Diabetes Mellitus Accelerates Progression of Osteoarthritis in Streptozotocin-induced Diabetic Mice by Deteriorating Bone Microarchitecture, Bone Mineral Composition and Bone Strength of Subchondral Bone

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Research

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Abstract

Background : The purpose of this study is to develop an optimal DM-OA mice model; to validate that diabetes aggravates OA; and to evaluate the microarchitecture, chemical composition and biomechanical properties of SB as a consequence of the damaged induced by diabetes OA.

Methods: Mice were randomly divided into three groups: DM-OA group, OA group and Sham group. Blood glucose levels, body weight and food intake of all animals were recorded. A semi-quantitative grading system (OARSI) was used to evaluated cartilage and SB degeneration. Microspectroscopy, microindentations, micro-CT imaging and fracture load of compression testing were also used to evaluated the properties of trabecular SB.

Results: Glycemic monitoring and pancreas pathological results indicated stable high blood glucose and massive destruction of pancreas and islet cells in the DM-OA group. The mineral-to-collagen ratio, microindentation elastic modulus, hardness, micro-architectural parameters, bone mineral density and fracture load of SB trabecular bone of the DM-OA group joint was lower than other two groups. On the other hand, The OARSI score, trabecular spacing and structural model index of the DM-OA group joint was higher than the other two groups.

Conclusions: The glycemic and pancreatic pathological results indicated the DM-OA model to be a simple and reliable model induced by STZ and surgery. The results showed the mechanisms in which diabetes accelerates OA by damaging and deteriorating the functions SB including microarchitecture, chemical composition and biomechanical properties.

Background

Osteoarthritis (OA), the most common form of degenerative joint disorder, is associated with a significant economy and health burden of disease on both an individual and global scale, as it is forecasted that 25% of the adult population(80% of the geriatric population over the age of 60), or more than 50 million people in the US, will be affected by this disease by the year 2020[1, 2]. AS a multifactorial disease, OA is characterized by pain, stiffness and functional impairment ultimately resulting in disability and impaired quality of life, especially in people 65 years and older. Although the precise etiology is still not clear, it is now considered that the whole joint is involved in the progression of OA. And growing evidence suggests subchondral bone plays a crucial role in the initiation and progression of OA[3]. The manifestations of subchondral bone exhibit dynamic remodeling parallel or even precede with cartilage degradation including architecture, biochemical composition, biomechanics and physiological function, which is widely considered as a hallmark a and a central feature of OA disease progress[4, 5]. The microarchitectural and histopathological changes not only deteriorate physiological function and integrity of subchondral bone, but also deteriorate the interaction (crosstalk) between cartilage and subchondral bone, which accelerate the progression of OA. In normal joints, subchondral bone exerts important stress bearing and structural support function just as a shock absorber and structural girder.

Subchondral bone can buffer approximately 30% of the intra-articular stress, can maintain the matching of the joint, and can prevent intra-articular stress concentration[6]. Heterogeneities in the architecture, density and rigidity of subchondral bone in OA, along with reduced elasticity, result in abnormal tensile stress and shear stress towards articular cartilage, thus facilitating secondary cartilage damage and degeneration. On the other hand, SB supplies nutrients to cartilage with about 50% of its glucose and oxygen requirements and facilitates the removal of metabolic waste products by the healthy crosstalk between SB and cartilage, which is also important to maintain the normal function of the joint [7]. In OA joints, abnormal SB remodeling and biomechanical properties could deteriorate the nutritional and biochemical interaction balance between SB and cartilage. what is more important, the transport of pain receptors, sensory nerves and biomolecule such as chemokines, cytokines, inflammatory mediators and proteases were surprisingly elevated by increased micropores, microcracks, fissures, vascular channels and neovascularization, which can delay cartilage repair, promote chondrocyte death and matrix degradation, finally enhance the clinic symptoms. In turn, cartilage damage could influence subchondral bone deterioration. This vicious circle leads to the progression of OA. Recently, animal and clinical trials have been proved that targeting SB via bone protective agents can break this vicious circle, relieve joint pain and can slow down articular cartilage degeneration[5]. Therefore, SB is currently recognised as a key to develop targeted and effective therapeutic strategies to treat OA.

Like OA, Diabetes Mellitus (DM) is also a common chronic disease with high morbidity and disability that threaten the global public health. An interesting point is that DM and OA share common risk factors represented by age, diet, obesity and lifestyle and the multifactorial etiology characterized by a combination of both genetic and environmental factors. Accumulating epidemiological and experimental findings have proved that DM is an independent risk factor and prognostic predictor for OA[8, 9]. In a cross-sectional study of the US Third National Health and Nutrition Examination Survey (NHANES III) has demonstrated that 11% of the patients with OA had DM, which was a significantly higher prevalence compared to the prevalence of 6% in the general population[10]. Similar results were derived from the Japanese Research on Osteoarthritis Against Disability study[11]. The association between OA and DM is also true for hand, that the patients with type 2 diabetes had a 2 times higher rate of hand OA than non-diabetics and it seems to be associated with pain in erosive hand OA[12]. Furthermore, a 20-year longitudinal cohort study of 927 individuals revealed that DM has an independent role in the pathogenesis and advancement of OA, controlling age, sex, and body mass index[9]. Additionally, diabetes increases the complications and revision rate of arthroplasty procedures[13]. Recently, the epidemiological association between OA and DM has been further confirmed in two independent meta-analyzes studies which showed stronger evidence is accumulating in favor of an independent contribution of DM to the development and progression of OA[14, 15]. However, the mechanisms underlying the connection between both diseases remain unclear. There is also an unfortunate paucity of quality research studies in animal models of DM relevant to OA[16]. While recent statistics show that diabetes affects 382 million adults worldwide, and this number is estimated to rise to 592 million by 2035 which means the prevalence of DM-OA will be widely and abruptly growing all over the world[17–19]. A

better understanding of the mechanisms of diabetic osteoarthritis becomes essential to provide opportunities to identify new therapeutic targets that might prevent and predict this disease.

By now, it has been well established that DM extremely and negatively affect bone health, and it is advocated that diabetes be incorporated as risk factors for fractures at all ages in both male and female individuals and increases further above that of the general population with ageing[20]. In particular, in subjects with T1D there is an about sixfold increase in hip fracture risk and an about twofold increase in vertebral fracture risk compared to individuals without diabetes [21]. Type 2 diabetes (T2D) is also associated with an about two- and threefold increase in hip fracture risk and 50–80% increased extremity fracture risk compared with non-diabetic subjects[22]. Moreover, individuals with diabetes experience worse fracture outcomes than normoglycemic individuals[23]. Diabetes could impact the bone quality and strength by unbalancing several processes and systems: bone formation, bone resorption, collagen formation and collagen cross-linking, secretion of inflammatory cytokines, skeletal muscle, incretin system, bone marrow adiposity, calcium metabolism[24]. Consequently, we hypothesized the DM could also interfere with subchondral bone remodeling, and aggravate the severity of OA. Unfortunately, there is a paucity of quality research in the effect of DM on SB and its role in DM-OA progression both in clinic and animal study.

Therefore, we performed this comprehensive study to assess change of physiological properties and functions of SB and cartilage during the progression of diabetes OA and the purpose of this study had three folds: 1) to develop a DM-OA mice model which would allow for clinical relevant and biological research; 2) to validate that diabetes aggravates OA pathogenesis in-vivo; and 3) to evaluate the microarchitecture, chemical composition and biomechanical properties of SB as a consequence of the damaged induced by diabetes OA.

Materials And Methods

Animal model and treatment

8 wk old male C57BL/6J mice from Guangdong Medical Lab Animal Center, (Guangzhou, China) were used in the present study. The mice were divided randomly in three groups: DM-OA group, OA group and Sham group. After 1 week of acclimatization, the DM-OA group was injected intraperitoneally (IP) with 150 mg/kg streptozotocin (STZ, Sigma, St Louis, MO, USA) at a single injection to induce diabetes, while the OA group and Sham group were given PBS vehicle at the same time. On the third day after STZ injection, blood glucose was measured in whole blood collected from the tail vein by a portable Glucometer (Accu-Check Active, Roche Diagnostics Limited, Germany). Only mice with a basal blood glucose level above 300 mg/dL for two consecutive days were considered diabetic otherwise the rest of the animals of DM-OA group were excluded from the study.

After quarantine and conventional health measures, OA was surgically induced in DM-OA group and OA group by transection of the anterior cruciate ligament (ACLT) of the right knee, as previously described[25]. Briefly, the ACL was transected to induce abnormal mechanical loading-associated

osteoarthritis, and make sure that no other structures were damaged. For the Sham group, the similar incision was closed after exposing the ACL, being used as a control. Mice were observed daily to verify healing and to ensure that they were using their right limb.

The second day after surgery was defined as day 1. The weekly blood glucose, body weight and food intake of all animals were recorded during the entire experimental period. The animals were group housed at temperature-controlled quarters (20–22°C), on a 12-hour/12-hour light/dark cycle, and ad libitum access to water and standard chow. All cages contained wood shavings, bedding and a cardboard tube for environmental enrichment. The Animal Care and Experiment Committee of Jinan University School of Medicine approved all experimental protocols. (Fig.1 exhibited the timeline of the study.)

Follow-up

Animals were sacrificed at 4, 8, and 12 weeks after the operation. After overnight fasting and mild ether anaesthesia, blood and pancreas were sampled. Blood samples were withdrawn by venipuncture from the retrobulbar veins into plastic tubes with appropriate anticoagulant (ethylenediaminetetraacetic acid-EDTA) to measure Glycated hemoglobin (GHb, HbA1c) and serum samples without anticoagulant to measure glucose (Glu) by an automatic analyzer (Roche Cobas 6000 c501). The pancreas was placed in 4% paraformaldehyde and stained with hematoxylin–eosin (HE) for histopathological study

The entire right knee joints from each group at every time point were dissected and fixed in 4% paraformaldehyde for 48 h. Then the joints were decalcified in 10% ethylenediaminetetraacetic acid for 3 weeks and were embedded in the same paraffin block. The samples were cut into 5-µm sections and prepared for histological analysis and immunohistochemistry to evaluate cartilage and subchondral bone degeneration. The other undecalcified joints were used to evaluate the properties of subchondral bone, including micro-CT imaging, confocal Raman microspectroscopy, and microindentation.

Intraperitoneal glucose tolerance test (IPGTT)

At about one week before termination, IPGTT was conducted to evaluate the glycogenic response to exogenously administered glucose in unanaesthetized animals. After an overnight fast (12–16 hours), the mice were injected intraperitoneally with 40% glucose (2 g/kg body weight). Tail blood samples were collected at 0, 30, 60, and 120 minutes after administration of glucose for measurement of glucose.

histological analysis and assessment

After knees were decalcified for 4 weeks in 4% paraformaldehyde and processed for standard paraffin embedding. On the basis of the OARSI recommendations for the histological assessment of osteoarthritis in the mice[24], three sections from each knee with 100 µm intervals were stained with Safranin O and Fast green, and subsequently cartilage damage was scored using the semi-quantitative grading and staging system (Pritzker et al. 2006). Briefly, grade 0 corresponded to normal articular cartilage; grade 0.5: loss of Safranin-O and/or cellular changes without structural changes to the articular surface; grade 1: small fibrillations without loss of cartilage; grade 2: vertical clefts down to the layer immediately below

the superficial layer and some loss of surface lamina; grade 3: vertical clefts/erosion to the calcified cartilage extending to <25% of the articular surface; grade 4: vertical clefts/erosion to the calcified cartilage extending to 25-50% of the articular surface; grade 5: vertical clefts/erosion to the calcified cartilage extending to 50-75% of the articular surface; grade 6: vertical clefts/erosion to the calcified cartilage extending >75% of the articular surface. Grades were assigned to the medial and lateral tibial plateau, and medial and lateral femoral condyles. Slides were blinded and Grades for all slides from each location of each sample were averaged, and grades from the three readers were averaged for each location.

Micro-CT imaging and analysis

To monitor the structural alterations and the tissue mineral content in the subchondral bone, the knee joints were scanned by micro-CT (Skysan, Belgium) with isotropic voxel size of 9 μm , as described previously. Regions of interest (ROI) within the trabecular bone restricted to the distal femoral epiphysis and proximal tibial epiphysis were created (Fig. 3), and trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th), trabecular mineralization density (BMD), and other trabecular bone parameters were determined using SkyScan software. All measured parameters are represented as the mean $\pm$ SD. Mimics software (Materialise, Plymouth, MI) was used to create three dimensional reconstructions for qualitative analysis.

Confocal Raman microspectroscopy

The chemical composition of the subchondral bone trabecular (mineral-to-collagen, inorganic (mineral) to organic (matrix) ratio, $\text{PO}_4^{3-} \nu_1/\text{proline}$) was characterized by confocal Raman microspectroscopy (Jobin Yvon, Horiba, France) as described previously[8,36]. Joint samples were dehydrated in a graded alcohol series and embedded in PMMA. The surface of subchondral bone trabecular was exposed by slicing in the frontal plane using a hard tissue cutter (Exakt 300CP, Exakt Technologies, Germany). The section surface was ground on successive grits of silicon carbide paper and polished with 1-mm alumina slurry. The $\times 50$ objective focused the laser (785-nm laser diode source) on a 3 μm region within the trabecular, and inelastic light was acquired by a spectrograph with 0.5cm^{-1} spectral resolution. The measured spectra consisted of three accumulations with an integration time of 10 seconds each. Using vendor-supplied software (Jobin Yvon), background fluorescence in the spectra was subtracted by a modified polynomial fitting algorithm. Spectra were collected from 10 trabecular locations within the distal femoral epiphysis and proximal tibial epiphysis respectively. The mineral-to-collagen ratio was calculated as the ν_1 phosphate peak intensity (962cm^{-1}) per proline peak intensity (856cm^{-1}) and averaged per bone.

Microindentation testing

Subchondral bone trabecular material properties were analyzed with microindentation testing using the micro-indentation testing system (Microhardness testing machine, FUTURE-TECH CORP, Kanagawa, Japan) equipped with a Berkovich diamond tip indenter as previously described (23, 24). Briefly, samples

were mounted and set in low temperature curing epoxy resin (EpoFix cold setting resin, Struers) and then ground through a series of silicon carbide grinders followed by diamond polishing. Specimens were examined visually under an optical microscope, and at least 10 indentations were made on each sample. A typical indentation experiment resulted in a loading and unloading force–displacement curve. The Vickers diamond pyramidal indenter compressed at 25g compression load onto the trabecular for a dwell time of 10s. An indentation mark was formed on the trabecular after loading. The diagonal length of the indentation mark was measured immediately (within 1 min) after the indentation to avoid the fast recovery of the indentation mark on the tissue matrix. The elastic properties were calculated between 50% and 95% of the initial unloading curve and the Oliver-Pharr method was used to calculate elastic modulus and hardness.

Mechanical compression of the SB

The proximal portion of the tibial epiphysis was grinded and mounted perpendicular to the tibial shaft for a punch-type compression test to measure the compression strength of the SB. The tibia was kept moist by wrapping in saline soaked gauze from the time of collection up to the time of testing. Then the tibia was positioned and centered under a 0.8 mm diameter flat-tipped cylindrical punch fixed to the actuator of the materials testing machine (Enduratec ELF 3200, Bose Corp., Eden Prairie, MN). The axial compression was produced via the flat cylindrical punch at a displacement rate of 1 mm/min. During each test, the punch was centered on the trabecular bone while avoiding contact with the cortical bone. The displacement and compressive force were recorded until it peaked on the load-displacement diagram.

Statistical analysis

All data are presented as mean and standard deviation (SD) and were analyzed using the Statistical Package for Social Sciences (SPSS, Chicago, IL; version 22.0). Descriptive statistics was used to describe the demographic and measurement variables of all the scapulae. Categorical variables were expressed as frequencies and percentages for each variable. Continuous variables were presented as mean values $\pm$ SD. The factorial design ANOVA and Student Newman-Keuls was applied to compare types. A P-value <0.05 was considered statistically significant.

Results

Induction of Diabetes and physiological parameters in experimental animals.

Induction of diabetes with STZ was both effective and sustained for DM-OA group. As depicted in Figure 2A, glycemic monitoring showed that blood glucose markedly increased in DM-OA group at all time points from 3 days after STZ administration. Consistent with the elevation in blood glucose levels, HbA1c level was also significantly greater in DM-OA group compared to OA group and Sham group ($8.24\pm0.46\%$, $3.91\pm0.41\%$ and $4.18\pm0.35\%$, respectively). In contrast, analysis of the AUC demonstrated showed an impaired glucose control for DM-OA group by using an IPGT test. Mean AUC of DM-OA group was

significantly greater than OA group and Sham group ($P < 0.05$). Pathological and histological results (Fig.2D-1) from DM-OA group indicated massive destruction of pancreas which exhibited a decreased number of islets, disruption of islet cytoarchitecture, and weaker insulin staining of granulated β cells compared with OA group and Sham group. While no significant differences in the physiological parameters were observed between OA group and Sham group.

Histological analysis and assessment

Osteoarthritis was accelerated significantly in DM-OA group assessed by histological analysis

The cartilage integrity in the tibial plateau and femoral condyle at 4, 8 and 12 weeks post operation was assessed histologically and scored according to the OARSI scoring system. As indicated by safranin-O/fast green staining (Figure 3 and 4A), cartilage matrix and chondrocyte loss occurred and worsened in DM-OA group than OA group by a time-dependent manner from 4 to 12 weeks post operation, whereas the sham group showed no obvious changes at three time points. At 4 weeks, the DM-OA group and OA group joints exhibited minor cartilage degeneration; matrix and chondrocyte loss affected the superficial and upper middle zone, and the histological score was no statistical difference. Subsequently, moderate articular cartilage degeneration reached the middle or deep zone and extended more substantially into the cartilage at 8 weeks. Cartilage histological scores of DM-OA group and OA group were significantly higher than the sham group. At 12 weeks, severe cartilage degeneration occurred more widespread; matrix and chondrocyte loss deepened to the tidemark. Cartilage histological score of DM-OA group exhibited significantly higher than OA group and sham group.

Micro-CT imaging and analysis

DM-OA group have a significant decrease in subchondral bone mass and rapid destruction of subchondral bone trabecular architecture

Representative three-dimensional pictures and morphometric parameters of the tibial SB from three groups obtained by micro-CT scanning confirmed that diabetes mellitus deteriorated trabecular bone architecture and accelerated progression of OA shown in Figure 5 and 6. In general, subchondral bone of tibial SB showed dynamic alterations following OA induction from bone resorption at the early stages (4 to 8 weeks) to bone accretion at the advanced stages (8 to 12 weeks) in OA group. In contrast, DM induced significant losses and resorption of trabecular bone result in destruction of SB structure in DM-OA group throughout the whole process. Specifically, compared with the Sham group, the BV/TV, Tb.Th and BMD in OA group showed decreased from 4 to 8 weeks ($P > 0.05$), then increased significantly from 8 to 12 weeks ($P < 0.05$). These changes were accompanied by an increase Tb.Sp and SMI from 4 to 8 weeks ($P > 0.05$), then a decrease from 8 to 12 weeks ($P < 0.05$). On the other hand in DM-OA group, there was a greater raise in BV/TV, Tb.Th and BMD ($P < 0.05$). This was paralleled by a decline in Tb.Sp and SMI during the whole process ($P < 0.05$).

Confocal Raman microspectroscopy

Declined Trabecular Bone Mineralization in DM-OA group

Raman spectral parameters of mineral and matrix composition were compared between three groups to assess DM effects on SB composition illustrated in Figure 4B. Analysis of Raman spectra revealed that although no significant difference in mineral-to-collagen ratio was observed at 4 weeks, a reduced mineral-to-collagen ratio was found in DM-OA group and OA group at 8 and 12 weeks, especially a significant decline in DM-OA group that were aggravated with the progression of the DM. Specifically, the mineral-to-collagen ratio of SB in DM-OA group was reduced from 9.19 ± 2.03 at 4 weeks to 4.89 ± 1.97 at 12 weeks. Simultaneously, the mineral-to-collagen ratio of SB in OA group was reduced from 9.06 ± 1.96 at 4 weeks to 7.43 ± 1.63 at 12 weeks, whereas in sham group the ratio was 9.13 ± 1.87 and 9.32 ± 2.11 respectively.

Microindentation testing

DM-OA group have reduced material mechanical strength of trabecular bone in SB

To further elucidate the intrinsic bone tissue mechanical properties, we investigated the mechanical response of the trabecular bone matrix at the tissue-level displayed as elastic modulus E_r and hardness H quantified by microindentation (Figures 7). The mechanical properties of the subchondral bone in DM-OA group showed statistically reduced compared with OA group as the DM progressed following the operation, whereas there seem no statistically significant differences in sham group throughout the course of the experiment. Specifically, the elastic modulus of trabecular bone in DM-OA group was reduced from 19.9 ± 1.2 at 4 weeks to 15.1 ± 1.5 at 12 weeks. Simultaneously, the mineral-to-collagen ratio of SB in OA group was reduced from 665.06 ± 42.27 at 4 weeks to 392.25 ± 34.61 at 12 weeks.

Mechanical compression of the SB

DM-OA group Exhibited a deterioration of mechanical Properties of SB

Last, the biomechanical properties and strength of SB was assessed by materials testing machine. Of note, although the difference of fracture load in 4 weeks was insignificant, there was a significant decrease in DM-OA group and OA group in 8 weeks. Moreover, the fracture load of SB trabecular bone in DM-OA group exhibited continuously diminished than that OA group and Sham group in 12 weeks. As shown in the Fig 4C, the fracture load of SB trabecular bone in DM-OA group was reduced from 4.83 ± 0.64 N at 4 weeks to 1.71 ± 0.18 N at 12 weeks.

Discussion

In this longitudinal research, we accurately quantified and compared the changes to joint derangement at three different time points of three mice groups during the initiation, progression, and end stages (or joint failure) of OA and DM development. More importantly, our study firstly investigated quantitative change

of subchondral bone on the architecture remodel, biochemical composition and biomechanics and physiological function in a DM mice model. This work successfully proved that diabetes aggravated OA pathogenesis in a novel DM-OA mice model which could be helpful for clinical and basic science research. Furthermore, the results showed the mechanisms in which diabetes accelerates OA by damaging and deteriorating the functions SB including microarchitecture, chemical composition and biomechanical properties. To our knowledge, this work is the first to describe the pathogenesis of diabetic osteoarthritis through diabetes and SB.

Diabetes mellitus (DM) and osteoarthritis (OA) are the most common coexistent diseases which are associated with a significant economy and health burden on both an individual and global scale. As early as in the 1980s, many doctors and researchers had noticed this phenomenon[26]. Now a burgeoning body of evidence proved that DM adversely affect articular tissues and exacerbate OA[8,9]. Most notably a 20-year longitudinal cohort study of 927 individuals revealed that DM has an independent role in the pathogenesis and advancement of OA, controlling age, sex, and body mass index[9]. Additionally, diabetes increases the complications and revision rate of arthroplasty procedures[13]. Recently, the epidemiological association between OA and DM has been further confirmed in two independent meta-analyzes studies which showed stronger evidence is accumulating in favor of an independent contribution of DM to the development and progression of OA[14,15]. Nevertheless, the effect has not been validated in vivo cartilage yet, but using clinic follow up information and statistical results[16]. In this study, we found a higher histological OARSI score in DM-OA group compared to OA group and Sham group, which is consistent with previous clinic reports.

However, the mechanisms underlying the connection between both diseases remain unclear[16]. By far most basic science studies focalized potential mechanisms linked to DM-influenced end-organ joint damage. Mediators such as hyperglycemia, AGEs, sorbitol, adipokines, and cytokines act through oxidative, osmotic, and inflammatory mechanisms to produce tissue damage[16]. For example, one group found that human OA chondrocytes from donors cultured in high glucose are unable to decrease GLUT1 protein or reduce glucose transport activity in comparison with normal chondrocytes. They also found that high glucose conditions favored production of oxidants and promoted matrix catabolism which would accelerate OA[27]. Nevertheless, the majority of researches are vitro experiment with the chondrocytes in a hyperglycemic environment, which still have a big gap from vivo study with the complicated and inherent characteristic[13]. There is an unfortunate paucity of quality research studies in animal models of DM relevant to OA[16]. Relatively few studies using STZ induced DM in mice or rats suggested the presence of cartilage damage because of hyperglycemia. What is a pity, those studies were always cross-section and showed slight and mild joint symptoms identified early stages of OA[8,9]. In our longitudinal study about a 4 month period, we were able to accurately quantify and compare the changes to joint derangement at three different time points of three mice groups during the initiation, progression, and end stages (or joint failure) of OA and DM development. The present findings firstly demonstrated that DM aggravated cartilage degeneration and OA pathogenesis occurred in a time-dependent manner in instability-induced model. These findings further strengthen the concept of a strong metabolic

component in the pathogenesis of OA. Therefore, this validation study sets the precedent for future investigations into possible mechanisms or pharmacological interventions using our longitudinal model.

In normal joints, subchondral bone exerts important stress bearing and structural support function just as a shock absorber and structural girder. Subchondral bone can buffer approximately 30% of the intra-articular stress, can maintain the matching of the joint, and can prevent intra-articular stress concentration[3]. On the other hand, SB supplies nutrients to cartilage with about 50 % of its glucose and oxygen requirements and facilitates the removal of metabolic waste products by the healthy crosstalk between SB and cartilage, which is also important to maintain the normal function of the joint[6]. The manifestations of subchondral bone exhibit dynamic remodeling parallel or even precede with cartilage degradation including architecture, biochemical composition, biomechanics and physiological function, which is widely considered as a hallmark and a central feature of OA disease progress[7]. Therefore, SB is currently recognised as a key to develop targeted and effective therapeutic strategies to treat OA. By now, it has been well established that DM extremely and negatively affect bone health, including cortical bone and cancellous bone[5]. DM could deteriorate the bone quality and strength, eventually lead to drastically rise fracture risk. In particular, in subjects with T1D there is an about sixfold increase in hip fracture risk and an about twofold increase in vertebral fracture risk compared to individuals without diabetes[21]. Nevertheless there is almost no report about the impact of DM to the subchondral bone. Therefore, to better reveal the role of subchondral bone in diabetic OA pathogenesis, our study firstly showed quantitative longitudinal data change on the architecture remodel, biochemical composition and biomechanics and physiological function in a DM mice model, which fill out the research gap between articular cartilage degeneration and subchondral bone abnormality, which are the primary concern in knee OA.

It is widely accepted that the microarchitecture and bone mass of subchondral bone is relate directly to the mechanical properties and as determinants of physiological function which exerts important stress bearing and structural support function[6]. Although few studies are about the subchondral bone, there are abundant research demonstrated the DM have adverse effects on cancellous bone, leading to microarchitectural deterioration[20]. More recent studies employing advanced three-dimensional imaging techniques have confirmed that total volumetric BMD in people with T1DM was significantly lower 22 to 37% in comparison to individuals without diabetes at various skeletal sites, including the femoral neck, lumbar spine, distal radius, and distal tibia[21]. MRI studies in young women with childhood-onset T1DM also have shown clear deficits in the trabecular microarchitecture of the tibia [28]. An animal study conducted on rats showed 60% of reduction in proximal tibial trabecular bone of rats affected by T1DM when compared with nondiabetic controls, with an additional 30% reduction concerning the moments of inertia at the ulnar or femoral diaphysis, these findings were consistently confirmed in other clinic reports[29]. In this study, we found lower total and trabecular volumetric BMD and substantial microarchitectural abnormalities, including lower trabecular thickness and decreased trabecular number and greater trabecular separation and network inhomogeneity compared with other two groups mice. Especially the much higher SMI indicated that the trabeculae in diabetes group were markedly more rod-like compared with non-diabetic group. Rod-like trabeculae possesses lower mechanical strength and

stiffness than plate-like trabeculae. In clinic, the bone loss at subchondral plate in knee osteoarthritis patients with hypertension and type 2 diabetes mellitus has been documented compared to nondiabetic controls, which is consistent with our result[30]. These microarchitectural deterioration were associated with more severe cartilage destruction in DM-OA group, supporting the paradigm of focal interactions between subchondral bone and cartilage in pathogenesis of knee OA.

DM not only lead subchondral bone loss and microarchitectural deterioration, but also decrease trabecular bone mineral mass including the mineral and organic component, eventually, invoke the intrinsic material mechanical strength[23]. From the pathophysiological point of view, bone metabolism in T1D patients is characterized by low bone turnover, and in particular by reduced bone formation due to decreased osteoblastogenesis, low levels of osteocalcin and reduced mineral apposition rate[31]. Additionally, slow and short osteoblastic cycle is accompanied by decreased bone mineralization[20]. The major experimental models of T1D indeed showed that diabetic bones display specific defects of bone mineralization, including decreased hydroxyapatite crystal perfection, decreased calcium-to-phosphate composition of the ash, and decreased ash content in certain bones such as the tibial metaphysis[32]. In this study, we found a significant reduced mineral to collagen ratio of subchondral bone trabecular in DM-OA group at 8 and 12 weeks by confocal raman microspectroscopy test at the microscopic level. Our result further confirmed reduced mineralization in subchondral bone trabecular which is a good complement and consistent with previous studies in other locations. To better reveal the change of subchondral bone trabecular in diabetic OA pathogenesis, we also examined subchondral bone trabecular mechanical strength by microindentation test. Consequently, the elastic modulus and hardness of subchondral bone trabecular significant reduced in DM-OA group, which is influenced by intrinsic bone material properties including mineralization and collagen integrity. Microindentation test has been used in vitro to evaluate variations among different anatomical sites, the effect of pathologies on the mechanical properties of bone structural units, the bone matrix quality close to implants and the effect of tissue condition (hydrated, dried or embedded) during the test[33,34]. However, to the best of my knowledge, this is the first time to demonstrate DM contributes to a reduction of subchondral bone strength at the macro-level. Recently, another kind of microindentation using the OsteoProbe technique has gained acceptance as a vivo research tool for estimating bone material strength in human beings non-invasively[35,36]. Following local anesthesia, this device creates microindents over the shaft of the tibia, which provides a measure of bone material strength (bone material strength index [BMSi]). Using this device, Farr et al. found that compared to controls, postmenopausal women with DM had significantly lower BMSi for 11.7%, which is consistent with our finding on mice and also pointed to impaired bone material properties as a potential cause of the increased fracture risk observed in these patients[37].

Besides providing subchondral bone trabecular strength, mineral and organic component at the macro-level, we also studied subchondral bone mechanical properties in apparent level in order to reflect the combined effects of microarchitecture, turnover, damage accumulation (e.g., microfractures), and mineralization. Due to mice small size, a 0.8 mm diameter flat-tipped cylindrical punch was fixed to the actuator of the materials testing machine and compressed directly to record the mechanical properties data. Uma Sankar and many studies developed and improved this method to measure the axial

compressive strength of the cancellous bone in femur of mice, which is more precise and visual than finite element modeling[38-43]. The present result in Sham group was consistent with form studies. What is difference is that we continuously tested at three different time points in our longitudinal study and the ultimate stress parameter of DM-OA group was significantly lower than the OA and Sham group, which was corresponding to the significantly worse parameters of the cartilage histological score, subchondral bone architecture and tissue properties in DM-OA group. These data further illustrate DM aggravated cartilage degeneration and OA pathogenesis by deterioration the stress bearing and structural support function of subchondral bone. The form studies also demonstrated DM extremely and negatively affect bone quality and strength from various anatomic sites[22]. Whereas threepoint bending can still be used to study long bone, methods such as direct compression that are commonly used to measure cancellous bone mechanical properties of the spine[24]. As a consequence, the fracture risk of diabetes patients was increased by approximately 70%[21].

Conclusion

In conclusion, for the first time, the present study proved that diabetes aggravated OA pathogenesis in a novel DM-OA mice model which could be helpful for clinical and basic science research. More importantly, the results showed the mechanisms in which diabetes accelerates OA by damaging and deteriorating the functions SB including microarchitecture, chemical composition and biomechanical properties.

Abbreviations

DM: Diabetes mellitus; OA: Osteoarthritis; SB: Subchondral Bone; ACL: Anterior cruciate ligament; STZ: Streptozotocin; T1D: Type 1 diabetes ; T2D: Type 2 diabetes; IPGTT: Intraperitoneal glucose tolerance test; BMD: Trabecular mineralization density; Tb.N: Trabecular number; Tb.Sp: Trabecular separation; Tb.Th: Trabecular thickness; ROI: Regions of interest; SD: Standard deviation.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The data that support the findings of this study are available within the article from the corresponding author upon request.

Competing Interests: The authors have no conflicts of interest to disclose.

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Figures

Figure 1

Timeline of the animal model and treatment before follow-up.

Figure 2

Induction of Diabetes and physiological parameters in experimental animals. (A) After the injection of STZ, the mean level of blood glucose was greater in DM-OA group compared to OA group and Sham group, $*P < 0.05$. (B) HbA1c level was also significantly greater in DM-OA group compared to OA group and Sham group, $*P < 0.05$. (C) Results of intraperitoneal glucose tolerance tests (IPGTTs) revealed a significant reduction in glycemic control in DM-OA group. Area under curve (AUC) was significantly greater in DM-OA group, $*P < 0.05$. (D) Histological study of the pancreas from DM-OA group indicated a dramatic reduction in size and cellularity of islets. H and E stained sections. (DM-OA group, Fig.1D-1, OA group, Fig.1D-2, Sham group, Fig.1D-3). The data are expressed as the means $\pm$ SD and compared using an ANOVA and the Student Newman Keuls post-hoc test between the groups. $*p < 0.05$ was considered significant.

Figure 3

Cartilage degeneration during diabetes mellitus and osteoarthritis progression. Histology images of knee joint from DM-OA group (A), OA group (B) and Sham group (C) at each time point of sacrifice (4, 8 and 12 weeks after operation). Grading of histological sections revealed more serious deterioration of articular cartilage and progression of OA in DM-OA group, compared to OA group and Sham group. Specifically, we observed focal loss of Safranin-O staining indicating decreased proteoglycan concentration, fissuring of articular cartilage, frequent loss and the flattened elongated chondrocytes, cell death in the articular cartilage, and atrophy of articular chondrocytes.

Figure 4

A Histologic scores of knee joint from DM-OA group, OA group and Sham group at 4, 8 and 12 weeks after operation, as determined using the Osteoarthritis Research Society International (OARSI) scoring system. Grading of OA using the OARSI scale showed statistically significant progression of OA in DM-OA group, compared to OA group and Sham group. The data are expressed as the means $\pm$ SD and compared using an ANOVA and the Student Newman Keuls post-hoc test between the groups. $*p < 0.05$ was considered significant. B: Changes in the mineral to collagen ratio of the subchondral bone among three groups. The mineral to collagen ratio was significantly lower in DM-OA group compared with OA group

and sham group at 8 and 12 weeks. The data are expressed as the means $\pm$ SD and compared using an ANOVA and the Student Newman Keuls post-hoc test between the groups. * $p < 0.05$ was considered significant. C Fracture load of compression testing of subchondral bone for three groups at 4, 8 and 12 weeks after operation. Graph showing that the fracture load of SB trabecular bone in DM-OA group exhibited continuously diminished than that OA group and Sham group. The data are expressed as the means $\pm$ SD and compared using an ANOVA and the Student Newman Keuls post-hoc test between the groups. * $p < 0.05$ was considered significant.

Figure 5

Changes in subchondral bone microarchitecture of DM-OA group (A), OA group (B) and Sham group (C) assessed by Micro CT at each time point of sacrifice (4, 8 and 12 weeks after operation). DM-OA group exhibited higher trabecular bone mass loss as compared with OA group and Sham group.

Figure 6

Quantitative analysis includes parameters derived from the geometric micro-structure of tibial SB. The data are expressed as the means±SD and compared using an ANOVA and the Student Newman Keuls post-hoc test between the groups. *p < 0.05 was considered significant.

Figure 7

Intrinsic mechanical properties of the subchondral bone trabecular. As early as 8 weeks, alterations in the trabecular bone led to an decrease in hardness and elastic modulus in DM-OA group. (A) Representative microindentation load displacement curve from the subchondral bone trabecular. (B) Optical microscope image of subchondral bone trabecular showing approximate microindentation positions as a function of measured. (C) Results produced by using the loading protocol for the tissue properties of the mouse bones. The data are expressed as the means±SD and compared using an ANOVA and the Student Newman Keuls post-hoc test between the groups. *p < 0.05 was considered significant.