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MiR-29c-3p reduces bone loss in rats with diabetic osteoporosis *via* targeted regulation of Dvl2 expression

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Abstract. – OBJECTIVE: The aim of this study was to investigate the influence of micro ribonucleic acid (miR)-29c-3p on rats with diabetic osteoporosis (DOP) and its underlying mechanism.

MATERIALS AND METHODS: A total of 30 specific pathogen-free (SPF)-grade male Wistar rats aged 6-week-old were randomly selected and divided into three groups according to different intervention means, including: NC group (control rats only injected with normal saline), DOP group (rats with DOP induced by injection of streptozotocin), and ME group (DOP rats injected with miR-29c-3p agonist for 4 consecutive weeks). The changes in blood glucose and body weight were recorded in the rats of each group every week. Enzyme-linked immunosorbent assay (ELISA) was applied to detect the content of bone turnover markers (BTMs) in serum, such as alkaline phosphatase (ALP), osteocalcin (OC), and procollagen type I N-terminal propeptide (PINP). The variations in serum calcium (Ca) and phosphorus (P) levels in the abdominal aorta were determined using an atomic absorption spectrometer in the three groups. Meanwhile, bone mineral density (BMD) of femur and lumbar vertebra (L1-L4) were examined. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to measure the changes in messenger RNA (mRNA) expressions of miR-29c-3p and Disheveled 2 (Dvl2) in the bone tissues of intervened rats. In addition, the staining and expression changes of Dvl2 protein in bone tissues were determined *via* immunohistochemistry.

RESULTS: The rats in NC group had normal behavioral activities, normally increased body weight, sensitive responses, as well as normal and stable blood glucose. In DOP group, the rats manifested clinical symptoms of diabetes mellitus (DM) (i.e., polydipsia, polyphagia, polyuria, and weight loss), lackluster hairs, decreased behavioral activities, slow responses, and blood glucose at a concentration higher than 16.7 mmol/L. However, the blood glucose rose first, and then, declined and it was maintained at a level higher than normal concentration in ME group. Meanwhile, the rate of weight loss significantly decreased. The results of qRT-PCR indicated that the relative expression level of miR-29c-3p in bone tissues of DOP group was remarkably lower than that in NC group ($p<0.01$). However, it was significantly higher in ME group than that in DOP group ($p<0.05$). DOP group exhibited significantly upregulated serum BTMs (ALP, CTX-1, OC, TRACP-5b, and PIPN) when compared with NC group ($p<0.05$) and ME group ($p<0.05$). Furthermore, femoral BMD decreased in DOP group ($p<0.05$) while increased in ME group, showing statistically significant difference between the two groups ($p<0.05$). Immunohistochemistry results indicated that the bone tissues of DOP rats were deeply stained, and protein expression of Dvl2 protein was significantly higher in comparison with NC group. The bone tissues were lightly stained in ME group, and the protein expression of Dvl2 was lower than that in DOP group. Besides, qRT-PCR results demonstrated that the mRNA expression changes of Dvl2 were consistent with its protein expression trends.

CONCLUSIONS: MiR-29c-3p reduces bone loss in rats with DOP *via* targeted regulation of Dvl2 expression.

Key Words:
MiR-29c-3p, Dvl2, Diabetic osteoporosis (DOP).

Introduction

In recent years, the risk of diabetes mellitus (DM) becomes higher and higher due to the impacts of people's poor eating habits and other social factors. According to the data from the International Diabetes Federation (IDF) in 2019,

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there were 425 million DM patients aged 18-99 years old around the globe. Meanwhile, the figure will increase to 629 million in 2045 based on the current prevalence rate[1]. Diabetic osteoporosis (DOP) is a systemic metabolic osteopathy affected by genetic and environmental factors. DOP is characterized by bone destruction and decline in bone mineral density (BMD)[2,3], belonging to secondary OP. Currently, the prevalence rate of DOP is as high as 60% among DM patients[4,5]. Fracture and bone pain are the most common clinical manifestations of DOP, seriously impairing patients' life quality and bringing heavy economic burdens. Therefore, more attention has been paid to the research and treatment of DOP. There are many differences between DOP and postmenopausal OP (PMOP), of which the former is mainly related to metabolic disorders triggered by DM. Elevation of blood glucose inhibits osteoblast proliferation, decreases bone formation and osteoprotegerin synthesis, as well as promotes osteoclast differentiation and bone resorption. This may ultimately result in severe calcium (Ca) loss and reduce BMD[4]. As for the treatment of DOP, blood glucose should be controlled first. Once DM is under control, the indexes of DOP can be greatly improved. Therefore, its course of treatment can be relatively short. However, PMOP needs to be treated for a long term[6]. In recent years, a large number of micro ribonucleic acids (miRNAs) have been proven to regulate bone formation and regeneration as well as implant osseointegration[7,8].

Most RNAs *in vivo* do not have encoding functions, such as miRNAs and lncRNAs. However, they play important regulatory roles in various diseases. For example, miRNAs participate in the occurrence and development of diseases by silencing the gene expression by complementary binding in the 3'-UTR of target messenger RNAs (mRNAs). Previous studies have demonstrated that the expression of miR-29c-3p is downregulated in nasopharyngeal carcinoma, gastric carcinoma, and hepatocellular carcinoma. *In vitro* functional experiments have indicated that miR-29c-3p exerts anti-cancer effects in tumors, serving as a vital tumor suppressor in the miRNA family[6,9-11]. As a cytoplasmic molecule in Wnt/β-catenin signal transduction, Disheveled 2 (Dvl2) protein is widely involved in signal transduction. Besides, the activation of the Wnt/β-catenin pathway enhances osteogenesis[12]. In view of the crucial role of this pathway in osteogenesis, the downstream target genes of miR-29c-3p mediated by the Wnt/β-catenin pathway were screened using online software in this study. It was shown that Dvl2 was one of the target genes of miR-29c-3p. Nevertheless, the expression and regulatory mechanism of miR-29c-3p in DOP have rarely been investigated and reported. Hence, understanding the regulatory pattern of miR-29c-3p in DOP is conducive to the development of therapeutic drugs for the disease.

Materials and Methods

Experimental Materials

This study was approved by the Animal Ethics Committee of Xiangyang Central Hospital Animal Center. Specific pathogen-free (SPF)-grade Wistar rats were provided by the Animal Research Center of our hospital. MiR-29c-3p agonist was purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China), blood glucose meter from Roche (Basel, Switzerland), and TRIzol reagent from Invitrogen (Carlsbad, CA, USA).

Experimental Groups

A total of 30 Wistar rats were selected as research objects in this study. All rats were assigned into NC group, DOP group, and ME group based on different intervention measures. In NC group, healthy control rats were only injected with normal saline. DOP rats in DOP group were injected with normal saline, while those in the ME group were administered with miR-29c-3p agonist by tail vein injection (30 μg/4 days).

Experimental Methods

SPF-grade male Wistar rats aged 6 weeks old were first fed in a suitable environment for 4 days. Subsequently, they were utilized to establish the laboratory animal model. Specifically, streptozotocin (STZ; 60 mg/kg) was injected intraperitoneally to induce DM. Blood glucose concentration higher than 16.7 mmol/L suggested successful establishment of the model of DM in rats. Meanwhile, healthy control rats in NC group were injected with normal saline. After that, the changes in BMD in NC group and DM group were detected every 4 weeks, and a statistical difference in BMD between the two groups indicated successful DOP modeling. During the experiment, the variations in body weight and blood glucose of rats were examined every week.

Sampling After Intervention

Following experimental observation, the rats in the three groups were anesthetized and sacrificed. Femoral tissues were isolated, placed in cryotubes, and immediately put into liquid nitrogen. 30 min later, the tissues were transferred into a refrigerator at -80°C. In the meantime, the blood was collected from the abdominal aorta to measure the content of bone formation markers and blood glucose.

Detection of Rat Bone Mineral Density (BMD)

BMD of rat femur and lumbar vertebra (L1-L4) were determined using a dual energy X-ray absorptiometry scanning system.

Detection of Bone Turnover Markers (BTMs) in the Serum

Blood samples were drawn from the abdominal aorta of rats after fasting for 12 h. Collected samples were centrifuged at 4°C for 15 min to separate the serum. Next, the serum levels of Ca and phosphorus (P) levels in rats were detected by an atomic absorption spectrometer. The content of bone turnover markers (BTMs), such as alkaline phosphatase (ALP), osteocalcin (OC), and procollagen type I N-terminal propeptide (PINP) in the serum was examined through enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Femoral tissues were fully ground in liquid nitrogen, and total RNA was extracted according to the instructions of TRIzol reagent. Subsequently, 1 μg of total RNA was synthesized into complementary deoxyribonucleic acid (cDNA) using PrimeScript™ II cDNA synthesis kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was then performed, and the relative expression of miR-29c-3p and Dvl2 were measured *via* $2^{-\Delta\Delta Ct}$ method. U6 was used as the internal reference in the quantitative analysis of miR-29c-3p expression, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference in the quantitative analysis of the Dvl2 expression. Primer sequences used in this study were as follows: MiR-29c-3p-F: CATAGCAGGTGGCATCAGAA; MiR-29c-3p-R: AGGGCAAAGGTATCCTGCTT. Dvl2-F: TCCACCATTACCCCCTTTGC; Dvl2-R: GCCATGCTCACTGCTGTCT. U6-F: CTCGCTTCGGCAGCACA, U6-R: AACGCTTCACGAATTTGCGT. GAPDH-F: TGATGGGTGTGAACCACGAG; GAPDH-R: CTGATGGGTGTGAACCACGAG.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) Statistics 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. The differences between two groups were analyzed by using the Student's *t*-test. One-way ANOVA was conducted to compare the differences among different groups, followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Changes in Blood Glucose and Body Weight of Rats in Each group

Wistar rats with DM manifested polydipsia, polyphagia, polyuria, weight loss, slow response, and behavioral activities. The rats in NC group had regular diets, normally increased body weight, and sensitive and rapid responses. In DOP group, blood glucose concentration remained above 16.7 mmol/L during the whole monitoring process, which was higher than the normal blood glucose concentration. However, the body weight decreased continuously. Blood glucose rose first and then declined in the ME group, which was always kept higher than the normal concentration. Additionally, the body weight was reduced constantly, while the rate of weight loss decreased in the late stage of monitoring (Figure 1).

Differentially Expression of MiR-29c-3p in Femoral Tissues after Intervention

In order to investigate the changes in miR-29c-3p expression, qRT-PCR was adopted to detect the expression of miR-29c-3p in femoral tissues of DOP rats. The results indicated that the relative expression level of miR-29c-3p in femoral tissues of DOP group was remarkably lower than that in NC group ($p<0.01$). However, it was notably higher in ME group than that in DOP group ($p<0.05$) (Figure 2).

MiR-29c-3p Reduced Serum Bone Turnover Markers (BTMs) and Raised Serum Ca and P Content in DOP Rats

Bone turnover markers (BTMs) can be applied to evaluate the therapeutic effect of OP, play a role in OP diagnosis, and reflect bone formation

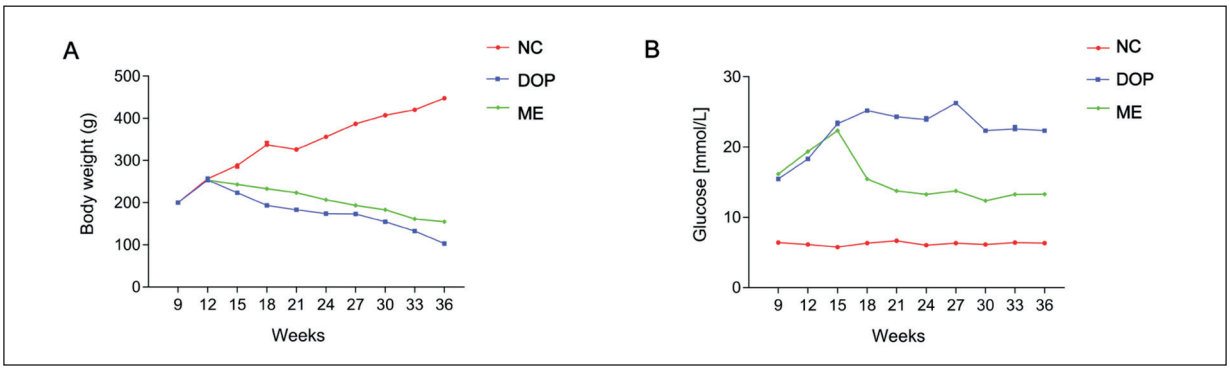

Figure 1. Changes in body weight and blood glucose of the rats during experiment. **A,** Changes in body weight of rats, **B,** Changes in blood glucose of rats.

and resorption. BTMs consist of bone resorption markers and bone formation markers. Osteoblasts can synthesize osteoblast markers, such as OC, ALP, and PINP. Moreover, they can reflect the osteogenic function in human body. Both cross-linked C-telopeptide of type I collagen (CTX-1) and tartrate-resistant acid phosphatase-5b (TRACP-5b) are bone resorption markers. Therefore, in the present study, the expression changes of BTMs were detected to represent the degree of bone turnover. The results indicated that DOP group exhibited significantly upregulated serum BTMs (ALP, CTX-1, OC, TRACP-5b and PIPN) compared with NC group ($p<0.05$). However, ME group manifested the opposite change trends, and as a consequence, ME group had markedly reduced markers of osteoblast and bone resorption in contrast with DOP group ($p<0.05$). All the results illustrated that miR-29c-3p was capable of downregulating the levels of serum BTMs and delaying DOP progression. Hyperglycemia has been found to induce osmotic diuresis. It was discovered in this study that the urine volume remarkably increased in DM rats, which induced the discharge of Ca and P and elevated Ca and P concentrations in the blood. Moreover, the concentrations of Ca and P in the blood rose significantly in ME group (Figure 3).

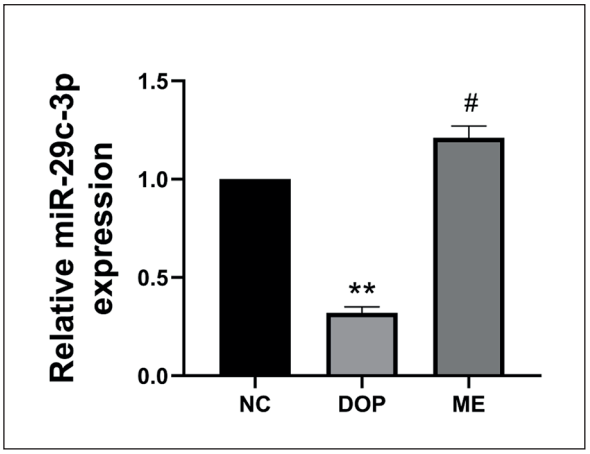

Figure 2. Relative expression of miR-29c-3p in each group of rats. The expression of miR-29c-3p is lower in DOP group than that in NC group (**$p<0.01$), while it is notably higher in ME group than that in DOP group ($^{\#}p<0.05$). (**$p<0.01$ *vs.* NC group, $^{\#}p<0.05$ *vs.* DOP group).

MiR-29c-3p Increased BMD in DOP Rats

BMD analysis revealed that BMD of lumbar vertebra (L1-L4) and femur decreased remarkably in DOP group when compared with NC group ($p<0.05$). However, it was restored in ME group after injection with miR-29c-3p agonist. Meanwhile, there was a significant difference between ME group and DOP group ($p<0.05$). It could be seen that miR-29c-3p was able to increase the BMD of DM rats (Figure 4).

MiR-29c-3p Weakened Protein and mRNA Expressions of Dvl2 in Bone Tissues

To explore the molecular mechanism of miR-29c-3p in reducing bone loss in DOP, the changes in the protein expression of Dvl2 in femoral tissues were examined *via* immunohistochemistry. It was shown that bone tissues in DOP group were deeply stained, and Dvl2 protein was highly expressed in comparison with NC group ($p<0.05$). In contrast, bone tissues in ME group were lightly stained, and the protein expression of Dvl2 was distinctly lower than that in DOP group ($p<0.05$). Meanwhile, the same variation trends of the mRNA expression of

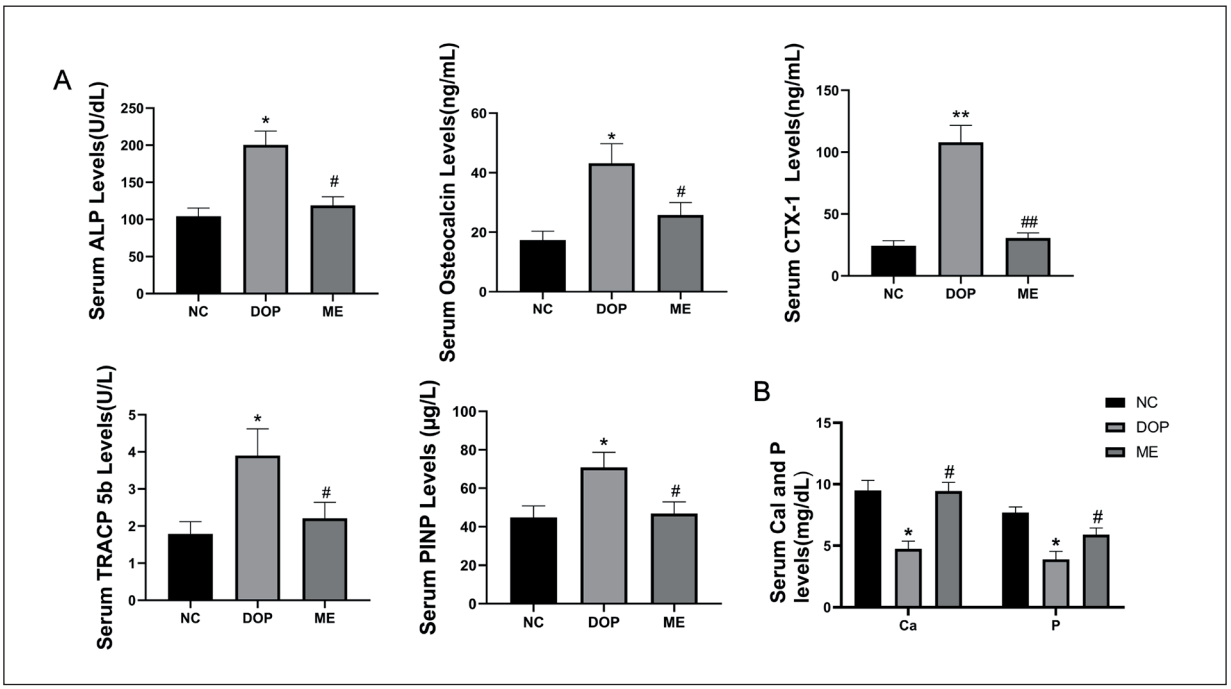

Figure 3. Changes in content of serum BTMs, Ca and P in each group of rats. **A,** Changes in content of BTMs detected *via* ELISA. The content of serum BTMs increases evidently in DOP group (*p<0.05) but decreases clearly in ME group ($^{\#}p$<0.05). **B,** Content of serum Ca and P in three groups of rats. The concentrations of serum Ca and P are prominently lower in DOP group than those in NC group, while they are higher in ME group than those in DOP group (p<0.05). (*p<0.05 *vs.* NC group, $^{\#}p$<0.05 *vs.* DOP group, **p<0.01, $^{\#\#}p$<0.01).

Dvl2 were observed (p<0.05). All these findings suggested that miR-29c-3p repressed the protein expression of Dvl2, thereby participating in DOP progression (Figure 5).

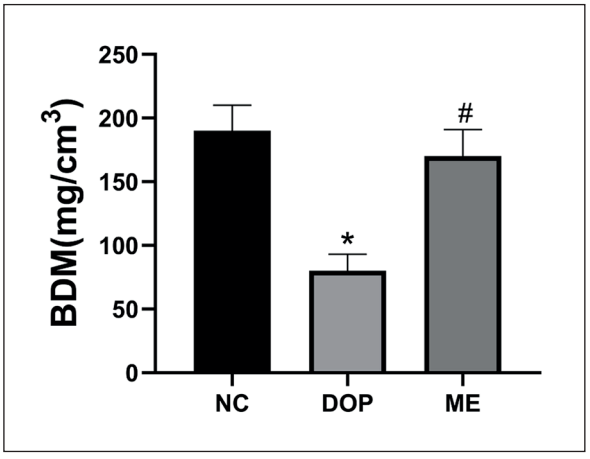

Figure 4. BMD of lumbar vertebra and femur in each group of rats. BMD decreases remarkably in DOP group compared with that in NC group (*p<0.05). However, it is higher in ME group than that in DOP group ($^{\#}p$<0.05). (*p<0.05 *vs.* NC group, $^{\#}p$<0.05 *vs.* DOP group).

Discussion

Decreased MBD, increased bone fragility and fracture risk are the most important features of DOP[13,14]. Multiple studies[15-17] have elucidated that DM affects bone renewal and integrity. Moreover, the bone loss increases, and the bone renewal is accelerated in DM patients[18]. Persistent hyperglycemia inhibits osteoblast differentiation and induces osteoblast apoptosis[19], considered as a leading cause of diabetic osteopenia[20,21]. Currently, there have been no studies on the expression changes of miR-29c-3p in DOP and its influence on the disease. In this study, our results manifested that highly expressed miR-29c-3p could relieve bone loss in DM rats.

According to the results of the present study, DM rats exhibited significantly reduced BMD, and raised blood glucose, BTMs, and osteoblast counts. This implied that the rat model of DOP was successfully established. The above indexes were ameliorated in ME group, illustrating that miR-29c-3p had a protective effect against DM-induced bone loss in rats.

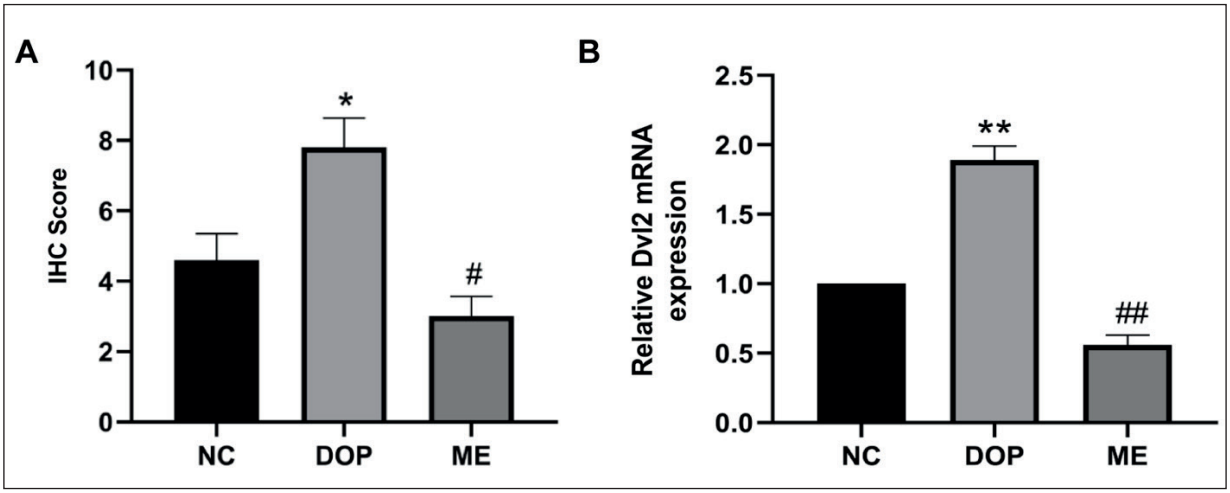

Figure 5. Protein and mRNA expressions of Dvl2 in bone tissues of rats. **A,** The immunohistochemistry score of Dvl2 protein in bone tissues is markedly raised in DOP group in comparison with NC group (*p<0.05) and ME group ($^{\#}p$<0.05). **B,** Changes in mRNA expression of Dvl2 in each group detected *via* qRT-PCR. The mRNA expression of Dvl2 in bone tissues is notably higher in DOP group than that in NC group (*p<0.05), but evidently lower than that in ME group ($^{\#\#}p$<0.01). (*p<0.05 *vs.* NC group, $^{\#}p$<0.05 *vs.* DOP group, $^{\#\#}p$<0.01).

Hyperglycemia has been confirmed to induce osmotic diuresis. In this study, it was found that urine volume significantly increased in DM rats. Moreover, increased urine volume resulted in Ca and P discharge and lowered Ca concentration in the blood. Therefore, activated osteoclasts can promote the mobilization of bone Ca and P, enhance bone resorption and reduce bone mass[22,23]. Upregulated blood glucose represses the proliferation of osteoblasts and facilitates the differentiation of osteoclasts. Moreover, it is held that abnormally increased differentiation of osteoclasts is the pathogenesis of DOP[23]. Our findings demonstrated that blood glucose decreased evidently in DOP rats injected with miR-29c-3p agonist. Therefore, the hypoglycemic effect of miR-29c-3p was a vital reason for its ability to resist DOP.

BTMs can be applied to evaluate the therapeutic effect on OP, meanwhile, they work in OP diagnosis to reflect bone formation and resorption. BTMs include bone resorption markers and bone formation markers. Current studies have found that osteoblasts can synthesize bone markers. Notably, OC, ALP and PINP can reflect osteogenic function in human body. As bone resorption markers, the higher levels of CTX-1 and TRACP-5b are, the lower BMD will be. It was discovered in the present study that the content of bone formation and resorption markers increased remarkably in DM rats, signifying the enhancement of bone turnover. The main cause of increased bone formation markers in the serum of DM rats might be that osteoblasts attempted to compensate the bone loss triggered by type I DM. Additionally, these BTMs were significantly downregulated in ME group, indicating that highly expressed miR-29c-3p in the femur could prevent DOP and reduce bone loss.

Dvl2 protein is a pivotal player in the Wnt/β-catenin signal transduction. However, activated Wnt/β-catenin pathway strengthens the osteogenetic effect of osteoblasts[23]. Furthermore, Dvl2 is also implicated in the pathogenesis of OP. In this study, the protein expression of Dvl2 in bone tissues in DM rats was significantly elevated. However, it decreased after the injection of miR-29c-3p agonist, suggesting that miR-29c-3p was able to modulate Dvl2 expression. Based on the results of BMD in rats of each group, exogenous overexpression of miR-29c-3p could restore the BMD of DOP rats. However, multiple parameters could participate to DOP and this pattern presented in this study justifies only in part this metabolic disorder. More research are still needed in future.

Conclusions

In summary, miR-29c-3p exerts protective effects in DOP by suppressing the protein expression of Dvl2. Our findings can provide a potential strategy for the treatment of DOP.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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