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Analysis of Effects and Mechanism of N6022 on Diabetic Lower Extremity Vascular Dysfunction

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Abstract: Objective: To analyze the effects of S-nitrosoglutathione reductase (GSNOR) inhibitor (N6022) on diabetic lower extremity vascular dysfunction and its mechanism. Methods: Sixty male C57BL/6 mice were chosen and randomly assigned to one of three groups: controls, models, or N6022. No intervention was made on the comparison group. Type 1 diabetes mellitus models and lower leg ischemia models were used to create the control group and the N6022 group, respectively. The N6022 group was injected with N6022 through tail vein. After two weeks of intraperitoneal anesthesia, mice were slaughtered, and their vein blood was extracted. Serum triglyceride (TG) and total cholesterol (TC) levels in rodents were measured enzymatically. The number of neovascularization of mice in each group was observed by staining skeletal muscle tissue sections. Vascular endothelial progenitor cells of mice were extracted and cultured. The number of EPCs involved in the tube like structure in the three groups was compared by tube like formation test, and β- Galactosidase staining was used to detect the senescence of endothelial progenitor cells in the three groups. The cell migration assay was used to detect the migration number of endothelial progenitor cells in the three groups, and clone formation assay was used to detect the number of vascular endothelial progenitor cell clones in the three groups. Pt-PCR and qPCR were used to identify the mRNA and protein expressions of vascular endothelial growth factor (VEGF-) and angiopoietin-1 (Ang-1). **Results:** Mouse serum TC and TG levels were markedly higher in the model and N6022 groups compared to the control group, and serum TC and TG levels were substantially reduced in the N6022 group compared to the model group (P<0.05). Compared to the control group, mice in the model group and the N6022 group had significantly greater serum fasting blood glucose and insulin levels, while mice in the N6022 group had significantly reduced serum fasting blood glucose and insulin levels, the difference was statistically significant (P<0.05).Both the model group and the N6022 group showed significantly reduced neovascularization and endothelial progenitor cell participation in tubular structure compared to the control group, while the N6022 group showed significantly increased neovascularization and endothelial progenitor cell participation in tubular structure compared to the model group (P<0.05). Mice in the model and N6022 groups had significantly fewer arterial endothelial progenitor cells migrating and clones, and substantially more aging cells, compared to mice in the control group. Numbers of migratory arterial endothelium progenitor cells and clones of endothelial progenitor cells in the blood vessels of N6022-treated animals were considerably greater than those of model-treated mice, while the number of aging cells was significantly reduced (P<0.05).The number of vascular endothelial progenitor cells migrating and the number of clonal clumps of vascular endothelial progenitor cells were significantly lower in the model group and N6022 group than in the control group. The number of aging cells was significantly higher in the model group and N6022 group than in the control group. The number of vascular endothelial progenitor cells that moved and the number of clonal clumps of these cells were both significantly higher in the N6022 group than in the model group, while the number of senescent cells was significantly lower in the N6022 group than in the model group (P<0.05). **Conclusion:** N6022 can improve the lipid metabolism status of diabetic mice, and has certain therapeutic effects on lower limb vascular dysfunction. Some researchers believe that N6022's role in controlling the growth and other cellular activities of arterial endothelium precursor cells is at the root of this phenomenon, aging, migration and mediating the expressions of angiogenesis genes.

Keywords: N6022, Diabetes Mellitus, Lower Extremity Vascular Dysfunction, Impact, Mechanism

INTRODUCTION

Diabetes is a chronic metabolic disease with high incidence worldwide, which can be manifested as polyuria, polydipsia, polydipsia and emaciation, accompanied by long-term hyperglycemia [1-2]. However, hyperglycemia is also associated with an increased chance of arterial illness and stroke. Long term hyperglycemia can lead to macrovascular and microvascular lesions, while chronic progressive lesions of the body's blood vessels can lead to complications such as coronary heart disease, renal failure and lower extremity vascular dysfunction [3-4]. Chronic progressive lesions of the body's blood vessels have been found to be the primary cause of disability and death in patients with diabetes, which not only has a profound impact on the patients' quality of life and health but also places a significant financial and emotional burden on their families and communities. Vascular endothelial dysfunction has been hypothesized to be at the root of the onset and progression of diabetic vascular disease. In this condition, vascular endothelial progenitor cells have the potential to differentiate into endothelial cells, at which point they might contribute to vascular endothelial injury and boost angiogenesis [5-6]. N6022 is an effective, selective and reversible S-Nitrosoglutathione reductase (GSNOR) inhibitor. At present, it has relatively good application effects in lateral root development and disease resistance of plants. In recent years, studies have found that N6022 can participate in diabetic vascular endothelial dysfunction [7], but its specific mechanism is still unclear. In this experiment, 60 C57BL/6 male mice were selected as the observation objects, and the purpose was to analyze the effects and mechanism of N6022 on diabetic lower extremity vascular dysfunction.

DATA AND METHODS

General Data

Sixty male C57BL / 6 rodents, ages 6-8 weeks, 10-16g, were bought from Jackson Aite Biotechnology (Beijing), Co., Ltd.

Methods

Twenty rodents were used for each of the three groups (control, model, and N6022). The untreated group served as the comparison. Both the type 1 diabetes mellitus model and the lower leg ischemia model were used to create the N6022 cohort. (1) A solution of STZ was made. Fasting blood glucose levels were measured in the model and N6022 groups after a 12-hour fast. Mice in the model group and the N6022 group received intraperitoneal injections of STZ solution (50mg / kg) for 5 days in a row, with each treatment being followed by feeding 1 hour later.And if the fasting blood glucose was greater than 12.0mmol/l,

it meant that the model was successfully established. (2) After 1 diabetes model was successfully established, the mice in model group and N6022 group were anesthetized intraperitoneally, the skin of the left lower limb was cut 5cm below the knee joint, the common femoral artery, superficial femoral vein, deep femoral vein and accompanying vein were separated and ligated, a ligature was placed at the extremity of the common femoral artery, the superficial femoral artery and vein, and the deep femoral artery and vein, the related vessels at the ligation point were removed, the incision was closed and disinfected. Mice in N6022 group were injected with N6022 via tail vein at a dose of 20mg / kg, once a day for 2 weeks.

Observation Indicators

Two weeks later, the mice were killed by intraperitoneal anesthesia, and the venous blood was collected. The serum levels of total cholesterol (TC) and triglyceride (TG) were detected by ELISA. Specific steps: the supernatant was carefully collected, and centrifuged again in case of precipitation during storage. Samples should be extracted as soon as possible after collection, and extracted according to relevant documents. After extraction, experiments should be carried out as soon as possible. If the test cannot be carried out immediately, the samples can be stored at -20 ℃, but repeated freezing and thawing should be avoided.ELISA was used to measure the levels of serum PINP, β-CTX, and BGP in the two groups of people. Specific steps: standard sample addition: The standard hole and the sample hole were set, and 50L of standards with different concentrations were added to the standard hole. The used antigen coating diluent was diluted to an appropriate concentration (generally, the amount of antigen coating required is 20-200μ l per well). With 100 μ l antigens per well, and set at 37 ℃ for 4h, or 4 ℃ for 24h. The liquid in the hole was discarded. 5% serum was sealed at 37 ℃ for 40min. When sealing, filled the reaction holes with sealing solution and removed the bubbles in the holes. After sealing, washed the holes with washing solution for three times, each time for 3min. Samples to be tested were added (Establishment of an appropriate concentration gradient): generally, the dilution of 1:50 ~ 1:400 should be used for testing, and a relatively large dilution volume should be used. Generally, sample absorption should be ensured to be >20μl. The diluted samples were added into the enzyme labeled reaction well, and added at least two wells for each sample, with 100 μl of each well. Then placed at 37℃ for 40～60min. The wells were washed with washing solution for three times, each time for 3min. Enzyme labeled antibody was added, and at 37℃ for 30~60min. Shorter than 30min often leads to unstable results. Added 100μ L per hole. Added substrate solution 100μ l per hole. And placed at 37℃ in the dark for 3 ~ 5 minutes, and the termination solution was added for color development. The experimental results were measured within 20min.

Indicators of neovascularization in muscle tissue on the operated side of mice: The number of neovascularization of mice in each group was observed by staining skeletal muscle tissue sections. Vascular endothelial progenitor cells of mice were extracted and cultured. Tube-like creation assay was used to evaluate the three groups' arterial endothelium precursor cell counts.

Biological activity index of vascular endothelial progenitor cells: β -galactosidase staining was used to detect the aging of vascular endothelial progenitor cells in three groups; Cell migration experiment was used to detect the number of cell migration of vascular endothelial progenitor cells in three groups; Clonal formation experiment was used to detect the number of clonal clumps of vascular endothelial progenitor cells in three groups.

Angiopoietin-1 and vascular endothelial growth factor (VEGF-) transcripts were measured using polymerase chain reaction (PT-PCR) and quantitative polymerase chain reaction (qPCR) (Ang-1). Specific steps: RNA extraction and identification were performed, total RNA was extracted by Trizol method, RNA concentration was determined by spectrophotometry, and RNA integrity was identified by agarose gel electrophoresis. b: Reverse transcription: To generate cDNA, we used a Primescript RT-kit and harvested 1 g of RNA, random primers 2μ1 (1μg) was added, M-M LV 200u, and cDNA was synthesized according to random hexadeoxynucleotide primer synthesis method. c: 2μ1 of the above reverse transcription product was used for PCR expansion in a 25μ1 reaction system. The cDNA was used as the template and GAPDH was used as the internal control for PCR amplification. PCR conditions: 40 cycles were amplified, additionally, each cycle was comprised of a pre-denaturation stage at 95 °C for 20s, a denaturation stage at 95 °C for 10s, an annealing stage at 57 °C for 20s, and an elongation stage at 72 °C for 15s: The amplified products were electrophoresed on 2% agarose gel, the results were observed and photographed, and the absorbance (a) ratio of the VEGF- α and Ang-1 mRNA band to GAPDH band of each case was calculated.

Statistical Methods

Spss20.0 software was used to analyze the data in this experiment. The TC, TG, VEGF- α and other measurement data were expressed as（□x±s ）. Analysis of variance, or the F test, was used to compare samples from different groups. When the conditions of variance analysis can't be met, the Kruskal-Wallis test was used to analyze the data, and the SNK-q test was used to compare two groups. The results of the statistics were statistically significant (P<0.05).

RESULTS

Comparison of Lipid Metabolism Indicators of Three Groups of Mice

Serum TC and TG levels in the model group and N6022 group were considerably higher than those in the control group (P<0.05), while the levels in the N6022 group were significantly lower than those in the model group. Please refer to Figure 1 and Table 1 for details.

Table 1: *Lipid metabolism comparison of three mouse types (□x±s)*

Grouping	Example	TC (mmol/l)	TG (mmol/l)
Control Group	20	1.69 ± 0.12	0.46 ± 0.08
Model group	20	2.85 ± 0.16 [a]	2.15 ± 0.24[a]
N6022 group	20	2.14 ± 0.23[ab]	1.52 ± 0.19[ab]
F		220.900	437.220
P		＜0.001	＜0.001

Note: compared with the control group, A indicates P<0.05; compared with the model group, b indicates P<0.05.

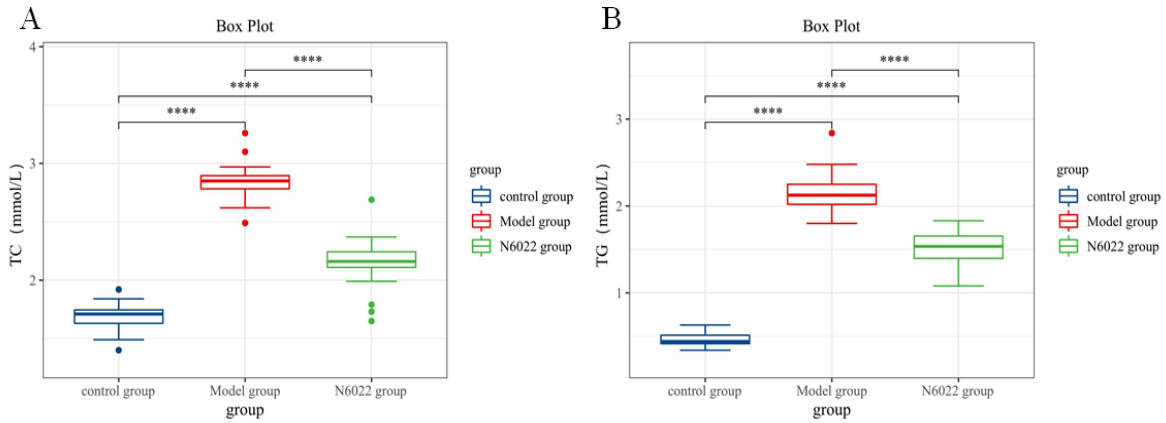

Figure 1. *Comparison of lipid metabolism indices of three groups of mice*

A: Comparison of TC levels of the two groups; B: Comparison of TG levels between the two groups

Note: **** indicates P<0.0001

Comparison of Glucose Metabolism Indicators of Three Groups of Mice

Serum fasting blood glucose and insulin levels were significantly higher in the model and N6022 groups compared to the control group, and substantially lower in the N6022 group compared to the model group (P0.05). Refer to Figure 2 and Table 2.

Table 2: *Comparison of glucose metabolism indices of three groups of mice* （□x±s）

Grouping	Example	Fasting blood glucose (mmol/l)	Insulin (mu/l)
Control group	20	5.12±0.27	15.88±1.73
Model group	20	20.12±1.35[a]	19.81±2.93[a]
N6022 group	20	12.44±2.14 [ab]	18.12±1.89 [ab]
F		49.53	15.39
P		＜0.001	＜0.001

Note: compared with the control group, A indicates P<0.05; compared with the model group, b indicates P<0.05.

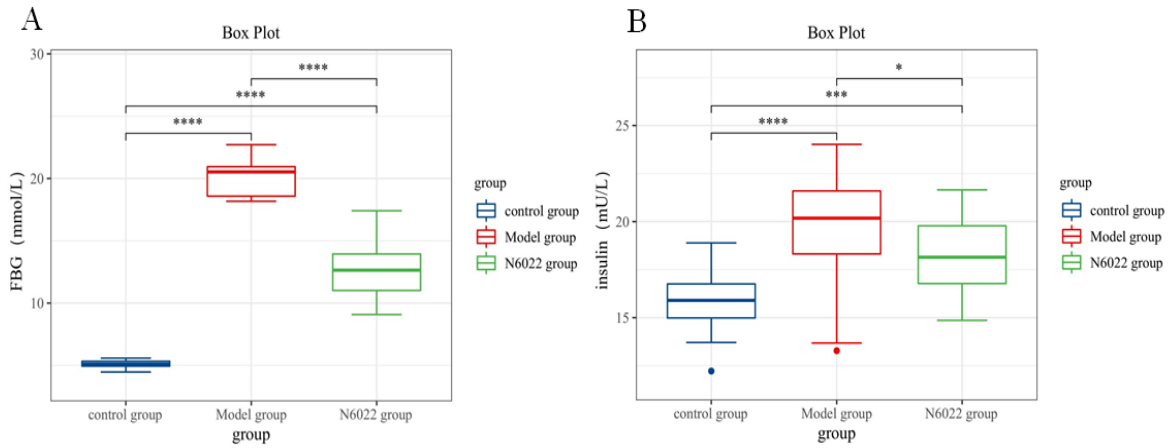

Figure 2. *Comparison of glucose metabolism indices of three groups of mice*

A: Comparison of fasting blood glucose levels between the two groups; B: Comparison of insulin levels between the two groups

Note: * indicates P<0.05; *** indicates P<0.001; **** indicates P <0.0001

Muscle Tissue Neovascularization Markers in Three Mouse Types

Neovascularization and vascular endothelial progenitor cell levels in tubular structures of mice were significantly lower in the model and N6022 groups compared to the control group, and significantly higher in the N6022 group compared to the model group, the difference was statistically significant (P<0.05). For examples, see Table 3 and Figure 3.

Table 3: *Muscle tissue neovascularization on the surgery side of three mouse groups (□x±s)*

grouping	Example	Neovascularization	Endothelial progenitor cells involved in tubular structures
Control group	20	126.52 ± 20.13	82.16 ± 14.23
Model group	20	53.85 ± 5.74[a]	41.75 ± 5.19[a]
N6022 group	20	101.23 ± 26.14[ab]	64.43 ± 10.75[ab]
F		72.810	71.360
P		＜0.001	＜0.001

Note: compared with the control group, A indicates P<0.05; compared with the model group, b indicates P <0.05.

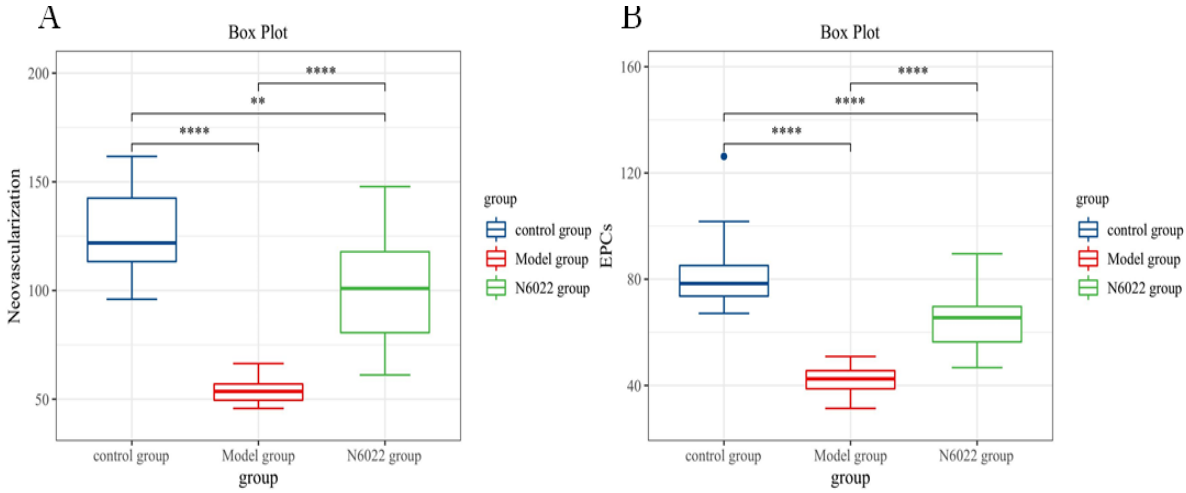

Figure 3. *Muscle tissue neovascularization markers in three mouse types*

A: Comparison of neovascularization between the two groups; B: Comparison of endothelial progenitor cells involved in tubular structure between two groups

Note: ** indicates P<0.01; **** indicates P<0.0001

Comparing Arterial Endothelium Precursor Cell Metabolic Activity in Three Mouse Groups

Mice in the model and N6022 groups had significantly fewer arterial endothelial progenitor cells migrating and clones, and substantially more aging cells, compared to mice in the control group. Mice in the N6022 group had a substantially greater number of arterial endothelium precursor cells migrating to new blood vessels and a dramatically reduced number of senescence cells compared to mice in the model group,the difference was statistically significant(P<0.05). See Table 4 and Figure 4.

Table 4: *Comparing arterial endothelium precursor cell metabolic activity in three mouse groups* （□*x*±*s*）

Grouping	Example	Number of senescent cells	Migration of endothelial progenitor cells	Number of clonal clumps of endothelial progenitor cells
Control group	20	24.30 ± 3.79	63.55 ± 7.37	42.15 ± 5.56
Model group	20	46.60 ± 4.14[a]	23.45 ± 6.43[a]	18.85 ± 1.46[a]
N6022 group	20	33.65 ± 5.91[ab]	36.15 ± 8.43[ab]	23.50 ± 3.91[ab]
F		113.260	151.15	188.76
P		＜0.001	＜0.001	＜0.001

Note: compared with the control group, A indicates P<0.05; compared with the model group, b indicates P<0.05.

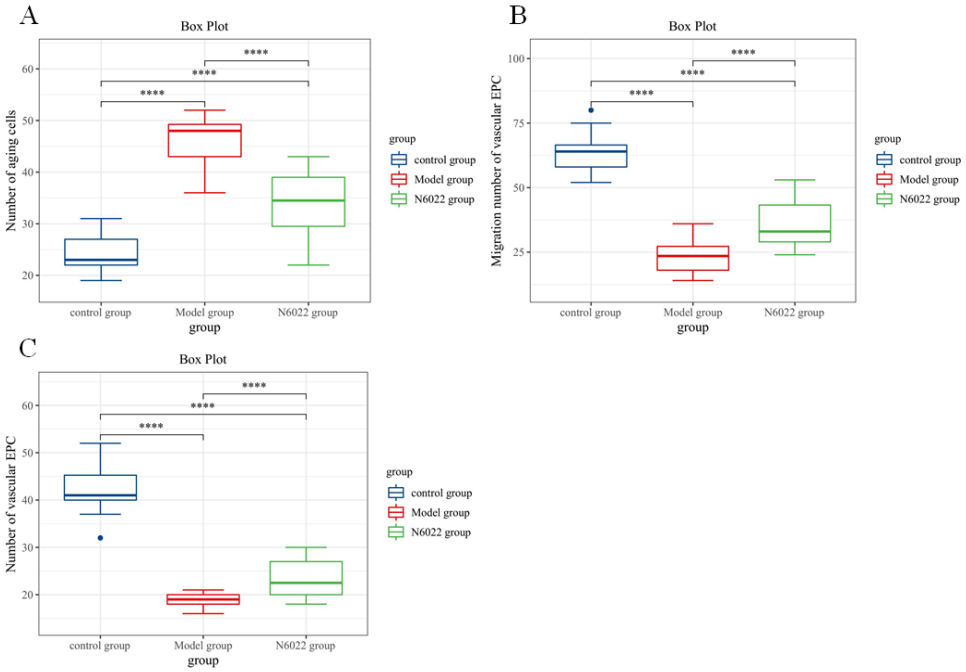

Figure 4. *Comparison of biological activity indices of vascular endothelial progenitor cells in three groups of mice*
A: Comparison of the number of senescent cells between the two groups; B: Comparison of the migration of vascular endothelial progenitor cells between the two groups; C: Comparing the two groups' arterial endothelium precursor cell clones.
Note: **** indicates P<0.0001

Three Mouse Groups' Angiogenic Genes of Arterial Endothelium Precursor Cells

Mice in the model group and the N6022 group had a considerably reduced VEGF- mRNA level compared to the control group, while mice in the control group had a significantly greater Ang-1 mRNA level. Mice in the N6022 group had substantially increased VEGF- and Ang-1 mRNA levels compared to mice in the model group, the difference was statistically significant (P<0.05). Refer to 5 Chart and 5 Image.

Table 5: *Comparison of angiogenic genes of endothelial progenitor cells in three groups of mice* （□*x*±*s*）

Grouping	Example	VEGF- α MRNA	Ang-1 mRNA
Control group	20	685.42 ± 120.36	1045.86 ± 230.15
Model group	20	345.12 ± 56.23[a]	2315.26 ± 345.12[a]
N6022 group	20	475.16 ± 35.49[ab]	4152.44 ± 315.02[ab]
F		93.570	539.500
P		＜0.001	＜0.001

Note: compared with the control group, A indicates P<0.05; compared with the model group, b indicates P<0.05.

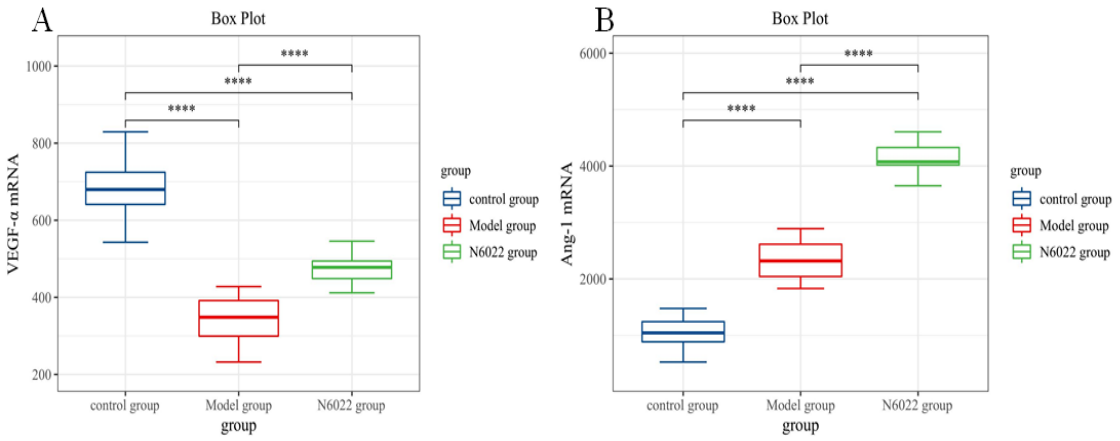

Figure 5. *Comparison of angiogenic genes of vascular endothelial progenitor cells in three groups of mice*
A: Comparison of VEGF- α mRNA between the two groups; B: Comparison of Ang-1 mRNA between the two groups
Note: **** indicates P<0.0001

DISCUSSION

Lower extremity vascular dysfunction is one of the common complications of diabetes mellitus and one of the most important pathogenesis of diabetic foot. The early symptoms mainly show pain, swelling, paralysis, cold feeling, etc., but also acute lower extremity ischemia, limited activity and other conditions, severe cases can lead to necrosis of the lower limbs and diabetic foot disease, leading to death or disability of patients, which brings serious treatment and economic burden to patients and society. As one of the most urgent unmet needs in the field of diabetes, the disease should receive extremely high attention [8-10]. Lower extremity atherosclerosis is the main cause of lower extremity vascular obstruction. Under normal circumstances, the arterial wall of normal people will have varying degrees of atherosclerotic changes, and diabetes can accelerate the process of atherosclerosis and aggravate its progress [11]. Diabetic patients have chronic venous lesions of the lower limbs, which is a group of comprehensive symptoms due to any morphological and functional abnormalities of the venous system, which can cause discomfort of the lower limbs and skin ulcers of the lower limbs and feet. Early screening of chronic venous lesions of the lower limbs in diabetic patients is conducive to reducing the occurrence of diabetic foot ulcers (DFU) as much as possible [12-15]. Therefore, analyzing the mechanism of diabetic patients complicated with lower extremity vascular dysfunction and looking for targeted and effective treatment measures have become the focus of the current research of medical staffs in the Department of chronic endocrinology.

N6022 is a GSNOR inhibitor. According to current studies, N6022 can participate in the effects such as the recovery of neurological function, antiviral, immune response regulation, bronchiectasis, and reduction of cardiac ischemia-reperfusion injury [16-17]. Jiao L and other researchers [18] have showed that GSNOR is a key denitrifying enzyme, which can regulate protein S-nitrosation and participate in the pathogenesis of Parkinson's disease (PD). N6022 treatment can alleviate PD-like pathology and neurotoxicity by inhibiting MPTP induced Cdk5 kinase activity and Cdk5 mediated autophagy. Khan M and other researchers [19] have believed that the metabolic group of dysregulation of nitric oxide (NO) and the NO-based neurovascular defensive mechanism occurs after stroke. S-nitrosoglutathione (GSNO), the primary NO molecule, plays a critical role in S-nitrosylation-based signaling processes. And N6022, an inhibitor of the S-nitrosoglutathione metabolic enzyme GSNO reductase (GSNOR), provides protection effect after myocardial ischemia.In addition, research has demonstrated that N6022 has the ability to stimulate the development of skeletal muscle and promote the recovery of blood flow, both of which are essential for the treatment of diabetic peripheral arterial disease[20-23]. In this experiment, the levels of serum TC, TG in N6022 group were significantly lower than those in model group, but significantly higher than those in the control group. The levels of neovascularization and vascular endothelial progenitor cells in N6022 group were significantly higher than those in model group, but significantly lower than those in the control group. These results suggest that N6022 may participate in neovascularization to improve lower extremity vascular dysfunction. In addition, N6022 can also improve lipid metabolism disorder, reduce atherosclerosis and improve lower extremity dysfunction.

Vascular endothelial progenitor cells are derived from adult stem cells in human bone marrow, which have the potential to undergo directional differentiation into functional arterial endothelial cells, thus contributing to the angiogenesis and blood vessel creation in the human body [24-26]. The ability of

human umbilical vascular endothelial cells to migrate and create tubes is inhibited when glucose levels are high; however, previous research has shown that N6022 can mitigate this impact. Therefore, N6022 may be beneficial for diabetic arterial disease as well as diabetic peripheral artery disease [27-30]. In this particular experiment, the number of clones of vascular endothelial progenitor cells and the number of migratory vascular endothelial progenitor cells were significantly higher in the N6022 group than they were in the model group, whereas they were lower in the control group. When compared to both the model group and the control group, the N6022 group had a noticeably lower percentage of cells that were showing signs of senescence. In comparison to their counterparts in the control group, the mice in the model and N6022 groups exhibited significantly lower levels of VEGF- α and higher levels of Ang-1. In comparison to the model mice, the rodents in the N6022 group exhibited significantly elevated amounts of both VEGF- α and Ang-1. Based on these findings, N6022 may be able to promote angiogenesis, contribute to the preservation of arterial tissue, and act as a stimulant for the development and expansion of vascular endothelial progenitor cells. In addition, because it mediates the generation of genes that are involved in the process of angiogenesis, N6022 is able to lend a hand in the process.

In conclusion, N6022 can improve the lipid metabolism status of diabetic mice and has certain therapeutic effects on lower limb vascular dysfunction. It's possible that the mechanism has something to do with how N6022 controls the biochemical activity of arterial endothelium progenitor cells, like multiplication, aging, migration and mediating the expressions of angiogenesis genes. However, due to the relatively short time of this experiment, the effects of N6022 on vascular endothelial progenitor cells have not been analyzed at the cell level, and the research time will be increased in the future for further exploration.

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