The effect of Jiedu Huoxue decoction on rat model of experimental nonbacterial prostatitis via regulation of miRNAs

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Context: The underlying mechanisms of Jiedu Huoxue decoction (JDHXD) in treating chronic prostatitis have not been fully explored.

Objective: This study investigates the miRNAs as potential biomarkers and the effect of JDHXD on the rat model of experimental nonbacterial prostatitis.

Materials and methods: Fifty-four Sprague-Dawley male rats were randomly divided into normal control, JDHXD low dose (0.5 g/kg/day), medium dose (1 g/kg/day), high dose (2 g/kg/day) and western medicine (cernilton 0.094 g/kg/day) groups, and intragastrically administered once daily for 30 days. The control and model (upon successful establishment) groups received distilled water. Differential expression of miRNAs was analysed with high-throughput miRNA sequencing and validated with qRT-PCR and Northern blot. Prediction of specific target genes and functional enrichment analysis were performed with bioinformatics.

Results: LD50 test showed no sign of toxicity with maximum feasible dose 4 g/kg JDHXD. Compared with control, 495 miRNAs showed expression changes in CAP/CPPS rats, of which 211 were significantly different and 37 were prostatic-related. There were 181 differentially expressed miRNAs between the model and high dose JDHXD groups, of which 23 were identical with the control and model groups. Compared with control, miR-146a, miR-423 and miR-205 expression increased significantly in the model group, and decreased dose-dependently in the JDHXD groups (p < 0.05), and vice-versa for miR-96 (p < 0.05). The effect of low dose JDHXD was comparable to cernilton (p > 0.05).

Discussion and conclusions: Future studies may explore the contributions of the active components in JDHXD. The study design is generalisable. The effect can be repeatedly verified in clinical trials.

Introduction

Chronic prostatitis (CP) refers to chronic inflammation of the prostate tissue caused by various factors. It is the most common disease in urology. According to the latest classification of prostatitis by the USA National Institutes of Health (NIH), type III prostatitis (chronic abacterial prostatitis/chronic pelvic pain syndrome [CAP/CPPS]) is the most common type of prostatitis, accounting for more than 90% of clinical prostatitis cases (Erickson et al. 2008; Polackwich and Shoskes 2016). Western medicine is still controversial regarding the pathogenesis of CAP/CPPS. Studies have shown that the pathogenesis of CAP/CPPS is mainly related to autoimmunity, infection, physical and chemical factors, oxidative stress and neuroendocrine factors. Abnormal immune regulation is considered to play an important role in the occurrence and development of CP/CPPS (Jang and Schaefer 2003). Recent clinical and animal studies have shown that the body’s immune status changes during the onset and progression of prostatitis. Local immune response can be induced by exogenous pathogenic factors acting on the prostate (Wang 2017). Abnormal expression of inflammatory genes and abnormal elevation of their expression products suggest that CAP/CPPS is a type of autoimmune disease with various inflammatory genes acting together (Zhou et al. 2007).

MicroRNAs (miRNAs) are small RNA molecules with a length of about 17–25 nucleotides which play an important role in regulating gene expression (Zhu et al. 2014). Although the number of miRNAs in the human genome is less than that of protein-coding genes, they are believed to regulate more than half of the human RNAs. miRNAs play an immunoregulatory role by regulating the expression of the immune regulatory genes, which is a new direction of immunology research in recent years (Ding et al. 2017; Chen et al. 2018).

There are different therapeutic interventions for CP, including α-blockers, antibiotics and anti-inflammatory drugs. Compared to these, one advantage of Chinese herbal medicine therapeutics is that multiple components in the herbal formula often play a synergistic role that is greater than individual drug. Monotherapy may not treat some complex diseases effectively, such as CP (Wang Z et al. 2016). Previous study suggested that directed multinodal therapy, rather than monotherapy should be considered for optimal management of CP/CPPS (Thakkinstian...
According to the pathogenesis of Traditional Chinese Medicine (TCM), performing treatment based on syndrome differentiation has a remarkable clinical effect, and has more obvious characteristics and advantages compared with Western medicine (Zhao and Zhu 2016). In his long-term clinical research, according to aetiology and pathogenesis, Professor Wenqiu Yu from our university developed the herbal formula of Jiedu Huoxue decoction (‘Polysatrin’) (JDHXD), an empirical prescription to treat CP, which is composed of ten herbs (Table 1).

Whether CAP/CPPS is also regulated by related miRNAs to exert its immunomodulatory effects is an important part of this study. In this study, the differential expression profiles of miRNAs in prostate homogenate of normal rats and rats with CAP/CPPS were analysed and detected with high throughput screening. Functional regulatory networks of miRNAs target genes were obtained by bioinformatics. The effect of JDHXD on the expression of differentially expressed miRNAs was observed via treatment on the experimental nonbacterial prostatitis in rats. Cernilton (Prostat tablet) was used as comparison in this study. The main components of cernilton are water-soluble pollen extract P5 and fat-soluble pollen extract EA10. It can reduce prostatic inflammation and has been used to treat nonbacterial prostatitis and prostate cancer (McClure Mark 2001).

This study explores the miRNAs as potential biomarkers for CAP/CPPS, the possible mechanism of JDHXD in the treatment of CAP/CPPS, and to provide theoretical and experimental basis for its prevention and treatment. The novelty of this study is to evaluate the therapeutic mechanism of JDHXD in CP via miRNAs by target gene prediction and differentiated miRNAs intersection analysis.

**Materials and methods**

**Experimental animals**

A total of 74 specific-pathogen free (SPF) male Sprague-Dawley (SD) rats, aged 4–6 months, weighed 170–210 g, purchased from the Experimental Animal Centre of Jiangxi University of Traditional Chinese Medicine, were kept in standard cages with five rats in each cage. They were given access to food and water ad libitum during the experiment period. The room was well ventilated, under natural light-dark conditions and kept between 18°C and 25°C.

This study was approved by the Animal Use and Care Committee of our university. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals (NIH, revised 2011).

**Preparation of JDHXD**

Main formula of JDHXD: *Rhizoma Smilacis Glabrae*, *Rhizoma Polygoni Cuspidati*, *Rhizoma Dioscoreae Colletti*, *Semen Plantaginis*, *Vaccariae Semen*, *Rhizoma Curcumae Aeruginosae*, *Radix Cyathulae*, *Radix Polygalae*, *Acorus tatarinowii*, and *Radix Glycyrrhizae* (Table 1) in ratio of 30:15:15:10:10:10:10:10:10:6.

The medicinal materials were produced by Guangdong Yifang Pharmaceutical Co., Ltd., Guangdong, P.R. China [product code: Rhizoma Smilacis Glabrae (9075821), Rhizoma Polygoni Cuspidati (9052551), Rhizoma Dioscoreae Colletti (9036741), Semen Plantaginis (8122581), Vaccariae Semen (9061413), Rhizoma Curcumae Aeruginosae (8071921), Radix Cyathulae (8105531), Radix Polygalae (8090381), Acorus tatarinowii (9036443), Radix Glycyrrhizae (9056401)], and provided by the Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine (1500 g, obtained in 2018). The herbal materials were identified by Prof. Wanchun Wang, and properly prepared. The voucher specimens were deposited in the Department of TCM Surgery, Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine.

The dry herbal materials were weighed and put into a regular stainless-steel pot. First, these herbal materials were soaked in distilled water for 40 min. Then, for the first cook, water, eight times the amount of the medicinal herbs by weight, was added, and boiled at 100°C for 40 min. The liquid was strained and the herbs were kept in the pot for the second cook. Then, water, six times the amount of the medicinal herbs by weight, was added, and boiled at 100°C for 30 min. The filtrate obtained was dried, and concentrated to 1:1 (1 mL of drug solution is equivalent to 1 g crude drug) and stored at 4°C for reserve after subpackaging. During intragastric administration, the drug was diluted in distilled water to 4 mL/rat, and given once a day. HPLC fingerprint analysis was conducted and the major compounds detected are shown in Figure 1.

**Main reagents and instrument**

Cernilton (Prostat Tablet) (Meirui Pharmacy, Co, Ltd., Nanjing, Jiangsu, China); RNA extraction kit (Beijing ComWin Biotech Co., Ltd., Beijing, P.R. China); SuperScript® III First Strand kits (Invitrogen Corp., Carlsbad, CA); pulversing machine (RT-02A, Beijing Hongquan Xianghe Machinery Technology Co., Ltd., Beijing, P.R. China); electronic balance (Sartorius AG, Göttingen, Germany); Agilent 1200 (Agilent Technologies, Inc., Santa Clara, CA, USA); real-time quantitative fluorescence polymerase chain reaction (PCR) kit (Bio-Rad Corp., Hercules, CA); NorthernMax® kit (Ambion Inc., Foster City, CA); nucleic acid quantifier (Nanodrop 2000, Nanodrop Technologies, LLC).

### Table 1. Medicinal herbs in JDHXD.

| Medicinal herbs | Family          | Genus       | Species | Variety | Authority                  |
|----------------|----------------|-------------|---------|---------|----------------------------|
| *Rhizoma Smilacis Glabrae* | Smilacaceae | Smilax | glabra |         | Wright, 1903                |
| *Rhizoma Polygoni Cuspidati* | Polygonaceae | Polygonum | cuspidatum | | Siebold and Zuccarini (1846) |
| *Rhizoma Dioscoreae Colletti* | Dioscoreaceae | Dioscorea | colletti | hypoglaucua | (Palibin) S.J.Pei & C.T.Ting, 176 |
| *Semen Plantaginis* | Plantaginaeae | Plantago | asiatica |         | Linnaeus, 1753              |
| *Vaccariae Semen* | Caryophyllaceae | Vaccaria | segetalis |         | (Neck) Garcke ex Aschers, 1864 |
| *Rhizoma Curcumae Aeruginosae* | Zingiberaceae | Curcuma | aeruginosa | officinalis | Roxburgh, 1810               |
| *Radix Cyathulae* | Amaranthaceae | Cyathula | tenuifolia |         | K.C.Kuan, 1976               |
| *Radix Polygalae* | Polygalaceae | Polygala |         | tatarinowii | Willdenow, 1802              |
| *Acorus tatarinowii* | Acoraceae | Acorus |         |         | Schott, 1859                |
| *Radix Glycyrrhizae* | Fabaceae | Glycyrrhiza |         | uralensis | Fischer ex de Candolle, 1825 |

**A total of 74 specific-pathogen free (SPF) male Sprague-Dawley (SD) rats, aged 4–6 months, weighed 170–210 g, purchased from the Experimental Animal Centre of Jiangxi University of Traditional Chinese Medicine, were kept in standard cages with five rats in each cage. They were given access to food and water ad libitum during the experiment period. The room was well ventilated, under natural light-dark conditions and kept between 18°C and 25°C.**

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Acute toxicity study

In the preliminary examination, the median lethal dose (LD_{50}) was not able to be found due to low toxicity. The maximum feasible dose (4 g/kg) was intragastrically administered to 20 male SD rats, which were kept fasting and given water only for overnight before administration. The rats were observed for toxic symptoms, body weight and mortality for 14 days after administration. At day 14, gross necropsies were performed.

Establishment of rat model of experimental nonbacterial prostatitis

The rat model of experimental nonbacterial prostatitis was established according to previous studies (Wei et al. 2006). The rats were randomly selected. After anaesthetised with intraperitoneal injection of 2.5% pentobarbital sodium (0.3 mL/100 g), castration was performed under aseptic conditions. Then, the skin was sutured, sterilised and bandaged, and the rats were put back into the cages and allowed food and water ad libitum. On day 2, the rats in the model establishment group were injected with oestradiol benzoate 0.25 mg/kg/day subcutaneously on the back at multiple points, for 30 days continuously. The rats in the normal control group were subcutaneously injected with the same amount of distilled water for 30 days.

After model establishment, three rats were randomly selected from the model establishment group and normal control group respectively for histopathological examination of the prostate (hematoxylin-eosin (HE) staining). The pathological changes of the prostatic parenchyma and stroma in the experimental rats were observed under microscope. The pathological manifestations of the prostate were graded as follows (Xu et al. 2002): grade 0: no inflammatory cell infiltration in the prostatic stroma, and there was uniform pink secretion in the glandular cavity; grade I: a small amount of inflammatory cell infiltration in the prostatic stroma and the endocrine secretion in the glandular cavity was decreased; grade II: a large number of inflammatory cell infiltration in the prostatic stroma and the endocrine secretion in the glandular cavity was obviously decreased and disappeared; grade III: A large number of inflammatory cell infiltration in the prostatic stroma, and the wall of the prostatic duct was destroyed.

Animal grouping and drug administration

In addition to the normal control group (n = 8), after successful establishment of rat model, the rats from the model establishment group were randomly divided into five groups (n = 8 in each group).

1. Model group
2. Chinese medicine group, which was further divided into low dose JDHXD (0.5 g/kg/day), medium dose JDHXD (1 g/kg/d) and high dose JDHXD (2 g/kg/day) groups.
3. Western medicine group (carnilton tablet 0.094 g/kg/day).

According to the ‘Experimental Methodology of Pharmacology’ (Xu et al. 2002), the dosage for rats is calculated by dose conversion between human and rat body surface area. According to the table of conversion for human vs. rats, if the clinical dose for human is X mg/kg [daily drug dosage of JDHXD (mg) per body weight (kg)], the dosage of rats is calculated by the formula (X mg/kg × 70 kg × 0.018)/200 g = (X mg/kg × 70 kg × 0.018)/0.2 kg = 6.3 X mg/kg. The above JDHXD doses were converted with reference to the adult dose: the medium dose was calculated according to the adult dose; the high dose was doubling of the medium dose while the low dose was half the medium dose.

All the above groups were intragastrically administered once a day for 30 days. The rats in the normal control and model groups were given distilled water similar amount to the drug intervention groups. At the end of treatment, 2.5% pentobarbital sodium was injected intraperitoneally (0.3 mL/100 g) for anaesthesia. Under aseptic condition, median incision of the lower abdomen was made, the prostate was removed, rinsed with normal saline and weighed. The prostatic tissue was divided into left and right parts. The left part was fixed in 10% neutral formaldehyde solution, dehydrated, paraffin embedded, sliced (4 μm), HE stained and observed under microscope.

Construction of cDNA library and high-throughput miRNA sequencing

Prostate tissue from the normal control group (group C), model group (group M), high dose JDHXD group (group H) and Western medicine group (group W) were taken. A pooled sample derived from all rats in the subgroups was used for constructing the cDNA library. Total RNA was extracted with Trizol method and Nanodrop 2000 was used to detect the
concentration and purity. The extracted RNA was digested with DNAase, and served as the template for cDNA synthesis with reverse transcription kit.

Polyacrylamide gel electrophoresis was used to enrich RNA molecules with the sizes of 18–30 nucleotide (nt). Then 3’ adapters were added and 36–44 nt RNAs were enriched, to which 5’ adapters were then ligated. The ligated products were reversely transcribed by PCR amplification, and 140–160 base pair (bp) PCR products were enriched to generate a cDNA library which was thereafter sequenced by using Illumina HiSeq™ 2500 (Gene Denove Biotechnology Co., Guangzhou, P.R. China).

Real-time PCR detection

With 20 μL reaction system of SuperScript® III First Strand Kits (Invitrogen Corp., Carlsbad, CA), mRNA was reverse transcribed into cDNA according to the manufacturer’s protocol and preserved at –20 °C before use. Primer Express 5.0 software was used to design gene-specific primers. The nucleic acid sequence of the primers is as shown in Table 2. The primers were synthesised by Sangon Biotech (Shanghai) Co., Ltd., Shanghai, P.R. China. The CT value was negatively correlated with the number of initial copies of DNA. By measuring the CT value of the gene amplification, the relative expression level of the target genes was calculated by double CT value method.

\[ \Delta CT = CT(\text{target gene}) - CT(\text{reference gene}) \]
\[ \Delta \Delta CT = \Delta CT(\text{target sample}) - \Delta CT(\text{control}) \]

The relative expression level of target genes = 2^(-\Delta \Delta CT).

The relative expression of the control group was 2^0 = 1 (Livak & Schmittgen 2001).

The parameters of PCR reaction: holding stage 95 °C~3min, cycling stage 94 °C~30s, 60 °C~30s, 72 °C~30s, number of cycles = 35.

Northern blot

Formaldehyde denatured gel was prepared. Pre-denatured was performed on the total RNA extracted from selected tissues. Denature loading buffer was added and denaturing gel electrophoresis was performed, followed by membrane transfer, baking and nucleic acid fixation. By using gene labelling system kit, Polyacrylamide gel electrophoresis was used to enrich RNA molecules with the sizes of 18–30 nucleotide (nt). Then 3’ adapters were added and 36–44 nt RNAs were enriched, to which 5’ adapters were then ligated. The ligated products were reversely transcribed by PCR amplification, and 140–160 base pair (bp) PCR products were enriched to generate a cDNA library which was thereafter sequenced by using Illumina HiSeq™ 2500 (Gene Denove Biotechnology Co., Guangzhou, P.R. China).

Data analysis

Statistical analysis was performed with SPSS 19.0 statistical software (SPSS Inc., Chicago, IL). All data were presented as mean± standard deviation, using (x ± s) as denotations. Differentially expressed miRNAs between two groups was performed according to the cut-off criteria of log_2 fold change (FC) > 1 and p < 0.05. One-way ANOVA and rank sum test were used for comparison between groups. The level of test significance at α = 0.05 was p < 0.05.

Results

Acute toxicity study

No mortality nor sign of toxicity were observed in 14 days after the administration of the maximum feasible dose (4 g/kg) of JDHXD. Gross necropsies indicated no apparent lesions in the major organs of rats. The doses up to 4 g/kg were considered as safe. Doses of 2, 1 and 0.5 g/kg were selected for further evaluation in this study.

Establishment of rat model of experimental nonbacterial prostatitis

According to the pathological grading criteria, the results showed that the inflammation grading for the model establishment group was grade III. A large number of inflammatory cells infiltrated the prostatic stroma and destroyed the wall of the prostate. The normal control group showed clear tissue margin with complete structure and no inflammatory cell infiltration was seen (Figure 2), suggesting that the model was successfully established.

Histopathological examination of the experimental groups

In the normal control group, a normal appearance of the stroma and glandular epithelium without inflammatory infiltration into the stroma and lumina was observed. Compared with the normal control group, the model group showed prostate atrophy, flat

| Genes   | Forward primers (5’–3’)          | Reverse primers (5’–3’)          |
|---------|----------------------------------|----------------------------------|
| miR-146a| CGCCAGGGTTCCTCCGTCACGACTGAAGCTGAATTTCCAATGGGTTT | CGCCAGGGGAATATTAGCTACACTGATATAACGGCGAACCCCAT |
| miR-96  | CGCCAGGGTTCCTCCGTCACGACTGGGATTTCCAATGGGTTT   | CGCCAGGGGAATATTAGCTACACTGATATAACGGCGAACCCCAT |
| miR-423 | CGCCAGGGTTCCTCCGTCACGACTGGGATTTCCAATGGGTTT   | CGCCAGGGGAATATTAGCTACACTGATATAACGGCGAACCCCAT |
| miR-205 | CGCCAGGGTTCCTCCGTCACGACTGGGATTTCCAATGGGTTT   | CGCCAGGGGAATATTAGCTACACTGATATAACGGCGAACCCCAT |
| U6      | CTGCGCTTCGGCAGCACA                  | ACGCTTCAGAATTTGGCT                |

Prediction and analysis of miRNAs target genes by bioinformatics

By using miRNA target gene database, sequence comparison of miRNA databases, RNA precursor and secondary structure prediction, and homology comparisons between miRNAs were performed to predict target genes. By using Gene Ontology (GO) database and multi-agent system (MAS), functional enrichment analysis and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis of target genes were performed to establish the miRNA-target gene-gene functional regulatory mechanism network. The enriched biological processes, molecular functions, cellular components, and signalling pathways were sorted.
glandular epithelial cells, reduced acinar cavity, a decrease in the number of acini, no secretion, a large number of inflammatory cells infiltration and vasoconstriction. Compared with the model group, the low dose JDHXD group showed increased acinar volume and inflammatory cell infiltration but marked atrophy; the medium dose JDHXD group showed relatively reduced prostate interstitial hyperplasia, acinar atrophy, and no inflammatory cells infiltration; the high dose JDHXD group showed no inflammatory cell infiltration of the prostate, and no obvious interstitial hyperplasia; while the western medicine group showed changes in prostate hyperplasia, cubic or columnar glandular epithelial cells, dilated acinar cavity, retained secretion, and the interstitial component proliferation was not significant (Figure 3). These data showed that different dosage of JDHXDs and western medicine mitigated the inflammation of CAP/CPPS.

Quality of sequencing data

Quality check of the sequencing data was performed to investigate whether sequencing results were affected by quality differences during the library preparation. First, we analysed the quality of sequencing data according to the length of the distribution of miRNAs (Figure 4(A)), and we can see that the tag length distribution of each group of samples has only one peak at 22 bp, which conforms to the tag length distribution of routine animal samples. Figure 4(B) showed the tag abundance statistics of different classifications in each group of samples. It can also be seen from the results that the quality of sequencing samples met the requirements of sequencing and this ensured the accuracy of the sequencing results.

Principal component analysis (PCA) and cluster analysis

The distribution of samples was investigated by PCA. PCA demonstrates the relationship between samples by dimension reduction (sample size required ≥3). In order to eliminate data noise, we filtered out the miRNAs with TPM <1 and performed PCA. From Figure 5(A), we can see that the experimental design is reasonable, and the two-dimensional spatial distribution of the same group of samples is relatively centralised, which shows that
Figure 4. Tag length distribution of miRNAs and tag abundance of different classifications of samples. (A) Histogram of length distribution (B) Pie chart for annotation (B-1) Normal control group (B-2) Model group (B-3) High dose JDHXD group (B-4) Western medicine group. C: Normal control group, M: Model group, H: High dose JDHXD group, W: Western medicine group.
the selection of these genes is representatively and the biological repetition is good.

Based on the expression of miRNAs, we performed hierarchical clustering on the relationship between the samples and miRNAs, calculated the distances between two samples of multiple samples, constructed the distance matrix, and merged the two nearest classes into a new class. Then we calculated the distance between the new and current classes, merged and calculated until there was only one class. Heat map was used to present the clustering results. In Figure 5(B), each column in the figure represents a sample, and each row represents a miRNAs. The expression of miRNAs in different samples was expressed in different colours. The redder the colour, the higher the expression level, and the greener the colour, the lower the expression level.

**Differentially expressed profile of miRNAs in prostate tissues**

Differential analysis of miRNAs was performed according to the expression of miRNAs. The screening criteria for difference was that the expression level changed more than twice and \( p < 0.05 \). Based on the statistical results, we analysed the differential expression of miRNAs among different groups. As shown in Figure 6(A), for model group compared with normal control group, 327 miRNAs were up-regulated while 168 were down-regulated \( (p < 0.05) \). We also analysed the effect of drug treatment on miRNA expression (comparisons between high dose JDHXD group, western medicine group and model group). It was found that 425 miRNAs showed expression changes in the high dose JDHXD group compared with the model group \( (p < 0.05) \), whereas only 151 miRNAs showed expression changes in the western medicine group \( (p < 0.05) \). Treatment with high dose JDHXD has more significant effect on the miRNAs expression compared to western medicine \( (p < 0.05) \). Further, we used scatter plots of sequencing data to evaluate the overall trend of centralised distribution of two groups of data (Figure 6(B)). It can also be concluded from the results that high dose JDHXD has significant intervention effect on miRNAs expression. In order to investigate the effect of drug treatment on the abnormal expression of miRNAs in rat model of experimental nonbacterial prostatitis, we further analysed the differences between the high dose JDHXD group, western medicine group and model group, and intersection of the differential molecules between the normal control group and model group. As shown in Figure 6(C), there
Figure 6. Analysis of differentially expressed miRNAs among samples. (A) Differential expressed miRNAs between groups. (B) Scatter plot of sequencing data (B-1) Expression level of M vs. C (B-2) Expression level of H vs. M (B-3) Expression level of W vs. M (B-4) Expression level of W vs. H (C) Venn diagram showing intersection of differential molecules between groups. C: Normal control group, M: Model group, H: High dose JDHXD group, W: Western medicine group.
were a total of 365 miRNAs in the intersection of the differential molecules between the high dose JDHXD group and model group and between the normal control group and model group, while there were 86 miRNAs in the intersection of the differential molecules between the western medicine group and model group and between the normal control group and model group.

**Bioinformatics analyses for differentially expressed miRNAs**

We used RNAhybrid (v2.1.2) (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) (Rehmsmeier et al. 2004) + svm\_light (v6.01) (Joachims 1999), miRanda (v3.3a) (http://www.microrna.org/microrna/home.do) (Betel et al. 2008), and TargetScan (Version: 7.0) (http://www.targetscan.org) (Agarwal et al. 2015) for target
gene prediction. The intersection of the predicted results of target genes obtained by the three methods was used as the predicted results of the target genes of miRNAs, and memorised the unpredicted target genes for functional annotation, to explore the main related functions of these candidate genes. Target gene prediction of the differential expressed miRNAs was performed. Database analysis of the relationship between the miRNAs and target genes with Human Genome Organisation (HUGO) gene terminology was carried out. Pathway-based analysis is helpful to further understand the biological functions of gene. KEGG is the main public database. For each functional enrichment and KEGG signal transduction pathway analysis (http://www.genome.jp/kegg/) (Kanehisa et al. 2008), the total number of functional centralised target genes was obtained. For GO analysis (http://www.geneontology.org) (Ashburner et al. 2000; The Gene Ontology Consortium 2019) of the specifically expressed miRNAs target genes of CAP/CPPS, based on the GO database, functional enrichment analysis of the target genes was performed.
to obtain the miRNA target gene functional regulatory network. The miRNA target gene network was visualised using Cytoscape (http://www.cytoscape.org/; Shannon et al. 2003). Figure 7 showed functional enrichment analysis performed on the differentially expressed miRNAs target genes. The intersection of the differential molecular target gene function/signalling pathway between the model group and normal control group, and between the model group and high dose JDHXD group included N-Glycan biosynthesis pathway, Oxytocin signalling pathway, Hippo signalling pathway, mTOR signalling pathway, FoxO signalling pathway and cell cycle. These signalling pathways were involved in cell proliferation, autophagy, immune regulation and other important biological functions.

**Validation with qRT-PCR and Northern blot**

According to the differential expression profiles obtained by sequencing, all the miRNAs expressed in the two groups of samples were calculated. We used edgeR to determine whether there were significant differences between the two groups of samples. There were 211 known miRNAs with significant differences between the normal control group and model group. According to the predicted results of target genes, 37 were prostate-related, and 181 were differentiated miRNAs between the model group and high dose JDHXD group, of which 23 intersected with the differentiated molecules of the normal control group and model group. We selected four molecules (miR-146a, miR-96, miR-423, miR-205) for further validation. As shown in Figure 8(A), the results of qRT-PCR validation were consistent with the sequencing trend. The expressions of miR-146a, miR-423 and miR-205 increased significantly in the model group ($p < 0.05$). After drug treatment, they showed a downward trend, and a dose dependence trend was noted in the Chinese medicine group. The expression of miR-96 decreased significantly in the model group ($p < 0.05$) and showed a recovery trend after drug intervention. Figure 8(B) is the exposure map and statistical analysis results of the Northern blot. The trends of miR-146a, miR-96, miR-423 and miR-205 were consistent with the results by qRT-PCR. These results suggested that drug intervention can induce significant changes in molecules in the model group towards normal levels and these miRNAs are likely to participate in the development of CAP/CPPS.

![Image](http://www.cytoscape.org/)
Discussion

With the in-depth study of CP, there are many new understandings about the pathological process, but the exact pathogenesis has not been fully elucidated (Koh et al. 2014; Riegel et al. 2014). In recent years, the relationship between autoimmune mechanisms and type III prostatitis has become a hot topic. A large number of studies have shown that inflammation changes of prostatic histology in patients with type III prostatitis are mainly T lymphocyte. The inflammation grade is positively correlated with the ratio of CD8+/CD4+ and level of cytokines in serum and semen. This indicates that the pathogenesis of type III prostatitis is related to autoimmune inflammation (Huang et al. 2015).

Some scholars have made some preliminary studies on the mechanism of TCM in the treatment of CP. For example, Lu et al. (2003) studied the effect of Danpu Granule on the pathological model of experimental chronic non-bacterial prostatitis and suggested that it may have the effect of inhibiting inflammatory immune response. It inhibits the release of similar growth factors by lymphocytes and monocytes, thereby reduces adhesion proteins, and the degree of prostatic inflammation and fibrosis, and restores abnormal biochemical indicators. Wen et al. (2010) studied the effect of Qianlie Huoxue decoction on inflammatory cytokines in rat prostate and autoimmune prostatitis and concluded that it can significantly alleviate the inflammatory reaction of prostate in rat model by regulating the local immune status of the prostate. However, due to lack of systematic and standardised research models and in-depth investigation on molecular mechanisms, most of the interpretations of results cannot be certified by modern medical science, and this has become an important reason that restrict the modernisation of TCM.

Lang and Xia (2015) found that Shugan Jieyu capsule can improve clinical symptoms of patients with type III B prostatitis and effectively alleviate their psychological problems such as anxiety and depression. Shao et al. (2017) showed that lower urinary tract symptoms of patients with type III A prostatitis can be effectively and safely improved with Saw palmetto fruit extract. However, there has been no in-depth study done to explore the relevant mechanisms. Elucidating the possible mechanism of TCM in the treatment of prostatitis can help in prevention and treatment of CP.

In this study, the model of experimental non-bacterial prostatitis was established via castrated rats induced by oestrogen. The principle of the model is that high dose of oestrogen can cause the increase of inducible nitric oxide synthase (iNOS), which accentuates the local immune mechanism of the prostate tissue, causes damage to the prostate tissue and develops CP. According to ‘Specifications for Preparation of Chronic Prostatitis Animal Models’ (Traditional Chinese Medicine Experimental Pharmacology Professional Committee, China Association of Chinese Medicine 2018) and Wei et al. (2006), the success rate of using this model is high, about 92.5%, and the model can be maintained for no less than 30 days. In this study, after establishment of the model of experimental non-bacterial prostatitis (determined by HE staining), the rats were further randomly divided into normal control group, model group, and treatment groups with JDHXDs and cernilton. Histopathological examination was again performed on the normal control and model group, as well as the treatment groups. Comparisons between groups were made, and similar differences were observed in the control and model groups, while treatment with JDHXDs or cernilton mitigated the inflammation as compared to the model group.

JDHXD comprises of 10 medicinal herbs and this combination can achieve satisfactory therapeutic effect. It can eliminate inflammation and effectively improve the symptoms of patients in clinical practice. Our previous studies showed that JDHXD can significantly reduce the levels of IL-6 and IL-8 in expressed prostatic secretions (EPS) of patients with type III prostatitis (Liu et al. 2008; Wang et al. 2010), alleviate or eliminate the inflammatory reaction of rat prostate, improve the destruction of its tissue structure, repair and protect prostatic tissue, reduce the levels of proinflammatory factors TNF-α and IL-8 in rat prostate, and increase the level of anti-inflammatory factor IL-10 (Wang et al. 2012). Although non-coding RNA is not directly involved in gene coding and protein synthesis, it plays an important role in post-transcriptional regulation, splicing and modification, and in many life activities (Bernardo et al. 2015). miRNAs as a large class of non-coding RNA exist widely in organisms. With in-depth study of miRNAs, it has been found that they have a variety of biological functions, and their mechanisms are extremely complex. In particular, some miRNAs involved in immune regulation provide new insights into the understanding of immune-related diseases (Magni et al. 2014). In this study, we established a rat model of experimental nonbacterial prostatitis and analysed the differential expression profiles of miRNAs in the prostatic tissue of rats by using second generation sequencing technology. We aggregated the miRNAs identified in each sample and calculated the tags per million (TPM) expression of each miRNA. The expression profiles of miRNAs in all samples were obtained, and a total of 1299 miRNAs were identified. Differential analysis was performed according to the expression of miRNAs. The screening criteria for the difference was that the expression level changed more than twice and p < 0.05. There were 495 miRNAs showed expression changes in the model group compared with the normal control group and 425 miRNAs showed expression changes in the high dose JDHXD group compared with the model group (p < 0.05). By comparing with the known animal miRNAs in mirbase, 211 known miRNAs with significant differences between the normal control group and model group were identified, of which 37 were prostatic-related, and 181 differentiatiated miRNAs between the model group and high dose JDHXD group, of which 23 were identical with the normal control group and model group. The intervention effect of high dose JDHXD on differentiatied miRNAs was significantly higher than that of western medicine Cernilton. By target gene prediction and differentiatied miRNAs intersection analysis, the intervention effect of JDHXD on the differentially expressed miRNAs in the model group can be studied, and its therapeutic mechanism in CP can be explored through miRNAs.

Through bioinformatics analysis and literature search, we selected 4 (miR-146a, miR-96, miR-423, miR-205) out of 23 molecules, which are closely related to prostatic inflammation and may be specific biomarkers, and performed further validation by qRT-PCR and Northern blot. The results were consistent with the sequencing trends. The expressions of miR-146a, miR-423 and miR-205 were significantly increased in the model group, showing a downward trend after drug treatment compared with the model group, and a dose-dependent trend in the Chinese medicine group. The expression of miR-96 decreased significantly in the model group, and showed a recovery trend after drug intervention. At the same time, we also investigated the intervention effect of cernilton on the above molecules. The results of qRT-PCR and Northern blot showed that the
intervention effect of cernilton on differentiated miRNAs was significantly lower than that of medium- and high-dose JDHXDs, and similar to that of low dose JDHXD.

miR-146 is a vital modulator of differentiation and biology function of cells for the innate and adaptive immunity (Yang and Wang 2016). By means of promoter analysis, miR-146a was found to be a NF-κB-dependent gene (Taganov et al. 2006). Previous study showed that miR-146a could negatively regulate signal transduction pathways leading to NF-κB activation (Rusca and Monticelli 2011). Our previous study found significant increase of NF-κB mRNA and protein expression in rat model of CAP/CPPS. Excessive activation of NF-κB can lead to overexpression of inflammatory factors, resulting in extensive damage to tissue structure and physiological function (Yan et al. 2019). In this study, the levels of miR-146a were significantly increased in the model group and this was significantly relieved by drug treatment.

Previous study found that overexpression of miR-96 decreased FoxO1 mRNA and protein levels and its effect on cell growth is mediated by FoxO1 (Hallidad et al. 2013). The FoxO pathway is considered to promote immune activity by negatively regulating the expression of immunosuppressive proteins including programmed death receptor 1 ligand (PD-L1) (Deng et al. 2018). miR-423-3p and miR-205 has been suggested as novel prognostic biomarkers for prostate cancer progression by regulating RAR expression (Long et al. 2019). Cell-cycle regulators have been found to have a unique effect in controlling certain immune functions (Balomenos and Martinez-A 2000). Thus, our previous study demonstrated that astragaloside can attenuate the progression of prostate cancer (Tang et al. 2018). Emodin has been shown to exhibit anti-inflammatory and anticancer properties in prostate cancer (Tu et al. 2019). Astilbin may serve as a potential anti-inflammatory agent (Huang et al. 2011), and has been found to inhibit cell proliferation and attenuate inflammation (Chen et al. 2018; Dong et al. 2018). However, the mechanism of these effective components on CP via regulation of miRNAs has not been fully studied yet.

There is likely a cooperative effect among these components involved in the therapeutic mechanism of JDHXD (Geary 2013; Fouqueri and Guedj 2015), and JDHXD may be an effective treatment option for CAP/CPPS via combination therapy of these major effective components.

KEGG pathway analysis enriched several immune-related pathways including N-Glycan biosynthesis pathway, Oxytocin signalling pathway, Hippo signalling pathway, mTOR signalling pathway, FoxO signalling pathway and cell cycle. N-Glycan is important in adaptive immune response (Demetriou et al. 2001; Dennis et al. 2009). Oxytocin can regulate immune functions (Clodi et al. 2008). It can also change the activity of other hypothalamic-pituitary-immune axes (Li et al. 2017). Hippo signalling pathway is important in innate immunity and autoimmunity (Zhang et al. 2018). mTOR is emerging as a critical regulator of immune function and proving to be a vital link between immune function and metabolism (Powell et al. 2012). FoxO could be critical in modulating immune homeostasis and lymphocyte selection (Birkenkamp and Coffer 2003). Cell-cycle regulators have been found to have a unique effect in controlling certain immune functions (Balomenos and Martinez-A 2000). Thus, our results suggested that immune factors are probably involved in CAP/CPPS regulation networks facilitated by miRNAs.

Through construction of the differentially expressed miRNAs, target genes and gene functional regulatory network, the miRNA core regulatory network, functional regulated core target gene and key gene function can be obtained, the impact on disease pathogenesis can be predicted and the role and mechanism of miRNAs in disease modification can be explored.

In this study, we identified several differentially expressed miRNAs that are potentially associated with prostatic inflammation and enriched several immune-associated pathways, indicating the possible immunological regulatory functions in CAP/CPPS progression and potential treatment of JDHXD. Our further step will elucidate the function of some of the miRNAs. A limitation of this study is that we only selected 4 molecules for validation and more molecules would be explored in subsequent studies.

miR-146a, miR-96, miR-423 and miR-205 can be potential biomarkers for CAP/CPPS. JDHXD may be an effective
treatment for CAP/CPPS via regulation of the differentially expressed miRNAs to suppress or activate the target gene expressions or their relevant signalling pathways. The study design can be generalised to a broader study population. The clinical efficacy of JDHXD has been tested on a number of studies in humans and proved to be effective (Wang and Yan 2006, 2007; Wang et al. 2010; Wang W et al. 2016), and we will continue to study in-depth later. The major components in JDHXD include baicalin, catechin, astragaloside, emodin, and astilbin. Further studies may focus on the contribution of these active components and its molecular mechanisms.

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No potential conflict of interest was reported by the author(s).

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