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Received: 24 July 2023 | Revised: 22 September 2023 | Accepted: 24 September 2023

DOI: 10.1111/dom.15319

WILEY

ORIGINAL ARTICLE

Sustained metabolic benefits of ΔTRTX-Ac1, a tarantula venom-derived peptide, when administered together with exenatide in high-fat fed mice

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Funding information
Diabetes UK; Ulster University

Abstract

Aim: The aim of the present study was to assess the long-term therapeutic efficacy of a recently discovered 28 amino acid peptide, Δ-theraphotoxin-Ac1 (Δ-TRTX-Ac1), originally isolated from venom of the *Aphonopelma chalcodes* tarantula. Δ-TRTX-Ac has previously been shown to improve pancreatic beta-cell function and suppress appetite.

Materials and Methods: Δ-TRTX-Ac1 was administered twice daily in high-fat fed (HFF) mice with streptozotocin (STZ)-induced insulin deficiency, namely HFF/STZ mice, for 28 days both alone and in combination with the venom-derived glucagon-like peptide-1 (GLP-1) mimetic, exenatide.

Results: Initial pharmacokinetic profiling of ΔTRTX-Ac1 revealed a plasma half-life of 2 h in mice, with ΔTRTX-Ac1 also evidenced in the pancreas 12 h post-injection. Accordingly, HFF-STZ mice received twice-daily injections of Δ-TRTX-Ac1, exenatide or a combination of both peptides for 28 days. As anticipated, HFF/STZ mice presented with hyperglycaemia, impaired glucose tolerance, decreased plasma and pancreatic insulin and disturbed pancreatic islet morphology. Administration of ΔTRTX-Ac1 reduced body weight, improved glucose tolerance and augmented pancreatic insulin content while decreasing glucagon content. Exenatide had similar benefits on body weight and pancreatic hormone content while also reducing circulating glucose. ΔTRTX-Ac1 decreased energy expenditure on day 28 whereas exenatide had no impact. All treatment regimens restored pancreatic islet and beta-cell area towards lean control levels, which was linked to significantly elevated beta-cell proliferation rates. In terms of benefits of combined ΔTRTX-Ac1 and exenatide treatment over individual agents, there was augmentation of glucose tolerance and ambulatory activity with combination therapy, and these mice presented with increased pancreatic glucagon.

Conclusion: These data highlight the therapeutic promise of ΔTRTX-Ac1 for diabetes, with suggestion that benefits could be enhanced through combined administration with exenatide.

KEYWORDS
diabetes, exenatide, GLP-1, obesity, spider venom-derived peptides

1 | INTRODUCTION

Type 2 diabetes mellitus (T2DM) is reaching epidemic proportions worldwide, with a predicted 454 million individuals to be diagnosed by 2045.[1] T2DM is linked to a progressive decline in pancreatic islet beta-cell function together with peripheral insulin resistance, leading to dysregulation of circulating glucose levels.[2] While several therapies have been approved for T2DM, none are able to address these underlying pathophysiological traits directly, which ultimately leads to polypharmacy and deteriorating glucose control.[3] In this regard, analogues of the incretin hormone, glucagon-like peptide-1 (GLP-1), represent second- or third-line intensification steps on the blood glucose-lowering pathway for patients with T2DM. GLP-1 is a gut-derived peptide known to potentiate glucose-stimulated insulin secretion and suppress appetite, alongside various other well-characterized extrapancreatic glucose-lowering actions.[4] The first clinically approved GLP-1 mimetic, namely exenatide, was initially discovered and isolated from the saliva of the venomous Gila monster (*Heloderma suspectum*) lizard,[5] with several other GLP-1 mimetics now being utilized within the clinic.[6] Although effective, compliance with the GLP-1 class of drugs can be limited because of gastrointestinal (GIT)-related adverse effects such as nausea and vomiting.[7] Strategies to augment the glucose-lowering efficacy of GLP-1 mimetics, while also minimizing the GIT side-effect profile, would therefore be highly sought after.[8]

In parallel with the venomous origin of exenatide,[5] we have recently characterized a novel peptide, Δ-theraphotoxin-Ac1 (ΔTRTX-Ac1), isolated from the venom of the Mexican blonde tarantula *Aphonopelma chalcodes*.[9] ΔTRTX-Ac1 was shown to exert noteworthy glucose-dependent insulinotropic actions consistent with other peptides isolated from venom of the *Grammostola rosea* and *Chilobrachys jingzhao* tarantulas.[10–12] Beyond insulin secretion, ΔTRTX-Ac1 also improved beta-cell proliferation and survival, as well as glucose handling and satiety in mice,[9] together representing a biological action profile highly advantageous in the setting of obesity-driven forms of diabetes such as T2DM. Importantly, ΔTRTX-Ac1 was also shown to exert additive appetite suppressive actions when administered co-jointly with exenatide in mice.[9] Encouragingly, ΔTRTX-Ac1 and exenatide would probably activate distinct cellular targets and cell signalling pathways, amplifying potential for additive, or even synergistic, benefits of the two venom-derived peptides.

To capitalize on this and directly probe the concept, we have investigated the impact of the 28-day twice-daily treatment with ΔTRTX-Ac1 alongside exenatide in high-fat fed (HFF) mice administered the beta-cell toxin streptozotocin (STZ), namely HFF/STZ mice, with STZ countering the classic beta-cell compensatory expansion induced by high-fat feeding.[13] Importantly, this creates a rodent model of diabetes that more closely resembles the beta-cell dysfunction present in human T2DM,[14] to help improve translatability of our findings. Initial studies investigated the pharmacokinetic (PK) profile of ΔTRTX-Ac1 in normal mice before progressing to chronic twice-daily injection regimen in HFF/STZ mice. Effects on food intake, body weight (BW), circulating glucose, insulin and glucagon, as well as glucose tolerance, insulin sensitivity, aspects of whole-body metabolism, pancreatic hormone content and morphology were then assessed in HFF/STZ mice.

2 | MATERIALS AND METHODS

2.1 | Peptides

ΔTRTX-Ac1 and exenatide were synthesized by Synpeptide at >95% purity, with peptide purity and identity confirmed in-house using high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), respectively, as previously described.[15] Briefly, HPLC was conducted on peptide samples (1 mg/ml) using a Phenomenex C-18 analytical column (250 × 4.6 mm) and flow rate of 1.5 ml/min, with acetonitrile as the eluting solvent. HPLC peaks were detected using a Thermoquest SpectraSystem UV2000 detector at 214 nm. For MALDI-TOF MS analysis of collected HPLC peaks, a PerSeptive Biosystems Voyager-DE Biospectrometer was employed, with the α-cyano-4-hydroxycinnamic acid matrix and subsequent detection of the mass/charge ratio.

2.2 | Animals

All mice were housed individually in an air-conditioned room (22 ± 2°C) with relative humidity of 51 ± 5% and a 12 h light/dark cycle (08:00-20:00 h). For PK studies, adult male C57BL/6 mice were maintained on standard rodent diet (10% fat, 30% protein, 60% carbohydrate; percentage of total energy 12.99 kJ/g; Trouw Nutrition) before experimentation at 14 weeks of age. For metabolic studies, adult male C57BL/6 mice (8 weeks old) were maintained on a high-fat diet (45% fat, 20% protein, 35% carbohydrate; percentage of total energy 26.15 kJ/g; Dietex International Ltd) for 3 weeks. Following

this period, mice received three once-weekly intraperitoneal (i.p.) injections of STZ (4-h fast, 50 mg/kg BW, freshly dissolved in citrate buffer, pH 4.5) while still being maintained on 45% high-fat diet throughout, with peptide administration commencing 1 week after the final STZ injection when mice were 15 weeks of age. Notably, as might be expected, STZ-induced insulin deficiency resulted in some weight loss in HFF mice,[16] with HFF/STZ mice having similar BW as lean controls (31.3 ± 0.9 vs. 30.2 ± 0.3 g; respectively) at the start of the experiment.

2.3 | Pharmacokinetic profiling of Δ-theraphotoxin-Ac1

Non-fasted C57BL/6 mice (n = 1) received ΔTRTX-Ac1 injections (10 mg/kg BW, i.p.) with blood withdrawn by cardiac puncture at 0, 2, 4, 6, 8 and 12 h post-administration and plasma extracted. A 10 mg/kg ΔTRTX-Ac1 injection was employed, based on previous in-house observations as being the minimum dose needed to ensure the peptide analyte was detectable in all samples. Plasma samples were then eluted on a C18 Kinetex polar HPLC column (3.0 × 100 mm, 2.6 μm; Phenomenex) at a flow rate of 0.35 ml/min using acetonitrile to extract proteins. ΔTRTX-Ac1 was detected using a Thermo Vantage QQQ mass spectrometer in positive ionization mode, with quantification by multiple reaction monitoring. For the pancreatic PK profile, tissues were extracted and immediately stored in isopentane for each time point, before cryosectioning on to indium tin oxide slides. MS imaging was conducted using a 5800 MALDI TOF/TOF instrument (AB Sciex). Acquisition was performed in positive reflector mode with a mass range of 250-1000 Da. The laser was operated at 29 μJ energy with a total of 100 accumulated shots/pixel acquired using α-ayano-4-hydroxycinnamic acid matrix. Resolution was set to 100 × 100 μm. Imaging reconstruction was then performed with TissueView® software and total ion current normalization.

2.4 | Studies in high-fat fed/streptozotocin mice

HFF/STZ mice were grouped (n = 8) based on BW and non-fasting blood glucose and received twice-daily i.p. injections (09:00 h and 17:00 h) of saline vehicle [0.9% (w/v) NaCl], ΔTRTX-Ac1 (25 nmol/kg BW), exenatide (2.5 nmol/kg BW) or a combination of both peptides at the same dose for 28 days. These mice were maintained on 45% high-fat diet throughout the study period with energy intake and non-fasting glucose assessed at regular intervals. A separate group of lean control mice were maintained on standard rodent diet throughout, for comparative purposes. At the end of the treatment period, glucose tolerance (18-h fasted, 18 mmol/kg BW; i.p.) and insulin sensitivity (non-fasted, 5 U/kg bovine insulin, i.p.) tests were performed. Locomotor activity, energy expenditure and respiratory exchange ratio were examined using the Complete Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments), as described previously.[15] In addition, immediately following euthanasia by lethal CO_2 inhalation and cervical dislocation, total body fat was measured by DXA scanning (Piximus Densitometer; Inside Outside Sales LLC). Terminal analyses involved collection of plasma for determination of insulin and glucagon levels as well as the dissection of pancreatic tissue that was either immediately snap frozen to measure hormone content or fixed in 4% paraformaldehyde for pancreatic islet histology assessment.[17] All animal experiments were conducted under the UK Animals (Scientific Procedures) Act 1986 & EU Directive 2010/63EU, approved by the UK Home Office under project licence PPL2902 and University of Ulster Animal Welfare and Ethical Review Body (AWERB).

2.5 | Biochemical analyses

Blood was obtained from the cut tip of the tail vein from conscious mice with glucose immediately recorded using a handheld glucometer (Ascencia Contour). Blood was also collected in heparin/fluoride coated microcentrifuge tubes and centrifuged at 1500 g for 15 min at 4°C in a microcentrifuge to extract plasma. Plasma insulin was measured by in-house radioimmunoassay,[18] while glucagon was measured by enzyme-linked immunosorbent assay (EZGLU-30K; Merck Millipore) according to the manufacturer's instructions.

2.6 | Pancreatic islet immunohistochemistry

Fixed pancreatic tissue were placed in an automated tissue processor, which involved dehydrating in 70%-100% ethanol followed by xylene immersion to remove wax before paraffin embedding. Embedded tissues were then cut at 5 μm sections on a microtome (Shandon Finesse 325; Thermo Scientific) and placed on poly-L-lysine coated slides. Islet morphology and beta-cell proliferation rates were then assessed by immunohistochemical staining as described previously.[19] Briefly, following overnight incubation with primary antibodies for insulin (1:400; ab6995; Abcam), glucagon (1:1000; ab92517; Abcam) or Ki-67 (1:400; ab15580; Abcam), slides were incubated for 45 min with the secondary antibodies Alexa Fluor 594 goat antimouse IgG (1:500; A-11005; Thermo Fisher) or Alexa Fluor 488 goat antirabbit (1:500; A-11008; Thermo Fisher), as appropriate. Nuclei were stained by incubation with 4′, 6-diamidino-2-phenylindole (DAPI) before mounting and imaging using a fluorescent microscope (Olympus system microscope, model BX51) fitted with DAPI (350 nm), fluorescein isothiocyanate (488 nm) and tetramethylrhodamine isothiocyanate (594 nm) filters and a camera system (Olympus XM10). For subsequent quantitative analysis, a 'closed polygon' tool was used in ImageJ software to identify areas of insulin and glucagon positive staining in μm^2.[20] Beta-cell proliferation was assessed by co-staining for insulin and Ki-67 and quantified by counting the number of insulin-positive cells expressing Ki-67.

2.7 | Statistical analyses

GraphPad PRISM (version 5.0) software was used to perform statistical analysis. Values are expressed as mean ± SEM. Comparative analyses between groups were conducted using a One-way ANOVA with Bonferroni post hoc correction for multiple comparisons. Groups of data are considered to be from different populations if $p < .05$.

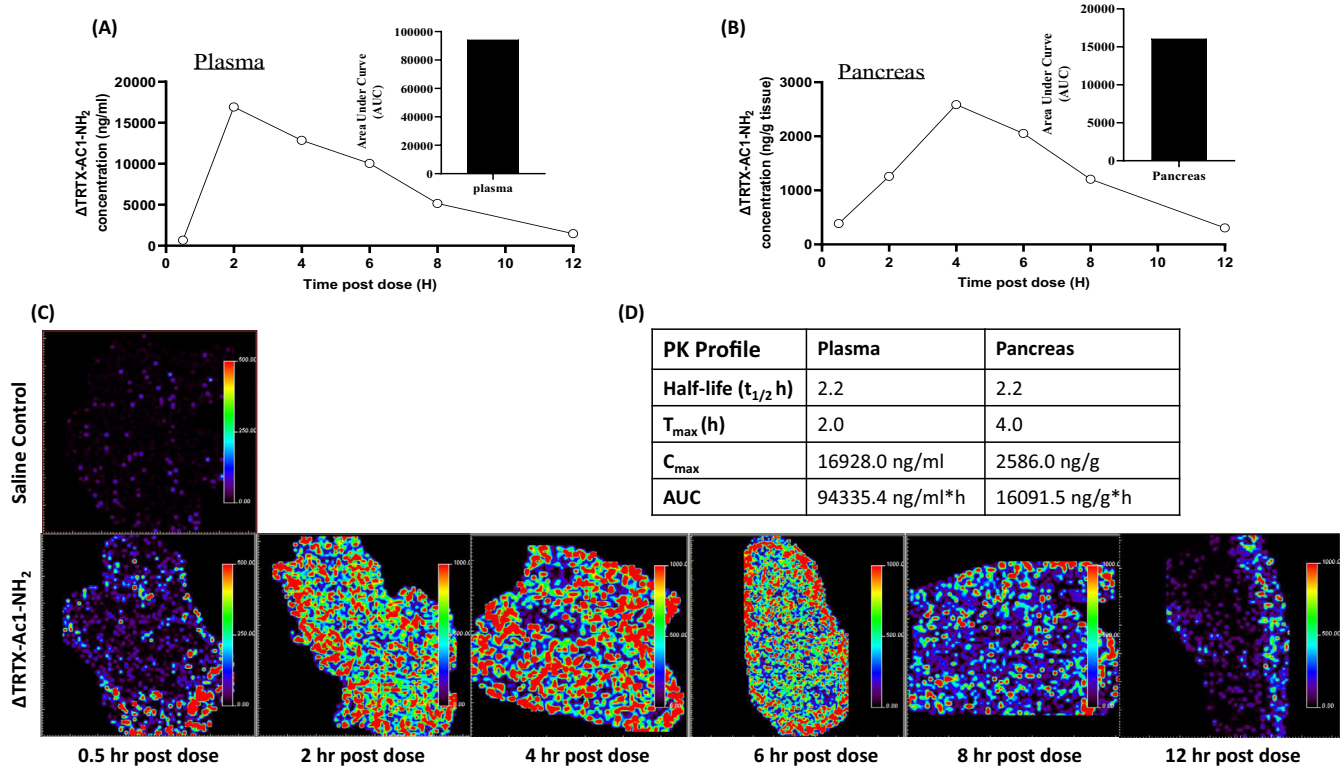
3 | RESULTS

3.1 | Pharmacokinetic profiling of Δ-theraphotoxin-Ac1 in mice

Normal healthy C57BL/6 male mice received a 10 mg/kg i.p. dose of ΔTRTX-Ac1, with the plasma half-life of the peptide calculated to be 2.17 h. Plasma C_{max} was 16 928 ng/g and observed at 2 h post-injection (Figure 1A,D). ΔTRTX-Ac1 was also clearly evidenced within the pancreas (Figure 1B,C), where the peptide half-life was quantified to be 2.16 h. In this setting, the C_{max} was 2586 ng/g tissue and observed at 4 h post-injection (Figure 1D). Pilot MS imaging studies confirmed that ΔTRTX-Ac1 was present within liver and kidney tissue, and passed the blood-brain barrier, following i.p. injection (data not shown).

3.2 | Effects of Δ-theraphotoxin-Ac1 and exenatide alone, or in combination, on food and fluid intake, body weight and circulating glucose in high-fat fed/streptozotocin mice

ΔTRTX-Ac1 and exenatide treatment alone, or in combination, lead to a significant reduction in percentage BW change on day 28 ($p < .05$; Figure 2A). In line with this, all exenatide-treated HFF/STZ mice had reduced percentage body fat when compared with saline control mice ($p < .01$; Figure 2B). Only combination treatment visibly reduced cumulative energy intake, with significant effects on days 6 and 9 when compared with HFF/STZ control mice ($p < .05$-.01; Figure 2C). Fluid intake was not altered in any of the groups of mice (Figure 2D). Saline-treated HFF/STZ mice presented with hyperglycaemia throughout the treatment period, with blood glucose levels of 23.7 ± 3.1 mmol/L on day 28 (Figure 2E). ΔTRTX-Ac1 had minimal influence on circulating glucose, and while exenatide did decrease blood glucose, only combination therapy significantly reduced circulating glucose to levels not different from lean control mice ($p < .05$; Figure 2E). As expected, terminal plasma insulin concentrations were decreased in HFF/STZ mice when compared with lean controls ($p < .05$; Figure 2F). All treatments increased circulating insulin when compared with HFF/STZ control mice, but this failed to reach significance (Figure 2F).



PK Profile	Plasma	Pancreas
Half-life ($t_{1/2}$ h)	2.2	2.2
T_{max} (h)	2.0	4.0
C_{max}	16928.0 ng/ml	2586.0 ng/g
AUC	94335.4 ng/ml*h	16091.5 ng/g*h

FIGURE 1 Plasma and pancreatic PK prolife of ΔTRTX-Ac1 in C57BL/6 mice. Mice were administered ΔTRTX-Ac1 (10 mg/kg body weight, i.p.; n = 1) and killed at time points indicated, with blood and pancreatic tissue extracted and processed for mass spectroscopy analysis. ΔTRTX-Ac1 was detected in the (A) plasma and (B) pancreas with a half-life of 2.17 and 2.16 h, respectively. (C) Representative mass spectroscopy images from pancreatic tissue. (D) Half-life, T_{max}, C_{max} and AUC PK data for ΔTRTX-Ac1 in plasma and pancreas. AUC, area under the curve; PK, pharmacokinetics; ΔTRTX-Ac1, Δ-theraphotoxin-Ac1.

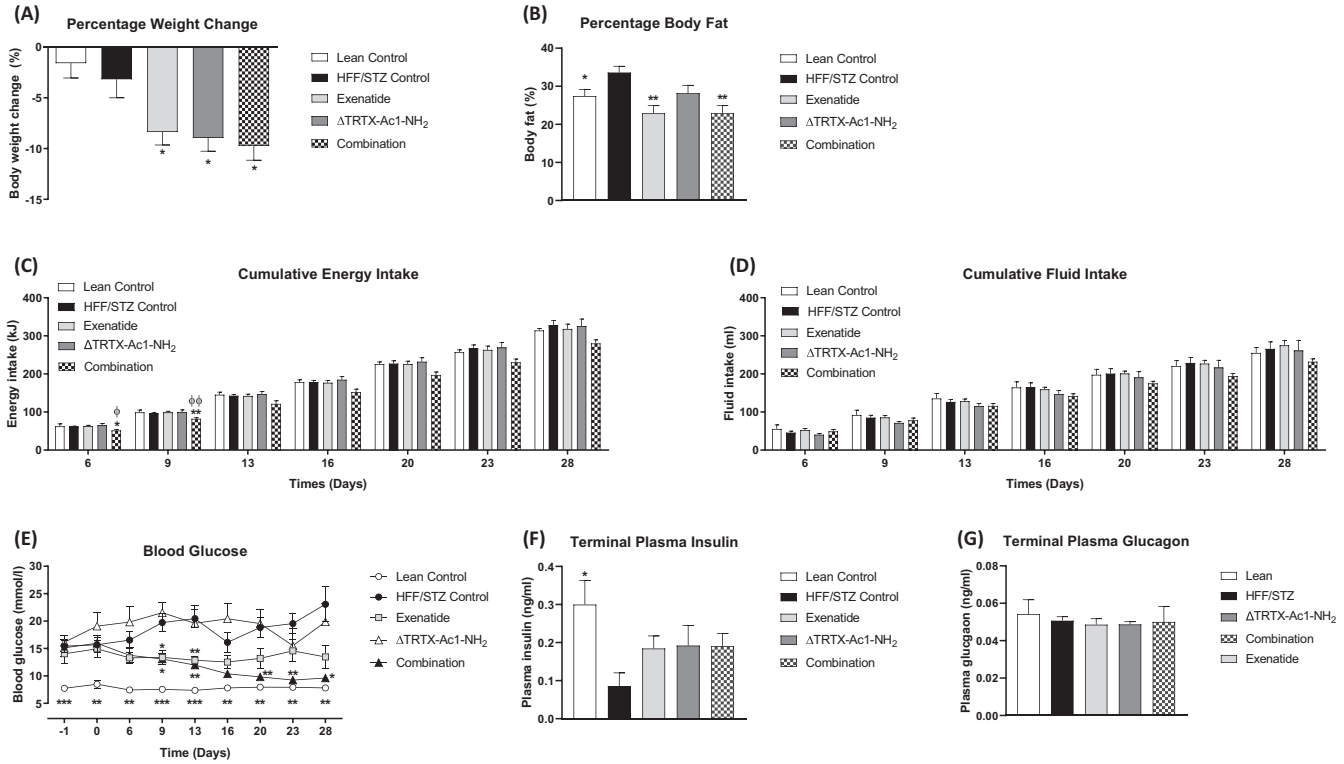


FIGURE 2 Effects of ΔTRTX-Ac1 and exenatide alone, and in combination on (A) percentage body weight change, (B) percentage body fat, cumulative (C) energy and (D) fluid intake, (E) circulating blood glucose, terminal plasma (F) insulin and (G) glucagon in HFF/STZ mice. (C-E) Parameters were measured at regular intervals over 28 days of treatment with twice-daily ΔTRTX-Ac1 (25 nmol/kg body weight, i.p.), exenatide (2.5 nmol/kg body weight, i.p.) or combination of both peptides at the same dose. (A,B,F,G) Parameters were assessed on day 28. Values are mean ± SEM, n = 8 mice/group. *$p < .05$, **$p < .01$, and ***$p < .001$ compared with HFF/STZ control. $^{\pi\pi}p < .01$ compared with ΔTRTX-Ac1 alone. HFF, high-fat fed; STZ, streptozotocin; ΔTRTX-Ac1, Δ-theraphotoxin-Ac1.

Plasma glucagon concentrations were not different between all groups of mice on day 28 (Figure 2G).

3.3 | Effects of Δ-theraphotoxin-Ac1 and exenatide alone, or in combination, on glucose tolerance, insulin sensitivity and pancreatic hormone content

Following a glucose challenge on day 28, treatment with ΔTRTX-Ac1 alone or in combination with exenatide reduced glucose both in terms of individual ($p < .05$-.001; Figure 3A) and 0-120 min AUC ($p < .01$-.001; Figure 3B) values. Exenatide treatment alone had no significant impact on glucose homeostasis, but values were visibly reduced when compared with HFF/STZ control mice (Figure 3A,B). Interestingly, combination treatment resulted in more effective glucose-lowering actions when compared with either treatment alone ($p < .05$; Figure 3B). Insulin sensitivity was unaffected by treatment interventions (Figure 3C,D), albeit ΔTRTX-Ac1-treated mice displayed greater overall effect (Figure 3D) probably because of higher starting blood glucose levels in these mice (Figure 3C). Pancreatic insulin content was reduced in HFF/STZ mice, with all treatments restoring insulin content to lean control levels ($p < .05$; Figure 3E). Pancreatic glucagon content was decreased by both ΔTRTX-Ac1 and exenatide treatment alone, when compared with HFF/STZ control mice ($p < .01$; Figure 3F), but notably combination therapy augmented pancreatic glucagon concentrations when compared with either of the individual treatment regimens ($p < .001$; Figure 3F).

3.4 | Effects of Δ-theraphotoxin-Ac1 and exenatide alone, or in combination, on energy expenditure, respiratory exchange ratio and locomotor activity

HFF/STZ mice presented with increased energy expenditure during both the light and dark phases when compared with lean controls ($p < .05$; Figure 4A-D). Treatment with ΔTRTX-Ac1 alone was able to reverse this effect fully (Figure 4A-D), with all other treatment interventions having no significant effect on energy expenditure (Figure 4A-D). The respiratory exchange ratio was not different between all groups of mice during the light phase (Figure 4E,F). However, in the dark phase mice treated with a combination of ΔTRTX-Ac1 and exenatide had an increased respiratory exchange ratio ($p < .05$; Figure 4G,H). HFF/STZ mice were consistently less active than lean controls in terms X-beam ambulatory activity line breaks

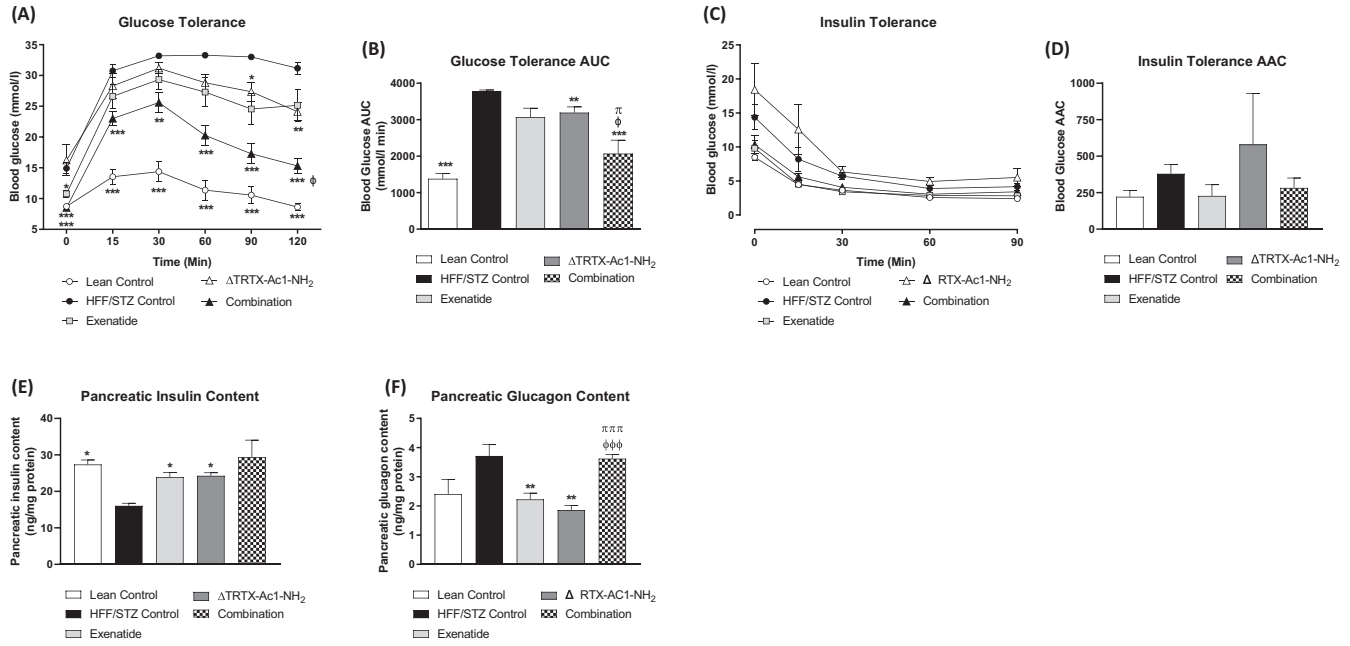


FIGURE 3 Effects of ΔTRTX-Ac1 and exenatide alone, and in combination on (A,B) glucose tolerance and (C,D) insulin sensitivity as well as (E,F) pancreatic insulin and glucagon content in HFF/STZ mice. Parameters were measured after the 28-day twice-daily treatment with ΔTRTX-Ac1 (25 nmol/kg BW, i.p.), exenatide (2.5 nmol/kg BW, i.p.) or combination of both peptides at the same dose. (A) Blood glucose was measured before and at regular intervals after glucose (18 mmol/kg, i.p.) administration at $t = 0$ min, with associated 0-120 min AUC values (B) also shown. (C) Blood glucose was measured prior to and at regular intervals after insulin (5 U/kg BW, i.p.) administration at $t = 0$ min, with associated 0-90 min AAC values (D) also shown. (E,F) Pancreatic insulin and glucagon content was quantified by radioimmunoassay or enzyme-linked immunosorbent assay respectively, following protein extraction. Values are mean ± SEM, n = 8 mice/group. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ compared with HFF/STZ control. $^{\Phi}p < .05$ and $^{\Phi\Phi\Phi}p < .001$ compared with exenatide alone. $^{\pi}p < .05$ and $^{\pi\pi\pi}p < .001$ compared with ΔTRTX-Ac1 alone. AAC, area above the curve; AUC, area under the curve; BW, body weight; HFF, high-fat fed; STZ, streptozotocin; ΔTRTX-Ac1, Δ-theraphotoxin-Ac1.

during both the light and dark phases ($p < .05$-$.001$; Figure 4I). The individual treatment intervention had no impact on this aspect of locomotor activity, but combination peptide therapy resulted in increased X-beam breaks ($p < .05$-$.001$; Figure 4I).

3.5 | Effects of Δ-theraphotoxin-Ac1 and exenatide alone, or in combination, on pancreatic islet morphology and beta-cell proliferation

HFF/STZ mice exhibited reduced ($p < .05$; Figure 5A,B) islet and beta-cell areas alongside a significant expansion ($p < .01$; Figure 5C) of alpha-cell area. All treatment interventions increased islet and beta-cell areas ($p < .05$-$.01$; Figure 5A,B), restoring levels to that of lean control mice (Figure 5A,B). The alpha-cell area was not different between saline control and all peptide-treated HFF/STZ mice (Figure 5C). In terms of alpha/beta-cell ratios, treatment with exenatide alone, or in combination with ΔTRTX-Ac1, returned this parameter towards lean control levels being significantly decreased when compared with saline or ΔTRTX-Ac1-treated HFF/STZ mice ($p < .05$-$.01$; Figure 5D). Islet number per mm^2 pancreatic tissue was decreased in HFF/STZ mice when compared with lean controls ($p < .05$; Figure 5E), with combination, but not individual, therapeutic intervention significantly augmenting this parameter ($p < .01$; Figure 5F). Interestingly, while HFF/STZ mice had similar beta-cell proliferative rates as lean controls, all peptide treatment groups had increased levels of beta-cell proliferation when compared with HFF/STZ control mice ($p < .05$; Figure 5F). Representative islet images showing insulin, glucagon and DAPI or insulin, Ki-67 and DAPI staining for each treatment group are shown in Figure 5G.

4 | DISCUSSION

Prominent glucose-lowering and appetite suppressive actions of ΔTRTX-Ac1, a novel peptide originally isolated from venom of the *A. chalcodes* tarantula, have recently been described.[9] These initial studies also showed additive beneficial effects following co-administration of ΔTRTX-Ac1 with exenatide in the acute in vivo setting.[9] Given the differing modes of action of these peptides, alongside the clinical need to improve antidiabetic efficacy and tolerability of currently approved GLP-1 mimetics,[21] we investigated the impact of individual and combined twice-daily treatment with ΔTRTX-Ac1 and exenatide for 28-days in HFF/STZ mice that represent a model of insulin resistance alongside compromised beta-cell function that is also seen in human T2DM.

Before examination of this experimental concept, in vivo plasma and pancreatic half-life of ΔTRTX-Ac1 was established.

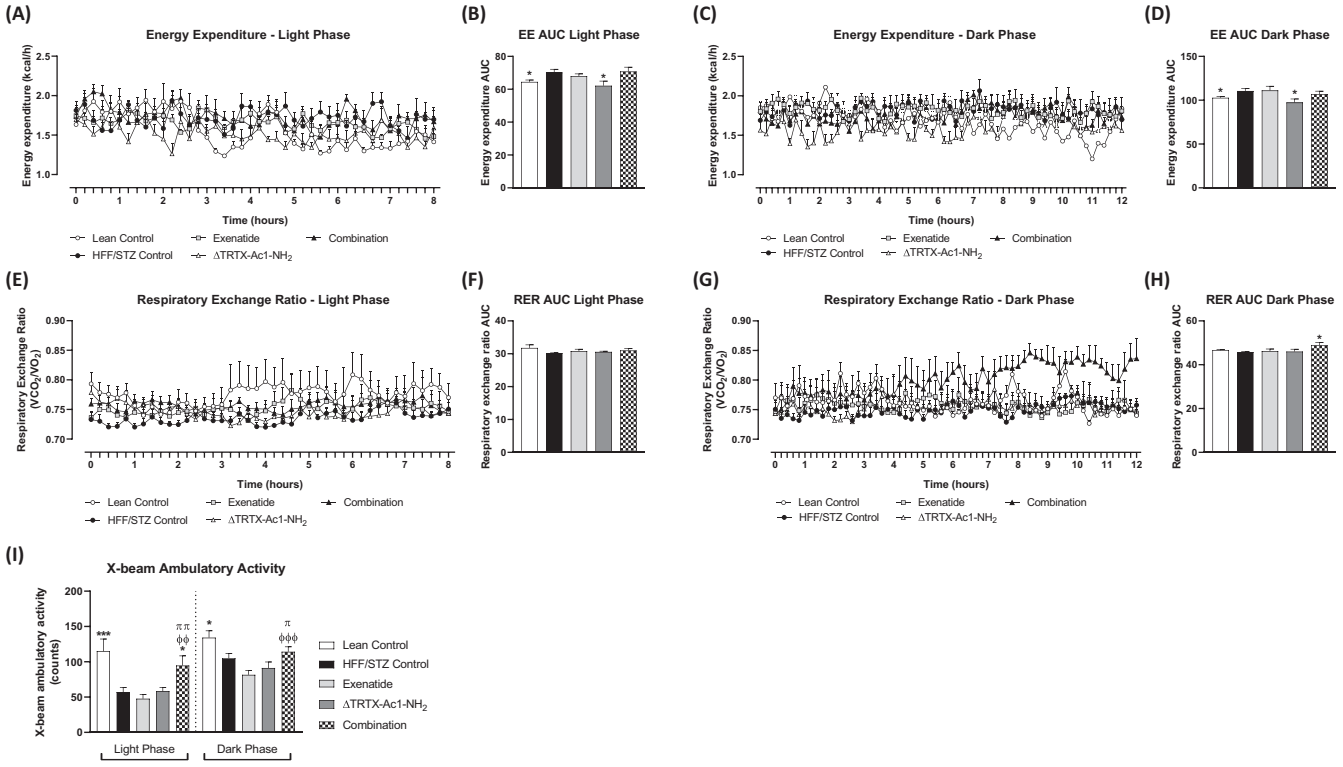


FIGURE 4 Effects of ΔTRTX-Ac1 and exenatide alone, and in combination on (A-D) EE, (E-H) RER as well as (I) locomotor activity in HFF/STZ mice. Parameters were measured after 28 days of treatment twice-daily with ΔTRTX-Ac1 (25 nmol/kg body weight, i.p.), exenatide (2.5 nmol/kg body weight, i.p.) or combination of both peptides at the same dose. EE, RER and x-beam ambulatory activity were assessed during both the (A,B,E,F,I) light and (C,D,G,H,I) dark phases using the CLAMS apparatus. Values are mean ± SEM, n = 8 mice/group. $^{*}p < .05$ and $^{***}p < .001$ compared with HFF/STZ control. $^{\Phi\Phi}p < .01$ and $^{\Phi\Phi\Phi}p < .001$ compared with exenatide alone. $^{\pi}p < .05$ and $^{\pi\pi}p < .001$ compared with ΔTRTX-Ac1 alone. AUC, area under the curve; EE, energy expenditure; HFF, high-fat fed; RER, respiratory exchange ratio; STZ, streptozotocin; ΔTRTX-Ac1, Δ-theraphotoxin-Ac1.

Comparable with exenatide,[22] ΔTRTX-Ac1 had a half-life of approximately 2 h in the bloodstream. The peptide was also detected in peripheral tissues, including the pancreas, following injection and shown to cross the blood-brain barrier. In that respect, the previously noted impact of ΔTRTX-Ac1 on appetite regulation may be centrally mediated.[9] In this regard, it would be interesting to assess c-Fos expression in brain regions linked to energy regulation following peripheral administration of ΔTRTX-Ac1. However, as with GLP-1 receptor activation,[6] the effects of ΔTRTX-Ac1 on peripheral tissues could also be linked to observed benefits on energy homeostasis and overall metabolism. Furthermore, we and others have previously shown that exenatide retains good bioactivity when injected at a dose of 2.5 nmol/kg in mice.[23] For ΔTRTX-Ac1, although the half-life is comparable with exenatide,[21] our previous investigations suggest that a dose of at least 25 nmol/kg is required to elicit bioactivity,[9] suggesting different therapeutic potencies of the two peptides despite their similar PK profiles. Thus, for preclinical testing we opted for a reduced dose of exenatide when compared with ΔTRTX-Ac1. Indeed, this may be more clinically relevant given the recent focus on treatment regimens that can reduce the dose of GLP-1 mimetic used, while retaining or even enhancing therapeutic efficacy, to help improve overall tolerability and patient compliance.[24] Endorsement of the choice of dose for exenatide was provided by our observed prominent efficacy of this regimen in HFF/STZ mice. Moreover, to add further to the translatability of findings, HFF/STZ mice were employed as a model of sustained high calorific dietary intake and insulin resistance together with pancreatic beta-cell secretory deficit, resembling the main pathophysiological characteristics of human T2DM.[14]

In keeping with the glucose homeostatic effects of a single injection of ΔTRTX-Ac1,[9] the peptide was able to improve glucose tolerance following a sustained 28-day twice-daily injection regimen in HFF/STZ mice. This not only corroborates bioactivity of ΔTRTX-Ac1 in both normal and diabetic rodents, but also suggests that desensitization to the benefits of ΔTRTX-Ac1 is not a concern. Although tachyphylaxis has been suggested as a possible limiting factor for GLP-1 drugs,[25] this now seems unlikely and methods to avoid that potential phenomenon are also well described.[26,27] Thus, there can be optimism in terms of persistent beneficial metabolic effects of combined administration of ΔTRTX-Ac1 with exenatide. Moreover, in the current study, combination peptide therapy resulted in notable additive improvements on glucose handling beyond that of either treatment alone, reinforcing this indication. Furthermore, while ΔTRTX-Ac1 had no impact on circulating glucose levels and exenatide decreased this parameter only on selected observation days, the combined treatment

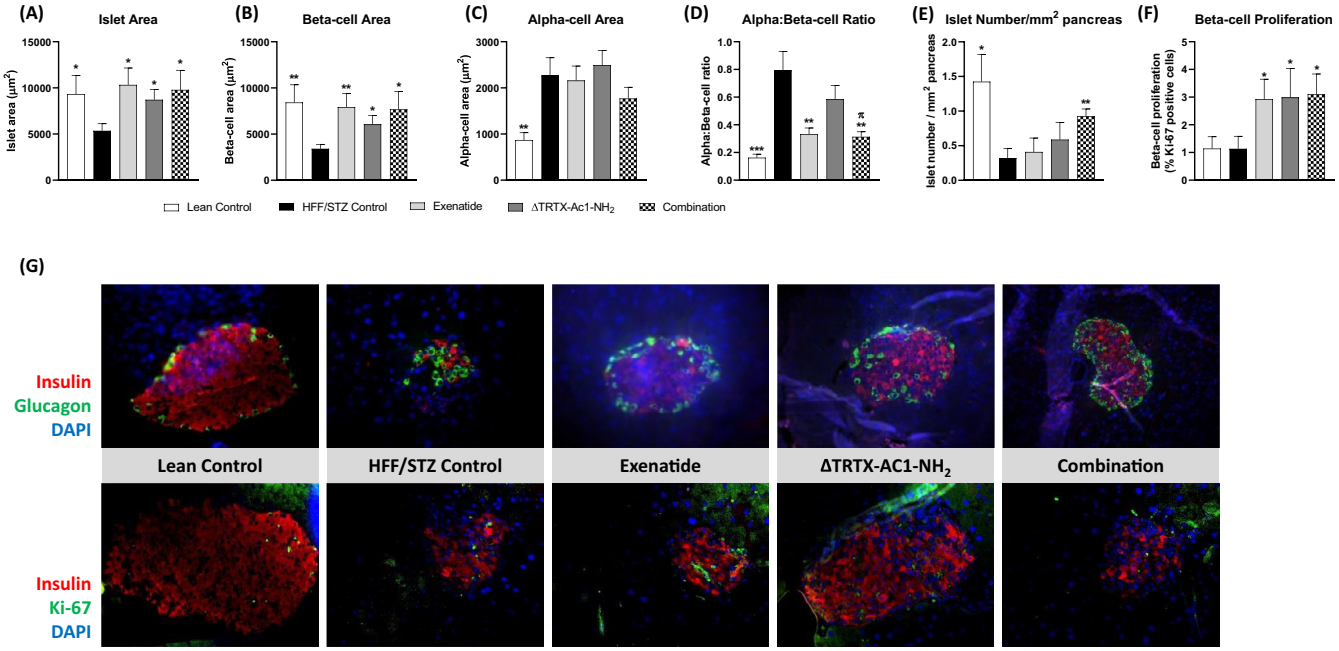


FIGURE 5 Effects of ΔTRTX-Ac1 and exenatide alone, and in combination on (A) islet area, (B) beta-cell area, (C) alpha-cell area, (D) alpha/beta-cell ratio, (E) number of islets per mm^2 and (F) beta-cell proliferation in HFF/STZ mice. Parameters were measured after the 28-day twice-daily treatment with ΔTRTX-Ac1 (25 nmol/kg body weight, i.p.), exenatide (2.5 nmol/kg body weight, i.p.) or combination of both peptides at the same dose. (A-E) Islet morphology was assessed using ImageJ software with (F) beta-cell proliferation measured by Ki-67 staining. (G) Representative islet images showing insulin, glucagon and DAPI or insulin, Ki-67 and DAPI staining are also shown. Values are mean ± SEM, n = 8 mice/group with >60 islets analysed/group. $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ compared with HFF/STZ control. $^{\pi}p < .05$ compared with ΔTRTX-Ac1 alone. DAPI, 4′, 6-diamidino-2-phenylindole; HFF, high-fat fed; STZ, streptozotocin; ΔTRTX-Ac1, Δ-theraphotoxin-Ac1.

approach was the most effective regimen. All HFF/STZ mice presented with some weight loss over the 28-day period probably because of STZ-induced insulin deficiency. Peptide interventions encouraged additional weight loss, and exenatide-treated mice had reduced body fat content in keeping with the anti-obesity actions of GLP-1 receptor activation.[28] Combined therapy led to reduced energy intake, in line with previously observed satiety effects of ΔTRTX-Ac1 and exenatide.[9,29] In that respect, we are unable to rule out entirely the elevated GIT symptoms with combined therapy, but this may seem unlikely as we did not observe any discernible changes of behaviour in these mice.

Indirect calorimetry was used to explore the impact of the separate treatment regimens on whole body metabolism. In keeping with advantages of combined therapy, a respiratory exchange ratio was augmented in these mice, suggesting increased carbohydrate utilization.[30] That said, combination treatment with ΔTRTX-Ac1 and exenatide also significantly increased locomotor activity, which could also be linked to increases of the respiratory exchange ratio.[31] Changes in locomotor activity and energy expenditure in rodents following exenatide injection have been reported previously,[31,32] but were not evident in our study possibly linked to differences in model and dosing regimens employed. The explanation for increased locomotor activity following combined ΔTRTX-Ac1 and exenatide administration is unknown, but does again highlight clear interactions between the two peptides. In this respect, the postulated cellular target for ΔTRTX-Ac1 is the Kv2.1 potassium channel,[9] that has previously been shown to augment GLP-1-mediated biological actions.[33] Thus, while we are unable to delineate fully the precise molecular interaction of ΔTRTX-Ac1, it may be that appropriate modulation of beta-cell Kv2.1 potassium channels by compounds such as ΔTRTX-Ac1 can enhance the metabolic benefits of GLP-1. However, there is also evidence that GLP-1 receptor activation can independently inhibit Kv2.1 channel activity,[34,35] which may question this hypothesis. Nonetheless, positive allosteric interactions of exenatide and ΔTRTX-Ac1 at Kv2.1 channels are also possible, which would allow for additive or synergistic effects of these peptides at the level of the beta-cell.[33] Ultimately, electrophysiology experiments would be required to confirm that theory. Despite this, given that the efficacy and adherence to GLP-1 mimetics in the clinic is often limited by dose-dependent GIT side-effects, alongside current issues around manufacture and supply of this class of drugs,[7] our findings are highly encouraging as a means of reducing GLP-1 dose requirements in humans. Interestingly, ΔTRTX-Ac1 also decreased energy expenditure in HFF/STZ mice, which is also difficult to rationalize given reduced BW but lack of significant change in energy intake, respiratory exchange ratio or ambulatory activity levels in these mice. As such, further studies are required to uncover underlying mechanisms, but it is clear that ΔTRTX-Ac1 exerts overall positive effects on metabolism that warrant consideration. Notably this effect on

energy expenditure was reversed by co-administration with exenatide, further promoting benefits of combination therapy.

Both ΔTRTX-Ac1 and exenatide have established direct positive effects on pancreatic beta-cell health,[9,36] prompting our more detailed analysis of islet histology in HFF/STZ mice. In the current study, 28-day twice-daily treatment with either peptide enhanced islet and beta-cell areas, reversing the detrimental impact of STZ on islet morphology.[37] In agreement with previous observations, this appeared to be linked, at least in part, to augmented beta-cell proliferative rates.[9,19] Moreover, pancreatic insulin content was also restored to normal levels by both ΔTRTX-Ac1 and exenatide. However, there was an apparent lack of additive benefits of combined therapy at the level of the beta-cell, which although a little surprising, may simply reflect the highly regulated nature within the local environment of pancreatic islets.[38] This perhaps helps to explain further the prominent benefits on glucose tolerance in the combined treatment group. In addition, combination therapy with ΔTRTX-Ac1 and exenatide resulted in augmented numbers of islet per mm^2 pancreatic tissue, which was not observed with either treatment alone. That said, there was no benefit of combined treatment on peripheral insulin action, but this may simply reflect the lower blood glucose levels in this group of mice before insulin administration and innate negative feedback pathways to prevent hypoglycaemia. Thus, euglycaemic-hyperinsulinaemic clamp studies may be required to uncover fully the treatment effects on insulin action. The alpha-cell area was unaltered in all HFF/STZ treatment groups of mice, despite there being markedly elevated circulating glucagon concentrations with combined, but not individual, treatments. To date there has been no investigation into the potential impact of ΔTRTX-Ac1 on alpha-cell secretory function, whereas it is well established the GLP-1 receptor activation inhibits glucagon release.[39] The interaction between the two peptides on alpha-cell function therefore necessitates further mechanistic insight, which is unfortunately outside the scope of the current study.

Collectively, these data confirm antidiabetic benefits of sustained administration of the tarantula venom-derived peptide, ΔTRTX-Ac1, in a rodent model of insulin resistance and beta-cell secretory dysfunction. While some bioactive venom-derived peptides fail to progress beyond preclinical evaluation because of safety and/or specificity issues,[40,41] it is encouraging to note that no adverse effects were observed following administration of ΔTRTX-Ac1 alone, or in combination with exenatide, in diabetic mice. As such, lack of malaise following sustained peptide injection in HFF/STZ mice suggests that reductions of BW are not linked to toxicity or a detrimental impact on behaviour. Prominently, benefits of ΔTRTX-Ac1 were augmented by concurrent treatment with the clinically approved GLP-1 mimetic exenatide, particularly in terms of overall glucose homeostasis, highlighting a particularly attractive therapeutic strategy for future consideration.

AUTHOR CONTRIBUTIONS

NI, VAG and SMcC conceived/designed the study. NI and AC-P drafted the manuscript. AC-P, DC, CMMcL and NT participated in the conduct/data collection and analysis and interpretation of data. All authors revised the manuscript critically for intellectual content and approved the final version of the manuscript.

ACKNOWLEDGMENTS

These studies were supported by a Diabetes UK funded PhD studentship (ACP) and Ulster University Research Funding support.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/dom.15319.

DATA AVAILABILITY STATEMENT

The authors declare that the data supporting the findings of this study are available within the article. Any additional raw data supporting the conclusions of this article will be made available by the lead author (ACP), without undue reservation.

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How to cite this article: Coulter-Parkhill A, Tanday N, Cobice D, et al. Sustained metabolic benefits of ΔTRTX-Ac1, a tarantula venom-derived peptide, when administered together with exenatide in high-fat fed mice. *Diabetes Obes Metab*. 2023;1-10. doi:10.1111/dom.15319