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Glucagon like peptide 2 has a positive impact on osteoporosis in ovariectomized rats

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ABSTRACT

Aims: In this study, we evaluate the effects of glucagon-like peptide 2 (GLP-2) on bone microarchitecture, bone turnover markers (BTMs) and inflammation markers in ovariectomized (OVX) rats.

Material and methods: In total, 31 Sprague-Dawley rats were divided into the following three groups: sham (control sham-operated with vehicle, n = 7), OV (OVX with vehicle, n = 12), and GLP-2 (OVX with GLP-2, n = 12). Intervention began at the 12th week after surgery and lasted for 4 weeks. The dosage of the GLP-2 was 160 μg/kg/d through subcutaneous injections, and normal saline was used as the vehicle agent. After 4 weeks of treatment, serum BTM and inflammation marker levels were measured by ELISA, and femora samples were analyzed by qRT-PCR, micro-CT, histology and histomorphometry.

Key findings: After 4 weeks of treatment, serum TRAcP-5b and RANKL levels as well as the CTX-1/P1NP ratio in the GLP-2 group decreased, and ALP activity, P1NP level, and OPG/RANKL ratio increased significantly; qRT-PCR analysis showed that mRNA levels of RANKL decreased, and Runx2, ALP, and Col-1 levels as well as the OPG/RANKL ratio increased significantly in the GLP-2 group compared with the OV group. In bone histology analysis, GLP-2 significantly decreased the AV/MV, Oc.N and Oc.S but increased the Ob.N, BFR and MAR. Analysis with μ-CT showed that the BMD, BV/TV, Tb.N and Conn.D increased significantly in the GLP-2 group compared with the OV group. The levels of serum inflammation markers TNF-α, IL-1β and IL-6 decreased, and TGF-β levels increased in the GLP-2 group compared with the OV group.

Significance: GLP-2 may have a positive impact on osteoporosis by promoting bone formation, inhibiting bone resorption and decreasing circulatory inflammation in ovariectomized rats.

1. Introduction

Osteoporosis is the reduction of bone mass and destruction of bone microarchitecture that is caused by the imbalance between bone formation and bone resorption. Postmenopausal osteoporosis is characterized by strong bone resorption relative to bone formation and bone mass loss accelerated during the late stage of menopause and is continued at a rapid pace for the next two decades [1]. At present, the treatment of osteoporosis is mainly to regulate bone resorption and bone formation balance; currently, the main treatments of osteoporosis are bone resorption inhibitors, and only full-length and truncated parathyroid hormones have been approved as anabolic agents for osteoporosis treatment [2]. Therefore, agents which could regulate bone resorption and bone formation simultaneously are needed in the future.

In recent years, some gastrointestinal hormones have been suggested as modulators of bone remodeling. Among these hormones, glucagon-like peptide 2 (GLP-2) plays crucial roles in this process. Glucagon-like peptide 2 (GLP-2) is a 33 amino acid single-chain peptide secreted by intestinal L cells, and it is a specific growth factor of gastrointestinal epithelium. The gastrointestinal growth-promoting effects of GLP-2 lead to significant enhancements in both small intestinal weight and crypt-villus height in mice [3].GLP-2 can also improve intestinal blood flow, improve absorption of nutrients [4], reduce intestinal permeability, reduce inflammatory reactions [5], and regulate glucose and lipid metabolism [6]. Therefore, GLP-2 plays a benefit role in gastrointestinal barrier, glucose and lipid metabolism. Because of the

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intimate connection between bone and gastrointestinal barrier, which the gastrointestinal barrier disruption could lead to the bone loss, GLP-2 might play a benefit role in bone turnover regulation [7]. Several studies have shown that GLP-2 transiently decreased the serum levels of C-telopeptide of type I collagen (CTX-1) [8–10]. Henriksen D.B. et al. found that after GLP-2 treatment, the serum level of CTX-1 decreased, but the levels of osteocalcin (OCN) and procollagen 1 amino-terminal propeptide (PINP) were unchanged in postmenopausal osteoporosis women [11,12]; after 4 months of continuous GLP-2 treatment, the bone mineral density (BMD) increased [13]. Our previous study showed that GLP-2 induced osteoclast apoptosis through the transforming growth factor β (TGF-β)-Smad 2/3 signaling pathway, inhibited the proliferation of osteoclasts [14] and promoted the transformation of osteosarcoma cells to osteoblasts in vitro [15]. However, until now, studies on the effects of GLP-2 in postmenopausal bone turnover were limited. Several studies from Henriksen D.B. et al. were limited by the small sample size; furthermore, their studies merely researched changes in the bone mineral density (BMD) and the serum levels of CTX-1, OCN and P1NP, but the detection of bone microstructure and other turnover markers (BTMs) were never investigated [12,13]. The experiments of Lu Y. et al. were only in vitro, but the effects of GLP-2 on osteoclasts or osteoblasts might be different in vivo and vitro. Therefore, the effect of GLP-2 on bone microstructure, BTMs and the anti-osteopenia mechanism in vivo are still unclear.

It was found that GLP-2 ameliorated inflammation in the treatment of Crohn's disease and inflammatory bowel disease (IBD) [16,17]. Furthermore, postmenopausal osteoporosis has been shown to be in close association with increased levels of inflammation [18,19]. Moreover, a number of studies have proved that an imbalanced ratio of nuclear factor kappa B receptor activator ligand (RANKL)/osteoprotegerin (OPG) caused by chronic inflammation is an important promoting factor of osteopenia [20–22]. Therefore, we suspected the anti-osteopenia effects of GLP-2 may be related to the amelioration of chronic low-grade inflammation. However, there has been no study on the anti-inflammation effects of GLP-2 in postmenopausal osteoporosis patients.

In this study, we used GLP-2 to detect changes in bone microstructure and morphology as well as serum levels of BTMs and inflammatory markers in ovariectomized rats and demonstrated the important role of GLP-2 in the treatment of osteoporosis.

2. Methods

2.1. Animals and material

2.1.1. Animal models

In total, 31 six-month-old Sprague-Dawley female rats (body weight 259.67 ± 15.42 g) were obtain from SIPPR/BK Laboratory (Shanghai, China) and raised at the experimental animal center of the Shanghai Medical College of Fudan University. The rats were housed under controlled ambient conditions (12-hour light/dark cycles, lights on at 6:00 a.m.) and had access to distilled water and the standard rodent diet ad libitum. All experimental procedures were approved by the Zhongshan Hospital Fudan University Animal Care and Use Committee, the ethical approval number is ZS20150212032.

After one week, the rats were randomly assigned into one of the following three groups: sham operation (sham, n = 7); ovariectomy (OVX) with GLP-2 (GLP-2) intervention (n = 12); and OVX with vehicle (OV, n = 12). All of the rats were anesthetized by an intraperitoneal (ip) injection of pentobarbital sodium (40 mg/kg) for the dorsal bilateral ovariectomy or control sham operation. The rats in the OV and GLP-2 group underwent bilateral ovary removal, and the sham group underwent a control sham operation that was the same as the ovariectomy surgical procedure but preserved the ovaries.

2.1.2. Intervention

Intervention began at the 12th week after surgery and lasted for 4 weeks. Rat GLP-2 was obtained from Tocris bioscience (Minneapolis, MN, USA) and was dissolved in normal saline (NS). The dosage of GLP-2 (160 μg/kg, daily subcutaneously injection) in the GLP-2 group were selected based on previous data in other trials [11]; NS was used as the vehicle agent. Equal volumes of NS were subcutaneously injected in the rats of the OV group and sham group. For the bone formation rate (BFR) and mineral apposition rate (MAR) analyses, the rats were treated with tetracycline (25 mg/kg, subcutaneous injection) 10 days before euthanasia and calcein (5 mg/kg, subcutaneous injection) 3 days before euthanasia. All of the interventions were performed at 10:00 a.m. After 4 weeks of treatment, one day before euthanasia, the animals were fasted for 12 h Then, rats in each group were anesthetized by pentobarbital sodium (50 mg/kg, ip injection) and then euthanatized by over collection of blood. The right femurs were prepared for micro-CT and dynamic histomorphometry analysis. The left femurs were prepared for HE staining, TRAP staining and toluidine blue staining. The bilateral tibias of all rats were prepared for qRT-PCR. The uterus was weighed to confirm the success of the ovariectomy. Centrifugation (3000 rpm, 15 min, 4 °C) was used to prepare the serum samples, and the serum/tissue samples were stored at −80 °C until they were needed for laboratory tests. A schematic timeline of the experimental procedure is shown in Fig. 1.

2.2. Biochemical analysis

Serum calcium (S-Ca) and phosphorous (S-P) concentrations were analyzed by an automated biochemistry analyzer (lei du biotechnology, Shen Zhen, China), Serum levels of BTMs and inflammation markers were measured by commercially available kits. Briefly, the following kits were included in this study: serum alkaline phosphatase (ALP) activity (BioAssay Systems, Hayward, CA USA); ELISA for procollagen 1 amino-terminal propeptide (P1NP, cusabio, Wuhan, Hubei, China); osteocalcin (OCN, ImmunoWay, Plano, TX, USA); serum C-terminal cross-linked telopeptides of type I collagen (CTX-1, ImmunoWay, Plano, TX, USA); tartrate-resistant acid phosphatase-5b (TRAcP-5b, AMEKO, Shanghai, China); osteoprotegerin (OPG, Cusabio, Wuhan, Hubei, China); and nuclear factor kappa B receptor activator ligand (RANKL Shanghai Jining, Shanghai, China). ELISA kits (Multisciences, Hangzhou, Zhejiang, China) of the following serum inflammatory markers were also included: tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and transforming growth factor β (TGF-β).

2.3. Bone analysis

2.3.1. Quantitative real-time polymerase chain reaction (qRT-PCR)

The bilateral tibias were dissected from the rats and cleaned with 0.1 M cold PBS (pH 7.2–7.4); the connective tissue and muscle were removed for RNA extraction and qRT-PCR, and then they were snap-frozen in liquid nitrogen to facilitate breakage. To collect the mRNA, all bones were homogenized separately in liquid nitrogen with a ceramic grinder. Total RNA was extracted using Trizol (Invitrogen, USA) according to the instructions and was reverse-transcribed using the Prime Script RT reagent kit with DNA Eraser (Takara Bio, Shiga, Japan); qRT-PCR was performed using SYBR-Green premix (Yeasen, Shanghai, China) and primers (Sangon Biotech, Shanghai, China) in a real-time detection system (ABI7500, USA). The relative expression level of mRNA was normalized to that of the endogenous control β-actin by using the $2^{-\Delta\Delta Ct}$ method. Table 1 shows the primer sequences of RANKL, OPG, RANK, OCN, ALP, runt-related transcription factor 2 (Runx2), collagen type I (Col-1), and β-actin.

2.3.2. Micro-computed tomography

All of the right femurs were dissected from the soft tissue, cleaned, fixed in 4% paraformaldehyde and transferred to 70% ethanol; then, they were studied by a SkyScan-1176 microcomputed tomography (μ-

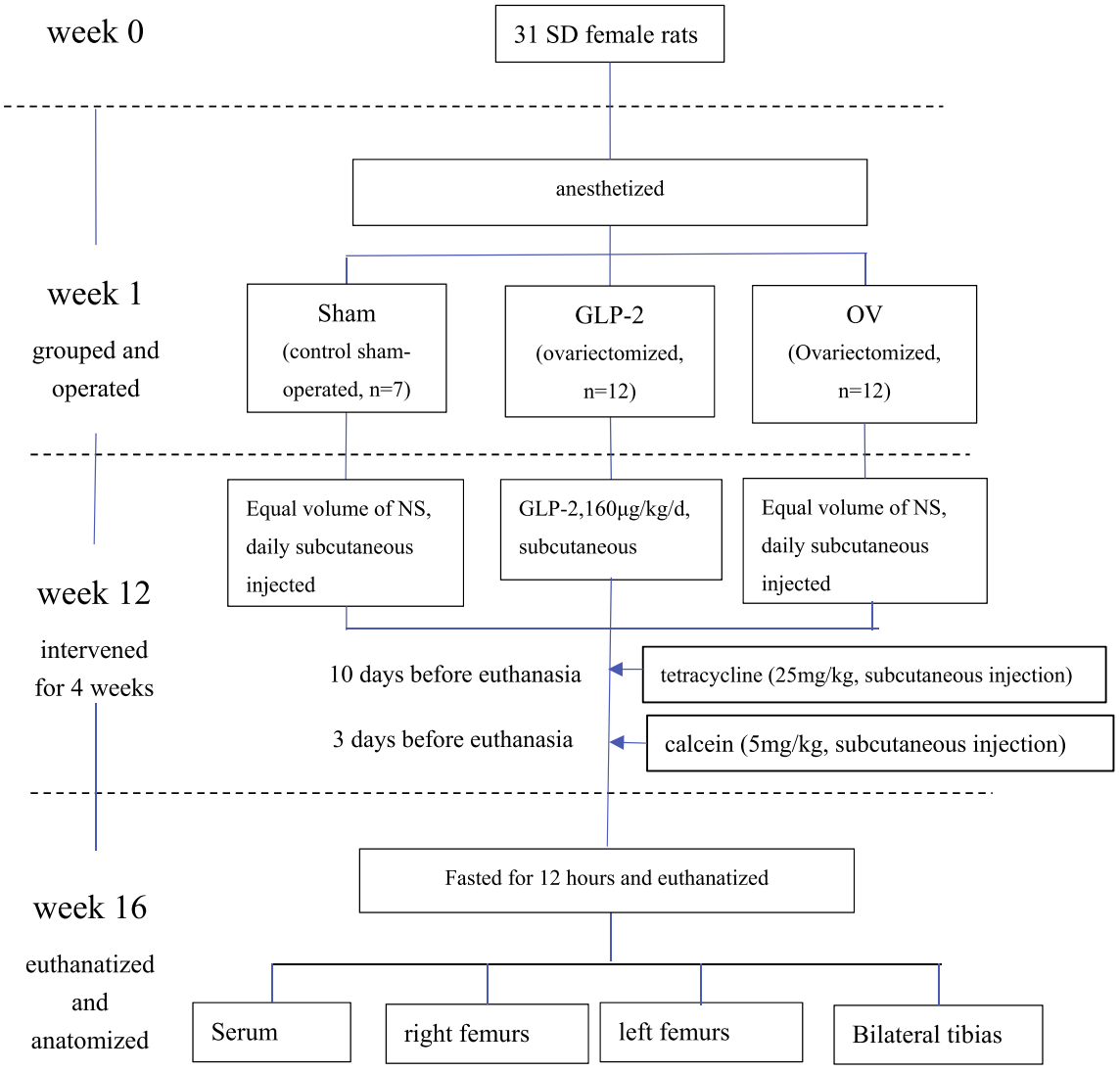

Fig. 1. Schematic timeline of the experimental procedure.
After raised in the animal center for one week, 31 six-month-old Sprague-Dawley female rats were divided into 3 groups: sham (sham-operated group, n = 7); OV (OVX with vehicle, n = 12); GLP-2 (OVX with GLP-2 intervention, n = 12). All rats in 3 groups were anesthetized (pentobarbital sodium, 40 mg/kg, ip injection) for operation. Sham group were underwent control sham operation, GLP-2 and OV group were bilateral ovariectomized. Then, all rats were raised in the same condition as before. Intervention began at 12th week after the surgery and lasted for 4 weeks. GLP-2 group were treated with GLP-2 (160 μg/kg, daily subcutaneous injected), OV group and sham group were treated with NS (Equal volume of vehicle, daily subcutaneous injected). 10 days before euthanasia rats were treated with tetracycline (25 mg/kg, subcutaneous injection) and 3 days before euthanasia, rats were treated with calcein (5 mg/kg, subcutaneous injection). At week 16, one day before euthanasia all rats in three groups were fasted for 12 h, then, the rats were anesthetized by pentobarbital sodium (50 mg/kg, ip injection) and euthanatized by over collection of blood. Serum was collected for ELISA, bilateral tibias for qRT-PCR analysis, right femurs for micro-CT and dynamic histomorphometry.

Table 1
The sequence of primers used in this study.

	Primers	Primer sequence (5′–3′)	Product length (bp)
ALP	Forward	ACTTCCCCAGCCCTTACTACCG	381
	Reverse	TCAGCACATAGCCCACACCG	
RANKL	Forward	TTGTGGAGCCTTAGCATGGA	134
	Reverse	TACAGGGTCGAGAGAGGACC	
OPG	Forward	GGAGAGTGAGGCAGGCTATTTGA	375
	Reverse	CTACAAATGGGATAAGGCTCGTG	
Runx2	Forward	AGTCCCAACTTCCTGTGCT	243
	Reverse	GGTGAAACTCTTGCCTCGTC	
Col 1	Forward	AACTTTGCTTCCCAGATGTCC	334
	Reverse	AGCCTCGGTGTCCCTTCA	
OCN	Forward	TGCTCACTCTGCTGACCCTG	109
	Reverse	TTATTGCCCTCCTGCTTG	
β-Actin	Forward	GCAGGAGTACGATGAGTCCG	74
	Reverse	ACGCAGCTCAGTAACAGTCC	

CT) system (Bruker micro-CT, Belgium). The scans were acquired using an 18 μm voxel size, 75 kV, 333 μA and 0.6 degree rotation steps (180 degrees angular range). For trabecular bone, the μ-CT evaluation was performed on a 2 mm^3 region of metaphyseal spongiosa in the distal femur. The regions were located 0.8 mm above the (femur) growth plate. NR econ software version 1.6 was used for the 3D reconstruction and visualization of the images. After the 3D reconstruction, version 1.13 of the CT software was used for bone analysis. The index of bone mineral density (BMD), bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular pattern factor (Tb.Pf), and connectivity density (Conn.D) were calculated or directly measured for the trabecular bone.

2.3.3. Histology and histomorphometry

For the bone histology and histomorphometry analyses, we used paraffin sections and double-labeled undecalcified bone sections. To

paraffinize the sections, all the left femurs from the rats were fixed in 4% paraformaldehyde (PFA), decalcified in 10% ethylenediaminetetraacetic acid (EDTA, pH 7.0, Sangon Biotech, China) as per the protocol, then embedded in paraffin and sectioned into five-μm-thick slices. The sections were stained with toluidine blue (solarbio, Beijing, China) to detect osteoblasts and calculated osteoblast number per millimeter trabecular surface (Ob.N), Tartrate-resistant acid phosphatase (TRAP, Sigma-Aldrich, USA) to detect osteoclasts, and calculated osteoclast number per millimeter trabecular surface (Oc.N) and osteoclast surface per millimeter trabecular surface (Oc.S), hematoxylin-eosin staining (HE, solarbio, Beijing, China) was used to evaluate the adipocyte volume per total bone marrow volume (AV/MV) [23]. These sections were photographed by an Olympus fluorescent microscope system.

For the bone formation rate (BFR) and mineral apposition rate (MAR) analyses, we used double-labeled undecalcified bones sections. The right distal femurs were separated, fixed in 70% ethanol, and embedded in methyl methacrylate. Finally, thirty-μm-thick double-labeled undecalcified sections were photographed by an Olympus fluorescent microscope system to analyze the MAR and BFR [23]. All of the primary measurements were measured by Imagine Pro Plus 6.0.

2.4. Statistical analysis

The data were expressed as the mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc., USA). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparisons test or the Kruskal-Wallis statistic followed by Dunn's post hoc multiple comparisons test. The sham group and GLP-2 group were each compared with the OV group. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Body weight and uterus index

As shown in Fig. 2, after OVX operation (1–12 week), the body weight increase rate in OV group and GLP-2 group was higher than that in sham group ($p < 0.01$), and there was no significantly difference between OV and GLP-2 group ($p > 0.05$). During the GLP-2 intervention (12–16 week), the body weight increase rate significantly increased at week 14, 15and 16 in GLP-2 group, compared with OV group ($p < 0.01$). The estrogen deficiency induced by OVX resulted in atrophy of the uterus, and the uterus index significantly decreased in the OV group and GLP-2 group compared with sham group ($p < 0.01$). GLP-2 treatment did not change the uterus index in the GLP-2 group compared with OV group.

3.2. The effects of GLP-2 on bone

3.2.1. Effects of GLP-2 treatment for 4 weeks on BTMs

Table 2 shows the effects of GLP-2 treatment on bone turnover markers. The S-Ca and S-P levels were not significantly changed in any group. Compared with the sham operation, the OVX nearly had no influence on serum bone formation markers, ALP activity and P1NP level in the OV group ($p > 0.05$) but increased the OCN level significantly compared with the sham group ($p < 0.05$). GLP-2 treatment in the GLP-2 group significantly increased the level of ALP activity and P1NP compared with the OV group ($p < 0.05$); however, GLP-2 treatment had almost no effect on OCN levels. The OVX significantly increased the levels of the bone resorption markers CTX-1, TRAcP-5b and RANKL as well as the CTX-1/P1NP ratio in the OV group compared with the sham group ($p < 0.05$ or $p < 0.01$), but the difference in the OPG level and OPG/RANKL ratio was not significant ($p > 0.05$). GLP-2 treatment had no influence on serum CTX-1 and OPG levels but significantly decreased the levels of TRAcP-5b and RANKL as well as the CTX-1/P1NP ratio and significantly increased the levels of ALP and P1NP as well as the OPG/RANKL ratio in the GLP-2 group compared with the OV group ($p < 0.05$ or $p < 0.01$).

3.2.2. GLP-2 affected the expression of genes related to bone formation and bone resorption

As shown in Fig. 3A, the expression of Runx2, ALP, Col-1 and OC mRNA were detected in all groups after 4 weeks of intervention. In the OV group compared with the sham group, the mRNA levels of Col1 and OCN were significantly increased, and the mRNA level of Runx2 was significantly decreased ($p < 0.05$ or $p < 0.01$), but the difference in ALP activity was not significant ($p > 0.05$). GLP-2 treatment significantly increased the mRNA levels of Runx2, Col1 and ALP in the GLP-2 group compared with the OV group ($p < 0.05$ or $p < 0.01$), but the difference in the mRNA level of OCN was not significant compared with the OV group ($p > 0.05$).

As shown in Fig. 3B, the expression of OPG and RANKL as well as the OPG/RANKL ratio were observed after 4 weeks of intervention. The mRNA levels of OPG and RANKL were significantly increased in the sham group compared with the OV group ($p < 0.05$ or $p < 0.01$), but the ratio of OPG/RANKL mRNA levels in the OV group was not significantly different from that of the sham group ($p > 0.05$). GLP-2 treatment reduced the mRNA level of RANKL, increased the mRNA level of OPG and increased the OPG/RANKL ratio in the GLP-2 group compared with the OV group ($p < 0.05$ or $p < 0.01$).

3.2.3. Bone histology and histomorphometry

As shown in Fig. 4, the effects of GLP-2 treatment on osteoblasts, osteoclasts and adipocytes were observed by histomorphometry. TRAP staining shows more positive cells in the OV group, compared with GLP-2 group (A). And toluidine blue staining shows more osteoblasts in

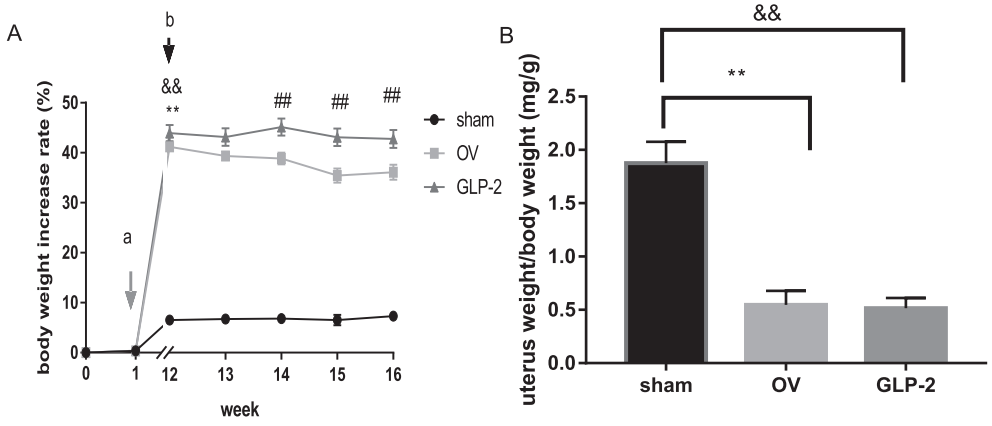

Fig. 2. Body weight and uterus index in ovariectomized rats. A: body weight increase rate Arrow a shows the time when the OVX began, arrow b shows the time when the GLP-2 treatment began. B: uterus index, uterus weight/body weight at week 16 (mg/g). 31 female SD rats were randomly divided into sham: sham-operated group. Values are means ± SEM OV: OVX treated with vehicle (n = 12); GLP-2: OVX treated with GLP-2 (n = 12). Values are expressed as means ± SEM. *p < 0.05, **p < 0.01, sham group compared with OV group, #p < 0.05, ##p < 0.01, GLP-2 group compared with OV group. & p < 0.05, && p < 0.01, GLP-2 group compared with sham group.

Table 2
Effects of 4 weeks treatment with GLP-2 on BTMs.

	Sham, n = 7	OV, n = 12	GLP-2, n = 12
S-Ca (mmol/L)	2.53 ± 0.0079	2.51 ± 0.00054	2.52 ± 0.0064
S-P (mmol/L)	1.92 ± 0.094	1.85 ± 0.075	1.91 ± 0.084
ALP activity (IU/L)	53.58 ± 6.33	57.220 ± 4.92	76.920 ± 8.78#
OCN (pg/ml)	2245 ± 166.7*	2832 ± 181.1	2669 ± 126.7
P1NP (pg/ml)	1306 ± 54.49	1185 ± 71.32	1497 ± 106.85#
CTX-1 (pg/ml)	4.946 ± 0.15**	6.685 ± 0.21	6.490 ± 0.25
TRAcP-5b (pg/ml)	104.200 ± 3.84*	116.400 ± 3.45	90.980 ± 4.18##
OPG (pg/ml)	84.730 ± 8.661	114.800 ± 30.510	171.800 ± 22.37
RANKL (pg/ml)	73.620 ± 5.83**	113.800 ± 4.10	89.790 ± 5.68##
OPG/RANKL	1.570 ± 037	1.236 ± 0.154	2.071 ± 0.36#
CTX-1/P1NP	0.003892 ± 0.00024**	0.005985 ± 0.00045	0.004593 ± 0.000282#

Values are mean ± SEM. S-Ca: serum calcium; S-P: serum phosphorous; ALP activity: serum alkaline phosphatase activity; OCN: osteocalcin; P1NP: procollagen 1 amino-terminal propeptide; CTX-1: type 1 collagen C-terminal peptide; TRAcP-5b: tartrate-resistant acid phosphatase-5b; OPG: osteoprotegerin; RANKL: receptor activating factor of nuclear κ B factor ligand; OPG/RANKL: OPG/RANKL ratio; CTX-1/P1NP: CTX-1/P1NP ratio. 31 female SD rats were randomly divided into sham: sham-operated group (n = 7) OV: OVX treated with vehicle (n = 12); GLP-2: OVX treated with GLP-2 (n = 12). *$p < 0.05$, **$p < 0.01$, sham group compared with OV group and #$p < 0.05$, ##$p < 0.01$, GLP-2 group compared with OV group.

GLP-2 group, compared with OV group (B), HE staining shows more and larger adipocytes in OV group, compared with GLP-2 group (C). After GLP-2 intervention, the width between the two labels increased (D). The quantified results show that GLP-2 treatment significantly decreased the Oc.N, Oc.S and AV/MV but increased the Ob.N, MAR, and BFR in the GLP-2 group compared with the OV group ($p < 0.05$ or $p < 0.01$) (E).

3.2.4. GLP-2 was beneficial to trabecular bone microarchitecture

Fig. 5 shows representative samples of the three-dimensional images of the femoral metaphysis, in which differences in the trabecular microarchitecture can be observed. In these results, the OVX group had a lower amount of trabecular bone and more broken trabecular bone in the distal femurs compared with sham group. After GLP-2 treatment, structure of the trabecular bone was significantly improved in the GLP-2 group compared with the OV group.

Table 3 shows the analysis of parameters that affect trabecular microarchitecture. Compared with the sham procedure, the BMD, BV/TV, Tb.N, and Conn.D in the OV group decreased significantly by 62.77%, 59.12%, 56.04%, and 69.68%, respectively; and the Tb.Sp and Tb.Pf increased significantly by 114.29% and 56.30%, respectively ($p < 0.01$). After GLP-2 treatment, the BMD, BV/TV, Tb.N, and Conn.D in the GLP-2 group increased significantly by 34.29%, 29.94%, 22.06%, and 38.3%, respectively, compared with the OV group ($p < 0.05$ or $p < 0.01$), The Tb.Pf decreased significantly by 15.73% ($p < 0.01$) in the GLP-2 group compared with the OV group, but the difference in Tb.Sp was not significant between the two groups ($p > 0.05$).

3.3. Serum inflammation markers

As shown in Table 4, there was no significant difference in the serum TNF-α , IL-1β , and IL-6 levels ($p > 0.05$) between the sham and OV groups, but the level of TGF-β was significantly decreased in the OV group compared with the sham group ($p < 0.01$). GLP-2 treatment significantly decreased the TNF-α, IL-1β, and IL-6 levels in the GLP-2 group compared with the OV group ($p < 0.05$) and increased the TGF-β level in the GLP-2 group compared with the OV group ($p < 0.05$).

4. Discussion

Some studies have shown that GLP-2 participates in the regulation of postprandial serum bone turnover markers [9,24]. However, associations between osteoporosis and GLP-2 are less established. So far, to the best of our knowledge, no study has analyzed the microarchitecture of bone tissue and detailed changes of BTMs after GLP-2 intervention in postmenopausal osteoporosis yet. Therefore, we performed a study to explore the relationship of GLP-2 on BTMs, microarchitecture of trabecular bone and inflammation markers that are related to osteoporosis.

In our study, the analysis of serum BTMs showed that GLP-2 treatment decreased serum bone resorption markers TRAcP-5b and RANKL but increased the ratio of serum OPG/RANKL levels. As far as we know, this is the first time these BTM levels have been measured after GLP-2 treatment. TRAcP-5b is considered as a marker of osteoclastic activity, and OPG is a decoy receptor that inhibits the activity of RANKL, which promotes osteoclast genesis; therefore, the ratio of OPG/RANKL expression is also believed to be a key in osteoclastogenic activity [25]. Our results showed that GLP-2 might inhibit osteoclast activity, which in turn inhibits bone resorption. However, to our surprise, we found that the level of CTX-1 remained unchanged in our study, which is different from a previous report that GLP-2 administration reduced serum CTX-1 levels [9,12,13]. According to Carsten Askov-Hansen et al. [11], serum CTX-1 concentrations were significantly decreased between 2 h to 6 h after subcutaneous injections of exogenous GLP-2. However, all of the rats in our experiment were anatomized 24 h after the last GLP-2 intervention; therefore, the transitional decrease of serum CTX-1 levels might not be observed. Furthermore, these three studies [9,12,13] were all conducted in humans; there has been no report on the effect of GLP-2 on CTX-1 suppression in rodents until now. The precise sites of GLP-2R expression in different animals and in humans are controversial, and the localization of GLP-2R in humans and rats might differ [26]; the different expression patterns of GLP-2R might lead to differences in the mechanism of GLP-2 between humans and rats, but the mechanism remain unclear and further studies are still be needed. In terms of the bone formation markers, our results showed GLP-2 increased the activity of bone formation markers P1NP and ALP and decreased the ratio of CTX-1/P1NP. This suggests that GLP-2 might increase bone formation; however, the expression of another bone formation marker, OCN, did not change significantly, which is a result that was also reported in the study from Henriksen D.B. et al. [13]. P1NP is related to the synthesis of bone collagen, while OCN is mainly related to bone mineralization [27,28]. Therefore, our results suggest that GLP-2 might only promote the synthesis of collagen in osteoblasts but does not promote bone mineralization. The ratio of CTX-1/P1NP is related to the relative rate of bone collagen degradation and synthesis and thus our result shows that the synthesis of bone collagen was promoted after GLP-2 treatment. Therefore, our analysis of serum levels of BTMs suggests that GLP-2 might participate in the anti-osteopenia process through both the suppression of bone resorption and promotion of bone formation.

To confirm the bone turnover-modulating effect of GLP-2 on gene expression level, we measured the mRNA levels of markers that are

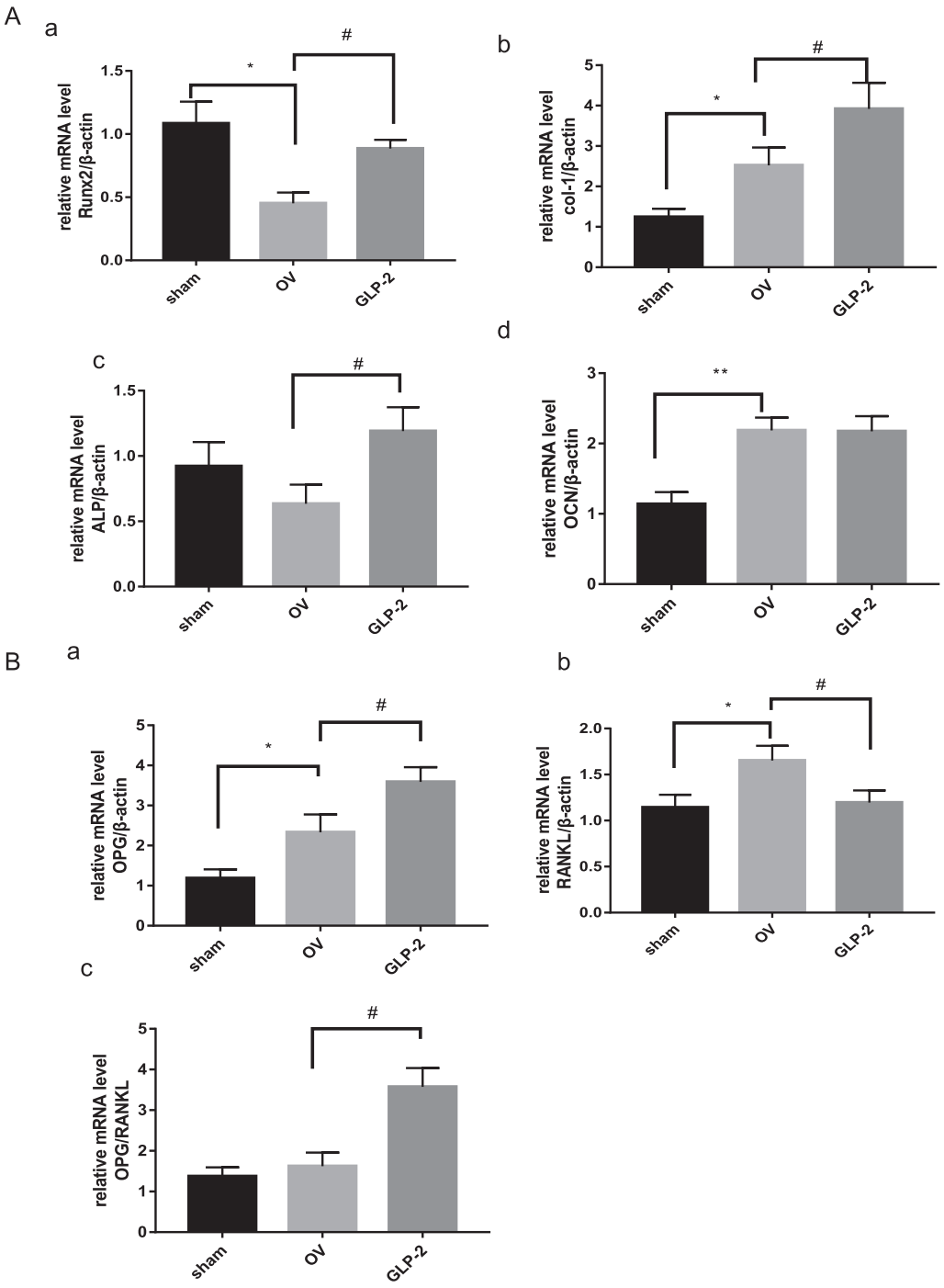

Fig. 3. mRNA level of bone formation and bone absorption related gene. After treated with GLP-2 for 4 weeks, rats' tibias were analyzed by qRT-PCR. A. (a) Runt-related transcription factor 2 (Runx2); (b) collagen type 1(Col1); (c) alkaline phosphatase (ALP); (d) osteocalcin (OCN); B. (a) (OPG) osteoprotegerin; (b) receptor activating factor of nuclear κB factor ligand (RANKL); (c) OPG/RANKL ratio. 31 female SD rats were randomly divided into sham: sham-operated group. Values are expressed as means ± SEM OV: OVX treated with vehicle (n = 12); GLP-2: OVX treated with GLP-2 (n = 12). Values are expressed as means ± SEM. *p < 0.05, **p < 0.01, sham group compared with OV group and #p < 0.05, ##p < 0.01, GLP-2 group compared with OV group.

related to bone formation and bone resorption by qRT-PCR. Our data show that GLP-2 decreased osteoclast genesis and the related ratio of OPG/RANKL mRNA levels and increased osteoblast genesis and the related mRNA levels of Runx2, Col1, and ALP but did not change OCN levels, which is consistent with the results from the analysis on serum BTM levels. Runx2 is a transcription factor that regulates osteoblast and lipocyte differentiation from BMMSCs [29]; ALP and Col1 are the early-stage markers of osteoblast genesis, and OCN is the late-stage marker [30]. Our results show that GLP-2 promotes bone formation in the early stage of osteoblast genesis, but GLP-2 may not have an effect in the late stage; furthermore, GLP-2 might inhibit osteoclast genesis by regulating the OPG/RANKL ratio at the mRNA level.

Inflammation is a factor in osteoporosis deterioration, but the anti-inflammation effect of GLP-2 in postmenopausal osteoporosis has not yet been reported. Therefore, we detected the changes in serum inflammatory marker levels in our subjects after 4 weeks of GLP-2 treatment. It was found that GLP-2 could decrease the serum levels of TNF-α, IL-1β, and IL-6 and increase the level of TGF-β, which is consistent with previous studies on the effect of GLP-2 on inflammatory bowel disease (IBD) [31–33]. Numerous previous studies have shown that increased levels of TNF-α, IL-1β, and IL-6 promotes bone resorption [20,21]. TGF-β is hypothesized to play an important role in promoting the development and proliferation of early osteoblasts, but inhibits osteoblast maturation and osteoclast proliferation [21]; therefore, the changes in serum inflammation marker levels may lead to the change of bone turnover-related gene expression in bone tissue. Thus, our results suggest that GLP-2 might benefit osteoporosis by regulated circulating inflammatory factors.

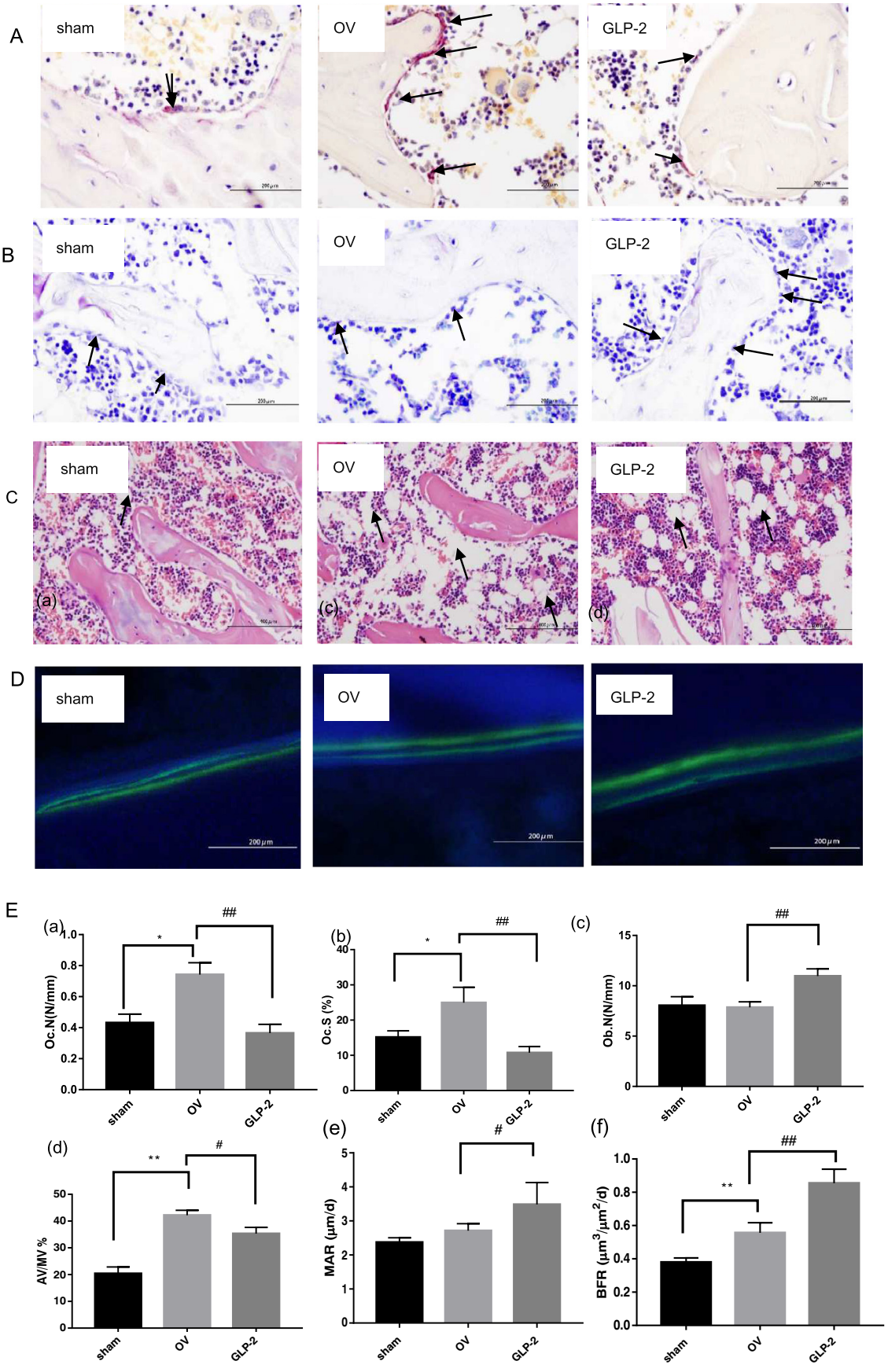

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Fig. 4. Bone histology and histomorphometry analyzation. (A) Tartrate-resistant acid phosphatase (TRAP) staining, osteoclasts were stained in red. Scale bar, 800 μm (B) toluidine blue staining Scale bar, 800 μm (C) hematoxylin and eosin staining, Scale bar,800 μm, (D) double-labeling with tetracycline and calcein. Scale bar, 400 μm (E): (a) Oc.N: the number of osteoclasts per millimeter trabecular surface was calculated (N/mm) (b): Oc.S: Oc. osteoclast surface per millimeter trabecular surface. (c) Ob.N: the numbers of osteoblasts per millimeter of trabecular bone surface (BS) were counted. (d) AV/MV: adipocyte volume per total bone marrow volume (e): mineral apposition rate (MAR); (f) bone formation rate (BFR). Values are expressed as means ± SEM. 31 female SD rats were randomly divided into sham: sham-operated group (n = 7). OV: OVX treated with vehicle (n = 12); GLP-2: OVX treated with GLP-2(n = 12). *p < 0.05, **p < 0.01, sham group compared with OV group and #p < 0.05, ##p < 0.01 GLP-2 group compared with OV group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

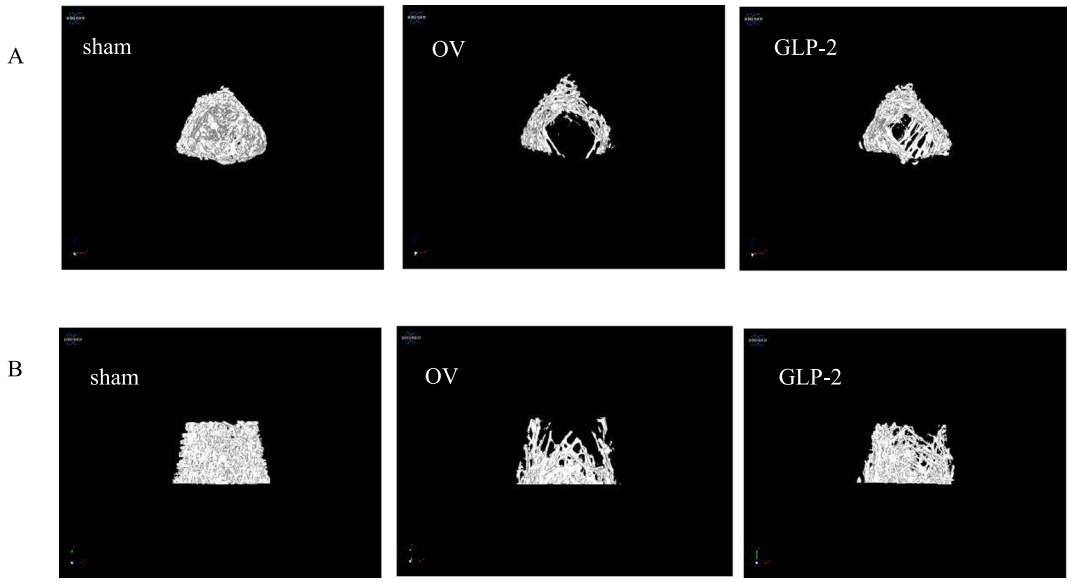

Fig. 5. 3-Dimensional reconstruction image of bone μ-CT. (A) Transverse scan of distal femur. (B) Coronal scan of distal femur. Sham: sham-operated group; OV: OVX treated with vehicle; GLP-2: OVX treated with GLP-2.

Table 3
μCT assessment of trabecular microarchitecture after GLP-2 treatment.

	Sham, n = 7	OV, n = 12	GLP-2, n = 12
BMD	0.094 ± 0.0015**	0.035 ± 0.0035	0.047 ± 0.0025#
BV/TV (%)	23.890 ± 0.34**	9.766 ± 0.80	12.690 ± 0.65#
Tb.Th (mm)	0.109 ± 0.00076	0.111 ± 0.0017	0.111 ± 0.0020
Tb.Sp (mm)	0.385 ± 0.0099**	0.825 ± 0.046	0.738 ± 0.024
Tb.N (1/mm)	2.186 ± 0.020**	0.961 ± 0.065	1.173 ± 0.057#
Tb.Pf (1/mm)	7.607 ± 0.33**	11.890 ± 0.53	10.020 ± 0.25##
Conn.D (mm^3)	0.00031 ± 0.000012**	0.000094 ± 0.0000087	0.00013 ± 0.0000090#

Values are mean ± SEM. BMD: bone mineral density, BV/TV: bone volume fraction; Tb.Th: trabecular thickness: Tb.Sp: trabecular spacing; Tb.N: trabecular number; Tb.Pf: trabecular pattern factor; Conn.D: connectivity density. Sham: sham-operated group(n = 7); OV: OVX treated with vehicle (n = 12); GLP-2: OVX treated with GLP-2 (n = 12) *p < 0.05, **p < 0.01, OV group compared with sham group and #p < 0.05, ##p < 0.01 GLP-2 group compared with OV group.

Table 4
Effects of 4 weeks treatment with GLP-2 on inflammation markers.

	Sham, n = 7	OV, n = 12	GLP-2, n = 12
TNF-α (pg/ml)	2.386 ± 0.11	2.574 ± 0.09	2.206 ± 0.072#
IL-1β (pg/ml)	304.800 ± 67.35	526.100 ± 118.44	208.400 ± 46.800#
IL-6 (pg/ml)	593.800 ± 37.62	924.200 ± 36.98	537.900 ± 21.59#
TGF-β (pg/ml)	10,945.000 ± 762.35**	7165.000 ± 445.14	8431.000 ± 280.1#

Values are mean ± SEM. Tumor necrosis factor α (TNF-α), interleukin 1 β (IL-1β), transforming growth factor β (TGF-β), interleukin 6 (IL-6). During the course of treatment, 31 female SD rats were randomly divided into sham: sham-operated group (n = 7) OV: OVX treated with vehicle (n = 12); GLP-2: OVX treated with GLP-2 (n = 12) *p < 0.05, **p < 0.01, sham group compared with OV group and #p < 0.05, ##p < 0.01, GLP-2 group compared with OV group.

To confirm the effect of GLP-2 on the bone density and microarchitecture of bone, we used μ-CT and found that the BMD increased 34.29% after 4 weeks of GLP-2 intervention; GLP-2 also improved the microstructure of trabecular bone and its parameters. The effects of GLP-2 on BMD were consistent with Henriksen D.B. et al. [13]; low BMD and deteriorated microarchitecture in trabecular bone were associated with aggravated osteoporosis and an increased bone fracture risk [34,35]. This μ-CT analysis indicated that GLP-2 protected against deterioration of the trabecular bone structure, which could be of benefit to osteoporosis. Then, we further examined the generation of

osteoblasts and osteoclasts on the surface of trabecular bone. GLP-2 decreased Oc.N, Oc.S and the AV/MV but increased Ob.N, BFR, and MAR in the bone histology analysis. These results might be caused by changes at the mRNA level of bone turnover-related genes that we measured above. Therefore, the analysis of the bone histology further confirmed that GLP-2 alleviates osteopenia by decreasing bone resorption and increasing bone formation.

Furthermore, we found that GLP-2could increase the body weight increase rate in ovariectomized rats, which is a result that was also reported in the study from Lu Y. et al. [14]. Weight increase has been considered as one of the positive factors for anti-osteopenia and increasing bone strength [36,37]. The reason may be that moderate mechanical loading can promote bone formation [38].

However, though we found GLP-2 could participate in the anti-osteopenia process, the mechanism was still unclear and whether GLP-2R exists on the surface of osteoblasts or osteoclasts remains uncertain [26]. According to our previous vitro study, GLP2 could decrease the number of osteoclasts induced from RAW264.7 cells and induce the apoptosis of osteoclasts [14]. The mechanism might be that GLP2 stimulates nitric oxide (NO) in osteoclasts and directly promotes osteoclast apoptosis through the TGF-β-Smad 2/3 signaling pathway [14]; if GLP-2R is located in enteric neurons in the wall of the small intestine, the GLP-2 signal might be transmitted via sympathetic or parasympathetic sensory neurons to activate neurons that innervate bone [11]. In addition, GLP-2 could improve intestinal barrier functions and absorption of nutrients [26]; an estrogen deficiency may cause the function of the intestinal barrier to deteriorate [19], so we suspected that GLP-2 may also alleviate postmenopausal osteoporosis by improving intestinal barrier functions and nutrition absorption. Therefore, GLP-2 may affect bone formation and resorption through direct or indirect pathways. The exact mechanism of GLP-2 still needs further investigation.

5. Conclusion

In conclusion, these findings show that GLP-2 treatment was beneficial to bone metabolism and its microstructure; GLP-2 may have a positive impact on osteoporosis in OVX rats by promoting bone formation and inhibiting bone resorption. We are also the first to show that GLP-2 decreased circulatory inflammation in OVX rats, which may benefit bone turnover. However, the anti-osteopenia mechanism of GLP-2 remains unclear, and further studies are still needed in the future.

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Author contribution

Study & experimental design, Bing'er Xu, Yu Hu, Weiying Ren;
Experimental operation & data analysis, Bing'er Xu, Yuting He, Yi Lu, Jiping Shen, Kefen Wu, Kan Xu, and Jiayu Wu;
Manuscript preparation, Bing'er Xu, Yu Hu;
Manuscript review & editing, Yu Hu.; Funding acquisition, Yu Hu.

Conflict of interest

All authors have no conflicts of interest.

References

[1] D. Lizneva, T. Yuen, L. Sun, S.M. Kim, I. Atabiekov, L.B. Munshi, S. Epstein, M. New, M. Zaidi, Emerging concepts in the epidemiology, pathophysiology, and clinical care of osteoporosis across the menopausal transition, Matrix Biol. 71-72 (2018) 70–81 l.
[2] A. Qaseem, M.A. Forciea, R.M. McLean, T.D. Denberg, Treatment of low bone density or osteoporosis to prevent fractures in men and women: a clinical practice guideline update from the American College of Physicians, Ann. Intern. Med. 166 (2017) 818–839 l.
[3] D.J. Drucker, P. Erlich, S.L. Asa, P.L. Brubaker, Induction of intestinal epithelial proliferation by glucagon-like peptide 2, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7911–7916 l.
[4] X. Guan, B. Stoll, X. Lu, K.A. Tappenden, J.J. Holst, B. Hartmann, D.G. Burrin, GLP-2-mediated up-regulation of intestinal blood flow and glucose uptake is nitric oxide-dependent in TPN-fed piglets 1, Gastroenterology 125 (2003) 136–147 l.
[5] W. Ren, J. Wu, L. Li, Y. Lu, Y. Shao, Y. Qi, B. Xu, Y. He, Y. Hu, Glucagon-like peptide-2 improve intestinal mucosal barrier function in aged rats, J. Nutr. Health Aging 22 (2018) 731–738 l.
[6] S. Baldassano, A. Amato, G.F. Caldara, F. Mule, Glucagon-like peptide-2 treatment improves glucose dysmetabolism in mice fed a high-fat diet, Endocrine 54 (2016) 648–656 l.
[7] N.D. Rios-Arce, F.L. Collins, J.D. Schepper, M.D. Steury, S. Raehtz, H. Mallin, D.T. Schoenherr, N. Parameswaran, L.R. McCabe, Epithelial barrier function in gut-bone signaling, Adv. Exp. Med. Biol. 1033 (2017) 151–183 l.
[8] D.B. Henriksen, P. Alexandersen, N.H. Bjarnason, T. Vilsboll, B. Hartmann, E.E. Henriksen, I. Byrjalsen, T. Krarup, J.J. Holst, C. Christiansen, Role of gastrointestinal hormones in postprandial reduction of bone resorption, J. Bone Miner. Res. 18 (2003) 2180–2189 l.
[9] L.S. Lopes, R.P. Schwartz, B. Ferraz-de-Souza, S.M. Da, P.H. Correa, M. Nery, The role of enteric hormone GLP-2 in the response of bone markers to a mixed meal in postmenopausal women with type 2 diabetes mellitus, Diabetol. Metab. Syndr. 7 (2015) 13 l.
[10] D.B. Henriksen, P. Alexandersen, I. Byrjalsen, B. Hartmann, H.G. Bone, C. Christiansen, J.J. Holst, Reduction of nocturnal rise in bone resorption by subcutaneous GLP-2, Bone 34 (2004) 140–147 l.
[11] C. Askov-Hansen, P.B. Jeppesen, P. Lund, B. Hartmann, J.J. Holst, D.B. Henriksen, Effect of glucagon-like peptide-2 exposure on bone resorption: effectiveness of high concentration versus prolonged exposure, Regul. Pept. 181 (2013) 4–8 l.
[12] D.B. Henriksen, P. Alexandersen, B. Hartmann, C.L. Adrian, I. Byrjalsen, H.G. Bone, J.J. Holst, C. Christiansen, Disassociation of bone resorption and formation by GLP-2: a 14-day study in healthy postmenopausal women, Bone 40 (2007) 723–729 l.
[13] D.B. Henriksen, P. Alexandersen, B. Hartmann, C.L. Adrian, I. Byrjalsen, H.G. Bone, J.J. Holst, C. Christiansen, Four-month treatment with GLP-2 significantly increases hip BMD: a randomized, placebo-controlled, dose-ranging study in postmenopausal women with low BMD, Bone 45 (2009) 833–842 l.
[14] Y. Lu, D. Lu, Y. Hu, Glucagon-like peptide 2 decreases osteoclasts by stimulating apoptosis dependent on nitric oxide synthase, Cell Prolif. 51 (4) (2018) e12443 l.
[15] Y. Lu, D. Lu, Y. Hu, GLP2 promotes directed differentiation from osteosarcoma cells to osteoblasts and inhibits growth of osteosarcoma cells, Mol. Ther. Nucleic Acids 10 (2018) 292–303 l.
[16] J. Gu, J. Liu, T. Huang, W. Zhang, B. Jia, N. Mu, K. Zhang, Q. Hao, W. Li, W. Liu, W. Zhang, Y. Zhang, X. Xue, C. Zhang, M. Li, The protective and anti-inflammatory effects of a modified glucagon-like peptide-2 dimer in inflammatory bowel disease, Biochem. Pharmacol. 155 (2018) 425–433 l.
[17] S.E. Alters, B. McLaughlin, B. Spink, T. Lachinyan, C.W. Wang, V. Podust, V. Schellenberger, W.P. Stemmer, GLP2-2G-XTEN: a pharmaceutical protein with improved serum half-life and efficacy in a rat Crohn's disease model, PLoS One 7 (2012) e50630 l.
[18] N.M. Al-Daghri, I. Aziz, S. Yakout, N.J. Aljohani, Y. Al-Saleh, O.E. Amer, E. Sheshah, G.Z. Younis, F.B. Al-Badr, Inflammation as a contributing factor among postmenopausal Saudi women with osteoporosis, Medicine (Baltimore) 96 (2017) e5780 l.
[19] F.L. Collins, N.D. Rios-Arce, S. Atkinson, H. Bierhalter, D. Schoenherr, J.N. Bazil, L.R. McCabe, N. Parameswaran, Temporal and regional intestinal changes in permeability, tight junction, and cytokine gene expression following ovariectomy-induced estrogen deficiency, Phys. Rep. 5 (2017) l.
[20] A.R. Moschen, A. Kaser, B. Enrich, O. Ludwiczek, M. Gabriel, P. Obrist, A.M. Wolf, H. Tilg, The RANKL/OPG system is activated in inflammatory bowel disease and relates to the state of bone loss, Gut 54 (2005) 479–487 l.
[21] M.N. Weitzmann, The role of inflammatory cytokines, the RANKL/OPG axis, and the immunoskeletal interface in physiological bone turnover and osteoporosis, Scientifica (Cairo) 2013 (2013) 125705 l.
[22] S.D. Brincat, M. Borg, G. Camilleri, J. Calleja-Agius, The role of cytokines in postmenopausal osteoporosis, Minerva Ginecol. 66 (2014) 391–407 l.
[23] D.W. Dempster, J.E. Compston, M.K. Drezner, F.H. Glorieux, J.A. Kanis, H. Malluche, P.J. Meunier, S.M. Ott, R.R. Recker, A.M. Parfitt, Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee, J. Bone Miner. Res. 28 (2013) 2–17 l.
[24] J.J. Holst, B. Hartmann, I.B. Gottschalck, P.B. Jeppesen, J. Miholic, D.B. Henriksen, Bone resorption is decreased postprandially by intestinal factors and glucagon-like peptide-2 is a possible candidate, Scand. J. Gastroenterol. 42 (2007) 814–820 l.
[25] A.E. Kearns, S. Khosla, P.J. Kostenuik, Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulation of bone remodeling in health and disease, Endocr. Rev. 29 (2008) 155–192 l.
[26] D.J. Drucker, B. Yusta, Physiology and pharmacology of the enteroendocrine hormone glucagon-like peptide-2, Annu. Rev. Physiol. 76 (2014) 561–583 l.
[27] Y.T. Tsao, Y.J. Huang, H.H. Wu, Y.A. Liu, Y.S. Liu, O.K. Lee, Osteocalcin mediates biomineralization during osteogenic maturation in human mesenchymal stromal cells, Int. J. Mol. Sci. 18 (2017) l.

[28] M.G. Vervloet, V.M. Brandenburg, Circulating markers of bone turnover, J. Nephrol. 30 (2017) 663–670 l.
[29] J. Wei, J. Shimazu, M.P. Makinistoglu, A. Maurizi, D. Kajimura, H. Zong, T. Takarada, T. Lezaki, J.E. Pessin, E. Hinoi, G. Karsenty, Glucose uptake and Runx2 synergize to orchestrate osteoblast differentiation and Bone formation, Cell 161 (2015) 1576–1591 l.
[30] F. Pagani, C.M. Francucci, L. Moro, Markers of bone turnover: biochemical and clinical perspectives, J. Endocrinol. Investig. 28 (2005) 8–13 l.
[31] J. Wu, K. Qi, Z. Xu, J. Wan, Glucagon-like peptide-2-loaded microspheres as treatment for ulcerative colitis in the murine model, J. Microencapsul. 32 (2015) 598–607 l.
[32] C.P. Ivory, L.E. Wallace, D.M. McCafferty, D.L. Sigalet, Interleukin-10-independent anti-inflammatory actions of glucagon-like peptide 2, Am. J. Physiol. Gastrointest. Liver Physiol. 295 (2008) G1202–G1210 l.
[33] H.A. Redstone, W.D. Buie, D.A. Hart, L. Wallace, P.J. Hornby, S. Sague, J.J. Holst, D.L. Sigalet, The effect of glucagon-like peptide-2 receptor agonists on colonic anastomotic wound healing, Gastroenterol. Res. Pract. 2010 (2010) l.
[34] B.A. Christiansen, S.L. Harrison, H.A. Fink, N.E. Lane, Incident fracture is associated with a period of accelerated loss of hip BMD: the study of osteoporotic fractures, Osteoporos. Int. 29 (10) (2018) 2201–2209 l.
[35] W.D. Leslie, S.R. Majumdar, S.N. Morin, D. Hans, L.M. Lix, Change in Trabecular Bone Score (TBS) with antiresorptive therapy does not predict fracture in women: the Manitoba BMD Cohort, J. Bone Miner. Res. 32 (2017) 618–623 l.
[36] D.J. van der Voort, P.P. Geusens, G.J. Dinant, Risk factors for osteoporosis related to their outcome: fractures, Osteoporos. Int. 12 (2001) 630–638 l.
[37] J. van Leeuwen, B.W. Koes, W.D. Paulis, M. van Middelkoop, Differences in bone mineral density between normal-weight children and children with overweight and obesity: a systematic review and meta-analysis, Obes. Rev. 18 (2017) 526–546 l.
[38] G.L. Galea, L.E. Lanyon, J.S. Price, Sclerostin's role in bone's adaptive response to mechanical loading, Bone 96 (2017) 38–44 l.