



## 1 **Abstract**

2 Neurotensin and xenin possess antidiabetic potential, mediated in part through augmentation  
3 of incretin hormone, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic  
4 polypeptide (GIP), action. In this study, fragment peptides of neurotensin and xenin, acetyl-  
5 neurotensin (8-13) and xenin-8-Gln, were fused together to create Ac-NT/XN-8-Gln.  
6 Following assessment of enzymatic stability, effects of Ac-NT/XN-8-Gln on *in vitro* beta-cell  
7 function were studied. Sub-chronic antidiabetic efficacy of Ac-NT/XN-8-Gln alone, and in  
8 combination with the clinically approved GLP-1 receptor agonist exendin-4, was assessed in  
9 high fat fed (HFF) mice. Ac-NT/XN-8-Gln was highly resistant to plasma enzyme degradation  
10 and induced dose-dependent insulin-releasing actions ( $p<0.05$  to  $p<0.01$ ) in BRIN-BD11 beta-  
11 cells and isolated mouse islets. Ac-NT/XN-8-Gln augmented ( $p<0.001$ ) the insulintropic  
12 actions of GIP, while possessing independent beta-cell proliferative ( $p<0.001$ ) and anti-  
13 apoptotic ( $p<0.01$ ) actions. Twice daily treatment of HFF mice with Ac-NT/XN-8-Gln for 32  
14 days improved glycaemic control and circulating insulin, with benefits significantly enhanced  
15 by combined exendin-4 treatment. This was reflected by reduced body fat mass ( $p<0.001$ ),  
16 improved circulating lipid profile ( $p<0.01$ ) and reduced HbA1c concentrations ( $p<0.01$ ) in the  
17 combined treatment group. Following an oral glucose challenge, glucose levels were markedly  
18 decreased ( $p<0.05$ ) only in combination treatment group and superior to exendin-4 alone, with  
19 similar observations made in response to glucose plus GIP injection. The combined treatment  
20 group also presented with improved insulin sensitivity, decreased pancreatic insulin content as  
21 well as increased islet and beta-cell areas. These data reveal that Ac-NT/XN-8-Gln is a  
22 biologically active neurotensin/xenin fusion peptide that displays prominent antidiabetic  
23 efficacy when administered together with exendin-4.

24

25

## 1 **Introduction**

2 Neurotensin and xenin are regulatory peptides that share amino acid sequence homology,  
3 activate similar receptors and possess some notable parallels in their bioactivity profiles (1,2).  
4 Both hormones stimulate appetite suppression (2-4) as well as modulate insulin secretion and  
5 glucose homeostasis (3,5). Positive effects of neurotensin and xenin have been observed also  
6 on pancreatic beta-cell growth and survival (4,6), together with prominent effects on metabolic  
7 regulation. In this regard, there is good evidence that neurotensin is co-expressed and co-  
8 released with the incretin hormone glucagon-like peptide-1 (GLP-1) from intestinal L-cells (4),  
9 whilst xenin is similarly secreted alongside glucose-dependent insulintropic polypeptide  
10 (GIP) from enteroendocrine K-cells (2). However, it should also be noted that neurotensin is  
11 also synthesised and secreted by enteroendocrine N-cells (7), suggesting that secretion of GLP-  
12 1 and NT are not fully paralleled *in vivo*. Nonetheless, neurotensin has recently been shown to  
13 augment the biological actions of GLP-1 (8), while xenin is well known to potentiate GIP  
14 mediated bioactivity (9-11). Thus, together neurotensin and xenin have the capacity to  
15 significantly enhance the biological actions of the incretin hormones. This is of particular  
16 interest in terms of the treatment of type 2 diabetes, given that a number of clinically approved  
17 antidiabetic therapeutics directly modulate the incretin system (12), with other agents believed  
18 to exert some of their benefits through this pathway (13,14).

19 Interestingly, the C-terminal portions of both neurotensin and xenin have been shown  
20 to retain similar bioactivity as their respective parent peptides (15,16). This would suggest that  
21 shorter neurotensin and xenin peptides which are easier to synthesise and formulate, may have  
22 therapeutic promise for diabetes. One potential hindrance to the use of such truncated  
23 neurotensin or xenin peptides relates to their relatively brief biological half-lives (16,17).  
24 However, enzymatic stability and subsequent prolongation of bioactivity, can be imparted  
25 through appropriate structural modification of these C-terminal truncated peptides (15,18). As

1 such, acetyl-neurotensin(8-13) and xenin-8-Gln represent fully characterised, enzymatic  
2 resistant, bioactive neurotensin and xenin peptide analogues (19,20). To increase therapeutic  
3 applicability of gut hormone derived peptides, generation of hybrid unimolecular peptides has  
4 been employed of late, through merging of the key bioactive amino acid sequences of the parent  
5 peptides (21-23). Based on this knowledge, we have constructed a novel neurotensin/xenin  
6 hybrid peptide, namely Ac-NT/XN-8-Gln, by fusing together the amino acid sequences of  
7 acetyl-neurotensin(8-13) and xenin-8-Gln. The xenin component of the fusion peptide was  
8 positioned at the C-terminus, as this has been employed with good success for other xenin  
9 hybrid peptides (24-26).

10 In the current study, we initially examined plasma stability of Ac-NT/XN-8-Gln  
11 followed by assessment of *in vitro* insulinotropic actions alone, and in combination with GIP  
12 or GLP-1. In addition, the impact of Ac-NT/XN-8-Gln on beta-cell proliferation and protection  
13 against apoptosis was also investigated in BRIN-BD11 beta-cells. Following confirmation of  
14 intact bioactivity of Ac-NT/XN-8-Gln, beneficial metabolic effects of twice daily  
15 administration alone, or in combination with exendin-4, were evaluated in high fat fed (HFF)  
16 mice.

17

## 18 **Materials and Methods**

### 19 **Peptides**

20 All peptides (Table 1) were purchased from Syn Peptide (Shanghai, China) at greater than 95%  
21 purity. In-house confirmation of peptide purity and molecular weight was carried out by RP-  
22 HPLC and MALDI-TOF MS, as previously described (27).

23

### 24 **Effects of peptides on *in vitro* insulin secretion**

1 The *in vitro* insulin secretory activity of test peptides was examined in BRIN-BD11 cells,  
2 cultured and maintained as previously described (28). For experimentation, BRIN-BD11 cells  
3 were seeded in 24-well plates at a cell density of 150,000 cells/well, and allowed to attach  
4 overnight at 37°C. Following pre-incubation with Krebs–Ringer bicarbonate buffer (KRBB)  
5 (pH 7.4) supplemented with 0.5% (w/v) BSA and 1.1 mM glucose (40 min; 37 °C), cells were  
6 then incubated with test peptides alone ( $10^{-6}$  –  $10^{-12}$  M), or in combination with either GIP or  
7 GLP-1 ( $10^{-7}$  M), at 5.6 or 16.7 mM glucose, as appropriate for 20 minutes. Relatively high  
8 concentrations of GIP and GLP-1 were employed for *in vitro* studies and these hormones have  
9 been shown to be synthesised and secreted locally within islets (29), with our previous studies  
10 confirming that augmentation of the insulinotropic actions of  $10^{-7}$  M GIP or GLP-1 is  
11 achievable (30,31). Aliquots of assay buffer (200 µl) were collected and stored at -20 °C prior  
12 to assessment of insulin concentrations by an in-house radioimmunoassay (32). To confirm  
13 insulin secretory activity of Ac-NT/XN-8-Gln, pancreatic islets were isolated from lean  
14 C57BL/6 mice by collagenase digestion, as described previously (33) and insulin secretion  
15 determined as outlined above (16.7 mM glucose), but over a 60 min test incubation period.

### 17 **Effects of peptides on *in vitro* beta-cell proliferation and protection against apoptosis**

18 BRIN-BD11 beta-cells were used to investigate effects of test peptides ( $10^{-8}$  and  $10^{-6}$  M) on  
19 beta-cell proliferation and protection against apoptosis. GLP-1 was employed as a positive  
20 control for all studies. Ki-67 immunostaining was used to assess effects on proliferation.  
21 Briefly, cells were seeded onto coverslips (40,000 cells per coverslip) and cultured overnight  
22 (18 h; 37 °C), in the presence of test peptides ( $10^{-8}$  and  $10^{-6}$  M). Cells were then washed with  
23 PBS, and fixed using 4% paraformaldehyde. Following antigen retrieval with citrate buffer  
24 (90°C for 20 min), tissues were blocked using 1.1% BSA for 30 min. Cells were then incubated  
25 with Ki-67 primary antibody (1:500; Abcam, ab15580), followed by Alexa Fluor® 488

1 secondary antibody (1:400, Invitrogen, A-11008). Coverslips were washed with PBS, mounted  
2 on slides for viewing using a fluorescent microscope (Olympus System Microscope) and  
3 photographed by DP70 camera adapter system. Proliferation frequency was expressed as  
4 percentage of total cells analysed. For analysis of the ability of test peptides ( $10^{-8}$  and  $10^{-6}$  M)  
5 to protect against apoptosis, cells were seeded as above, but apoptosis evoked through  
6 activation of caspase 3 and 7 activity by a luminogenic, tetrapeptide sequence DEVD Caspase-  
7 Glo® 3/7 substrate. Caspase induced cleavage of the substrate was then assessed by an  
8 ApoLive-Glow™ Multiplex assay kit, according to the protocol provided by the manufacturer  
9 (Promega, Wisconsin, USA).

10

## 11 **Animals**

12 Studies were carried out using male NIH Swiss mice (12-14 weeks of age, Envigo Ltd, UK),  
13 housed individually in an air-conditioned room at  $22 \pm 2$  °C with a 12 h light:12 h dark cycle.  
14 Prior to experiments, animals were maintained on a high-fat diet (45% fat, 35% carbohydrate  
15 and 20% protein, Special Diet Services, UK, with percent of total energy of 26.15 kJ/g) for 10  
16 weeks. This diet resulted in progressive body weight gain and hyperglycaemia compared to  
17 control mice. Lean control animals were maintained on a standard rodent chow diet (10% fat,  
18 30% protein and 60% carbohydrate, Trouw Nutrition, UK, with percent of total energy of 12.99  
19 kJ/g). All mice had *ad libitum* access to water and respective diets. Experiments were carried  
20 out in accordance with the UK Animal Scientific Procedures Act 1986 and approved by the  
21 University of Ulster Animal Welfare and Ethical Review Body (AWERB) under the UK Home  
22 Office project licence 2804. All animal studies were conducted in the Biomedical and  
23 Behavioural Research Unit (BBRU) at Ulster University, Coleraine.

24

## 25 **Sub-chronic studies**

1 Twice daily i.p. injections of saline vehicle (0.9% w/v NaCl), exendin-4, Ac-NT/XN-8-Gln or  
2 a combination of both peptides (each peptide at 25 nmol/kg bw) were administered at 09:30 h  
3 and 17:30 h for 32-days in HFF mice. Cumulative food intake, body weight, non-fasting  
4 glucose and insulin concentrations were monitored at regular intervals. HbA1c concentrations  
5 were measured on day 32. In addition, at the end of the treatment period, oral glucose tolerance  
6 (18 mmol/kg bw; p.o.), biological response to GIP (18 mmol/kg glucose in combination with  
7 native GIP (25 nmol/kg); i.p.) and insulin sensitivity (15 U/kg bw; i.p.) tests were performed.  
8 All test solutions were administered in a final volume of 5 ml/kg body weight.

9

### 10 **Terminal analyses**

11 All animals were euthanised via CO<sub>2</sub> gas inhalation and cervical dislocation. Percentage body  
12 fat mass was measured at study termination by DEXA scanning (PIXImus Densitometer; Inside  
13 Outside Sales, Fitchburg, WI, USA). Pancreatic insulin content was measured following  
14 hormone extraction (1.5% HCl, 75% ethanol and 23.5% H<sub>2</sub>O), as described previously (34).  
15 For histological analyses, tissue was excised, immediately fixed using 4% paraformaldehyde  
16 and embedded in paraffin wax. Tissue sections were then deparaffinised, rehydrated and  
17 probed with primary antibodies, namely mouse anti-insulin antibody (1:500; Abcam, ab6995)  
18 and guinea-pig anti-glucagon antibody (PCA2/4, 1:400; raised in-house). Sections were  
19 incubated with secondary antibodies, Alexa Fluor 594 goat anti-mouse IgG (1:400) and Alexa  
20 Fluor 488 goat anti-guinea pig IgG (1:400). The slides were viewed under a FITC (488 nm) or  
21 TRITC filter (594 nm) using a fluorescent microscope (Olympus system microscope, model  
22 BX51) and photographed using a DP70 camera adapter system. Islet parameters were analysed  
23 using Cell<sup>F</sup> image analysis software (Olympus Soft Imaging Solutions, GmbH).

24

### 25 **Biochemical analyses**

1 Blood samples were collected from the tail vein of conscious mice into chilled fluoride/heparin  
2 glucose microcentrifuge tubes (Sarstedt, Numbrecht, Germany) at the time points indicated in  
3 the Figures. Blood glucose was measured directly using a handheld Ascensia Contour blood  
4 glucose meter (Bayer Healthcare, Newbury, Berkshire, U.K.). HbA1c concentrations in whole  
5 blood were measured using A1cNow<sup>®</sup> kits (PTS diagnostics, Indiana, USA). Total and LDL-  
6 cholesterol, as well as blood triacylglycerol concentrations, were measured using a Hitachi  
7 Automated Analyser 912 (Boehringer Ingelheim, Mannheim, Germany). Plasma and  
8 pancreatic insulin were assayed by a modified dextran-coated charcoal radioimmunoassay, as  
9 described previously (32).

10

## 11 **Statistical analysis**

12 Statistical analysis was performed using GraphPad PRISM (Version 5). Results are expressed  
13 as means  $\pm$  SEM and data compared using repeated measures ANOVA followed by the  
14 Student-Newman-Keuls *post hoc* test. Unpaired student t-test was used where appropriate.  
15 Area under curve (AUC) and area above curve (AAC) analyses were calculated using  
16 trapezoidal rule with baseline subtraction. Groups of data were significantly different if  $p < 0.05$ .

17

## 18 **Results**

### 19 **Plasma enzyme stability**

20 When incubated together with murine plasma, Ac-NT/XN-8-Gln remained 100% intact up to  
21 and including 4 h, and over 90% peptide intact over an extended 8 h incubation period (Table  
22 1). Native GLP-1 was employed as a positive control in this system, and was fully degraded  
23 by 4 h (data not shown).

24

### 25 **Effects of Ac-NT/XN-8-Gln on insulin release from BRIN-BD11 cells**



1 The insulinotropic actions of Ac-NT/XN-8-Gln, and respective parent peptides, at 5.6 and 16.7  
2 mM glucose were investigated in BRIN-BD11 cells. At 5.6 mM glucose, xenin-8-Gln increased  
3 ( $p < 0.05$  –  $p < 0.001$ ) insulin secretion at concentrations of  $10^{-8}$  M and above, whilst acetyl-  
4 neurotensin(8-13) and Ac-NT/XN-8-Gln evoked a significant ( $p < 0.05$ ) increase of insulin  
5 secretion at  $10^{-6}$  M (Figure 1A). Interestingly, at 16.7 mM glucose only xenin-8-Gln retained  
6 significant insulinotropic actions, whereas acetyl-neurotensin(8-13) inhibited ( $p < 0.05$ )  
7 glucose-stimulated insulin secretion (Figure 1B). Xenin-8-Gln augmented ( $p < 0.05$  –  $p < 0.01$ )  
8 GIP-induced insulin secretion but had no impact on GLP-1 evoked insulin release, with Ac-  
9 NT/XN-8-Gln possessing a similar, but more prominent, bioactivity profile in the presence of  
10 GIP (Figure 1C,D). Interestingly, acetyl-neurotensin(8-13) had no effect on GIP-induced  
11 insulin release and appeared to inhibit ( $p < 0.05$ ) GLP-1 related insulin secretion (Figure 1C,D).  
12 Prominent ( $p < 0.01$ ) insulinotropic actions of Ac-NT/XN-8-Gln were then confirmed in  
13 isolated mouse islets at 16.7 mM glucose (Figure 1E).

14

### 15 **Effects of Ac-NT/XN-8-Gln on beta-cell proliferation and protection against apoptosis**

16 Similar to exendin-4, but with some reduced efficacy, acetyl-neurotensin(8-13) and Ac-  
17 NT/XN-8-Gln significantly ( $p < 0.001$ ) augmented BRIN-BD11 beta-cell proliferation at  
18 concentrations of  $10^{-8}$  and  $10^{-6}$  M (Figure 2A). Interestingly, in the current setting xenin-8-Gln  
19 was devoid of benefits on beta-cell proliferation, with the actions of Ac-NT/XN-8-Gln being  
20 significantly ( $p < 0.05$  –  $p < 0.01$ ) superior to xenin-8-Gln (Figure 2A). In relation to protection  
21 against apoptosis, all test peptides protected against ( $p < 0.01$  -  $p < 0.001$ ) BRIN-BD11 beta-cell  
22 apoptosis at  $10^{-8}$  and  $10^{-6}$  M when compared to control cultures (Figure 2B).

23

1 **Effects of twice-daily administration of Ac-NT/XN-8-Gln and exendin-4 alone, or in**  
2 **combination, on body weight, food intake, glucose, insulin and HbA1c concentrations in**  
3 **HFF mice**

4 Twice-daily administration of exendin-4 alone to HFF mice reduced ( $p < 0.05$  –  $p < 0.01$ )  
5 cumulative energy intake from day 17 onwards when compared to HFF control mice, an effect  
6 that was not observed with any of the other treatment modalities (Figure 3A). Body weight was  
7 significantly decreased ( $p < 0.05$  –  $p < 0.001$ ) in mice receiving exendin-4 alone, or in  
8 combination with Ac-NT/XN-8-Gln (Figure 3B), but there was no obvious benefit of adding  
9 Ac-NT/XN-8-Gln to exendin-4 therapy in terms of overall body weight change (Figure 3B).  
10 Moreover, body fat mass was especially reduced ( $p < 0.001$ ) in HFF mice treated with Ac-  
11 NT/XN-8-Gln in combination with exendin-4 (Figure 3C). Non-fasting blood glucose levels  
12 were declined to levels similar to that of lean controls in HFF mice treated with exendin-4  
13 alone, or in combination with Ac-NT/XN-8-Gln on day 4, and remained at normoglycaemic  
14 levels throughout in these groups of HFF mice (Figure 3D). In full agreement, HbA1c  
15 concentrations on day 32 were identical to lean controls in HFF mice treated with exendin-4  
16 alone, or in combination with Ac-NT/XN-8-Gln (Figure 3E). Similarly, circulating insulin was  
17 elevated ( $p < 0.05$  –  $p < 0.001$ ) only in HFF mice receiving exendin-4 alone, or in combination  
18 with Ac-NT/XN-8-Gln (Figure 3F).

19

20 **Effects of twice-daily administration of Ac-NT/XN-8-Gln and exendin-4 alone, or in**  
21 **combination, on glucose tolerance, metabolic response to GIP, insulin sensitivity and lipid**  
22 **status in HFF mice**

23 Following an oral glucose challenge, individual and 0-120 min AUC glucose levels were  
24 significantly decreased ( $p < 0.05$ ) only in mice treated with Ac-NT/XN-8-Gln in combination  
25 with exendin-4 when compared to HFF saline treated controls, with values similar to lean

1 control mice (Figure 4A,B). In this respect, combination treatment was more effective than  
2 either parent peptide alone. There was a tendency for glucose-induced insulin secretion to be  
3 augmented in all HFF treatment groups, but this failed to reach significance (Figure 4C,D).  
4 Strikingly similar observations were made in response to glucose plus GIP injection, with only  
5 the combination treatment group evoking significant ( $p<0.05$ ) metabolic benefits of GIP  
6 (Figure 4E-H), and superior to Ac-NT/XN-8-Gln or exendin-4 alone. These improvements in  
7 metabolism were associated with significantly improved ( $p<0.05$ ) insulin sensitivity in the  
8 combined treatment group, that was not apparent with either individual peptide treatment  
9 (Figure 5A,B). In keeping with benefits of combined therapy, treatment of Ac-NT/XN-8-Gln  
10 in combination with exendin-4 reduced ( $p<0.05$ ) circulating triacylglycerols (Figure 5C). In  
11 addition, both exendin-4 alone, and in combination with Ac-NT/XN-8-Gln, decreased ( $p<0.01$ )  
12 total-cholesterol concentrations (Figure 5D), with LDL-cholesterol also decreased ( $p<0.01$ ) in  
13 these groups of mice when compared to treatment with Ac-NT/XN-8-Gln alone (Figure 5E).

#### 15 **Effects of twice-daily administration of Ac-NT/XN-8-Gln and exendin-4 alone, or in** 16 **combination, on pancreatic insulin content and islet histology in HFF mice**

17 Treatment with exendin-4 alone and in combination with Ac-NT/XN-8-Gln for 32 days  
18 decreased ( $p<0.001$ ) pancreatic insulin content when compared to HFF control mice, with  
19 levels similar to that of lean controls (Figure 6A). HFF mice had significantly ( $p<0.001$ )  
20 increased islet area compared with lean controls (Figure 6B), that was associated with  
21 characteristic adaptive increases ( $p<0.001$ ) in beta-cell area (Figure 6C). Treatment with  
22 exendin-4 alone, and even more so in combination with Ac-NT/XN-8-Gln, further increased  
23 ( $p<0.05$  –  $p<0.01$ ) islet and beta-cell areas (Figure 6B,C). Interestingly, although there was a  
24 tendency for alpha-cell area to be decreased by each treatment regimen, there were no  
25 significant differences in alpha-cell area between all groups of mice (Figure 6D).

1

2 **Discussion**

3 Original early work demonstrated direct additive benefits of combined activation of GIP and  
4 xenin signalling pathways on pancreatic beta-cell function and metabolism (10), which have  
5 been largely confirmed by others (24,35). More recently, additive, or potentially synergistic,  
6 positive effects of combined GLP-1 and neurotensin signalling have also been evidenced (8).  
7 Indeed, there is also suggestion that xenin can directly affect GLP-1 secretion and action  
8 (25,36), with neurotensin having a potentially comparable effect on GIP activity (37). Taken  
9 together, neurotensin and xenin have the combined ability to modulate and augment the incretin  
10 effect, with obvious therapeutic implications for diabetes. This is even more apparent, given  
11 that highly prominent preclinical benefits of incretin enhancer drugs have not been fully  
12 translated to the clinic. Thus, the present study was designed to incorporate the metabolic  
13 advantages of neurotensin and xenin within a single unimolecular peptide, namely Ac-NT/XN-  
14 8-Gln, with subsequent characterisation of biological activity and assessment of ability to  
15 augment the antidiabetic efficacy of an established incretin mimetic drug.

16 As expected, Ac-NT/XN-8-Gln was highly resistant to enzymatic breakdown, in  
17 keeping with enhanced stability of the parent peptides acetyl-neurotensin(8-13) and xenin-8-  
18 Gln (19,20). We were unable to perform *in vivo* pharmacokinetic analysis of Ac-NT/XN-8 as  
19 the specific assay required for such experiments is currently unavailable. Encouragingly  
20 however, Ac-NT/XN-8-Gln displayed prominent *in vitro* and *ex vivo* insulin secretory actions,  
21 and unlike neurotensin related peptides (38), insulintropic actions were evident under  
22 hyperglycaemic conditions. Thus, xenin peptides have been consistently shown to promote  
23 insulin secretion at both basal and elevated glucose levels (39,40), whereas neurotensin  
24 possesses reduced insulin secretory effectiveness at increased glucose concentrations (41).  
25 Moreover, Ac-NT/XN-8-Gln imparted independent benefits on beta-cell growth and survival

1 as has been demonstrated previously with both native neurotensin and xenin (6,42). Positive  
2 effects on pancreatic beta-cell function and turnover are consistent with local synthesis and  
3 secretion of both hormones within the endocrine pancreas, and related essential physiological  
4 actions (2,4). Thus, immunohistochemical analyses confirm the presence of both neurotensin  
5 and xenin immunoreactivity locally within islets (6). Interestingly, in our experimental system,  
6 we were unable to recapitulate the potentiating effect of neurotensin on GLP-1 bioactivity (4,7),  
7 with acetyl-neurotensin(8-13) and Ac-NT/XN-8-Gln both appearing to perturb GLP-1 induced  
8 insulin secretion. However, in accord with previous observations (20), xenin-8-Gln clearly  
9 augmented GIP stimulated insulin secretion, with Ac-NT/XN-8-Gln fully replicating this  
10 beneficial effect, thus Ac-NT/XN-8-Gln may function more like xenin at the level of the  
11 pancreatic beta-cell. In this regard, the receptor activation profile of Ac-NT/XN-8-Gln requires  
12 further detailed investigation, especially since the exact receptor that xenin signals through has  
13 not been fully established to date (35). Thus, use of commercially available neurotensin  
14 receptor antagonists, or receptor knockout cell lines, may be useful to help uncover the  
15 receptors that are positively modulated by Ac-NT/XN-8-Gln. However, at the level of the  
16 pancreatic beta-cell, it is clear that Ac-NT/XN-8-Gln retains ability to activate similar cell  
17 signaling pathways as the parent peptides, and we assume that this is also true in other tissues  
18 that express neurotensin or xenin receptors (2).

19       Following confirmation of enzymatic stability and preserved bioactivity of Ac-NT/XN-  
20 8-Gln, a chronic 32-day investigation of Ac-NT/XN-8-Gln antidiabetic actions alone, and in  
21 combination with the clinically approved GLP-1 mimetic, exendin-4, was conducted in the  
22 HFF mice. An additional group receiving exendin-4 alone was included as control. Although  
23 neurotensin and xenin are known to exert appetite suppressive actions (4,20,43), similar to  
24 GLP-1 (44), only treatment with exendin-4 alone decreased energy intake in HFF mice in the  
25 current setting. This was associated with clear reductions in body weight, which were also

1 apparent, although to a lesser extent, with combined Ac-NT/XN-8-Gln injection. Indeed,  
2 exendin-4 induced benefits on circulating glucose levels were not improved by  
3 supplementation therapy with Ac-NT/XN-8-Gln. More interestingly however, the most  
4 prominent reduction of body fat content was noted in the combined treatment group, suggesting  
5 important benefits on lipid metabolism and body composition by co-activation of GLP-1,  
6 neurotensin and xenin signalling pathways in HFF mice. In agreement, whilst circulating lipid  
7 profile of HFF mice was improved by all treatment regimens, only combined therapy reduced  
8 triacylglycerol concentrations. In this regard, originally it was believed that xenin acts on  
9 adipose tissue to stimulate lipolysis (45), but more recent studies reveal direct lipogenic and  
10 adipocyte differentiation actions of xenin (46). In agreement, an elegant study has suggested  
11 that neurotensin can also promote lipid accumulation (47). However, activation of all three  
12 hormone signalling pathways appears to lead to reduced lipid deposition. In some agreement,  
13 interaction between xenin and GIP pathways within adipocytes has previously been noted to  
14 induce responses that contrast to effects of either hormone alone (46).

15         Decreased adiposity would be expected to improve insulin sensitivity (48), and this was  
16 very much apparent in HFF mice treated with a combination of Ac-NT/XN-8-Gln and exendin-  
17 4. Whilst GLP-1 is well recognised to improve insulin action (49), similar observations have  
18 also been made with xenin-based peptides (2). Moreover, neurotensin is believed to be an  
19 important driving factor behind the improvement of insulin sensitivity in obese patients after  
20 metabolic surgery (50). The same combined treatment group also displayed significantly  
21 improved glucose handling and glucose-lowering actions of GIP when compared to all other  
22 groups of HFF mice, despite no obvious augmentation of corresponding glucose- or GIP-  
23 induced insulin secretion, again suggesting improvement of insulin action in peripheral tissues.  
24 Additive benefits of Ac-NT/XN-8-Gln and exendin-4 to improve glucose disposal could also  
25 be linked with insulin-independent glucose-lowering actions of each hormone (20,49,51), but

1 this would require further detailed investigation. However, pancreatic insulin stores were  
2 decreased in HFF mice treated with Ac-NT/XN-8-Gln in combination with exendin-4,  
3 implying reduced metabolic demand. Despite this, the characteristic expansion of pancreatic  
4 beta-cells by sustained high fat feeding in mice (52) was amplified by combined therapy, and  
5 likely linked to the increase of circulating insulin witnessed in these mice, although it should  
6 be noted that circulating insulin levels were also similarly increased in mice receiving exendin-  
7 4 alone. Such observations are in harmony with GLP-1 mediated benefits on beta-cell mass  
8 (53), as well as in direct agreement with our *in vitro* observations with Ac-NT/XN-8-Gln.

9 In conclusion, the present study has demonstrated that the novel hybrid peptide Ac-  
10 NT/XN-8-Gln is biologically active and can substantially augment exendin-4 benefits in HFF  
11 mice, especially with regards to glucose handling, insulin action and lipid metabolism.  
12 Although further studies are required to uncover the underlying molecular mechanisms, it is  
13 evident that co-activation of GLP-1, neurotensin and xenin pathways represents an antidiabetic  
14 treatment option that merits further consideration.

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1 **Conflict of interest**

2 All authors declare no conflict of interest.

3

4 **Funding**

5 These studies were supported by Department for the Economy, Northern Ireland and Ulster  
6 University Selective Research Funding

7

8 **Author contributions**

9 NI and VAG conceived/designed the study. NI and SLC drafted the manuscript. RAP and SLC  
10 and participated in the conduct/data collection and analysis and interpretation of data. All  
11 authors revised the manuscript critically for intellectual content and approved the final version  
12 of the manuscript.

13

14 **Data availability statement**

15 The authors declare that the data supporting the findings of this study are available within the  
16 article. Any additional raw data supporting the conclusions of this article will be made available  
17 by the authors, without undue reservation.

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**Table 1. Amino acid sequence, MALDI-TOF MS analysis and enzymatic stability of xenin-8-Gln, acetyl-neurotensin(8-13) and Ac-NT/XN-8-Gln.**

Peptide	Sequence	Percentage intact peptide remaining (%)		
		2 h	4 h	8 h
<b>Xenin-8-Gln</b>	H-HIS-PRO-GLN-GLN-PRO-TRP-ILE-LEU-OH	100	98.3	94.1
<b>Acetyl-neurotensin(8-13)</b>	Ac-ARG-ARG-PRO-TYR-ILE-LEU-OH	98.2	94.4	89.6
<b>Ac-NT/XN-8-Gln</b>	Ac-ARG-ARG-PRO-TYR-ILE-LEU- HIS-PRO-GLN-GLN-PRO-TRP-ILE-LEU-OH	100	100	91.7

Peptide stability was assessed following 2, 4 and 8 h incubation in murine plasma at 37°C. Degradation products were separated using HPLC, analysed by MS, and percentage degradation calculated from peak areas.



## Figure legends

### Figure 1. Acute effects of acetyl-neurotensin(8-13), xenin-8-Gln, Ac-NT/XN-8-Gln alone and in combination with GIP or GLP-1, on insulin release from BRIN-BD11 cells and isolated mouse islets

BRIN-BD11 cells were incubated (20 min) with a range of concentrations ( $10^{-12}$  to  $10^{-6}$  M) of acetyl-neurotensin(8-13), xenin-8-Gln or Ac-NT/XN-8-Gln alone (A,B) or in combination with GIP (C) or GLP-1 (D) in the presence of (A,C,D) 5.6 or (B) 16.7 mM glucose. (E) Murine islets were incubated (60 min) with Ac-NT/XN-8-Gln ( $10^{-8}$  and  $10^{-6}$  M) in the presence 16.7 mM glucose. (A-E). Insulin release was measured using radioimmunoassay. Values represent means  $\pm$  SEM (n=8 for A-D and n=5 for E). \* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001 compared to respective glucose controls.  $\Delta p$ <0.05,  $\Delta\Delta p$ <0.01 and  $\Delta\Delta\Delta p$ <0.001 compared to respective  $10^{-7}$  M GIP or GLP-1 control.

### Figure 2. Effect of acetyl-neurotensin(8-13), xenin-8-Gln and Ac-NT/XN-8-Gln on beta-cell proliferation and protection against apoptosis.

BRIN-BD11 cells were incubated overnight (18 hours) with exendin-4, acetyl-neurotensin(8-13), xenin-8-Gln or Ac-NT/XN-8-Gln (each at  $10^{-8}$  and  $10^{-6}$  M). (A) Proliferation was measured using Ki-67 immunocytochemistry. (B) Caspase-3/7 activation was detected by luminescence. (A) Values represent means  $\pm$  SEM (n=3-4). \*\* $p$ <0.01 and \*\*\* $p$ <0.001 compared to respective media control.  $\Delta\Delta p$ <0.01 and  $\Delta\Delta\Delta p$ <0.001 compared to respective Ac-NT/XN-8-Gln. (B) Values are mean  $\pm$  SEM (n=3). \*\* $p$ <0.01 and \*\*\* $p$ <0.001 compared to untreated control culture.

**Figure 3. Effects of twice-daily administration of Ac-NT/XN-8-Gln and exendin-4 alone, or in combination, on body weight, food intake, glucose, insulin and HbA1c concentrations in HFF mice.**

(A,B,D,F) Parameters were measured for 6 days before and 32 days during (indicated by black horizontal line in panels B,D,F) twice-daily treatment with saline, exendin-4, Ac-NT/XN-8-Gln or a combination of both (each at 25 nmol/kg) in HFF mice. (C,E) Parameters were assessed on day 32. Values represent mean  $\pm$  SEM (n=6-8). \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 compared to HFF saline control mice.

**Figure 4. Effects of twice-daily administration of Ac-NT/XN-8-Gln and exendin-4 alone, or in combination, on glucose tolerance and metabolic response to GIP.**

Test were performed following 32 days twice-daily i.p. administration of saline, exendin-4, Ac-NT/XN-8-Gln or a combination of both peptides (each at 25 nmol/kg bw) in 10 h fasted HFF mice. (A-D) Blood glucose (A) and plasma insulin (C) were measured prior to and after oral administration of glucose alone (18 mmol/kg bw). (E-H) Blood glucose (E) and plasma insulin (G) were measured prior to and after i.p. administration of glucose (18 mmol/kg bw) in combination with GIP (25 nmol/kg bw). (B,D,F,H) Corresponding 0-120 min AUC values are also shown. Values represent mean  $\pm$  SEM (n=6-8). \*p<0.05 and \*\*p<0.01 compared to HFF saline control mice. <sup>+</sup>p<0.05 compared to Ac-NT/XN-8-Gln treated HFF mice. <sup>^</sup>p<0.05 compared to lean saline control mice. Where appropriate to aid interpretation, lines are used to indicate significance between groups on the bar graphs.

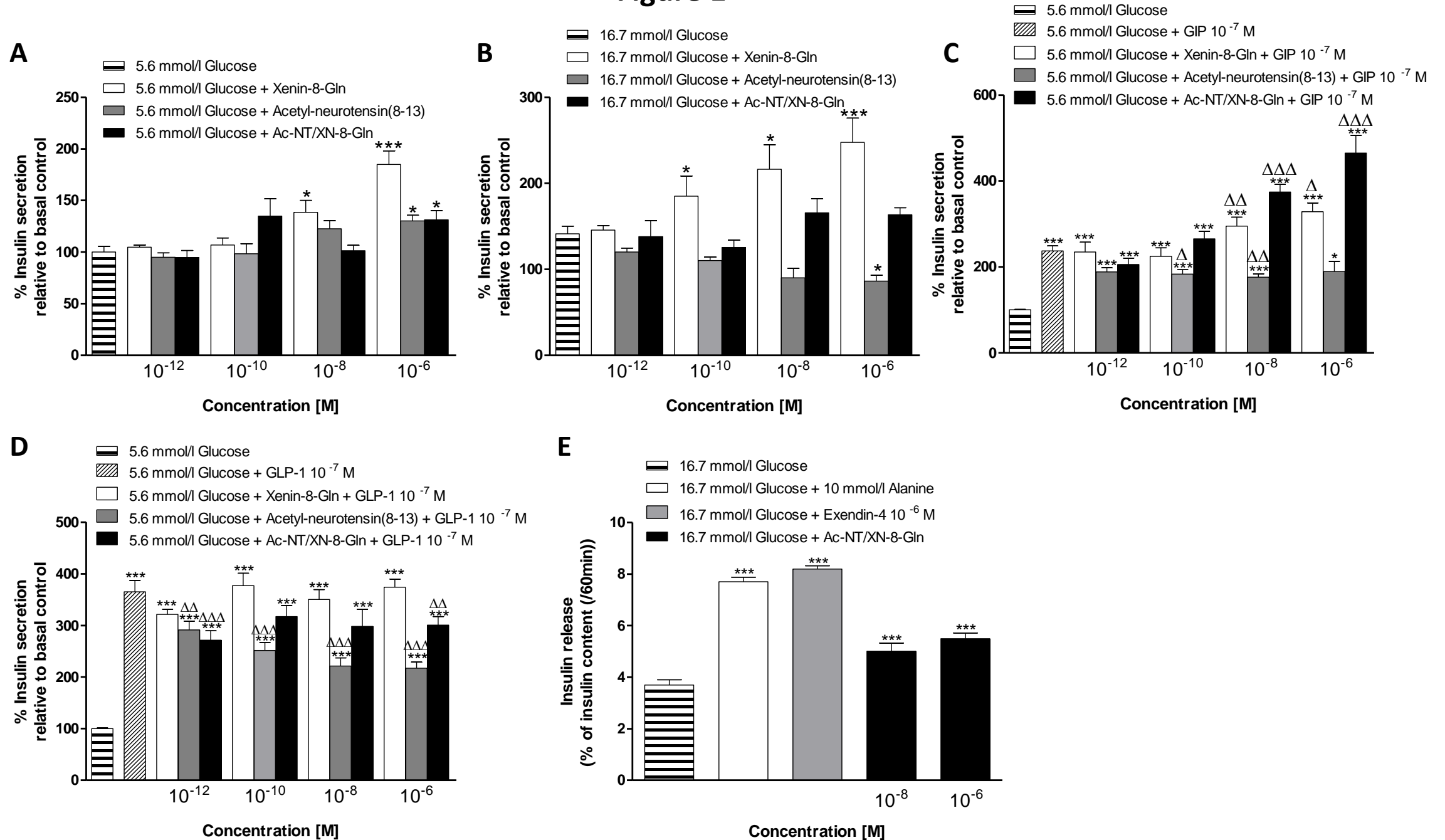
**Figure 5. Effects of twice-daily administration of Ac-NT/XN-8-Gln and exendin-4 alone, or in combination, on insulin sensitivity and lipid status in HFF mice.**

Test were performed following 32 days twice-daily i.p. administration of saline, exendin-4, Ac-NT/XN-8-Gln or a combination of both peptides (each at 25 nmol/kg bw) in HFF mice. (A,B) Blood glucose was measured prior to and after i.p. administration of insulin (25 U/kg bw), with 0-60 min AAC values also shown. (C-E) Effects of test peptides on circulating (C) triglycerides, (D) total- and (E) LDL-cholesterol concentrations. Values are mean  $\pm$  SEM (n=6-8). \*p<0.05 \*\*p<0.01 and \*\*\*p<0.001 compared to HFF saline control mice. <sup>+</sup>p<0.05 and <sup>++</sup>p<0.01 compared to Ac-NT/XN-8-Gln treated HFF mice. Where appropriate to aid interpretation, lines are used to indicate significance between groups on the bar graphs.

**Figure 6. Effects of twice-daily administration of Ac-NT/XN-8-Gln and exendin-4 alone, or in combination, on pancreatic insulin content and islet histology in HFF mice**

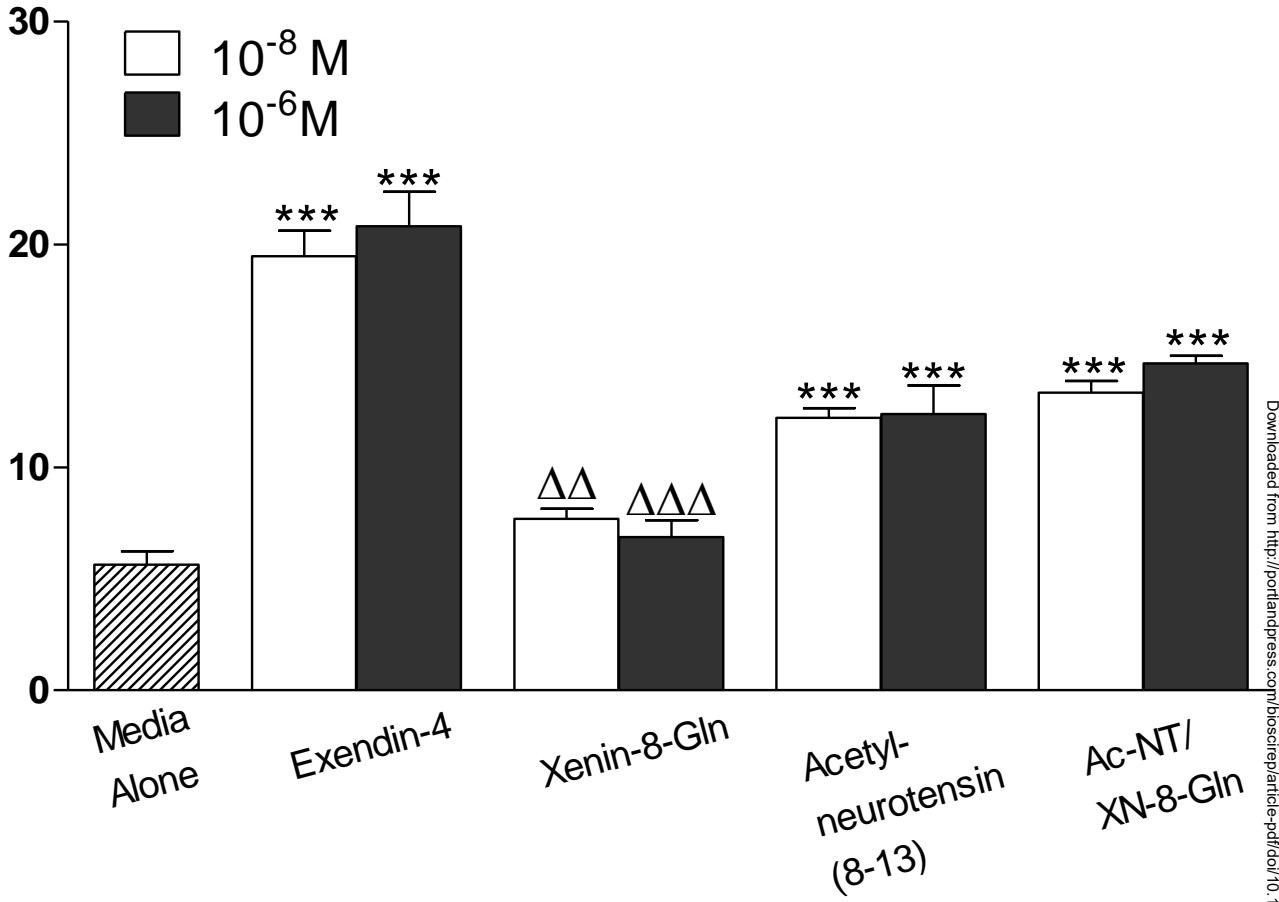
Effects of 32 days twice-daily i.p. administration of saline, exendin-4, Ac-NT/XN-8-Gln or a combination of both peptides (each at 25 nmol/kg bw) on (A) pancreatic insulin content as well as (B) islet, (C) beta-cell and (D) alpha-cell areas in HFF mice. Values represent mean  $\pm$  SEM (n=6-8). <sup>+++</sup>p<0.001 compared to lean control. \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 compared to HFF saline control mice.  $\Delta\Delta$ p<0.01 and  $\Delta\Delta\Delta$ p<0.001 compared to Ac-NT/XN-8-Gln treated HFF mice. Where appropriate to aid interpretation, lines are used to indicate significance between groups on the bar graphs.

**Figure 1**

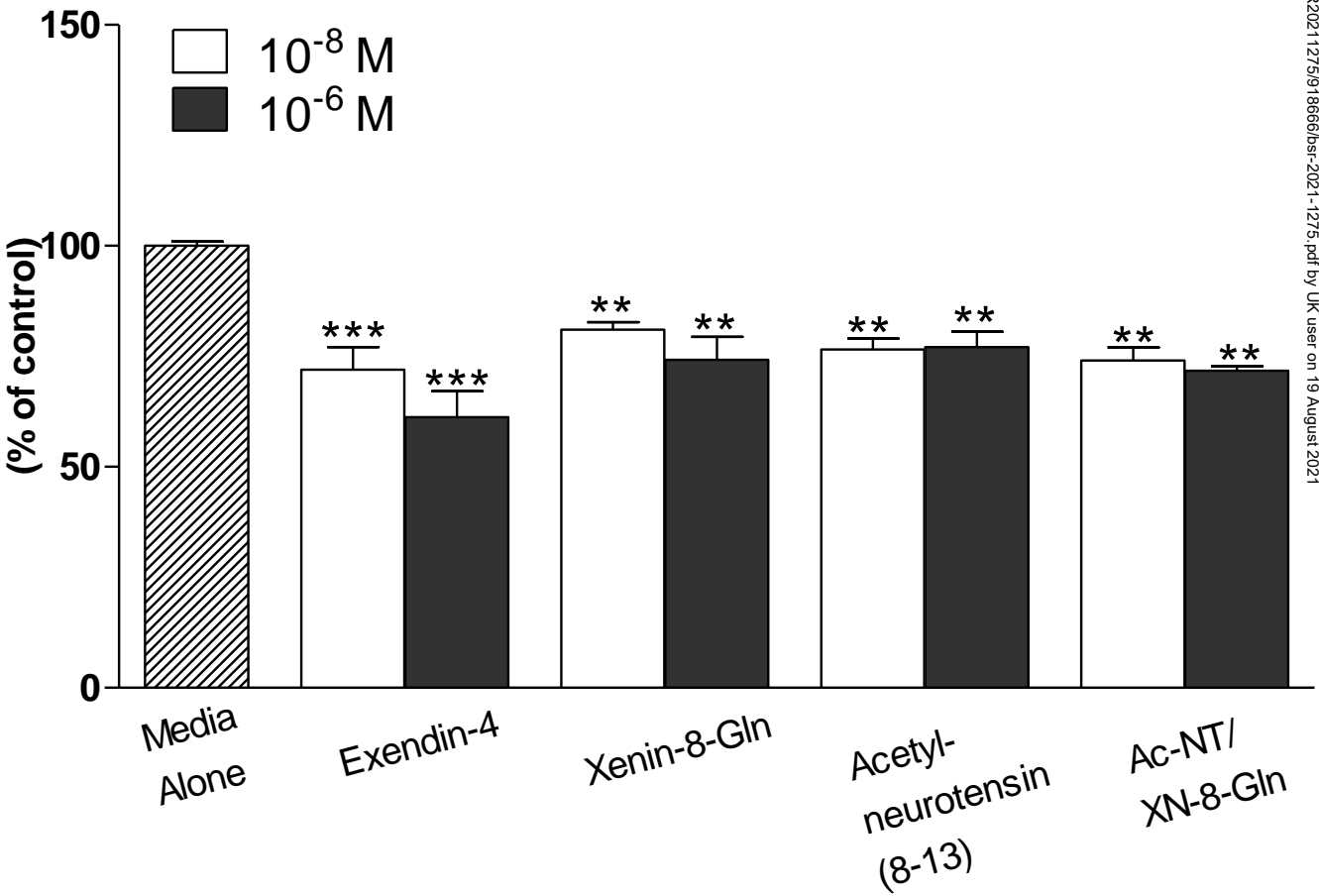


# Figure 2

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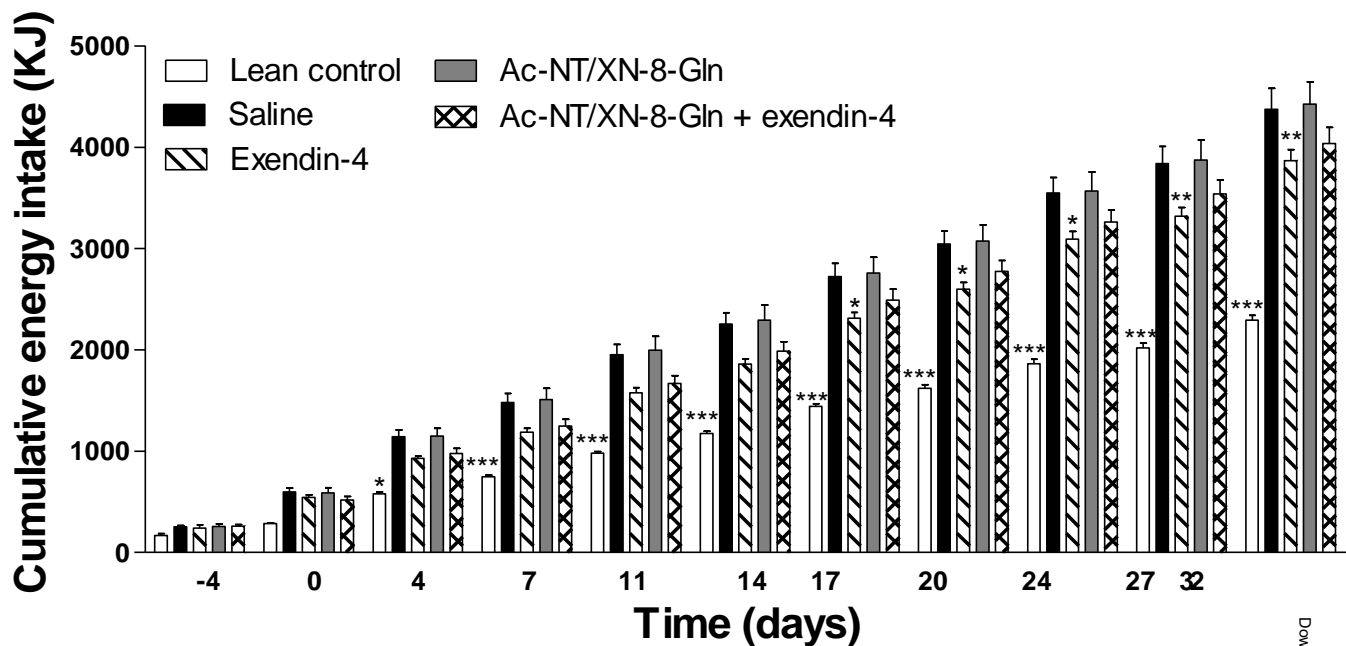


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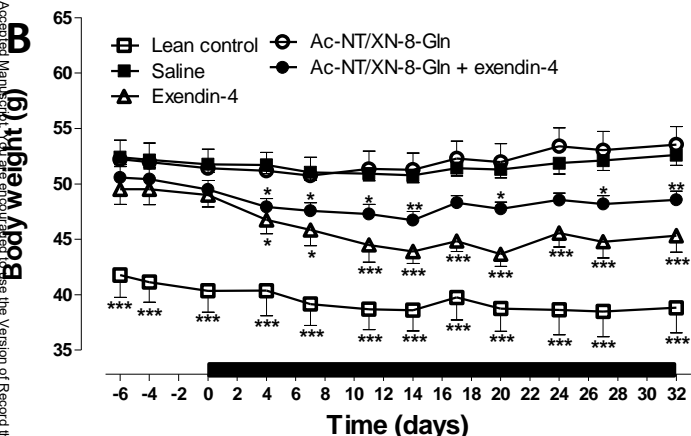


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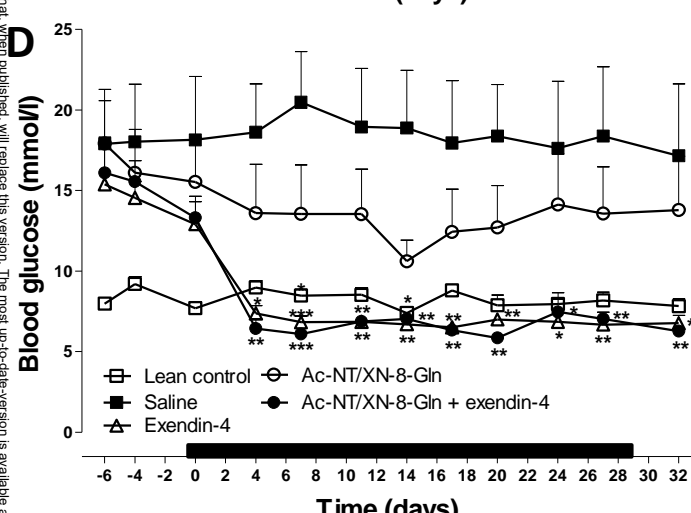
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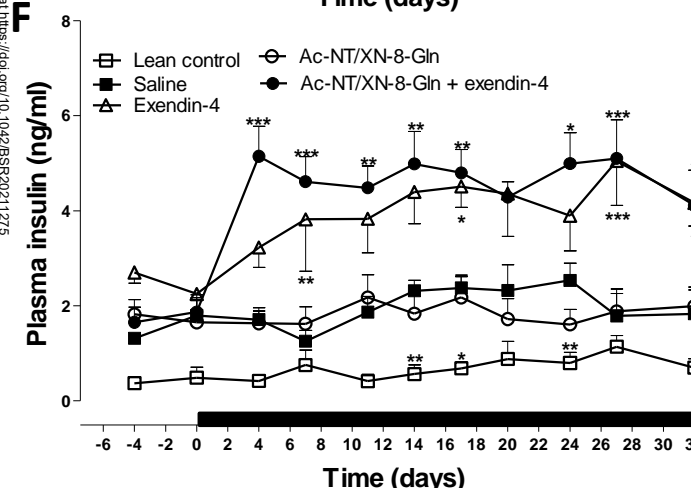
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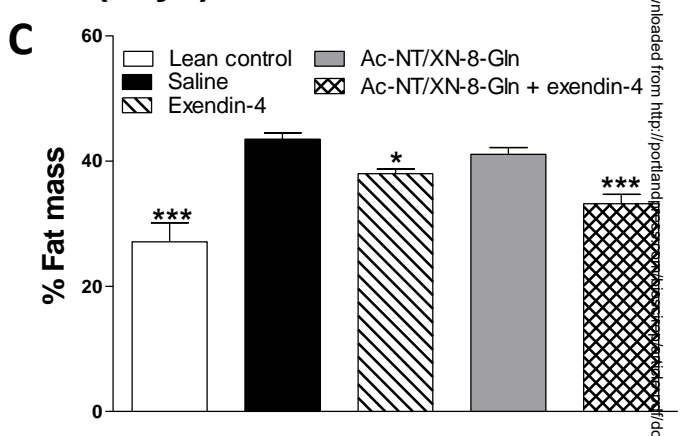
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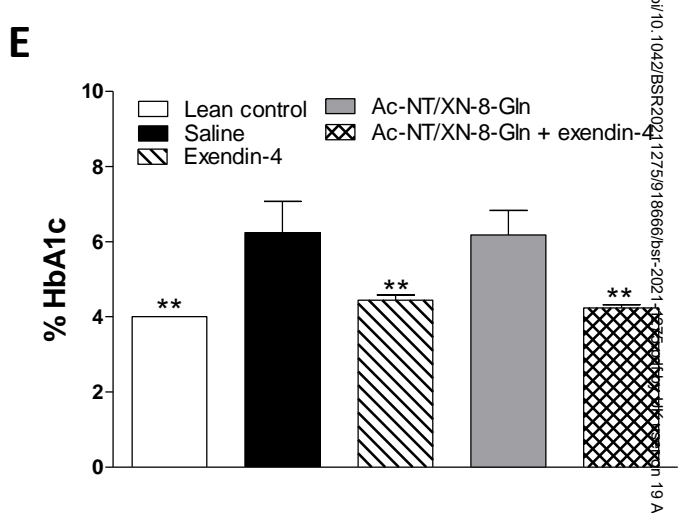
**E**



**C**



**F**

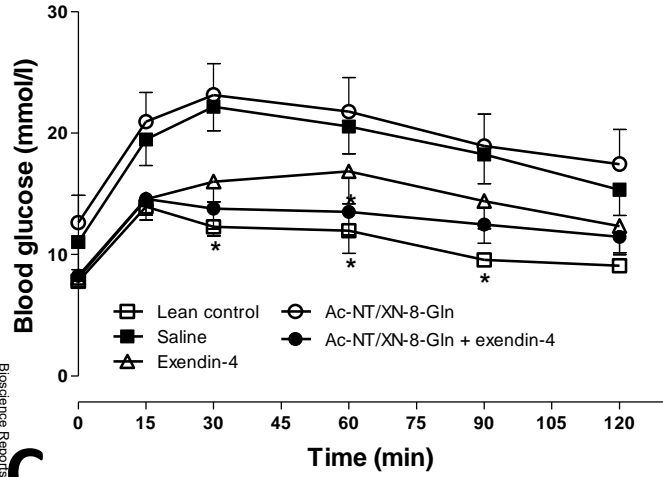


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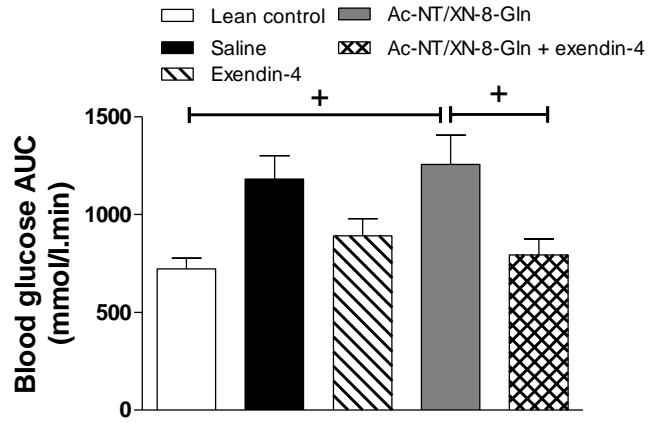
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# Figure 4

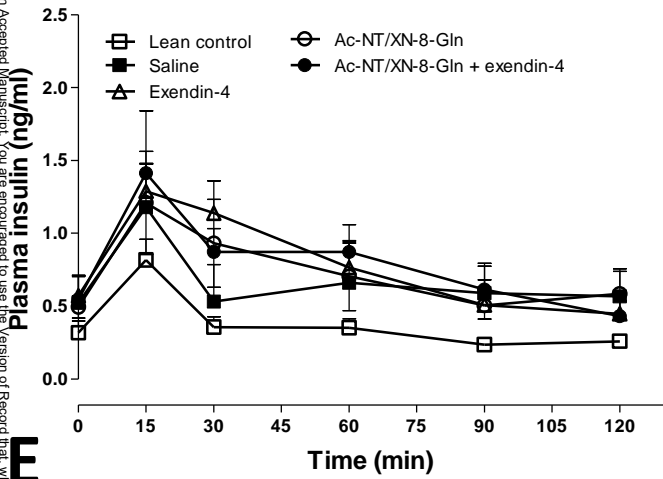
## A



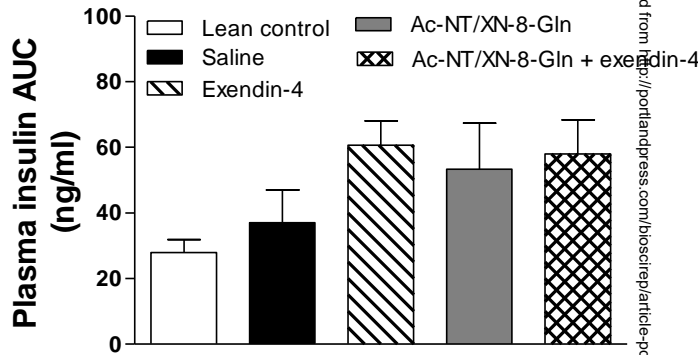
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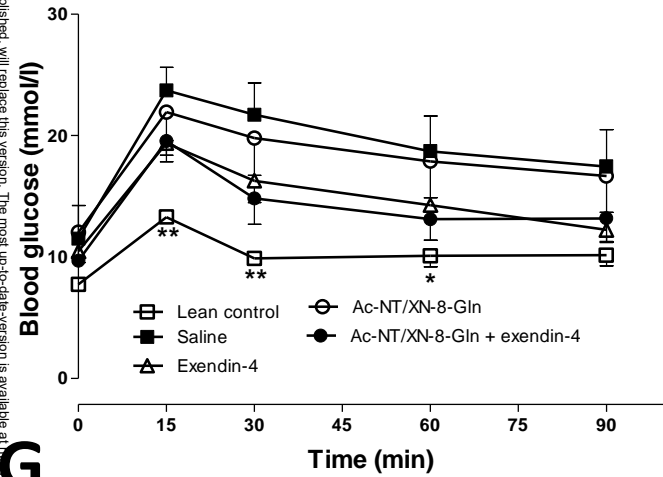
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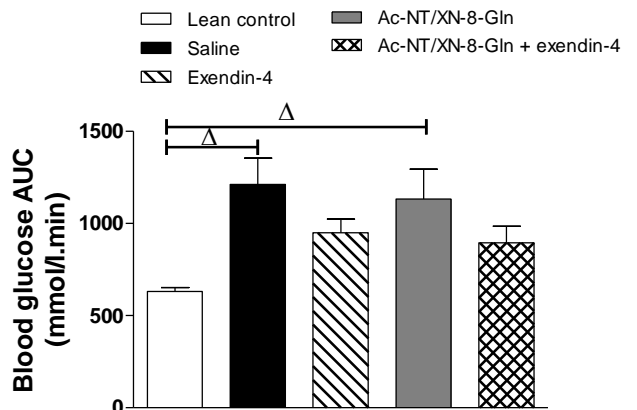
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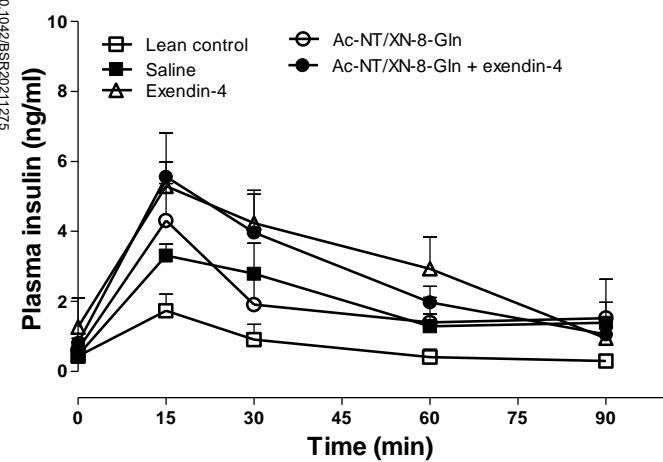
## E



## F



## G



## H

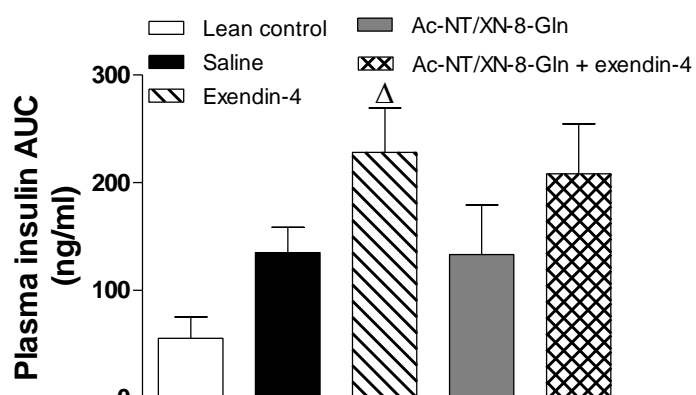
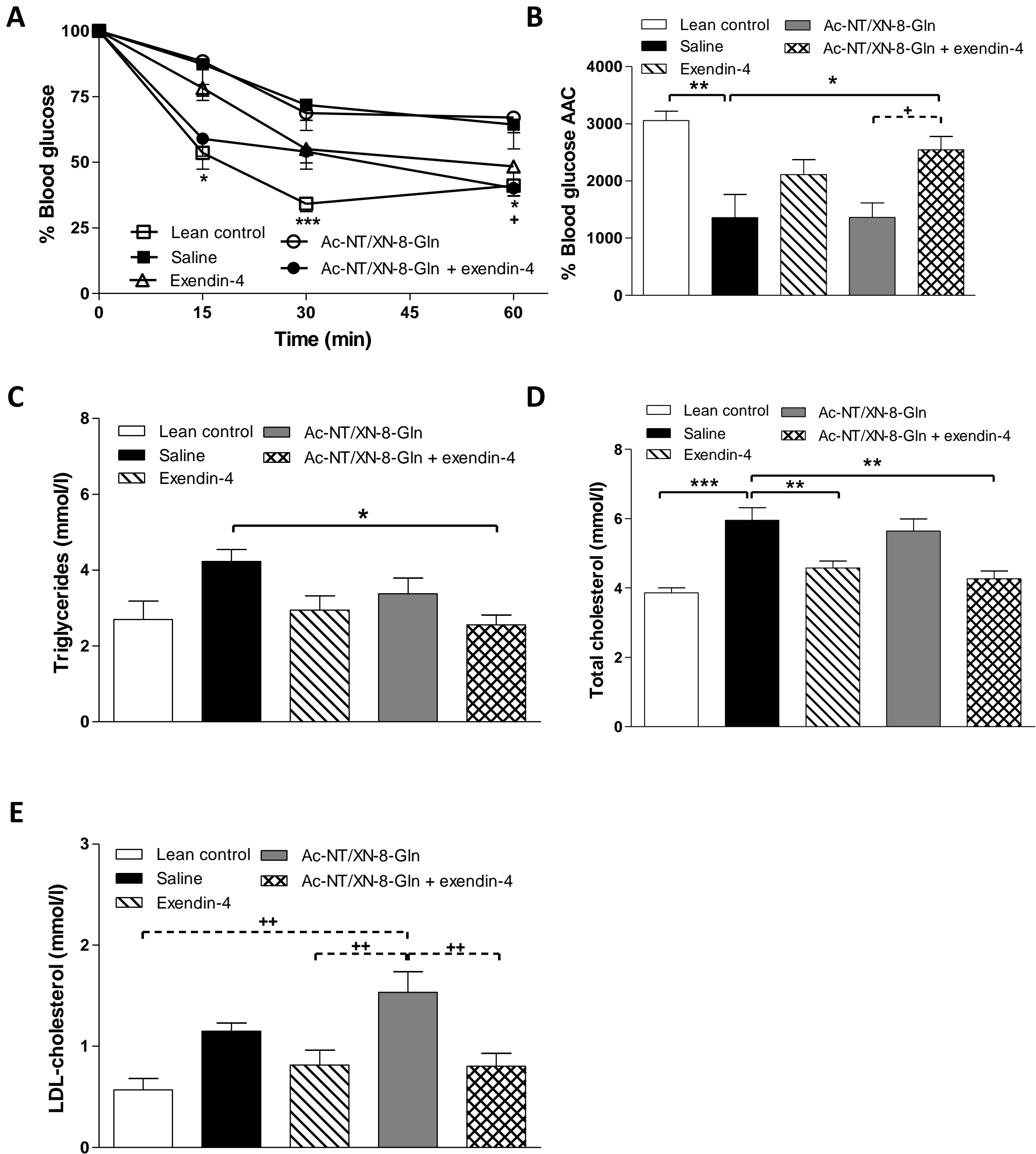


Figure 5





# Figure 6

