Reduning Injection prevents carrageenan-induced inflammation in rats by serum and urine metabolomics analysis

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Original Article

1. Introduction

Reduning Injection (RDN), a patented traditional Chinese medicine, is prepared from three commonly used traditional Chinese medicines, involving Artemisiae Annuae Herba (Artemisia carvifolia Buch.-Ham. ex R. Br.), Lonicerae Japonicae Flos (Lonicera japonica Thunb.) and Gardeniae Fructus (Gardenia jasminoides Ellis), and is administered by intravenous drip. Clinically, it effectively treats cold, cough, upper respiratory infection, acute bronchitis (Jiang et al., 2019; Xiong et al., 2018) and viral infection (Shahen et al., 2018; Tang et al., 2014). The modern pharmacological studies showed that it has anti-inflammatory and antipyretic effect. However, its previous drug efficacy research mainly focused on clinical evaluation or animal efficacy experiment (Chang et al., 2015; Gao et al., 2020; Zhang et al., 2013), the holistic anti-inflammatory mechanism of RDN has not been elucidated from a metabolic perspective yet.

Metabolomics can reflect the holistic functional state of living systems, and provide insights into the global metabolite profiles influenced by pathological stimuli or drug treatments in biological samples such as urine, plasma or tissue (Fan et al., 2016; Gika, Zisi, 2019). The objective of this study is to elucidate the anti-inflammatory mechanism of Reduning Injection (RDN) by analyzing the potential biomarkers and metabolic pathways of the carrageenan-induced inflammatory model from the overall metabolic level. UPLC-Q-TOF/MS was used to detect and analyze changes of endogenous metabolites in the serum and urine of carrageenan-induced inflammatory rats. Combined with multivariate analysis and databases analysis, inflammatory-related potential biomarkers were screened and identified to analyze possible metabolic pathways. The reliability and biological significance of these biomarkers was verified by metabolic network analysis and correlation analysis with pharmacodynamic indicators. The results showed that RDN has a good regulation of the metabolic disorder of endogenous components in carrageenan-induced inflammatory rats. And its anti-inflammatory mechanism is mainly related to the regulation of amino acid and lipid metabolism. This research method is conducive to the interpretation of the overall pharmacological mechanism of Chinese medicine.

Objective: To elucidate the anti-inflammatory mechanism of Reduning Injection (RDN) by analyzing the potential biomarkers and metabolic pathways of the carrageenan-induced inflammatory model from the overall metabolic level.

Methods: Rat inflammatory model was established by carrageenan. UPLC-Q-TOF/MS was used to detect and analyze changes of endogenous metabolites in the serum and urine of carrageenan-induced inflammatory rats. Combined with multivariate analysis and databases analysis, inflammatory-related potential biomarkers were screened and identified to analyze possible metabolic pathways. The reliability and biological significance of these biomarkers was verified by metabolic network analysis and correlation analysis with pharmacodynamic indicators.

Results: A total of 16 potential biomarkers were screened and identified by multivariate analysis and metabolite databases, among which 13 species could be adjusted by RDN. The metabolism pathway analysis revealed that histidine metabolism, sphingolipid metabolism, and tyrosine metabolism were greatly disturbed. Their biomarkers involved urocanic acid, sphingosine, and norepinephrine, all of which showed a callback trend after RDN treatment. The three biomarkers had a certain correlation with some known inflammatory-related small molecules (histamine, arachidonic acid, Leukotriene B4, and PGE2) and pharmacodynamic indicators (IL-6, IL-1β, PGE2 and TNF-α), which indicated that the selected biomarkers had certain reliability and biological significance.

Conclusion: RDN has a good regulation of the metabolic disorder of endogenous components in carrageenan-induced inflammatory rats. And its anti-inflammatory mechanism is mainly related to the regulation of amino acid and lipid metabolism. This research method is conducive to the interpretation of the overall pharmacological mechanism of Chinese medicine.

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Inflammation is a complex active defensive response induced by external substances in the body's evolutionary process. Local or systemic inflammatory responses will occur when the body is invaded by substances such as bacterial infection, entry of foreign particles and metastasis of malignant tumors. At present, lambda-carrageenan is widely used to induce the acute, strong, and sustained local inflammatory model by injection in rats, ruminants, horses, and swine. It can cause localized edema and infiltration of white blood cells, increase the levels of local prostaglandin E2 (PGE2), and stimulate the secretion and release of some cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Myers, Deaver, & Lewandowski, 2019). IL-1β, interleukin-6 (IL-6) and TNF-α are important inflammatory cytokines, which play important roles in regulating and mediating inflammation (Cavalcanti et al., 2020; Hu et al., 2017; Lopez-Castejon & Brough, 2011). PGE2 produced by the metabolism of arachidonic acid can dilate arterioles, increase histamine, and increase the permeability of blood vessel walls, thereby promoting inflammatory exudation, mediating local inflammatory responses, and causing edema. Therefore, inhibiting its synthesis may be one of the ways to reduce inflammation. (Kakavandi et al., 2020).

In this study, a rat inflammatory model was established by carrageenan. Inflammatory factor levels were applied to evaluate model establishment and drug efficacy. Serum and urine metabolomics based on UPLC-Q-TOF/MS was used to study the potential biomarkers, and disordered metabolic pathways of this model and explain the mechanism of RDN in the treatment of inflammatory.

2. Materials and methods

2.1. Materials and reagents

Redunying Injection (Lot number: 170522, 2.6 g crude drug per milliliter) was manufactured by Jiangsu Kanion Pharmaceutical Co., Ltd. (Jiangsu, China). Dexamethasone sodium phosphate injection (DMSP; Lot number: 51703211, 5 mg/ml) was bought from Hubei Tianyao Pharmaceutical Co., Ltd. (Xiangyang, China). Carrageenan was purchased from Liaoning Provincial Institute for Drug Control (Shenyang, China). 2-Chloro-4-phenylalanine was obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). Formic acid, acetonitrile, and methanol of MS grade were acquired from Merck (Darmstadt, Germany). Ultrapure water was purified by a Milli-Q system (Milford, MA, USA). Rat IL-6, IL-1β, PGE2, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Animals and inflammation model processes

Male Sprague-Dawley rats, weighing 180–220 g, were bought from Shanghai Jiesijie Lab. Animal Co., Ltd. (Shanghai, China), and the animal production license number was SCXK2013-0006. All rats were housed in an appropriate condition with constant temperature [(23 ± 2 °C)], relative humidity (60 ± 5)%, and 12 h light/dark cycle. After one week of adaptation, forty healthy rats were divided randomly into four groups, including the normal control group (NC-G), model control group (MC-G), RDN group (RDN-G) and positive drug group (PD-G). According to the clinical usage and dosage of RDN (intravenous drip, adult dose 20 mL each time, once a day), the RDN-G rats were intravenously injected with 6 mL/kg RDN (three times the human clinical equivalent dose) via tail vein once a day for three consecutive days. Correspondingly, the PD-G rats were intravenously injected with 0.4 mL/kg DMSP, the NC-G and MC-G rats were injected with 0.9 % saline, respectively. Fifteen minutes after the last administration, the rats’ right hind toe of the MC-G, RDN-G and PD-G were injected subcutaneously with 0.1 ml of 1 % carrageenan suspension to induce an inflammation model. An equal volume of 0.9 % saline was used for the NC-G rats. The volume below the toe joints of all rats was measured by the YLS-7B Toe Volume Measuring Instrument (Jinan Yiyuan Technology Development Co., Ltd., Jinan, China) before and after modeling. And the average swelling degree and inhibition rate of each group at 2 and 4 h after modeling was calculated and compared. When the inhibition rate is > 30 %, and there is a statistical difference in the swelling degree, it indicates that the dose has an anti-inflammatory effect (Wang et al., 2013). Toe swelling (mL) = volume below the toe joint after inflammation – volume below the toe joint before inflammation. Inhibition rate (%) = (average toe swelling of model group – average toe swelling of administration group) / average toe swelling of model group.

The research was conducted strictly in accordance with the recommendations in the guide for laboratory animal use and care of the National Institutes of Health (NIH Publication No. 85–23, revised in 1985). The animal experiments were performed under the rules and systems of the Experimental Animal Ethics Committee of Jiangsu Kanion Pharmaceutical (IACUC No. 2018052805).

2.3. Samples collection and preparation

2.3.1. Serum samples

After modeling for 4 h, the rats’ blood was collected through the orbit, stood quietly for 30 min at 4 °C, and centrifuged at 3 000 rpm for 15 min. The supernatant was divided into two parts for metabolite and pharmacological analysis and stored at −80 °C until analysis.

Before UPLC-Q-TOF/MS detection, 100 μL serum was added to 600 μL methanol (with 10 μg/mL 2-chloro-4-phenylalanine) to precipitate proteins. The samples were vortexed for 1 min with a XW-80A Micro Vortex Mixer (Huxi Analytical Instrument Factory, Shanghai, China) and centrifuged at 14 000 rpm for 10 min at 4 °C using a 5424 High-Speed Centrifuge (Eppendorf, Germany).

2.3.2. Urine samples

During 0–4 h after modeling, the rats’ urine was collected, centrifuged at 3 000 rpm for 5 min at 4 °C. And the supernatant was divided into two parts for metabolite and pharmacological analysis and stored at −80 °C until analysis.

Before UPLC-Q-TOF/MS detection, 100 μL urine was collected, centrifuged at 3 000 rpm for 5 min at 4 °C, stored at −80 °C. A total of 200 μL urine was accurately aspirated and added to 600 μL methanol containing 10 μg/mL 2-chloro-4-phenylalanine. Then it was vortexed and centrifuged at 14 000 rpm for 10 min at 4 °C. The supernatant was transferred and evaporated to dryness under nitrogen. After drying, 400 μL methanol was added, vortexed for 2 min, and centrifuged at 14 000 rpm for 10 min. Then the supernatant was again collected and evaporated to dryness. A total of 200 μL methanol was added, vortexed, and centrifuged at 14 000 rpm for 10 min. Finally, the supernatant was taken for UPLC-Q-TOF/MS analysis.

2.3.3. Quality control sample

To evaluate the stability and reproducibility of UPLC-Q-TOF/MS, 10 μL of every urine/serum sample was extracted and mixed to
generate a pooled quality control (QC) sample. Then QC samples of serum and urine were also prepared by the above protocol. Before the serum/urine samples were detected, the QC samples were injected five times to balance the analysis system. Then they were run after every ten samples for quality control of the chromatography (Zhu et al., 2018).

2.4. Pharmacodynamic assay

IL-6, IL-1β, PGE2, and TNF-α in serum was measured with commercially ELISA kits.

2.5. UPLC-Q-TOF/MS analyses

The metabolomics analysis of serum and urine samples was performed by An Agilent 1290 LC system equipped with a quaternary pump, an online degasser, a temperature-controlled autosampler and a thermostatic column compartment set (Agilent Technologies Inc., USA). An Agilent ZORBAX Eclipse Plus-C18 column (2.1 mm × 100 mm, 1.8 μm) was used to separate the analytes. The column was maintained at 30 °C with a flow rate of 0.4 mL/min. The injection volume was 1 μL. The mobile phases of serum and urine samples were water-0.1 % formic acid (A) and acetonitrile (B). The gradient system of serum samples was 2% B at 0–1 min, 2%–50% B at 1–3 min, 50%–60% B at 3–14 min, 60%–90% B at 14–18 min, 90%–100% B at 18–19 min, 100% B at 19–20 min, 100%–2% B at 20–22 min. The gradient program of urine samples was as follows: 2%–10% B at 0–5 min, 10%–20% B at 5–11 min, 20% B at 11–17 min, 20%–50% B at 17–22 min, 50%–100% B at 22–23 min, 100% B 23–24 min, 100%–2% B at 24–25 min.

Mass spectrometry detection was performed using an Agilent 6538 Q-TOF Mass (Agilent Technologies Inc., USA) in positive electrospray ionization source (ESI) mode. The conditions of source parameters were as follows: drying gas temperature, 350 °C; drying gas (N2) flow rate, 10.0 L/min; skimmer, 65 V; capillary, 4000 V; OCT1 RF Vpp, 750 V; fragmentor voltage, 100 V; nebulizer, 40 psig; acquisition rate, 1.5 spectrum/s. MS data were collected from m/z 100 to 1,000, and MS² data were collected from m/z 25 to 1 000.

2.6. Data analysis

Raw mass data from the UPLC-Q-TOF/MS system were converted into .cef format by MassHunter Profinder software (Agilent Technologies Inc., USA), then imported to Agilent Mass Profiler Professional software (MPP, Agilent Technologies Inc., USA) for further processing. Parameters including Experiment type (identified and unidentified), Organism (Rattus norvegicus), Minimum absolute abundance (3 000 counts), Baseline option (Baseline to median of all samples) were set. Principal component analysis (PCA), and partial least-squares-discriminant analysis (PLS-DA) were employed for multivariate data analysis. In general, the model is effective when $R^2_Y > 0.4$ and $Q^2 < 0.3$. Metabolites were identified by HMDB (https://www.hmdb.ca/), METLIN (https://metlin.scripps.edu/) and KEGG (https://www.kegg.com). Pathway analysis were performed with Metabo Analyst (https://www.metabolanaly.st.ca/). One-way analysis of variance (ANOVA) was used to compare multiple groups, and independent-sample t-test was applied to compare two groups by SPSS 20.0 software (SPSS Inc., Chicago, USA). $P < 0.05$ is significant difference, and $P < 0.01$ means extremely significant difference.

3. Results and discussion

3.1. Anti-inflammatory effect of RDN

Before and after model establishment, the rats’ toe swelling in each group were recorded at different time. As shown in Fig. 1, the rats’ toe swelling in MC-G was significantly increased at 2 h and 4 h after modeling ($P < 0.01$, vs NC-G), which showed that the carrageenan-induced inflammatory model was successful. After modeling for 4 h, the rats’ toe swelling markedly decreased from $(0.671 \pm 0.160)$ to $(0.345 \pm 0.273)$ ($P < 0.01$, vs MC-G) in RDN-G, and decreased to $(0.189 \pm 0.101)$ ($P < 0.01$, vs MG) in PD-G. The inhibition rate of RDN-G was 48.53%, which indicated that RDN had significant anti-inflammatory efficacy on carrageenan-induced inflammatory rats.

The concentrations of IL-6, IL-1β, PGE2, and TNF-α in each group were shown in Fig. 2. Compared with NC-G, the levels of IL-6, IL-1β, PGE2, and TNF-α were all increased in MC-G, among which IL-1β and TNF-α had statistical significance ($P < 0.05$), indicating the success of modeling from another perspective. The levels of IL-6, IL-1β, PGE2, and TNF-α were all reduced after administration of RDN and DMSO, which indicated that RDN had callback effects on the inflammatory factors in model rats. All of these results suggested a definite anti-inflammatory effect for RDN.

3.2. UPLC-Q-TOF/MS analysis and validation

UPLC-Q-TOF/MS was used to analyze the serum and urine samples, and data acquisition was performed in positive ion mode. The total ion chromatograms (TICs) of the serum and urine samples...
from NC-G, MC-G, RDN-G and PD-G were shown in the Fig. 3. The results of QC samples were shown in the Table 1 and Table 2.

For the metabolomics analysis, ten characteristic peaks and internal standard (IS) in serum/urine were selected as the quality control object of the obtained data, whose polarity and mass spectral response were highly different. The relative standard deviations (RSDs) of their peak areas were within ±15% in serum QC samples and urine QC samples, indicating that the UPLC-Q-TOF/MS system was stable. The obtained data of serum and urine samples were reliable and effective to further metabolomics analysis.

3.3. Multivariate statistical analysis

In the PCA and PLS-DA score plots of the serum samples (Fig. 4), the MC-G was completely distinguished from the NC-G, and four different groups also had a good separation. These results indicated that the endogenous metabolites’ content in the MC-G rats’ serum had deviated from the normal state; RDN and DMSP had certain anti-inflammatory efficacy. Relevant parameters of the PLS-DA model were as follows: \( R_{X}^{2} = 0.836, R_{Y}^{2} = 0.834, Q_{Y}^{2} = 0.788 \), illustrating the model had good stability and predictability.

In the PCA and PLS-DA score plots of the urine samples (Fig. 5), it was found that the NC-G and MC-G had a good separation on the first principal component, and the RDN-G and PD-G had a tendency to the NC-G. The PD-G partially overlapped the NC-G, indicating that the level of endogenous metabolites in the rats’ urine of PD-G was very close to that of NC-G. These results showed the inflammation model rats tended to normal after drug treatment, which was consistent with the serum. Relevant parameters of the PLS-DA model of the four groups were as follows: \( R_{X}^{2} = 0.878, 

Fig. 2. Difference of pharmacodynamics indexes, interleukin-6 (A), interleukin-1β (B), prostaglandin E2 (C), and tumor necrosis factor-α (D), in the normal control group (NC-G), model control group (MC-G), Reduning injection group (RDN-G) and positive drug group (PD-G). #P < 0.05 vs NC-G; *P < 0.05, **P < 0.01 vs MC-G.

Fig. 3. Typical total ion chromatograms (TICs) of serum samples (A) and urine samples (B) in positive ion mode. a: normal control group (NC-G), b: model control group (MC-G), c: Reduning injection group (RDN-G), d: positive drug group (PD-G).
Table 1

| No. | m/z | Before sample analysis | During sample analysis |
|-----|-----|------------------------|------------------------|
|     |     | Peak area | RSD (%) | Peak area | RSD (%) |
| 1   | 182.08 | 184 909 ± 3 562 | 1.93 | 175 272 ± 4 971 | 2.84 |
| 2   | 166.08 | 568 254 ± 9 527 | 1.68 | 571 831 ± 13 508 | 2.36 |
| 3   | 200.04 | 805 122 ± 17 602 | 2.19 | 796 789 ± 10 119 | 1.27 |
| 4   | 205.09 | 605 989 ± 12 954 | 2.14 | 600 783 ± 9 325 | 1.55 |
| 5   | 163.13 | 30 564 ± 2 206 | 7.22 | 30 460 ± 777 | 2.55 |
| 6   | 387.17 | 496 644 ± 10 298 | 2.07 | 398 929 ± 50 730 | 12.72 |
| 7   | 376.26 | 26 658 ± 2 215 | 8.31 | 25 271 ± 1 212 | 4.80 |
| 8   | 496.34 | 7 300 587 ± 397 469 | 5.44 | 7 366 617 ± 341 027 | 4.63 |
| 9   | 303.23 | 14 009 ± 1 162 | 8.30 | 13 424 ± 1 006 | 7.50 |
| 10  | 524.37 | 5 224 835 ± 237 717 | 4.55 | 5 385 825 ± 288 907 | 5.36 |
| 11  | 305.24 | 88 216 ± 5 644 | 6.40 | 84 338 ± 8 290 | 9.83 |

Note: IS, internal standard; SD, standard deviation; RSDs, relative standard deviations.

Table 2

| No. | m/z | Before sample analysis | During sample analysis |
|-----|-----|------------------------|------------------------|
|     |     | Peak area | RSD (%) | Peak area | RSD (%) |
| 1   | 132.07 | 883090 ± 12181 | 1.38 | 863582 ± 11155 | 1.29 |
| 2   | 166.08 | 357604 ± 4436 | 1.24 | 369138 ± 22293 | 6.04 |
| 3   | 200.04 | 1982890 ± 31303 | 1.58 | 2032243 ± 117062 | 5.76 |
| 4   | 180.06 | 6328110 ± 87581 | 1.38 | 5994004 ± 673457 | 11.24 |
| 5   | 209.06 | 50748 ± 4158 | 8.19 | 50490 ± 4459 | 8.83 |
| 6   | 220.13 | 46809 ± 2784 | 5.95 | 44492 ± 5540 | 12.45 |
| 7   | 443.27 | 19237 ± 597 | 3.10 | 20383 ± 1179 | 5.78 |
| 8   | 465.28 | 20950 ± 1041 | 4.97 | 20316 ± 1050 | 5.17 |
| 9   | 410.18 | 15259 ± 993 | 6.51 | 15336 ± 722 | 4.71 |
| 10  | 328.14 | 185610 ± 18501 | 9.97 | 189485 ± 14726 | 7.77 |
| 11  | 387.18 | 433722 ± 9502 | 2.19 | 372831 ± 41873 | 11.23 |

Note: IS, internal standard; SD, standard deviation; RSDs, relative standard deviations.

Fig. 4. PCA score plots (A, B) and PLS-DA score plots (C, D) of serum samples from normal control group (NC-G), model control group (MC-G), RDN group (RDN-G) and positive drug group (PD-G) at 4 h after modeling.
$R^2_Y = 0.953$, $Q^2_Y = 0.923$, indicating the PLS-DA model had good stability and predictability.

### 3.4. Identification of potential biomarkers

Based on the criteria of $P < 0.05$ and fold change (FC) $\geq 2$, the primary and secondary mass spectra information, Metabolite Databases (METLIN, HMDB and KEGG), a total of 16 metabolites in serum and urine samples were selected and identified as the potential biomarkers of inflamed rats. Their detailed information was shown in Table 3. Compared with NC-G, 13 of them increased significantly, and three of them decreased significantly in MC-G. RDN could correct 13 of them, and positive drugs could call back 14 of them. There were 12 biomarkers regulated by the two drugs. The results implied that RDN could regulate the metabolic disorder of inflamed rats to a certain degree.

### 3.5. Metabolic pathway analysis of potential biomarkers

MetaboAnalyst website was used to analyze the metabolic pathways of 16 potential biomarkers. The disturbed metabolic pathways in inflammatory rats were histidine metabolism, sphingolipid metabolism, tyrosine metabolism, and vitamin B6 metabolism (Fig. 6), which involved four biomarkers such as urocanic acid, sphingosine, norepinephrine, pyridoxine (Table 4). According to the analysis, both RDN and DMSP could regulate the above four metabolic pathways.

| No. | Metabolites                                      | Formula   | $t_a$ (min) | Mass      | HMDB     | KEGG     | MC-G vs NC-G | RDN-G vs MC-G | PD-G vs MC-G | Origins |
|-----|--------------------------------------------------|-----------|-------------|-----------|----------|----------|--------------|----------------|--------------|---------|
| 1   | Urocanic acid*                                   | C$_6$H$_6$N$_2$O$_2$ | 2.130       | 138.042   | HMDB00301 | C00785   |               |                |              | urine   |
| 2   | (Z, Z)-3,6-Nonadienal                            | C$_9$H$_{14}$O | 6.320       | 138.104   | HMDB31152 | C16323   |               |                |              |         |
| 3   | Tiglylglycine                                    | C$_7$H$_{11}$NO$_3$ | 3.030       | 157.073   | HMDB00959 | /        |               |                |              |         |
| 4   | 3,4-Dimethoxy-benzaldehyde                       | C$_9$H$_{10}$O$_3$ | 5.758       | 166.060   | HMDB32138 | C02201   |               |                |              |         |
| 5   | Pyridoxine                                       | C$_8$H$_{11}$NO$_3$ | 3.879       | 169.073   | HMDB00239 | C00314   |               |                |              |         |
| 6   | Norepinephrine*                                  | C$_8$H$_{11}$NO$_3$ | 3.845       | 169.177   | HMDB00216 | C00547   |               |                |              |         |
| 7   | Jasmonolone                                      | C$_{11}$H$_{16}$O$_2$ | 6.062     | 180.115   | HMDB30039 | /        |               |                |              |         |
| 8   | Jasmonic acid                                    | C$_{12}$H$_{18}$O$_2$ | 5.522     | 210.125   | HMDB32797 | C08491   |               |                |              |         |
| 9   | Pandamariactam 3x                                | C$_{12}$H$_{17}$NO$_3$ | 2.389     | 235.120   | HMDB33610 | /        |               |                |              |         |
| 10  | Ubiquinone-1                                     | C$_{14}$H$_{18}$O$_4$ | 8.209     | 250.120   | HMDB02012 | C00399   |               |                |              |         |
| 11  | Tetrathylin B                                    | C$_{12}$H$_{17}$NO$_2$ | 4.017     | 287.100   | HMDB329914| /        |               |                |              |         |
| 12  | 3-Methoxy-2-(4-methylbenzoyl)-4H-1-benzopyran-4-one | C$_{18}$H$_{14}$O$_4$ | 9.215     | 294.089   | HMDB311975| /        |               |                |              |         |

*Note: MC-G, model control group; NC-G, the normal control group; RDN-G, RDN group; PD-G, positive drug group; vs, versus * Represents that the metabolite has been compared with the reference substances.*
disordered metabolic pathways. It suggested that RDN could rectify the metabolism disorder of the carrageenan-induced inflamed rats.

The metabolic neural network was constructed between the above four biomarkers and the known inflammation-related small molecules, including histamine (Hasala et al., 2008; Sato et al., 2020), arachidonic acid (Higgs, Palmer, Eakins, & Moncada, 1981; Trostchansky, Moore-Carrasco, & Fuentes, 2019), leukotriene B4 (Wan, Tang, Stsiapanava, & Haeggstrom, 2017), PGE2. The results showed they were closely related (Fig. 7), indicating that the screened inflammation-related biomarkers had certain reliability.

3.6. Correlation analysis between biomarkers and pharmacodynamic indicators

According to the metabolic pathway results (Impact $\geq 0.10$), the vital biomarkers were urocanic acid, sphingosine and norepinephrine (Table 4). Table 5 displays the relationship between these biomarkers and the pharmacodynamic indicators, which were systematically characterized by Pearson correlation analysis. Urocanic acid and norepinephrine was both significantly correlated with IL-1$\beta$, IL-6 and PGE2. These results indicated that the selected inflammation-related biomarkers had certain biological significance, and further proved that RDN could exert anti-inflammatory effects by regulating these biomarkers.

3.7. Biological significance of obtained biomarkers

Urocanic acid has an immunosuppressive effect (Gibbs, Tye, & Norval, 2008). It is formed by histidine catalyzed by histidine ammonia lyase for deamination, and is divided into cis and trans forms. Trans urocanic acid has an inhibitory effect on the immune function of skin cells, which is mainly reflected in the suppression of delayed hypersensitivity, contact hypersensitivity and skin cancer (Beissert et al., 2001; Halliday, Damian, Rana, & Byrne, 2012; Walterscheid et al., 2006). cis-urocanic acid has anti-cancer and promotes apoptosis (Beissert et al., 2001; Laihia et al., 2010). The increase of its content in the inflammatory rats’ urine may be caused by histidin metabolism disorder or glutamate metabolism disorder in inflamed tissues.

Norepinephrine is an important neurotransmitter and endocrine hormone, mainly synthesized and secreted by sympathetic postganglionic neurons and adrenergic nerve endings in the brain. Dopamine is the precursor of norepinephrine. It is hydroxylated by $\beta$-hydroxylase to produce norepinephrine, and then to produce epinephrine through methylation. As the $\alpha$ receptor agonist, norepinephrine can regulate the activities of cardiovascular and respiratory organs in the body. In addition, it also plays a certain role in body temperature regulation, stress response and immune regulation (Fitzgerald, 2009).

Sphingosine plays important roles on the synthesis and metabolism of sphingolipids, and can be converted to ceramides, sphingosine, and sphingosine 1-phosphate. Ceramide participates in a variety of cell signal transduction; regulates cell growth, differentiation and programmed death; is also involved in physiological functions such as stress, immunity and inflammation. Sphingosine 1-phosphate is a recognized immunomodulator (Yin et al., 2009).

Pyridoxine is one of the three substances that synthesize vitamin B6. It can be quickly catalyzed to pyridoxal-5-phosphate (PLP) by pyridoxal kinase, and participates in protein and amino acid metabolism (Shiau & Wu, 2003). Pyridoxine can promote the synthesis of nucleic acids and proteins by enhancing the activity of serine hydroxymethyltransferase (Perry, Yu, Chen, Matharu, & Stover, 2007; Depeint, Bruce, Shangari, Mehta, & O’Brien, 2006). Pyridoxine participates in the carbon chain extension and desaturation of essential fatty acids, and plays an important role in

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**Table 4**

| Pathway names            | Match status | $p$   | $-\log(p)$ | Holm $p$ | FDR  | Impact |
|--------------------------|--------------|-------|------------|----------|------|--------|
| Histidine metabolism    | Urocanic acid | 0.112 | 2.1895     | 1.0      | 1.0  | 0.15   |
| Sphingolipid metabolism | Sphingosine   | 0.153 | 1.8742     | 0.9      | 1.0  | 0.14   |
| Tyrosine metabolism     | Norepinephrine| 0.285 | 1.2545     | 1.0      | 0.9  | 0.08   |
| Vitamin B6 metabolism   | Pyridoxine   | 0.069 | 2.6791     | 1.0      | 1.0  | 0.08   |

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![Fig. 6. Summary of pathway analysis with MetaboAnalyst. a: Histidine metabolism; b: Sphingolipid metabolism; c: Tyrosine metabolism; d: Vitamin B6 metabolism.](image1)

![Fig. 7. Neural network of inflammation-related biomarkers. The big solid circle represents the inflammation-related biomarkers; the big hollow circle represents the known inflammation-related small molecules.](image2)
increasing the content of unsaturated fatty acids (Cunnane, Manku, & Horrobin, 1984; Tsuge, Hotta, & Hayakawa, 2000). In addition, pyridoxine is also involved in the immune response process in the body (Rall & Meydani, 1993).

In summary, the anti-inflammatory mechanism of RDN is mainly related to the regulation of amino acid metabolism and lipid metabolism.

4. Conclusion

The UPLC-Q-TOF/MS analytical method applied to rat serum and urine metabolomics research was stable and reliable. After carrageenan-induced inflammation, rat model was successfully replicated, 16 inflammation-related potential biomarkers were identified from rat serum and urine by MPP software and Metabolite database search technology. The greatly disturbed metabolic pathways in inflammatory rats were histidine metabolism, sphingolipid metabolism, and tyrosine metabolism. The three pathways involved three biomarkers (urocanic acid, sphinganine and norepinephrine), which had a certain correlation with known inflammation-related small molecules and pharmacodynamic indicators. Both RDN and DMSP could regulate the above three disordered biomarkers. Therefore, anti-inflammatory mechanism of RDN was mainly related to the regulation of amino acid metabolism and lipid metabolism. These results clarified the anti-inflammatory mechanism of RDN from the overall metabolic level, and provided the theoretical and data support for better interpretation of its clinical efficacy.

In this study, the rationality of the anti-inflammatory mechanism was verified through metabolic network analysis, correlation analysis, and physiological significance of biomarkers. For further in-depth verification, targeted metabolomics will be used to verify these results, and the key biomarker sphingosine will be administered intragastrically to observe the recovery of inflammation model rats. In addition, methods such as inhibitor blocking and gene expression can also be used for verification.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 5: Pearson correlation coefficient between biomarkers and pharmacodynamic indicators.

| Pharmacodynamic indicators | Biomarkers         | Urocanic acid | Norepinephrine | Sphingosine |
|-----------------------------|--------------------|---------------|----------------|-------------|
| IL-1β                       | 0.856*             | 0.944*        | 0.112          |
| IL-6                        | 0.785*             | 0.871*        | -0.497         |
| PGE2                        | 0.887*             | 0.968*        | 0.024          |
| TNF-α                       | 0.878*             | 0.972*        | -0.134         |

Note: correlation between two compounds, *P < 0.05; |r| > 0.9, a significant correlation; 0.8 ≤ |r| < 0.9, high correlation; 0.5 ≤ |r| < 0.8, moderate correlation; 0.3 ≤ |r| < 0.5 low correlation; |r| < 0.3, irrelevant.

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