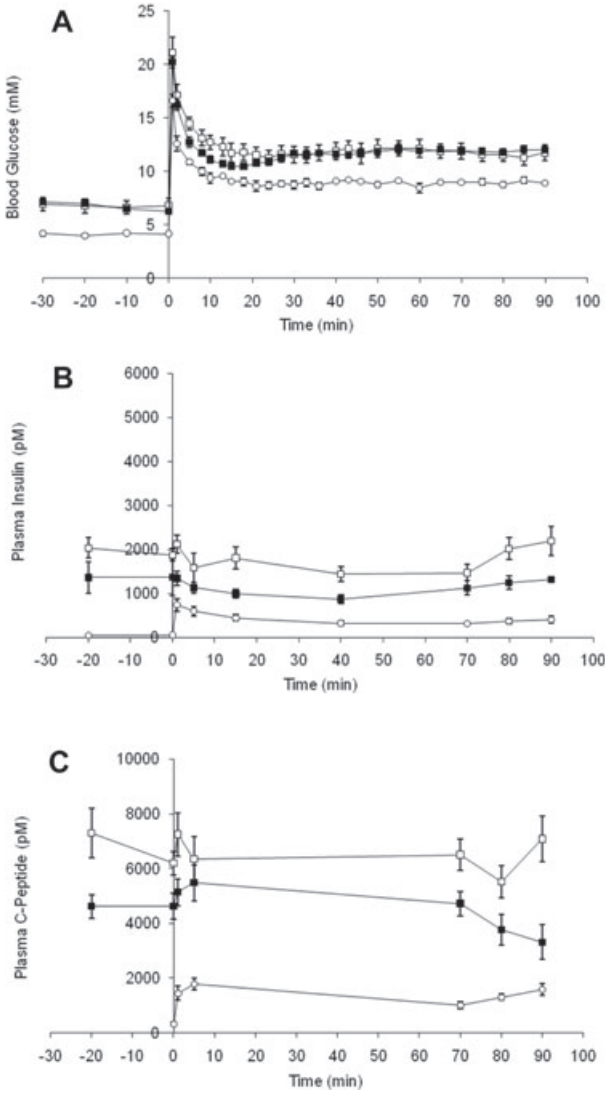

Figure 2. Glucose (A), insulin (B) and C-peptide (C) concentrations during the hyperglycaemic clamp in the prevention study. Open circles, female lean Zucker diabetic fatty (ZDF) rats; open squares, female obese, high fat-fed dosed with vehicle; solid squares, dapagliflozin 1 mg/kg, 33 days. Dapagliflozin treatment was initiated at the same time as the high-fat diet and discontinued 48 h before clamp studies.

Table 2. Dapagliflozin treatment of female ZDF rats reduces the development of insulin resistance and of impaired pancreatic function.

		Basal stage		Hyperglycaemic clamp					
Treatment group	n	Plasma glucose (mmol/l)	Plasma insulin (pmol/l)	Plasma glucose (mmol/l)	Glucose infusion rate (μmol/kg/min)	Plasma insulin (pmol/l)	Plasma C-peptide (pmol/l)	Insulin sensitivity index (μmol/kg/min)/ (pmol/l)	Disposition index (μmol/kg/min/pmol/l). (pmol/l)
Prevention study									
Lean vehicle	4	5.0 ± 0.1†	52 ± 9‡	10.8 ± 0.3	76.7 ± 2.7‡	363 ± 57†	1311 ± 189†	0.23 ± 0.04‡	0.28 ± 0.01‡
Obese vehicle	6	8.3 ± 0.7	1957 ± 180	14.1 ± 0.8	43.0 ± 2.5	1895 ± 240	6371 ± 613	0.02 ± 0.00	0.15 ± 0.01
Obese Dapa	5	8.4 ± 0.1	1369 ± 414	14.7 ± 0.4	85.8 ± 8.6‡	1229 ± 103*	3935 ± 233†	0.08 ± 0.02†	0.29 ± 0.04†
Intervention study									
Lean vehicle*	6	5.3 ± 0.1†	33 ± 1‡	10.6 ± 0.2†	79.6 ± 9.6†	427 ± 118*	305 ± 623*	0.26 ± 0.06‡	0.45 ± 0.05†
Obese vehicle	6	8.3 ± 0.9	1642 ± 184	13.8 ± 0.9	41.2 ± 3.9	2483 ± 631	9250 ± 1657	0.02 ± 0.01	0.18 ± 0.04
Obese Dapa	6	8.3 ± 0.3	1150 ± 149	14.4 ± 0.4	69.0 ± 8.2*	1436 ± 209	6569 ± 505	0.05 ± 0.01*	0.33 ± 0.04*

ZDF, Zucker diabetic fatty.
*$p \leq 0.05$ vs. corresponding obese vehicle.
†$p \leq 0.01$ vs. corresponding obese vehicle.
‡$p \leq 0.001$ vs. corresponding obese vehicle.

Effect of Dapagliflozin on Pancreatic Morphology

Representative sections of islets stained for insulin are illustrated in figure 3. Results from immuno-histochemistry image analysis of pancreatic sections from both prevention and intervention studies were similar (Table 3; figure 4). Obese female rats fed a high-fat diet had greater β-cell mass than lean control animals, whether determined by insulin staining area (Table 3; figure 4) or by the number of β-cells within the islet (as percentage of total islet cells). The variability of β-cell mass in obese animals was markedly reduced following dapagliflozin (figure 4); although the absolute β-cell mass increased compared to the vehicle-treated group, this did not reach statistical significance. The proportion of β-cells that stained brightly for insulin reflects insulin content at the time of necropsy; this was depleted in obese, insulin-resistant animals but maintained following dapagliflozin treatment (Table 3).

In the prevention study, for lean animals, where β-cells are closely associated, the morphology index was high ($1239 \pm 56\ \mu m^2$), whereas in insulin-resistant animals, where β-cells are more dispersed within an islet, the morphology index was low ($687 \pm 66\ \mu m^2$). Dapagliflozin maintained the islet morphology index, at a level similar to that of lean control animals ($1576 \pm 126\ \mu m^2$; Table 3).

Discussion

Dapagliflozin is a novel, selective inhibitor of the renal SGLT2, which elicits sustained glucose lowering in normal and type 2 diabetic subjects [11,25] and in animal models [9]. The present study was designed to investigate whether, by inhibiting renal glucose reabsorption, dapagliflozin has potential to address one of the core defects of type 2 diabetes, namely the progressive degeneration of pancreatic function.

The ZDF rat model was selected for this study because it develops diabetes as a result of inadequate compensatory insulin production for the degree of insulin resistance [26,27]. Other models of pancreatic failure, such as STZ rat and partial pancreatectomy, have a pancreatic defect without a background of insulin resistance. The male ZDF rat develops β-cell failure spontaneously and irreversibly at the age of about 6–8 weeks, whereas the female ZDF only develops hyperglycaemia when challenged with a high-fat diet [21]; the development of diabetes can thus be controlled. Furthermore, we have shown that reversal of the diet when animals are moderately hyperglycaemic results in improvement of the diabetic state [22]. Thus, as a model for pancreatic degeneration in type 2 diabetes, it is suitable for evaluation of therapeutic intervention. In this study, hyperglycaemia was evident from about 8 weeks of age, consistent with other studies in this and other laboratories [21,26,28], indicating failure of the pancreas to compensate for increasing insulin resistance. Surprisingly, plasma insulin concentration did not decline in association with hyperglycaemia in this study. Notwithstanding, in this study, female high fat-fed ZDF rats clearly showed inadequate islet compensation for increasing insulin resistance.

Dapagliflozin was well tolerated and significantly reduced the progression of hyperglycaemia; treatment was effective both in the prevention and intervention study arms. Disease progression was monitored by blood glucose concentration immediately prior to dapagliflozin administration. At the dose interval, dapagliflozin residual drug concentration was sufficient to decrease glucose by 0.9 mmol/l (from the preliminary pharmacokinetic study). Thus, the improvement in glycaemic control, indicated by glucose and by gHb levels, cannot be accounted for entirely by the presence of residual drug. Measurements of insulin sensitivity and pancreatic

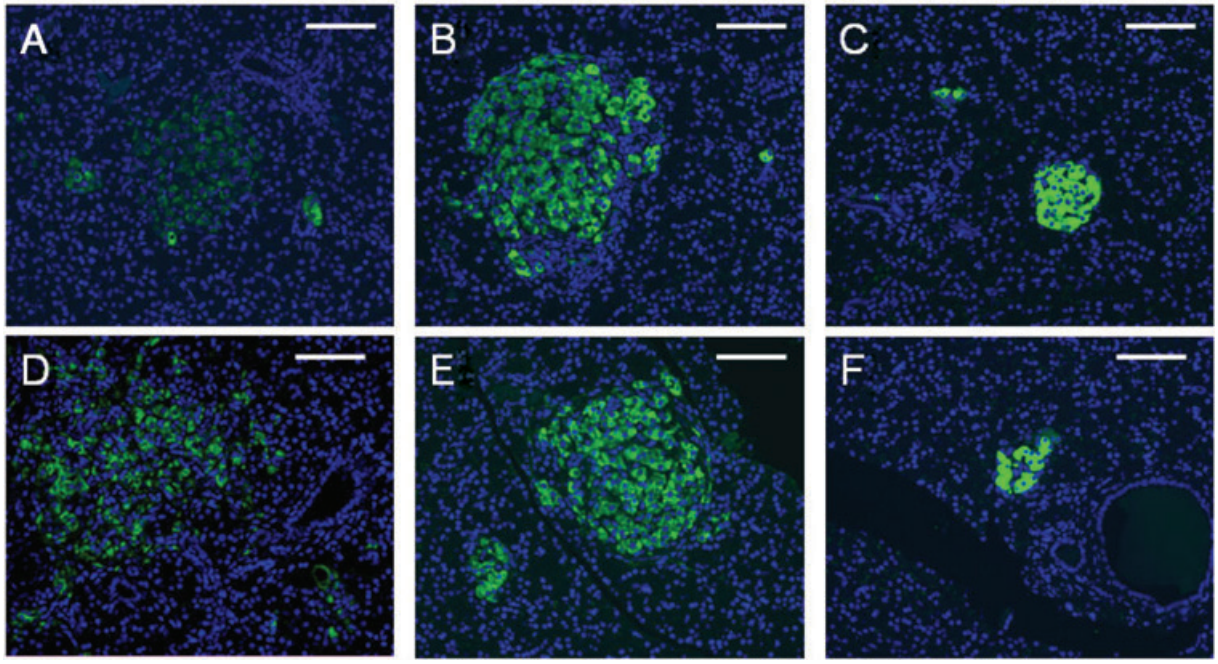

Figure 3. Islet insulin staining. Images (A–C) are from the prevention study and (D–F) are from the intervention study. These images are representative of eight animals from each group. Images were captured using a 10× objective on the imageXpress™ fluorescent microscope. Nuclei were stained using Hoechst (blue), β-cells were labelled with an anti-insulin antibody and fluorescein isothiocyanate (FITC) secondary (green). The scale bar represents 100 µm. (A and D) Vehicle control obese Zucker diabetic fatty (ZDF) islets, the islet morphology is poor, β-cells appear scattered and islet insulin staining is visibly reduced. (B and E) Representative images of obese ZDF rats: dapagliflozin-treated islet size is increased compared to lean animals, islet morphology and insulin staining intensity are clearly improved. (C and F) Lean ZDF rats, islets are smaller and more compact than in the obese animals and insulin staining intensity is much brighter.

Table 3. Dapagliflozin treatment of female ZDF rats improves pancreatic islet morphology.

Treatment group	n	Plasma insulin (pmol/l)	Plasma triglyceride (mmol/l)	β-Cell mass (% of pancreas area)	β-Cell number (% of total pancreas cells)	Brightly stained β-cells (% of β-cells)	β-Cell size (pixels)	Morphology index (µm^2)
Prevention study								
Lean vehicle	7	46 ± 9‡	0.75 ± 0.05‡	0.49 ± 0.08	0.69 ± 0.12	21.36 ± 1.87‡	194.8 ± 18.7	1239 ± 56*
Obese vehicle	8	1114 ± 144	10.79 ± 1.18	1.02 ± 0.34	1.15 ± 0.36	7.90 ± 1.85	222.0 ± 15.6	687 ± 66
Obese dapagliflozin	7	488 ± 88†	7.06 ± 0.23†	1.18 ± 0.17	1.49 ± 0.18	17.17 ± 2.88†	210.6 ± 14.2	1576 ± 126*
Intervention study								
Lean vehicle	8	141 ± 62‡	0.86 ± 0.14‡	0.44 ± 0.03†	0.59 ± 0.03*	18.93 ± 1.56†	213.1 ± 14.0	1375 ± 34†
Obese vehicle	8	1907 ± 384	11.28 ± 1.0	1.03 ± 0.18	1.23 ± 0.25	9.81 ± 2.59	211.3 ± 9.9	825 ± 49
Obese dapagliflozin	7	828 ± 146*	7.86 ± 0.8†	1.17 ± 0.15	1.47 ± 0.22	14.53 ± 2.06†	231.6 ± 21.2	1410 ± 72*

ZDF, Zucker diabetic fatty.
$^{*}p \leq 0.05$ vs. corresponding obese vehicle.
$^{\dagger}p \leq 0.01$ vs. corresponding obese vehicle.
$^{\ddagger}p \leq 0.001$ vs. corresponding obese vehicle.

function were made 48 h after the final dose of dapagliflozin, when preclamp plasma glucose concentrations indicated that there was no residual effect of dapagliflozin; any effects on these parameters may therefore be associated with changes in insulin sensitivity and β-cell function acquired through sustained inhibition of SGLT2. This is consistent with the previous report [9], where dapagliflozin, administered for 15 days to male ZDF rats, increased hepatic insulin sensitivity and supports the hypothesis that hyperglycaemia itself contributes to the development and maintenance of insulin resistance [29].

A hyperglycaemic clamp was used to explore the functional capability of the endocrine pancreas; DI indicates the ability of β-cells to respond to rising blood glucose concentrations by increasing insulin secretion and accounts for the compensation due to insulin sensitivity changes [24,30]. Preliminary investigation showed the potential of the hyperglycaemic clamp to explore pancreatic function in the female ZDF model. After only 2 weeks of high-fat feeding, DI decreased by approximately 50% in comparison to obese chow-fed controls, which themselves had reduced pancreatic function relative to their

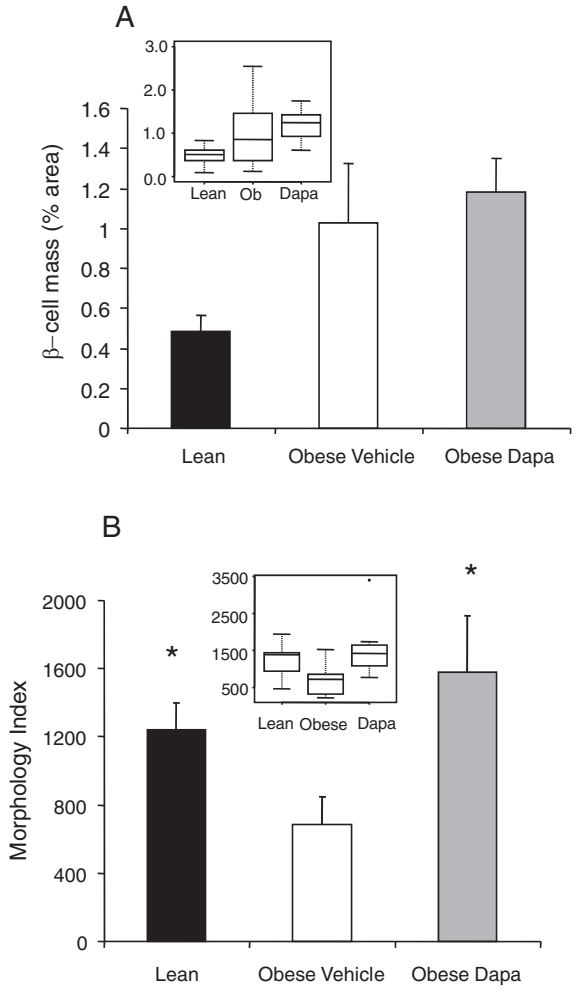

Figure 4. β-Cell mass and morphology index in the prevention study. Dapagliflozin has no effect on β-cell mass (A) and improves islet morphology (B). *p < 0.05 vs. obese fat-fed group. Insets are box plots of the same data.

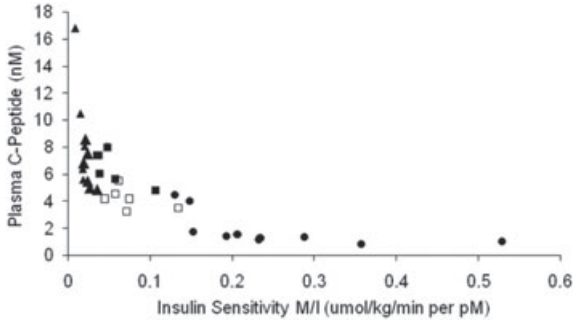

Figure 5. Hyperbolic relationship between insulin sensitivity and insulin secretion, from steady-state phase of the hyperglycaemic clamp. Solid circles, female lean Zucker diabetic fatty (ZDF) rats; solid triangles, female obese, high fat-fed dosed with vehicle; dapagliflozin 1 mg/kg 33 days; open squares, 'prevention study' initiated at the same time as high-fat diet; solid squares, 'intervention study' when animals were moderately hyperglycaemic.

lean counterparts. We have also previously shown disrupted islet morphology (reduced β-cell mass, increased β-cell scattering) in obese female rats fed on high-fat diet compared with normal chow [22]. Therefore, in the current studies we used lean controls as comparators to explore the ability of dapagliflozin to normalize pancreatic function and morphology. In the 'prevention study', dapagliflozin increased DI to that of lean control rats, indicating a marked improvement in pancreatic responsiveness (indicated by C-peptide concentration) to the residual insulin resistance. A similar, although slightly less marked effect was seen when dapagliflozin was administered to hyperglycaemic animals. A hyperbolic curve relates insulin release to insulin sensitivity in the absence of hyperglycaemia in man [30]. In our study, animals treated with dapagliflozin exhibited greater insulin sensitivity, resulting therefore in lower β-cell function required to control blood glucose than for vehicle-treated rats (figure 5).

To differentiate the separate factors contributing to plasma insulin response during the clamp, we examined plasma C-peptide concentration in addition to insulin. Plasma C-peptide concentration increased in response to glucose in lean and dapagliflozin-treated groups, but not in obese controls. Importantly, this increase in C-peptide response was not associated with elevated plasma insulin in dapagliflozin-treated animals. In insulin-resistant subjects, reduced insulin hepatic clearance insulin contributes to fasting hyperinsulinaemia [31]; decreased insulin : C-peptide ratio may be a result of improved hepatic insulin sensitivity [32].

Immunohistochemical analysis also showed a reduction in pancreatic islet insulin content in obese vehicle controls when compared with lean animals. As the animals were fasted before pancreas excision, this indicates a potential defect in insulin biosynthesis that warrants further investigation. Interestingly, dapagliflozin treatment increased insulin staining intensity when compared with obese vehicle controls, fully restoring the staining pattern seen in lean animals. In *Psammomys obesus*, lowering blood glucose with phlorizin for only 2 days restored pancreatic insulin content [33], but our data would suggest that prevention or sustained reversal of hyperglycaemia is required for the maintenance of normal islet morphology.

In insulin-resistant animals, β-cell mass is increased in response to elevated insulin demand [34–36]. This was evident in our study; although we did not correct for pancreas weight, as in our previous studies with this model we saw no difference in pancreas weight between obese (fed either chow or high-fat diet) and lean female ZDF rats [22] and therefore, changes in proportional area of β-cells reflects changes in β-cell mass. Decreased β-cell mass has been observed in association with rosiglitazone treatment; putatively because of increased insulin sensitivity and consequent reduced insulin demand [37]. As dapagliflozin treatment also increased insulin sensitivity, it would be expected to reduce β-cell mass. However, β-cell mass showed a trend to increase, although the considerable variation of β-cell mass seen in obese vehicle-treated rats meant this did not reach a statistical significance. The variation in β-cell mass was reduced with dapagliflozin. Importantly, islets of Langerhans, which are fibrotic and disrupted in fat-fed obese ZDF rats [23], were restored to a healthy appearance by dapagliflozin. Both gluco- and lipotoxicity have been

proposed as protagonists of β-cell failure [38]. In this study, dapagliflozin markedly lowered plasma glucose, supporting our view that these improvements were mediated by decreased hyperglycaemia and increased insulin sensitivity, consistent with the findings of Kjørholt et al. and Harmon et al. [14,39]. In addition, reduced insulin requirement due to the renal, insulin-independent glucose excretion would be expected to reduce stress on the β-cell. We did not measure plasma free fatty acids in the current study, although plasma triglyceride, in addition to glucose concentration, was reduced by dapagliflozin. So it is not possible to address categorically the relative contribution of the lipid toxicity to the improvement seen in the pancreas. Others have reported that hyperglycaemica and not hyperlipidaemia correlates best with the progressive loss of islet function in the ZDF rat model [39]. Improved insulin secretion with phlorizin has been shown previously [16], but ours is the first study to quantify both changes in β-cell morphology and functional changes *in vivo* in response to selective SGLT2 inhibition.

In conclusion, we showed that sustained treatment with the SGLT2 inhibitor dapagliflozin improved insulin sensitivity and islet function in an insulin-resistant, animal model of type 2 diabetes. Islet morphology was markedly improved. This suggests that reduction of hyperglycaemia by dapagliflozin, through an insulin-independent mechanism, may improve core defects present in type 2 diabetes. Whether the effects of lowering blood glucose actually prevented the loss of β-cell function or merely delayed the response would need to be investigated in longer studies. Future work will need to be undertaken to determine the relative contribution of proliferation or apoptosis effects on β-cell actions of dapagliflozin.

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Conflict of Interest

F. R. M. contributed to design, conduct, analysis and writing; J. E. P. to conduct and analysis; H. B. J. to conduct; R. M. M. to design and writing; L. W. to conduct and analysis; J. M. W. to design and analysis and S. M. P. to design, analysis and writing. At the time of completing the studies, all authors were employees of either AstraZeneca Pharmaceuticals or Bristol-Myers Squibb Pharmaceuticals.

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