STABLE INCRETIN MIMETICS COUNTER RAPID DETERIORATION OF BONE QUALITY IN TYPE 1 DIABETES MELLITUS

Sity Aishah Mansur\textsuperscript{1,2}, Aleksandra Mieczkowska\textsuperscript{3}, Béatrice Bouvard\textsuperscript{3}, Peter R Flatt\textsuperscript{1}, Daniel Chappard\textsuperscript{3,4}, Nigel Irwin\textsuperscript{1}, Guillaume Mabilleau\textsuperscript{3,4*}

\textsuperscript{1} Ulster University, School of Biomedical Sciences, Coleraine, United Kingdom; \textsuperscript{2} University Tun Hussein Onn Malaysia, Johor, Malaysia; \textsuperscript{3} LUNAM Université, GEROM-LHEA, Institut de Biologie en Santé, Angers, France; \textsuperscript{4} LUNAM Université, SCIAM, Institut de Biologie en Santé, Angers, France

Type 1 diabetes mellitus is associated with a high risk for bone fractures. Although bone mass is reduced, bone quality is also dramatically altered in this disorder. However, recent evidences suggest a beneficial effect of the glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) pathways on bone quality. The aims of the present study were to conduct a comprehensive investigation of bone strength at the organ and tissue level; and to ascertain whether enzyme resistant GIP or GLP-1 mimetic could be beneficial in preventing bone fragility in type 1 diabetes mellitus. Streptozotocin-treated mice were used as a model of type 1 diabetes mellitus. Control and streptozotocin-diabetic animals were treated for 21 days with an enzymatic-resistant GIP peptide ([D-Ala²]GIP) or with liraglutide (each at 25 nmol/kg bw, ip). Bone quality was assessed at the organ and tissue level by microCT, qXRI, 3-point bending, qBEI, nanoindentation and Fourier-transform infrared microspectroscopy. [D-Ala²]GIP and liraglutide treatment did prevent loss of whole bone strength and cortical microstructure in the STZ-injected mice. However, tissue material properties were significantly improved in STZ-injected animals following treatment with [D-Ala²]GIP or liraglutide. Treatment of STZ-diabetic mice with [D-Ala²]GIP or liraglutide was capable of significantly preventing deterioration of the quality of the bone matrix. Further studies are required to further elucidate the molecular mechanisms involved and to validate whether these findings can be translated to human patients.

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a severe disorder that persists through the entire life of affected individuals. T1DM is characterized by the inability of pancreatic beta-cells to secrete insulin and therefore results in hyperglycemia. Several complications including bone loss and elevated fracture risk have been reported in T1DM (Kemink et al., 2000; Krakauer et al., 1995). Increased fracture risk could be partially related to diabetic complications such as retinopathy and neuropathy that increase the incidence of fall (Forsen et al., 1999; Vestergaard et al., 2009). Another more likely explanation could reside in a direct deleterious effect of diabetes on bone tissue. Bone mineral density (BMD) has been reported to be lower in T1DM patients as compared to age-matched control subjects (Danielson et al., 2009; Tuominen et al., 1999). However, elevated fracture risk among T1DM patients still persists even after adjusting for hip BMD (Stromeyer et al., 2005). Furthermore, the low BMD observed in T1DM does not fully explain the high fracture risk in this population (Stromeyer and Cauley, 2007). Thus, there is a supposition that
diabetic bone is more fragile than non-diabetic bone for a given BMD and that T1DM not only affects bone microarchitecture but also tissue material properties.

Rodent models of T1DM can be generated by injection of streptozotocin (STZ), which is specifically toxic to insulin-producing pancreatic beta-cells, and leads to hypoinsulinemia and hyperglycemia (Szkudelski, 2001) similar to that observed in T1DM patients. Several studies using the STZ-induced diabetic mouse model have been published in the last decade, reporting inconsistent effects of T1DM on bone material properties, including the mineral and organic component of the bone matrix (Facchini et al., 2006; Reddy et al., 2001; Silva et al., 2009; Zhang et al., 2007).

Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are gut hormones secreted by entero-endocrine cells that are well known for their incretin effect, i.e. potentiation of insulin secretion from the endocrine pancreas (Drucker, 2013). A role for gastro-intestinal hormones in controlling bone remodeling has been suggested as changes in the profile of serum markers of bone remodeling after a meal coincides with a peak in gastro-intestinal hormone secretion (Einenæi et al., 2010; Henriksen et al., 2003). Furthermore, we and others have reported the presence of functional incretin receptors on bone cells and alterations of bone microarchitecture in genetically-modified incretin receptor animals (Gaudin-Audrain et al., 2013; Mieczkowska et al., 2014; Seino and Yabe, 2013; Tsukiyama et al., 2006; Xie et al., 2005; Xie et al., 2007; Yabe and Seino, 2012; Yamada et al., 2008). Recently, a role for GIP and GLP-1, in controlling tissue material properties has also been highlighted (Mabilleau et al., 2013; Mabilleau et al., 2014; Mieczkowska et al., 2013; Mieczkowska et al., 2014). However, the molecular mechanism of action of these gut hormones on bone is unclear. Due to the anabolic action of insulin on bone and the incretin effect of GIP and GLP-1, one could wonder whether the action of gut hormones on bone could be mediated by increased insulin secretion. Indeed, little is known about the capacity of these gut hormones to improve bone material properties in diabetic animals.

The aims of the present study were (i) to conduct a comprehensive investigation of trabecular and cortical bone strength at the organ and tissue level in streptozotocin-diabetic mice; and (ii) to ascertain whether enzyme resistant GIP or GLP-1 mimetic could be beneficial in preventing bone fragility in this animal model of T1DM.

**MATERIAL AND METHODS**

**GIP and GLP-1 mimetic**

Peptides used in this study were all purchased from GL Biochem Ltd. (Shanghai, China). All peptides were identified and characterized by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Briefly, 1.5 µl of peptide solution was individually dispensed into different well of a 100-well stainless steel plate and allowed to dry at room temperature. 1.5 µl of matrix solution (10mg/ml of α-cyano-4-hydroxycinnamic acid in acetonitrile) (Sigma-Aldrich, Poole, UK) was added to the dried peptides and the mixture was again left to dry. The molecular mass of peptide was determined using Voyager-DE BioSpectrometry Workstation (PerSeptive
BioSystems, Framingham, MA, USA) and recorded as mass-to-charge (m/z) ratio vs percentage intensity of peak. The observed molecular mass for [D-Ala²]GIP was 4984.3 Da corresponding to theoretical value of 4983.6 Da. The observed molecular mass for liraglutide was 3752.6 Da corresponding to theoretical value of 3751.3 Da.

**Animals**

The chronic effect of [D-Ala²]GIP or liraglutide on metabolic control and bone quality were performed using streptozotocin (STZ)-induced diabetic mice. Young male Swiss T0 mice (n=30, 8 weeks old) initially received 150 mg/kg bw of STZ to induce diabetes. Six days post-STZ introduction, the mice were divided into three groups (n=10) and started receiving once daily intraperitoneal injections of [D-Ala²]GIP or liraglutide (each at 25 nmol/kg bw) or saline vehicle (0.9% NaCl) for 21 days. These doses of [D-Ala²]GIP and liraglutide have been chosen based on our previous studies (Kerr et al., 2011; Martin et al., 2013; Porter et al., 2010). Age- and sex-matched normal mice (n=6) were also injected with saline vehicle (0.9% NaCl) once daily for the same period of time. Animals were maintained on a 12h:12h light-dark cycle in a temperature-controlled room (21.5 ± 1°C). Animals received standard rodent maintenance diet and water ad libitum. Blood samples were obtained from the cut tip of the tail vein of conscious mice and samples were collected with fluoride coated microvette tubes (Sarstedt, Germany). The samples were immediately kept in ice and centrifuged using microcentrifugate (Beckman Instruments, Galway, Ireland) for 3 min at 13 000 g. The separated plasma was aliquoted into 500 μl eppendorf tubes and kept at -20°C. All experiments were conducted according to the Guidelines of the NIH regarding animal care (Guide for the Care and Use of Laboratory Animals, 1996), the United Kingdom Office regulations (UK Animals Scientific Procedures Act 1986) and European Union laws. The use of these animals was approved by our Animal Care and Use Committee. The mice were also intraperitoneally injected with calcein (10 mg/kg bw) both at 7 and 2 days before being killed by inhalation of CO₂. After necropsy, tibias and femurs were collected and cleaned of soft tissue. Tibias were fixed with a formalin-based fixative whilst femurs were stored in 70% ethanol. As described previously, this storage does not alter the mechanical behavior (Mieczkowska et al., 2014).

**Intraperitoneal glucose tolerance test**

Eighteen hours prior to administration of glucose, food was withheld. In order to get the baseline of plasma glucose concentrations, pre-injection blood samples were collected (0 min) and then at 15, 30 and 60 min after intraperitoneal injection of glucose. Plasma was separated at 13,000g for 3 min using a microcentrifuge (Beckman Instruments, Galway, Ireland). Supernatants were then aliquoted into 500 μl eppendorf tubes and stored at -20°C for plasma glucose and insulin measurements as previously reported (Flatt and Bailey, 1981).

**MicroCT**

MicroCT analysis was performed on tibias with a Skyscan 1172 microtomograph (Bruker MicroCT, Kontich, Belgium) equipped with an X-ray tube operating at 69 kV/100 μA. The isotropic pixel size was fixed at 3.75 μm, the
rotation step at 0.25° and exposure was done with a 0.5-mm aluminium filter. Trabecular parameters were assessed in the proximal secondary spongiosa 0.5mm below the growth plate and extending 2mm down. Bone volume (BV/TV, in %), trabecular number (Tb.N, /mm), trabecular thickness (Tb.Th, in µm) and trabecular separation (Tb.Sp, in µm) were determined. Cortical parameters were assessed at the midshaft tibia. Cortical VOIs extended on 2-mm centered at the midshaft tibia. External bone diameter (B.Dm, in µm), marrow diameter (Ma.Dm, in µm) and cortical thickness (Ct.Th, in µm) were measured with a lab-based routine made with ImageJ 1.45s (NIH, Bethesda, MD). All these parameters were determined according to guidelines and nomenclature proposed by the American Society for Bone and Mineral Research (Bouxsein et al., 2010).

**Quantitative X-ray imaging (qXRI)**

Bone mineral content at the midshaft femur was determined using qXRI as previously reported (Mieczkowska et al., 2013). Digital X-ray images of the left femur were recorded at a 12-µm pixel resolution using a Faxitron MX20 device (Edimex, Angers, France). The region of interest was located at the midshaft femur and represented a height of 2 mm. GL\_mean, the mean grey level of each bone, was determined.

**Three-point bending**

Three-point bending experiments were performed on the right femur. Prior to mechanical testing, femurs were rehydrated in saline overnight at 4°C. Bone strength at the whole bone scale was investigated by 3-point bending. Measurements were done with a constant span length of 10 mm on an Instron 5942 (Instron, Elancourt, France). The press head as well as the two support points were rounded to avoid shear load and cutting. Femurs were positioned horizontally with the anterior surface facing upward, centered on the support and the pressing force was applied vertically to the midshaft of the bone. Each bone was tested with a loading speed of 2 mm.min\(^{-1}\) until failure with a 500N load cell. The load-displacement curve was acquired with the Bluehill 3 software (Instron). Ultimate load, yield load, ultimate displacement, post-yield displacement, stiffness and total absorbed energy were computerized according
to previously published equations (Turner and Burr, 1993).

**Fourier-transform infrared microscopy (FTIRM)**

After three-point bending experiments, femurs were cross-sectionally cut at the midshaft using a diamond saw (Accutom, Struers, Champigny sur Marne, France) and embedded undecalcified in polymethylmethacrylate at 4°C as previously reported (Blouin et al., 2008). Cross-sections (4µm thickness) of the midshaft femur were cut dry on a heavy duty microtome equipped with tungsten carbide knives (Leica Polycut S) and sandwiched between BaF₂ optical windows. Spectral analysis were obtained on a Bruker Vertex 70 spectrometer (Bruker optics, Ettlingen, Germany) interfaced with a Bruker Hyperion 3000 infrared microscope as previously reported (Mabilleau et al., 2013). For FTIR analysis, 12 spectra on each bone were acquired between double calcein labelling and analyzed with the Opus Software (release 6.5, Bruker). The contribution of the embedding polymethylmethacrylate (pMMA) and water vapor were corrected for each spectrum prior to baseline correction. Individual spectra were then subjected to curve fitting using a commercially available software package (Grams/AI 8.0, Thermofisher scientific, Villebon sur Yvette, France) as previously reported (Mieczkowska et al., 2014). The collagen maturity index, determined as the relative ratio of subbands located at 1660 cm⁻¹ and 1690 cm⁻¹ of the amide I peak, was assessed (Paschalis et al., 2001). Then, bone sections were demineralized with EDTA and reanalyzed by FTIRM (Boskey and Pleshko Camacho, 2007). The collagen glycation index was investigated as the intensity ratio of the 1032 cm⁻¹ over the Amide I region (Guilbert et al., 2013). The collagen integrity index was determined as the area ratio of the 1338 cm⁻¹ band over the amide II region (West et al., 2005).

**Nanoindentation**

Nanoindentation tests evaluated the mechanical properties of the bone matrix. As nanoindentation assesses volume of material at a length scale less than that of individual microstructural features in bone, this technique avoids confounding factors such as bone microarchitecture and porosity that affect material properties at larger length scales such as 3-point bending. PMMA blocks used for FTIRM sections were polished to a 1-µm finish with diamond particles (Struers, France) and the same subregions as used for FTIRM were analyzed by nanoindentation (Gaudin-Audrain et al., 2013). Prior to nanoindentation testing, blocks were rehydrated overnight in saline. Twelve indentations, at distance from canals, osteocyte lacunae and/or microcracks were randomly positioned in cortical bone with a NHT-TTX system (CSM, Peseux, Switzerland) equipped with a Berkowitch diamond probe. Indentations were made up to a depth of 900 nm with a loading/unloading rate of 40mN/min. At maximum load, a holding period of 15 seconds was applied to avoid creeping of the bone material. The following material properties at the tissue-level, maximum load, indentation modulus, hardness and dissipated energy, were determined according to Oliver and Pharr (Oliver and Pharr, 1992).

**Quantitative backscattered electron imaging (qBEI)**
Quantitative backscattered electron imaging was employed to determine the bone mineral density distribution (BMDD) as previously reported (Mabilleau et al., 2014). QBEI experiments were performed on the same blocks and same regions as nanoindentation and FTIRM. Polymethylmethacrylate blocks were carbon-coated and observed with a scanning electron microscope (EVO LS10, Carl Zeiss Ltd, Nanterre, France) equipped with a five quadrant semi-conductor backscattered electron detector. The microscope was operated at 20 kV with a probe current of 250 pA and a working distance of 15 mm. The backscattered signal was calibrated using pure carbon (Z=6, mean grey level = 25), pure aluminum (Z=13, mean grey level =225) and pure silicon (Z=14, mean grey level =253) standards (Micro-analysis Consultants Ltd, St Ives, UK). The cortical bone area was imaged at a 200X nominal magnification, corresponding to a pixel size of 0.5 µm per pixel. Four images per samples were taken. Three variables were obtained from the bone mineral density distribution: \( \text{Ca}_{\text{peak}} \) as the most frequently observed calcium concentration, \( \text{Ca}_{\text{mean}} \) as the average calcium concentration and \( \text{Ca}_{\text{width}} \) as the width of the histogram at half maximum of the peak.

**Statistical analysis**

Results were expressed as mean ± standard error of the mean (SEM). Non-parametric Mann-Whitney U-test was used to compare the differences between the groups using the Systat statistical software release 13.0 (Systat software Inc., San Jose, CA). Differences at p<0.05 were considered to be significant.

**RESULTS**

**Short term treatment with [D-Ala²]GIP or liraglutide did not affect body weight or glucose or insulin levels**

Injection of STZ resulted in significant modifications in metabolic parameters (Figure 1). Body weight was significantly reduced by 26% in untreated STZ animals as compared with CTRL animals (p<0.001). Treatment of STZ-diabetic mice with [D-Ala²]GIP or liraglutide did not restore a normal weight. Glucose levels were significantly higher in STZ-diabetic mice by 2-fold as compared to CTRL animals (p<0.001). Neither [D-Ala²]GIP nor liraglutide decreased glucose in STZ-diabetic mice. Insulin levels were significantly lower in STZ-diabetic animals by 4-fold as compared to CTRL mice (p<0.001). [D-Ala²]GIP and liraglutide did not affect insulin levels in STZ-diabetic mice.

**Short term treatment with [D-Ala²]GIP or liraglutide exhibited differential effects on osteoclast and osteoblast activity**

Trabecular microarchitecture was assessed by microCT. As represented in table 1, diabetic animals exhibit a slight but insignificant reduction in BV/TV and Tb.N. Although not significant, administration of [D-Ala²]GIP seemed to prevent the reduction in BV/TV and Tb.N. On the other hand, liraglutide did not modify the pattern of bone loss observed in diabetic mice.

Dynamic bone formation parameters (MAR, MS/BS and BFR/BS) were all significantly lower in diabetic mice (Figure 2). Furthermore, the number of osteoclast (N.Oc/B.Pm) and osteoclast surface (Oc.S/BS) were also significantly lower in diabetic animals. Treatment with [D-Ala²]GIP prevented reductions in MAR, MS/BS, BFR/BS and
Oc.S/BS. On the other hand, treatment with liraglutide did not prevent reductions in MAR, MS/BS, BFR/BS and Oc.S/BS.

**Short term treatment with \([D-Ala^2]GIP\) or liraglutide did not prevent loss of whole bone strength and cortical microstructure**

Cortical bone mineral density was assessed by quantitative X-ray imaging. As represented Figure 3A, GLmean was significantly lower by 8% in STZ-diabetic animals as compared with CTRL mice \((p=0.0024)\). On the other hand, \([D-Ala^2]GIP\) or liraglutide treatment did not improve this parameter \((Figure\ 3B)\). Three-point bending assessment of cortical bone revealed that STZ-diabetic mice presented with lower mechanical response, at the whole bone scale, except for post-yield displacement where significant augmentation by 40% and 34% were evidenced in \([D-Ala^2]GIP\) and liraglutide-treated animals, respectively.

Three dimensional models of cortical bone were computerized and are represented Figure 4A. Cortical thickness appeared reduced in diabetic animals. Indeed, as represented Figure 4B, we evidenced a significant higher value for Ma.Dm in STZ-diabetic mice as compared with CTRL animals. As B.Dm was unchanged, Ct.Th was significantly lower in STZ-diabetic animals as compared with CTRL mice. \([D-Ala^2]GIP\) or liraglutide treatments did not significantly improve cortical bone microstructure in STZ-diabetic mice.

**[D-Ala^2]GIP and liraglutide treatment improved tissue material properties**

Another explanation for reduced mechanical response at the whole bone level may be due to alteration of tissue material properties. We first examined by nanoindentation whether the mechanical response, at the tissue level, was affected by STZ. As represented Figure 5, STZ-diabetic mice presented with reductions in maximum force \((24\%, p=0.0027)\), hardness \((30\%, p=0.006)\), indentation modulus \((18\%, p<0.001)\) and absorbed energy \((23\%, p=0.021)\) as compared with CTRL animals. Diabetic mice treated with \([D-Ala^2]GIP\) presented with significant improvements in maximum force \((34\%, p<0.001)\), hardness \((35\%, p=0.015)\), indentation modulus \((31\%, p<0.001)\) and absorbed energy \((41\%, p=0.005)\) as compared to STZ-treated controls. Similarly, liraglutide treatment in STZ-diabetic mice significantly improved these parameters as compared to untreated STZ-diabetic mice with augmentations in maximum force \((42\%, p<0.001)\), hardness \((49\%, p=0.008)\), indentation modulus \((32\%, p<0.001)\) and absorbed energy \((39\%, p=0.006)\).

We then tried to ascertain whether the bone mineral compartment of the bone matrix was modified in STZ-diabetic mice \((Figure\ 6)\). \(Ca_{peak}\), \(Ca_{mean}\) and \(Ca_{width}\) were not significantly different in STZ-diabetic mice as compared to CTRL animals. Treatment of STZ-diabetic mice with either \([D-Ala^2]GIP\) or liraglutide did not modify these parameters.

The collagen compartment was studied by FTIRM. As represented Figure 7, collagen maturity index was significantly increased in
STZ-diabetic mice. Furthermore, the collagen glycation index was substantially higher in STZ-diabetic mice whilst the collagen integrity index was significantly lower. Treatment of STZ-diabetic mice with [D-Ala²]GIP did not significantly modify the profile of collagen maturity index or the collagen glycation index as compared with untreated STZ-diabetic animals. On the other hand, collagen integrity index was significantly increased compared with untreated STZ-diabetic animals. Similarly, collagen maturity index and collagen glycation index were not significantly modified in liraglutide-treated STZ animals as compared to untreated STZ animals. However, collagen integrity index was significantly higher after liraglutide treatment as compared to untreated STZ animals.

DISCUSSION

Whilst living with T1DM is manageable with lifestyle changes and adequate insulin therapy, diabetic complications still occur. Among them, bone fracture is a severe complication that greatly alters the quality of life. In the present study, we evidenced that in STZ-diabetic mice, cortical microstructure, bone cell activities and tissue material properties were altered, ultimately resulting in lower bone strength. Interestingly, although the enzyme resistant GIP and GLP-1 mimic had no effect on trabecular and cortical microstructure in diabetic mice, they significantly improved tissue material properties and especially the collagen integrity index resulting in an enhancement of tissue-level bone strength.

It is generally accepted that bone turnover is reduced in T1DM (McCabe, 2007) and in the present study, a reduction in osteoclast activity and bone formation were observed. Furthermore, the observed alterations of trabecular and cortical microarchitectures evidenced in STZ-diabetic mice were similar to those reported in several mouse models of T1DM (Motyl et al., 2012; Nyman et al., 2011; Thrailkill et al., 2005). In the present study, [D-Ala²]GIP or liraglutide treatment did not alleviate hyperglycemia or hypoinsulinemia due to destruction of pancreatic beta cells. Taken together, it is likely that the observed reduction of cortical microstructure seen in STZ-diabetic mice (untreated and treated) could be linked to the observed hypoinsulinemia. Indeed, Thrailkill et al demonstrated that insulin therapy of diabetic animals was capable of reversing this cortical phenotype (Thrailkill et al., 2005). Insulin has been described as an anabolic agent in bone (Thomas et al., 1998) and, as such, one could hypothesize that the observed hypoinsulinemia could have directly caused in the observed reduction in cortical thickness. Another possibility is that it could arise as an indirect consequence of hypoinsulinemia. Indeed, in control and treated STZ-diabetic mice, we also observed a significant hyperglycemia that ultimately resulted in higher formation of advanced glycation end (AGE) products as evidenced by the increase collagen glycation index. AGEs have been shown to dramatically affect osteoblast and osteoclast biology (Franke et al., 2011; Yoshida et al., 2009) and as such, could have participated in the modification of cortical microstructure. Furthermore, in the present study, T1DM was initiated 6 days before administration of [D-Ala²]GIP or liraglutide. The duration of [D-Ala²]GIP or liraglutide treatment was relatively short (21 days) and it is also possible that this
time-course did not allow sufficient time to offset the negative consequences of the induction of T1DM.

One could argue that the study duration should be extended to observe more pronounced effects on bone microarchitecture. The window of opportunity to study STZ diabetic mice depends on extent of beta cell destruction, which dictates severity of diabetes and its complications. In the present study, we used a protocol involving single injection of 150 mg/kg bw STZ. Administration of a single large dose of STZ initiates immediate beta cell destruction due to rapid uptake by insulin-producing cells. Due to the rapid onset and severity of beta cell loss in this experimental model, it was not possible to keep STZ diabetic mice for longer period without implementing insulin therapy. This protocol contrasts with multiple dose of STZ (40-50 mg/kg bw) administered on each of 5 consecutive days that have previously been used is some animal studies (Motyl and McCabe, 2009; Motyl et al., 2012; Nyman et al., 2011). The use of such multiple low doses of streptozotocin results in slow onset of diabetes due to progressive lymphocytic infiltration of islets (insulitis) and cell mediated chemical attack that is not observed in humans (Motyl and McCabe, 2009; Reddy et al., 1995). That is why we chose to administer a large dose of STZ to demonstrate clear effects of insulin-deficient diabetes on bone without need to implement insulin therapy (which itself may affect bone) or cause undue stress to the animals. It is also notable that Nyman et al. stated ‘a few diabetic animals expired during the extended course of the study’ illustrating the value of shorter-term studies using mice not in terminal decline. Of course, this approach might not be enough long to observe significant effect of immediate diabetes on bone microarchitecture (trabecular and cortical) and might represent a limitation in this study. Nevertheless, T1DM is suspected to deteriorate “bone quality” and based on our previous studies in animal models, we hypothesized that deterioration of tissue material properties may appear early after the onset of diabetes.

Tissue material properties were also markedly reduced in STZ-diabetic mice as suggested by reduction in post-yield displacement and mechanical behavior at the tissue-level. Noteworthy, [D-Ala²]GIP or liraglutide treatment in STZ-diabetic mice was capable of restoring similar mechanical response at the tissue level despite hypoinsulinemia and hyperglycemia. In addition, it restored post-yield displacement values to those observed in control animals at the organ level. Bearing in mind that hypoinsulinemia was not corrected by [D-Ala²]GIP or liraglutide in diabetic mice, these results suggest that [D-Ala²]GIP and liraglutide acted on bone tissue independently of the insulin axis and that tissue material properties might be the primary target of incretin therapies in diabetic animal model. The collagen glycation index in newly-formed bone was markedly higher in untreated STZ-diabetic mice and is probably a consequence of the observed hyperglycemia. Since [D-Ala²]GIP and liraglutide also failed to reduce hyperglycemia in STZ-diabetic mice, the collagen glycation index was also unchanged. The collagen maturity, measured spectroscopically, was higher in untreated- and [D-Ala²]GIP- or liraglutide-treated STZ animals.
Previously Sato et al reported a lower value for immature collagen cross-link in T1DM (Saito et al., 2006). As the collagen maturity index reflects the level of mature over immature collagen cross-link ratio, it could be an explanation as to why this parameter is increased in the present study.

The observed degradation of the collagen triple helix is worthy of comment. The collagen integrity index has been previously validated as a measure of collagen integrity and is affected by enzymatic degradation of the collagen by matrix metalloproteinases (MMPs) as observed in cartilage samples harvested from osteoarthritic joints (West et al., 2005). In the present study, STZ-diabetic mice exhibited a marked reduction in this parameter suggesting an enzymatic degradation of the triple helix. T1DM is associated with a chronic inflammation and several reports have been made regarding higher matrix MMP activities in connective tissues, other than bone (Ning et al., 2012; Ryan et al., 1999; Symeonidis et al., 2013; Takahashi and Takasu, 2011). To the best of our knowledge, MMP activity has never been assessed in bone tissue of diabetic animals. However, it is plausible that MMP activities could be increased in T1DM due to the chronic inflammation state, and this could potentially participate to the reduced collagen integrity by cleaving the triple helix. Reduced collagen integrity index could also be linked in the reduction of post-yield displacement observed in 3-point bending experiment and tissue material properties investigated by nanoindentation. On the other hand, [D-Ala²]GIP or liraglutide treatments were capable of improving the collagen integrity index, not totally but by 43% and 52% respectively. The effects of [D-Ala²]GIP or liraglutide on MMP expression or activities are unknown. Nevertheless, it is possible that both molecules could interfere with either the expression or activity of MMPs pathways and might contribute to the reduced rate of collagen breakdown. However, further studies are needed to elucidate the mechanisms behind improvement of collagen integrity index.

In conclusion, we evidenced that STZ-diabetic mice presented with significant alterations of cortical microstructure and bone strength at the whole bone and tissue level in a relative short period. Treatment of STZ-diabetic mice with [D-Ala²]GIP or liraglutide was inefficient in hampering either cortical microstructure degradation or whole bone mechanical properties. On the other hand, these treatments significantly improved mechanical properties at the tissue level as well as collagen integrity within 21 days. Further studies are required to further elucidate the molecular mechanisms involved and to validate whether these findings can be translated to human patients.

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Figure 1: Metabolic and compositional parameters in CTRL and diabetic animals. (A) Body weight. CTRL (filled circle), untreated STZ mice (filled square), STZ mice treated with [D-Ala²]GIP (open circle) and STZ mice treated with liraglutide (open square). The bar represents the treatment period. (B) Fat mass. (C) Glucose and (D) Insulin levels. GIP: [D-Ala²]GIP, Lira: liraglutide. Values are means ± SEM ***: p<0.0001 vs. CTRL animals.
Figure 2: Histomorphometrical analysis in CTRL and diabetic animals. GIP: [D-Ala²]GIP, Lira: liraglutide. Values are means ± SEM for 6-8 mice. **p < 0.01, *p < 0.05 vs. CTRL group. $$: p<0.01, $: p<0.05$ vs. STZ-diabetic group. $$: p<0.01, #p<0.05$ vs. STZ+GIP group.
FIGURE 3

Figure 3: Effects of T1DM and once-daily administration of [D-Ala²]GIP or liraglutide on cortical bone mineral content and strength in STZ-induced diabetic mice. (A) GLmean values. (B) Bone strength was evaluated by three-point bending test. GIP: [D-Ala²]GIP, Lira: liraglutide. Values are means ± SEM for 6-8 mice. **P < 0.01, *P < 0.05 vs. CTRL. #: p<0.05 and ##: p<0.01 vs. STZ-diabetic animals.
FIGURE 4

Figure 4: Effects of T1DM and once-daily administration of [D-Ala²]GIP or liraglutide on cortical bone microstructure in STZ-induced diabetic mice. (A) Three-dimensional models of the midshaft tibia and (B) Cortical bone parameters. GIP: [D-Ala²]GIP, Lira: liraglutide. Values are means ± SEM for 6-8 mice. ***P < 0.001, *P < 0.05 vs. CTRL.
FIGURE 5

Figure 5: Effects of T1DM and once-daily administration of [D-Ala²]GIP or liraglutide on tissue material properties in STZ-induced diabetic mice. GIP: [D-Ala²]GIP, Lira: liraglutide. Values are means ± SEM for 6-8 mice. *: p<0.05, **: p<0.01, ***: p<0.001 vs. CTRL. #: p<0.05, ##: p<0.01 and ###: p<0.001 vs. STZ-diabetic animals.
Figure 6: Effects of T1DM and once-daily administration of [D-Ala²]GIP or liraglutide on tissue mineral density distribution in cortical bone. GIP: [D-Ala²]GIP, Lira: liraglutide. Values are means ± SEM for 6-8 mice.
**Figure 7**

Effects of T1DM and once-daily administration of [D-Ala²]GIP or liraglutide on collagen properties. GIP: [D-Ala²]GIP, Lira: liraglutide. Values are means ± SEM for 6-8 mice. ***p < 0.001, **p < 0.01, *p < 0.05 vs. CTRL group. ##: p<0.01 vs. STZ-diabetic group.