NOVEL CANINE MODELS OF OBESE PRE-DIABETES AND OF MILD TYPE 2 DIABETES

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ABSTRACT

Human type 2 diabetes (T2DM) is often characterized by obesity-associated insulin resistance (IR) and beta-cell function deficiency. Development of relevant large animal models to study T2DM is important and timely, because most existing models have dramatic reductions in pancreatic function and no associated obesity and IR, features that resemble more T1DM than T2DM. Our goal was to create a canine model of T2DM in which obesity-associated IR occurs first, followed by moderate reduction in beta-cell function leading to mild diabetes or impaired glucose tolerance. Lean dogs (n=12) received a high-fat diet that increased visceral (52%, p<0.001) and subcutaneous (130%, p<0.001) fat and resulted in a 31% reduction in insulin sensitivity (SI) (5.8±0.7·10^{-4} to 4.1±0.5·10^{-4} uU/ml \cdot min^{-1}, p<0.05). Animals then received a single low dose of streptozotocin (STZ) (range 30-15 mg/kg). The decrease in beta-cell function was dose dependent and resulted in three diabetes models: a) frank hyperglycemia (high STZ dose); b) mild T2DM with normal or impaired fasting glucose (FG), 2h glucose >200 mg/dl during OGTT and 77-93% AIRg reduction (intermediate dose); and c) pre-diabetes with normal FG, normal 2h glucose during OGTT and 17-74% AIRg reduction (low dose). 12 weeks after STZ animals without frank diabetes had 58% more body fat, decreased β-cell function (17-93%) and 40% lower SI. We conclude that high fat feeding and variable dose STZ in dog results in stable models of obesity, insulin resistance and a) overt diabetes or b) mild T2DM or c) impaired glucose tolerance. These models open new avenues for studying the mechanism of compensatory changes that occur in T2DM and for evaluating new therapeutic strategies to prevent progression or to treat overt diabetes.
Keywords: type 2 diabetes, obesity, animal models, streptozotocin, insulin secretion
INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a highly prevalent disease with an enormous public and individual health impact. According to the Centers for Disease Control National Diabetes Fact Sheet, in 2007 23.6 million people in the U.S. (7.8% of population) had diabetes and an estimated 57 million people had prediabetes (IFG or IGT or both) (10). The disease’s increasing prevalence requires adequate and strong intervention for prevention of new cases and new or improved therapeutic tools for the existing cases (11). T2DM is characterized by a combination of resistance to insulin action and an inadequate compensatory insulin secretory response, and is usually associated with obesity (15). Increased body fat and especially visceral fat accumulation have been shown to be risk factors for the development of impaired glucose tolerance or diabetes (14). The mechanisms of the initial alterations in the development of T2DM, related to fat deposition and the associated changes in liver, muscle and adipocyte, are not fully elucidated. Studying the relationship between insulin resistance and hyperinsulinemic compensation (or failure thereof) and the change from normal glucose tolerance to impaired glucose tolerance and finally to T2DM requires complex interventional, closely controlled studies that are not always possible in humans.

The search for better understanding of complex mechanisms of human T2DM and need for new therapy emphasizes the role of appropriate animal models that reproduce the natural history and the characteristics and causes of T2DM in humans. Rodent models have provided useful insights in the physiopathology of T2DM (26). However, there remains a need for large animal models that reproduce the natural history of T2DM and also allow for invasive clinical measurements which can be followed longitudinally.
Canine, swine (pig or minipig) and non-human primate animal models of diabetes are currently available. In most of these models pancreatectomy or chemical alteration of beta cells (streptozotocin, alloxan) are used to induce diabetes ((as reviewed in (38), (3), (33)). The resulting phenotype is often characterized by frank hyperglycemia with dramatic reduction in insulin secretion, but without obesity and associated insulin resistance. Such models can be of substantial value in studying the complications of diabetes and the effect of prolonged hyperglycemia, but they may be less suitable for studying the milder changes that occur in the pre-clinical stages of type 2 diabetes; i.e., “prediabetes” and the factors involved in progression to overt fasting hyperglycemia and loss of glucose tolerance.

We have previously developed a high-fat high-calorie diet model of canine obesity and insulin resistance. Although weight gain is modest (~10-15%), the animals exhibit a significant 76% increase in body fat (both in the visceral and in the subcutaneous depots) and approximately 30% decrease in insulin sensitivity. The reduced insulin sensitivity is usually compensated adequately by an increase in insulin secretion, such that normal glycemia is maintained (22). Similarly, in many obese individuals, insulin resistance is compensated by hyperinsulinemia. Only when less than appropriately reduced insulin output occurs, is glycemic control lost, resulting in hyperglycemia (4).

The goal of our current study was to create a canine model of mild T2DM in which obesity-associated insulin resistance is first induced, followed by a streptozotocin-induced decrease in pancreatic β-cell function, yielding a syndrome that more closely resembles T2DM, or alternatively, a mild “pre-diabetic” state.
MATERIALS AND METHODS

Animals

We studied 12 male dogs (initial BW: 29.0 ± 0.9 kg), housed under controlled kennel conditions in the University of Southern California Medical School Vivarium. All surgical and experimental procedures were approved by the University of Southern California Institutional Animal Care and Use Committee.

Experimental design

The experimental design is presented in Figure 1.

After a period of acclimatization of 2-3 weeks in which animals were presented once a day with a standard diet containing 3684 kcal/day (27% protein, 38% carbohydrate and 35% fat), in each animal an initial metabolic assessment was performed to evaluate insulin sensitivity and pancreatic function. All animals were deprived of food for 18 h before the experiments, and were studied in the conscious relaxed state. The metabolic assessment consisted of three protocols: 1) a hyperinsulinemic euglycemic clamp (EGC), 2) a stepped hyperglycemic clamp (HGC) and 3) an intravenous glucose tolerance test (IVGTT). Additionally, 4) in some of the animals oral glucose tolerance tests (OGTT) were performed to assess the possible contribution of gastro-intestinal hormones to glycemic control. In all animals abdominal MRI was performed to assess body composition. After the initial characterization animals were placed on a high fat hypercaloric diet (5110 kcal/day, 20% protein, 27% carbohydrate, 53% from fat) and they remained on this diet for the rest of the study. After 6-8 weeks of the hypercaloric diet, the animals underwent a second period of metabolic assessment (IVGTT, EGC, HGC and OGTT) and an MRI. Animals then received one dose of streptozotocin (STZ)
by intravenous injection, followed by: IVGTTs at 1 week and 2 weeks post-STZ and full
metabolic assessment and MRI at 4 and 10 weeks after STZ. Weekly fasting plasma
samples were taken to measure glucose, insulin, C-peptide and other parameters of
glucose metabolism. Food intake was measured daily and body weight was recorded once
per week.

Metabolic Assessments

**IVGTT:** Blood samples were drawn from a peripheral vein at -20, -10 and -1 min.
Fasting values were defined as the average of the three basal samples. At t=0, 0.3 g/kg
BW glucose (50% dextrose, 454 mg/ml, B Braun, Irvine, CA) was injected into a
peripheral vein. Additional samples were taken at 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19 min.
At t=20 min a bolus of 0.03U/kg porcine insulin was injected into a peripheral vein
followed by sampling at 22, 23, 24, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160 and
180 min.

**Euglycemic hyperinsulinemic clamps:** in the morning of the experiment, after a
basal plasma sample, a continuous infusion of tritiated glucose (0.25 uCi/min) was
initiated in a peripheral vein and maintained for the entire duration of the experiment.
After 120 min, peripheral infusions of somatostatin (1 µg/kg/min) and insulin (0.75
mU/kg/min) were started and continued for 180 min. Plasma glucose was maintained at
euglycemic levels via a variable rate peripheral infusion of glucose spiked with 3-3H-
glucose (to avoid large fluctuations in plasma specific activity).

**Stepped hyperglycemic clamp:** After basal plasma sampling, peripheral glycemia
was sequentially raised via an exogenous glucose infusion to three glycemic levels: 100
mg/dl (t=0-60 min), 150 mg/dl (t= 60-150 min) and 200 mg/dl (t= 150-240 min). Plasma
samples were obtained every 10 minutes. Hyperglycemic clamp data was not used for a
single animal in the control period.

Euglycemic hyperinsulinemic clamps and hyperglycemic clamps were not
performed in animals if they exhibited fasting hyperglycemia over 200 mg/dl.

**OGTT:** Basal blood samples were drawn from a peripheral vein at -20, -10 and -1
min. At t=0, 25 g glucose (55 ml 50% dextrose, 454 mg/ml) was given as a bolus by oral
gavage. Additional blood samples were taken at 15, 30, 45, 60, 90, 120 and 180 min.

**MRI:** Abdominal MRIs were performed in animals fasted overnight using a 1.5 T
Gemsow scanner (General Electric). Dogs were pre-anesthetized with s.c 1.35 mg of
atropine sulfate (Phoenix Pharmaceuticals, Burlingame, CA) plus 5 mg of acepromazine (Boehringer Ingelheim, St Joseph, MO), then anesthetized with a single i.v. dose of a
mixed solution of 10 mg/kg ketamine (Bioniche Pharma, Lake Forest, IL) and 0.5 mg/kg
diazepam (Bioniche Pharma, Lake Forest, IL). The scanning consisted of 30 contiguous
slices 1-cm thick (T1-weighted, repetition time = 500 ms (TR 500), echo time = 14.0 ms
(TE:14), placing the first slice at the level of the junction between the inferior-limb and
the trunk. The left renal hilum was chosen as midpoint landmarks for all sessions.

**Induction of pancreatic defect with streptozotocin (STZ)**

Animals were fasted overnight and brought to laboratory where a peripheral vein
catheter was placed. Streptozotocin powder (Sigma-Aldrich, St Louis, MO) was
dissolved in citrate buffer solution (HPCE pH 4.5, Sigma-Aldrich, St Louis, MO)
immediately before injection, to obtain a 62.5 mg/ml STZ-citrate solution. One of four
STZ doses were used in each animal: 30, 22.5, 18.5 or 15 mg/kg (n=2 animals for the 30
and 22.5 mg/kg dose, n=4 for the 18.5 and 15 mg/kg doses). STZ solution was
administered within 1 h of mixing via intravenous injection. All animals reacted to STZ
administration by vomiting within 1-2 hours and reduction of food intake for the
following 24-48h. All animals recovered their food intake and normal behavior 1-7 days
after STZ administration. Animals were monitored frequently during the first 48h to
avoid episodes of hypoglycemia.

Monitoring and assessment

Before the STZ injection the animals were monitored by daily measurement of
food intake and weekly measurement of body weight, glucose, insulin and other plasma
measurements. After the STZ injection plasma glucose was measured daily or twice
daily, as necessary, until plasma glucose stabilized. Ketone bodies and glucosuria were
monitored using urine test strips (Keto-Diastix, Bayer, White Plains, NY). Renal and
hepatic function were assessed before and after treatment with streptozotocin by
measurement of serum creatinine, blood urinary nitrogen (BUN) and serum alanine
aminotransferase (ALT) and aspartate aminotransferase (AST). Exocrine pancreatic
function was assessed by measuring serum amylase and lipase (Antech Diagnostics,
Irvine, CA). After STZ administration some animals developed overt diabetes, with a
fasting plasma glucose of over 200 mg/dl. In these animals insulin therapy was initiated
two days after STZ administration. Intermediate acting insulin (Humulin®, Eli Lilly,
Indianapolis, IN) was administered subcutaneously once daily at meal time to maintain
glycemia below 200 mg/dl.

Pancreas histology and imaging

Animals were euthanized at the end of the monitoring period with pentobarbital
(350mg/ml;20cc iv injection, Virbac, Carros, France) and pancreatic tissue was fixed in
10% formalin. Paraffin-embedded 5 µm sections were immunostained with guinea pig anti-human insulin antibody (Millipore, St. Charles, MO) followed by Cy 2-conjugated donkey anti-guinea pig (Jackson ImmunoResearch, West Grove, PA) as described (19). Fluorescent images were acquired and analyzed using Ariol SL-50 (Applied Imaging Corp., San Jose, CA). The Ariol scanner is based on an Olympus BX61 microscope with motorized stage and autofocus capabilities, equipped with a black and white video camera (Jai CVM2CL). Slides were scanned at 20X objective magnification with the DAPI and FITC filters. Optimal exposure times were determined before automated scanning. After scanning, threshold levels of the individual signals were optimized before final analysis. Analytical readouts of user defined areas included: area of signal (area above threshold) and total area analyzed was calculated by the software. Scanning and analyses were performed through the Translational Pathology Core Laboratory, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA. Islet images were taken at the Multi-Photon Core at the USC Keck School of Medicine, using a Leica TCS SP5 AOBS Multi-photon confocal microscope system.

**Blood sampling and assays**

Blood samples were collected in heparinized tubes containing EDTA (for glucose, insulin, lactate), EDTA and Trasylol (for peptides) or EDTA and DPP-IV inhibitor (for active GLP-1). Plasma glucose concentration was determined using the YSI 2300 autoanalyzer (Yellow Springs Instruments, Yellow Springs, OH) and remaining plasma was stored at -80º C for further analysis. Insulin was measured using a human insulin enzyme-linked immunoassay kit (Millipore, St. Charles, MO) adapted in our laboratory for dog plasma. C-peptide and glucagon were measured using RIA kits (Millipore, St.
Charles, MO. Active GLP-1 was measured by ELISA (Millipore, St. Charles, MO). Free fatty acids were determined using a colorimetric assay (Wako, Neuss, Germany). The radioactivity of plasma glucose and tracer infusates was determined as follows: samples were diluted and deproteinized with barium hydroxide and zinc sulfate. After drying overnight, the samples were resuspended in water, scintillation cocktail was added, and then the samples were measured in a scintillation counter.

Calculations

MRI analysis 11 slices (5 above and 5 below the landmark) were used for analysis of total trunk body fat, visceral fat and subcutaneous fat. Images were analyzed using Scion Image for Windows (Alpha 4.0.3.2., Scion Corporation, Frederick, MD, USA). Fat was differentiated from non-fat tissue based on pixel intensity. Visceral adipose tissue (VAT) was defined as the fat located in the intra- and retro-peritoneal region, and this region was separated from the subcutaneous adipose tissue (SAT) by manual trace. For each slice the area was calculated in cm² (area of 226 x 256 pixels equivalent to 34.9 x 34.9 cm²) and multiplied by the thickness of the slice to obtain the volume.

IVGTT derived indices - Calculation of Minimal Model parameters Insulin sensitivity (SI) and glucose effectiveness (Sg) were calculated by the application of the minimal model of glucose kinetics to the time course of glucose and insulin from the IVGTT using MINMOD Millennium software (Version 6.02, 2004). The acute insulin response to glucose (AIRg) was calculated as the AUC of the insulin concentrations above the average of the basal values, from 0 to 10 min. The disposition index (DI) was calculated as the product SI x AIRg for each experiment. Kg (glucose tolerance) was
calculated as the slope of the linear regression of the natural log of plasma glucose concentration versus time from 10 to 19 min.

**Euglycemic hyperinsulinemic clamp derived indices** Endogenous glucose production (EGP) and glucose disappearance (Rd) were calculated using Steele’s equation modified for use with labeled glucose infusion (16) after data smoothing using OOPSEG (9). Whole body insulin sensitivity (SI_{clamp}) was calculated as SI_{clamp} = ΔGinf / (ΔIns x Glu_{ss}), where ΔGinf is the increase in glucose infusion from basal to steady state, ΔIns is the increase in insulin from basal to steady state and Glu_{ss} is the steady state glucose level. Peripheral insulin sensitivity (SI Rd) was calculated as SI Rd = ΔRd / (ΔIns x Glu_{ss}), where ΔRd is the change in Rd from basal to steady state. Hepatic insulin sensitivity (SI EGP) was calculated as ΔEGP / (ΔIns x Glu_{ss}), where ΔEGP is the change in EGP from basal to steady state.

**Stepped hyperglycemic clamp** Steady state was defined as the last 30 min of each hyperglycemic step. Beta cell response to glucose was calculated as the slope of the line relating plasma insulin to plasma glucose concentration for each of the three steps of the clamp.

**OGTT data analysis** Post STZ OGTT glucose and insulin data was compared to glucose and insulin plasma levels from OGTT performed in a lean group of dogs (n=13, including 5 of the 12 dogs used in the current study) (OGTT-CON).

**Fasting parameters** were calculated (unless otherwise indicated) as the average of all the fasting values in experiment days for the specific timepoint.

**Statistical analysis**
RESULTS

INDUCTION OF OBESITY AND INSULIN RESISTANCE VIA A HIGH FAT-HYPERCALORIC DIET

Body weight and body composition

Initiation of fat feeding resulted in a significant increase in body weight (Fig.2A), an increase that was maintained for the duration of the fat feeding. After 6-8 weeks of fat feeding the average BW was 31.4 ± 0.8 kg, an 8.3 % increase from baseline (29.0 ± 0.9 kg, p<0.0001). This modest increase in overall weight belied a striking 74% increase in total body fat (from MRI, from 613 ± 51 cm$^3$ to 1025 ± 60 cm$^3$ (Fig 2B)). There were increases in both visceral fat (52 % increase, from 418 ±29 cm$^3$ to 617 ± 36 cm$^3$) and subcutaneous fat (130 % increase, from 195 ± 28 cm$^3$ to 408 ± 35cm$^3$).

Glucose tolerance, insulin sensitivity and pancreatic beta cell function after fat feeding

Intravenous glucose tolerance, as reflected in the Kg, was not changed by fat feeding (Table 1). This was due to reciprocal changes in insulin sensitivity, which declined by about 30% (-29% by IVGTT and -27% by euglycemic clamp) and insulin response, which increased correspondingly. The decline in insulin sensitivity was accounted for by decreased peripheral sensitivity, with no measureable change in insulin sensitivity of the liver (Table 1). The peripheral insulin resistance was exquisitely
compensated by an increase in insulin response, as measured by first phase insulin IVGTT AIRg (+45%), IVGTT C-peptide AUC (+58%) as well as hyperglycemic clamp (+23%), such that neither glucose tolerance (Kg) nor secretory function (DI) was reduced (Table 1). There were no changes in GLP-1 or glucagon with fat-feeding and body fat accumulation (data not shown).

This data recapitulates the remarkable ability of the intact gluco-regulating system in the dog to compensate appropriately with hyperinsulinemia for insulin sensitivity. It also speaks to the normal adaptability of insulin kinetics in this animal model.

BETA CELL DEFECT IN OBESE INSULIN RESISTANT ANIMALS WITH STREPTOZOTOCIN

Fat fed obese animals were subsequently administered a single dose of streptozotocin, ranging from 15-30 mg/kg Figure 3 shows the dose-dependency of AIRg reduction induced by STZ administration. Just two animals received 30 mg/kg – they showed a almost total reduction in pancreatic beta cell function (98 and 99%), 1 week after streptozotocin, with fasting hyperglycemia over 200 mg/dl, requiring insulin therapy (Fig 4). Two additional animals received a lower 22.5 mg/kg STZ dose. One likewise exhibited fasting hyperglycemia (259 mg/dl), and a 99% depletion of AIRg immediately after STZ (Fig 3 and Fig 4). The second animal that received the 22.5 mg/kg dose showed 84% reduction in first phase insulin response, without fasting hyperglycemia. Because we were interested in developing a model of mild diabetes, and based upon these pilot results, we turned to lower doses of STZ in the fat-fed obese models.

Doses 15 mg/kg and 18.5 mg/kg STZ produced variable reduction in AIRg: between 17- 74% for the lowest dose, and 76- 93% for the 18.5 mg/kg dose (Fig 3).
Despite the latter reductions, fasting plasma glucose was within normal range after both doses (Fig 4).

Thus, the two highest streptozotocin doses (22.5-30 mg/kg) produced a dramatic reduction in pancreatic function in the 4 animals studied, and fasting hyperglycemia in 3 of 4. The overweight animals that received the lower doses (15-18.5 mg/kg) had a more modest decreases in pancreatic beta cell function. As were we searching for a model of mild T2DM, we chose to phenotype in more detail animals on the lesser two doses. These animals were followed for period of 12 weeks and underwent full metabolic assessment, as well as measurements of body weight, food intake and body fat.

Characteristics of 18.5 mg/kg STZ dose, “the obese mild T2DM model.”

Body weight decreased immediately following STZ, in parallel with decreased post-streptozotocin food intake (data not shown), but BW remained higher than the control period (Figure 5A). Despite the STZ injection, by week 4 post-STZ animals had on average recovered their pre-injection body weight and still showed increased in both visceral and subcutaneous fat over pre-STZ control (23% and 108%, respectively (Figure 5B). Over the following weeks, as the animals continued to consume the high-fat diet, body weight remained almost constant (32.3 ± 1.2 kg at week 4 and 32.0 ± 1.2 at week 10), masking large increases in body fat (103% increase in visceral fat and 187% increase in subcutaneous fat at 10 weeks). Total body fat increased from 21% during control to 28% at 4 weeks and 33% at 10 weeks after STZ.
Figure 6 shows plasma glucose during IVGTT in the control period and at 1, 2, 4 and 10 weeks after the 18.5 mg/kg STZ injection. Kg decreased 75% after streptozotocin and remained virtually identical over the following weeks (Table 2).

With this 18.5 mg/kg STZ dose, pancreatic beta cell function assessed by IVGTT was decreased 77-93%, as shown by the lower AIRg and C-peptide AUC (Table 2). At week 10 AIRg was the same as at week 4, indicating the STZ-induced beta cell defect was not transient. The hyperglycemic step clamp likewise reflected the decrease in pancreatic beta cell function (Table 2).

Insulin sensitivity post-STZ showed similar patterns by IVGTT or by euglycemic clamp (Table 2). SI IVGTT decreased 33% with fat feeding, from 5.5 ± 0.5 to 3.7 ± 1.0 [(μU/ml)^{-1} * min^{-1}], for this group. Post streptozotocin SI further decreased and remained lower than control for the duration of the monitoring period, such that at the end of 10 weeks it was 40% lower than the control period. Euglycemic hyperinsulinemic clamp analysis revealed a similar trend, with the animals being 44% and 50% less sensitive at 4 and 10 weeks, respectively, after streptozotocin.

In all animals oral glucose tolerance was measured at 4 and 10 weeks after streptozotocin. Even though most animals had normal FPG or slightly higher IFG, glucose and insulin patterns during an OGGT indicated that these animals could be classified as diabetic based on their oral glucose tolerance test (Fig 7A). Glucose AUC was significantly higher and insulin AUC was significantly lower compared to a control group (OGTT-CON), both at 4 weeks (glucose: 20283 ± 1944 mg/dl * min in STZ group vs. 2029 ± 368 mg/dl *min OGTT-CON, p<0.01; insulin 7598 ± 2267 pM*min in STZ group vs. 15137 ± 2271 pM *min OGTT-CON, p<0.05) and at 10 weeks (glucose: 19277
± 1620 vs. 2029 ± 368 mg/dl *min OGTT-CON, p<0.01; insulin 4934 ± 1661 vs. 15137 ± 2271 pM *min OGTT-CON, p<0.01) (Fig 7B). Fasting glucagon levels tended to be higher after STZ, without reaching significance (53 ± 8 ng/l at 10 weeks STZ vs. 43 ± 5 ng/l before STZ, p=0.08). There were no significant changes in active GLP-1 levels during OGTT (data not shown).

The animals that received the 18.5 mg/kg STZ dose did not have any signs of hepatic or renal damage, as assessed by measurements of AST, ALT, BUN and creatinine; serum amylase and lipase values were also normal, indicating normal exocrine pancreas function (data not shown).

Thus, the animals that received the 18.5 mg/kg dose of streptozotocin exhibited metabolic characteristics highly reminiscent of human obese insulin resistant T2DM. At the 4 week monitoring timepoint these animals had 43% more body fat (with 108% increases in subcutaneous fat and 23% increases in visceral fat), their insulin sensitivity was 48% decreased compared to control and they had on average 83 % reduction in pancreatic beta cell function. Fasting plasma glucose varied between 102 and 114 mg/dl. During the OGTT glycemia was significantly higher compared to a control group, and the 120 min glucose was above 200 mg/dl. The model remained stable over the next weeks, with the exception of one animal that became hyperglycemic 10 weeks after streptozotocin.

Characteristics of the “obese impaired glucose tolerance model” (15 mg/kg STZ dose)

In the lowest streptozotocin dose group body weight did not decrease after streptozotocin, and continued to increase until the end of the study (Fig 8A). We
measured large increases in total body fat: by 85% at 4 weeks (135% in increase in subcutaneous fat and 65% increase in visceral fat) and by 144% at 10 weeks (185% increase in subcutaneous and 130% increase in visceral fat) (Fig 8B).

The 15 mg/kg dose of streptozotocin produced a less dramatic reduction in intravenous glucose tolerance than all other STZ doses. Kg was reduced by 44% at week 1 after streptozotocin (p<0.05). Though it appeared to ameliorate over the following weeks (by week 4 it was similar to the control period) at 10 weeks it was reduced again (Figure 9)(Table 3).

AIRg was reduced on average by 50% at week 1 (range 24 to 75%) and remained remarkably stable over the follow up period of 12 weeks. Hyperglycemic clamp data also indicated a reduction in beta cell function, but of lower magnitude, on average 25%.(Table 3). Euglycemic clamp measured SI was decreased by 15% at week 4 and 33% at week 10. In contrast, we measured a surprising apparent increase in SIIVGTT (43% and 34% at 4 and 10 weeks, respectively)(Table 3).

Oral glucose tolerance was decreased compared to a control group, both at 4 weeks (glucose AUC 5245 ± 1084 vs. 2029 ± 368 mg/dl*min OGTT-CON, p=0.05) and at 10 weeks (glucose AUC 5873 ± 1975 vs. 2029 ± 368 mg/dl*min OGTT-CON, p=0.14). In contrast to the IVGTT results, during OGTT plasma insulin concentration was not significantly lower compared to the control group (insulin AUC: 15792 ± 5318 pM* min at 4 weeks, and 12420 ± 2267 pM* min at 10 weeks vs. 15137 ± 2271 pM* min OGTT-CON) (Fig 10).

All animals that received 15 mg/kg STZ had AST, ALT, BUN, creatinine, serum amylase and lipase within the normal range (data not shown).
In brief, the animals that received our lowest streptozotocin dose (15 mg/kg) showed increased body fat (by 85% at 4 weeks and by 144% at 10 weeks), were mildly insulin resistant (as indicated by the euglycemic hyperinsulinemic clamp, but by IVGTT), and had a stable reduction in beta cell function, of variable range (between 24 and 75%). Oral glucose tolerance remained reduced compared to normal controls.

Pancreas histology

Immunohistochemical staining for insulin performed at sacrifice (12 weeks post STZ) showed decreased insulin fluorescence compared with pancreatic tissue from lean, non-STZ treated animals (n=4) (Fig 10): % FITC stain $0.630 \pm 0.174$ for control, $0.115 \pm 0.029$ for 15 mg/kg STZ ($p<0.05$ vs. control) and $0.144 \pm 0.069$ for 18.5 mg/kg STZ ($p<0.05$ vs. control).

DISCUSSION

A variety of animal models are used in the study of T2DM, ranging from rodents to feline, pig, dog and primates, from genetic to chemically induced (26). However, few of these models mirror the pathophysiology and natural history of T2DM, especially obesity-related T2DM.

In our study we report the development of a canine model that resembles the metabolic defects in human T2DM and a model similar to defects of impaired glucose tolerance associated with obesity and insulin resistance. Animals were first made obese by feeding them a highly palatable elevated fat-high carbohydrate diet. After 6-8 weeks animals had gained a modest amount of weight (8%) which was due to dramatic increases in body fat (130% subcutaneous and 52% visceral) and was associated with reduction in
insulin sensitivity (reduced 31%). With fat deposition alone, the insulin resistance was
offset by a compensatory increase in insulin secretion, such that glucose tolerance was in
the normal range. This time-course of hyperinsulinemic compensation in the face of
insulin resistance in the dog model was described by Mittelman et al. in a moderate fat-
induced model of insulin resistance in dog (30). Insulin resistance appeared in the first
week of fat feeding and remained present for the duration of the study (12 weeks). Insulin
resistance was initially compensated by increased insulin secretion and subsequently by
decreased hepatic insulin clearance. The fat-fed dog model thus confirms the hyperbolic
relationship insulin sensitivity – insulin secretion observed in humans (4)(21) and
elegantly exemplifies the coordinated intervention of several organs (pancreas, liver,
adipose tissue) in order to maintain euglycemia.

The second step in the development of our diabetes-like model was administration
of a single i.v injection of streptozotocin, in low doses ranging from 15-30 mg/kg. STZ
has been used to induce diabetes in various species including mouse, rat, guinea pig,
hamster, dog, pig and non-human primates (32) (38). There is large variability in the
diabetogenic dose among species. In large mammals dose ranging from 20 mg/kg (29) to
300 mg/kg (31) have been used. In the dog the LD50 is 1500 mg/m2 (50 mg/kg), very
close to the diabetogenic dose (2) (32), such that repeated administration of low STZ
doses (37), a combination of STZ and alloxan (8) or STZ and pancreatectomy (18) have
been used to induce diabetes. In other species nicotinamide has been administered to
protect the pancreas, liver and kidney from STZ-induced damage (25). Due to the marked
destruction of pancreatic beta cells most canine and pig models of diabetes using
streptozotocin resemble not T2DM, but T1DM. For example, in pilot studies our highest
streptozotocin dose of 30 mg/kg left virtually no residual pancreatic function and was overtly diabetogenic. The animals’ glucose homeostasis deteriorated rapidly and even with insulin therapy glycemic control was difficult to obtain. Of the two animals in the second highest group (22.5 mg/kg) one had immediately post-injection frank diabetes that required insulin therapy while the other had normal fasting glycemia that was maintained until the end of the follow up period (4 weeks). None of the animals showed any signs of hepatic or renal damage. The insulin secretion was reduced 80 - 99 % and insulin sensitivity was reduced 53%. Thus this high-intermediate dose of STZ (22.5 mg/kg) results in a model that has a dramatic reduction in insulin secretion and low insulin sensitivity, possibly overt diabetes, and is suitable for studying the long term complications of T2DM. It has the advantage over established models of T2DM that the STZ dose used to produce a beta cell defect is low enough that it is very unlikely to have a negative effect on other organs. An interesting aspect of our study is that with the relatively low doses of streptozotocin of 22.5 or 30 mg/kg we obtain a phenotype that has overt diabetes. It has been previously shown that higher doses are required to induce diabetes in the canine species(32). It is very possible that our result of a lower diabetogenic dose is due to the fact that at the time of STZ administration animals are obese and insulin resistant. In rats an i.p. dose of 35 mg/kg STZ resulted in hyperglycemia in obese fat-fed rats, but not in their lean counterparts. Only a higher dose of 45 mg/kg STZ produced hyperglycemia in both groups (34).

In our obese insulin resistant canine model a carefully selected dose of 18.5 mg/kg STZ produced a variable 76% to 93% decrease in insulin secretory function. By week 4 post STZ all animals had impaired fasting glucose, FPG ranging from 104 to 114
mg/dl. Additionally, the 2h OGTT plasma glucose over 200 mg/dl fulfills the human criteria for diagnosis of diabetes mellitus in humans (1). In fact, there are few guidelines for diagnosing diabetes in the dog. Canine diabetes diagnosis is usually made in the presence of any casual glucose over 200 mg/dl and symptoms of diabetes (35), much in the line with human criteria. The upper limit of the normal glucose interval is reported as 100 mg/dl, so we can infer that the range of FPG obtained in our study with the 18.5 mg/kg dose are in the IFG category (20). This IFG was a distinguishing feature of the 18.5 mg/kg STZ dose group; the animals receiving the lowest STZ dose (15 mg/kg) did not routinely show FPG over 100 mg/dl, and 2 h OGTT glycemia was below 200 mg/dl.

Insulin sensitivity of the animals receiving 18.5 mg/kg STZ was decreased 30% after fat feeding pre-STZ and further to 50% at 4 weeks after STZ, as indicated both by IVGTT and the euglycemic hyperinsulinemic clamp. The decremental reduction in SI after STZ could be due to the continuation of fat feeding per se or to the acute effect of STZ. It has been reported that administration of STZ alone induces insulin resistance (7; 23), though other studies have not confirmed this finding (37). In our study, due to temporarily decreased food intake, animals lost weight and body fat immediately post-STZ, which would favor the explanation of STZ induced insulin resistance.

STZ-induced weight loss was only temporary. Animals recovered their pre-injection food intake within a week. At 4 weeks post-STZ they exhibited more body weight and body fat (both visceral and subcutaneous) than in the control period. For dogs, unlike for diabetes or IFG/IGT, there are well defined obesity criteria. Widely used is the Body Condition Score (BCS)(either 5 point or 9 point) that classifies dogs as ideal, overweight or obese (24). Though the ability of the score to predict body fat has been
shown in one study (27), this BCS assessment is subjective and relies on the personal experience and training of the person making the assessment. Obesity in dogs has been defined as body weight 15% (12) or 20% (27) over ideal, but in mixed breed dogs ideal body weight is hard to establish. No specific criteria for definition of obesity based on % body fat or size of specific fat depots exists; however, in our sample of dogs body fat increased from 21% during the control period to 28% and 33% at 4 and 10 weeks after STZ, respectively, which would qualify the animals as overweight or obese.

The animals that received the lowest STZ dose (15 mg/kg) presented an interesting pattern of metabolic changes. STZ reduced beta cell function as assessed by both IVGTT (50%) and by hyperglycemic clamp (25%). Intravenous glucose tolerance decreased 50% in the first week after STZ, and it appeared to slowly recover, but by 10 weeks it was reduced again by 40%. The increased glucose tolerance was not due to increased insulin secretion, since AIRg and C-peptide AUC remained remarkably stable. Spontaneous recovery of pancreatic function post-STZ has been previously described, but only in very young animals (36). SI IVGTT increased post-STZ, and the DI improved as well. In contrast, the euglycemic clamp data showed a decrease in SI clamp post-streptozotocin. We believe that the apparent increase in SI IVGTT is an artifact due to the small group size. The increase in SI IVGTT is mostly driven by a large SI increase in one of the four animals. In contrast, the clamp SI decreased in all animals. Moreover, in the animal in which SI IVGTT shows an apparent increase, the finding is not supported by euglycemic clamp data, which shows a decrease in SI. Lastly, a decrease (but not increase) in insulin sensitivity would correlate with the continuation of fat-feeding and with streptozotocin administration, both known to induce insulin resistance. While
phenotyping the 15 mg/kg dose would be of interest, the purpose of the current study was to choose the appropriate STZ dose for a mild type 2 diabetic animal model; the 15 STZ dose was a stepping stone in this process, but we believe it is not necessary for our current goal to completely phenotype the animals (additional 14 animals needed based on conservative power calculation), since the change beta cell function is the primary outcome of the STZ treatment.

In summary, we present in the current study the development of a new large animal model of obesity, insulin resistance and beta cell deficiency. While none of the animal models of T2DM can reproduce the human disease in its complexity, our model has many features resembling human T2DM: adult onset, association with obesity and insulin resistance, residual insulin secretion with disappearance of phase 1 and natural history in which insulin resistance and compensatory hyperinsulinemia occur first, followed by a decline in pancreatic function. Our canine model offers the advantage of an easy induction: a period of fat feeding is followed by one injection of STZ at a low dose that is well tolerated and has no effects on the exocrine pancreas, liver or kidney. Animal welfare is a critical consideration in developing large animal models of diseases and in our case, because of the mild form of diabetes, animals do not require insulin therapy, the recovery after injection is fast and food intake and body weight are maintained and even increased. Our model is thus suitable for longitudinal studies with multiple assessments and testing for pharmacological intervention, as well as mechanistic insights in the pathophysiology of T2DM.

ACKNOWLEDGEMENTS
This work was supported by a research grant from Amylin Inc. R.N.B. is supported by the National Institutes of Health (DK 27619 and DK 29867). The USC Multiphoton Imaging Core was supported by the shared instrument grant 1S10RR024754-01 from NCRR.

We are especially grateful to Dr. Joyce Richey and Dr. Marilyn Ader for insightful comments; we wish to thank Rita Thomas for technical assistance, Edward Zuniga and Edgardo Paredes for animal care and experiment assistance, Dr. Clara Magyar and Dr. Janos Peti-Peterdi for assistance with pancreas imaging and Dr. Vincent Poitout for the immuno-histochemistry protocol.

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11. Ref Type: Abstract


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23. **Koopmans SJ, Mroz Z, Dekker R, Corbijn H, Ackermans M and Sauerwein H**.


25. **Larsen MO, Wilken M, Gotfredsen CF, Carr RD, Svendsen O and Rolin B**.


Table 1. Parameters of glucose homeostasis after fat-feeding and weight gain

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>After weight gain</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IVGTT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kg (min⁻¹) (*10⁻³)</td>
<td>3.3 ± 0.3</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>DI</td>
<td>2946 ± 498</td>
<td>2917 ± 353</td>
</tr>
<tr>
<td>SI [(μU/ml)⁻¹ * min⁻¹]</td>
<td>5.8 ± 0.7</td>
<td>4.1 ± 0.5*</td>
</tr>
<tr>
<td>AIRg [μU/ml * min]</td>
<td>497 ± 50</td>
<td>719 ± 47**</td>
</tr>
<tr>
<td>C-peptide₀-10 min [pM * min]</td>
<td>4306 ± 390</td>
<td>6837 ± 554**</td>
</tr>
<tr>
<td><strong>Euglycemic hyperinsulinemic clamp</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SI [dl/kg/min/pM] (*10⁻⁴)</td>
<td>7.1 ± 0.8</td>
<td>5.2 ± 0.5†</td>
</tr>
<tr>
<td>SI (_{EGP}) [dl/kg/min/pM] (*10⁻⁴)</td>
<td>1.0 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>SI (_{Rd}) [dl/kg/min/pM] (*10⁻⁴)</td>
<td>5.9 ± 0.6</td>
<td>4.4 ± 0.5†</td>
</tr>
<tr>
<td>FFA suppression [ΔmM]</td>
<td>0.53 ± 0.06</td>
<td>0.48 ± 0.07</td>
</tr>
<tr>
<td><strong>Hyperglycemic clamp</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta cell function [pM/mg/dl]</td>
<td>4.8 ± 0.5</td>
<td>6.2 ± 0.5*</td>
</tr>
</tbody>
</table>

* p<0.05, **p<0.01, † p=0.05
### Table 2. Parameters of glucose homeostasis for the 18.5 mg/kg STZ dose group (n=4)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Fat</th>
<th>STZ wk1</th>
<th>STZ wk2</th>
<th>STZ wk4</th>
<th>STZ wk10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IVGTT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kg (min-1) (*10⁻²)</td>
<td>3.2 ± 0.5</td>
<td>4.0 ± 1.0</td>
<td>0.8 ± 0.0*</td>
<td>0.7 ± 0.1**</td>
<td>1.1 ± 0.2*</td>
<td>1.0 ± 0.1*</td>
</tr>
<tr>
<td>DI</td>
<td>2504 ± 449</td>
<td>2783 ± 798</td>
<td>69 ± 28**</td>
<td>127 ± 36**</td>
<td>203 ± 51**</td>
<td>234 ± 52**</td>
</tr>
<tr>
<td>SI [(μU/ml)⁻¹ * min⁻¹]</td>
<td>5.5 ± 0.5</td>
<td>3.7 ± 1.0</td>
<td>2.7 ± 0.4*</td>
<td>2.5 ± 0.3**</td>
<td>2.9 ± 0.3*</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>AIRg [μU/ml * min]</td>
<td>441 ± 51</td>
<td>752 ± 93**</td>
<td>25 ± 10**</td>
<td>48 ± 8**</td>
<td>68 ± 11**</td>
<td>63 ± 14**</td>
</tr>
<tr>
<td>C-peptide AUC₀⁻¹₀ min [pM*min]</td>
<td>5224 ± 1011</td>
<td>7574 ± 184</td>
<td>n/a</td>
<td>n/a</td>
<td>1005 ± 149*</td>
<td>889 ± 425*</td>
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<tr>
<td><strong>Euglycemic hyperinsulinemic clamp</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SI [dl/kg/min/pM] (*10⁻²)</td>
<td>7.1 ± 0.8</td>
<td>3.6 ± 0.4</td>
<td>n/a</td>
<td>n/a</td>
<td>4.0 ± 0.6</td>
<td>3.6 ±0.5</td>
</tr>
<tr>
<td>SI_EGP [dl/kg/min/pM] (*10⁻⁴)</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>n/a</td>
<td>n/a</td>
<td>0.5 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>SI_Rd [dl/kg/min/pM] (*10⁻⁴)</td>
<td>4.6 ± 0.3</td>
<td>2.8 ± 0.5</td>
<td>n/a</td>
<td>n/a</td>
<td>3.6 ± 0.6</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>FFA suppression [Δ mmol/l]</td>
<td>0.56 ± 0.08</td>
<td>0.46 ± 0.05</td>
<td>n/a</td>
<td>n/a</td>
<td>0.63 ± 0.13</td>
<td>0.51 ± 0.07</td>
</tr>
<tr>
<td><strong>Hyperglycemic clamp</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta cell function [pM/mg/dl]</td>
<td>5.7 ± 0.7</td>
<td>6.0 ± 0.6</td>
<td>n/a</td>
<td>n/a</td>
<td>0.8 ± 0.4**</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

*p<0.05 and ** p<0.01 vs. control
Table 3. Parameters of glucose homeostasis for the 15 mg/kg STZ dose group

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Fat</th>
<th>STZ wk1</th>
<th>STZ wk2</th>
<th>STZ wk4</th>
<th>STZ wk 10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IVGTT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kg (min-1) (*10^-2)</td>
<td>3.0 ± 0.5</td>
<td>3.5 ± 0.5</td>
<td>1.7 ± 0.3*</td>
<td>2.4 ± 0.6</td>
<td>3.0 ± 0.6</td>
<td>2.1 ± 1.0</td>
</tr>
<tr>
<td>DI</td>
<td>2619 ± 1055</td>
<td>2493 ± 591</td>
<td>1504 ± 255</td>
<td>1597 ± 297</td>
<td>1517 ± 208</td>
<td>1928 ± 474</td>
</tr>
<tr>
<td>SI [(μU/ml) -1 * min^-1]</td>
<td>4.1 ± 1.2</td>
<td>3.2 ± 0.4</td>
<td>5.2 ± 1.1</td>
<td>5.7 ± 1.6</td>
<td>5.9 ± 1.8</td>
<td>5.6 ± 2.0</td>
</tr>
<tr>
<td>AIRg [μU/ml * min]</td>
<td>600 ± 114</td>
<td>742 ± 88</td>
<td>306 ± 54*</td>
<td>311 ± 54*</td>
<td>313 ± 69*</td>
<td>383 ± 59</td>
</tr>
<tr>
<td>C-peptide [pmol/l*min]</td>
<td>3754 ± 199</td>
<td>4707 ± 381</td>
<td>n/a</td>
<td>n/a</td>
<td>3415 ± 1005</td>
<td>3456 ± 904</td>
</tr>
<tr>
<td><strong>Euglycemic hyperinsulinemic clamp</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SI [dl/kg/min/pM] (*10^-4)</td>
<td>6.5 ± 1.8</td>
<td>6.0 ± 0.5</td>
<td>n/a</td>
<td>n/a</td>
<td>5.5 ± 1.4</td>
<td>4.3 ± 1.1</td>
</tr>
<tr>
<td>SI EGP [dl/kg/min/pM] (*10^-4)</td>
<td>1.1 ± 0.5</td>
<td>0.8 ± 0.1</td>
<td>n/a</td>
<td>n/a</td>
<td>0.8 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>SI Rd [dl/kg/min/pM] (*10^-4)</td>
<td>5.6 ± 1.6</td>
<td>5.1 ± 0.4</td>
<td>n/a</td>
<td>n/a</td>
<td>4.7 ± 1.4</td>
<td>3.9 ± 1.1</td>
</tr>
<tr>
<td>FFA suppression [Δ mmol/l]</td>
<td>0.39 ± 0.05</td>
<td>0.35 ± 0.06</td>
<td>n/a</td>
<td>n/a</td>
<td>0.61 ± 0.10</td>
<td>0.53 ± 0.09</td>
</tr>
<tr>
<td><strong>Hyperglycemic clamp</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta cell function [pM/mg/dl]</td>
<td>4.0 ± 0.9</td>
<td>5.1 ± 1.0</td>
<td>n/a</td>
<td>n/a</td>
<td>2.9 ± 1.0</td>
<td>3.1 ± 0.9</td>
</tr>
</tbody>
</table>
Figure legends

Figure 1. Experimental design. MA: full metabolic assessment (euglycemic hyperinsulinemic clamp, stepped hyperglycemic clamp, IVGTT and OGTT); MRI: magnetic resonance imaging; STZ: streptozotocin injection.

Figure 2. (A) Body weight and (B) Total, omental and subcutaneous fat volume, during the control period and after the hypercaloric high fat diet (** p<0.01; ***p<0.001 vs. control).

Figure 3. Dose-response relationship between STZ dose and IVGTT-AIRg at 1, 2, 4 and 10 weeks after the STZ injection.

Figure 4. Dose-response relationship between STZ dose and IVGTT fasting plasma glucose during control period and 1, 2, 4 and 10 weeks after the STZ injection.

Figure 5. (A) Body weight and (B) Total, VAT and SAT volume, during the control period, after fat feeding before STZ and 4 weeks and 10 weeks after the STZ injection (dose 18.5 mg/kg). **p<0.01, *p<0.05 and # p=0.05 vs. control.

Figure 6. Plasma glucose during IVGTT in the control period and at 1, 2, 4 and 10 weeks post 18.5 mg/kg STZ.

Figure 7. (A) Glucose and (B) insulin during an OGTT at 4 weeks and 10 weeks after the STZ injection (dose 18.5 mg/kg), compared to a control group (OGTT-CON).

Figure 8. (A) Body weight and (B) Total, VAT and SAT volume, during the control period, after fat feeding before STZ and 4 weeks and 10 weeks after the STZ injection (dose 15 mg/kg). ***p<0.01, **p<0.01 and *p<0.05 vs. control.

Figure 9. Plasma glucose during IVGTT in the control period and at 1, 2, 4 and 10 weeks post 15 mg/kg STZ.

Figure 10. (A) Glucose and (B) insulin during an OGTT at 4 weeks and 10 weeks after the STZ injection (dose 15 mg/kg), compared to a control group (OGTT-CON).

Figure 11. Immunohistochemical staining for pancreatic insulin (FITC) in control dogs (A) and animals that received streptozotocin (B) Magnification 10x.
The graph shows the glucose levels over time for different groups labeled as control, stz wk 1, stz wk 2, stz wk 4, and stz wk 10. Glucose levels are measured in mg/dl, and the x-axis represents time in minutes ranging from -30 to 180.
The graph shows the glucose levels (mg/dl) over time (min) for different groups.

- **Control**
- **Stz wk 1**
- **Stz wk 2**
- **Stz wk 4**
- **Stz wk 10**

The glucose levels are measured at various time points, with a notable peak at zero time for the control group. The graph indicates a decline in glucose levels over time for all groups, with some variation between them.