Transcriptome Sequencing and Metabolome Analysis Reveal the Mechanism of Shuanghua Baihe Tablet in Treatment of Oral Mucositis

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Research

Keywords: Oral mucositis, Shuanghua Baihe tablet, transcriptomics, metabolomics, IL-17/TNF signal pathway

Posted Date: June 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-656833/v1>

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Version of Record: A version of this preprint was published at Chinese Journal of Natural Medicines on December 1st, 2021. See the published version at [https://doi.org/10.1016/S1875-5364\(22\)60150-X](https://doi.org/10.1016/S1875-5364(22)60150-X).

Abstract

Background: Oral mucositis (OM) caused by chemotherapy is the most common complication in the radiotherapy of head and neck tumors. In severe cases, it can lead to the interruption of treatment, which can affect the control of the disease and the life quality of patients. Shuanghua Baihe tablet (SBT) is a traditional Chinese medicine (TCM) formula, which is administered to treat OM in China. It has been clinically effective for more than 30 years, but its mechanism is still unclear. With the rise of multiple omics, it is possible to study the mechanism of Chinese herbal compound prescriptions.

Methods: Based on transcriptomics and metabolomics, we explored the mechanism of SBT in the treatment of OM. The OM model of rats was established by 5-FU induction, and SBT was orally administered at dosages of 0.75 and 3g/kg/day. In order to search for SBT targets and related metabolites, the dysregulated genes and metabolites were detected by transcriptomics and metabolomics. Immune related indicators such as interleukin-17 (IL-17) and tumor necrosis factor- α (TNF- α) were detected by ELISA. Treg cell disorders was analyzed by flow cytometry.

Results: Our results showed that SBT significantly alleviated the symptoms of OM rats and the inflammatory infiltration of ulcer tissues. After SBT administration, inflammatory related metabolic pathways including linoleic acid metabolism, valine, leucine and isoleucine biosynthesis were significantly altered. Furthermore, the production of proinflammatory factors like IL-17 and TNF- α , was also dramatically reduced after SBT administration. Besides, the infiltration degree of Treg cells in the spleen of OM model rats was significantly improved by SBT administration, thus maintaining the immune balance of the body.

Conclusions: This study shows that SBT regulates inoleic acid metabolism, Glycerophospholipid metabolism and amino acid metabolism, and inhibits IL-17/TNF signal transduction to restore Treg and Th17 cell homeostasis in OM rats, thereby alleviating chemotherapy-induced OM.

1 Introduction

It's necessary for patients with malignant tumors to receive radiotherapy and chemotherapy, but they can cause a series of serious complications, among which oral mucositis (OM) is one of the most common complications. In particular, the incidence of OM in patients with head and neck malignant tumors is relatively high in the process of radiotherapy and chemotherapy, reaching more than 75% [1]. OM caused by radiotherapy or chemotherapy is an inflammatory response to chemotherapy or radiotherapy in tumor patients, which is mainly characterized by thinning and damage of oral mucosa, sometimes accompanied by fibrinoporous pseudomembrane overburden and spontaneous bleeding [2]. OM can seriously affect the life quality of patients, including pain, malnutrition and infection. Especially, the inability to eat and infection can significantly restrict treatment for tumor patients and reduce patients' confidence in treatment [3]. By reason of the foregoing, it is particularly significant to improve the treatment of OM caused by tumor radiotherapy and chemotherapy in clinical practice for improving the life quality of tumor patients.

Traditional Chinese Medicine (TCM) has a history of more than one thousand years in Asia. With the discovery and use of TCM monomers such as artemisinin and curcumin, more and more attention has been paid to the research and application of TCM [4, 5]. Shuanghua Baihe tablet (SBT) is a TCM prescription which has been used for the treatment of OM over 30 years in China, containing 10 kinds of medicinal materials, namely, Coptidis Rhizoma, C. Bungeanae Herba, Isatidis Radix, Arnebiae Radix, Lonicerae Japonicae Flos, Lophatheri Herba, Rehmanniae Radix, Liliibulbus, Asari radix et rhizoma and Snake Bile [6]. SBT has been approved by the Chinese Food and Drug Administration (Approval identifier: No. Z20123033) for the treatment of recurrent OM ulcers. A double-blind, placebo-controlled study also showed that it can reduce the occurrence, latency, and severity of OM in patients with nasopharyngeal carcinoma undergoing radiotherapy and chemotherapy [7]. However, there has been no systematic analysis of SBT's mechanism in the treatment of OM, since TCM usually works *in vivo* through multiple components, multiple pathways and multiple targets. As one of the most significant characteristics of TCM compound in the clinical application, synergy determines the necessity of exploring the mechanism of action of TCM compound from a holistic and systematic perspective [8].

As the high-throughput sequencing technology evolves, it is increasingly common to use omics as a new technology for gathering biological data. The main advantage of multi-omics joint analysis is that these integrated data can be used to conduct analyses aimed at the subtle changes in organisms and lay the reliable data foundation for the prediction of biological function targets from a holistic and systematic perspective [9]. Combined transcriptome and metabolome analysis is the most common research method in the study of TCM mechanism. Transcriptomics can bring a deeper insight into the mechanisms of TCM at the transcriptional and post-transcriptional level. Integrated with transcriptomic data, the results of metabolomic analysis would be more comprehensive and credible [10, 11]. Many studies have shown that combined transcriptome and metabolome analysis is an effective approach to evaluating the efficacy of TCM and confirming the gene expression, metabolites and metabolic pathways that significantly change under the treatment of Chinese medicine [12].

In this study, based on transcriptome and metabonomics, combined with existing molecular biology techniques, we investigated the synergistic effects of the main active components of SBT and explored its potential mechanism. Transcriptome and metabolomics analysis showed that SBT plays a vital role in the treatment of OM by affecting immune-related pathways such as IL-17 signaling pathway, TNF signaling pathway and metabolic pathways like linoleic acid metabolism. Therefore, these results prove the rationality of TCM compounds, and indicate that several weak Chinese medicines can be combined together to exert strong efficacy (Fig. 1).

2 Materials And Methods

2.1 Instrument, Materials and Animal

UHPLC-Q-Orbitrap System: Ultimate 3000 UHPLC (Dionex, USA), Q Exactive high resolution mass spectrometry (Thermo Fisher Scientific, USA). Methanol and acetonitrile (HPLC) (Thermo Fisher Scientific Inc.). Formic acid (HPLC) (Aladdin Industrial Co., Ltd.).

Shuanghua Baihe tablets (Lot No.42070405, 0.6 g/tablet, Yangtze River Pharmaceutical Group Co., Ltd., China) was dissolved in a 0.5% Carboxymethylcellulose sodium (CMC-Na) aqueous solution. Dexamethasone (Zhejiang Xianju Pharmaceutical Co., Ltd., China) were administered at a dose of 1 mg/kg. 5-Fluorouracil $\geq 99\%$ (HPLC) (Sigma-Aldrich) was administered at a dose of 40 mg/kg at a concentration of 50 mg/ml. Ultra-pure water was prepared with the Milli-Q water purification system (Millipore, MA, USA).

Male Sprague-Dawley (SD) rats (6–8 weeks, 180–220 g) were purchased from experimental animal center of Henan province (Zhengzhou, China). All animals were fed under constant standard environmental conditions ($23 \pm 1^\circ\text{C}$, $55\% \pm 5\%$ humidity and a 12/12 h light/dark cycle) for 1 week before the experiment. Ethical approvals for the animal experiments were obtained from the Animal Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Permit No.2020-ky-336, data 11/08/2020).

2.2 OM model

In order to replicate the immunosuppressive effect induced by chemotherapeutics, rats were given 40 mg/kg 5-FU solution on the – 3 and – 1 days of the experiment. The normal control (NC) group was intraperitoneally injected with equal proportion saline (i.p.). On day 0, the rats were anesthetized with 10% chloral hydrate (3 mL/kg, i.p.), and subsequently 0.1 mL of the 10% acetic acid was injected into the left side of the rat's mouth buccal mucosa, the NC group was injected with the same amount saline. The rats injected with 10% acetic acid can form oral ulcers after 24 hours [13, 14]. Eventually, after ten days of the process, all the animals were euthanized with thiopental (80 mg/kg, i.p.) [15] (Fig. 2A).

2.3 Experimental design and sample collection

Thirty SD rats were randomly assigned to normal control (NC) group, model control (MC) group, SBT 0.75, 3g/kg group (S1, S3) (0.75g/kg as clinical equivalent dose), positive drug (D) group (Dexamethasone 1 mg/kg), with 6 rats in each group. The NC group, not subjected to OM, received intragastric administration of 0.5% CMC-Na aqueous solution, once a day, for 10 days; the MC group, subjected to OM by 5-FU and 10% acetic acid, received daily 0.5% CMC-Na aqueous solution (i.g.) for 10 days. The S1, S3 and D group, subjected to OM, were given the corresponding drugs (i.g.) once a day until euthanasia.

About 4ml of blood was drawn from the abdominal artery of rats and divided into two parts. One part was coagulated at room temperature for 1 h, and the serum was separated after centrifugation at 1000g 4°C for 10 min. The other part was stored in a heparinization EP tube, and the plasma was separated after centrifugation at 1000g 4°C for 10 min. The plasma and serum were stored at 80°C until required for experiments. After blood was collected, the rats were dissected and the spleen and thymus of rats were weighted to calculate their indexes. Spleen index = spleen mass / body mass ($\text{mg} \cdot \text{g}^{-1}$); Thymus index = thymus mass / body mass ($\text{mg} \cdot \text{g}^{-1}$). Then, OM tissues of rats were collected and divided into two parts: one was stored in 80°C and the other was immersed in 10% (w/v) neutral buffered formalin solution for histological analysis.

2.4 Biochemistry Tests

2.4.1 Macroscopic and histopathological analysis

The oral mucosae of rats were exposed, photographed, and scored as the following scoring criteria: 0, was normal mucosa, without chyme or vascular dilation; 1, erythema, mucosa without erosion; 2, severe erythema, vascular dilatation and surface erosion; 3, one or more ulcers, the total area of the ulcers less than 25%, severe erythema and vascular dilation; 4, the total number of cheek pouch ulcers was about 50%; 5, the mucosa of cheek pouches is almost completely ulcerated and the mucosa has lost its flexibility [16].

For histological examination, the oral mucosa was embedded in paraffin, and three consecutive 3- μm -thick sections were cut from each specimen. Slides were prepared and stained with hematoxylin and eosin (HE). The evaluation of histopathology also uses the scoring method, which determines the inflammatory cell infiltration, vasodilation, bleeding areas, edema and abscesses in a single blind manner. The scoring criteria were as follows: 0, normal epithelium and connective tissue; 1, Divided into discontinuous areas of vasodilation or reepithelialization, mild inflammation Disease of infiltration; 2, moderate vascular dilatation, epithelial degeneration, and neutral Granulocyte inflammatory infiltrates, showing bleeding areas, edema, and ulcers, but no abscess; 3, severe vasodilation and neutrophilic inflammation embellish [17].

2.4.2 Flow Cytometry

After the rats were dissected, the spleens were removed from the abdominal cavity immediately, which were sufficiently ground and filtered with a 40 μm filter to prepare splenic cell suspension. Red blood cells (RBCs) in the splenic cell suspension were lysed fully with RBC lysis solution. Splenic cell single cell suspension was incubated with FITC anti-rat CD4 and APC anti-rat CD25 (Biolegend Biotechnology Co., Ltd, USA), while PE anti-rat Foxp3 antibody was used as intracellular staining following the manufacturer's instructions (Biolegend Biotechnology Co., Ltd, USA). Then, samples were collected by flow cytometry (ACEA Novocyte3130, USA) according to the instructions, and data analysis were conducted with FlowJO software version 10.0 (Tree Star, Inc. Ashland, OR, USA).

2.4.3 Cytokine quantification

Rat TNF- α enzyme-linked immunosorbent assay kit (MULTISCIENCES BIOTECH, CO., LTD, China) and Rat IL-17 ELISA Kit (Thermo Fisher Scientific CO., LTD, USA) were used to perform cytokine quantification by reading the absorbance at 450 nm. The stored rat serum was taken out of the refrigerator at -80°C and melted on ice. Then according to the operating steps in the Rat TNF- α ELISA Kit, the TNF- α content in the plasma of different groups rats was detected.

2.5 Transcriptome analysis

2.5.1 Total RNA extraction

Total RNA was extracted from the tissues using Trizol (Invitrogen, Carlsbad, CA, USA) in accordance with instruction manual. About 60 mg of tissues were ground into powder after freezing in liquid nitrogen in a 2 mL tube, subsequently homogenized for 2 minutes and rested horizontally for 5 minutes. The mix was centrifuged for 5 minutes at 12,000×g at 4°C, then the supernatant was transferred into a new EP tube containing pre-filled 0.3 mL chloroform / isoamyl alcohol (24:1). The mix was shaken vigorously for 15s, and then centrifuged at 12,000×g for 10 minutes at 4°C. After centrifugation, the upper aqueous phase where RNA remained was transferred into a new tube with equal volume of supernatant of isopropyl alcohol, and then centrifuged at 13,600 rpm for 20 minutes at 4°C. After desorting the supernatant, the RNA pellet was washed twice with 1 mL 75% ethanol, then the mix was centrifuged at 13,600 rpm for 3 minutes at 4°C to collect residual ethanol, followed by the pellet air dry for 5 minutes in biosafety cabinet. Finally, 25 μ L ~ 100 μ L of DEPC-treated water was added to dissolve the RNA. Subsequently, total RNA was qualified and quantified using a Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA).

2.5.2 mRNA Library Construction

Oligo (dT)-attached magnetic beads were utilized to purified mRNA. Purified mRNA was fragmented into small pieces with fragment buffer at appropriate temperature. Then, First-strand cDNA was generated in First Strand reaction system by PCR, and the second-strand cDNA was generated the same way. After the reaction product was purified by magnetic beads, A-Tailing Mix and RNA Index Adapters were added and incubated until the end is repaired. The cDNA fragments with adapters were amplified by PCR, and the products were purified by Ampure XP Beads. The qualified library was amplified on cBot to generate the cluster on the flowcell, and the amplified flowcell was sequenced single end on the HiSeq4000 or HiSeq X-ten platform (BGI-Shenzhen, China).

2.5.3 Data processing and analysis

The sequencing data was filtered with SOAPnuke (v1.5.2) [18] by (1) Removing reads containing sequencing adapter; (2) Removing reads of which the low-quality base ratio (base quality less than or equal to 5) was over 20%; (3) Removing reads whose unknown base ('N' base) ratio was above 5%; afterwards clean reads were obtained and stored in FASTQ format. The clean reads were mapped to the reference genome using HISAT2 (v2.0.4) [19]. Apply Bowtie2 (v2.2.5) (Langmead and Salzberg, 2012) to align the clean reads with the reference coding gene set, and then calculate the gene expression level by RSEM (v1.2.12) [20]. Then, differential expression analysis was performed using the limma voom (v3.36.1) [21] with P value < 0.05 and $|\log_2(\text{fold change})| \geq 1$ for screening the differential expression genes (DEGs) in differential groups. Then, the Gene Ontology (GO), KEGG pathway enrichment analysis and Gene Set Variation Analysis (GSVA) were performed to reveal functional modules and signal pathways of interest.

2.6 Metabolomics Analysis

2.6.1 Sample Preparation

The plasma samples obtained in NC group, MC group and SBT (Six samples with the best therapeutic effect were selected from S1 and S3 groups as the SBT group) group (6 in each group) were taken out of the hypothermia refrigerator (-80°C) and melted on ice. Plasma (50 μ L) was spiked with 150 μ L acetonitrile solution and 10 μ L internal standard (200ng/ml Erlotinib-d6 and CA-d4, dissolved in methanol). After vortexing for 2 min, the mixture was centrifuged at 14,000 rpm at 4°C for 10 min. The 150 μ L supernatant was extracted and volatilized for 2h. Then it was reconstituted with 100 μ L acetonitrile water (1:1), centrifuged at 14,000 rpm at 4°C for 10 min. 90 μ L supernatant was extracted and transferred to automatic sampling bottle for analysis. Pooled plasma was a mix composed of 10 μ L plasma of each sample, which was regarded as the quality control (QC) samples and processed the same way.

2.6.2 Instrument operating conditions

The UHPLC system was utilized to separate metabolites in the plasma, and 5 μ L aliquot from the per sample was injected into a ACQUITY UHPLC® BEH C₁₈ (50×2.1mm, 1.7 μ m) chromatographic column (Waters, USA) preserved at 40°C. Phase A was acetonitrile and phase B was 0.1% formic acid aqueous solution. The gradient elution was as follows at a flow rate of 0.3 mL/min: 0~1.0min, 95% A; 1.0~9.0min, 95~0% A; 9.0~12.0min, 0~0% A; 12.0~12.1min, 0~95% A; 12.1~15.0min, 95% A.

Spray voltage of Electrospray ionization (ESI) source was set to +3.5 kV and -2.8 kV in positive and negative ion mode, respectively. The data were scanned in Full / ddms patterns with a range of 80.00-1200.00 m/z, a first-order MS resolution of 70000, a second-order MS Resolution of 17500. The gradient collision energy was set to 20, 30 and 40 eV. The capillary temperature and the auxiliary gas flow were 320°C and 30 arb. For the sake of the

reliability of the results, we used random sampling method to inject samples, and balance the instrument with 5 QC samples before testing. Then one QC sample was inserted between every 5 samples to evaluate the stability of the apparatus.

2.6.3 Data processing and analysis

The original data from plasma samples were imported into the Compound Discoverer (Version 3.0, Thermo Scientific) for peak detection, calibration, and normalization pretreatment. After the data was preprocessed, principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed on the 2-dimensional data matrix with the SIMCA software (version 14.1, Umetrics AB, Umea, Sweden). Then, the risk of model overfitting was evaluated by a 200-time permutation test. Aimed to further screen the potential markers between different groups, we performed Student's t-test on all detected metabolite peaks and calculated their variable importance in the projection (VIP) and fold change values. VIP > 1 and $P < 0.05$ metabolites are used as potential markers, which are identified by searching the HMDB, MoNA and KEGG databases and comparing their m/z value, retention time and MS/MS spectra using MassFrontier. A heat map was drawn to show the variation trend of the identified key metabolites with the pheatmap package (R version 3.3.0). Finally, the KEGG and MetaboAnalyst database were chosen to uncover the metabolic pathways associated with SBT.

2.7 Statistical Analysis

SPSS16.0 software was utilized to conduct statistical analysis. Student T test was used for comparison between groups. Measurement data were expressed as mean $\pm$ standard deviation ($\bar{X}S$), and $P < 0.05$ indicated statistical significance.

3 Results

3.1 Macroscopic and histopathological analysis

Before intragastric administration, there was no obvious difference in the ulcer area and erosion degree of the rats' oral mucosa in each group. After the experiment, we continued to observe the general morphology of the oral ulcer surface of the rats in each group, and the ulcer mucosa tissue was taken for HE staining. The oral mucosa of rats in NC group was completely healthy and there was no inflammatory cell infiltration; the rats of the MC group showed about 50% oral mucosal ulcers, oral mucosal damage, squamous epithelium breakage and shedding, and the surface covered the necrotic group (Fig. 2B, C). HE staining showed that there was serious inflammatory infiltration of neutrophils, pyknosis, rupture, and deep staining of nuclei; rats in the S1 group showed no signs of mucosal erosion, superficial erosion, mild abscess, and discrete inflammatory cell infiltration. Compared with the MC and S1 groups, the ulcer area in the S3 and D groups was significantly reduced, with mild superficial erosion, discrete inflammatory cell infiltration, and no bleeding, edema or abscess ($P < 0.05$, Fig. 2D, E).

3.2 Effects of SBT on Thymus and Spleen Indexes of OM rats.

The spleen is the largest immune organ of the human body and plays a vital character in regulating immune function of the body. Therefore, we further calculated the spleen index and thymus index of rats. Spleen index = spleen mass / body mass ($\text{mg}\cdot\text{g}^{-1}$); thymus index = thymus mass / body mass ($\text{mg}\cdot\text{g}^{-1}$). Compared with the NC group, the thymus index and spleen index of the rats in the MC group were significantly increased ($P < 0.01$); contrasted with the MC group, the thymus index and spleen index of the rats in the S1 and S3 groups reduced to varying degrees. Among them, contrasted with the NC group, the thymus index and spleen index of rats in the S3 group were lower, and the difference was significant with $P < 0.05$ (Table 1). It indicates that SBT may relieve splenic and thymus hypertrophy, and reduce inflammation stress response.

Table 1 Effects of SBT on spleen index and thymus index of rats with OM ($\bar{X}S$, n = 6).				
	NC	MC	S1	S3
Spleen index	1.97 $\pm$ 0.12	4.89 $\pm$ 0.11**	3.35 $\pm$ 0.44##	2.55 $\pm$ 0.40##
Thymus index	0.95 $\pm$ 0.06	1.97 $\pm$ 0.14**	1.58 $\pm$ 0.11##	1.23 $\pm$ 0.16##
Note: Compare with NC group,* $P < 0.05$,** $P < 0.01$; Compare with MC group,## $P < 0.05$,### $P < 0.01$				

3.3 Transcriptomic analysis reveals alterations in gene expression in OM that are restored by SBT treatment

3.3.1 Transcriptomics alteration in NC, MC and SBT groups

In view of the better treatment results of the SBT high-dose group, we further used its tissue samples for transcriptomics analysis to study the gene expression and pathways changes caused by SBT. Firstly, we compared the gene expression of different samples, which indicated that the distribution of gene expression of different samples was basically the same (Fig. 3A). To identify transcriptome changes in NC, MC and SBT groups, principal component analysis (PCA) was conducted to examine the gene expression relationships among NC, MC and SBT groups. Each replicate from the

same group was clustered closely together, which implied that the repeatability of each treatment met our expectation. MC and SBT groups were clustered far from NC group, which indicated that MC and SBT groups decreased obvious changes in gene expression (Fig. 3B). Then, differential expression analysis between MC and NC group identified 590 significantly dysfunctional genes (Fig. 3C), of which 393 genes were reversed by SBT. The heat map shows the reversed genes with significant differences (Fig. 3D, $P < 0.01$).

3.3.2 Enrichment analysis of reversed genes

To understand the pathophysiological relevance of the reversed genes and the impact of SBT on their expression, we subjected the reversed genes to GO and pathway analysis. Interestingly, biological processes (BP) enrichment analysis carried out by ClueGO showed that the reversed genes mainly participated in processes related to inflammatory and immune response, which play a key role in the occurrence and development of OM (Fig. 3F). KEGG pathway enrichment analysis was performed to illuminate the functions of these reversed genes as well (Fig. 4A). These reversed genes were highly enriched in the following functions and pathways: IL-17 signaling pathway, leukocyte transendothelial migration, glycosphingolipid biosynthesis - lacto and neolacto series, cytokine-cytokine receptor interaction, tight junction, chemokine signaling pathway, TNF signaling pathway, intestinal immune network for IgA production, T cell receptor signaling pathway and cAMP signaling pathway. Considering that GO and KEGG enrichment analyses tend to focus on genes with significant differences and omit some genes with insignificant differential expression but biological significance, GSVA was performed to screen out important differential pathways. The results indicated that ether lipid metabolism, glycine serine and threonine metabolism, alpha linolenic acid metabolism and valine leucine and isoleucine degradation were restored by SBT treatment (Fig. 4B-E). These results confirmed that inflammatory, immune and metabolites pathway may play crucial parts in the treatment of OM with SBT.

3.4 Metabolite expression profile among NC, MC and SBT groups

3.4.1 Target metabolites analysis

We further explored the expression of metabolites for NC, MC and SBT groups with a metabolome method. The data processed by the Compound Discovery software was submitted to the SIMCA software and All samples was performed Principal component analysis (PCA). It is observed that the QC samples stays together in the positive ion and negative ion mode, indicating that the metabolic spectrum analysis method has good stability and repeatability (Fig. 5A, B). Besides, the PCA result showed that there is a certain degree of difference among MC, NC and SBT group. In order to establish the metabolic markers for discrimination between NC and MC, an OPLS-DA model was established, of which the results demonstrated that the separation between NC and MC group presented in the scatter plot of the model was quite evident (Figures S1A, D). After a 200 times' permutation, the resulting R^2Y and Q^2 values of the obtained model ($R^2Y = 1$, $Q^2 = 0.625$, Figure S1B; $R^2Y = 1$, $Q^2 = 0.552$, Figure S3E), showed that the model is stable and reliable, without overfitting. Moreover, the value above 1.0 was considered to be significant in the VIP plot (Figures S1C, F). Then, in order to ensure the statistical significance and the concentration changes status, student's t-test and fold change values were also calculated. Based on the filter of $VIP > 1.0$ and $P < 0.05$, a total of 32 metabolites were identified including cholic acid, linoleic acid, 4-Pyridoxic acid, LysoPC, etc. by referencing to some literatures [22–25] and comparing with several databases (Table 2). A heat map was drawn from 32 differential metabolites presented in NC and MC groups, indicating there is an obvious demarcation line between NC group and MC group (Fig. 5C). Among the identified substances, 18 of them were reversibly regulated by SBT in SBT group and even attained the level in the NC group (Table 2), which were also shown in a heat map (Fig. 5D).

Table 2
Different metabolites identified between NC group vs. MC group and reversed in SBT group.

No.	HMDB ID	KEGG	Formula	Molecular Weight	RT [min]	Name	VIP	MC vs NC	SBT vs MC	ΔPPM
1	HMDB0000673	C01595	C18H32O2	280.23993	8.366	Linoleic acid	1.99151	** (↑)	** (↓)	-0.913
2	HMDB0011756	C02710	C8H15NO3	173.10441	4.685	N-Acetyllecine	1.57028	* (↓)	** (↑)	0.173
3	HMDB0061714	C16533	C22H40O2	336.30306	11.423	Docosadienoate (22:2n6)	1.84682	** (↓)		-3.48
4	HMDB0004667	C14762	C18H32O3	296.23518	9.216	13-HODE	1.65088	* (↓)		-1.72
5	HMDB0000355	C03761	C6H10O5	162.05292	1.065	3-Hydroxymethylglutaric acid	1.78904	* (↑)	** (↓)	-0.368
6	HMDB0010386	C04230	C26H50NO7P	519.3333	9.08	LysoPC(18:2(9Z,12Z)/0:0)	1.91877	** (↑)	** (↓)	-1.145
7	HMDB0000637	C05466	C26H43NO5	449.31397	5.969	Chenodeoxycholic acid glycine conjugate	1.84056	** (↓)	* (↑)	-2.413
8	HMDB0000017	C00847	C8H9NO4	183.05237	1.079	4-Pyridoxic acid	1.79113	* (↑)	* (↓)	0.466
9	HMDB0003464	C01035	C5H11N3O2	145.0853	0.999	4-Guanidinobutanoic acid	1.73135	* (↑)	** (↓)	0.252
10	HMDB0010388	C04230	C26H48NO7P	517.31743	7.818	LysoPC (18:3(9Z,12Z,15Z)/0:0)	1.611	* (↑)		-4.738
11	HMDB0001123	C00108	C7H7NO2	137.04785	0.991	2-Aminobenzoic acid	1.75502	* (↑)	* (↓)	0.398
12	HMDB0005060	C16525	C20H36O2	308.27149	10.767	Eicosadienoic acid	1.71393	* (↓)		-1.436
13	HMDB0000827	C01530	C18H36O2	284.27134	11.372	Stearic acid	1.80805	* (↓)		0.221
14	HMDB0010392	C04230	C28H54NO7P	547.36411	9.075	LysoPC (20:2(11Z,14Z)/0:0)	1.69018	* (↑)	** (↓)	-0.394
15	HMDB0000064	C00300	C4H9N3O2	131.0696	1.193	Creatine	1.57437	* (↓)	* (↑)	0.431
16	HMDB0010383	C04230	C24H48NO7P	493.31692	8.025	LysoPC (16:1(9Z)/0:0)	1.851	** (↑)	** (↓)	-1.947
17	HMDB0002343	C14772	C20H34O4	355.27212	6.905	5,6-DHET	1.71683	* (↓)		-1.335
18	HMDB0000094	C00158	C6H8O7	192.02619	1.229	Citric acid	1.76173	* (↓)		4.615
19	HMDB0010385	C04230	C26H52NO7P	521.3481	8.872	LysoPC (18:1(11Z)/0:0)	1.79277	* (↑)	** (↓)	-0.49
20	HMDB0014842	C07253	C20H23NO4	341.1628	3.965	Naltrexone	1.68317	* (↑)	* (↓)	3.494
21	HMDB0000054	C00486	C33H36N4O6	584.26406	11.004	Bilirubin	1.75945	* (↓)		-3.426
22	HMDB0009213	C00350	C39H70NO7P	695.4872	1.001	PE (18:4(6Z,9Z,12Z,15Z)/P-16:0)	1.87356	* (↑)	* (↓)	4.527
23	HMDB0002725	C07254	C18H26O2	274.19313	7.721	Nandrolone	1.91112	* (↓)		4.259
24	HMDB0061714	C16533	C22H40O2	336.30306	11.423	Docosadienoate (22:2n6)	1.88109	* (↓)		3.852
25	HMDB0029737	C08493	C9H7NO	145.05291	4.482	Indole-3-carboxaldehyde	1.99942	** (↑)		0.969
26	HMDB0010403	C04230	C30H52NO7P	569.34844	8.664	LysoPC (22:5(7Z,10Z,13Z,16Z,19Z)/0:0)	1.7017	* (↑)	** (↓)	2.723

No.	HMDB ID	KEGG	Formula	Molecular Weight	RT [min]	Name	VIP	MC vs NC	SBT vs MC	ΔPPM
27	HMDB0002231	C16526	C20H38O2	310.28713	11.361	11Z-Eicosenoic acid	2.05284	** (↓)		3,567
28	HMDB0001149	C00430	C5H9NO3	131.05839	1.558	5-Aminolevulinic acid	1.82588	* (↓)	** (↑)	-1.458
29	HMDB0010394	C04230	C28H52NO7P	545.34803	8.591	LysoPC (20:3(8Z,11Z,14Z)/0:0)	1.82476	* (↑)	** (↓)	3.063
30	HMDB0000626	C04483	C24H40O4	392.29261	7.718	Deoxycholic acid	1.78683	* (↓)		3.562
31	HMDB0000619	C00695	C24H40O5	408.28758	6.212	Cholic acid	1.85065	* (↓)		0.686
32	HMDB0000687	C00123	C6H13NO2	131.09468	1.095	L-Leucine	1.56564	* (↓)	* (↑)	0.037

3.4.2 Pathway Analysis

Based on the differential metabolites between NC group and MC group, a biology analysis on the metabolic pathways has been conducted, and the results demonstrated that the majority of metabolic disorders induced by OM mainly concluded linoleic acid metabolism, porphyrin and chlorophyll metabolism, biosynthesis of unsaturated fatty acids, glycine, serine and threonine metabolism, arginine and proline metabolism, valine, leucine and isoleucine biosynthesis, vitamin B6 metabolism etc. (Fig. 5E). After the treatment of SBT, 18 disordered metabolites were adjusted in reverse, which mainly participated in linoleic acid metabolism, glycerophospholipid metabolism, valine, leucine and isoleucine biosynthesis and glycine, serine and threonine metabolism (Fig. 5F). These results were in line with the results of transcriptional sequencing enrichment analysis, further proofing that SBT may play an excellent therapeutic character for OM patients by regulating the metabolic pathways.

3.5 Inflammation-related indicators

In accordance with the results of KEGG enrichment analysis, we focus on the IL-17 signaling pathway and the TNF signaling pathway. In this study, the IL-17 signaling pathway and the TNF signaling pathway was sorted out as the interest target for further study on the basis of these following reasons: (1) IL-17 is an important pro-inflammatory cytokine secreted by helper T cells (Th17) and innate immune cells, and plays a key role in a variety of inflammatory responses and the pathological process of autoimmune diseases [26]; (2) TNF-α, mainly secreted by macrophages, is a cytokine involved in systemic inflammation and one of many cytokines responsible for the acute response [27]. We tested the IL-17 and TNF-α content in rats' serum. Compared with the NC group, the serum IL-17 and TNF-α level in the MC group was evidently increased ($P < 0.01$), indicating that the MC group had significant inflammatory symptoms. Compared with the MC group, the IL-17 and TNF-α level in serum of rats in the S1 and S3 groups was evidently reduced ($P < 0.05$, Fig. 6A, B). The results showed that SBT can lower the level of serum TNF-α in OM rats, thereby reducing the inflammatory response. This may be one of the mechanisms of SBT to treat oral ulcers. Given that KEGG analysis results included cytokine-cytokine receptor interaction and T cell receptor signaling pathway, we further tested the proportion of Treg cells with immunosuppressive function in the spleen. Compared with MC group, the proportion of Treg cells in the spleen of rats in groups S1 and S3 significantly increased ($P < 0.01$, Fig. 6C), which further suggested that SBT could maintain immune balance of rats and play an essential character in the recovery of OM rats.

3.6 Biological model integrating transcriptome and metabolome data

Our metabolome data showed that SBT treatment can reverse the disorder of linoleic acid metabolism, glycerophospholipid metabolism, valine, leucine and isoleucine biosynthesis and glycine, serine and threonine metabolism. Besides, the enrichment results of transcriptome sequencing data indicated that glycosphingolipid biosynthesis, ether lipid metabolism, glycine serine and threonine metabolism, alpha linolenic acid metabolism and valine leucine and isoleucine degradation also participated in SBT treatment, which was consistent with metabonomics alterations. Then, with an integrative correlation network of the transcriptome (reversed genes, $P < 0.05$) and metabolome (reversed metabolites, $P < 0.05$), we explored the underlying mechanisms that occur in the progress of SBT treatment on OM. This network revealed three clusters of genes and metabolites, including linoleic acid metabolism, glycerophospholipid metabolism and amino acid metabolism, and several DEGs involved in metabolism (Fig. 7). The reversed genes in the network, such as BCAT2, PLA2G5, SAT1 etc., were closed to the process of metabolism. These comprehensive results validate that both genes and metabolites of linoleic acid metabolism, glycerophospholipid metabolism and amino acid metabolism are crucial factors in the progression of SBT, and that disorders of metabolism are correlated with the occurrence and development of the disease.

4 Discussion

At present, the mechanism of OM is not completely illustrated. The incidence of OM is increasing globally, and more and more patients with malignant tumors suffer from oral mucositis when receiving radiotherapy or chemotherapy [1, 28]. Therefore, improving the prevention and treatment of OM caused by tumor radiotherapy and chemotherapy is clinically significant to prolong the survival time of patients, improving the quality of life of patients, reducing the economic burden of patients and improving the connotation of tumor care [29, 30]. As a TCM formula, Shuanghua Baihe Tablet

has the functions of regulating the body's immunity, anti-inflammatory and analgesic, which can effectively alleviate the symptoms of mucosal inflammation and oral odor. In recent years, a series of studies have proved that SBT has a good effect in the treatment of chemotherapy-induced OM [7]. It not only reduces the pain of patients, but also significantly promotes the healing of congestive erosions. However, due to the complex active ingredients of TCM compound and the unclear target of action, the mechanism of SBT in the treatment of OM has not been elucidated [31]. With the development of multi-omics technology, it is possible to explore the mechanism of Chinese herbal compound prescriptions [32].

In this study, we investigated the synergistic effects of the main active components of SBT and explored its potential mechanism. The comprehensive treatment effect of SBT was observed from ulcer score, histopathology, biochemistry and other indexes, which indicated that SBT has a good therapeutic effect on OM caused by chemotherapy. In order to investigate the mechanism of SBT treatment on OM, we compared the transcriptional profiles of NC and MC groups' tissues with identified dysregulated genes of OM. After the treatment of SBT, several of dysregulated genes were reversed. Gene ontology and pathway enrichment analysis indicated that these reversed genes had notable correlation with immune response, cytokine – cytokine receptor interaction, leukocyte transendothelial migration, IL – 17 signaling pathway, TNF signaling pathway and biological metabolic process. With the treatment of SBT on OM, the immune and inflammation process of cells was also severely affected, thus leading to IL – 17, TNF- α and Treg cells disturbances. The results were verified by Flow Cytometry and Cytokine quantification. Previous studies have shown that IL – 17, TNF- α and Treg cells are closely related to the progression of OM, whose proportion determine the recovery rate and recurrent rate of OM [33–35]. Besides, some disordered metabolic processes contribute to prenaternal accumulation of many important metabolites severely affecting cellular behavior.

From the perspective of central dogma, expression genes are upstream products of genetic information while expression metabolites are downstream ones. Therefore, gene expression changes can naturally cause the alteration of metabolites in tumor progression. Transcriptomics analysis verified that many pivotal cellular metabolic pathways are restored in the treatment of SBT on OM. Here, the metabolic profile of linoleic acid metabolism, glycerophospholipid metabolism, valine, leucine and isoleucine biosynthesis and glycine, serine and threonine metabolism pathway were further studied through metabolomics to determine metabolic biomarkers and establish biological model of the treatment of SBT on OM. It's underscored from our data that the metabolites involving Linoleic acid metabolism, Glycerophospholipid metabolism, Valine, leucine and isoleucine biosynthesis and Glycine, serine and threonine metabolism pathway play indispensable parts in the treatment of SBT on OM. Some essential compounds of these pathways, i.e. LysoPC (18:1(11Z)/0:0), Linoleic acid, 4-Guanidinobutanoic acid, 5-aminolevulinic acid, PE (18:4(6Z,9Z,12Z,15Z)/P-16:0), creatine and L-leucine, were regarded as the significant targets. Linoleic acid is the most highly consumed PUFA found in the human diet, whose content is close to the serum inflammatory factors [36]. Previous study also showed that Glycerophospholipid metabolism, Valine, leucine and isoleucine biosynthesis and Glycine, serine and threonine metabolism pathway can affect the body's immune system and ability to deal with inflammation [37, 38]. These results confirm that metabolomics can explore metabolic profile of metabolic pathway, verifying transcriptional variability, and thus help biologists comprehend the mechanism of the treatment of SBT on OM.

In conclusion, this study is the first to research the therapeutic effect of SBT on OM rat model by combining transcriptomics and metabolomics. The results showed that SBT can improve the inflammatory symptoms of oral mucosa, and the mechanism may be related to its anti-inflammatory function, maintaining immune balance of the body and repairing the metabolic pathways of many disorders. Especially, immune-related pathways like IL-17 signaling pathway, TNF signaling pathway and metabolic-related pathways such as Linoleic acid metabolism play essential roles in the SBT treatment on OM.

Abbreviations

Oral mucositis, OM; Shuanghua Baihe tablet, SBT; traditional Chinese medicine, TCM; interleukin-17, IL-17; tumor necrosis factor- α , TNF- α ; Carboxymethylcellulose sodium, CMC-Na; normal control, NC; model control, MC; hematoxylin and eosin, HE; real-time quantitative PCR, RT-qPCR; Gene Ontology, GO; Gene Set Variation Analysis, GSVA; Electrospray ionization, ESI; variable importance in the projection, VIP; Principal component analysis, PCA

Declarations

Acknowledgments

We thank the reviewers for their insightful comments.

Authors' contributions

Q. G. is responsible for the acquisition of the data, analysis and interpretation of the data, drafting of the manuscript, and statistical analysis. R. L., W. X. and J. Z. participated in experimental design. Others contributed to the critical revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding

This study was supported by the Clinical Research Special Fund of Wu Jieping Medical Foundation (320.6750.2020-04-9). This work was supported by the National Key Research and Development Program of China (Grant No. 2017YFC0909900). The National Natural Science Foundation of China

(Grant No. 82002433), and Science and Technology Project of Henan Provincial Department of Education (Grant No. 18A320044 and 21A320036), Henan Province Medical Science and Technology Research Project Joint Construction Project (Grant No. LHGJ20190003, LHGJ20190055).

Availability of data and materials

Processed data is contained within the article. Raw data is available from the corresponding author upon request.

Ethics approval and consent to participate

Ethical approvals for the animal experiments were obtained from the Animal Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Permit No.2020-ky-336, data 11/08/2020).

Consent for publication

Not applicable.

Competing of interest

The authors declare no conflict of interest.

Supplementary material

Figure S1: The OPLS-DA model of the different groups. The OPLS-DA plot of the different groups in positive ion mode (A) and negative ion mode (D); the permutations test of the MC vs. NC group in positive ion mode (B) and negative ion mode (E); the VIP plot of the MC vs. NC group in positive ion mode (C) and negative ion mode (F), Table S1: Different genes identified between NC group vs. MC group and reversed in SBT group.

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Figures

Figure 1

The flow diagram of this study.

Figure 2

Protective effect of SBT against OM rats. (A) OM rat model establishment and administration program; SBT improved the macroscopic analysis (B) and scores (C) of the OM rats; Histopathological analysis (D) and scores (E) of the OM rats. (n=8, *P<0.05, **P<0.01).

Figure 3

Transcriptomics analysis in NC, MC and SBT groups. (A) the distribution of gene expression in different samples; (B) The PCA plot of the different groups; (C) The volcano plot of the MC vs. NC group; (D) The heat map of the reversed genes with significant differences; (E) the biological processes enrichment analysis of the reversed genes.

Figure 4

The pathway enrichment analysis. (A) The significant KEGG pathways of the reversed genes. (B-E) The important differential metabolomics pathways were screen out by gene-set variation analysis (GSVA). $P < 0.05$

Figure 5

Metabolomics analysis in NC, MC and SBT groups. (A) The PCA plot of the different groups in positive ion mode, (B) The PCA plot of the different groups in negative ion mode; (C) The heat map of the disordered metabolites in NC vs. MC group, (D) The heat map of the metabolites adjusted by SBT in NC, MC, and SBT group; (E) Pathway enrichment analysis related to the differential metabolites between NC and MC group; (F) The pathway analysis of the reversed metabolites.

Figure 6

The biochemistry analysis in NC, MC and SBT groups. The concentration level of IL-17(A) and TNF-α(B) in different groups; (C) the proportion of Treg cells in the spleen of different groups. (*P<0.05, **P<0.01)

Figure 7

Metabolite pathways, metabolites and genes perturbations in NC, MC and SBT groups. (The histogram indicated the content of metabolites in different groups; The point shown the Log2FC value log2FC values for the difference analysis of different groups)

Supplementary Files

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