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Study on the antiendotoxin action of Pulsatillae Decoction using an Affymetrix rat genome array

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ABSTRACT

A high-throughput and efficient Affymetrix rat genome array was used to investigate the pharmacological mechanism of the traditional Chinese medicine, Pulsatillae Decoction (PD), used for the treatment of diseases induced by lipopolysaccharide (LPS). Rat intestinal microvascular endothelial cells (RIMECs) were challenged with 1 μg/ml LPS for 3 h, and then treated with PD at a concentration of 1 mg/ml for 24 h. Total RNA from each treatment group was extracted from cultured RIMECs for detection by the Affymetrix Rat Genome 230 2.0 Array. The results showed that 36 genes were upregulated and 33 genes were downregulated in the LPS group vs. the blank control group; 566 genes were upregulated and 12 genes were downregulated in the PD-treated group vs. the LPS group; and 93 genes were upregulated and 29 genes were downregulated in the PD-treated group vs. the blank control group. The analysis of these data suggested that PD specifically and effectively reduce damage induced by LPS, and improved physiological and biochemical responses to counteract the effects of LPS.

1. Introduction

Swine colibacillosis is an acute multi-type infectious disease of the alimentary canal caused by pathogenic *Escherichia coli*. The disease spreads quickly and induces high mortality rates [1], resulting in great harm and loss to the pig industry. Multivalent and gene vaccines are used in pregnant swine to prevent the disease. However, maternal antibodies are not always sufficiently effective to protect neonatal piglets. Although antibiotics or antisera can be used as alternatives, the issues of drug resistance, drug residues and high costs are of concern.

Lipopolysaccharide (LPS), a component of the endotoxins released by Gram-negative bacteria, is the main pathogenic factor of colibacillosis, and is largely responsible for the morbidity and mortality associated with the disease [2]. LPS activates a series of signal-transduction pathways in cells and causes pathological effects [3]. LPS released by *E. coli* can damage the microcirculation and vascular endothelial cells and promote excessive secretion of various kinds of cytokines, thereby contributing to the inflammatory cascade response and others that ultimately triggers multiple organ dysfunction syndromes, eventually leading to death.

Many Chinese herbal medicines can effectively suppress and eliminate bacterial pathogens, in addition to alleviating fever and clearing toxic materials. They are widely used to prevent and cure infectious diseases, and show high efficacy, lower toxicity, fewer side effects, less drug resistance and lower residual levels than drugs. Many studies have focused on the use of Chinese herbal medicines for LPS-induced damage responses [4,5].

Pulsatillae Decoction (PD) is a representative prescription of Chinese herbal medicine for treatment of diseases caused by bacteria. It is composed of four herbs, namely *Radix Pulsatillae*, *Rhizoma Coptidis*, *Cortex Phellodendri* and *Cortex Fraxini*. Previous studies have confirmed that Anemoside B4 and Anemonin are isolated from root of *Radix Pulsatillae* [6–8]. *Rhizoma Coptidis* [9–11] and *Cortex Phellodendri* [12–14] contain Berberine, Jateorhizine and Palmatine in their root barks. Aesculin and Esculetin are the main active compounds in bark of *Cortex Fraxini* [15–17]. PD possesses the ability to alleviate fever and clear toxic materials, alleviate pain and improve blood circulation. Several clinical trials have shown that the preparation had prominent curative effects on enteritis and bacillary dysentery [18,19].

Chinese herbal medicines can regulate the balance of cellular functions and improve the physiological conditions of the body in many ways, especially by regulating the function of endothelial cells. Regarding the initiation and development of pathological damage, intestinal microvascular endothelial cells can be activated by LPS, leading to changes in the expressions of various genes that modulate physiological and biochemical responses of the body [20]. Studies have confirmed that intestinal microvascular endothelial cells are important primary targets for gastrointestinal and systemic diseases [21]. Rat intestinal microvascular

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endothelial cells (RIMECs) represent a good model for such studies.

Gene chip technology, with its characteristics of high-throughput simultaneous analysis, automatic rapid analysis and multiparameter sensitive analysis, is useful for in the study of gene function and the interactions between genes [22]. In the present study, changes in the levels of gene expression in RIMECs treated with LPS and PD were evaluated using the Affymetrix Rat Genome 230 2.0 Array. This array covers 30,200 transcripts and their mutants, representing more than 28,000 functional rat genes [23]. The purpose of this study was to investigate the pharmacological mechanism of PD in the treatment of diseases induced by LPS. At same time, we hoped to provide a theoretical basis and technological support for further research on PD, such as effective ingredients screening, identification of target genes, and personalized administration.

2. Materials and methods

2.1. Main reagents

Radix Pulsatillae (100 g), *Rhizoma Coptidis* (40 g), *Cortex Phellodendri* (80 g) and *Cortex Fraxini* (80 g) (Beijing Tongrentang Pharmacy Products) were soaked in 10 volumes of water for 1 h in a round flask, the fluid was then heated for 1 h before being filtered while still hot. The dregs of the medical decoction were sequentially extracted into eight and six times the volume of water following the same procedure described above. The three filtrates were combined and condensed by decompression to give a solution (PD) with an approximate drugs concentration of 1 g/ml. PD was diluted to 10 mg/ml with maintenance medium containing 15% fetal bovine serum (FBS; Sigma), 100 U/ml penicillin and 10 μg/ml streptomycin (Gibco). The diluted solution was filtered through a 0.22 μm microporous membrane and stored at 4 °C. LPS from *Escherichia* O55:B5 (Sigma; L-2880, 10 mg/vial) was also diluted to working concentrations with maintenance medium.

2.2. Experimental methods

Fifth-generation RIMECs cultured in our laboratory were inoculated into 12-well plates [24]. RIMECs were identified by the expression of the factor VIII-related antigen using fluorescence immunocytochemistry (model DMB5 fluorescence microscope; Motic, USA) and only cells showing positive responses were considered as experimental cells. In the blank control group, the complete medium was replaced with maintenance medium without LPS or PD. In the LPS group, the complete medium was replaced with maintenance medium containing 1 μg/ml LPS. In the PD group, the cells were initially challenged with maintenance medium containing 1 μg/ml LPS, followed by the addition of PD to a final concentration of 1 mg/ml after 3 h. The cells were then incubated at 37 °C in a cell incubator (MCO-17AC; Sanyo, Japan) for 12 h.

2.3. RNA isolation and cDNA, cRNA synthesis

Total RNA was isolated from RIMECs in each group according to the manual of the Trizol reagent (Invitrogen Corporation, Carlsbad, California, USA) [25] and then purified following the RNeasy protocol [26]. The quality of the total RNA samples was assessed by optical density (OD) measurement at 260/280 nm and agarose electrophoresis (180 V, 0.5 h) with a 2:1 ratio of 28S rRNA to 18S rRNA intensities [27]. Five microgram of total RNA was used as a template for cDNA synthesis. cDNA purification, biotinylated cRNA synthesis, and purification were performed by following the manufacturer's instructions (Affymetrix) [28]. The quality and concentration of cDNA and cRNA were examined using a previously reported procedure [27].

2.4. cRNA fragmentation and microarray detection

Fifteen microliter (1 μg/μl) of cRNA was incubated with 5× fragmentation buffer (Affymetrix Inc., Santa Clara, CA, USA) at 94 °C for 35 min to digest the cRNA into 35–200 bp fragments. The hybridization buffer (prepared according to Affymetrix protocol) was added to the Rat Genome 230 2.0 microarray (Affymetrix Inc., Santa Clara, CA, USA), and then hybridization was carried out at 45 °C for 16 h on a rotary mixer at 60 rpm. The microarray was washed and stained on a GeneChip fluidics station 450 (Affymetrix Inc., Santa Clara, CA, USA), and scanned by GeneChip Scan 3000 (Affymetrix Inc., Santa Clara, CA, USA) [29]. Raw data representing the signal values of gene expression were processed with the GeneChip Operating Software (GCOS) 1.4 [30].

2.5. Microarray data analysis

The normalized signal values, signal detections (P, A, M), and experiment/control (Ri) were obtained by quantifying and normalizing the signal values, using GCOS 1.4. The probe signal values were scaled to evaluate gene expression (p-value < 0.05), marginal expression (0.05 < p-value < 0.065), and no expression (p-value > 0.065). The signal values of each chip were normalized and evaluated for whether a gene's expression had changed by the ratios that compared the normalized p-value of the treated group to that of the control group, e.g. a gene whose ratio value $\geqslant 2$ was regarded as upregulated expression; a gene whose ratio value $\leqslant -2$ was regarded as downregulated [31]. Statistics and cluster analyses were conducted on these values with GeneMath, GeneSpring (Silicon Genetics, San Carlos, CA, USA) and Microsoft Excel Software (Microsoft, Redmond, WA, USA) [32].

2.6. Real-time RT-PCR

Primer and probe sequences were designed by primer express 2.0 software according to the mRNA sequences of five target genes: interleukin 1 alpha (Il1a), interleukin 6 (Il6), endothelin 1 (Edn1), tumor necrosis factor superfamily 11 (Tnfsf11), prostaglandin E synthase (Ptges) and the internal control actin gene (GenBank number: AJ245643, NM_012589, NM_012548, NM_057149, AF280967 and NM_031144), which were synthesized by Shanghai GeneCore BioTechnologies Co. Ltd. The samples from the LPS group and the blank control group were subjected to real-time RT-PCR, using the chimeric fluorescence (SYBR Green I) method. Detection of PCR amplification and data processing was carried out according to previously published procedures [33,34].

3. Results

3.1. Total RNA quality control

28S rRNA and 18S rRNA intensities were clearly visible on an agarose gel electrophoresis image of total RNA for each group. The ratios of OD 260/280 in blank, LPS and PD groups were 1.83, 1.88 and 1.86, respectively. The quality and quantity of total RNA in each group completely satisfied the experimental requirements of the gene chip.

3.2. Differences in gene expression

Thirty six genes were upregulated and 33 genes were downregulated out of a total of 69 genes whose expressions showed signif-