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RESEARCH ARTICLE

Exenatide Treatment Alone Improves β-Cell Function in a Canine Model of Pre-Diabetes

Viorica Ionut[1☯†], Orison O. Woolcott[1☯], Hasmik J. Mkrtchyan[1], Darko Stefanovski[1], Morvarid Kabir[1], Malini S. Iyer[1], Huiwen Liu[2], Ana V. B. Castro[1], Qiang Wu[1], Josiane L. Broussard[1], Cathryn M. Kolka[1], Isaac Asare-Bediako[1], Richard N. Bergman[1]*

1 Diabetes and Obesity Research Institute, Cedars-Sinai Medical Center, Los Angeles, California, United States of America, **2** Department of Physiology and Biophysics, University of Southern California, Los Angeles, California, United States of America

† Deceased.
☯ These authors contributed equally to this work.
* Richard.Bergman@cshs.org

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Abstract

Background

Exenatide's effects on glucose metabolism have been studied extensively in diabetes but not in pre-diabetes.

Objective

We examined the chronic effects of exenatide alone on glucose metabolism in pre-diabetic canines.

Design and Methods

After 10 weeks of high-fat diet (HFD), adult dogs received one injection of streptozotocin (STZ, 18.5 mg/kg). After induction of pre-diabetes, while maintained on HFD, animals were randomized to receive either exenatide (n = 7) or placebo (n = 7) for 12 weeks. β-Cell function was calculated from the intravenous glucose tolerance test (IVGTT, expressed as the acute insulin response, AIR_G), the oral glucose tolerance test (OGTT, insulinogenic index) and the graded-hyperglycemic clamp (clamp insulinogenic index). Whole-body insulin sensitivity was assessed by the IVGTT. At the end of the study, pancreatic islets were isolated to assess β-cell function *in vitro*.

Results

OGTT: STZ caused an increase in glycemia at 120 min by 22.0% (interquartile range, IQR, 31.5%) (P = 0.011). IVGTT: This protocol also showed a reduction in glucose tolerance by 48.8% (IQR, 36.9%) (P = 0.002). AIR_G decreased by 54.0% (IQR, 40.7%) (P = 0.010), leading to mild fasting hyperglycemia (P = 0.039). Exenatide, compared with placebo, decreased body weight (P<0.001) without altering food intake, fasting glycemia, insulinemia, glycated hemoglobin A1c, or glucose tolerance. Exenatide, compared with placebo, increased both OGTT- (P = 0.040) and clamp-based insulinogenic indexes (P = 0.016),

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improved insulin secretion *in vitro* ($P = 0.041$), but had no noticeable effect on insulin sensitivity ($P = 0.405$).

Conclusions

In pre-diabetic canines, 12-week exenatide treatment improved β-cell function but not glucose tolerance or insulin sensitivity. These findings demonstrate partial beneficial metabolic effects of exenatide alone on an animal model of pre-diabetes.

Introduction

Insulin resistance and β-cell dysfunction play fundamental roles in the pathogenesis of type 2 diabetes. Impairment of β-cell function to compensate to for insulin resistance accelerates the progression to type 2 diabetes [1].

Exenatide, a synthetic analogue of exendin-4, a natural ligand of the glucagon-like peptide 1 receptor, has been extensively used for type 2 diabetes treatment. Exenatide has been shown to reduce hyperglycemia, promote body weight loss, and improve insulin sensitivity and β-cell function, resulting in lower hemoglobin A1c levels [2]. However, it is unclear whether these multiple effects are related to exenatide *per se* or concomitant interventions such as lifestyle changes or combined antidiabetic drugs. Numerous studies have explored the therapeutic effects of exenatide in type 2 diabetes [3], but few studies have examined its metabolic effects on pre-diabetes. Impaired fasting glucose and impaired glucose tolerance are well established risk factors for type 2 diabetes [4–7]. Treatment of these pre-diabetic conditions has been associated with delayed progression to diabetes [6]. Previous clinical studies have explored the effect of exenatide on glucose tolerance (in combination with lifestyle changes) [8], insulin sensitivity, and β-cell function (in combination with pioglitazone and metformin) [9], and the homeostasis model assessment-insulin resistance index [10]. However, none of these studies has systematically explored the effect of exenatide alone on glucose homeostasis in the pre-diabetes state.

The prevalence of pre-diabetes (impaired fasting glucose or impaired glucose tolerance) in the United States has been estimated in ~35% [11]. Given the high prevalence of pre-diabetes, a high risk factor for type 2 diabetes [4], and the widely use of exenatide in the clinical practice, we thought it is relevant to further study the metabolic effects of exenatide alone in a canine model of pre-diabetes. In the present study, we hypothesize that chronic treatment with exenatide alone improves glucose homeostasis in the pre-diabetic state. Thus, we determined the effects of exenatide on glucose tolerance, β-cell function, and insulin sensitivity in a canine model of pre-diabetes.

Materials and Methods

Animals and diet regimen

Experiments were conducted in adult male mongrel dogs, 1–2 years old. Dogs were supplied by Antech, Inc. (Barnhart, MO). Animals were single housed in stainless steel kennels in the vivarium of the Keck School of Medicine, University of Southern California (Los Angeles, CA). Kennels had gates between runs, fiberglass slatted floors or plastic coated expanded metal floors (24 square feet of floor space), and stainless steel feeders. Animals were permitted social contact between the runs through a steel mesh wall. Animals were provided with environmental enrichment. Dogs received positive interactions with animal care staff on a daily basis. Dogs were exercised within the room during room cleaning. Before the commencement of the study,

animals received a standard diet consisted of 825 g of dry chow and one canned food (Hill's Pet Nutrition, Topeka, KS) for 2–3 weeks [12]. Thereafter, animals were fed a hypercaloric high-fat diet (HFD) until the end of the study. HFD diet consisted of 825 g of dry chow and one canned food supplemented with lard (6 g/kg of baseline body weight). Total daily food presented (09:00–12:00 h) contained 5,527 kcal (53.0% from fat). Daily food intake was assessed by subtracting the weight of food presented from the weight of food left in the bowl or dropped on the floor. Water was provided *ad libitum*.

Study design

All *in vivo* experiments were performed in the morning, after 12–16 hours of fasting. Biopsies from liver and pancreas for *in vitro* experiments were obtained at the end of study, prior to euthanasia, under general inhalant anesthesia (3% isoflurane). The full study protocol was approved by the Institutional Animal Care and Use Committees from the University of Southern California and the Cedars-Sinai Medical Center (Los Angeles, CA).

After an initial 10 weeks of HFD, starting at week −15, animals received a single intravenous dose of streptozotocin (STZ, 18.5 mg/kg of body weight) at week −5. This dose was previously found to be effective in fat-fed canines to induce pre-diabetes [13]. Five weeks after STZ administration, animals were randomized to receive either exenatide (n = 7; 10 μg subcutaneously b.i.d. during the weekdays and once daily during weekends) or placebo (n = 7). Drug treatment started at week 0 and continued until the end of the study (week 12, our primary endpoint). HFD was initiated at week −15 and was maintained until the end of the study. The oral glucose tolerance test (OGTT) and the intravenous glucose tolerance test (IVGTT) were performed in all animals at the beginning of the study, prior to the commencement of exenatide treatment (week 0), and at the end of the study. Body weight was assessed weekly. Daily food intake was recorded starting at week 0. IVGTT was also performed at weeks 3, 6, and 9. Fasting plasma glucose and insulin were also measured during IVGTT experiments. Hyperglycemic clamp and magnetic resonance imaging (MRI) of the abdominal region were performed at weeks 0 and 12. Likewise, assessment of fasting plasma glucagon, glycated hemoglobin A1c, and energy expenditure (resting metabolic rate) were performed at weeks 0 and 12.

Exenatide was kindly provided by Amylin Pharmaceuticals Inc. Streptozotocin was purchased from Sigma-Aldrich (St Louis, MO), dissolved in citrate buffer solution (pH 4.5; Sigma-Aldrich) immediately before injection [13].

Experimental procedures

MRI. Fat distribution in the abdominal region was assessed under general anesthesia by MRI using a 1.5-T Siemens Scanner [12]. MRI scan included eleven transverse 10-mm thick slices. For each slice, major regions of the abdominal anatomy (extra- and intra-abdominal compartments) were defined by manual drawing. Fat and non-fat tissues were distinguished based on pixel intensity using an imaging software (sliceOmatic 4.3, Tomovision, Magog, QC, Canada). Total abdominal fat volume included the sum of subcutaneous and visceral fat obtained from the 11 slices [12].

OGTT. Glucose 50% was given as a bolus by oral gavage. Blood samples were taken at 15, 30, 45, 60, 90, 120, and 180 min [14]. Glucose tolerance was measured as the glucose concentration at t = 120 min. β-Cell function was measured as the OGTT insulinogenic index: the ratio of the over-basal area under the curve (AUC) of plasma insulin (mU/L) to over-basal AUC of plasma glucose (mg/dL). AUCs of plasma glucose ($AUC_{GLUCOSE}$), insulin ($AUC_{INSULIN}$), and glucagon ($AUC_{GLUCAGON}$) were calculated using the trapezoidal rule (Prism 4.0, GraphPad Software, Inc.).

IVGTT. β-Cell function (acute insulin response, AIR_G), whole-body insulin sensitivity (S_I), the disposition index (DI), glucose effectiveness (S_G), intravenous glucose tolerance (K_G), and hepatic insulin clearance were calculated from the IVGTT, performed as previously described [15, 16]. Hepatic insulin clearance was expressed as the fractional clearance rate of insulin (FCR) and the metabolic clearance rate of insulin (MCR) [17]. A bolus of glucose 50% (0.3 g/kg of body weight) was given intravenously at 0 min and a bolus of insulin (0.03 U/kg of body weight) at 20 min. Blood samples were taken at t = 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 140, 160, and 180 min. AIR_G, S_I, DI, and S_G were calculated using MINMOD (Minmod Millennium version 6.02, MINMOD Inc., Los Angeles, CA). K_G was calculated as the negative slope of the natural log of glucose versus time from t = 10 to t = 19 min. FCR and MCR were calculated using WINSAAM (version 3.3.0, University of Pennsylvania) [17]. FCR was calculated from the decay of plasma insulin after the intravenous bolus. Insulin from t = 22–80 min was fit to the following exponential decay curve. MCR was calculated as the ratio of the dose of injected insulin during the IVGTT to the over-baseline area under the curve of insulin after time of injection to infinity.

Graded-hyperglycemic clamp. Glucose 50% was infused intravenously in a peripheral vein at variable rates so as to maintain blood glucose constant at three sequential concentrations: 100 mg/dL (t = 0–59 min), 150 mg/dL (t = 60–149 min) and 200 mg/dL (t = 150–240 min) [18]. β-Cell function was calculated as the slope of the linear relation between insulin (mU/L) and glucose (mg/dL) during the steady-state at each glucose clamp value (100 mg/dL, 150 mg/dL, and 200 mg/dL) [18]. β-Cell function was also measured as the clamp insulinogenic index: the ratio of the over-basal $AUC_{INSULIN}$ to over-basal $AUC_{GLUCOSE}$.

Resting metabolic rate. Resting metabolic rate was assessed as previously described [12] using indirect calorimetry. Data were analyzed using TurboFit (VacuMed, Ventura, CA).

Islet static incubation. Islets were isolated from the pancreas tail immediately before euthanasia [18, 19]. All *in vitro* experiments were performed after 18–24 h culture in 5-cm petri dishes. Prior to static incubation experiments, islets were washed three times in Krebs-Ringer bicarbonate buffer modified with HEPES [19, 20]. Islets were hand-picked and transferred to 24-well culture plates containing Krebs-Ringer buffer. After 1-h equilibrium period at 37°C with 3 mmol/L glucose, separate batches of islets (15 islets per well, three-replicate experiments) were exposed to either 3, 7, 10, 15, 20, or 27 mmol/L glucose for one additional hour (glucose solution was added to reach such concentrations). β-Cell function was calculated as 1) the total insulin secreted at the end of the static incubation (pmol•L^{-1}/islet/h) and 2) the stimulation index: the ratio of the insulin secreted at high glucose concentration (second hour of the static incubation) to the basal insulin (equilibrium period) [19].

Liver gene expression. RNA was extracted from biopsied hepatic tissues using the Tri-Reagent Kit (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized according to manufacturer's protocol, using 1 μg of total RNA using Superscript II (Invitrogen, Carlsbad, CA). Real-time polymerase-chain reaction (RT-PCR) was performed using a Light-Cycler 4.8 instrument (Roche Applied Science, Indianapolis, IN). cDNA was amplified using the 'universal probe system' on a Roche microplate with a final volume of 10 μL reaction mix containing 2.5 μL, 100-fold diluted cDNA, 7 μL LightCycler TaqMan Master Mix buffer (Roche Probes Master kit, Roche Applied Science, Indianapolis, IN), 1 μmol/L specific forward-reverse primers and 0.5 μL specific universal probes. Primers and universal probes are shown in Table 1. Quantification of 18S rRNA was used for sample normalization using TaqMan probes. RT-PCR was performed according to manufacturer's protocol (Roche Applied science, Indianapolis, USA). The specificity of amplification was determined by melting curve analysis. The results were analyzed by relative quantification, ΔΔC method.

Blood sampling and biochemical assays. Blood samples were collected, stored, and analyzed as previously described [13, 18]. Insulin from plasma and *in vitro* experiments were determined by ELISA (human kit, Millipore) [19]. Glucagon concentration was measured by radioimmunoassay (canine kit, Millipore).

Statistical analyses

Since data were non-normally distributed, non-parametric tests were used. Data were expressed as medians and their interquartile range (IQR). Wilcoxon matched-pairs signed-rank test and Mann-Whitney U test were used for comparison within and between groups, respectively. Quade's test was used to compare the metabolic effects of exenatide versus placebo adjusting for pretreatment values. Mixed-effects linear regression [21]) was used to compare groups when repeated measures were performed, testing for treatment X time interaction. Friedman ANOVA was used if interaction was found to be significant. Differences were statistically significant if P was less than 0.05. All analyses were performed using IBM SPSS Statistics version 20.0 and Stata/SE 10.0 for Windows (StataCorp LP, College Station, TX).

Results

Pre-diabetes induction

STZ treatment resulted in a decrease in glucose tolerance (Table 2). Glycemia at 120 min post-oral glucose challenge increased by 22.0% (IQR, 31.5%) (P = 0.011). K_G decreased by 48.8% (36.9%) (P = 0.002). Fasting glycemia increased by 3.4% (IQR, 4.9%); P = 0.039), from 99.1 mg/dL (6.6 mg/dL) to 103.7 mg/dL (7.4 mg/dL). Fasting plasma insulin remained unchanged (P = 0.972). OGTT insulinogenic index decreased by 73.6% (IQR, 41.3%) (P = 0.011) and AIR_G fell by 54.0% (IQR, 40.7%) (P = 0.010). Since insulin sensitivity remained unchanged (P = 0.875), *DI* decreased accordingly (P = 0.006). S_G also decreased (P = 0.008). Body weight increased by 5.8% (IQR, 6.9%) (P = 0.046).

Effect of exenatide on food intake and body composition

Exenatide's effect on food intake was not different from placebo throughout the study (P = 0.494; mixed model regression) (P = 0.603, Quade's test) or at week 12 (Fig 1 and Table 3).

Table 1. Primers and probes used for RT-PCR.

Gene	Forward	Reverse	UPL
FAS	ATGCTGGGCATGGAGTTC	CACCAGTCCCATCACACG	59
SREBP1c	TGCTTCTGACAACCATGAAAA	GGCCAGGGAGCTGATACC	8
CPT1	ATGGGCATGAACGCAGAG	CAGGACGTACTCCCACAGGT	26
PPAR-α	GGAGCTAGATGACAGCGACA	GCGATCTCCACAGCAAATG	5
CEACAM1	TTCCAGAACATCACCCTGAA	AGTGCAGTTTCAAATTTTTGGTT	47
GK	GTGGCTGGAAAAGTTCAGGA	CACTCAGCACCACCAGTCC	161
GLUT2	AGCATCTTCGAACCTTGTCAC	TCATTCCACCAATTGCAAAG	93
GLUT4	CCCTATGTCTTCCTTCTGTTCG	CGGGTTTCAGGCACTTTTAG	15
G6Pase	AAGCCAATGACTGTGCCAAT	ACCTCTGGCCTCAAAATGG	83
PEPCK	GCTCCGAGGAGGAGAACC	CCTCTGATCATGCCCTGTC	67
18S rRNA	GCGGCTTTGGTGACTCTAGATA	TTGATAGGGCAGACGTTCG	141

18S rRNA, 18S ribosomal RNA; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; CPT1, carnitine palmitoyltransferase I; FAS, Fas cell surface death receptor; G6Pase, glucose-6-phosphatase; GK, glucokinase; GLUT2, glucose transporter type 2; GLUT4, glucose transporter type 4; PEPCK, phosphoenolpyruvate carboxykinase; PPAR-α, peroxisome proliferator-activated receptor alpha; RT-PCR, real-time polymerase-chain reaction; SREBP1c, sterol regulatory element-binding protein 1c; UPL, Universal Probe Library.

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In fact, there was a high variability in food intake (Fig 1A). In contrast, exenatide, compared with placebo, did promote mild body weight loss throughout the study ($P<0.001$; mixed model regression) (Fig 1B) with no effect on abdominal fat distribution (Table 3).

Effect of exenatide on fasting plasma glucose, insulin, glucagon, and glycated hemoglobin A1c

Analysis of repeated measures showed no differences between groups in fasting plasma glucose (P = 0.663; mixed model) or insulin (P = 0.349) (Fig 2). However, since time interacted with treatment for insulin (P = 0.034) outcomes, Friedman ANOVA was performed independently. We found no changes in plasma insulin with placebo (P = 0.725) or exenatide (P = 0.150). Likewise, we found no changes in glycated hemoglobin A1c in the exenatide or placebo group (Fig 2).

Effects of exenatide on glucose metabolism, insulin clearance, and energy expenditure

Exenatide treatment had no effect on oral glucose tolerance (Fig 3 and Table 4). Glycemia at 120 min post-oral glucose challenge remained unchanged (P = 1.00). Exenatide was not better than placebo in improving $AUC_{GLUCOSE}$ (P = 0.063; Fig 3A and Fig 3B), $AUC_{INSULIN}$ (P = 0.133; Fig 3C and Fig 3D) or $AUC_{GLUCAGON}$ (P = 0.618; Fig 3E and Fig 3F). Likewise, K_G was not different between groups (P = 0.658, mixed model). However, compared with placebo, exenatide had a beneficial effect on OGTT insulinogenic index (P = 0.040, Quade's test) (Table 4), even while adjusting for body weight (P = 0.032, mixed model).

Data calculated from the IVGTT (Fig 4) showed no exenatide effects on AIR_G, S_I, DI or S_G when compared with placebo (Table 4). Likewise, we found no differences between groups in insulin clearance throughout the study (MCR: P = 0.952; FCR: P = 0.499; mixed model). The

Table 2. Changes in metabolic parameters after induction of pre-diabetes following a single dose of streptozotocin (STZ) in high-fat-fed canines (n = 13).

	Before pre-diabetes	Pre-diabetes	Delta change	P value
OGTT				
Glucose 60 min ($mg \cdot dL^{-1}$)	110.7 (17.8)	148.0 (62.2)	43.1 (71.9)	0.002
Glucose 120 min ($mg \cdot dL^{-1}$)	97.0 (4.4)	123.1 (56.6)	18.6 (30.2)	0.011
$AUC_{GLUCOSE}$ ($mg \cdot dL^{-1} \cdot min^{-1}$)	1972.0 (1589.0)	5337.0 (4817.0)	3365.0 (6071.0)	0.002
$AUC_{INSULIN}$ ($mU \cdot L^{-1} \cdot min^{-1}$)	1707.0 (1402.0)	2040.0 (1269.0)	−154.0 (518.3)	0.422
Insulinogenic index ($mU \cdot L^{-1} \cdot min^{-1}$)/($mg \cdot dl^{-1} \cdot min^{-1}$)	0.84 (0.67)	0.25 (0.31)	−0.58 (0.94)	0.011
IVGTT				
Fasting glucose ($mg \cdot dL^{-1}$)	99.1 (6.6)	103.7 (7.4)	−3.4 (5.1)	0.039
Fasting insulin ($mU \cdot L^{-1}$)	42.0 (30.0)	53.5 (32.3)	−1.3 (27.7)	0.972
K_G ($\% \cdot min^{-1}$)*	3.3 (1.2)	1.6 (0.3)	−1.7 (1.6)	0.002
AIR_G ($mU \cdot L^{-1} \cdot min$)*	481.9 (282.0)	178.8 (124.2)	−262.4 (200.8)	0.010
S_I ($mU^{-1} \cdot L^{-1} \cdot min^{-1}$)*	4.7 (3.7)	4.7 (2.6)	−0.7 (5.1)	0.875
DI*	2476.4 (1586.1)	978.2 (520.2)	−1356.8 (1565.1)	0.006
S_G ($\% \cdot min^{-1}$)*	4.2 (2.7)	2.7 (0.6)	−1.7 (2.3)	0.008
Body weight (kg)	27.1 (1.9)	27.8 (1.4)	1.5 (1.8)	0.046

Values are medians (interquartile range). Delta change column represents changes from baseline (before pre-diabetes).

*Due to partial missing insulin data, only 12 dogs were included for analyses.

P values were determined by Wilcoxon matched-pairs signed-rank test. AIR_G, acute insulin response to glucose; AUC, area under the curve; DI, disposition index; IVGTT, intravenous glucose tolerance test; K_G, intravenous glucose tolerance; OGTT, oral glucose tolerance test; S_I, insulin sensitivity; S_G, glucose effectiveness.

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insulin/glucose slope calculated from the graded-hyperglycemic clamp was not different between groups (P = 0.498) (Table 4). Likewise, clamp insulinogenic index from t = -20 to t = 240 was not different between groups (P = 0.534) (Fig 5). However, we noted that the clamp insulinogenic index estimated from t = 40 to t = 110 (period when the median plasma glucose actually increased from ~100 mg/dL to ~150 mg/dL), improved with exenatide (P = 0.043 vs. baseline; P = 0.016, between groups, Quade's test) (Fig 5 and Table 4). We did not see changes in β-cell function from t = −20 to t = 30 (P = 0.492, Quade's test). Likewise, we did not see changes in β-cell function from t = 120 to t = 240 (when the median glucose increased from ~150 mg/dL to 200 mg/dL) (P = 0.753, Quade's test).

Although we found no differences in resting metabolic rate (Table 4), exenatide group showed an increase in carbohydrate oxidation (P = 0.046). However, this effect was not different compared with placebo (P = 0.867, Quade's test).

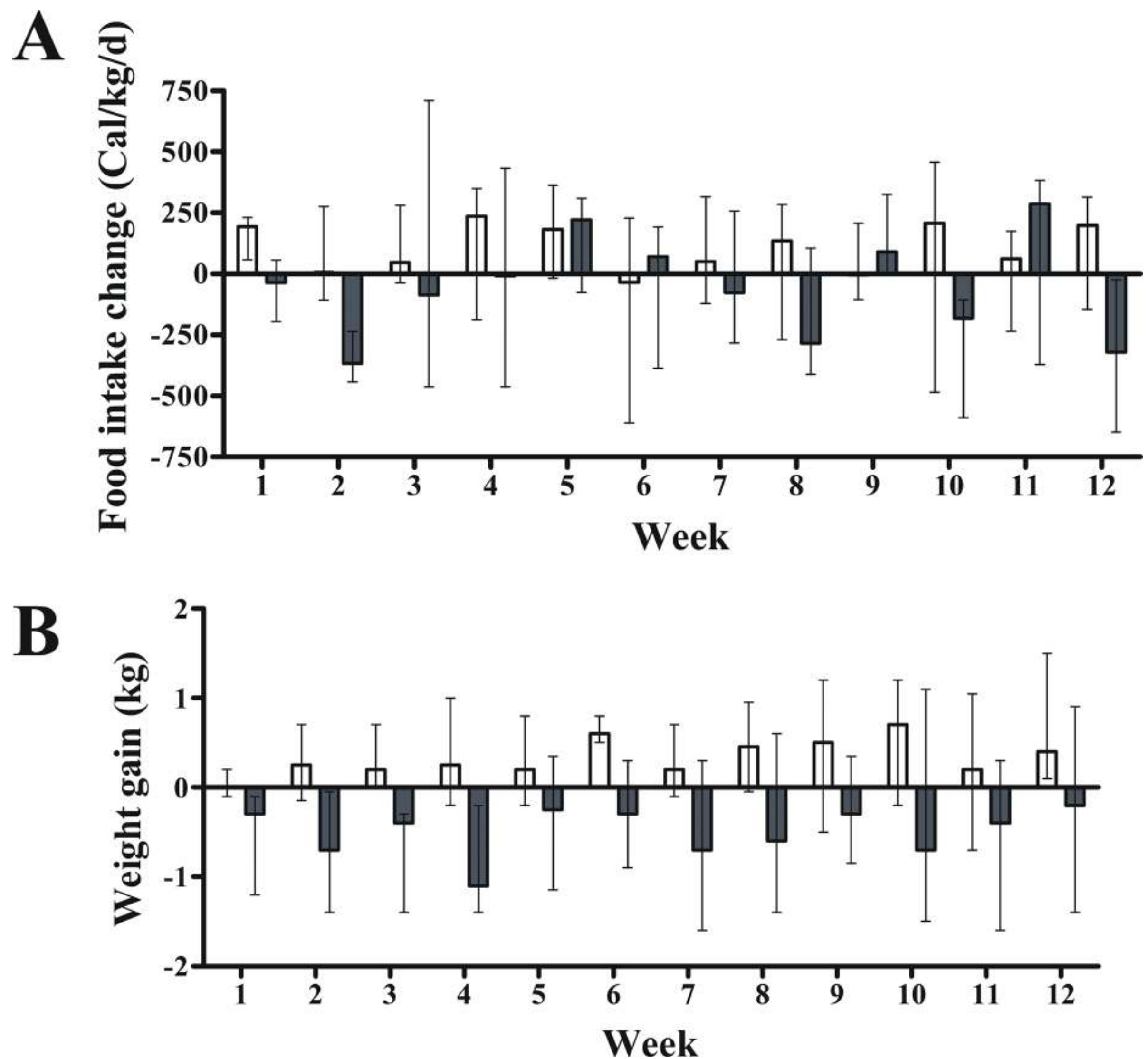

Fig 1. Effect of exenatide on food intake and body weight in pre-diabetic canines (n = 14). Overall, compared with placebo, 12-week treatment with exenatide had no effect on food intake (A) but did reduce body weight (B) throughout the study (P<0.001; mixed model regression). Gray and white columns indicate exenatide and placebo groups, respectively. Top of columns are medians. Bars are upper and lower quartiles.

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Table 3. Changes in food intake and body composition after exenatide treatment for 12 weeks in pre-diabetic canines.

	Exenatide (EXE) (n = 7)			Placebo (PBO) (n = 7)			P value (EXE vs. PBO)
	Baseline	Endpoint	Delta change	Baseline	Endpoint	Delta change	
Food intake (Cal/d)	2679.9 (1241.0)	2320.5 (579.6)	−322.2 (622.8)*	2320.8 (973.9)	2246.7 (1031.8)	−198.4 (459.0)	0.603
Food intake (Cal/kg/d)	98.5 (30.0)	84.4 (16.0)	−4.0 (18.7)*	84.7 (33.3)	79.8 (40.4)	6.4 (17.1)	0.624
Body weight (kg)	27.7 (3.5)	27.5 (3.1)	−0.2 (2.3)	27.9 (1.5)	28.8 (2.1)	0.4 (1.4)	0.217
SAT (cm^3)	166.1 (63.1)	135.3 (73.4)	−2.1 (45.3)	227.2 (131.0)	191.3 (59.7)	−7.5 (56.4)	0.395
VAT (cm^3)	310.2 (208.4)	312.5 (192.3)	−10.2 (68.8)	499.0 (194.4)	400.3 (316.8)	−0.8 (40.2)	0.445
Total fat (cm^3)	504.5 (224.9)	492.5 (286.9)	−21.1 (72.5)	736.3 (185.7)	588.8 (371.5)	−6.3 (17.6)	0.325

Values are medians (interquartile range). Delta change columns represent changes from baseline in each group. VAT: visceral adipose tissue; SAT: subcutaneous adipose tissue.

* P<0.05, Wilcoxon matched-pairs signed-rank test. P values between groups were determined by Quade's test, adjusting for baseline differences.

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Effect of exenatide on β-cell and liver function *in vitro*

Exenatide, compared with placebo, improved glucose-stimulated insulin secretion *in vitro* (P = 0.041, mixed model) (Fig 6), likely influenced by increased basal insulin in the exenatide group (P = 0.046) (Fig 6A). When glucose-stimulated insulin secretion was adjusted for basal insulin, the differences between groups disappeared (P = 0.184). β-Cell function expressed as the stimulation index was not different between groups (P = 0.523; Fig 6B).

Since an increase in carbohydrate oxidation occurred with exenatide, we measured mRNA expression of liver genes related to fat and glucose metabolism. We found no significant differences between groups in genes related to fatty acid synthesis (Fas cell surface death receptor: P = 0.291; sterol regulatory element-binding protein 1c: P = 0.685), glucose oxidation (glucose transporter type 2: P = 0.223; glucose transporter type 4: P = 0.391; glucokinase: P = 0.062), fat oxidation (carnitine palmitoyltransferase I: P = 0.123; peroxisome proliferator-activated receptor alpha: P = 0.062); or insulin clearance (carcinoembryonic antigen-related cell adhesion molecule 1: P = 0.223). Although we found lower mRNA expression of glucose-6-phosphatase (P = 0.028) in the exenatide group, as compared with placebo, mRNA expression of phosphoenolpyruvate carboxykinase was not significantly different (P = 0.116); thus, our data do not conclusively support an inhibitory effect of exenatide on gluconeogenic activity.

Discussion

Our findings indicate that a 12-week treatment with exenatide alone, compared with placebo, improves β-cell function but not glucose tolerance or insulin sensitivity in pre-diabetic canines fed a HFD. Improvement of β-cell function did not translate in lower fasting glycemia or reduced glycated hemoglobin A1c concentration. These results indicate only partial beneficial metabolic effects of exenatide alone on pre-diabetes.

Numerous studies have explored the therapeutical effect of exenatide in type 2 diabetes [3]. However, a few studies have explored the effect of exenatide on glucose homeostasis on pre-diabetes. Exenatide has been shown to improve glucose homeostasis in individuals with pre-diabetes in combination with lifestyle changes or other antidiabetic drugs [8–10]. However, whether the beneficial effects of chronic exenatide treatment on pre-diabetes are mainly due to exenatide *per se* have remained unclear. In fact, no previous study has specifically explored the effect of exenatide alone on glucose tolerance, β-cell function, and insulin sensitivity in the pre-diabetes state, using well accepted methods, under controlled conditions. In the present study, we used a canine model of pre-diabetes, characterized by impaired glucose tolerance as evidenced by a ~20% increase in plasma glucose at minute 120 during OGTT and a reduction in

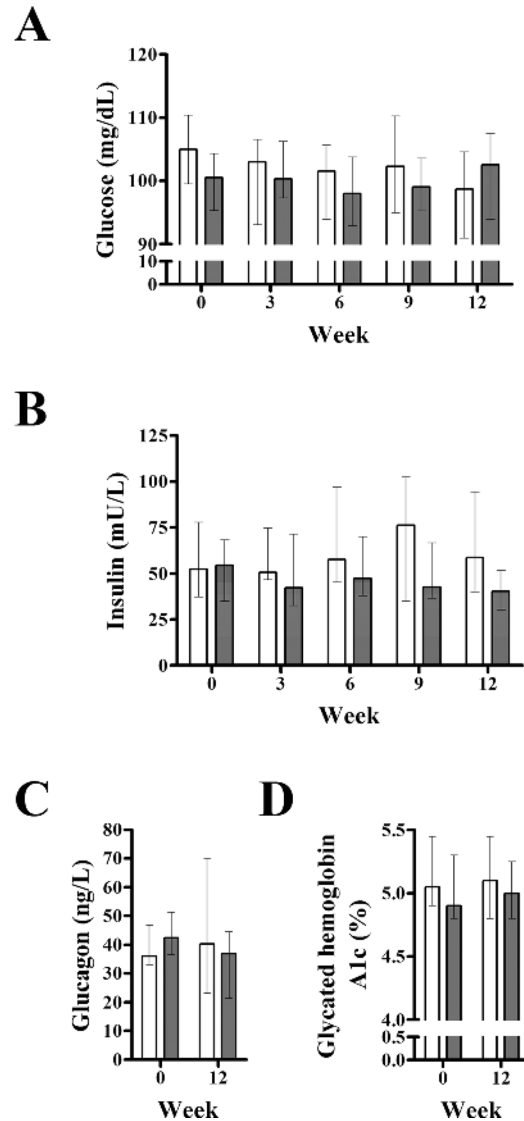

Fig 2. Effect of exenatide on fasting plasma glucose, insulin, glucagon, and glycated hemoglobin A1c. Compared to placebo (n = 7), exenatide (n = 7) did not alter fasting plasma glucose (A), insulin (B), glucagon (C) or glycated hemoglobin A1c (D) in pre-diabetic canines. Gray and white columns indicate exenatide and placebo groups, respectively. Top of columns are medians. Bars are upper and lower quartiles. In the placebo group, glucagon and hemoglobin A1c data were available only in 6 dogs.

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K_G by ~50%. In addition, our pre-diabetic canines showed mild impaired fasting glucose, as evidenced by a ~3% increase in fasting glycemia.

Chronic exenatide treatment, compared with placebo, caused a reduction in body weight while animals where maintained on a HFD. These results are consistent with previous findings in individuals with impaired fasting glucose or impaired glucose tolerance [8–10]. However, we found no beneficial effect of exenatide on abdominal fat mass. Exenatide alone given for 12 weeks has been shown to promote weight loss in obese individuals by reducing appetite rather than increasing energy expenditure [22]. We did not find changes in food intake or resting metabolic rate in pre-diabetic canines. Thus, the mechanisms by which chronic treatment with exenatide induced weight loss in the pre-diabetic canines remain elusive, though physical activity or energy expenditure were not measured, also plausible mechanisms for weight loss.

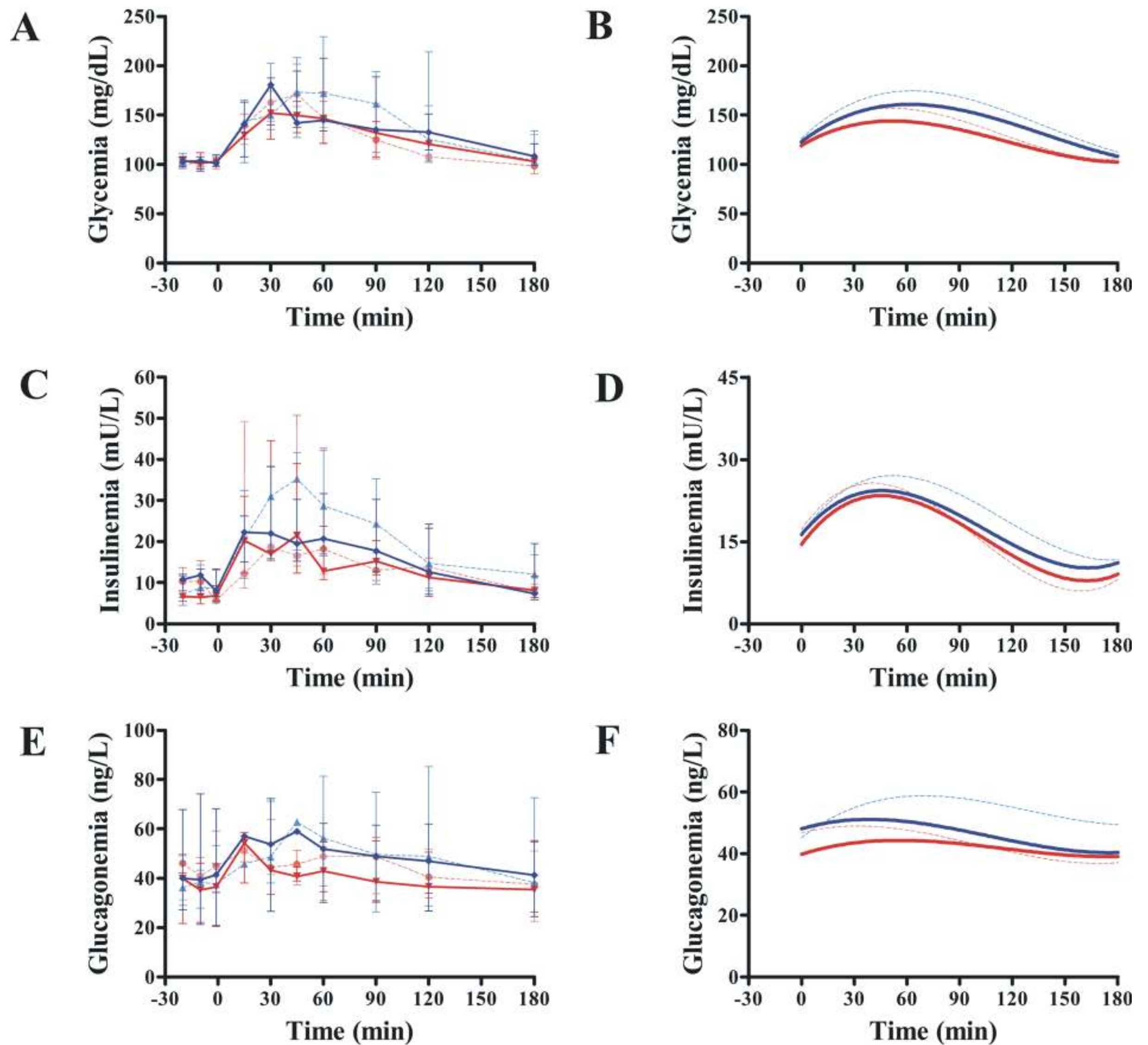

Fig 3. Effect of exenatide on oral glucose tolerance. Compared with placebo (n = 7), exenatide (n = 7) was not different in modifying $AUC_{GLUCOSE}$ (A and B), $AUC_{INSULIN}$ (C and D) or $AUC_{GLUCAGON}$ (E and F). Continuous red and blue lines indicate exenatide and placebo groups, respectively, after 12 weeks. Dash red and blue lines indicate exenatide and placebo groups, respectively, at baseline (week 0). B, D, and F show the nonlinear fits (polynomial of third order) for curves represented in A, C, and E, respectively. Plots are medians. Bars are upper and lower quartiles. Glucagon data were available only in 6 dogs.

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Nevertheless, exenatide alone appears to cause a very modest body weight loss in individuals with pre-diabetes [10], and in individuals with severe obesity [22], which is consistent with our findings (Fig 1B). Exenatide combined with diet and physical exercise may well have a stronger effect in reducing body weight in individuals with pre-diabetes [8].

We did not find changes in plasma fasting glucose or glycated hemoglobin A1c, which is consistent with findings from large clinical studies with exenatide in pre-diabetic individuals [8, 10]. In individuals with type 2 diabetes, a significant but small decrease in glycated

hemoglobin A1c has been reported after exenatide treatment concomitant with metformin (−0.4%) for 102–113 weeks [23] or with sulfonylurea (−0.8%) over a period of 30 weeks [24].

We found an improvement of β-cell function during OGTT (independent of body weight) but not during the IVGTT after exenatide treatment. These differences could be related, at least

Table 4. Changes in metabolic parameters after exenatide treatment for 12 weeks in pre-diabetic canines.

	Exenatide (EXE) (n = 7)			Placebo (PBO) (n = 7)			P value (EXE vs. PBO
	Baseline	Endpoint	Delta change	Baseline	Endpoint	Delta change	
OGTT							
Glucose 60 min (mg•dL^{-1})	147.0 (26.9)	146.6 (52.2)	−17.3 (35.2)	172.2 (107.7)	144.9 (73.7)	−6.6 (42.1)	0.956
Glucose 120 min (mg•dL^{-1})	107.5 (57.8)	120.7 (5.0)	13.6 (63.4)	125.4 (110.8)	132.5 (36.7)	−11.3 (74.7)	0.298
$AUC_{GLUCOSE}$ (mg•dL^{-1}•min^{-1})	5337.0 (4225.0)	4367.0 (3623.0)	−1815.0 (5068.0)	6617.0 (11311.0)	7523.0 (6729.0)	92.(6186.0)	0.063
$AUC_{INSULIN}$ (mU•L^{-1}•min^{-1})	1139.0 (2047.3)	1517.0 (1994.0)	−39.8 (994.3)	2075.0 (980.0)	1377.0 (1507.3)	−576.9 (1540.4)	0.133
$AUC_{GLUCAGON}$ (ng•L^{-1}•min^{-1})	0.17 (0.20)	0.39 (1.41)	0.36 (0.96)	0.25 (0.35)*	0.44 (0.37)*	0.02 (0.70)	0.618
Insulinogenic index (mU•L^{-1}•min^{-1})/(mg•dl^{-1}•min^{-1})	0.20 (0.51)	0.40 (0.74)	0.18 (0.32)	0.29 (0.31)	0.18 (0.11)	−0.05 (0.18)	0.040
IVGTT							
K_G (%•min^{-1})	1.5 (0.5)	1.5 (0.9)	0.3 (0.8)	1.6 (0.4)	2.5 (1.7)	0.9 (2.0)	0.377
AIR_G (mU•L^{-1}•min)	158.7 (280.3)	278.4 (200.7)	−25.0 (175.2)	183.5 (104.4)	209.9 (154.7)	24.9 (87.4)	0.266
S_I (mU^{-1}•L^{-1}•min^{-1})	4.3 (2.4)	5.2 (2.7)	0.7 (2.4)	5.2 (3.4)	3.3 (5.0)	−0.5 (4.2)	0.274
DI	1058.7 (574.3)	1094.7 (597.7)	106.2 (441.4)	946.7 (1002.8)	840.8 (858.6)	77.2 (398.6)	0.521
S_G (%•min^{-1})	2.4 (0.8)	3.0 (1.1)	0.5 (0.4)†	2.9 (0.8)	3.3 (1.6)	0.2 (1.4)	0.297
MCR (mL•min^{-1}•kg^{-1})	8.5 (5.4)	11.8 (3.1)	0.5 (4.9)	10.1 (2.4)	8.7 (1.7)	−1.4 (3.5)	0.116
FCR (min^{-1})	0.47 (0.20)	0.40 (0.07)	−0.09 (0.20)	0.47 (0.10)	0.42 (0.24)	−0.15 (0.31)	0.851
Graded-Hyperglycemic clamp							
Insulin/Glucose slope (mU•L^{-1})/(mg•dl^{-1})	20.5 (29.5)	25.5 (17.3)	−6.9 (21.7)	22.5 (13.9)	23.1 (13.9)	0.6 (8.7)	0.498
$AUC_{GLUCOSE}$ (baseline-200 mg•dL^{-1})	11894.0 (5430.0)	11592.0 (2312.0)	−365.0 (3075.0)	11716.0 (1988.0)	10450.0 (1656.0)	−1019.0 (2970.0)	0.335
$AUC_{INSULIN}$ (baseline-200 mg•dL^{-1})	2296.0 (1949.0)	2663.0 (2150.0)	−73.0 (849.0)	2407.0 (2352.0)	1839.0 (2059.0)	27.0 (1981.0)	0.586
Insulinogenic index (baseline-200 mg•dL^{-1})	0.16 (0.29)	0.22 (0.28)	−0.00 (0.19)	0.21 (0.28)	0.18 (0.22)	0.02 (0.20)	0.534
$AUC_{GLUCOSE}$ (100–150 mg•dL^{-1})	1143.0 (327.5)	822.8 (428.4)	−177.6 (755.9)	854.1 (387.6)	1103.0 (601.4)	72.0 (531.3)	0.381
$AUC_{INSULIN}$ (100–150 mg•dL^{-1})	365.1 (178.5)	294.3 (505.8)	−28.2 (317.1)	272.8 (672.6)	212.3 (261.5)	−56.9 (184.1)	0.567
Insulinogenic index (100–150 mg•dL^{-1})	0.30 (0.15)	0.41 (0.48)	0.11 (0.25)†	0.32 (0.61)	0.19 (0.19)	−0.05 (0.19)	0.016
Energy expenditure							
Resting metabolic rate (Cal/kg/d)	42.2 (9.0)	40.0 (16.3)	−3.9 (9.7)	49.0 (13.2)	48.2 (7.6)	−1.0 (13.5)	0.667
Energy from carbohydrate oxidation (%)	25.1 (29.8)	43.2 (14.9)	17.0 (27.1)†	38.6 (37.8)	45.5 (41.1)	9.0 (62.7)	0.867

Values are medians (interquartile range). Delta change columns represent changes from baseline in each group.

* Data available only for 6 dogs.

† P<0.05, Wilcoxon matched-pairs signed-rank test.

AIR_G, acute insulin response to glucose; AUC, area under the curve; *DI*, disposition index; *FCR*, fractional clearance rate of insulin; IVGTT, intravenous glucose tolerance test; K_G, intravenous glucose tolerance; *MCR*, metabolic clearance rate of insulin; OGTT, oral glucose tolerance test; S_I, insulin sensitivity; S_G, glucose effectiveness.

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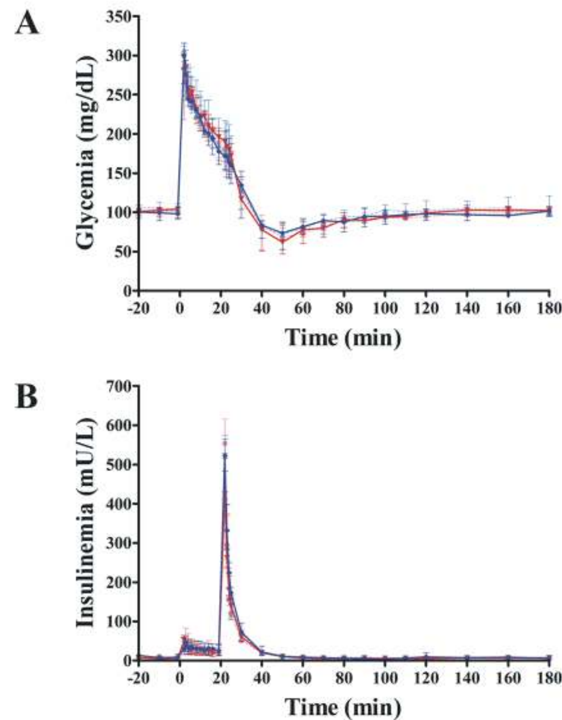

Fig 4. Effect of exenatide on intravenous glucose tolerance. Glucose (A) and insulin (B) profiles are identical in exenatide (n = 7) and placebo groups (n = 7). Continuous red and blue lines indicate exenatide and placebo groups, respectively, after 12 weeks. Dash red and blue lines indicate exenatide and placebo groups, respectively, at baseline (week 0).

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in part, to exenatide's incretin effect [25]. This finding is consistent with those from clinical studies conducted in pre-diabetic [9] and diabetic individuals [26]. However, we also found an improvement of β-cell function assessed by the hyperglycemic clamp at more moderated intravenous glucose incursions (up to 150 mg/dL). This might suggest an incretin-independent effect of exenatide on the β-cells or reduced insulin clearance. However, the latter possibility is unlikely since we did not detect changes in *MCR* or *FCR*.

It is surprising that improvement of β-cell function by exenatide did not translate in an improvement in glucose tolerance. Previous studies have shown improved glucose tolerance with exenatide in pre-diabetic individuals [8, 9]; however, it is not clear in the latter studies if the beneficial effect on glucose tolerance was due to exenatide *per se* or due to the effect of lifestyle intervention (diet and exercise) [8] or the combination with pioglitazone and metformin [9]. It is possible that improvements in insulin sensitivity are necessary to observe substantial improvement in glucose tolerance. However, insulin resistance and β-cell dysfunction, independently, appears to be predictive of glucose intolerance in humans [27].

We did not observe changes in insulin sensitivity, arguing the findings from a previous clinical study in pre-diabetic patients [9]; however, in the latter study exenatide was given in combination with pioglitazone and metformin, the former drug known to be an insulin sensitizer [28]. Individuals with new onset type 2 diabetes receiving exenatide for 24 weeks improved insulin sensitivity, while following a diet and exercise regimen [29]. Positive effect in canines [30] or no effect in humans [31] on insulin sensitivity have been reported after acute administration of exenatide. Rodent studies have shown a beneficial effect of exenatide on insulin

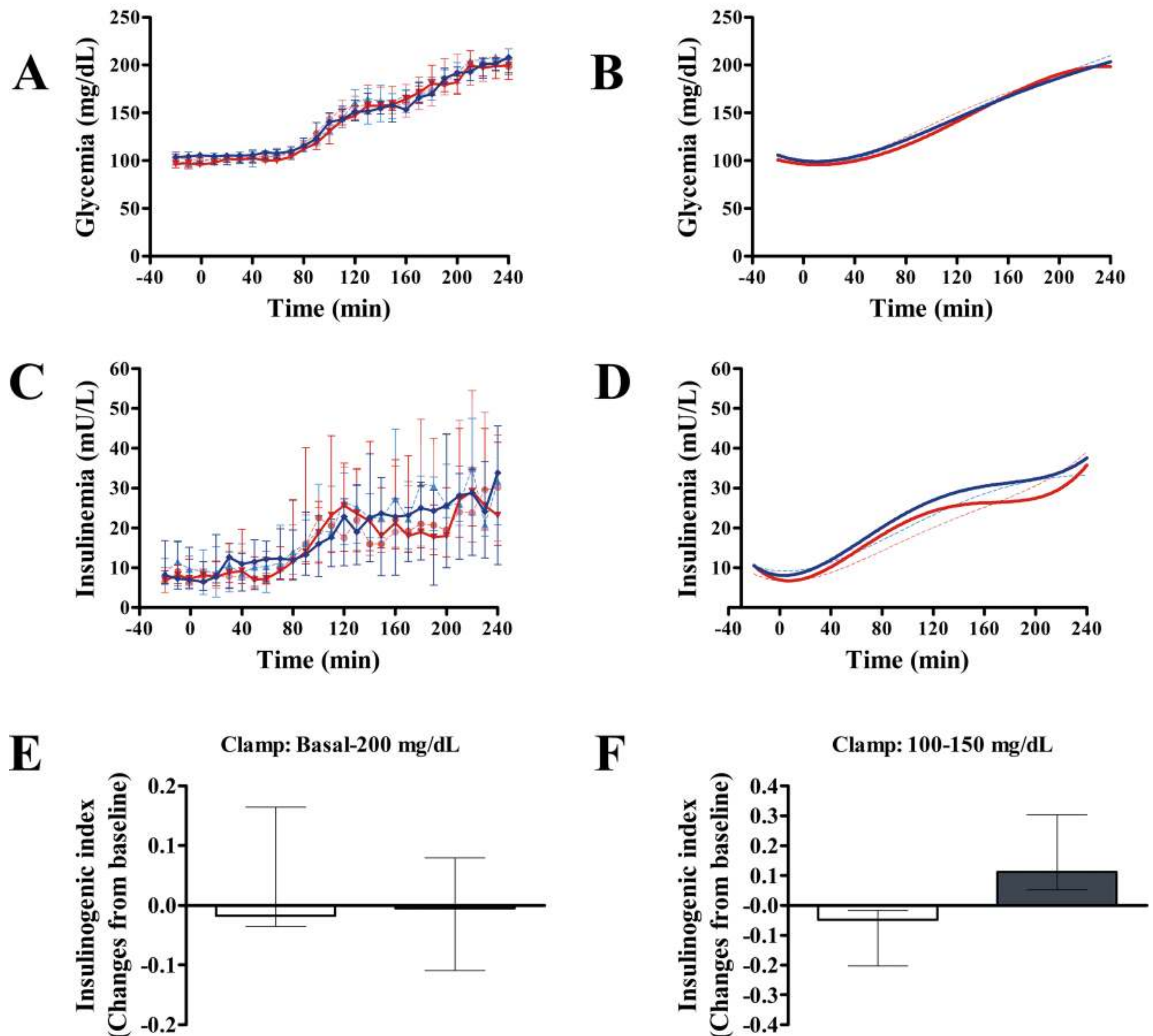

Fig 5. Effect of exenatide on β-cell function during graded-hyperglycemic clamp. After 12 weeks, compared with placebo (n = 7), exenatide (n = 7) was not different in modifying the area under the curve of glucose ($AUC_{GLUCOSE}$) (A and B) or $AUC_{INSULIN}$ (C and D) during the whole test. In E and F, β-cell function is expressed as the insulinogenic index: the ratio of $AUC_{INSULIN}$ to $AUC_{GLUCOSE}$. Clamp at very high glucose concentrations does not discriminate the stimulatory effect of exenatide on β-cell function, as compared with placebo (E). In contrast, clamp period at more physiological glucose concentrations (F) shows a significant increase in β-cell function as compared with placebo (P = 0.016, Quade's test). In A-D, continuous red and blue lines indicate exenatide and placebo groups, respectively. Dash red and blue lines indicate exenatide and placebo groups, respectively, at baseline (week 0). B and D show the nonlinear fits (polynomial of third order) for curves represented in A and C, respectively. In E and F, gray and white columns indicate exenatide and placebo groups, respectively. Columns are medians. Bars are upper and lower quartiles.

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sensitivity at very high concentrations after 6–10 weeks [32, 33]. *In vitro* studies also show contradictory results [34, 35]. It is unclear whether the conflicting results are due to differences in exenatide dose, duration of treatment, health conditions of study participants or species-related differences. Thus, the possible beneficial effect of exenatide on insulin sensitivity remains

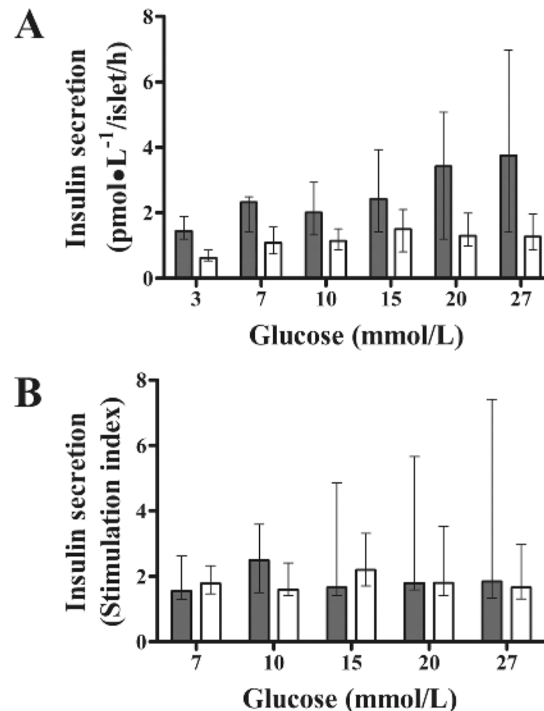

Fig 6. Effect of chronic systemic treatment with exenatide on β-cell function assessed *in vitro*. Compared with placebo, 12-week systemic treatment with exenatide improved total insulin secretion in isolated pancreatic islets from pre-diabetic canines (A) (P = 0.041, mixed model). Each glucose concentration was tested in different islet batches (exenatide: n = 5; placebo: n = 4; three-replicate experiments) (A). Note that the differences in insulin secretion between groups disappeared when insulin secretion was normalized to basal insulin (P = 0.523, mixed model) (B). Gray and white columns indicate exenatide and placebo groups, respectively. Top of columns are medians. Bars are upper and lower quartiles.

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controversial. It should be noted that HFD did not decrease insulin sensitivity in our pre-diabetic canines, possibly explained by a reduced anabolic effect of insulin post-STZ, potentially preventing further improvement of insulin sensitivity by exenatide.

Exendin-4 has a direct insulinotropic effect in rat islets [36, 37], mouse insulinoma cells [37], and islets from type 2 diabetic subjects [38]. Isolated human islets cultured in presence of exenatide for 48 h also showed increased insulin secretion [39, 40]. However, the effect of chronically administered exenatide on isolated islets has not previously been explored in a model of pre-diabetes. Activation of the glucagon-like peptide 1 receptor may also promote β-cell proliferation and prevent β-cell apoptosis, particularly in rodents [41, 42], whereas those effects may not necessarily translate to humans [42, 43]. Since we did not assess β-cell mass, we cannot prove or disprove a possible contribution of increased β-cell mass to explain the increased in vitro insulin secretion in the present study. However, supporting a potential contribution of increased β-cell mass is the fact that we did not find differences in glucose-stimulated insulin secretion in vitro when data were normalized to basal insulin secretion. Since the in vitro insulinotropic effect of exenatide appears to be transient while exenatide is present in the media [40, 44], the increased insulin secretion in vitro in our study does not appear to be explained by an acute effect of exenatide since islets were tested 18–24 h after isolation in the absence of exenatide in vitro. Nevertheless, the argument in favor of increased β-cell mass after chronic exenatide treatment in canines is purely speculative.

The present study has strengths. No previous study has specifically explored the chronic effect of exenatide alone on glucose tolerance, β-cell function, and insulin sensitivity in an

animal model of pre-diabetes. Chronic exenatide therapy in combination with lifestyle has been reported to have beneficial effects on pre-diabetes [8–10]. However, whether these effects are mainly due to exenatide *per se* or lifestyle changes have remained unclear. In the present study we demonstrated that chronic exenatide treatment alone improves β-cell function but not glucose tolerance or insulin sensitivity. Strength of the present study is that we used a relatively homogenous population of animals with a pre-diabetic state induced ~5 weeks prior to the commencement of the exenatide treatment. Previous studies [8–10] have explored exenatide's effects on glucose homeostasis in individuals with pre-diabetes of variable duration.

Our findings should be interpreted according to the limitations of the study. Our experiments were performed in a small number of animals. Unlike the frequent association of insulin resistance with impaired glucose tolerance in humans [45], our canine model of pre-diabetes did not develop insulin resistance, even while animals were fed a HFD. In addition, our canine model of pre-diabetes was very mild, therefore, diagnostic criteria for human pre-diabetes [46] cannot be applied. However, although small, we induced a significant increase in fasting glycemia and glycemia 120 min post-OGTT (Table 2), and a marked decrease in intravenous glucose tolerance or K_G [47]. Another limitation is the relatively short duration of exenatide treatment (12 weeks). It is uncertain whether a more prolonged exenatide treatment in pre-diabetic canines would have resulted in normalization of glucose intolerance and fasting hyperglycemia.

Because we used STZ, it can be argued that our model of pre-diabetes may resemble a pre-diabetic model of type 1 diabetes, rather than a pre-diabetic model of type 2 diabetes. However, we administered a single low dose of STZ (18.5 mg/kg), much lower than the high total STZ dose used in rodents to induce type 1 diabetes (typically ranging from 150–200 mg/kg [48]), aiming to partially impair β-cell function and to induce glucose intolerance and mild hyperglycemia. Moreover, the animals studied were fed a HFD to resemble obesity, a well-established risk factor for type 2, but not type 1, diabetes. Thus, our data put in perspective the partial beneficial metabolic effects of chronic exenatide treatment alone, a widely used clinical drug, in a canine model of pre-diabetes. However, clinical studies are required to determine whether exenatide alone would be appropriate as a pre-diabetes therapy.

In conclusion, in our pre-diabetic canine model, 12-week exenatide treatment improved β-cell function but not glucose tolerance or insulin sensitivity. These findings demonstrate partial beneficial metabolic effects of exenatide alone on an animal model of pre-diabetes.

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Author Contributions

Conceived and designed the experiments: VI RNB CMK. Performed the experiments: VI OOW HJM DS MK MSI HL AVBC QW JLB. Analyzed the data: VI OOW RNB DS MK IAB. Contributed reagents/materials/analysis tools: DS. Wrote the paper: OOW VI RNB. Performed the statistical analysis: OOW. Had full access to all the data in the study and takes full responsibility for the integrity of the data and the accuracy of the data analysis: OOW. Helped revise the manuscript critically for important intellectual content: MK DS CMK.

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