Effect of Zoledronic Acid Combined With Metformin on Bone of Db/db Mice

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Abstract

Background Diabetic Osteoporosis (DO) is a complication of diabetes. As a first-line medication for diabetes, metformin combined with the anti-osteoporosis drug zoledronic acid is still unclear whether it is effective in treating DO. In this work, we explored the effect of metformin combined with zoledronic acid in the treatment of DO.

Methods 12-week-old db/db mice were randomly divided into db/db group, ZA (zoledronic acid) group, MET (metformin) group, CMT (combination therapy group) group, Set WT (wild-type) mice of the same strain and age as the blank control group. Body weight and blood glucose were measured every 2 weeks. After 10 weeks, samples were taken for pathology and imaging related tests.

Results (1) The body weight of db/db mice treated with metformin was stable and blood glucose was decreased. (2) After HE staining, db/db mice treated with metformin showed decreased adipocytes, increased blood cells and blood vessels in bone marrow cavity; db/db mice treated with zoledronic acid showed increased trabeculae. The number of bone trabeculae and blood vessels in the bone increased after the combined use of the two drugs. (3) By OPG staining and semi-quantitative analysis, db/db mice had the lowest osteoblastic activity. db/db mice treated with metformin had higher osteoblastic activity than those treated with zoledronic acid, and the osteoblastic activity of CMT was higher than that treated with ZA group or MET group. (4) After TE staining, semi-quantitative analysis showed that the number of bone lacunae in WT group was the least, the number of bone lacunae in ZA group and CMT group was significantly lower than that in db/db group, but the number of bone lacunae in MET group was not significant. (5) The bone morphological analysis of db/db mice in the CMT group was significantly better than that of MET or ZA alone by micro-CT scanning and bone tissue parameters determination.

Conclusion ZA combined with MET has better anti-bone loss effect than zoledronic acid or metformin alone in db/db mice.

Introduce

China has the largest diabetic population in the world, and it is estimated that the number of type 2 diabetic patients will reach 440 million in 2030[1]. Diabetes patients have a variety of complications, such as cardiovascular and cerebrovascular diseases, eye diseases, osteoporosis and so on. Among them, diabetic patients with osteoporosis have reduced bone mass, increased bone fragility, and damaged bone microstructure, which is called Diabetic Osteoporosis (DO). DO can significantly increase the risk of patients with brittle fracture, long-term severe pain, motor dysfunction, disability and mortality[2,3].

Today, bisphosphonates as a first line drug for treatment osteoporosis in postmenopausal women, also is used for treatment hypercalcemia, multiple myeloma and osteolytic bone metastases. Some studies showed that bisphosphonates can prevent bone loss for short-term; while long-term using can improve bone density, in the same time inhibit bone formation in mice[14]. Zoledronic acid (ZA) is one of bisphosphonates drug. Some studies showed that ZA also prevent bone loss and increase bone density

in diabetic animal model[12]. Meanwhile, ZA has also been shown to promote the proliferation of bone marrow mesenchymal stem cells (BMSCs) and promote their differentiation into osteoblasts, and increase the expression of OCN, RANKL, OPG and type II collagen[13]. In addition, a can inhibit the migration, differentiation and bone resorption of osteoclasts through the AMPK pathway[15].

Metformin is the first-line oral anti-diabetes drug for type 2 diabetes patients[4]. But some studies showed that metformin has a positive effect on osteoporosis. Metformin promotes osteogenic differentiation, bone matrix synthesis, and osteoblastic proliferation by activating AMPK in vitro[34,35]. Meanwhile, metformin blocks the harmful effects of osteoblasts by glycation end products (AGEs), hyperosmotic and hyperglycemic environments; and reverses alkaline phosphatase (ALP) activity, calcium deposition, decreased reactive oxygen species (ROS) and apoptosis by high glucose[36]. Metformin inhibited the AGE receptor (AGER) expression and activation AMPK pathway to decreased AGE level[37,38]. Shao et al found that the metformin increase the expression of collagen I (Col I), osteocalcin (OCN), osteoprotegerin (OPG); and decrease the expression of metal matrix proteinase 1 (MMP1) and metal matrix proteinase 2 (MMP2)[39]. In clinical data, L Joseph et al. followed up more than 23,000 patients and found that diabetic patients who took metformin for hypoglycemic therapy had a lower fracture risk than diabetic patients who did not take metformin [41]. LU et al. followed up more than 30,000 diabetic patients in Taiwan for 15 years and found that the osteoporosis rate of DO patients who received metformin treatment was lower than that of patients who did not receive metformin treatment [45]. Of course, there are also some reports that metformin has anti-osteogenesis effects [6]. J Jeyabalan et al. performed ovariectomy (OVX) on female C57BL/6 mice, and intervened with metformin, and found that there was no significant change in bone mass in the mice. At the same time, they gave metformin to female Wistar rats with femoral bone defects and found that it was effective. There is no significant effect on fracture healing [42]. There is no clear conclusion about the effect of metformin on bone quality.

For DO patients, zoledronic acid injection has been proven to improve patients' bone condition[43], and has no effect on patients' body weight and blood glucose[44]. Whether zoledronic acid combination with metformin can better improve the bone condition is what we want to explore. Therefore, this study intends to use micro-CT and pathological staining to observe the changes of bone in the animal model with treatment of zoledronic acid combined with metformin, to explore the influence two drugs on bone metabolism with the view to providing basis for the elderly patients with type 2 diabetes with osteoporosis to choose drug therapy, reduce fracture risk, reduce pain, reduce social and economic burden.

Materials And Methods

Animals and diets

Twelve-week-old male mice of BKS-LEPR^{EM2CD479} / GPT DB/DB with genotype (LEPR) KO/KO and the same strain with genotype (LEPR) WT/WT were selected and included in the study (Jiangsu Jicuiyaokang). All the mice are SPF class and living in SPF Animal Experimental Center of Chengdu

Medical College (temperature:20-25°C, light: 12h). Mice had free access to pure water and food for the duration of the study. The 30 mice have been divided into 5 groups by according to the random number table: WT group, DB/DB group, ZA group, metformin (MET) group and metformin combination with ZA group (Combination Therapy group, CMT). WT group and DB/DB group were administrated intraperitoneally 0.9% saline every day. ZA group were administrated intraperitoneally 100 g•kg⁻¹ of ZA once[16], and next were administrated intraperitoneally 0.9% saline every day. MET group were administrated intraperitoneally 200 mg•kg⁻¹ metformin every day[32]. CMT group were administrated intraperitoneally 100 g•kg⁻¹ of ZA once, and next were administrated intraperitoneally 200 mg•kg⁻¹ of metformin every day. After 10 weeks of drug treatment in each group of mice, the mice were sacrificed by cervical dislocation, and samples were taken for pathology and imaging related tests.

Measurement of Body weights and blood glucose level

Body weights and blood glucose level data were collected at the conclusion of the experiment. Body weights and blood glucose level were measured every 2 weeks. And the mice were fasted for 8h. The blood glucose level was recorded as 33.3 mmol•L⁻¹ when the blood glucose level was higher than the maximum of the glucose meter.

Pathological staining

Hematoxylin-eosin (HE) staining

The contralateral tibia was taken for pathological examination, fixed in 4% paraformaldehyde for 48 h, then decalcified, gradient alcohol dehydration, paraffin embedding, sectioning and HE staining. The section thickness was 5µm. Observed under a microscope and photographed. After sacrifice of mice, the tibias were isolated and immediately fixed with formalin. The bone was then dehydrated and embedded in paraffin. Paraffin-embedded sections were performed for Hematoxylin-eosin (HE)

Immunohistochemical staining of OPG

The OPG antibody (abacam, USA) was used to stain the tibial tissue. Photos were taken and saved from 5 randomly fields of mirror. Image-Pro Plus 6.0 software was used for semi-quantitative analysis of the positive results.

Trypan blue - eosin (TE) staining

The trypan blue staining reagent (Suolaibao, China) and eosin staining reagents (Suolaibao, China) were used to stain the tibial tissue. The microscopic observation of bone cells, bone morphology pit and osteoclasts were conducted after dyeing. Photos were taken and saved from 5 randomly fields of mirror and the number of bone morphology pit was recorded and then semi-quantitative analysis of the number of bone morphology pit was conducted.

Micro- CT scanning

The tibia was scanned by micro-CT (model: ZKKS-MCT-SHARP), the energy/intensity was 70kVP, 100μA 7 W, the exposure time of 4 frames was 100ms, and the scanning time was 12min. The region of interest (ROI) of cancellous bone for 3D reconstruction which contains bone tissue 0.7mm away from the distal end of the growth plate and 1.5mm thick, the image information was extracted with the lowest threshold of 66. Data processing software: ZZKS-MICROCT4.1. Quantitative analysis of the reconstruction was performed using its software (Sanco Medical AG). Parameters are as follows: Connectivity density (Conn.D), structure model index (SMI), bone mineral density (BMD), bone volume fraction (bone volume/tissue volume, BV/TV), trabecular bone Quantity (trabecular number, Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp).

Statistical analysis

All the numeric parameters were recorded as mean $\pm$ standard deviation. The SPSS 25.0 statistical software was used for normality and one-way ANOVA. If Levene tested homogeneity of variance, LSD test was used for analysis. If the variances were not uniform, Welch correction and Dunnett's T3 multiple was used for analysis. A P value <0.05 was considered statistically significant unless otherwise stated.

Results

3.1 Body weight and blood glucose measurement results

The body weight of WT group increased slowly and blood glucose remained stable. The body weight of DB/DB group increased as slowly as the WT group but blood glucose level in DB/DB group and ZA group was higher than the WT group. The body weight and blood glucose of MET and CMT group decreased slightly. (Fig. 1 and Fig. 2)

HE staining results

There were more bone trabeculae and blood cells in bone marrow cavity in WT group while more fat cells, less bone trabeculae with sparse, small and fractured bone trabeculae were found in DB/DB group. In the ZA group, bone trabeculae were broken, small number, disorganized structure and enlarged space, including a large number of fat cells. The Met and CMT groups had more bone trabeculae, more blood cells in the marrow cavity, and fewer adipocytes. Show in Figure 3.

OPG staining results

OPG expression level was the highest in WT group, and the lowest in DB/DB group. OPG expression level in CMT group was higher than DB/DB group, ZA group and MET group. Compared with DB/DB group, ZA group, MET group and CMT group, WT group has all $P < 0.05$ (Figure 4), which has statistical significance. Compared with MET group and CMT group, respectively, DB/DB group has $P < 0.05$, which has statistical significance. Compared with ZA group DB/DB group showed $P > 0.05$, which has no statistical significance. Compared with MET group, ZA group showed $P > 0.05$, which has no statistical significance.

Compared with CMT group ZA group showed $P < 0.05$, which has statistical significance. Compared with CMT group MET group showed $P > 0.05$, which has no statistical significance.

Figure 5, the positive result of OPG staining showed that the cytoplasm was brown, black or brown, and the nucleus was blue.

TE staining results

There were bone lacunae on bone trabeculae, and some of them were empty bone lacunae in every group (Figure 7). There are a few osteoclasts on the surface of the bone, which are different in shape and multinucleate. Compared with DB/DB group and MET group WT group showed $P < 0.05$, which has statistical significance. The number of bone lacunae in WT group was the least. And the number of bone lacunae in ZA group and CMT group was lower than that in DB/DB group and there was no significant decrease in MET group.

Micro-CT scan results

Conn.D, SMI, BMD, Tb.N, Tb.Th, Tb. Conn.D, SMI, Tb.N, Tb. SP in DB/DB group had significant differences compared with WT group ($P < 0.05$) (Figure 8). Compared with DB/DB group ZA group showed the Conn.D, SMI, BMD, Tb.N, Tb.SP had significant differences ($P < 0.05$). MET group compared with DB/DB group shown only SMI had significant differences ($P < 0.05$). CMT group compared with DB/DB group shown the BV/TV, Conn.D, SMI, BMD, Tb.N, Tb.SP had significant differences ($P < 0.05$). The 2D imaging of the samples shown in Figure 9. WT group had more bone trabeculae, and DB/DB group had a large number of defects and cavities in the proximal tibia. ZA group, MET group and CMT group shown the number of trabeculae was significantly increased. The ROI bone trabeculae was imaged in the SEG diagram. The image shown WT group had the most bone trabeculae, which were concentrated and ordered; and bone trabeculae were small and scattered in DB/DB group. Trabecular thickness (TH) shows the distribution of bone trabeculae. WT group had more bone trabeculae, and more than DB/DB group. The trabeculae of the ZA group, MET group and CMT group more than DB/DB group, but less than WT group. There were almost no coarser bone trabeculae in DB/DB group; the coarser bone trabeculae was the largest in WT group.

Discussion

Some studies already found that 4 weeks DB/DB mice as type II diabetes animal model because the mice have symptoms similar to type II diabetes patients, such as hyperglycemia, hyperlipidemia, obesity, polyuria, polydipsia and other physiological manifestations[17]. Some studies shown that DB/DB mice are secondary to decreased bone mass[18], osteocalcin level and bone cortical strength[19]. Therefore, DB/DB mice were used as an animal model of type II diabetes mellitus with osteoporosis. Since the incidence of osteoporosis in postmenopausal women has been greatly increased[33], in this study the db/db mice were used for the test effects so we can factor out the variants of menopause and sex hormone changes on osteoporosis, and simply observe the effects of drugs on bone.

Some clinical and basic experimental studies focused on the metformin anti-osteoporosis effect, but there was no clear conclusion. Metformin has the anti-osteoporosis effect[5], but a retrospective study showed that metformin cannot reduce the risk of vertebral fracture[10]. Further studies showed that metformin could neither promote osteogenesis nor prevent bone loss[11]. When osteoporosis occurs, the body will also decrease bone mass and increase fat cells in the bone marrow.[20].

It has been found that BMSCs can differentiate into cells with different functions according to different media and environments. In vitro study metformin was found to promote the proliferation and differentiation of BMSCs into osteoblasts, and inhibit BMSCs into adipocytes[21]. In addition, other studies have found that metformin can promote differentiation and mineralization of osteoblasts[6]. Some animal experiments showed that metformin can prevent bone loss in ovariectomy (OVX) rats, increase the number of bone trabeculae, inhibit the osteoclasts growth[7,9]. Metformin can increase the gene expression of osteogenesis at the molecular level, such as RUNX2 and ALP; and also inhibit the gene expression, such as PPAR γ and C/EBP α [7]. Meanwhile, some studies found that metformin also inhibits osteoclast differentiation in RAW264.7 macrophages induced by nuclear factor κ B ligand (RANKL)[8]. Different results and conclusions in the studies mentioned above may result from the metformin dosage, duration of medication and different statistical methods. Metformin has been shown the positive effect on the treatment of osteoporosis, and we suspected that zoledronic acid combined with metformin would have a better effect on the treatment of osteoporosis.

In our study, metformin made the body weight and blood sugar decrease slightly in DB/DB mice (Figure 1 and Figure 2). Lachin et al. found that metformin results in weight loss, which contributes to a reduction in the incidence of type II diabetes in high-risk group [22]. Metformin results in weight loss in all people that have diabetes or not, but the mechanism of weight loss is not clear. The mechanism of metformin results in weight loss may be associated with acting on the central nervous system to reduction the activity of AMPK in the hypothalamus for inhibit appetite [23]. But there is a common consensus that the mechanism of metformin results in lower blood glucose, including reduction glucose production in the liver, enhancing insulin sensitivity, and inhibiting the glucose absorption in the gastrointestinal tract [24]. Osteoblasts and osteoclasts were rarely found in both HE and TE staining sections of the DB/DB mice tibia, which could be due to the fact that high glucose and hyperosmotic environment promote their growth [25-26]. And after metformin treatment the DB/DB mice, HE staining found the number of adipocytes was decrease in the medullary cavity of bone tissue, the increase of blood vessels and hematopoietic cells, which could be due to the fact that metformin results weight loss and lower lipid, decrease of adipocytes [27-28]. The high glucose level in diabetic patients leads to a series of changes in the tiny blood vessels, which is also the main complication of diabetes. However, blood vessels are the nutrient transporters of bone tissue, so the damage of blood vessels will also affect bone mass and bone structure [29]. In addition, Isoda, et al found that metformin can exert a direct vascular anti-inflammatory effect and protect vascular endothelial cells and repair blood vessels [30-31]. We also found the increase of blood cells in the bone marrow cavity which might be the result from the increase of blood vessels associated to blood cells, or blood cells proliferation caused by metformin.

On the other hand, the number of osteoblasts and osteoclasts in HE staining remained low in metformin treatment. Although metformin treatment can promote the proliferation and differentiation of bone-oriented BMSCs, and inhibit the differentiation of lipo-oriented BMSCs, promote the differentiation and mineralization of osteoblasts in vitro [6]. But in our study, metformin cannot inhibit negative effects of the high glucose and hyperosmotic environment on osteoblasts.

Recent studies found that metformin can decrease the progression of osteoarthritis and repair cartilage. Feng et al. found that there were fewer MMP-13 positive cells in metformin treated DMM mice, and that metformin reduced chondrocytes p16 levels in OA mice; and increase AMPK polarization and inhibited mTORC1 expression in OA mice [46]. Li et al. found the metformin treatment of the DMM mice by intra-articular injection and lavage can reduce the cartilage injury, OARSI scores and the matrix metalloproteinases 13 levels; and production of α collagen can be increased by activating the AMPK pathway[47]. Furthermore, some studies showed that metformin can up-regulate the expression of phosphorylated AMPK and total AMPK in articular cartilage tissue in DMM mice; its certain protection through AMPK signaling pathway. It has been demonstrated that metformin treatment after joint injury can inhibit the development of OA in injury-induced OA animal models [48].

ZA has been proved to prevent bone loss and increase bone density in diabetic animal model [12]. Basso FG et al. found that zoledronic acid have cytotoxic effects on osteoblasts by reducing cell viability (CV), Total protein production (TPP), alkaline phosphatase (ALP) activity, mineral nodular formation (MNF), ALP, osteocalcin (OCN) gene expression and strong cellular morphological changes [49]. Patntirapong S et al. found that zoledronic acid can reduce the formation of bone nodules and damage the cellular activity of osteoblasts/osteoblasts (damage the cell proliferation and differentiation ability of osteoblasts and preosteoblasts, and mediate defective bone mineralization) [50]. However, zoledronic acid can promote their proliferation, stimulate their osteogenic differentiation, and increase the expression of OCN, RANKL, OPG and type II collagen for BMSCs [13]. The number of osteoclasts increased and their activity increased in the occurrence of osteoporosis. Osteoclasts are an important anti-osteoporosis therapy breakthrough. The mechanism of anti-osteoporosis using zoledronic acid is to inhibit osteoclasts activity and reduce bone loss [11]. Lin et al. demonstrated that zoledronic acid inhibited NF- κ B and JNK signaling pathways by cell activity assay, osteoclast generation, immunofluorescence and bone resorptive lacunae test for inhibiting the generation and absorption of osteoclasts [40]. Other studies also demonstrated that zoledronic acid inhibits osteoclast migration, differentiation and bone resorption through the AMPK pathway [15]. More studies demonstrated the inhibit effect of zoledronic acid on osteoblasts and osteoclasts, but a few studies found the positive effect which could be caused by the different zoledronic acid dosage, treatment time, experimental scheme and cell lines. So, zoledronic acid as anti-osteoporosis drug, also has an effect on fat cells? These require our further experimental research.

ZA injection is an effective anti-osteoporosis drug combined with metformin. HE staining found that there were a large number of adipocytes in the zoledronic acid treated DB/DB mice bone marrow cavity, and the number of bone trabeculae was slightly more than those without zoledronic acid. Metformin

treatment DB/DB mice had lower adipocytes, more hematopoietic cells, and slightly increase trabeculae in bone marrow compared with those without antidiabetic drug. However, ZA combined with metformin treatment in DB/DB mice results in reduction of fat cells in the bone marrow cavity and the increase of the number of bone trabeculae.

OPG immunohistochemical staining of osteoblasts demonstrated the expression level of osteoblasts in CMT group was higher than in ZA group and MET group. The TE staining and semi-quantitative analysis were used and found that more bone lacunae in the osteoporosis DB/DB mice with some cases of empty bone lacunae. The number of bone lacunae ZA group was comparable to CMT group in DB/DB mice, but the number of bone lacunae MER group in DB/DB mice had not increased. Micro-CT scanning demonstrated that the bone mass in ZA group DB/DB mice was higher than without ZA. The number of bone trabeculae and SMI were higher before using metformin in the DB/DB mice treatment, which indicated that the ZA treatment with metformin could do better to prevent bone loss and osteoporosis than ZA alone. This result could be due to the fact that metformin inhibits the proliferation of adipocytes and promote the repair of blood vessels, which further provides blood supply to bone.

Of course, our research also has certain limitations. The sample size is small, the difference in bone microstructure indicators is not too obvious, and there is a certain statistical deviation.

In conclusion, zoledronic acid combined with metformin reduced bone mass loss in DB/DB mice to a certain extent and was superior to zoledronic acid or metformin alone for anti-osteoporosis therapy.

Declarations

Acknowledge

Not applicable.

Author Contributions

All authors were responsible for experimental design, animal feeding, and data processing. LYS and ZW were responsible for experimental design and article writing. YXY completed feed and pathological staining. Data statistics and analysis are handled by MWJ.

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Availability of data and materials

Please contact the correspondence author for the data request.

Ethics approval and consent to participate

All procedures were approved by the Experimental Animal Care and Use Committee of the first affiliated Hospital of Chengdu Medical College and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

Figure 1

The weight of mice

Figure 2

Blood glucose of mice

Figure 3

Observation under 50 times microscope of HE staining

Figure 4

Average area value of positive results after OPG staining Remark *P<0.05 vs WT, †P<0.05 vs DB/DB, ‡P<0.05 vs ZA, §P<0.05 vs MET

Figure 5

Observation under 400 times microscope of OPG staining. A positive result is that the cytoplasm is brown or brown, and the cytoplasm is blue. The red arrow points to positive cells

Figure 6

Bone lacuna count after TE staining Remake *P $\leq$ 0.05 vs WT, †P $\leq$ 0.05 vs DB/DB, ‡P $\leq$ 0.05 vs ZA, §P $\leq$ 0.05 vs MET

Figure 7

Observation under 400 times microscope of TE staining Remake The red arrow points to osteoclasts (nucleus>3)

Figure 8

The results of 7 sets of data of mouse tibia after Micro-ct scanning, Data are expressed as mean $\pm$ standard deviation (SD) Remark * $P \leq 0.05$ vs WT, $^{\dagger}P \leq 0.05$ vs DB/DB, $^{\ddagger}P \leq 0.05$ vs ZA, $^{\S}P \leq 0.05$ vs MET

Figure 9

The tibia of mice was scanned by Micro-CT Remake. The two-dimensional image (2D image) in the figure shows the number and shape of trabecular bone in the bone marrow cavity. The segmented (SEG) image reflects the number of trabecular bone. The trabecular thickness (TH) image reflects the distribution of trabecular bone. The color is determined by Green to red, reflecting the trabecular bone from thin to thick.