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NLRP3 inflammasome inhibitor glyburide expedites diabetic-induced impaired fracture healing

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ABSTRACT

Localized inflammation is accompanied by the diabetic-induced fracture. The present study aims to investigate the therapeutic effects of glyburide, an NLRP3 inflammasome inhibitor, in a diabetic-induced fracture model. An animal model of diabetic-induced fracture was established and the mice were administrated with metformin or glyburide for 3 weeks. Quantitative polymerase chain reaction (qPCR) and Western blotting were used to evaluate the relative expressions of IFN-γ, TNF-α, and IL-6. Micro-computed tomography (μCT) scanning was applied to evaluate bone callus formation. Histopathology examinations of fractured femur sections were performed using Tartrate-resistant acid phosphatase (TRAP) staining and Alcian blue and orange G staining. Bone strength was evaluated using Torsional testing. Our results showed that treatment of glyburide significantly decreased the expressions of IFN-γ, TNF-α, and IL-6 in the fracture calluses in diabetic-induced fracture model, while bone callus volume and bone volume fraction were increased. Additionally, our results also demonstrated that treatment of glyburide rescued the increase of osteoclasts in the bone-cartilage interface. Apart from decreasing a percentage of cartilage area and increasing the percentage of bone and fibrotic tissue area, treatment of glyburide increased the maximum torque and yield torque of fractures. These results implied that glyburide might be used as a potential drug candidate for diabetic-induced fracture.

1. Introduction

Diabetes is a metabolic disorder disease, which is characterized by an abnormally high blood sugar levels caused by insufficient production of insulin or cells improperly responding to insulin (American Diabetes, 2014). Diabetes induces a series of complications including diabetic retinopathy and kidney diseases as well as increases the risks of stroke (American Diabetes, 2014; Lebovitz, 2001). As the 7th leading cause of death disease, there are approximate 1.6 million deaths directly caused by diabetes in 2016. In the following year, there are over 420 million patients diagnosed with diabetes (Petersmann et al., 2018; Harding et al., 2019). Many studies have revealed diabetes associated with bone fractures, which is supported by the evidence that diabetes increases the risk of bone fractures and impairs the process of bone healing (Cortet et al., 2018; Compston, 2018). It is worth noting that patients with diabetes experience a significant delay in fracture healing as compared to non-diabetic patients (Compston, 2018; Kanazawa and Sugimoto, 2018). Although a growing body of evidence suggests that accumulation of advanced glycosylation end-products (AGEs) caused by diabetes is correlated to impaired fracture healing (Karim et al., 2018), the underlying mechanisms of diabetic-induced impaired fracture healing remain unclear.

Numerous studies have demonstrated a close link between diabetes and inflammation (Wellen and Hotamisligil, 2005; Dandona et al., 2005). Patients with diabetes are always accompanied by high levels of inflammatory cytokines and mediators as compared to non-diabetic patients (Dandona et al., 2005). For instance, overexpression of tumor necrosis factor (TNF)-α in adipose tissue is a hallmark of obesity and an inducer of insulin resistance (Dandona et al., 2005). The increase of inflammatory cytokines caused by diabetes partially promotes the formation of osteoclast and affects the process of fracture healing (Jiao et al., 2015). Additionally, inflammatory cascade accelerates the formation of AGEs, increases the production of reactive oxidative species, and leads to lipid peroxidation, thereby increasing bone resorption and causing a delay of fracture healing in diabetics (Wellen and Hotamisligil, 2005; Jiao et al., 2015). Thus, inhibition of inflammation might provide an option to accelerate fracture healing caused by diabetics.

Inflammasomes are multimeric complexes, which are responsible for the regulation of inflammation caused by a series of factors

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including pathogens, virus, or toxic compounds (Schroder and Tschopp, 2010). NLRP3 is one of the best-characterized inflammasomes and has been reported to be associated with many diseases including cardiovascular diseases, diabetes, multiple sclerosis and cancer (Schroder and Tschopp, 2010; Khare et al., 2010). NRLP3 inflammasome inhibitor represents a significant improvement toward the development of strategies against NLRP3 inflammasome-associated diseases. In 2009, Lamkanfi and colleagues have identified that glyburide is able to inhibit NLRP3 activation in murine and human macrophages (Lamkanfi et al., 2009). In 2017, Liu and colleagues have further revealed that glyburide therapy inhibits NLRP3 activation in colon tissue and alleviates chronic colitis in interleukin (IL)-10 deficient mice (Liu et al., 2017). In addition to serving as an NLRP3 inflammasome inhibitor, glyburide is also a diabetes medicine used to treat type II diabetes. In the present study, for the first time, we identified that glyburide expedited diabetic-induced impaired fracture healing. We believe that the investigation of other NRLP3 inflammasome inhibitors will provide an alternative option for the treatment of impaired fracture healing induced by diabetics.

2. Materials and methods

2.1. Animals and protocols

Male BALB/c mice (12-week old) were purchased from Beijing Vital River Company (Beijing, China). The animals were housed in a 12 -h light-dark cycle and fed under experimental conditions with a temperature of 22–24 °C and humidity of 50 ± 5%. The experimental protocols used in this study was reviewed and approved by Jiangsu Province Hospital Animal Care and Use Committee.

An animal model of diabetic-induced fracture was established, as shown in Fig. 1. The BALB/c mice were intraperitoneally (*i.p.*) injected with 40 mg/kg streptozotocin for 5 consecutive days. After that, on the 15th day, the mid-diaphyseal femur fracture model was constructed according to a previously reported method. Briefly, an 8 mm long skin incision was made on the femur, and blunt dissection of muscle was used to expose the mid-shaft of the femur. Mid-diaphyseal femur fractures were created using a rotary Dremel with a diamond blade attachment. A 25-gauge needle was inserted into the medullary canal of the femur from the distal end. Next, the animals were administrated metformin or glyburide at a dose of 100 mg/kg/d for 21 days. The bone fracture healing was examined on the 37th day. A Faxitron system (Faxitron X-ray, Wheeling, IL) was used to take X-ray images at the time of surgery and every week following surgery until sacrifice.

2.2. Quantitative polymerase chain reaction (qPCR)

Trizol reagent was used to extract total RNA from the fracture calluses, according to the instructions of the manufacturer. Isolation of total RNA was performed under sterile condition. To remove DNA contamination, RNase-free DNase I was used. Primers for IFN-γ, TNF-α, IL-6, and β-actin were used for amplification of these genes. To analyze the accuracy of the PCR reaction, the Melt curves were used. To evaluate the expressions of genes, 2-$^{\triangle\triangle}$Ct values were calculated. The mRNA expression values of IFN-γ, TNF-α, IL-6 were normalized to that of β-actin. Real time PCR primers for mRNA expression as follows. IFN-γ, forward: 5′-AAGCTGATGGCCCTAAACAG -3′, reverse: 5′-AGGTGCA TCGTGCACATAAG -3′; TNF-α, forward: 5′-CCTTCCAGGATGAGGACA TGA -3′, reverse: 5′-TGAGTCACAGAGGATGGGCTC -3′; IL-6, forward: 5′-GAATGGGGTGATGAGCAGTT -3′, reverse: 5′-CAGAAGGGCAGGATA CAGC -3′; GAPDH, forward: 5′-ACCCACTCCTCCACCTTTGA-3′, reverse: 5′-CTGTTGCTGTAGCCAAATTCGT-3′.

2.3. Western blotting

Extraction and qualification of proteins were performed according to a previously reported method (Yang et al., 2017). Briefly, a RIPA buffer was used to lysis the fracture calluses. Next, the lysate was centrifuged at 13,000 *g* to remove insoluble material. The concentrations of protein were qualified by using a BCA protein assay kit (Beyotime, Jiangsu, China).

Protein samples were loaded at equal amounts for each sample and 10% SDS gel was used to separate the samples. Next, the SDS gel was then transferred to a PVDF membrane and 5% non-fat milk was used to block the membrane. The membrane was probed with the primary antibodies followed by an appropriated secondary antibody. An imaging system (Bio-Rad, CA, USA) was used to examine chemiluminescence. The expressions of protein were normalized to that of β-actin.

2.4. Micro-computed tomography (μCT) scanning

A non-destructive imaging toolViva CT40 μCT scanning system was applied to evaluate bone callus formation. The parameters for scanning the fractured femurs were 114 mA and 70 kVp. The integration time is 200-ms. Viva CT40 software was used to construct a three-dimensional image. Segmentation and countering were acquired in manual. Bone callus volume and bone volume fraction (bone volume/total volume) were measured according to a previously reported method (Alharbi et al., 2018).

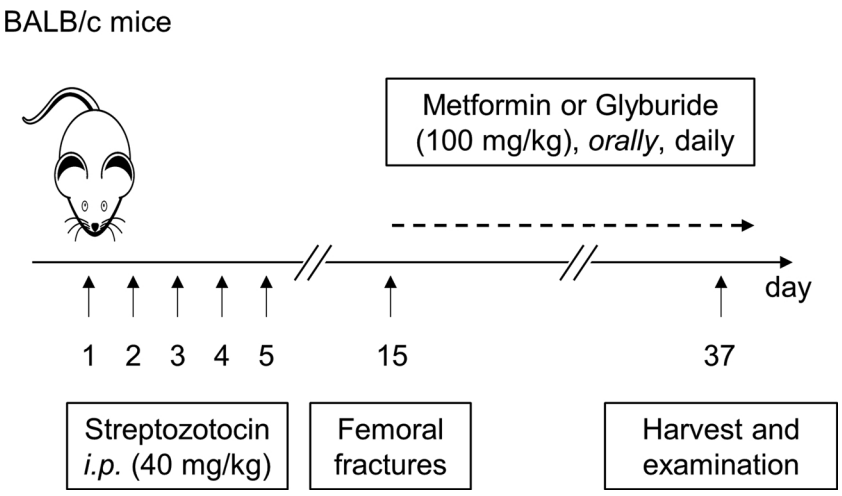

Fig. 1. Schematic depicts for the experiment protocol.
Male BALB/c mice (12-week old) were used to establish diabetic fracture model by intraperitoneal (i.p.) injection with streptozotocin (STZ) (40 mg/kg) for 5 consecutive days. And then, and the mid-diaphyseal femur fracture model was constructed on the 15th day. The animals were gavage with metformin or glyburide at a dose of 100 mg/kg/d lasting until the 37th day. At the end of the experimental period, the animals were sacrificed and bone fracture healing was examined.

2.5. Tartrate-resistant acid phosphatase (TRAP) staining

On the 37th day, all animals were sacrificed and histologic sections were obtained. TRAP staining was used for identifying osteoclasts. Osteoclasts adjacent to the bone-cartilage were counted as multi-nucleated TRAP-positive cells.

2.6. Alcian blue and orange G staining

Histological examination of fractured femur sections was also evaluated using Alcian blue and orange G staining. Visiopharm software (Visiopharm, Westminister, CO, USA) was used for the qualifying area of cartilage, fibrotic tissue, and bone in the histologic sections according to a previously reported method (Zhang et al., 2016).

2.7. Torsional biomechanics analysis

Bone strength was assessed using Torsional testing according to a previously reported method. Briefly, the proximal and distal ends of harvested femur specimens were cemented into 6.35 mm^2 aluminum tubes using bone cement prepared to the manufacturers' specifications (DePuy Endurance; Warsaw, IN). A custom jig was used to ensure axial alignment and the maintenance of approximately 7 mm gauge length for all samples. Specimens were submerged in PBS at room temperature for 2 h after potting to allow for rehydration of the tissue and hardening of the bone cement. Samples were mounted on an EnduraTec TestBench™ system (200 N mm torque cell; Bose Corp., Minnetonka, MN). In this study, Maximum torsion and Yield torque were used as two crucial parameters to evaluate biomechanical stability of fractured femurs.

2.8. Statistical analysis

SPSS (SPSS, Chicago, IL, USA) was used to statistical analysis. All Data were expressed as mean ± S.D. To evaluate the significance, one-way analysis of variance with multiple comparisons and Student-Newman-Keuls (SNK) test were performed. A *P*-value less than 0.05 was considered as a statistical significance.

3. Results

3.1. Treatment of glyburide inhibited the expressions of IFN-γ, TNF-α, and IL-6 in the fracture calluses

To investigate the effects of glyburide on the localized inflammation in the fracture calluses, the expressions of inflammatory cytokines including IFN-γ, TNF-α, and IL-6 were determined using qPCR and Western blotting. We observed that the protein and mRNA levels of IFN-γ and TNF-α were significantly increased in fracture calluses in the diabetic mice with a femoral fracture. Treatment of glyburide or metformin significantly decreased the levels of IFN-γ (Fig. 2A and B) and TNF-α (Fig. 2C and D) at both the transcriptional and post-transcriptional levels in comparison to the control group ($p < 0.01$). Similarly, the levels of IL-6 were significantly increased in the diabetic mice with femoral fracture, whereas treatment of glyburide or metformin significantly decreased IL-6 at both the transcriptional and post-transcriptional levels ($p < 0.05$, Fig. 2E and F). Notably, treatment of glyburide showed stronger inhibitory effects on IFN-γ ($p < 0.01$), TNF-α ($p < 0.01$), and IL-6 ($p < 0.05$) than those in the metformin-treated group. These results demonstrated that glyburide exerted localized anti-inflammatory effects in the fracture calluses.

3.2. Treatment of glyburide accelerated callus formation in fractures

We then assessed the bone callus formation. As shown in Fig. 3A, we observed an increased bone callus formation in glyburide-treated mice.

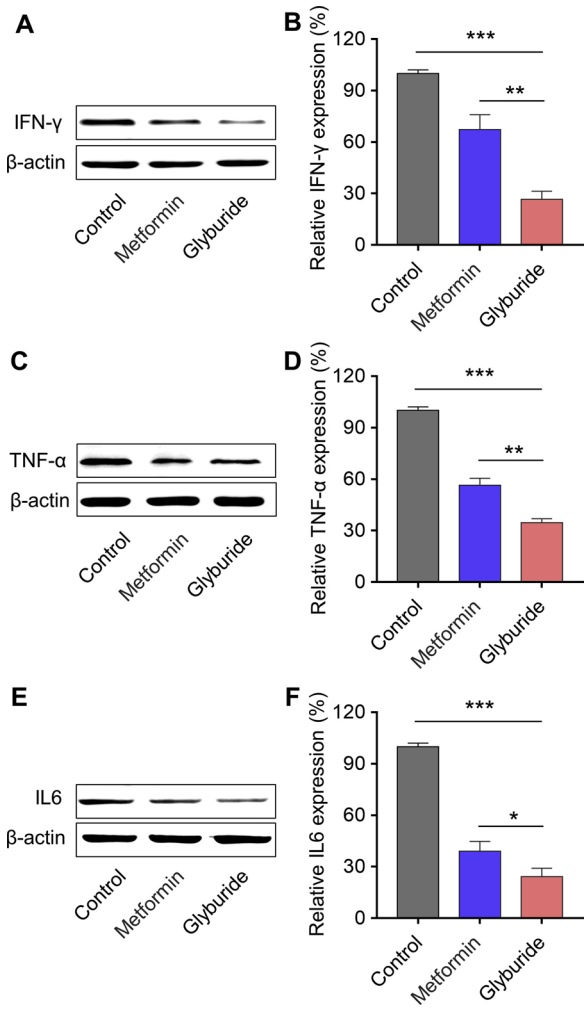

Fig. 2. Treatment of glyburide significantly inhibited inflammation in the fracture calluses of diabetic mice with a femoral fracture.
After treatment of glyburide or metformin for 21 days, the fracture calluses were harvested and the mRNA and protein expressions of IFN-γ (A and B), TNF-α (B and C) and IL-6 (E and F) in fracture calluses were determined using qPCR and Western Blotting. β-actin was used as a control (Each group contains 8 mice). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

μCT was then used for qualification of bone callus volume and bone volume fraction (bone volume/total volume). We found that treatment of glyburide or metformin significantly increased bone callus volume and bone volume fraction in comparison to the control group ($p < 0.05$, $p < 0.01$, Fig. 3B and C). Additionally, glyburide-treated group showed higher bone callus volume and bone volume fraction than those in metformin-treated group. These results supported that treatment of glyburide accelerated callus formation in fractures ($p < 0.05$, $p < 0.05$).

3.3. Treatment of glyburide rescued the increase of osteoclasts caused by diabetes

Osteoclasts are one of the crucial cell types for maintaining and remodeling of bones. To evaluate the effects of glyburide on the numbers of osteoclast, TRAP staining was performed on the bone-cartilage interface, as shown in Fig. 4A, we observed the accumulation of TRAP-positive cells in fractures in the control group. Treatment of glyburide or metformin reduced the accumulation of TRAP-positive cells in comparison to the control group. The numbers of osteoclasts adjacent to cartilage were counted. As expected, treatment of glyburide or

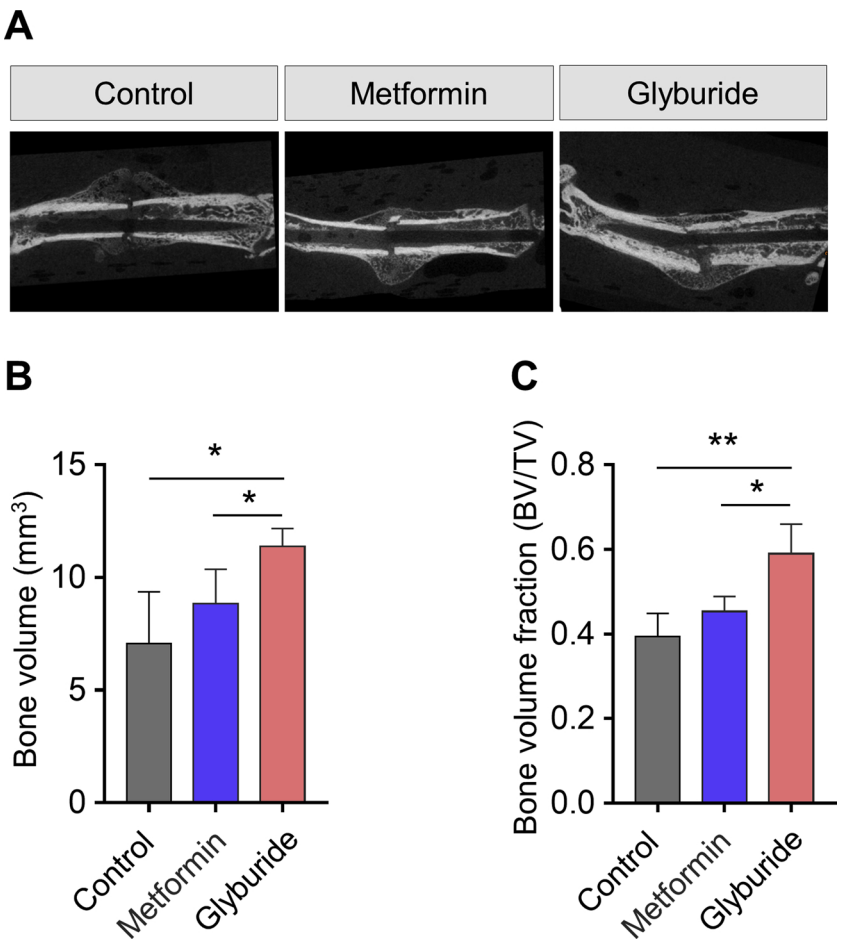

Fig. 3. Treatment of glyburide accelerated callus formation in fractures.
(A) Micro-computed tomography (μCT) scanning revealed increased *in vivo* bone callus formation with treatment of glyburide. μCT scanning were taken 3 weeks after first treatment to assess *in vivo* bone callus formation. Quantification showed glyburide significantly increased bone callus volume (B) and (C) bone volume fraction (bone volume/total volume) as compared to control or metformin-treated groups. Data were presented as mean ± SD (Each group contains 8 mice). * $p < 0.05$, ** $p < 0.01$.

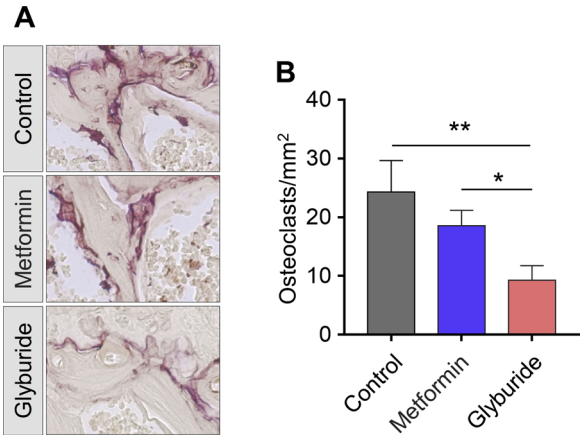

Fig. 4. Treatment of glyburide rescued the increase in osteoclasts caused by diabetes.
(A) Sections were stained with tartrate resistant acid phosphatase (TRAP) to evaluate the effects of glyburide on the numbers and distribution of osteoclasts. (B) Osteoclasts adjacent to cartilage were counted as multi-nucleated TRAP-positive cells. Data were presented as mean ± SD (Each group contains 8 mice). * $p < 0.05$, ** $p < 0.01$.

metformin significantly decreased the numbers of osteoclasts ($p < 0.01$, $p < 0.01$, Fig. 4B). Additionally, treatment of glyburide showed stronger inhibitory effects on the accumulation of TRAP-positive cells in comparison to the metformin-treated group ($p < 0.05$, Fig. 4B).

3.4. Treatment of glyburide resulted in expedited endochondral bone formation and mineralization

Moreover, we investigated the effects of glyburide on endochondral bone formation and mineralization using alcian blue and orange G staining. As shown in Fig. 5A, we observed that glyburide-treated group showed a decrease of a percentage of cartilage (blue area) and an increase of a percentage of mature bone (orange area) in comparison to control or metformin-treated group.

Visiopharm software was used to qualify the percentage of cartilage, bone, and fibrotic tissue area. The results demonstrated that treatment of glyburide dramatically decreased the percentage of cartilage and fibrotic tissue area, increased a percentage of bone area in comparison to the control group ($p < 0.01$, $p < 0.01$, $p < 0.01$, Fig. 5B-D). Notably, when compared with the metformin-treated group, treatment of glyburide also showed significant differences when it comes to the percentage of cartilage, bone, and fibrotic tissue area ($p < 0.01$, $p < 0.05$, $p < 0.05$, Fig. 5B-D).

3.5. Treatment of glyburide increased biomechanical strength of fractures

We further evaluated the effects of glyburide on the biomechanical strength of fractures using Torsion testing. As shown in Fig. 6A, treatment of glyburide significantly increased the maximum torque of fractures in comparison to the control group ($p < 0.01$, Fig. 6B). Similarly, yield torque was also significantly increased in glyburide-treated group in comparison to the control group ($p < 0.01$, Fig. 6B). When compared with the metformin-treated group, glyburide-treated group showed the increase of maximum torque and yield torque with the significant differences ($p < 0.05$, $p < 0.01$, Fig. 6A-B).

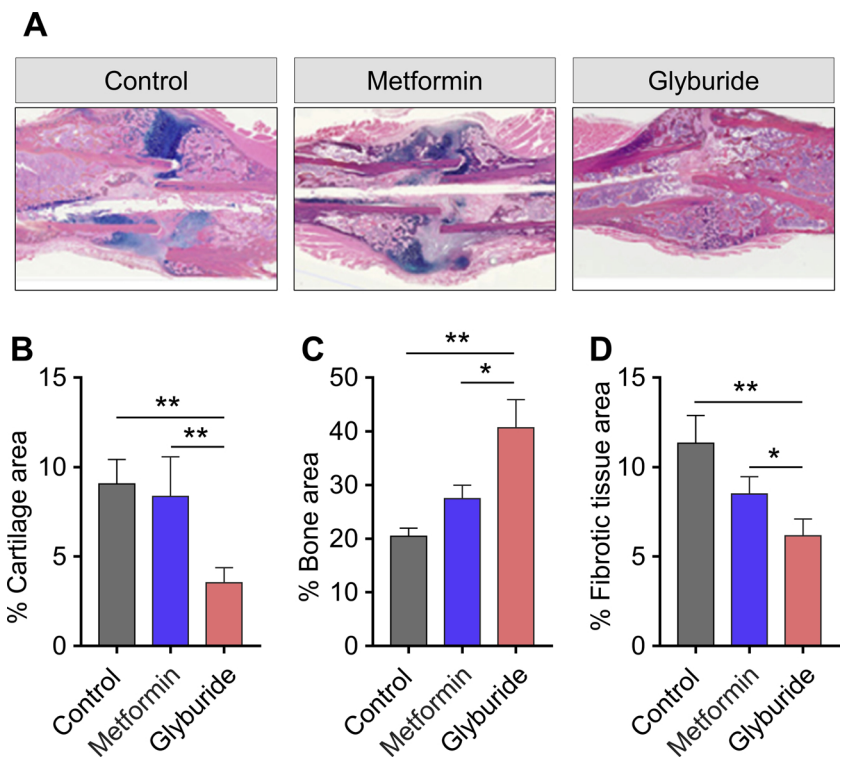

Fig. 5. Treatment of glyburide resulted in expedited endochondral bone formation and mineralization.
Histological examinations of fractured femur sections were performed using Alcian blue and orange G staining (A). Visiopharm software was used to qualify the percentage area of (B) cartilage, (C) bone and (D) fibrotic tissue. Data were presented as mean ± SD (Each group contains 8 mice). * $p < 0.05$, ** $p < 0.01$.

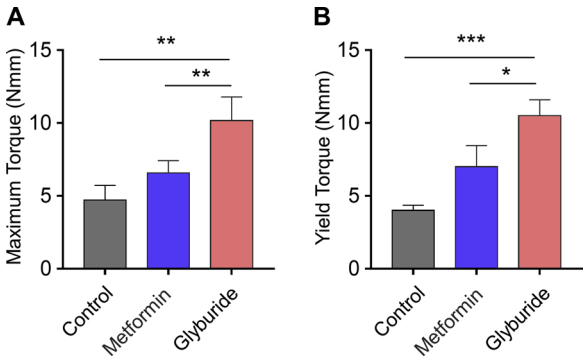

Fig. 6. Treatment of glyburide resulted in an increase of biomechanical strength.
Maximum torsion (A) and Yield torque (B) were used to evaluate effects of glyburide on the strength of fractured femur sections. Data were presented as mean ± SD (Each group contains 8 mice). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

In the present study, we identified glyburide, an NLRP3 inflammasome inhibitor, possessing properties to accelerate fracture healing induced by diabetes. Treatment of glyburide suppressed the localized inflammation by inhibition of inflammation cytokines including IFN-γ, TNF-α, and IL-6. Additionally, treatment of glyburide also accelerated callus formation in fractures and rescued the increase of osteoclasts caused by diabetes. Furthermore, treatment of glyburide resulted in expedited endochondral bone formation and mineralization and increased biomechanical strength of fractures. These results implied that glyburide might be used as a potential drug candidate for impaired fracture healing induced by diabetes.

Glyburide is used for the treatment of type II diabetes in the clinical (Simonson et al., 1984). In addition to serving as an anti-diabetic drug, glyburide also demonstrated biological activities for the treatment of other diseases including melioidosis, leishmaniasis, and inflammatory bowel diseases (Simonson et al., 1984; Koh et al., 2011; Rub et al., 2019). Notably, glyburide has been identified to be an NLRP3 inflammasome inhibitor. Liu and colleagues have revealed that glyburide treatment inhibits NLRP3 activation in colon tissue and alleviates chronic colitis in interleukin (IL)-10 deficient mice (Liu et al., 2017). This study, for the first time, explored the effects of glyburide on the treatment of impaired fracture healing induced by diabetes and identified that treatment of glyburide expedited impaired fracture healing induced by diabetes.

Inflammation plays an important role in bone tissue regeneration (Mountziaris and Mikos, 2008). In the current study, the increase of inflammatory cytokines including IFN-γ, TNF-α, and IL-6 was observed in the fracture calluses, suggesting that localized inflammation occurs in the process of fracture healing. Interestingly, treatment of glyburide significantly decreased the expressions of IFN-γ, TNF-α, and IL-6 when compared with those in the control group. It has been reported that glyburide does not suppress in the secretion of TNF-α and IL-6 in murine macrophage induced by lipopolysaccharides and ATP (Lamkanfi et al., 2009). Another study performed by Bruun and colleagues has demonstrated that glyburide decreases the expressions of TNF-α, IL-6, and IL-8 in adipocytes (Bruun et al., 2007). Our results are in agreement with an *in vivo* study, glyburide therapy shows inhibitory effects on the expressions of inflammatory cytokines including IFN-γ, TNF-α, and IL-6 in patients with melioidosis (Koh et al., 2011). However, the target cells of glyburide in the development of fracture healing induced by diabetes should be investigated in further study.

It is worthwhile noting that treatment of glyburide accelerated callus formation, rescued the increase in osteoclasts, and accelerated the bone formation and mineralization in impaired fracture healing induced by diabetes. Osteoclasts are responsible for maintaining homeostasis of bone formation by secretion lysosomal enzymes that dissolve the bone tissue (Sasaki, 2003). In the process of callus formation, pro-inflammatory cytokines including TNF-α and IL-6 active the expressions of receptor activator of nuclear factor kappa-B ligand, which is a key receptor for induction of maturation of osteoclasts (Sasaki, 2003; Miyamoto and Suda, 2003). As expected, in the control group, we observed the accumulation of the osteoclasts accompanied

by an increase of inflammatory cytokines including IFN-γ, TNF-α, and IL-6 in fractures. Treatment of glyburide leaded to less number of osteoclasts in fractures, suggesting that glyburide treatment rescued the increase in osteoclasts induced by diabetes. Apart from exerting anti-inflammatory effects and inhibitory effects on the increase of osteoclast, we also evaluated the effects of glyburide on endochondral bone formation and mineralization using Alcian blue and orange G staining. The results demonstrated that the percentage of the bone area was significantly decreased in the process of endochondral bone formation in the control group. However, treatment of glyburide dramatically increased the percentage of bone and fibrotic tissue area. Additionally, treatment of glyburide dramatically also decreased a percentage of cartilage area. All of these implied that treatment of glyburide expedited impaired fracture healing induced by diabetes. However, one limitation of this study is that we did not investigate the effects of glyburide on non-diabetic mice. Therefore, non-diabetic mice should be used in the further study to document explicit effects of glyburide on non-diabetic mice.

In the present study, in addition to evaluate the effects of glyburide therapy on mechanical properties of bone formation, the biomechanical strength of bone was also assessed. The results demonstrated that treatment of glyburide significantly increased the maximum torque and yield torque of fractures, indicating that glyburide therapy increased bone strength.

The present study, for the first time, identified that an NLRP3 inflammasome inhibitor, glyburide, expedited the impaired fracture healing induced by diabetes. Not only the localized inflammation was suppressed by glyburide, but also the formation of endochondral bone and biomechanical strength of fractures were affected by glyburide. We speculated that the activation of NLPR3 inflammasome is crucial for regulating localized inflammation in the fracture calluses. Therefore, inhibition of NLPR3 inflammasome might provide an alternative option to treat diabetic-induced impaired fracture healing. Base on the findings showed in the present study, it is worthwhile investigating the therapeutic potential of other NLPR3 inflammasome inhibitors on diabetic-induced impaired fracture healing. Also, we believe that our finding will shed more light on the investigation of other NRLP3 inflammasome inhibitors for the treatment of a delay of fracture healing in diabetics.

5. Conclusion

In summary, this study identified that glyburide accelerated fracture healing induced by diabetes. Treatment of glyburide suppressed the localized inflammation in the fracture calluses. Besides, treatment of glyburide accelerated callus formation in fractures and rescued the increase of osteoclasts caused by diabetes. Moreover, treatment of glyburide resulted in expedited endochondral bone formation and mineralization and increased biomechanical strength of fractures. These results implied that glyburide might be used as a potential drug candidate for impaired fracture healing induced by diabetes.

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

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