Original Article

Randomized research on the mechanism of local oxygen therapy promoting wound healing of diabetic foot based on RNA-seq technology

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Background: High purity oxygen therapy has good clinical efficacy in the treatment of diabetic foot (DF), but its mechanism of promoting wound healing has been unclear.

Methods: Patients with DF were randomly divided into an experimental group and a control group. The experimental group was given local oxygen therapy (LOT) by a micro-oxygen therapy instrument, which administered uninterrupted >95% pure oxygen for 24 h at a flow rate of 3 mL/h. Six skin samples from the experimental group before and after treatment underwent RNA sequencing (RNA-seq), and the differentially expressed genes (DEGs) were screened.

Results: The clinical results showed that the mean wound healing time of the experimental group was 26 days ($P<0.05$); the healing area of the experimental group was 3.1–15.3 cm^3, with a mean of 8.8 cm^3, and that of the control group was 2.4–10.4 cm^3 ($P<0.05$). LOT promoted the healing of DF wounds mainly through the tumor necrosis factor (TNF) signaling pathway and the apoptosis pathway.

Conclusions: According to our results, LOT can promote DF healing mainly by inhibiting the local oxidative stress reaction of wound skin and by inhibiting the inflammatory and apoptotic pathways. The molecular markers and pathways screened warrant further study.

Keywords: Tissue construction; diabetic foot (DF); high-concentration oxygen; local oxygen therapy (LOT); transcription group

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Introduction

Diabetes has become an important endocrine and metabolic disorder disease worldwide. With the ageing global population, the number of people with diabetes is increasing. In 2015, approximately 8.8% of the world's adults aged 20–79 years (415 million people) had diabetes. It is estimated that by 2040, the global prevalence of diabetes will rise to 10.4% (642 million people) (1). Moreover, in the late stage of diabetes, patients often exhibit a variety of complications, such as chronic organ damage and dysfunction (2), which seriously affect their quality of life. Therefore, diabetes has become a major public health problem. Diabetic foot (DF) is the most common complication of patients with diabetes (3), and its incidence increases with the rise of diabetes (4). The pathogenesis of DF includes neuropathy (5), vascular disease and Charcot

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disease (6). With the occurrence of foot ulcers, patients often face the risk of subsequent osteomyelitis, amputation or even death (7). Accelerating the healing of DF wounds is a critical factor for relieving DF symptoms and reducing clinical injury.

Routine DF treatment in the clinic includes: (I) medication (8): with blood pressure, blood glucose and blood lipids controlled, anticoagulant and antithrombotic drugs are administered to protect the circulatory system, and antibiotics are administered to control infection; mecobalamin is used for patients with nerve injury. However, medication therapy is only suitable for patients with mild DF, where surgery is the main choice for patients with more complex clinical symptoms. (II) Artery bypass grafting: Tukiainen (9) reported that raising the radial forearm flap significantly accelerated vein graft flow, and graft patency at 2 years was 89%. However, a series of factors, including age, glucose metabolism, end-stage renal disease and blood vessel length and quality, contribute to the prognosis of infrapopliteal bypasses, which limits its utilization in the clinic (10). Peripheral neurolysis is another DF surgical therapy, as reported by Palaniappan (11), where 90% of patients with DF experienced no pain after treatment, and the tibial nerve was stimulated in 69% of patients, resulting in plantar flexion and inversion, thereby improving the symptoms of peripheral neuropathy in patients with DF effectively. (III) Interventional therapy: Georgakarakos summarized endovascular treatment in DF (12), and successful revascularization in diabetic patients decreased the recurrence of ischemic ulcers (13). Although some researchers have evaluated self-expandable (14) or drug-eluting (15) stents in DF treatment, and have reported higher clinical efficacy compared to simple angioplasty (15), additional larger trials are required to draw safer conclusions for these new methods. (IV) Stem cell therapy: some researchers have attempted to repair DF ulcer tissue with stem cells (16), as it has been proven that mesenchymal stem cells (17) and bone marrow mononuclear cells (18) have the potential for treating DF. However, although the appearance of immune rejection can be avoided, it is unsuitable for patients with severe anemia. In addition, bone marrow stem cell transplantation is not suitable for patients with hereditary diseases, limiting its application in the clinic. (V) Skin replacement therapy: compared with stem cell therapy, skin grafting could be proposed to treat noninfected diabetic wounds directly. A meta-analysis (19) indicated that, after a mean of 5.35 weeks, the rate of healed DF skin grafting was 85.5%, with recurrence, infection and regraft rates of 4.2%, 4.4% and 12.1%, respectively. However, considering the skin basis of patients with DF, the longer healing time limits the use of this method, spawning skin regeneration matrices for DF skin grafting, such as Integra, Nevelia, MatriDerm, Pelnac and Renoskin (20), but the high cost is a barrier to the application of these biological matrices. A low-cost approach coupled with high clinical efficacy, hyperbaric oxygen therapy is also classified as skin replacement therapy. Based on the theory of hyperoxia-promoted wound healing (21), we used local oxygen therapy (LOT) to treat the wounds of patients with DF at our hospital, and obtained good clinical efficacy.

Most existing studies on LOT focus on its clinical application (22). Although some have analyzed its mechanism (23), considering the complexity of DF, single pathway or marker regulation is insufficient for the mechanistic study of LOT. Deeper systematic molecular pathology research is needed to uncover the overall clinical changes in DF skin. Considering that specific biological processes are often accompanied by RNA changes during treatment, systematic mRNA analysis can quickly reveal specific biological processes and molecular mechanisms in the process of disease occurrence (24). With the development of high-throughput technology, RNA sequencing (RNA-seq) technology uses transcriptome sequencing for comprehensive and in-depth detection of the expression profiles of sample tissues. With bioinformatics, the different genes of samples can be screened, a database for functional enrichment can be built and finally the process of biological sample change can be determined. Therefore, RNA-seq can be used to analyze the mechanism of LOT in DF wound healing. In the present study, we evaluated the clinical effect of LOT on DF and conducted transcriptome analysis of DF pathological samples before and after treatment. Finally, we analyzed the differential expression spectrum and identified the specific bioprocesses and pathways involved in the process. The results reveal the potential mechanism of the treatment and provide a preliminary basis for further molecular research. We present the following article in accordance with the CONSORT reporting checklist (available at http://dx.doi.org/10.21037/apm-20-295).

Methods

Clinical data

From January 2018 to January 2019, we enrolled 58

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patients with DF at our hospital for the study. According to the random number distribution principle, the patients were randomly divided into the LOT group and the control group. The LOT group included 22 men and 7 women aged 55–90 years, with a mean age of 74.33±6.47 years and a mean disease course of 15.70±6.61 years. The control group included 20 men and 9 women aged 57–88 years, and the mean age was 71.34±5.32 years; the mean disease course was 16.23±5.54 years. Age, sex, wound area or disease course were not significantly different between the groups (P>0.05). All patients who were enrolled fully understood the content and purpose of the study and signed the corresponding informed consent form. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics committee of Beijing Shijitan Hospital (No. lc-TNB-20180106) and informed consent was taken from all the patients.

Inclusion and exclusion criteria

Inclusion criteria: (I) clinical diagnosis of DF; (II) duration of wound non-union before admission ≥16 weeks; (III) ischemic DF of the lower limbs; (IV) blood glucose after admission could be controlled within 6–10 mmol/L; (V) age ≥40 years; (VI) wound area within 8 cm × 8 cm.

Exclusion criteria: (I) DF with renal failure; (II) the granulation tissue of the wound after debridement was not fresh or exudate was present; (III) albumin <30 g/L; (IV) blood vessels, tendons, nerves and bone were exposed in the wound; (V) patient could not adhere to the device usage or had poor compliance.

Clinic treatment

The patients' blood glucose was maintained at 6–10 mmol/L, and they were treated with conventional anti-infective drugs. The wounds were cleaned until there was fresh granulation tissue. The patients in the control group were treated with conventional nursing according to conventional treatment methods. In the LOT group, along with conventional treatment, LOT was administered with a micro-oxygen instrument (Wuxi Guoying Technology Co., Ltd., Wuxi, China) with plastic dressing, and an infusion tube was placed on the wound surface to provide >95% pure oxygen with a flow of 3 mL/h for 24 h. The treatment period was 4 weeks. In addition, the treatment was terminated if the wound area healed completely.

Observation indicators

Healing area

A tablet computer equipped with Kinect 2.0 image software was used to rapidly measure the wound depth and volume. The DF defect volume was recorded after debridement and after the 4-week treatment. The wound healing area was defined as the reduction area.

Healing time

Wound healing was defined as covering or scabbing of the epithelium and no obvious granulation tissue exposure.

Bacterial culture

After treatment, the wound exudate was collected for bacterial culture (the healed wound was regarded as negative). The Z-shaped pharyngeal tester was evenly applied from the upper to lower end of the wound to collect the wound base exudate. The obtained clinical samples were sent to the microorganism room of the clinical laboratory for bacterial isolation and culture, and the results were obtained after 5 days.

Pain score

A visual analogue scale (VAS) score of 0–10 points was used to score the pain value of the two groups, and were compared before and after treatment. The pain scores of the patients who healed within 4 weeks were collected when the study was terminated.

Transcriptome data

Sample collection

Samples were collected from six patients in the experimental group before and after treatment. Selection criteria: (I) all patients' skin healed within 4 weeks. (II) The wound healing area was >8.8 cm^3, and the wound healing area in the above six patients was 10.3±1.4 cm^3. (III) Pain score <4. Before treatment, the patients were selected for conventional debridement until the granulation tissue was fresh and disinfected. The granulation skin was selected, and the total area of skin tissue collected using sterilized scissors was approximately 0.5 cm^3. After treatment, the total area of skin tissue was 0.5 cm^2. The skin tissues collected twice were immersed in RNA later solution and stored in a refrigerator at –70 ℃.

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Sample pre-treatment

The sample was placed in liquid nitrogen and ground into powder, and 100 mg/1,000 mL TRIzol was added and fully mixed. Then, the sample was placed in a centrifuge tube, an equal volume of chloroform was added, and the tube was shaken for 30 s. The tube was incubated for 10 min at room temperature and centrifuged at 12,000 rpm at 4 ℃ for 15 min. The supernatant was removed, an equal volume of isopropanol was added to the tube and fully mixed, and the tube was incubated for 20 min at room temperature. Then, the sample was centrifuged at 12,000 rpm at 4 ℃ for 10 min, the supernatant was removed, and 1 mL 75% diethylpyrocarbonate (DEPC) ethanol was added to the sediment, which was then centrifuged twice at 7,500 rpm at 4 ℃ for 5 min. The supernatant was removed, and the remaining precipitate was evaporated at room temperature, yielding the total RNA sample for the subsequent research.

RNA-seq analysis

Total RNA samples extracted by sample sequencing were sent to Lianchuan Bio, Hangzhou, for transcriptome sequencing. The main steps were: DNase was used. The RNA samples were treated with enzyme I to eliminate DNA contamination, and oligo (dT) magnetic beads were added to the purified samples to enrich the mRNA; an appropriate amount of division buffer was added to the samples as a disruption agent to form short fragments under high temperature. The obtained mRNA fragments were used as a template to synthesize complementary DNA (cDNA), which was purified using a QIAquick PCR kit and eluted by EB buffer. The cDNA ends were repaired and connected with a poly (A) tail for sequencing. After the first step, PCR amplification was carried out to establish the RNA library for the study. After the library had passed quality inspection, it was sequenced with an Illumina HiSeq 4000 unit.

Bioinformatics analysis

The sequencing data were compared with the reference genome (human skin tissue) using HISAT software, and the alignment results were used to assemble the transcripts. The known gene models of the genome and the gene models predicted by StringTie were classified and expressed by ASprofile software. The difference in gene expression between the two groups was evaluated using fragments per kilobase of transcript per million mapped reads (fpkm). The gene location of differential expression was false discovery rate (FDR) <0.05, and the differential expression multiple was >1.5 times. The differentially expressed genes (DEGs) were analyzed for further biofunction enrichment. To investigate the biological function of DEGs, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) and Genetic Ontology (GO) analyzes with the WebGestalt databases (http://www.webgestalt.org/).

Statistical methods

The data were analyzed using SPSS 18.0. The obtained data are expressed as the mean ± SD. The measurement data were analyzed using the independent samples *t*-test, the grade data were compared using the rank sum test, and the chi-square test was used for count data. P<0.05 was deemed statistically significant.

Results

Typical LOT cases

All patients in the LOT group had a large amount of granulation tissue. *Figure 1* shows three typical cases.

Case 1: male, 65 years old; diabetes disease course of 13 years. He was admitted to the hospital due to 'left foot rupture and non-union more than 4 months'. The wound was debrided until the granulation tissue was fresh and then disposed of for iodophor dressing. After daily dressing change, micro-oxygen treatment was applied, and the wound was filled with alginate. *Figure 1A* shows the wound condition and administration of micro-oxygen after surgery. There was a 4×1×2 cm^3 cavity in the wound. After 3 days of LOT, granulation tissue grew rapidly from the bottom of the wound (*Figure 1B*). With 15 days of LOT, the local wound healed without obvious exudation (*Figure 1C*).

Case 2: male, 51 years old; diabetes disease course of 10 years. He was admitted mainly because of 'rupture of left sole for more than 2 years'. Bed trimming revealed fresh granulation tissue in the wound surface of the bottom of the foot (*Figure 1D*). After daily iodophor disinfection and administration of micro-oxygen, granulation tissue gradually accumulated in the wound, and was regularly trimmed. After 10 days of LOT, the wound healed (*Figure 1E*).

Case 3: (this patient was not enrolled for evaluation in the present study) female, 42 years old; diabetes disease course of 10 years. She was admitted to the hospital mainly because of 'rupture of right heel with fever for more than 1 week'. After the soft tissue and ligament in the wound had been removed, the sole bone was exposed,

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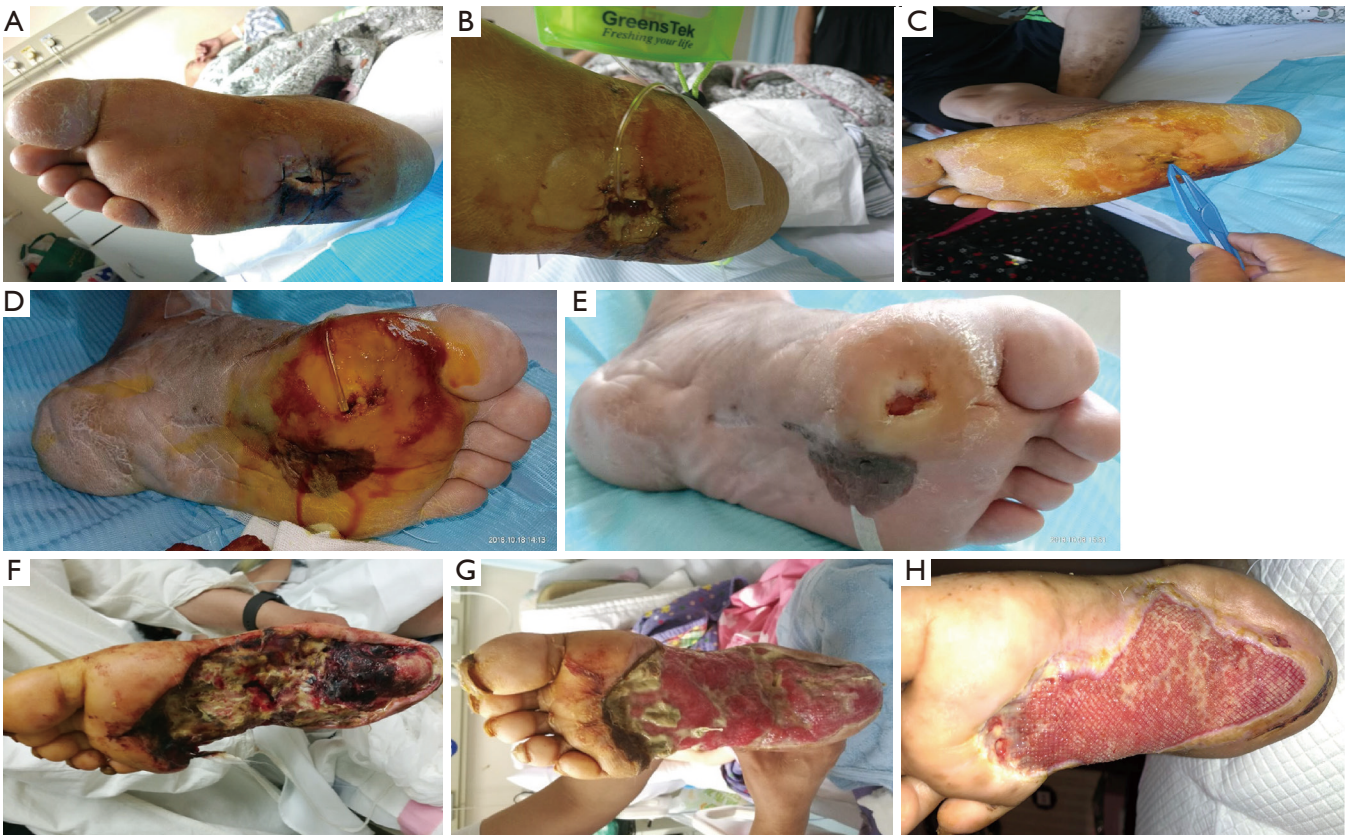

Figure 1 Clinical case of LOT for DF. (A,B,C) exhibited wound condition of case 1 in 0, 3 and 15 d, respectively; (D,E) exhibited wound condition of case 2 in 0 and 10 d; (F,G,H) exhibited wound condition of case 3 in 0, 30 and 60 d, respectively. LOT, local oxygen therapy; DF, diabetic foot.

and exhibited obvious local infection (*Figure 1F*). As the patient refused amputation and was given alginate, micro-oxygen was administered. After 30 days of treatment, the patient underwent re-operation to remove the necrotic fascia and suspected necrotic tissue (*Figure 1G*). After daily iodophor disinfection and administration of micro-oxygen, the necrotic tissue on the skin edge was trimmed, covered with gauze and treated with micro-oxygen for 60 days. The wound healed after 60 days (*Figure 1H*).

Clinical effects of LOT on DF

As shown in *Table 1*, the wound healing time of the LOT group was 5–30 d, with an average of 19 days, while the wound healing time of the control group was 13–30 d (with three cases that did not heal), with an average wound healing time of 26 days, which was significantly longer than that of the LOT group (P<0.05); the healing area of the LOT group was 3.1–15.3 cm^3, with an average healing area of 8.8 cm^3, and that of the control group was 2.4–10.4 cm^3, with an average of 6.2 cm^3, which was significantly smaller than that of the LOT group (P<0.05). According to the bacterial detection results, the main pathogenic bacteria were *Staphylococcus aureus* (five cases) and *Pseudomonas aeruginosa* (one case). The pain score of the LOT group was 0–6 points, with an average of 4.1 points, and that of the control group was 0–8 points, with an average of 4.5 points. However, there was no significant difference between the two groups (P>0.05).

Transcriptome sequencing results

Gene quantitative analysis was conducted using the RSEM tool, where we identified 28,620 genes before treatment and 28,678 genes after treatment. Before and after treatment, the first quarter (1/4 of fpkm) was 0.28 and 0.24,

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Table 1 Clinical effects of topical oxygen therapy for patients with DF

Group	N	Wound healing time (d)	Wound healing area (cm^3)	Bacterial culture		Pain score
				Positive	Negative	
LOT group	29	19±5.9	8.8±3.4	0	28	4.1±1.6
Control group	29	26±3.3*	6.2±2.2*	6*	23*	4.5±1.2

Student *t*-test was conducted for statistical analysis. *, P<0.05. DF, diabetic foot; LOT, local oxygen therapy.

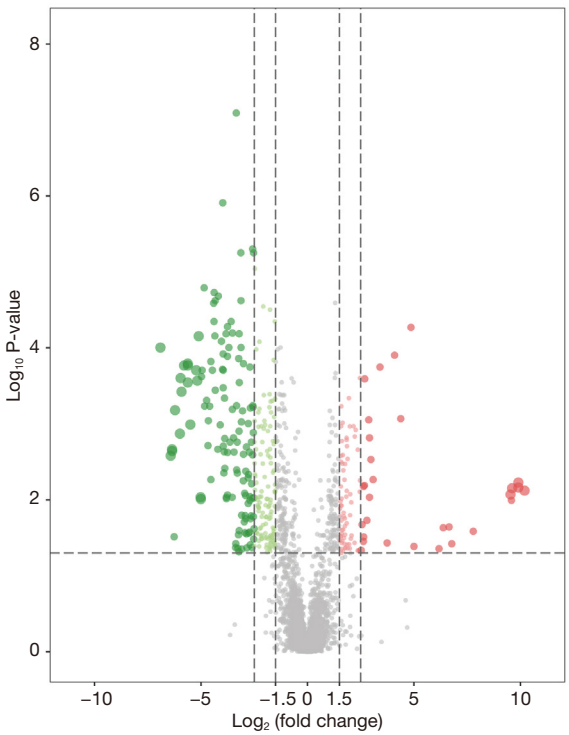

Figure 2 mRNA expression of DF skin samples before and after LOT treatment. We screened the DEGs of the StringTie-assembled and -quantified genes (P<0.05 or Q<0.05), and the differential expression multiple was screened as >1.5. A total 577 DEGs were identified, where green (379 DEGs) represents down-regulated gene expression, while red (198 DEGs) represents up-regulated gene expression. DF, diabetic foot; LOT, local oxygen therapy; DEG, differentially expressed gene.

respectively, and the median (median of fpkm) was 1.03 and 0.89, respectively.

Regarding the large amount of data obtained by RNA-seq, we conducted a screen of the patients' skin genes, where we utilized the coverage value to eliminate numerous genes with low coverage, and considering the depth of transformation sequencing, we eliminated reads with coverage values of <10. Accordingly, we obtained 6,691 (23.38%, before treatment) and 6,054 (21.11%, after treatment) genes, and screened 5,118 co-expressed genes for further analysis. We utilized the ballgown package of R to analyze the DEGs of the StringTie-assembled and -quantified genes (P<0.05 or Q<0.05), and the differential expression multiple was screened as >1.5. Then, we obtained 577 DEGs: 198 were upregulated and 379 were downregulated (*Figure 2*), where *RAB2B*, *SMAD4*, *SON*, *PER1*, *SMAD5* and *GADD45A* were significantly downregulated and *MCM5*, *CHD1*, *RRA*s and *PPP1R3C* were significantly upregulated. The detail of the DEGs was listed in https://cdn.amegroups.cn/static/application/a3245cb70e05a054c5116a58648624e2/apm-20-295-1.pdf.

Biological function analysis

To analyze the biofunctions of the selected DEGs, we identified 570 genes in WebGestalt database, the result of Biological function analysis was listed in https://cdn.amegroups.cn/static/application/4024145a3de92529d4903c275f0a0bb6/apm-20-295-2.pdf. According to the GO enrichment results (see *Figure 3*), the biological process (GO-BP) is mainly focused on the metabolic process, biological regulation, response to stimulus and localization. The main cellular components (GO-CC) include the cell membrane and nucleus. The molecular function (GO-MF) includes protein binding and ion binding (see Figures S1-S3 for further analysis results). The KEGG pathway enrichment results indicated that 10 pathways with FDR of <0.05 participated in the LOT clinical treatment process (*Figure 4*). Eliminating pertussis, legionellosis and Kaposi sarcoma-associated herpesvirus infection, another seven pathways, such as the tumor necrosis factor (TNF) signaling pathway and the apoptosis pathway, were deemed the main pathways in LOT of DF.

Discussion

Patients with DF often have arteriovenous stenosis

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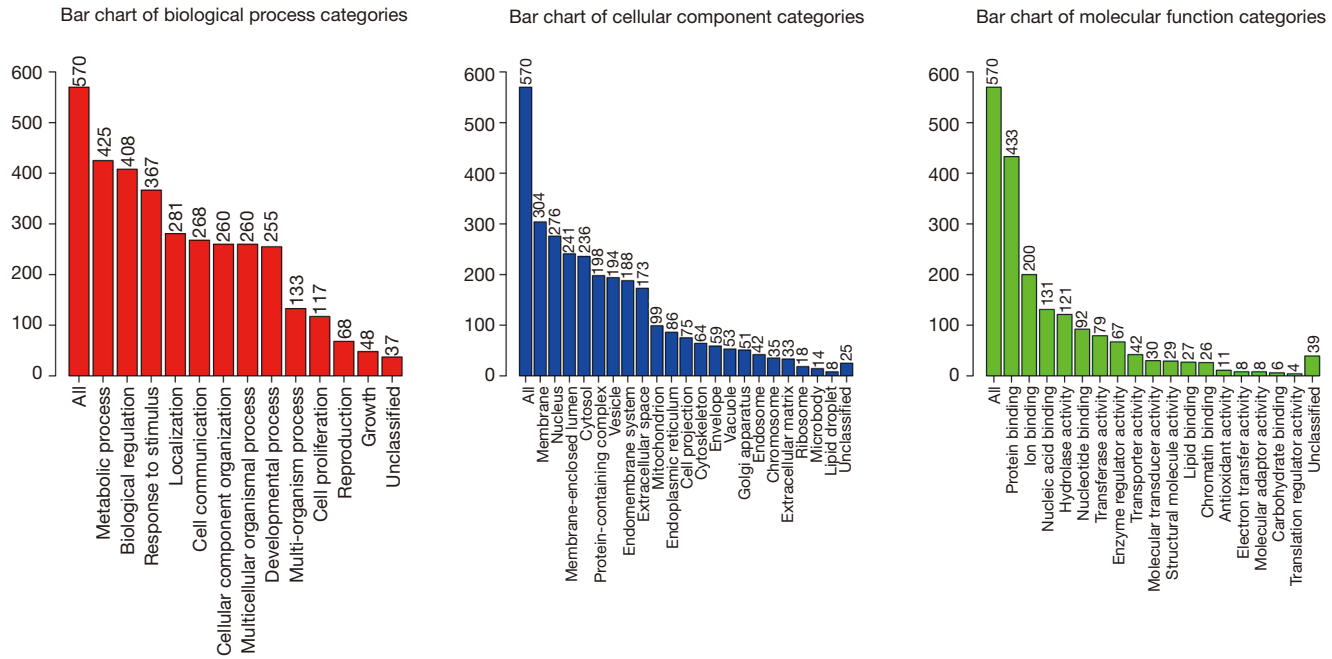

Figure 3 GO enrichment of different expressed genes, and red, blue and green represent the categories of BP, CC and MF, respectively. GO, Genetic Ontology; BP, biological process; CC, cellular component; MF, molecular function.

of the lower limbs, especially the feet and lower legs. Approximately 90% of reported clinical DF cases are of vascular origin. In DF treatment, blood metabolism and other factors lead to the skin at the wound site often being difficult to heal (25,26). Therefore, accelerating wound healing at the DF wound site is the key to improving the prognosis and restoring the physiological function of patients with DF. According to the American Board of Wound Management, the complete wound treatment process should include TIME: debridement (T), anti-infection or inflammation (I), wet balance (M) and edge trimming (E). In addition, the function of oxygen in the clinic has also been widely studied, and the concept of oxygen has been introduced to the TIME principle, as the TIMEO2 principle (27) has been considered a solution to the hypoxic condition of DF wounds. Previous research has described the mechanism of high-purity oxygen in promoting wound healing, which includes the following: (I) with fibroblast migration and replication, collagen assembles and its tensile strength increases (28); (II) angiogenesis and vascular reconstruction are promoted (29); (III) the phagocytic function of inflammatory cells is activated with the respiratory burst process, enhancing the anti-infection ability of the cells (30,31); (IV) the analgesic effect (32,33): as an efficient oxygen treatment compared with the hyperbaric oxygen chamber, LOT is more flexible in operation and has a longer effective treatment time (34). The clinical cases in the present study demonstrate that, compared with traditional therapy, LOT shortens the wound healing time and increases the healing area, improving the clinical prognosis effectively. However, most studies on LOT focus on the clinical characteristics of high-purity oxygen, and limited studies concentrate on its molecular mechanism (35,36). In the present study, we utilized high-throughput sequencing to analyze the mRNA changes in the wound skin of patients with DF before and after LOT and explain the mechanism of LOT via DEG functional enrichment.

We obtained 577 DEGs: 198 were upregulated and 379 were downregulated; the most significantly downregulated genes were *RAB2B*, *SMAD4*, *SON*, *PER1*, *SMAD5* and *GADD45A*, and the most significantly upregulated genes were *MCM5*, *CHD1*, *RRA*s and *PPP1R3C*. SMAD4 and SMAD5 are important signal transduction molecules in the transforming growth factor (TGF) signaling pathway, playing an important role in mediating the occurrence and aggravation of inflammation (37). Similar to SMAD4, PER1 inhibits the upstream targets of a

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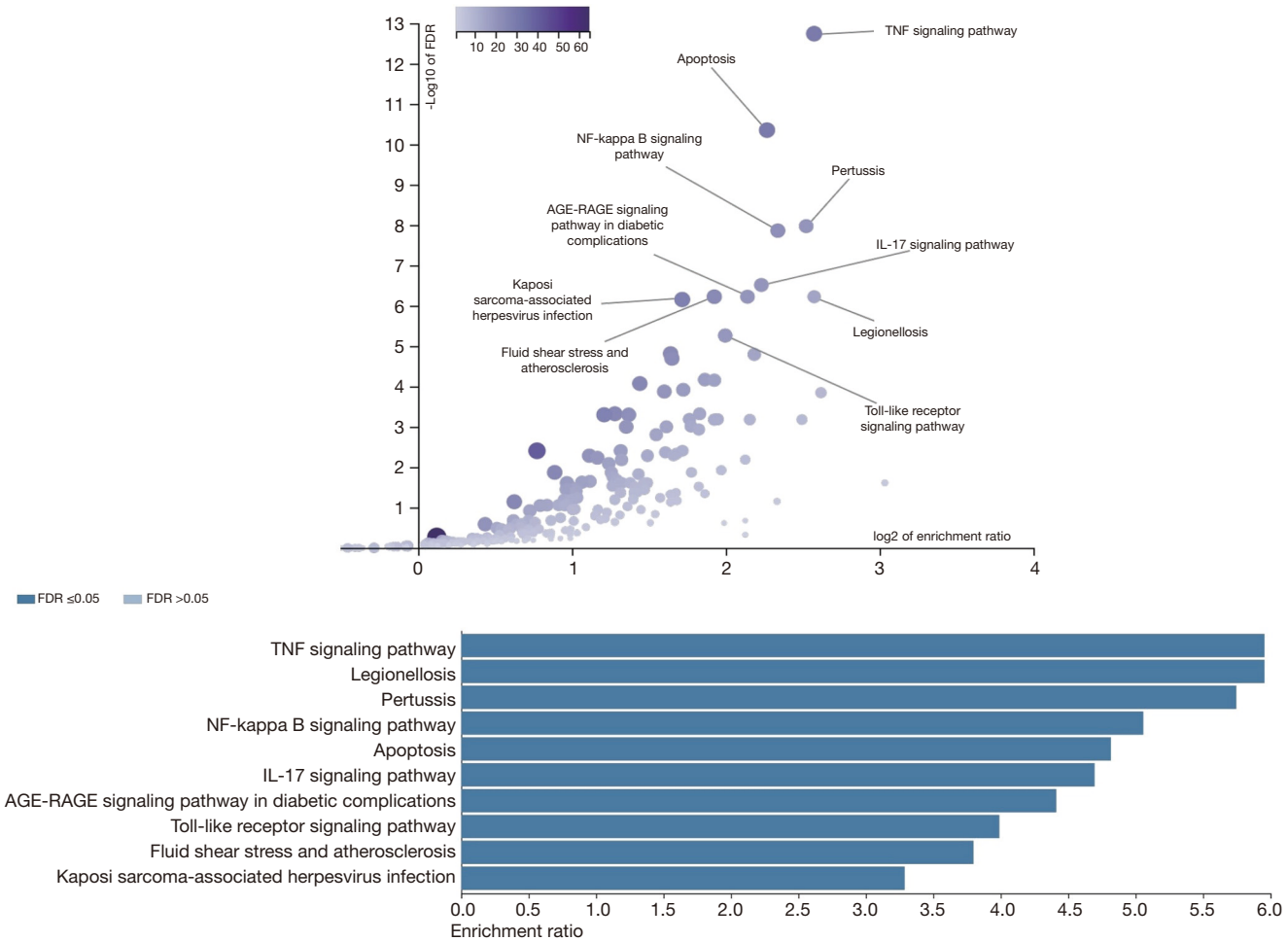

Figure 4 KEGG enrichment of DEGs. The volcano figure above represents the distribution of different pathway. Where the X-axis represents the enrichment rate and Y-axis represents the difference level (FDR value). The bar chart below represents the distribution of DEGs screened in different pathways. KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed gene; FDR, false discovery rate.

series of inflammatory factors. For example, in BV2 cells, deletion of the *PER1* gene blocks the release of TNF-α, interleukin (IL)-6 and other inflammatory factors in culture medium significantly (38). MCM5 is a minichromosome maintenance protein whose main function in cells is to promote cell proliferation (39), while RRAs mainly mediate leucocyte generation. In addition, RRA upregulation is also related to downstream mitogen-activated protein kinase (MAPK) signal transduction (40). Briefly, most of the downregulated genes focus on the inflammatory and apoptosis pathways, while the upregulated genes can stimulate cell proliferation and accelerate wound healing. We may preliminarily speculate that LOT can delay the apoptosis of skin cells in DF wounds and alleviate the occurrence of inflammation.

In LOT, the most influential pathway is the TNF signaling pathway. TNF can antagonize the role of cytokines, such as uncoupling protein 2 (UCP2), and aggravate vascular damage by expressing upstream TNF-α (41). Our results indicate that, with the suppression of the TNF signaling pathway, LOT can downregulate TNF-α, AKT and other key signaling factors. The apoptosis pathway is another important pathway in LOT. According to Rao (42), the accumulation of oxygen free radicals in DF wounds can directly activate the apoptosis process of skin cells, inhibiting the formation and proliferation of wound

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granulation tissue cells. In the present research, LOT inhibited caspase 3 (CASP3) and CASP8 expression in the apoptosis pathway, where CASP3 can mediate downstream apoptosis directly. According to Patel (43), the nuclear factor kappa B (*NFKB*) gene is closely related to the pathogenesis of diabetes and its associated complications, mainly through regulating the oxidative stress response to stimulate the onset of diabetes. IL-17 is an important inflammatory signaling pathway that participates in the pathogenesis of DF (44,45). IL-17 activation can stimulate the expression of inflammatory factors and promote the progression of local inflammation. Considering the inflammatory pathological state of patients with diabetes (46), IL-17 signaling pathway activation would intensify the inflammatory response in DF wounds. Our results show that LOT can reduce the inflammatory response by inhibiting the IL-17 signaling pathway. Therefore, the main mechanisms of LOT for treating DF are limiting the inflammatory response and apoptosis, and blocking a series of oxidative stress reactions. LOT also inhibits the molecular markers of the age-related diabetic syndrome pathway. We may speculate that LOT has the potential to significantly relieve diabetes directly. Moreover, its inhibition of vascular shear force and the atherosclerotic pathway is also interpreted as improvement in the DF microvasculature.

In conclusion, the therapeutic mechanism of LOT in DF is multi-level, multi-channel and holistic. It can accelerate granulation tissue growth and wound healing by regulating the expression of multiple molecular markers and by inhibiting multiple signaling pathways. Compared with the standard treatments, LOT suppressed a series of inflammatory signaling pathways by inhibiting the oxidative stress response. However, deep-level regulation, such as the importance of different pathways in this process, the regulatory relationship between different pathways and the relationship between the expression of key molecular markers and the above pathways, requires confirmation.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/apm-20-295). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics committee of Beijing Shijitan Hospital (No. lc-TNB-20180106) and informed consent was taken from all the patients.

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