Exercise Improves Bone Formation by Upregulating the Wnt3a/β-catenin Signalling Pathway in Type 2 Diabetic Mice

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Abstract

Bone formation in type 2 diabetes mellitus (T2DM) is inhibited by decreased bone formation capacity. Exercise can significantly improve bone metabolism; however, the effect and mechanism of exercise on bone formation in T2DM is not known. In this study, we used the method of a high-fat diet and injecting STZ to build a T2DM mouse model, and the mice were treated with swimming and downhill running for 8 weeks. Their body weights were obtained using an electronic scale. Their bone length and wet weights were measured by Vernier callipers and an electronic balance. The bone microstructure of the distal femur was analysed by Alizarin red staining of paraffin sections and micro-CT. An ALP dye was used to detect the changes in ALP activity in the mouse skull and osteoblasts. Alizarin red staining was applied to stain the osteoblasts produced by BMSCs. The mRNA expression of related factors were detected by RT-PCR. Our results showed that the body weights of T2DM mice were significantly increased by exercise. When the Wnt3a/β-catenin pathway in the bones of T2DM mice is inhibited, their bone formation ability is significantly reduced, resulting in the degradation of the bone tissue morphology and structure. Except for the significant increase in body weight and Runx2 mRNA expression, swimming had no significant effect on bone formation-related indicators in T2DM mice. However, downhill running could significantly increase their body weight, while the tibia length, wet weight, and the trabecular morphological structure of the distal femur and the indexes of bone histomorphology were significantly improved by activating the Wnt3a/β-catenin pathway. Compared with swimming, downhill running can activate the Wnt pathway to improve bone formation ability, thereby improving bone histomorphology. These findings suggest that exercise promotes OB differentiation, osteogenic capacity and enhances bone formation metabolism by regulating Wnt3a/β-catenin signalling in T2DM mice.

1 Introduction

Type 2 diabetes mellitus (T2DM) is an energy metabolic disorder characterized by high blood glucose and reduced insulin secretion, a pathogenesis closely related to insulin resistance and impaired islet β cell function (Ge et al., 2019). Metabolic disorders in type 2 diabetes lead to many complications, such as microangiopathy, diabetic neuropathy, etc. (Gu et al., 2019). Deborah et al. found that patients with T2DM have a lower bone quality and a higher risk of bone fracture (Sellmeyer et al., 2016). Moreover, the bone tissue microstructure, such as the trabecular number (Tb.N), biomechanical properties, bone mineral density (BMD) and bone volume fraction (BV/TV) are significantly lower in T2DM mice than in normal mice (Shi et al., 2017; Huang et al., 2016). Huang L et al. reported that the BMD, BV/TV, and Tb.N of the femur were significantly decreased in T2DM mice (Huang et al., 2016). These results revealed that T2DM caused a decrease in bone mass and the degeneration of bone tissue morphology.

Osteoblasts (OB) are differentiated from bone marrow mesenchymal stem cells (BMSCs), and there is a close positive correlation between the OB number, osteogenic capacity and bone formation metabolism (Oliveira et al., 2014). However, there are few related studies about how T2DM inhibits OB differentiation and bone formation. Park JS et al. (2014) confirmed that the number of OBs differentiated by BMSCs in T2DM mice and their bone formation decreased. In this process, many signalling pathways or key

molecules play an important regulatory role. Wnt3a/β-catenin is a key pathway regulating bone formation, which can regulate OB differentiation and osteogenic capacity (Hong et al., 2019; Fujita et al., 2019). As a key subtype of Wnt proteins, Wnt3a, when activated, leads to the phosphorylation of β-catenin, which activates the expression of downstream target genes Runx2, Osx, etc., and regulates OB differentiation and bone formation (Wu et al., 2019). However, whether the Wnt3a/β-catenin pathway has a regulatory role in T2DM inhibition of OB differentiation and bone formation remains to be revealed.

Exercise is an important method to improve bone metabolism, which can promote OB differentiation and osteogenic capacity, and improve bone formation metabolism (Gardinier et al., 2019; Iura et al., 2015). However, the mechanical stimulation of the bone by different modes of exercise (divided into the direct force, that is, the reaction of the ground to the bone and the indirect force, that is, the pulling force of the muscle on the bone) are quite different (Herbst et al., 2017). A related study found that the effect of direct forces on bone formation is significantly better than indirect forces (Akpinar et al., 2019). Although there are many studies on the improvement of bone metabolism in T2DM by exercise, and some studies have focused on bone phenotypic indicators such as BMD and bone biomechanics (Gushiken et al., 2015; Bello et al., 2014), related studies on the effects of different exercises on the expression of Wnt3a/β-catenin signalling pathway-related molecules and bone formation in T2DM mice have not been reported.

In this study, we employed a high-fat-diet (HFD) combined with a one-time injection of streptozotocin to induce a T2DM mouse model to evaluate the effects of different exercises on OB differentiation, osteogenic capacity and bone tissue microstructure. The results showed that downhill running activated the Wnt3a/β-catenin pathway in the bones of T2DM mice to promote OB differentiation and bone formation capacity, promote bone formation metabolism in type 2 diabetic mice, improve the microstructure of the bone tissue, and its effect was better than swimming.

2 Materials And Methods

2.1 Animals

Forty four-week-old C57BL/6 mice were provided by B&K Laboratory Animal Company (Shanghai, China). The mice were housed under standard conditions and the room was maintained on a cycle of 12 hours light and 12 hours darkness, at a temperature of 25±3°C and with free access to food and water. All animal experiments were approved by the Ethics Committee on Animal Use of Yangzhou University.

2.2 Establishment of the T2DM Model

All animals were adaptively fed for 1 week and then randomly divided into a normal group (ZC, n=10) and a T2DM model (n=30). The T2DM model mice were fed a high-fat diet (31.7% lard, 25.8% casein 30 mesh, 16.3% maltodextrin 10, 9% sucrose, 6.5% cellulose BW200, 3.4% soybean oil, 2.3% potassium citrate, 1% H_2O, 1.6% mineral mix S10026, 1.4% dicalcium phosphate, 1.3% vitamin mix V10001, 0.8% calcium carbonate, 0.4% L-cystine, and 0.3% choline bitartrate, purchased from Jiangsu Xietong Pharmaceutical

Bio-engineering Co., Ltd.) for 6 weeks, and then STZ (80 mg/kg) was injected 12 hours after fasting, while the normal group mice were injected with a citric acid-sodium citrate solution. The blood glucose concentration of the mice was detected after 12 hours of fasting 2 weeks later. The blood glucose concentration was above 8 mmol/L for the T2DM mice (Kanazawa et al., 2011; Zheng et al., 2015), and 27 models were successfully established and randomly divided into the T2DM control group (TC, n=9), the T2DM swimming group (TS, n=9) and the T2DM downhill running group (TD, n=9). Normal mice were fed a normal diet, and T2DM mice continued to have a high-fat diet, both drinking water freely.

2.3 Training Protocol

TS group and TD group mice were trained in swimming and downhill running, respectively. For the swimming group, the mice were placed in a (42 cm long × 40 cm wide × 36 cm water depth) container, for 50 min/day, 8 weeks in total. The first week was adaptive training, 30 minutes a day for the first two days, 40 minutes a day for the third and fourth day, then 50 minutes a day for the fifth and sixth day, while normal training started in the second week. Downhill running: 0.8 km/h, 50 minutes, slope −9 degrees, 6 days/week, a total of 8 weeks. The first week was adaptive training, 30 min/day for the first two days, 40 minutes a day for the third and fourth day, then 50 minutes a day for the fifth and sixth day. Later, normal training started in the second week.

2.4 Body Weight

The body weight of each group of mice was weighed using an electronic scale.

2.5 Oral Glucose Tolerance Test (OGTT)

After the last exercise intervention in the eighth week, the mice were fasted for 12 hours and gavaged 2 g/kg glucose for the OGTT experiment. After gavage at 0, 30, 60, and 120 minutes, blood glucose was measured through the tail vein, and we calculated the area under the curve. The calculation method of the area under the time of the blood glucose curve was AUC (h·mmol·L-1)=1/4 A+1/2 B+1/2 C+1/4 D (A, B, C, and D were the blood glucose values measured after 0, 30, 60 and 120 minutes of intragastric glucose administration) (Lokman et al., 2013).

2.6 Measurement of Bone Length and Wet Weight

We used a Vernier calliper to measure the left tibia length of the mice in each group. We used an electronic balance to measure the left tibia wet weight of the mice in each group.

2.7 Alizarin Red Staining

After fixing with 4% PFA for 24 hours, the left femurs were decalcified in 4% EDTA for 30 days after washing with PBS. Then, we carried out alcohol gradient dehydration and embedded it in paraffin, and sagittally cut it into 6 μm sections. After placing on a baking sheet, the slices were dewaxed with xylene, and rehydrated with an alcohol gradient (from a high to a low concentration). We washed them in PBS solution and used Alizarin red to stain the slices for 5 minutes followed by washing with PBS, dehydrated it with an alcohol gradient (a low to a high concentration), xylene I and II for 5 minutes each, and sealed it with neutral gum. Finally, we used a Leica microscope to take photographs.

2.8 Microcomputed Tomography (micro-CT) Analysis

The left femur of each group was fixed in 4% paraformaldehyde (PFA) for 24 hours, washed with PBS and then scanned by a micro-CT scanner (Skyscan, Aarselaar, Belgium) with a resolution of 18 μm per pixel. Cross-sectional images of the distal femur were used for 3-dimensional histomorphometric analysis of the trabecular and cortical bone. The region of interest (ROI) of the distal femur selected for analysis was 5% of the femoral length from 0.05 mm below the growth plate to determine the trabecular and cortical BMD, BV/TV, bone surface area to volume ratio (BS/BV), ratio of bone surface area to tissue volume (BS/TV), trabecular thickness (Tb.Th), Tb.N and trabecular separation (Tb.Sp).

2.9 ALP Staining of the Skull

After washed with PBS, the skull was fixed in 4 degrees 4% PFA for 24 hours. Then, we used 0.1% Triton-X100 (for permeabilization) and PBST to treat it in a 4 degree environment for 24 hours, and it was stained with an ALP dye solution (0.002 g AS-MAX and 0.006 g Fast Red Violet LB Salt dissolved in 5 ml pH 8.3 of Tris-HCl and 5 ml of ddH2O) for 1 hour. We took photographs with a Canon camera and used Photoshop software to take a screenshot of the seam locations.

2.10 ALP and Alizarin Red Staining of OB

After euthanizing the mice under aseptic conditions, bilateral femurs and tibias were taken from each mouse and cleaned of all attached soft tissues. The bone marrow cavity was exposed, and the bone marrow was isolated. A single-cell suspension was prepared by repeated aspiration. The total bone marrow cells were counted in haemocytometer and cultured in alpha minimal essential medium (α-MEM; GIBCO, USA) supplemented with 10% foetal calf serum (GIBCO, USA), 1000 U/ml ciprofloxacin (Sigma, USA). The cells were disseminated into 6-well plates (Costar, USA) at a concentration of 1×107 cells/well in 2 mL α-MEM, and incubated at 37°C in a humidified atmosphere of 5% CO2 in air. The medium was changed every other day to remove the non-adherent cells. On the seventh day of culture, to the α-MEM we added 100 U/ml glycerophosphoric acid (Sigma) and 1000 U/ml ascorbic acid (Sigma) to induce the bone marrow cells to differentiate into osteoblasts. The medium was changed every other day. On the seventh day since differentiation, the alkaline phosphatase positive colony forming units-fibroblastic

(ALP CFU-f) were fixed with 10% PFA for 10 minutes at RT. After washing with PBS, the cells were stained with ALP and Alizarin red dye solution. We used a camera (Canon, Japan) to photograph the staining results.

2.11 Quantitative PCR

A portion of the right femur (with bone marrow removed) of the same weight from each mouse was placed in a grinding tube, and pre-soaked steel beads in DEPC water and TRIzol (Takara, Shiga, Japan) were added. Tissue grinding was performed to extract the total RNA. Then, using a reverse transcriptase cDNA kit (Takara, Shiga, Japan) we reverse transcribed the total RNA into cDNA. The mRNA expression of related factors was evaluated with a Real-Time PCR System (Applied Biosystems 7500, Waltham, MA, USA) by using a SYBR Premix Ex Taq kit (Takara, Shiga, Japan). The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Wnt3a, Forward: 5'-AGGACCCATCTGATTCCCCA-3' and Reverse: 5'-CTTGTGGCAGATGGGCTG TA-3', β-catenin, Forward: 5'-AGACAGCTCGTTGTACTGCT-3' and Reverse: 5' GTGTCGTGATGGCGTAGAAC-3', Runx2, Forward: 5'-GTCCTATGACCAGTCTTA CC-3' and Reverse: 5'-GATGAAATGCCTGGGAACTG-3', Osx, Forward: 5'-GTTCACCTGTCTGCTCTGCTC-3' and Reverse: 5'-AGCTCCTTAGGG CCACTTGG-3', β-actin: Forward: 5'-ACCCAGAAGACTGTGGATGG-3' and Reverse: 5'- TTCAGCTCAGGGATGACCTT-3'). Each gene was analysed in three repetitions. We used the method of 2-△△Ct to calculate the expression of each gene's mRNA.

2.12 Western Blot

Extraction of total bone tissue protein and the procedures and methods for detecting the cytokines protein expression in bone were consistent with a previous study (Luo et al., 2016). The antibodies involved in the experiments were directed against Wnt3a (CST, USA, RRID:AB_2215411), β-catenin (CST, USA, RRID:AB_11127855), Runx2 (CST, USA, RRID:AB_10949892) and Osx (abcam, USA, AB_22552), derived from rabbits. The dilution ratio of all of the antibodies was 1:1000. Blots were tested by the Alpha gel imaging system (AlphaImager HP, USA) and used Quantity One software (Bio-Rad Inc., USA) to determine the grey values. The ratio of grey values of target proteins to the internal reference is defined as the expression of the target proteins.

2.13 Statistical Analysis

The experimental results are presented as the mean±standard deviation (SD). The data were statistically analysed by SPSS 20.0 (ZC group and TC group were subjected to independent sample t-tests. One-way analysis of variance was used for the TC, TS and TD groups), $P<0.05$ and $P<0.01$, respectively, indicate that the experimental results have significant differences.

3 Results

3.1 Effect of Exercise on OGTT in T2DM Mice

Oral glucose tolerance tests (OGTT) reflect the body's ability to regulate blood sugar (Brede et al., 2020). It could be seen from Figure 1A that the blood glucose concentration of mice in the ZC group reached a maximum of 13.7 mmol/L after 30 minutes of intragastric administration, and within 120 minutes, the blood glucose concentration was restored to the basic level of 10.06 mmol/L. The blood glucose concentration of the mice in the TC group rose to a maximum of 29.87 mmol/L within 30 minutes, and there was no significant drop to 23.72 mmol/L at 120 minutes, indicated that the islet function of the mice in the TC group was impaired and their ability to regulate blood glucose was decreased (Figure 1A). Glucose tolerance was severely impaired. Compared with the TC group, the glucose tolerance of the TS group did not change significantly. After intragastric glucose, the blood glucose concentration rose to a maximum of 28.47 mmol/L within 30 minutes. However, there was no significant decrease to 22.23 mmol/L at 120 minutes (Figure 1A). Interestingly, compared with the TC group, the glucose tolerance in the TD group was significantly improved ($p<0.01$). The AUC analysis confirmed this result. We found the AUC in TD group is significantly decreased ($p<0.01$) compared with TS group, indicating that downhill running could reduce the rate of glucose absorption and improve the impaired glucose tolerance of diabetic mice (Figure 1B).

3.2 Exercise Decreased the Body Weight, Bone Length and Bone Wet Weight of the T2DM Mice

To clarify the changes of body weight, bone length and wet weight of the T2DM mice, electronic scales, electronic balances and Vernier callipers were used to measure the above indexes. The body weight of the TC group was significantly increased relative to the ZC group ($P<0.01$). Compared to the TC group, the body weight of the TS group was obviously lower ($P<0.01$), while that of the TD group was further reduced ($P<0.01$) (Figure 2A). The bone length of the TC group was less than that of the ZC group ($P<0.05$), which indicated that downhill running could significantly promote the tibia to grow longer ($P<0.05$), but the effect of swimming on tibia length was not obvious ($P>0.05$) (Figure 2B). The wet weight of the tibia in the TC group was significantly lower than in the ZC group ($P<0.01$). Compared to the TC group, the tibia wet weight of the TS group had no significant difference, but the TD group was significantly increased (Figure 2B).

Taken together, these results showed that T2DM could promote the body weight and affect the bone length and wet weight. Exercise could significantly reduce the body weight of T2DM mice, and downhill running had a better effect on bone length and wet weight than swimming.

3.3 Exercise Improved the Bone Histomorphology of the T2DM Mice

Bone histomorphology is an important index of the evaluation of bone metabolism, which is damaged by T2DM (Gallagher et al., 2014). To evaluate the effect of exercise on bone histomorphology, Alizarin red staining and micro-CT of the distal femur were performed. The trabecular bone histomorphology of the TC group was significantly degraded compared to the ZC group. Furthermore, the BMD ($P<0.05$), BS/BV ($P<0.05$), Tb.Th ($P<0.05$) of the cortical bone and BMD ($P<0.01$), BV/TV ($P<0.01$), BS/BV ($P<0.01$), BS/TV ($P<0.01$), Tb.Th($P<0.01$), Tb.N ($P<0.01$) and Tb.Sp ($P<0.01$) of the trabecular bone of the TC group were significantly changed, while the BV/TV ($P>0.05$), BS/TV ($P>0.05$), Tb.N ($P>0.05$), and Tb.Sp ($P>0.05$) of the cortical bone had no significant difference (Figure 3). The above results showed that the morphology of cancellous bone tissue in T2DM mice was significantly degraded, but the cortical bone changes were not significant.

Exercise had a significant impact on bone histomorphology, and exercise could increase BMD and improve the related indicators of bone tissue microstructure (Fang et al., 2019; Troy et al., 2018). In this study, there were no significant differences between the TC and TY group. The trabecular bone histomorphology of the TD group was notably improved. Compared to the TC group, the BMD ($P>0.05$), BV/TV ($P>0.05$), BS/BV ($P>0.05$), BS/TV ($P>0.05$), Tb.Th ($P>0.05$), Tb.N ($P>0.05$), and Tb.Sp ($P>0.05$) of the trabecular and cortical bone in the TY group showed no significant changes, while the BV/TV ($P<0.01$), BS/BV ($P<0.05$), and Tb.Th ($P<0.05$) of the cortical bone and BMD ($P<0.01$), BV/TV ($P<0.01$), BS/BV ($P<0.01$), BS/TV ($P<0.01$), Tb.Th ($P<0.05$), and Tb.N ($P<0.01$) of the trabecular bone in the TD group were all significantly changed; however, the BMD ($P>0.05$), BS/BV ($P>0.05$), Tb.N ($P>0.05$), and Tb.Sp ($P>0.05$) of the cortical bone and Tb.Sp ($P>0.05$) of the trabecular bone were not changed (Figure 3). The BS/TV ($P<0.05$) of the trabecular bone in the TD group was significantly improved relative to the TY group, and other relevant indicators had no significant differences. The above results indicated that downhill running could improve the bone histomorphology of T2DM mice significantly, but the effect of swimming was not obvious.

3.4 Exercise Significantly Promoted OB and the Osteogenic Capacity of T2DM Mice

OB are differentiated from BMSCs, and the osteogenic capacity of OB will directly determine bone metabolism (Elango et al., 2019). Type 2 diabetes is an energy metabolic disease that causes a significant decrease in OB differentiation and osteogenic capacity (Nomura et al., 2019). In our study, skull and OB Alizarin red staining and OB ALP staining showed that the number of OBs and the osteogenic capacity of type 2 diabetic mice were significantly decreased. Exercise plays an important role in promoting OB differentiation and improving osteogenic capacity (Mohan et al., 2015). However, there are few studies on the effects of exercise on OB differentiation and osteogenic capacity in type 2 diabetic mice. After 8 weeks of training, the changes in the ALP staining of the skull and OB and the Alizarin red staining of OB in the TS group were not significant, but the related results of the TD group were significantly improved (Figure 4). Furthermore, there was a significant difference between the TS group and TD group. The above research results reflected that T2DM resulted in a significant decrease in the

number of OBs and osteogenic capacity. Exercise could promote BMSCs to differentiate into OBs and improve the osteogenic capacity, and the effect of downhill running was better than swimming (Figure 4).

3.5 Exercise Caused a Significant Increase of the Related Factors in the Wnt3a/β-catenin Pathway of T2DM Mice

To investigate the mechanism of T2DM and exercise on bone formation capacity and bone histomorphology, we tested the expression of the related factors in the Wnt3a/β-catenin pathway which was a key signalling regulating bone formation. T2DM inhibits OB differentiation and bone formation by inhibiting the Wnt3a/β-catenin pathway, leading to the degradation of the bone morphological structure and the initiation of osteoporosis. In this study, the mRNA and protein expression of Wnt3a ($P<0.05$) (Figure 5A), β-catenin ($P<0.05$ or $P<0.01$) (Figure 5B), Runx2 ($P<0.05$ or $P<0.01$) (Figure 5C) and Osx ($P<0.05$ or $P<0.01$) (Figure 5D) were significantly down-regulated. Therefore, this study focused on the effects of exercise on the expression of Wnt3a/β-catenin pathway factors in the bones of T2DM mice. In the TS group, the mRNA expression of Runx2 was significantly increased ($P<0.05$) (Figure 5C), but the mRNA expression of Wnt3a ($P>0.05$) (Figure 5A), β-catenin ($P>0.05$) (Figure 5B), and Osx ($P>0.05$) (Figure 5D), and the protein expression of Wnt3a ($P>0.05$) (Figure 5A), β-catenin ($P>0.05$) (Figure 5B), Runx2 ($P>0.05$) (Figure 5C), and Osx ($P>0.05$) (Figure 5D) had no significant changes. Compared to the TC group, the mRNA and protein expression of Wnt3a, β-catenin, Runx2, and Osx in the bones of the TD group mice were not significantly changed ($P<0.05$ or $P<0.01$) (Figure 5). In addition, also, the expression of Wnt3a/β-catenin pathway related factors in the bones of the TD group were significantly higher than in the TS group except for the Wnt3a mRNA and Osx protein (Figure 6). These results indicated that the Wnt3a/β-catenin pathway was suppressed in T2DM mice, and downhill running activated the Wnt3a/β-catenin pathway in the bone of T2DM mice.

4 Discussion

T2DM is associated with skeletal system complications characterized by degenerated bone tissue morphology and osteoporosis (Ding et al., 2019; Xu et al., 2019). In this study, using a high-fat diet plus STZ we established the T2DM mouse model to investigate the effects of different exercises on bone formation. We concluded that inhibition of the Wnt3a/β-catenin pathway in the bones of T2DM mice leads to decreased OB differentiation and bone formation, resulting in the degeneration of the bone morphological structure and decreased BMD. This showed that the Wnt3a/β-catenin pathway plays a key role in T2DM inhibiting bone formation by decreasing OB differentiation and osteogenic capacity. It also indicated the success of modelling of the T2DM mice in this study. After 8 weeks of training, downhill running could improve the bone morphological structure and bone mass through promoted OB differentiation and the osteogenic capacity, which is regulated by activating the Wnt3a/β-catenin pathway, and its effect on the bone formation of T2DM mice was better than swimming.

Osteoporosis is a common complication in T2DM patients, which is characterized by a degeneration of bone morphological structure and decreased BMD (Liu et al., 2018). At present, exercise as a green and healthy intervention method, could promote bone formation and inhibit bone resorption, which improves bone metabolism (Gardinier et al., 2018; Friedman et al., 2016; Banu et al., 2006). However, current studies focus on the impact of exercise on the T2DM bone phenotype (such as BMD, biomechanical indicators, etc.). After 20 weeks of voluntary running, the maximum breaking force and stiffness of bone histology indexes in type 2 diabetic rats were improved (Takamine et al., 2018), and 6 weeks treadmill exercise (10 m/min, 60 min/day, 5 days/week) could improve femoral BMD (Akagawa et al., 2018). There are few reports on the effects of different kinds of exercise on the morphological structure of bone tissue in T2DM mice. Therefore, this study used swimming and downhill running to train the T2DM mice. After 8 weeks training, compared to the TC group, the body weight was significantly decreased, but the other bone morphological structure indexes, such as tibia length, tibia wet weight, BMD, BV/TV, Tb.Th, Tb.N and Tb.Sp of cortical bone and trabecular bone in the TS group did not show significant changes. However, the body weight, tibia length, tibia wet weight, and morphological structure of trabecular bone in the distal femur of the TD group were all changed significantly.

This study used the method of micro-CT to scan the distal femurs and found that the BMD, BV/TV, BS/BV, BS/TV, Tb.Th, and Tb.N of trabecular bone in the TD group were all improved, but the cortical bone only showed changes in BV/TV, BS/BV and Tb.Th. It is suggested that downhill running could evidently improve the morphological structure of bone tissue in T2DM mice, especially trabecular bone, while the effect of swimming was not significant. This is related to the higher-strength ground reaction (also known as direct mechanical stimulation) in the bone of T2DM mice during downhill running (Shalumon et al., 2018). Direct mechanical stimulation promotes OB differentiation and bone formation, and inhibits OC differentiation, nucleation and bone resorption ability (Song et al., 2016), and then improves the morphological structure of bone tissue, which is first manifested in trabecular bone (Neumann et al., 2015). There were no significant changes in the bone histomorphometry of the trabecular and cortical bones in the TS group, indicating that the effect of swimming on the morphological structure of the bone tissue in T2DM mice was not significant. The reason for this is related to the mechanical stimulation on bone produced by swimming in this study. Because pre-experiments found that the indirect mechanical stimulation of swimming on bone promotes bone formation to improve the morphological structure of bone tissue is not significant, the mechanical stimulation of the bone of T2DM mice (i.e., muscle pulling) failed to reach the threshold of bone metabolism, which leads to the bone tissue morphology not improving significantly.

OB is a major cell that is differentiated from BMSCs and regulates bone formation metabolism (Neumann et al., 2015). T2DM inhibits OB differentiation and osteogenic capacity; however, exercise could promote T2DM mice's OB differentiation and osteogenic capacity (McGee-Lawrence et al., 2017). After 8 weeks of exercise, we performed ALP and Alizarin red staining on OB differentiated from the T2DM mice's BMSCs. It found that the number of OBs and the osteogenic capacity in TS group were not significantly changed, while the TD group was significantly changed. Moreover, the number of OBs and the osteogenic capacity in the TD group were significantly higher than those in the TS group. This

indicated that downhill running significantly promoted OB differentiation and bone formation in T2DM mice, but the swimming effect was not significant. This is related to the direct mechanical stimulation of the bones produced by downhill running on T2DM mice, which can promote the phosphorylation of stress-activated protein kinases/Jun amino-terminal kinases (pSAPK-JNK) in bone (da Rocha et al., 2015). After the above signal pathway or cytokine expression is activated, it promotes the differentiation of BMSCs into OBs with strong osteogenic capacity (Kim et al., 2019). The effect of swimming is not significant, and the muscle pulling force on the bone of T2DM mice is small, which cannot be related to the formation of effective mechanical stimulation of BMSCs, thereby inhibiting their differentiation into OBs.

The Wnt3a/β-catenin pathway, which is a key signalling pathway regulating bone formation and metabolism, plays an important regulatory role in OB differentiation and its osteogenic capacity (Salazar et al., 2013). Ayse BC et al. (2010) studied the effects of interstitial fluid shear and tensile strain on bone cells and found that both mechanical stimuli promoted Wnt3a/β-catenin pathway activation. However, current studies on the promotion of the Wnt3a/β-catenin pathway by exercise and the regulation of OB differentiation and bone formation in T2DM mice have yet to be completed. This study used RT-PCR and western blotting to test the mRNA and protein expression of related genes in the Wnt3a/β-catenin pathway in T2DM's mice bone. This study found that the mRNA expression of Runx2 was significantly increased in the TS group, but the mRNA expression of Wnt3a, β-catenin, and Osx and the protein expression of Wnt3a, β-catenin, Runx2, and Osx had no significant change, while the mRNA and protein expression of Wnt3a, β-catenin, Runx2, and Osx in the bones of the TD group mice were significantly upregulated. This indicates that downhill running can activate the Wnt3a/β-catenin pathway in the T2DM mice's bone, but swimming has no effect. The expression of related factors in the bone of the TD group was significantly higher than in the TS group except for the mRNA expression of Wnt3a and Osx. This is related to the role of Wnt and Osx at the translation level. The reasons for the differences in the above results are closely related to the large-scale direct mechanical stimulation of the bones produced by downhill running in T2DM mice. Large-scale direct mechanical stimulation can significantly upregulate BMP-2. When BMP-2 is activated, the interaction between Smad1 and DVL promotes β-catenin activity, thereby promoting Wnt3a-mediated OB differentiation and bone formation (Liu et al., 2006). The mechanical stimulation of downhill running can also activate miR-495 expression in T2DM mice's bone (Sun et al., 2018), and miR-495 activates downstream dishevelled 2 (DVL-2), while the expression of upregulated DVL will activate Wnt3a and downstream phosphorylation of β-catenin and Runx2 expression (Du et al., 2019). Limited by the few current studies, the molecular mechanism of exercise affecting the Wnt3a/β-catenin pathway in T2DM bone is still not clear, and additional study is warranted.

5 Conclusion

Bone formation is inhibited in T2DM mice, leading to osteoporosis. Downhill running activates the Wnt3a/β-catenin pathway in the bones of T2DM mice, promotes OB differentiation and osteogenic capacity, enhances bone formation metabolism, and improves the bone morphological structure.

Abbreviations

T2DM	Type 2 diabetes mellitus
BMD	Bone mineral density
OB	Osteoblasts
BMSCs	Bone marrow mesenchymal stem cells
HFD	High-fat-diet
OGTT	Oral glucose tolerance test
PFA	Paraformaldehyde
micro-CT	Microcomputed tomography
ROI	Region of interest
SD	Standard deviation
pSAPK-JNK	Phosphorylation of stress-activated protein kinases/Jun amino-terminal kinases

Declarations

1 Ethics Approval and Consent to Participate

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers): All animal procedures were performed according to the guidelines of the Experimental Animal Care and Use Committee at Yangzhou University (license NO: YZU-TYXY-31)

2 Conflicts of Interest

The authors have declared that no competing interests exist.

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5 Author Contributions

CXH was responsible for topic selection, writing and experimental design; SP was responsible for the revision of the later thesis; the Masson staining and RT-PCR experiments were completed by LB; the ELISA and Western Blot experiments were completed by YK; the later data summary processing and mapping were completed by LPC. All authors have read and agreed to the published version of the manuscript.

6 Consent for publication

Written informed consent for publication was obtained from all participants.

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Figures

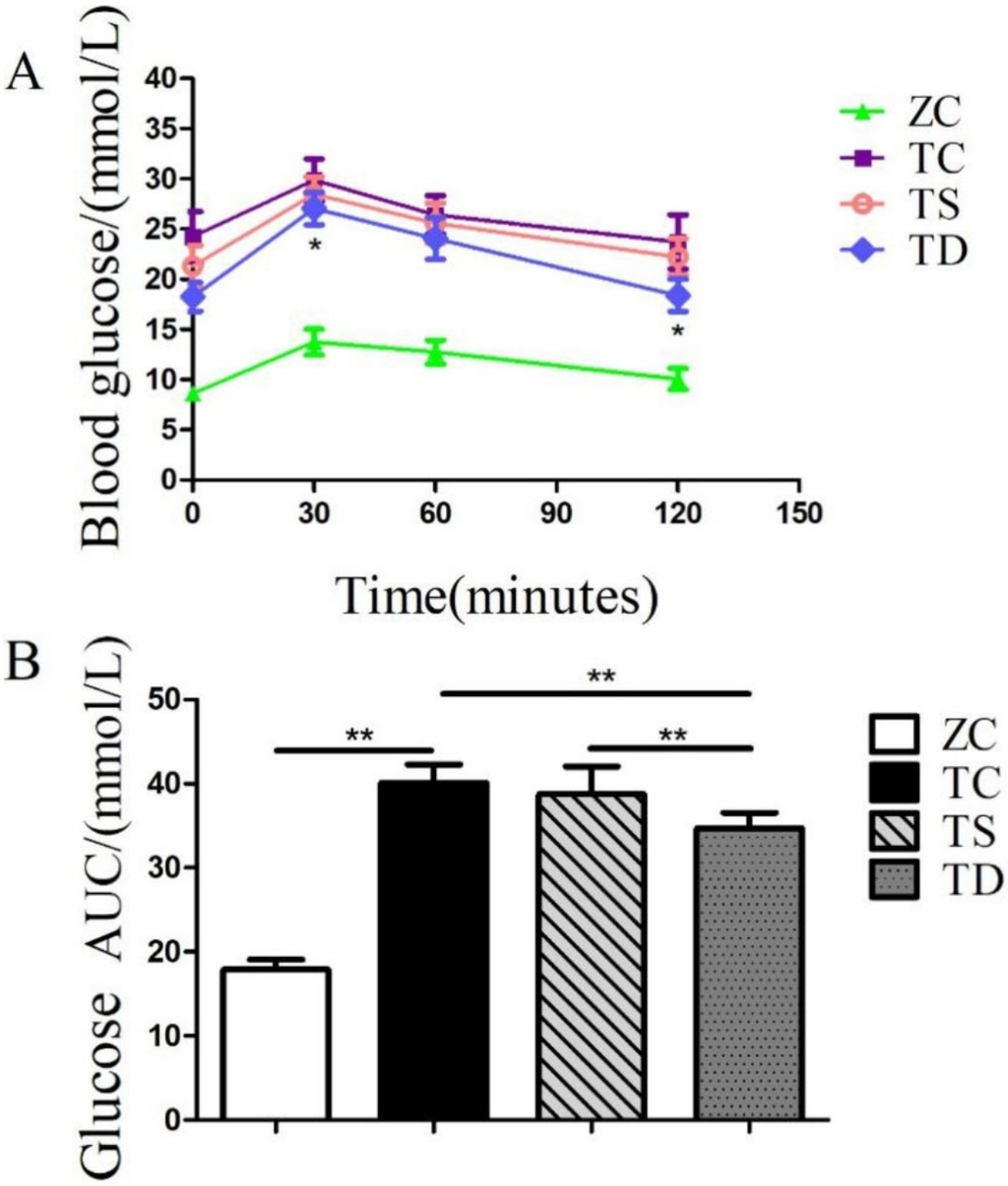

Figure 1

The effect of exercise on oral glucose tolerance test (OGTT) in diabetic mice. (A) Oral glucose tolerance curve in ZC (n=10) , TC (n=9) , TS (n=9) , TD (n=9) group. (B) Area under the glucose curve in A. * $p<0.05$, ** $p<0.01$.

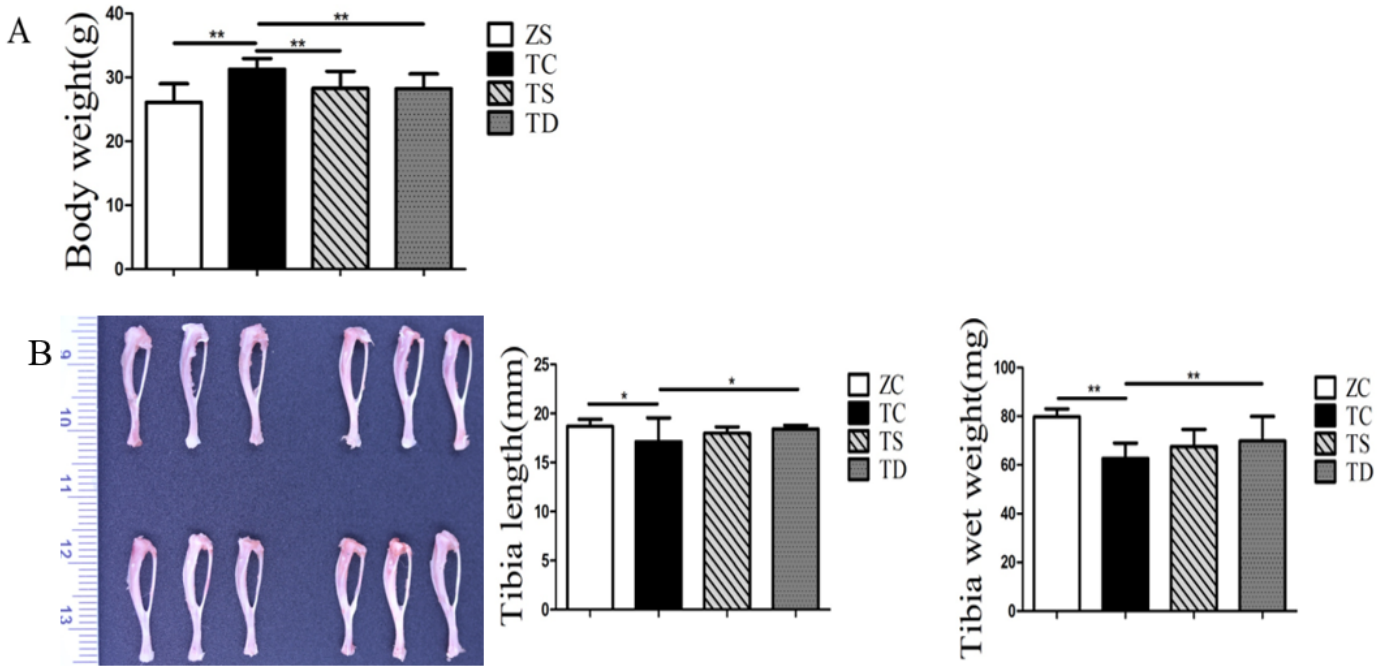

Figure 2

Effects of exercise on body weight and tibia length and wet weight. (A) The effects of T2DM on the body weight of mice and the effects of swimming and downhill running on the body weight of T2DM mice. (B) The effects of T2DM on tibia length and wet tibia weight in mice. Swimming and downhill running interventions for 8 weeks, tibia length and tibia wet weight changes in T2DM mice. * $p<0.05$, ** $p<0.01$.

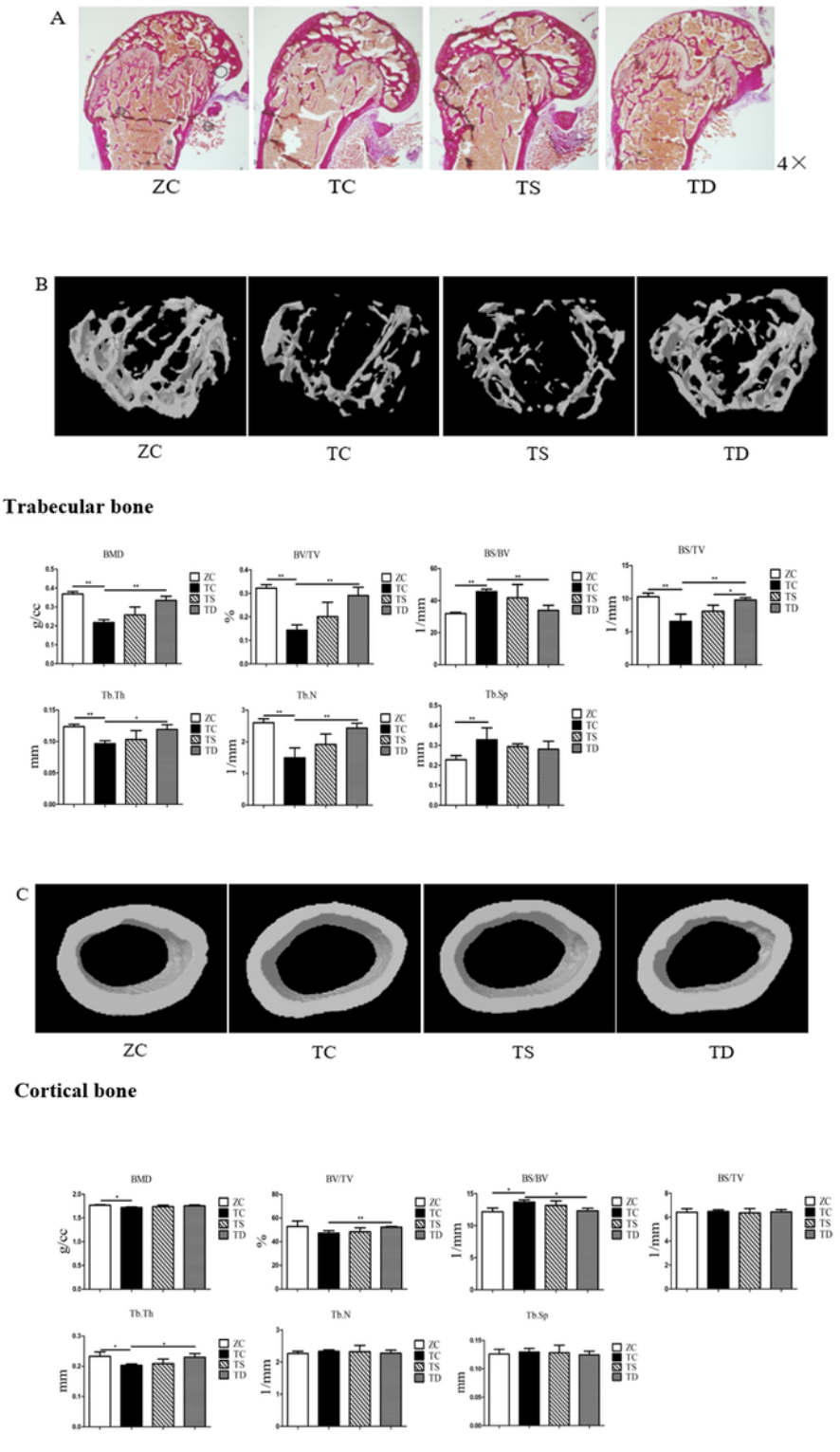

Figure 3

Effects of exercise on the morphological structure of bone. (A) Using Alizarin red staining to observe the effects of two kinds of exercise on the changes of trabecular bone in the distal femoral cancellous bone of T2DM mice (4×). (B) Representative images of micro-CT analysis of the microstructure of the trabecular bone at the mouse distal femur and related indicator changes. BMD: bone mineral density, BV/TV: Bone volume fraction, BS/BV: Bone surface area to bone volume ratio, BS/TV: Ratio of bone

surface area to tissue volume, Tb.Th: Trabecular thickness, Tb.N: Trabecular number, Tb.Sp: Trabecular separation. (C) Representative images of micro-CT analysis of the microstructure of the cortical bone at the mice distal femur and related indicator changes. * p<0.05, ** p<0.01.

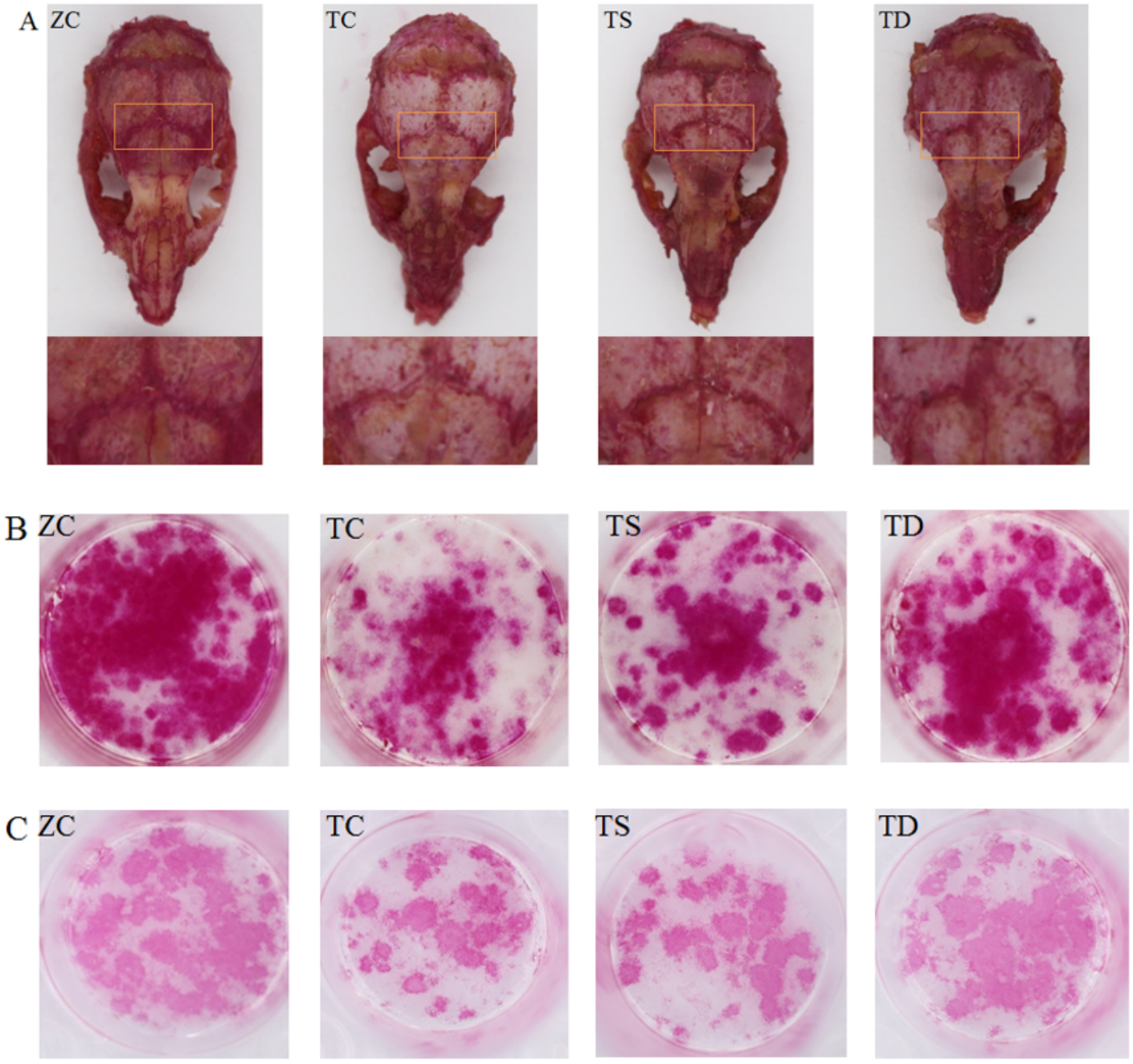

Figure 4

Effects of exercise on bone formation capacity. (A) ALP staining was performed on the skulls of mice in each group to observe the changes in ALP activity of bone tissues at the skull herringbone sutures. The stronger the ALP activity, the darker the colour will be. (B) After inducing BMSCs to differentiate into OB, we performed ALP staining to observe the changes in the number of OBs and ALP activity produced by

differentiation. (C) Alizarin red staining of the OB produced by induced differentiation to observe the changes in the number of OB produced by differentiation and bone formation ability.

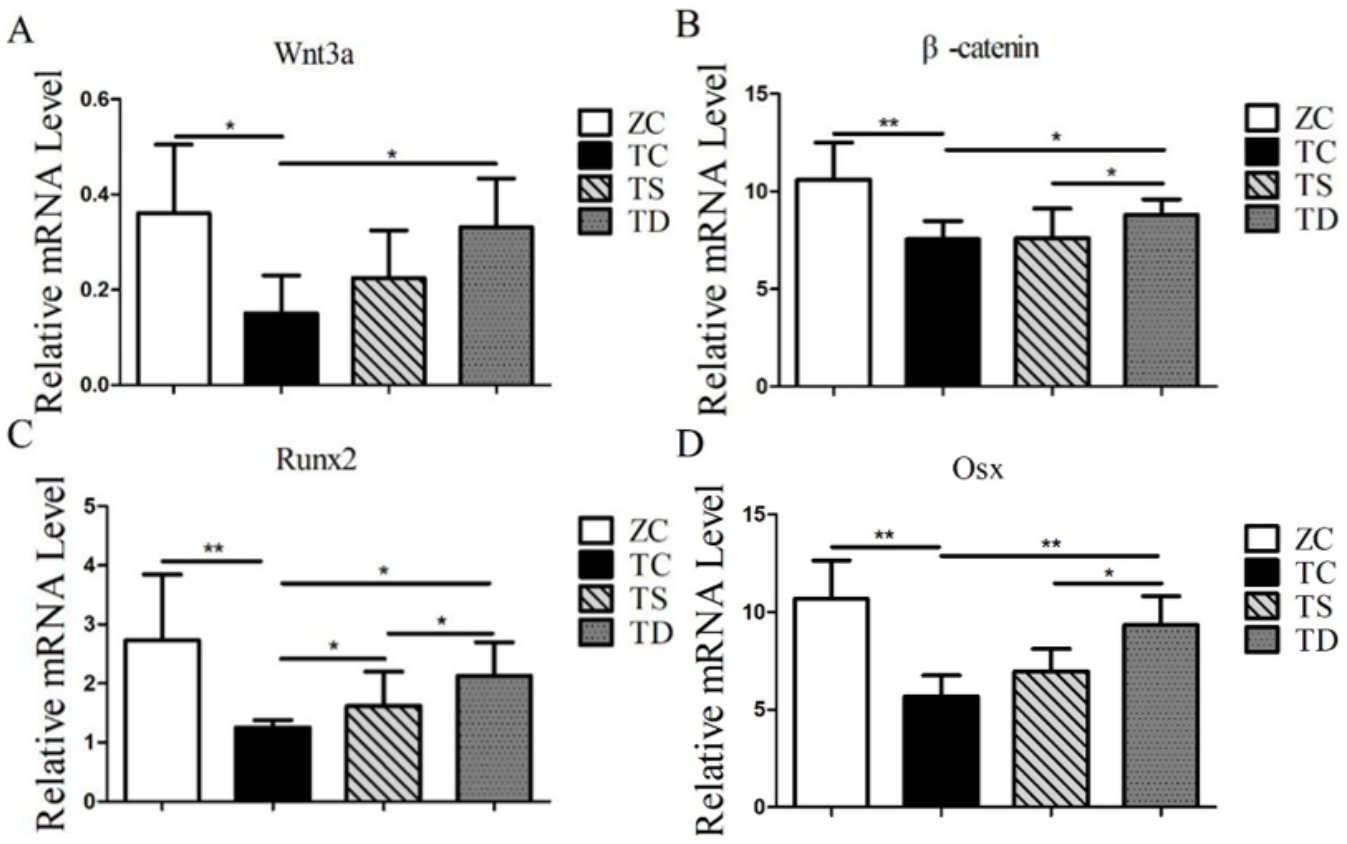

Figure 5

mRNA expression of related factors in Wnt/β-catenin signalling pathway. Relative mRNA expression levels of Wnt3a, β-catenin, Runx2 and Osx in the right tibia of each group of mice. * denotes $p<0.05$, ** indicates $p<0.01$.

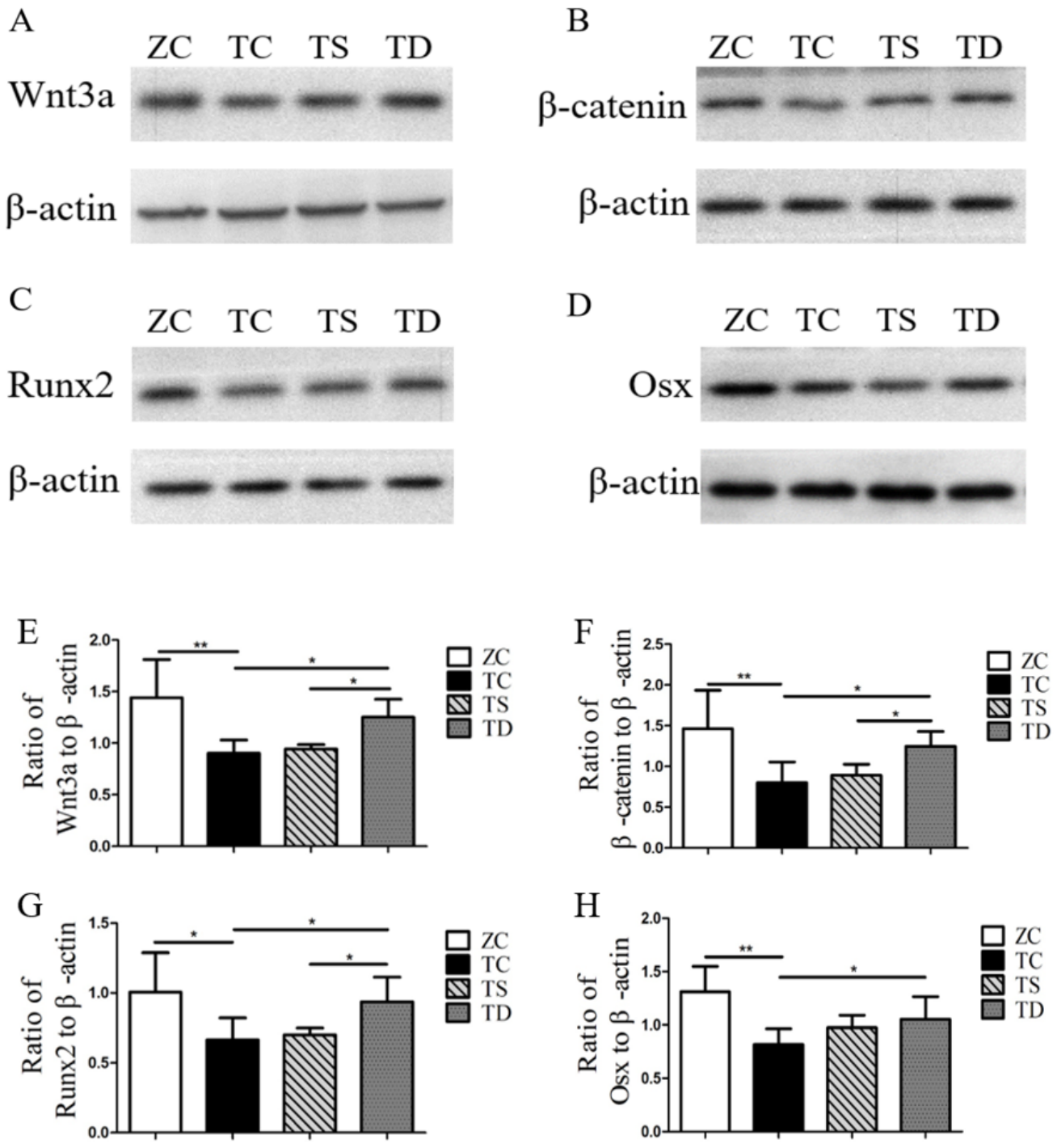

Figure 6

Protein expression of related factors in the Wnt/β-catenin signalling pathway. Femurs from six mice of each group mice were pooled for protein preparation. We used the method of western blotting to test the relative protein expression levels of Wnt3a, β-catenin, Runx2 and Osx. * $p<0.05$, ** $p<0.01$.