Original Article
GF109203X attenuates RANKL-induced osteoclastogenesis and suppresses osteolysis in a mouse model

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Abstract: Aseptic loosening after joint replacement is primarily caused by wear particle-induced osteolysis, which shortens the life of the prosthesis. Research shows that there are many osteolytic cytokines around prostheses that are loosened due to wear particles. Among these, receptor activator of nuclear factor κB ligand (RANKL) is the only factor that can directly stimulate the formation and functional activation of osteoclasts. This study aimed to inhibit the signaling path of RANKL-induced osteoclastogenesis is a promising prevention and treatment strategy for wear particle-induced osteolysis. Based on our previous research, it was confirmed that GF109203X attenuates osteoclastogenesis by inhibiting RANKL-induced osteoclast differentiation. We established a mouse model of polyethylene (PE) particle-induced osteolysis to study the effect of a protein kinase C (PKC) inhibitor on osteoclastogenesis in vivo. We found that by inhibiting the combination of RANKL and receptor activator of nuclear factor κB (RANK), which mediates the signaling paths of osteoclastogenesis, GF109203X acted in a dose-dependent manner to suppress the differentiation and functional activity of osteoclasts, reduce osteoclast formation, decrease the expression of osteoclast-associated receptor (OSCAR) and cross linked C-telopeptide of type I collagen (CTX-1), and promote the expression of osteoprotegerin (OPG). In addition, the degree of bone destruction was inhibited, bone resorption pitting and area were reduced, and the symptoms of osteolytic disease were lessened. This research helped clarify that GF109203X may provide a new option for the treatment of osteolytic disease.

Keywords: PKC inhibitor, GF109203X, RANKL, osteoclast, osteolysis

Introduction

At present, joint replacement therapy is the primary treatment option for osteoarthritis. However, the most important complication after joint replacement surgery is loosening of the prosthesis, which has no ideal treatment [1, 2]. Although the most common method of treating aseptic loosening of artificial joints is joint revision surgery, this method cannot guarantee the stability of the prosthesis and even affect the patient's daily activity and quality of life. Researchers have increasingly studied the wear particle-induced aseptic loosening of the prostheses, since finding and developing a specific drug to overcome bone dissolution by inhibiting or blocking the progress of wear particle-induced osteolysis [3], would reduce patients' pain and economic burden greatly.

Aseptic loosening of the prosthesis is mainly due to mechanical and biological factors and results from a biological inflammatory mediator reaction [4-6]. Recent studies consider the release of wear particles into the surrounding prosthesis and the resultant bone dissolution to be the key factor in triggering prosthetic aseptic loosening [7, 8]. Crotti et al [9] and Kim et al [10]. found that loosening of the prosthesis is not only associated with a large number of inflammatory cells and cytokines produced by surrounding tissue, such as interleukelin-6 (IL-6), interleukelin-1 (IL-1), tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE-2), etc., but also is associated with the expression of RANKL, RANK and OPG. Moreover, studies have suggested that these cells, especially macrophage, produce a variety of cytokines which are the activator of RANKL [11]. The combination of

RANKL and RANK promotes osteoclast differentiation and maturation and prevents apoptosis. It is the key factor leading to osteolysis around the prosthesis. The osteoblast and marrow stromal cells produce OPG, which combined with RANK and RANKL, blocks RANKL-induced osteoclast differentiation and maturation and benefits bone reconstruction [12, 13]. Therefore, inhibiting the signaling pathways that lead to RANKL-induced osteoclastogenesis and reducing the biological effects induced by wear particles are crucial steps for aseptic loosening of the prosthesis.

RANKL is the key cytokine in osteoclast differentiation. It is a member of the tumor necrosis factor (TNF) super family, and is primarily secreted by the ossification cells, T lymphocytes, and B lymphocytes [14]. The preliminary research identified that in the RANKL-mediated signaling pathways, RANKL links to RANK and causesinhibitor kappa B kinase β (IKKβ) phosphorylation. It then induces the degradation of IκB, eventually leading to the release of nuclear factor kappa B (NF-κB) into the nucleus, which prevents osteoclast apoptosis and maintains normal differentiation [15, 16]. In the osteoclast precursors (OCPs), Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) is activated by calcineurin dephosphorylation. It participates in all aspects of osteoclast formation and activation; extracellular regulated protein kinases (ERK) signal pathways are involved in regulating the expression of NFATc1 gene transcription factors, participate in OCPs proliferation, differentiation, and survival together with NFATc1, and enhance the expression of RANK [17-19]. Because the NF-κB, NFAT, and ERK signaling pathways are involved in the formation and differentiation of osteoclasts, whose activation and phosphorylation is mediated by RANKL and RANK in combination, targeting the RANKL-mediated signal pathway by attenuating the signal transduction of osteoclastogenesis should available to bone resorption disease caused by osteoclasts.

PKC regulates the proliferation, differentiation, and survival of several cellular components. The PKC family includes more than 10 kinds of isomers. Among these, PKC-β has two isomers: PKC-βI and PKC-βII. The expression of these two PKC-β isomers increases during the process of osteoclast differentiation. Studies have shown that PKC-β is a key factor in regulating

osteoclast differentiation by participating in the RANKL-mediated downstream signaling pathway of NF-κB and ERK [20, 21]. Previous studies found that GF109203X acts as a PKC inhibitor by intervening in the activity of NF-κB, the NFAT signaling pathway, and then inhibits RANKL-induced osteoclastogenesis functional activation, suggesting that GF109203X has therapeutic potential in osteolytic disease [22]. However, these studies on GF109203X were only at the cellular level, and have not yet been applied to animal models. Therefore, we set up a mouse model of particle-induced surface bone erosion to observe the degree of damage to the calvarium and detect the expression of osteoclasts and related specific cytokines of osteoblasts. We inferred that GF109203X can inhibit osteoclast formation, explored the potential protective effect of GF109203X against osteolysis, and addressed clinical applications for the prevention and treatment of bone dissolution disease.

Materials and methods

Polyethylene particle preparation

Pure PE particles were purchased from Clariant (Gersthofer, Germany) [23]. To remove adherent endotoxins, the particles were soaked in 100% ethanol for 48 h, prepared for washing [24], and then resuspended in a mixture of phosphate-buffer solution and 1% normal mouse serum at a concentration of 1000 mg/mL. The mean particle size was 1.84 ± 1.50 μm (range 0.14-12.1); more than 32% of the particles were smaller than 1 μm [25].

Reagent preparation and groups of mice

GF109203X was purchased from Sigma (Castle Hill, New South Wales, Australia). Eight-week old LPS-resistant C57BL/6 mice were purchased from the Laboratory Animal Center, Academy of Military Medical Sciences, PLA, China (mean weight 18.3 ± 0.6 g, range 17.5-19.2 g) and randomly divided into four groups of six: Control group (no PE particle-induced and injected with PBS); Vehicle group (PE particle-induced and injected with PBS); GF109-203X-low group (PE particle-induced and treatment with 100 μl GF109203X); and GF1092-03X-high group (PE particle-induced and treatment with 200 μl GF109203X). The studies were completed at the Regenerative Medicine

and Pathology Laboratory. Ethical approval was ratified in accordance with the principles and procedures by the Animal Care Committee of Guangxi Medical University, China [No.201509105].

Model of polyethylene particle-induced calvarial osteolysis

The mouse model of PE particle-induced calvarial osteolysis was based on a model established by Wedemeyer et al [23]. Mice were raised for two weeks before being sacrificed. Two percent pentobarbital (0.01 mL/g) was used to anesthetize the mice. Two mm of calvarial skin was exposed and the cranial periosteum was dissected. A control group with no PE particle implantation was also injected with PBS (100 μl). In the other three groups, PE particles (30 μl, 1000 mg/mL) were implanted under the periosteum at the middle incision of the calvarium, and the skin was stapled. Two days after implantation of PE particles, the positive group was injected with 100 μl PBS, and the other two groups were treated with GF109203X; these were separately injected with a 100 μl or 200 μl dosage into the periosteum. PBS or GF109203X was injected every other day for 14 days. Blood was collected from the femoral artery after the mice were anaesthetized and then sacrificed by cervical dislocation for ELISA detection. The calvaria were stored in 10% formaldehyde and prepared for micro-CT analysis and histological evaluation (tartrate-resistant acid phosphatase [TRAP] staining). No adverse effects or mortality occurred.

Micro-computed tomography (micro-CT) scanning

PE particles were removed by mixing in 10% formaldehyde for 24 h before scanning to exclude interference. The mice calvaria were constructed by a high-resolution micro-CT system (Skyscan 1076; Skyscan, Kontich, Belgium) using the following settings: X-ray voltage, 75 kV; electric current, 120 μA; rotation step, 0.5°. Representative three-dimensional images of the mouse calvaria in each group were exported to the computer for scanning. In order to analyze the reconstructed micro-CT images quantitatively, a square region of interest (ROI) 6 mm long × 6 mm wide around the area of PE particle implantation was selected for further qualitative and quantitative analysis. Bone vol-

ume to tissue volume (BV/TV), number of pits, and percentage of porosity of each sample were measured.

Histomorphometric analysis of osteocytes

The mouse calvaria were recycled and decalcified in 10% EDTA (pH 7.4) at 4°C for one month, and then embedded in paraffin. The eroded section of each calvarium was prepared for TRAP staining. A high-powered microscope was used to examine and photograph the specimens for quantitative analysis; the number of TRAP+ multinucleated osteoclasts was counted, and the bone erosion area was measured.

Pathological analysis of the liver and the small intestine

In addition, the liver and the small intestines of the mice were collected and stored in 10% formaldehyde after sacrifice, and then hematoxylin and eosin- (H&E-) stained and TUNEL staining. Each section was evaluated microscopically.

Serum analysis

Blood collected in the Eppendorf tubes was centrifuged for extraction of serum to analyze the levels of RANKL, OSCAR, CTX-1, and OPG, as per ELISA kit protocols (Wuhan Hi-tech Med, Hubei, China).

Statistical analysis

Values are presented as the mean ± SD of results obtained from three or more experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test (GraphPad Prism 6 for Windows). $P < 0.05$ was considered significant.

Results

GF109203X attenuated the bone destruction of PE particle-induced osteolysis in C57BL/6 mice

In order to analyze the therapeutic effect of GF109203X on particle-induced bone dissolution and confirm the function of GF109203X in suppressing calvarial osteolysis in an animal model, we set up a murine mouse calvarial osteolysis model of PE particle-induced osteolysis to evaluate the effect of GF109203X on bone dissolution in vivo. The calvaria were observed under micro-CT scanning, and the area
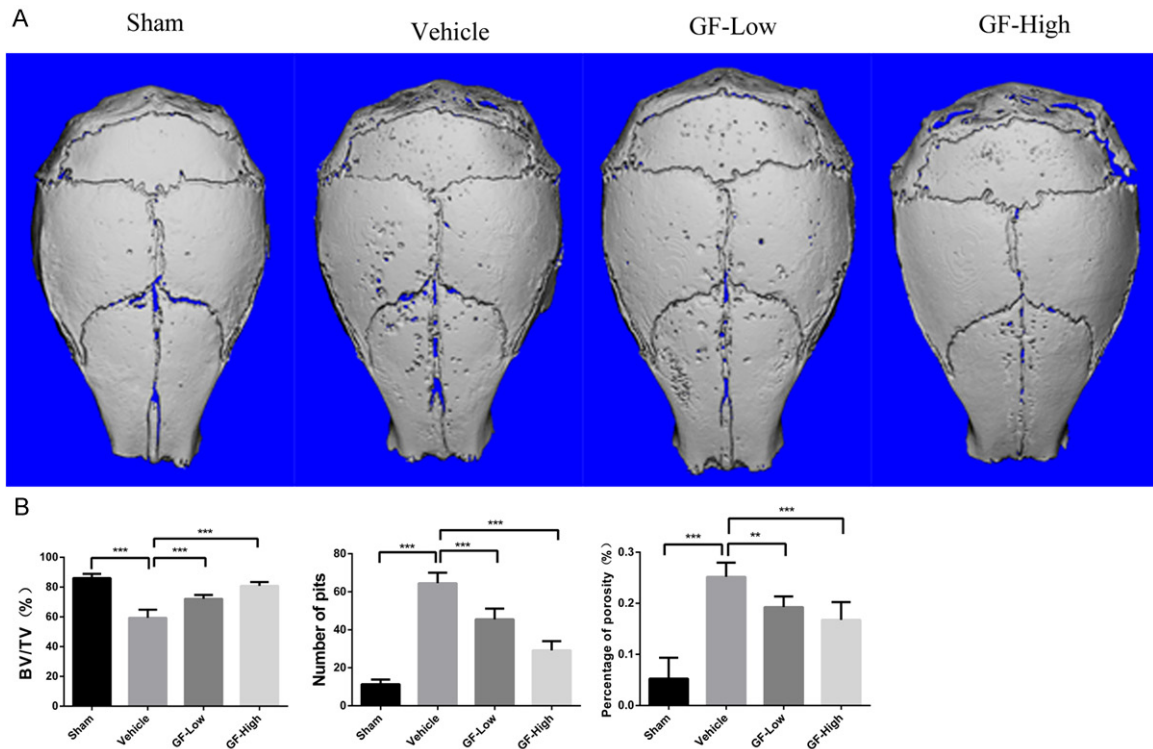
Figure 1. Comparison of bone destruction at day 14 after surgery. A. Representative micro-CT scanning images of PE particle-induced calvarial osteolysis in each group. B. Quantitative micro-CT analysis of the ROI (6 mm long × 6 mm wide) of the murine calvaria. Ratios of bone volume to tissue volume (BV/TV), number of pits, percentage of porosity of each sample was measured (n = 6). Results are presented as mean ± SD (*p*-value < 0.05, **p*-value < 0.01, ***p*-value < 0.001).

of PE particle implantation showed severe osteolysis, as evidenced by the extensive surface erosion on the calvaria in the vehicle group as compared to the sham group. By contrast, treatment with GF109203X significantly reduced the extent of PE particle-induced bone destruction, as the scan images showed that bone loss of mice in the high GF109203X group was markedly less than that of mice in the low GF109203X group (**Figure 1A**). The quantitative analysis of bone mass parameters was further identified, and confirmed these results. The significant increase in BV/TV and marked reduction in the number of resorptive pits and percentage of porosity of the calvaria (**Figure 1B** and Supplementary Table 1) led to the conclusion that GF109203X effectively induced and ameliorated particle-induced calvarial dissolution.

GF109203XGF reduced the number of osteoclasts and suppressed PE particle-induced calvarial inflammatory response

To investigate the inhibitory function of GF109-203X on particle-induced osteolysis and confirm that GF109203X can reduce the number of

osteoclasts and the inflammatory reaction, we then analyzed the pathological changes in the mouse skulls by TRAP staining. In the area where PE particle injection resulted in bone destruction, inflammatory cells, such as lymphocytes, macrophages, and particularly osteoclasts, were aggregated along the bone surface erosion, as revealed by TRAP staining (10 ×) (**Figure 2A**). In addition, consistent with micro-CT quantitation, histological assessment and histomorphometric analysis demonstrated that both low-dose and high-dose GF109203X markedly ameliorated PE particle-induced osteoclastogenesis in a dose-dependent manner, as revealed by the reduced number of TRAP (+) osteoclasts and bone erosion area in the treatment groups (**Figure 2B** and Supplementary Table 2). Thus, our results confirmed that GF109203X can attenuate the formation of osteoclast.

GF109203X suppresses the expression of RANKL and OSCAR

Previous studies have shown that GF109203X can inhibit the signaling pathways of RANKL-
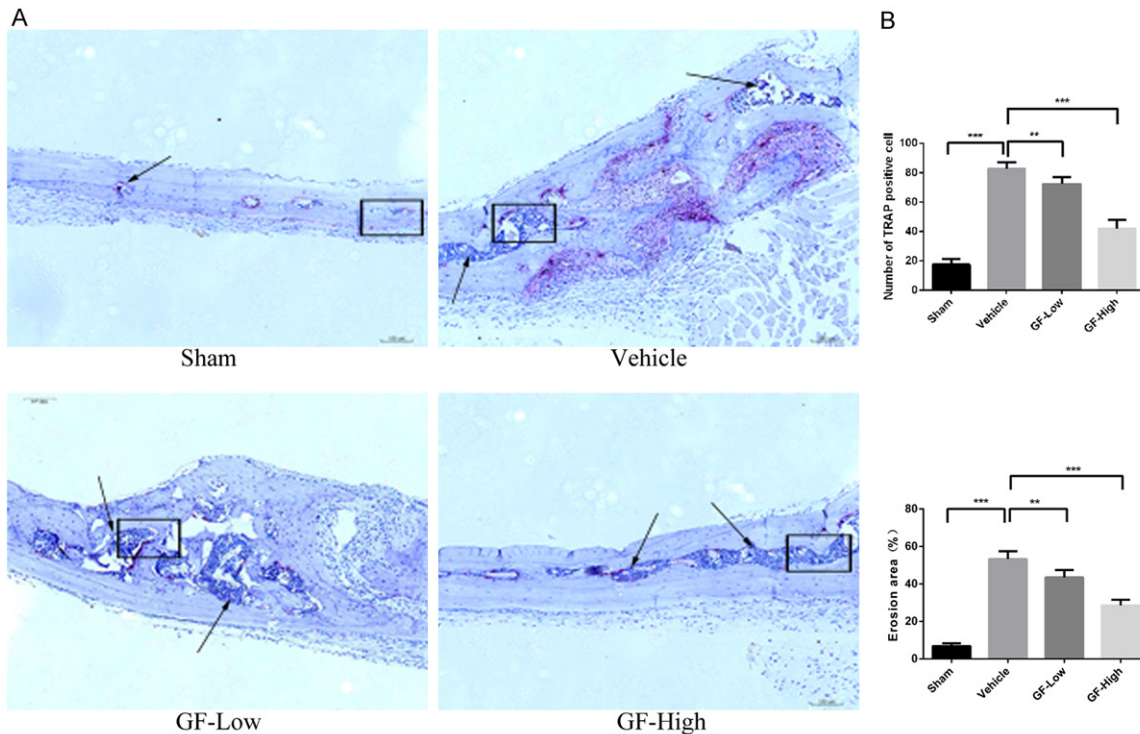
Figure 2. Histological staining of calvaria sectionsin each group, respectively. A. TRAP staining images. B. The number of TRAP-positive cells per wide field (a fixed-size region in the picture) and erosion area was measured at the sature site of the calvaria (n = 6). Values are mean ± SD (*p-value < 0.05, **p-value < 0.01, ***p-value < 0.001 compared to the vehicle control).

induced osteoclastogenesis. To confirm that the primary mechanism of GF109203X inhibition of osteoclast formation is interference with the RANKL-mediated downstream signaling pathways and attenuation of RANKL expression feedback, we used ELISA kits to detect the expression of RANKL and OSCAR for quantitative analysis. The results indicated that expression of RANKL and OSCAR was higher in the vehicle group than in the sham group. Moreover, the levels of serum RANKL and OSCAR conspicuously decreased under treatment with GF109203X, and their concentration in the GF109203X-high group was significantly lower than in the GF109203X-low group (**Figure 3A**, **3B** and Supplementary Table 3A, 3B). Expression of RANKL and OSCAR was reduced after treatment with GF109203X, which is associated with osteoclast differentiation.

GF109203X inhibits bone resorption and decreases bone dissolution

To further explore whether GF109203X is also effective for particle-induced bone destruction in animal models and to evaluate the amelioration of bone dissolution after treatment with GF109203X. We detected the expression of CTX-1 using ELISA. The data showed that the concentration of CTX-1 was higher in the vehicle group than in the sham, low-dose, and high-dose groups. Treatment with GF109203X markedly reduced CTX-1 expression in a dose-dependent manner, the higher the medication dosage, the lower the expression of CTX-1 (**Figure 3C** and Supplementary Table 3C). These results indicated that GF109203X may ameliorate osteoclast-induced bone resorption.

GF109203X promotes the expression of osteoblast cytokine OPG

The OPG expressed in the osteoblasts is a protective factor in bone reconstruction. We measured OPG quantitatively using ELISA for studying whether it has an effect on the expression of osteoblasts. The results showed that the concentration of OPG was lower in the vehicle group than in the sham group. Collectively, treatment with GF109203X significantly increased OPG expression as compared to the vehicle group, and the higher the dosage, the high-
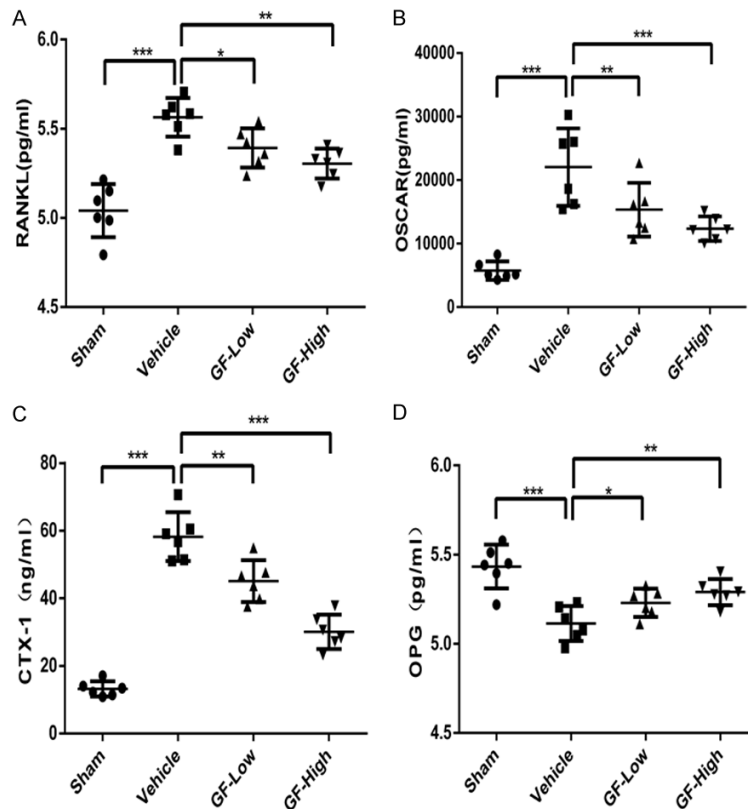
Figure 3. Biochemical analyses of the murine serum. A. RANKL concentration in particle-induced mouse model of osteolysis (n = 6). B. OSCAR concentration in particle-induced mouse model of osteolysis (n = 6). C. CTX-1 concentration in particle-induced mouse model of osteolysis (n = 6). D. OPG concentration in particle-induced mouse model of osteolysis (n = 6). The values are presented as mean ± SD. Significance was considered as P < 0.05 (*p-value < 0.05, **p-value < 0.01, ***p-value < 0.001).

er the concentration of OPG (**Figure 3D** and Supplementary Table 3D). Taken together, these data imply that GF109203X regulates the balance of osteoclast and osteoblast differentiation in bone destruction, and the expression of osteoblasts get promotion is mainly related to the inhibition of osteoclast.

Assessment of GF109203X damage to the liver and the small intestine

PKC participates in a variety of cell processes. GF109203X, as a PKC inhibitor, has been widely used in research on anti-tumor drugs. In order to further understand the metabolism of GF109203X in C57 mice, and whether it causes damage to the visceral organs, we extracted the liver and the small intestine for pathological study. H&E pathological staining demonstrated that the pathology of the liver and the small intestine did not significantly differ between the groups (**Figure 4A**). The TUNEL staining (**Figure**

4B) and the apoptosis cell count (**Figure 4C** and Supplementary Table 4) were further examined in the liver and the small intestine, and confirmed that there was no difference in pathology between each groups. These results show that GF109203X causes no damage to the body's metabolism.

Overall, our results suggest that GF109203X attenuates RANKL-induced osteoclast formation and suppresses wear particle-induced osteolysis in vivo. This is mainly reflected in the reduced expression of RANKL, OSCAR, and CTX-1 and increased expression of OPG, which is beneficial to the reconstruction of bone. Finally, the number of osteoclasts was reduced and bone destruction was ameliorated.

Discussion

Joint replacement is the most common clinical surgical treatment for osteoarthritis; however, both particle-induced osteolysis around the prosthesis and increasing mechanical wear and tear between the implant and bone can cause prosthetic loosening [26, 27]. Since the generation of wear particles is inevitable, researchers have studied the signaling pathways that result in osteolysis and used drugs to inhibit or block the cytokines and signaling pathways that induce osteoclast formation. Such as, Jimi et al suppressed the signaling pathways of NF-κB by inhibiting NEMO (IKKγ)-binding domain peptide, and found that this could reduce osteoclast formation and bone erosion in arthritis, confirming that the signaling system of NF-κB plays a crucial role in the process of osteoclast formation [28, 29]. Similarly, we do research into GF109203X suppresses the signaling pathways that RANKL mediates osteoclast formation.

Ultra-high molecular weight polyethylene (UHMWPE) particle-induced bone dissolution is
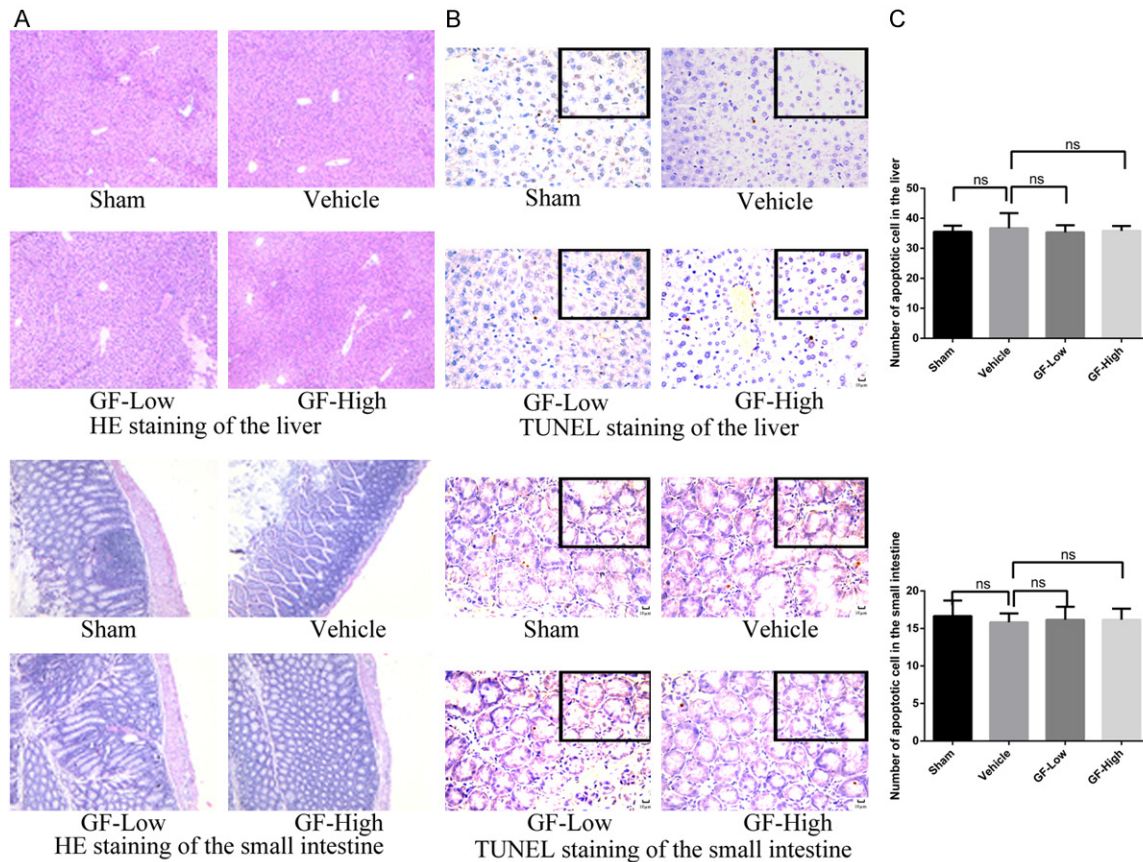
Figure 4. Histopathological staining in each group, representatively. A. Hematoxylin and eosin (H&E) staining (10 ×) of the liver and the small intestine. B. TUNEL staining of the liver and the small intestine. To analyze the TUNEL-stained images of cell apoptosis, a rectangular region was selected for counting of the apoptotic cells (box at top right in the picture). C. The number of apoptotic cells in each group (n = 6). Significance was considered as $P < 0.05$; ns denote no significance.

commonly used in osteolysis models, and plays a large role in the process of bone erosion surrounding prosthetics compared to metal particles and bone cement particles [30]. Similar to other researches on Ti particle-induced osteolysis [31], we established a mouse model of PE particle-induced calvarial osteolysis and simulated the organizational environment of prosthetic aseptic loosening to study how GF10-9203X inhibits osteoclast formation induced by RANKL and observe and analyze the curative effect of GF109203X on osteolysis. The results of micro-CT and TRAP staining showed that GF109203X inhibited osteoclast formation in a dose-dependent manner and reduced the number of osteoclasts and bone resorption pits, so in the area of bone resorption, the level of the mice calvarial damage decreased.

During osteoclast differentiation, RANKL binds to osteoclast receptor RANK and can mediate the downstream signal pathway of NF-κB, NFAT, and ERK phosphorylation in the process of osteoclastogenesis [29, 32, 33]. In the present study, in accordance with previous signaling pathway studies on inhibition of RANKL-induced NF-κB and NFAT transcription factor activity, GF109203X was shown to attenuate RANKL-induced osteoclast differentiation, suppress osteolysis in C57 mice, and promote the protective effect of osteoblast formation, finally appeared as decreasing the expression of RANKL, OSCAR, and CTX-1 and increasing the expression of OPG. Collectively, the severity of bone dissolution got improvement. Furthermore, pathological staining of the liver and the small intestine showed that GF109203X did not damage the metabolism of the mice. In view of GF109203X's effective reduction and amelioration of particle-induced calvarial osteolysis, it will provide a therapeutic option for

osteolytic disease. However, the key to bone reconstruction lies in the balance between bone resorption of osteoclasts and bone formation of osteoblasts. Whether GF109203X has influence on the conduction of signaling pathways in osteoblast formation and how it affects, the effect of other subtypes of PKC inhibitor on bone dissolution disease still require further study.

In conclusion, the results showed that polyethylene particles can induce osteolysis, while GF109203X can suppress bone dissolution in a dose-dependent manner by attenuating RANKL-induced osteoclastogenesis and functional activation of osteoclasts. Therefore, GF-109203X may have clinical applications for the prevention and treatment of wear particle-induced aseptic prosthetic loosening, and is likely to provide a new treatment option for osteolytic diseases in the future.

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Disclosure of conflict of interest

None.

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