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ORIGINAL RESEARCH

Expression Profiling of L5-S2 Spinal Cord Dorsal Horn in a Rat Model of Chronic Pelvic Pain Syndrome Uncovers Potential Mechanism of Electroacupuncture Mediated Inflammation and Pain Responses

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Purpose: We aim to explore expression profiles of genes in SCDH of CPPS model rat relevant to pain and inflammation by RNA-Seq and to investigate the mechanism of anti-inflammatory and analgesic of EA.

Methods: Thirty-six SD male rats were randomly divided into three groups (n = 12): sham operation, model, and EA. The rat CPPS model was established by injecting CFA into the ventral lobes of the prostate. The rats in EA group were treated at Guanyuan (CV4), Zhongji (CV3), Sanyinjiao (SP6) and Huiyang (BL35) for a total of 20 times, with a frequency of 2/100Hz. Mechanical allodynia, H&E staining and ELISA were used to detect the changes of pain threshold and tissue inflammation; RNA-Seq technique was used for profiling gene changes in SCDH and qRT-PCR was used for further validation.

Results: Persistent mechanical allodynia and severe tissue inflammatory reaction both occurred in CPPS rats. After EA therapy, the pain sensitivity and inflammatory response of CPPS rats decreased significantly. RNA-Seq identified that a total of 46 DEGs were significantly up-regulated and 65 DEGs down-regulated after EA. GO enrichment showed that EA was mainly reflected in the regulation of the immune system by participating in the regulation of leukocyte, neutrophil cellular processes and cytokine metabolism. KEGG enrichment demonstrated that signal transduction and immune system were the most significant pathways. We further identified that the expressions of Pik3r2, Akt1, and Casp9 were significantly up-regulated and Jak2 and Stat3 down-regulated in the PI3K-AKT/JAK-STAT signal pathway.

Conclusion: Our study revealed that immune and inflammatory responses are the main biological events that induce chronic pelvic pain in rats, and EA can exert anti-inflammatory and analgesic effects by regulating the expression of related genes on PI3K-AKT/JAK-STAT signal pathway in SCDH. This study provided putative novel targets of EA, which may have anti-inflammatory and analgesic effects of CPPS.

Keywords: electroacupuncture therapy, chronic pelvic pain, anti-inflammatory and analgesia, RNA-seq technique, cytokine, spinal cord dorsal horn

Introduction

Chronic pelvic pain syndrome (CPPS) is a complex clinical entity. Among patients with chronic pelvic pain who visit a male genitourinary specialist, 90–95% may be diagnosed with category III of prostatitis syndromes, which based on the 1999 version the prostatitis classification by the National Institutes of Health (NIH).[1] CPPS is often associated with

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various symptoms of lower urinary tract symptoms, sexual dysfunction, as well as with emotional consequences.[2] These symptoms usually lasted for at least 3 months in the past 6 months.[3] Pain is one of the most typical symptom of CPPS. In particular, the pain including chronic pelvic pain, testicular pain, and pain or discomfort during voiding or ejaculation, which seriously affect the quality of patients' life.[1,4] Surgeons are more likely to recommend antibiotics, anti-inflammatory drugs, α-blockers and neuromodulators as treatment options for CPPS patients. However, the compliance of patients is poor due to the slow onset of drugs.[3,5] With the development of modern medicine, it had been proved that sacral nerve stimulation therapy is effective in the treatment of some patients with chronic pelvic pain. Sacral nerve stimulation is mainly through the special electrical stimulation device implanted into the patient's body, so as to implement continuous low-frequency electrical stimulation to the sacral nerve, mainly stimulate the nerves innervating the anal sphincter and pelvic floor muscles, and to guide the reconstruction of the patient's pelvic floor function,[6] which had been proved to be effective in chronic pelvic floor pain syndrome, lower urinary tract dysfunction, sexual dysfunction and spastic pelvic floor syndrome.[7–10] However, as a minimally invasive treatment, the safety and high cost of sacral nerve stimulation therapy are the main contradictions considered by patients, and the treatment will be accompanied by complications such as pain, dislocation of electrode, intestinal dysfunction and infection.[6] Therefore, based on the treatment principle of sacral nerve stimulation, many clinicians try to replace sacral nerve stimulation with continuous EA stimulation. And to explore a treatment with integrated Chinese and Western medicine, less complications, low cost and high curative effect.[6] Acupuncture is a non-pharmacological treatment option for chronic pain. Currently, there is some clinical evidence supporting the use of acupuncture in alleviating pain and urinary symptoms in patients with CPPS, and the research results were published in the "Annals of internal medicine", which provided a strong evidence for the acupuncture treatment of CPPS.[11,12] However, the question of why acupuncture could be efficacious for CPPS patients remains unanswered.

Spinal cord dorsal horn (SCDH) is an important integration center of various sensory transmission and a key part of pain regulation. It receives pain signal inputs from the peripheral sensory neurons and plays a critical role in integrating pain signals and central pain sensitization.[13] Recent studies have reported that acupuncture had anti-inflammatory, immunomodulatory and neuroregulatory effects.[14] Acupuncture signals enter the spinal cord along the peripheral afferent nerve and interact with nociceptive signals from the pain site, thus exerting anti-inflammatory and analgesic effects.[15] According to the review published earlier, we found that the commonly used acupoints are Guanyuan (CV4), Huiyang (BL35), Zhongji (CV3), Ciliao (BL32), Xingjian (LR2), Qugu (CV2), Sanyin points (Jiayin 1, Jiayin 2, and Chong yin), Shenshu (BL23), Zhibian (BL54), and Sanyinjiao (SP6).[16] In the treatment of diseases, acupuncture usually follows the principle of "local point + far point" compatibility. Neuroanatomy shows that CPPS pain is mainly dominated by ilioabdominal sulcus nerve, pudendal nerve and perineal nerve, the nerve fibers mainly originate from lumbar and sacral plexus of spinal cord segment.[17] The nerves distributed under Guanyuan (CV4), Zhongji (CV3), Sanyinjiao (SP6) and Huiyang (BL35) overlap with the nerves distributed at the pain site of CPPS. Tibial nerve is distributed under the Sanyinjiao (SP6), the electrical stimulation can reach the posterior root of the spinal cord through the tibial nerve and regulate the function of the corresponding viscera through central feedback.[18] Guanyuan (CV4) and Zhongji (CV3) are located in the body surface projection area of the prostate, under them, parasympathetic nerves are distributed. EA can regulate prostate activity by activating parasympathetic nerves. Huiyang (BL35) is located in the projection area of prostate tissue on the back, EA can directly stimulate the pelvic nerve and pudendal nerve to improve the function of prostate.[19] So, we chose these acupoints to investigate the efficacy of EA on CPPS.

Furthermore, in order to explore the analgesic mechanism of EA, we proceed to carry out genome-wide expression profiling of the SCDH by using RNA sequencing (RNA-Seq). RNA-Seq technology can study gene function and structure from the overall level, reveal gene function, phylogeny and evolution related to life activities, as well as interaction with other organisms, etc. Its advantage lies in its high resolution, which can be accurate to a single base and reveal the precise position of transcription boundary. In addition, the dynamic range of quantification of gene expression level is larger, and it is easy to detect genes with low expression level and high accuracy. It has a good correlation with qRT-PCR results, and the results of RNA-Seq can show a high level of repeatability in both technical and biological repetition. This method may provide further insights into identifying novel and effective therapeutic targets against CPPS.[20,21]

Materials and Methods

Experimental Animals

Thirty-six male specific-pathogen-free (SPF)-bred Sprague-Dawley (SD) rats (200–220 g) were obtained from *Beijing Vitong Lihua Laboratory Animal Technology Co.,Ltd*, Licence: *SYXK (Beijing) 2016–0006*. Rats were reared in stainless steel cages under standard conditions (12h light/dark cycle at 23±2 °C, 45–55% humidity). The rats had free access to food and water, and were acclimated for one week before experiments. All the experiments followed the *Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals*.

CPPS Rat Model Establishment

Thirty-six rats were randomly divided into three groups (sham operation, model, and EA), with 12 rats in each group. The rats were all anesthetized with intraperitoneal injection of 0.35mL/100g 1% sodium pentobarbital (*Beijing BioDee BioTech Corporation Ltd*). Then, a longitudinal incision (1cm beside the anterior midline) was made in the right lower abdomen of rat to expose the bilateral ventral lobes of prostate. The rat model of CPPS was established as previously described.[22] Equal volume (0.1 mL) of complete Freund's adjuvant (*CFA, Beijing Benovir Biotechnology Co.,Ltd*) was injected into the ventral prostate of rats in model and EA groups. And the same volume of saline was injected into the ventral prostate of rats in sham operation group. Then, 16×10^5U penicillin sodium solution was injected intraperitoneally to prevent infection. All the animals were kept with a free access to water and food. The general physical conditions such as activities, the gloss of hair, water intake and urine output were observed throughout the period. After 14 days, 2 rats in each group were randomly selected to evaluate whether the model was successfully prepared by hematoxylin-eosin (H&E) staining. If there were a large number of inflammatory cells infiltrating in the prostate and the pain threshold was significantly reduced, the model was successfully established.

Acupoints Location and Intervention Methods

The acupoints Guanyuan (CV4) and Zhongji (CV3) are located at points 3/5 and 4/5 down the ventral midline connecting the umbilicus to the pubic tubercle.[23] Sanyinjiao (SP6) is located at 10 mm proximal to the prominence of the medial malleolus.[24] Huiyang (BL35) is located at anteromedial of the transverse process of the 6th lumbar spine.[25]

The rats in sham operation and model groups were loosely immobilized every day without other intervention. In the EA group, the Guanyuan (CV4), Zhongji (CV3) and bilateral Sanyinjiao (SP6) acupoints were vertically inserted at a depth of 3 mm with disposable acupuncture needles (0.18×13 mm, *Beijing Zhongyan Taihe Medical Instrument Co., Ltd*) in supine position. Then, bilateral Huiyang (BL35) acupoints were inserted at a depth of 6 mm in the prone position. The two ipsilateral needles were connected to the output terminals of the HANS-200E instrument with a 2/100 Hz alternating frequencies. The treatment began at the 15th day after modeling, 20 min for each acupoint, once daily for four therapeutic courses (five days as a course), 1 day break between the two courses to detect the mechanical pain. The time course of the experiment is presented schematically in Figure 1.

Measurement of Threshold to Mechanical Stimulus

Mechanical pain threshold was assessed using von Frey filaments (*Aesthesio, RWD Life Science Co., Ltd*).[26] Rats were acclimated in the testing apparatus for at least 30 min before behavioral testing. A series of von Frey filaments with increasing bending forces (range 0.4 to 15 g) were applied one by one to the skin between the 3rd and 4th toes of the left hind foot. Each filament was applied for 4–6 s until the filaments are slightly bent in a "C" shape. Withdrawal of paw or licking during the application was considered as a positive response to fibre stimulation, marked it as "X". On the contrary, if there is no response, mark it as "O". If the rat responded positively, the less stiff one less stiff one. When no withdrawal or licking was observed, the stiffer filament was used for the next trial. Then, a series of "XO" combinations are obtained, and an interval of 10 seconds between each application. The bending force of the filament to which the animal responded was taken as the baseline threshold to mechanical stimulus. But if the rat did not show any withdrawal or licking response to the application of a 15 g von Frey filament,

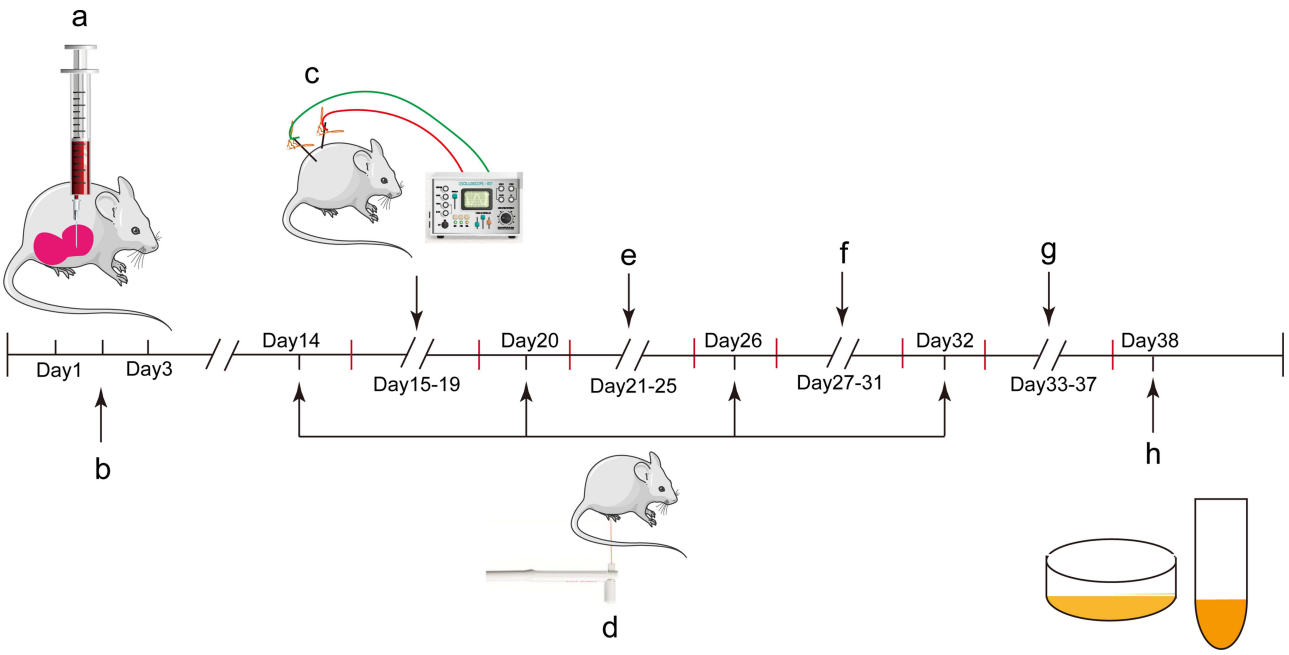

Figure 1 Experimental time flow. (**A**) Injection of CFA into prostate tissue; (**B**) intraperitoneal injection of penicillin sodium; (**C**) schematic diagram of EA intervention (1st course); (**D**) mechanical pain threshold measurement; (**E**) EA intervention (2nd course); (**F**) EA intervention (3rd course); (**G**) EA intervention (4th course); (**H**) pain measurement and Index detection. On the 1st day, CFA was injected into the ventral lobe of the prostate tissue of rats to establish CPPS model. Penicillin sodium was injected intraperitoneally for 3 consecutive days after the operation. Acupuncture intervention was started on the 15th day, 5 days as a course. Take a rest for 1 day between each course of treatment to detect mechanical pain for 4 consecutive courses.

the mechanical pain threshold for the rat was 15 g. Finally, enter the table formula to calculate the 50% mechanical pain threshold for the rats.[27]

H&E Staining Analysis

For histological evaluation, both lateral lobe of prostate was dissected. Then, the prostate tissue was fixed in 4% paraformaldehyde (*Beijing Coolaber Co.,Ltd*) for two days at 4°C. Then, the tissue was dehydrated in ethanol (75–100%), cleared in xylene and embedded in paraffin. Small pieces were sectioned serially at 4 μm and stained by H&E Staining Kit (*Jiangsu KeyGEN Biotechnology Co.,Ltd*). The pathological change and morphology of prostate were observed by microscope.

Measurement of PGE2 and SP by ELISA

After the rats were anesthetized, abdominal aortic blood was taken. The blood samples were centrifuged at 3000 rpm for 20 minutes at 4°C. The levels of PGE2 and SP were measured by special commercial ELISA kits (*Nanjing Jiancheng Bioengineering Institute*). ELISA calc software was used for calculation. Logistic curve (four parameters) was used to determine the levels of PGE2 and SP in serum of rats.

Library Preparation for Transcriptome Sequencing

Total mRNAs from five rats in each group were isolated and used to construct sequencing libraries. The cDNA library of each sample was prepared and sequenced by *Novogene Technology Co., Ltd* (*Beijing, China*). A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Then, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. First-strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. In order to select cDNA fragments of preferentially 370–420 bp in length, the library fragments were purified with AMPure XP system (*Beckman Coulter, Beverly, USA*). The kit for library building is NEBNext® Ultra™ RNA Library Prep Kit for Illumina®.

Bioinformatics Analysis

Primary sequencing data produced by RNA-Seq were subjected to quality control (QC). The raw data obtained by sequencing contained a small number of reads with sequencing connectors or low sequencing quality. In order to ensure the quality and reliability of data analysis, the raw data should be filtered. The filtering content is: remove the reads with adapter; Remove the reads containing N (N indicates that base information cannot be determined); Remove low-quality reads (reads with base number of Qphred≤20 accounting for more than 50% of the whole read length). After original data filtering, sequencing error rate inspection and GC content distribution inspection, clean reads for subsequent analysis are obtained. If the alignment result passed QC, then the downstream analysis such as Gene Ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were proceeded with. Table 1 briefly summarized the information of sequencing data from each sample.

GO and KEGG Enrichment Analysis of DEGs

Cluster Profiler R package and online tools (http://www.geneontology.org and http://www.genome.jp/kegg) were used to test the statistical enrichment of differential expression genes, and genes with a p-value≤0.05 and a |log2foldchange|≥1 were considered differentially expressed.

Quantitative Real-Time PCR (qRT-PCR) Analysis

The relative mRNA expression level of L5-S2 SCDH target gene was detected by qRT-PCR. After grinding, the spinal cord tissue was placed in tubes with the Trizol reagent (*Sigma-Aldrich,St.Louis, MO, USA*). Then, RNA was isolated from the samples by chloroform extraction and isopropanol precipitation methods.[28] The First-Strand Synthesis Master Mix (*Beijing LABLEAD Biotech Co.,Ltd*) were used for reverse transcription to obtain cDNA. Primer sequences are listed in Table 2. The reaction mixture (10 μL) was prepared using cDNA (1 μL), forward and reverse primers (0.5 μL), PowerUp™ SYBR™ Green Master Mix (5 μL, *Life Technologies*) and Nuclease-Free Water (3 μL). The amplification was carried out with an initial denaturation step at 95°C for 3 min, followed by 44 repeated thermal cycles (95°C for 30s, 60°C for 30s, 72°C for 30s, 95°C for 2 min). The expression of each target gene was normalized using GAPDH, as a reference gene, and the relative levels of genes were quantified by measuring the $2^{-\Delta\Delta Ct}$ method.

Table 1 The Summary of Raw RNA-Sequencing Data Set

Sample	Raw Reads	Clean Reads	Error Rate (%)	Q20 (%)	Q30 (%)	GC pct (%)	Total Map (%)
Sham1	46,276,670	45,533,214	0.02	98.50	95.55	48.20	44,053,575(96.75%)
Sham2	46,794,948	45,985,902	0.02	98.52	95.55	48.34	44,373,887(96.49%)
Sham3	45,506,614	44,607,242	0.02	98.71	96.14	48.61	43,201,831(96.85%)
Sham4	46,064,614	44,145,720	0.02	98.11	94.85	49.63	42,425,355(96.10%)
Sham5	46,084,372	43,677,846	0.02	98.27	95.22	50.13	41,953,196(96.05%)
M1	47,770,326	46,594,146	0.03	97.37	92.63	50.28	44,684,470(95.90%)
M2	45,584,270	44,661,068	0.02	98.37	95.30	49.05	43,066,746(96.43%)
M3	41,820,320	39,885,264	0.02	98.09	94.73	49.08	38,508,016(96.55%)
M4	41,910,576	40,058,854	0.02	98.16	94.88	49.04	38,641,189(96.46%)
M5	46,646,784	43,752,486	0.02	98.12	94.81	48.05	42,229,602(96.52%)
EA1	45,817,160	43,449,002	0.02	98.03	94.66	49.74	41,710,426(96.00%)
EA2	45,460,046	42,942,912	0.02	98.09	94.76	49.72	41,283,785(96.14%)
EA3	47,419,586	46,189,258	0.03	97.65	93.36	50.25	44,364,123(96.05%)
EA4	45,372,718	43,458,436	0.02	98.12	94.86	49.95	41,826,125(96.24%)
EA5	45,710,400	44,238,144	0.02	98.16	94.92	50.05	41,861,490(94.63%)

Notes: It showed the summary of RNA-sequencing data of five samples of each group, including raw reads number, clean reads number, error rate, as well as Q20 (Phred quality scores Q), Q30 GC pct and total map. Raw reads: Number of reads in raw data; Clean reads: Number of reads filtered from raw data; Error rate: Overall data sequencing error rate; Q20: Percentage of bases with Phred value greater than 20 in total bases; Q30: Percentage of bases with Phred value greater than 30 in total bases; GC pct: Percentage of G and C in four bases in clean reads.

Table 2 Sequence of the Primers for Real-Time Quantitative Reverse Transcriptase PCR

Gene	Primer Sequence (5'-3')
Pik3r2	Forward primer: CATGCTCTGTGGTGGTGGATGG
	Reverse primer: ACAAGTGATGCGTGCTGGTAGTG
Akt1	Forward primer: GCCACGGATACCATGAACGAC
	Reverse primer: CGTGGCCGCCAGGTTTTAATA
Casp9	Forward primer: AGCTGGCCCAGTGTGAATAC
	Reverse primer: GCTCCCACCTCAGTCAACTC
JAK2	Forward primer: GGGAAGGCCGATGTTCTGAAA
	Reverse primer: TGTGCAGGAGATGTGGAGGTT
Stat3	Forward primer: GTTTCCGGAGCTGCAGTTTAG
	Reverse primer: GCGGGATTGTACCTCAGCGAT
GAPDH	Forward primer: ACCACAGTCCATGCCATCAC
	Reverse primer: TCCACCACCCTGTTGCTGTA

Statistical Analysis

Data analysis were statistically performed by using IBM SPSS 20.0. Firstly, a normality test was performed. One-way analysis of variance (ANOVA) for multiple comparisons followed by Tukey's post hoc test was performed for the data with normal distribution. All values were given as means ± standard deviations (SD). If the data with non-normal distribution, the Kruskal–Wallis test was performed for the data. The relative expression of mRNA was calculated by $2^{-\Delta\Delta Ct}$, and we used GraphPad prism 6 software to make chart. The results were considered statistically significant at P value<0.05.

Results

Effect of EA on Mechanical Pain Threshold in Rats

The data showed that the mechanical stimulation pain threshold was kept at a stable level throughout the periods in the sham operation group. And during the modeling period, the mechanical pain threshold was significantly decreased ($p<0.01$). Compared with the model group, the pain threshold was gradually increased in the EA group, and significantly increased ($p<0.01$) after the third course of treatment. (Figure 2).

Histological Analyses

Visual observation after dissection: The prostate tissue of the sham operation group was pink, soft and smooth, with uniform texture and no adhesion with surrounding tissues. Compared with sham operation group, the prostate tissue of the model was dark red, with protuberances and hard nodules on the surface, which adhered to the surrounding tissues. By comparison, the histopathological condition of prostate in EA group was improved.

Observation under microscope: The prostate tissue structure of the sham operation group was clear, the glandular cavity was regular and filled with uniform pink secretion. There was no or only a small amount of inflammatory cell in the stroma. Compared with the sham operation group, there were different degrees of inflammatory cell infiltration in the stroma of the model group, which was the most important histopathological feature of CPPS rats. Besides, the acinar lumina was significantly narrowed and atrophied, number of acini and secretions in acinus were reduced. EA treatment significantly alleviated the CFA-induced inflammation in rat's prostate. The shape and edema of acini were improved in varying degrees. (Figure 3).

The Expression of PGE2 and SP in Serum

As shown in Figure 4A and B, the CPPS rats induced by CFA caused a significant increase in the level of PGE2 and SP as compared to the sham operation group ($P<0.01$). EA caused a significant decrease in the level of PGE2 and SP as compared to model group ($P<0.01$).

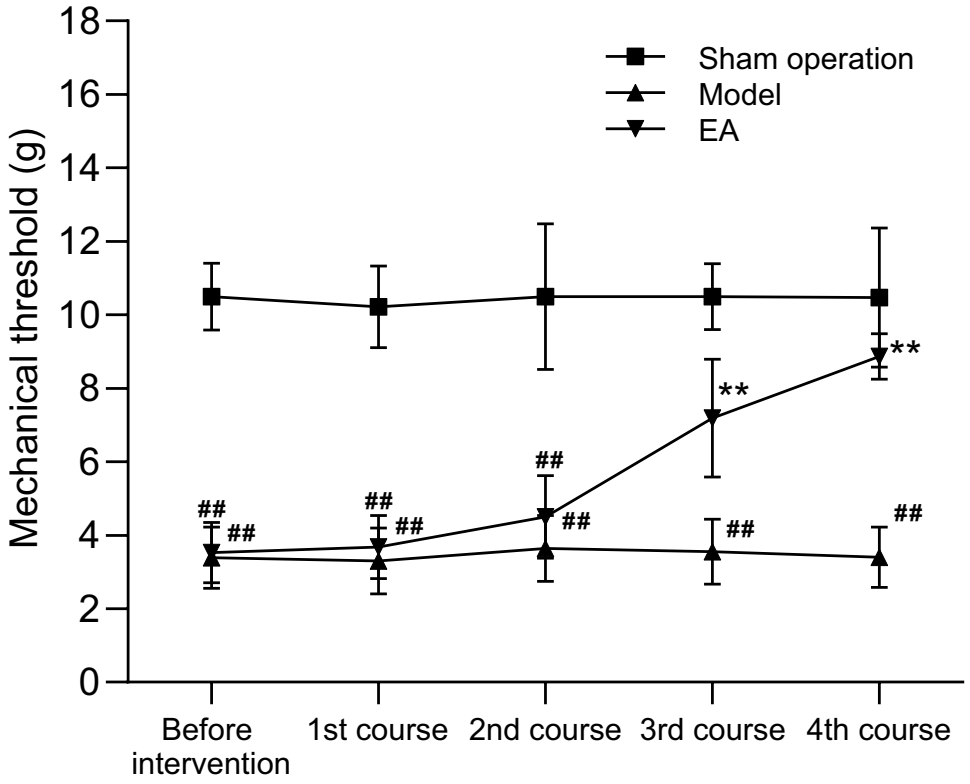

Figure 2 Mechanical pain threshold test before intervention and after each course of treatment. n=10 rat per group for each time point, Values represent mean±SD.
Notes: ##P<0.01 versus sham operation group; **P<0.01 versus model group.

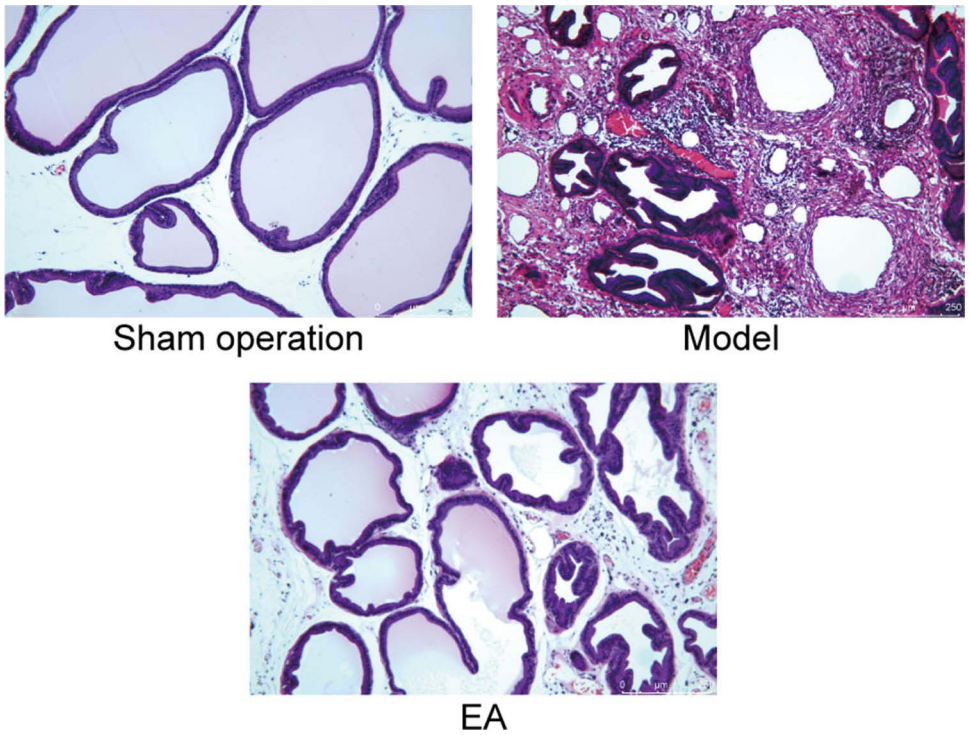

Figure 3 Comparison of prostatic histomorphology in rats. Scale bar=250 μm, HE×100 times. There were inflammatory cell infiltration and atrophic glandular cavity in the model group.

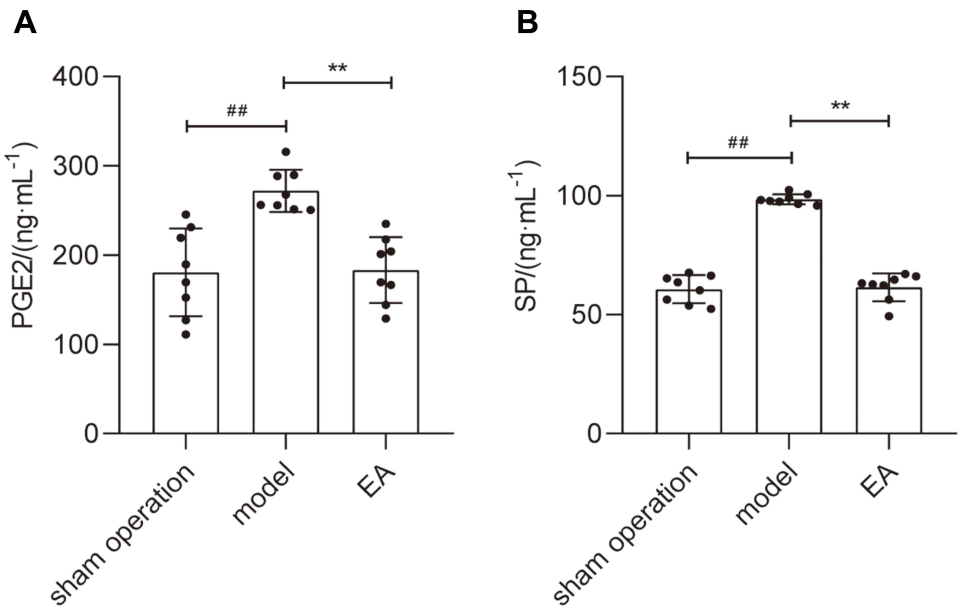

Figure 4 The expression levels of PGE2 and SP in serum of rats in each group (ng·mL^{-1}). (**A**) The expression of PGE2; (**B**) the expression of SP. n = 8 rat per group. ##P<0.01 versus sham operation group; **P<0.01 versus model group.

QC Analysis of Gene Expression Profile of L5-S2 SCDH in Rats

In order to better explore the analgesic mechanism of EA on CPPS, we sequenced and screened the differentially expressed genes in the L5-S2 SCDH of rats in sham operation group, model group and EA group by RNA-Seq. The data showed that a total of about 680 million readings were obtained, and the average reading of each sample is about 45.61 million. The sequencing error rate of all samples were less than 0.03%; Q20 ranged from 97.37% to 98.71%; Q30 ranged from 92.63% to 96.14%; The Total Map indicated that about 94.63–96.85% of the readings of each sample can be mapped to the reference genome (Table 1). The correlation of gene expression level among samples is also a key criterion to test whether the experiments are reliable and whether the samples chosen are reasonable. Therefore, we calculated the correlation value between samples based on normalized expression results (Figure 5A) and drew the correlation heat map (Figure 5B).

Examining Gene Expression Profiles of SCDH

Then, we compared the DEGs of SCDH between sham operation and model group, EA and model group with criteria of |log2FoldChange|≥1 and P value≤0.05, and further displayed in the scatter plot graph. Based upon the criterion, we identified a total of 460 DEGs, including 321 up- and 139 down-regulated compared the model group to sham operation group (Figure 6A). This showed that 460 genes were significantly expressed in SCDH without the interference of surgery. And a total of 559 DEGs, including 250 up- and 309 down-regulated compared EA group to model group (Figure 6B). The genes that were significantly down-regulated after modeling but significantly up-regulated after EA intervention, which were the target up-regulated genes to be sought. Similarly, the genes that were significantly up-regulated after modeling but significantly down-regulated after EA intervention, which were the target down-regulated genes to be sought. The Venn diagram showed that there were 46 DEGs significantly up-regulated and 65 DEGs down-regulated after EA therapy (Figure 6C).

Gene Ontology Analysis of the Differential Genes

To better understand the associated functions of the DEGs in SCDH, GO analysis was used to perform an enrichment analysis and classifications. First, we compared the model group with the sham operation group. The result showed that

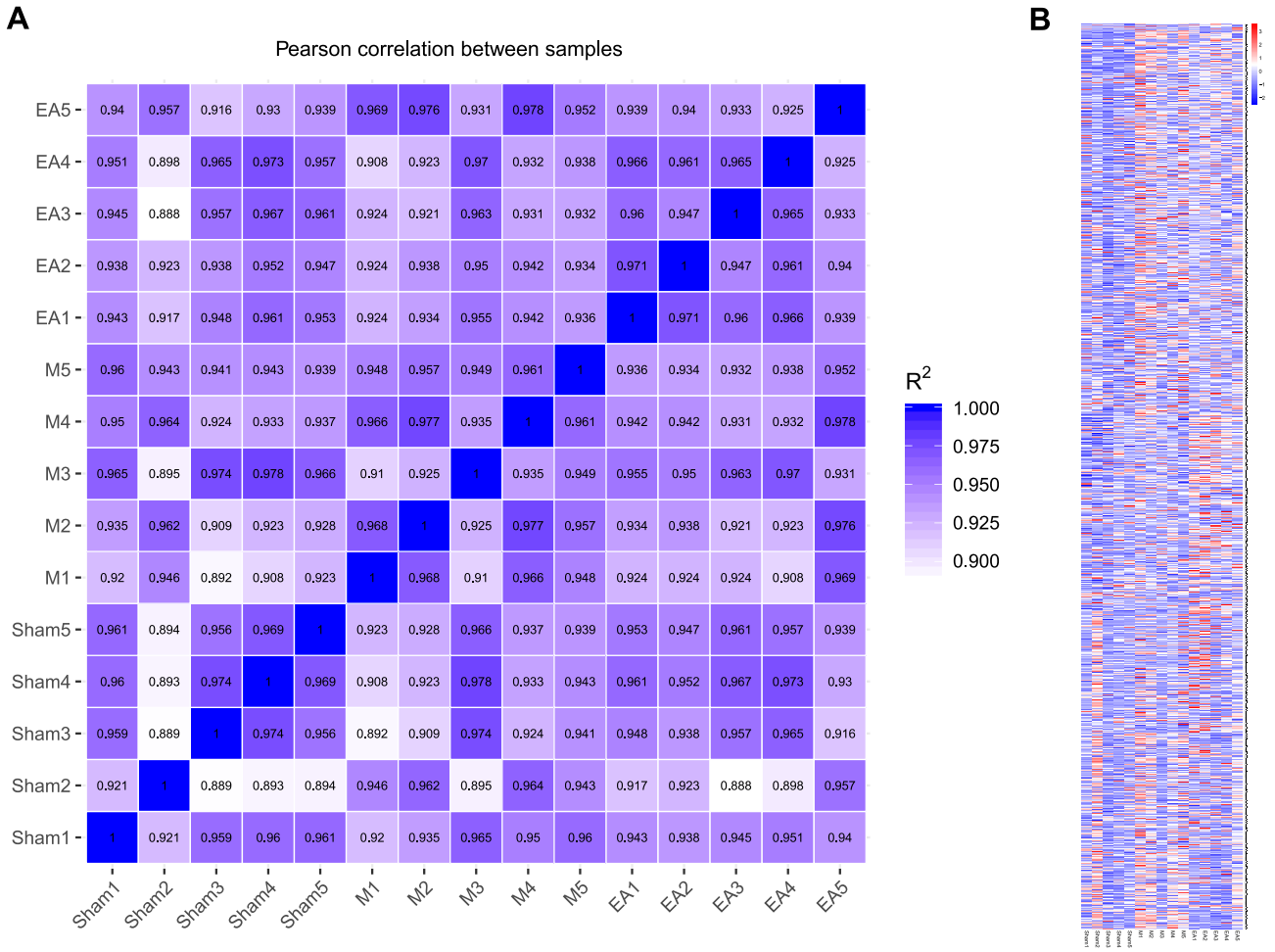

Figure 5 Correlation coefficient diagram and heat map between samples. (**A**) Pearson correlation between samples of sham operation, model and EA groups. The darker the color, the higher the correlation between samples. (**B**) Heat map displaying the hierarchical clustering of DEGs from sham operation, model and EA groups.

GO secondary classification terms involved in biological processes mainly include "immune system process", "localization", "response to stimulus" and "cellular process", mainly enriched in "neutrophil chemotaxis", "leukocyte migration", "neutrophil migration", "granulocyte chemotaxis", "cytokine metabolic process", "leukocyte chemotaxis", etc. Cellular component terms associated with "extracellular region", "organelle" and "cell", mainly enriched in "extracellular matrix", "proteinaceous extracellular matrix", "extracellular matrix component", "axoneme part", etc. Which suggested that organelle and some extracellular components are mainly involved in the transduction of disease-related signals. Moreover, molecular functions were associated with "binding" and "molecular function regulator", mainly enriched in "dynein intermediate chain binding", "receptor ligand activity", "cytokine activity", "receptor regulator activity", etc. Which implying that the activities of cytokines and receptor ligands in SCDH of CPPS rats were significantly affected. Therefore, it was visible that CPPS induced by CFA can activate the immune system, mediate the chemotaxis and migration of leukocytes and neutrophils, and affect the metabolic process of cytokines. Immune dysfunction may be involved in the generation and maintenance of inflammation and pain to a certain extent (Figure 7A). To investigate whether EA has anti-inflammatory and analgesic effects on CPPS by regulating the immune system, we compared the EA group with the model group. It showed that GO can also be enriched in these processes such as "neutrophil chemotaxis", "neutrophil migration", "granulocyte chemotaxis", etc. (Figure 7B). Which suggested that EA may modulate the immune system to produce anti-inflammatory and analgesic effects on CPPS, so as to restore the function of prostate tissue.

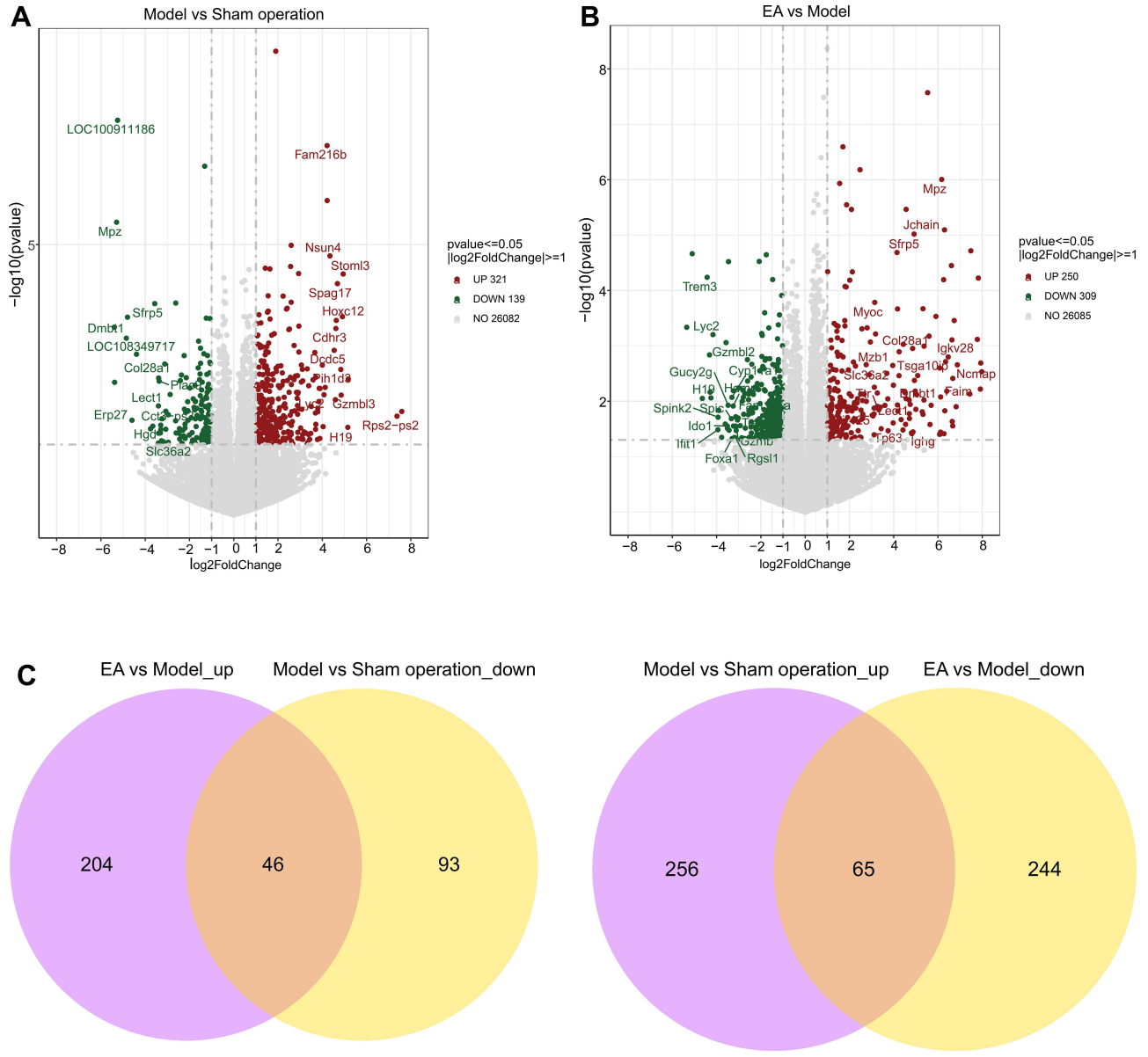

Figure 6 Volcano plot and Venn diagrams. (**A**) Volcano plot of samples with different mRNA expression between sham operation and model group. (**B**) Volcano plot of samples with different mRNA expression between model and EA group. Log2 (Fold Change) is plotted as the abscissa and log10 (p value) is plotted as the ordinate. Significantly up-regulated genes are indicated in red and down-regulated genes are indicated in green. (**C**) Venn shows 46 DEGs were significantly upregulated and 65 DEGs down-regulated after EA therapy.

Analysis of Important KEGG Pathways

Next, we performed KEGG pathway enrichment analysis of the DEGs. The first thing we compared the model group with the sham operation group. We found that 204 pathways were significantly enriched, indicating that the mechanism of chronic pelvic pain caused by CFA may be related to these 204 pathways. We further analyzed these pathways and drew the classification histogram of signal pathways (Figure 8A). The figures indicated the most enriched pathways were focused on "signal transduction" (23 pathways), followed by the "immune system" (16 pathways). And then, we compared the EA group with the model group (Figure 8B). We found that 208 pathways were significantly enriched, and the most enriched pathways were also focused on "signal transduction" (20 pathways), followed by the "immune system" (18 pathways) and "endocrine system" (17 pathways). These results indicated that the effect of EA on CPPS was mainly achieved by affecting the signal transduction of related cells in SCDH and regulating the immune and endocrine

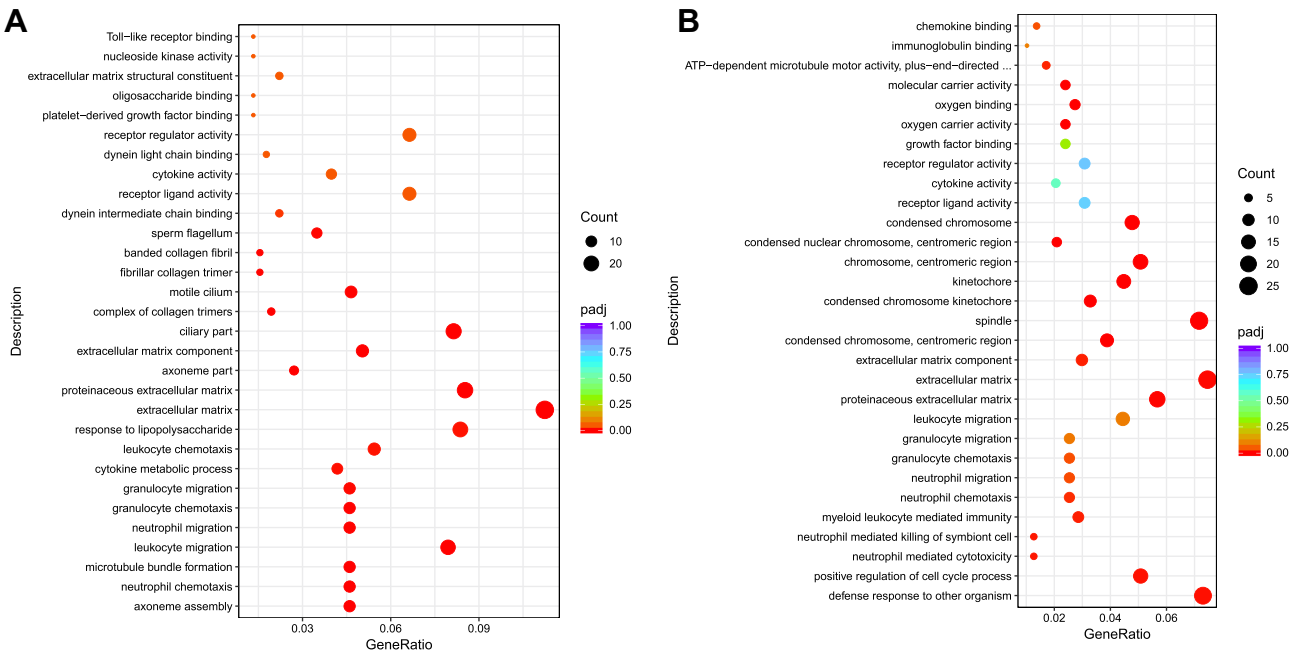

Figure 7 Bubble chart of GO pathway analysis. (**A**) GO pathway analysis of DEGs compared between sham operation and model. The top 20 significant biological processes, cellular components, and molecular functions of DEGs were shown. (**B**) GO pathway analysis of DEGs compared between EA and model.The figure showed that some significant enrichment processes in figure a are still significantly expressed after EA. The overlapping part of the two figures is the target gene function of this study.

system functions. Scatter diagrams of KEGG measured the degree of enrichment by rich factor, q value and gene number of the enriched pathway. Based upon the enrichment analysis of KEGG pathway, we found that among the differential pathways related to this study, the differential genes in the model group were significantly enriched in the classifications of "IL−17 signaling pathway", "Cell adhesion molecules (CAMs)", "TNF signaling pathway", "JAK−STAT signaling pathway" and "PI3K−Akt signaling pathway" compared with the sham operation group (Figure 9A). Similarly, these pathways were also significantly enriched in the comparison between EA group and model group (Figure 9B). This demonstrated that EA can activate or inhibit the expression of the above pathways to a certain extent to alleviate related symptoms of CPPS.

Identifying PI3K-AKT/JAK-STAT Signaling Pathway in SCDH as a Key Role in EA Mediating the Pain and Inflammation Responses in CPPS Rat Model

KEGG results showed that the high enrichment of PI3K-Akt signaling pathway may be an important target signaling pathway for EA regulation of inflammation and pain in CPPS. A series of signal transduction pathways such as Toll like receptor, B cell receptor and JAK/STAT can be used as upstream pathways to transmit PI3K-Akt signals. Because JAK/STAT signaling pathway is also highly expressed in CPPS and is critical to the immune response, we focused on the JAK/STAT pathway. JAK can be activated by cytokines, and activated JAK further activates PI3K, which then activates the key metabolite PIP3 (phosphatidylinositol-3, 4, 5-diphosphate). Phosphorylation of PIP3 then promotes the activation of AKT, which further activates downstream regulatory pathways (Figure 10). Pik3r2, Akt1 and Casp9 are the key genes in PI3K-AKT pathway, Jak2 and Stat3 are the key genes in JAK/STAT pathway. We detected the up regulation genes of Pik3r2, Akt1 and Casp9 and the down regulation genes of Jak2 and Stat3 after EA by RNA-Seq.

Verification of the Genes from RNA-Seq Data by qRT-PCR

Then, we set to validate the reliability of RNA-Seq data via qRT-PCR. Our results revealed that the mRNA expression levels of Pik3r2, Akt1 and Casp9 were all significantly upregulated, whereas Jak2 and Stat3 were both significantly downregulated in SCDH after EA, which confirmed the consistent and validation of our data (Figure 11A–E).

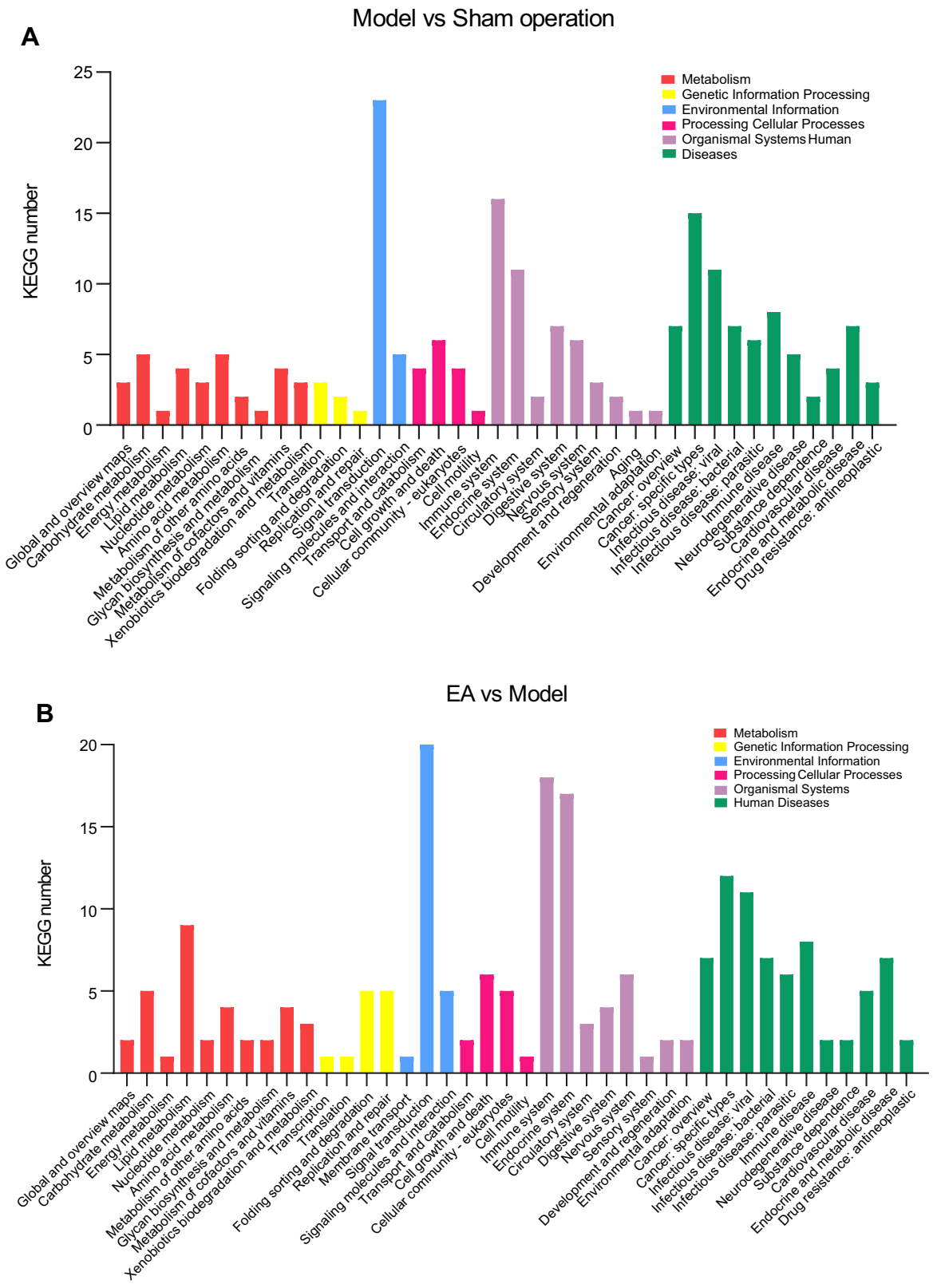

Figure 8 KEGG secondary classification histogram. (**A**) KEGG classified statistical histogram of model vs sham operation. (**B**) KEGG classified statistical histogram of EA vs model. Classification description terms is plotted as the abscissa and KEGG number is plotted as the ordinate.

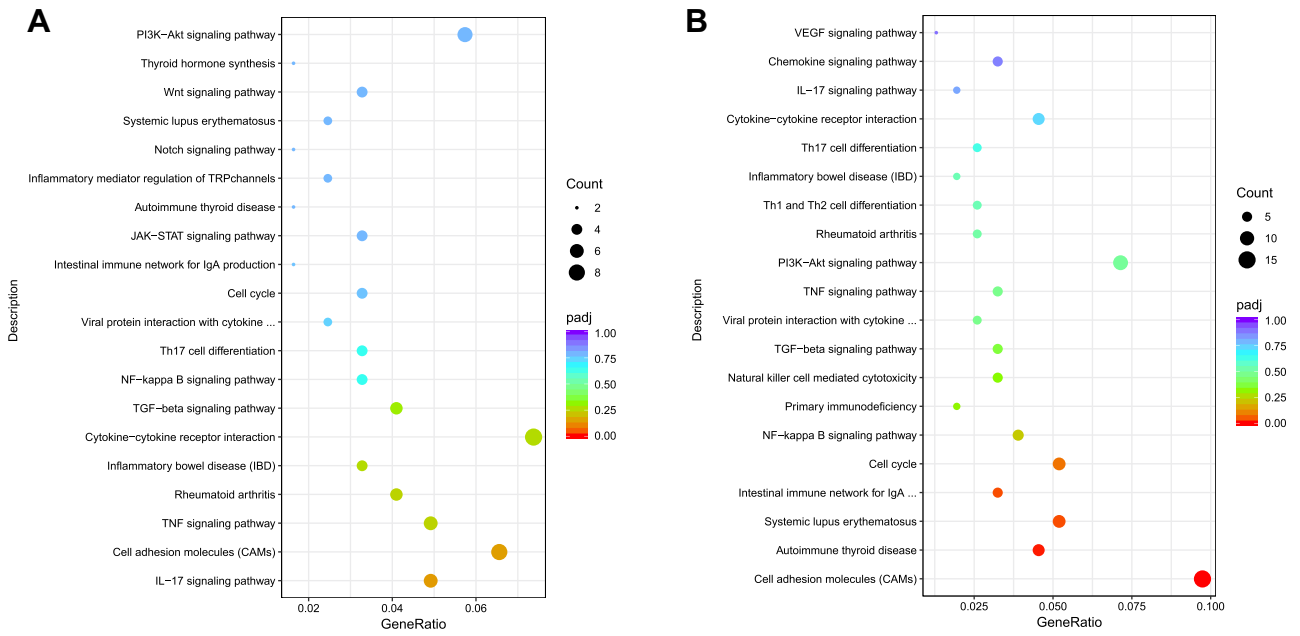

Figure 9 Bubble chart of KEGG pathway analysis. (**A**) KEGG pathway analysis of DEGs of model vs sham operation. (**B**) KEGG pathway analysis of DEGs of EA vs model. Bubble plots showing the significant pathways for up- and down-regulated DEGs. Larger bubbles indicate higher number of genes. The color of each bubble reflects significance (p value). The top 20 disease-related pathways were shown.

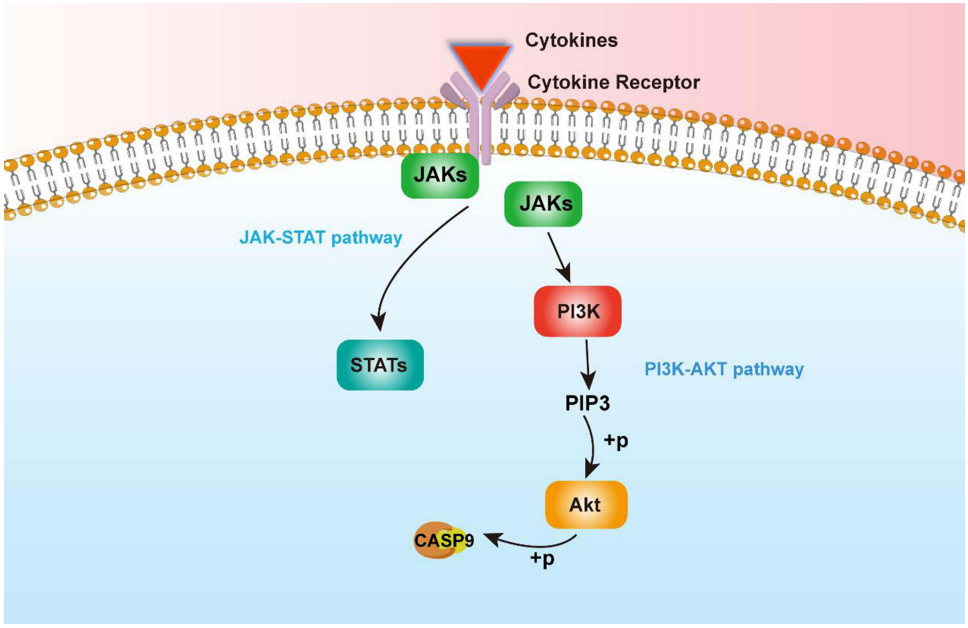

Figure 10 The diagram of PI3K-Akt/JAK-STAT pathway.

Discussion

Pelvic pain is the most prominent symptom of CPPS and is characterized by chronic, persistent, site variability and refractory.[4] Inflammation is an important factor in inducing pain and is the most common type of pain in clinical practice. A variety of cytokines will be released locally to continuously stimulate peripheral pain receptors to induce pain, which is the interaction between inflammatory response mediated immune response and nociceptive receptor sensitization.[29] As in this study, we detected that the mechanical pain threshold of CPPS rats decreased significantly, the expressions of PGE2 and SP in serum increased significantly, and a large number of inflammatory cells infiltrated in prostate tissue. At present, more and

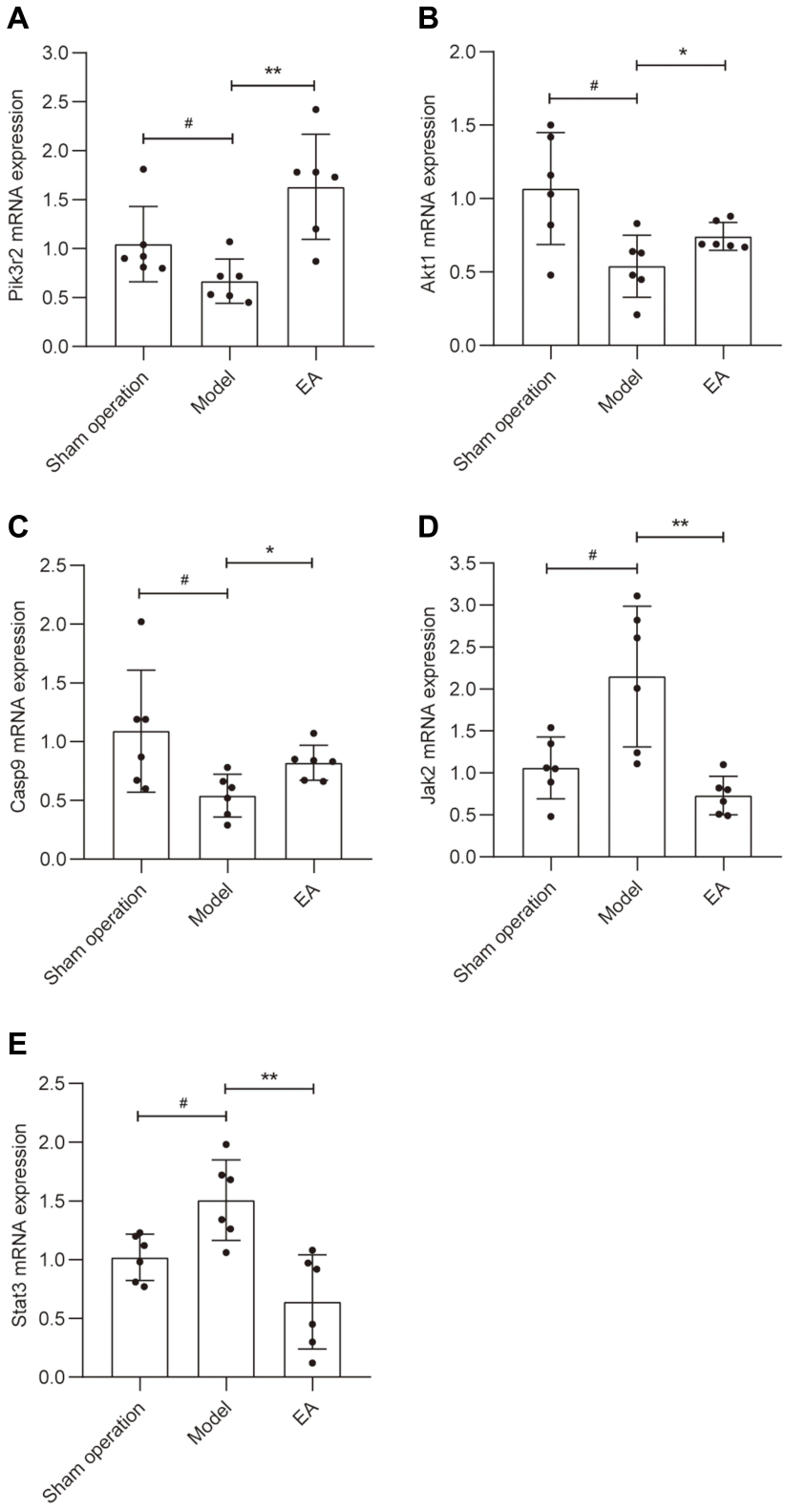

Figure 11 Relative mRNA expression levels of the rats in the SCDH. (**A**) Pik3r2; (**B**) Akt1; (**C**) Casp9; (**D**) Jak2; (**E**) Stat3. GAPDH was used as a housekeeping gene. n = 6 rat per group. #P<0.05 versus sham operation group; *P<0.05, **P<0.01 versus model group.

more studies have confirmed that central sensitization is the cause of neuropathic pain and visceral related chronic pain.[30] Central sensitization is neuropathic pain driven by neuroinflammation in peripheral and central nervous systems, hyperexcitability of spinal dorsal horn neurons and dorsal root ganglion is considered to be an important cause of pathological pain.[31] Neuroanatomy shows that CPPS pain is not only localized in the prostate but also involved in lumbosacral, lower abdomen, perineum, posterior urethra, testicles, penis and anus, these areas are mainly innervated by the ilioinguinal and perineal nerves, and nerve fibers are mainly derived from the lumbosacral plexus in the spinal cord.[19] The spinal dorsal horn is the first integrated regulation center of nociceptive information transmission. The secondary lesion of L5-S2 spinal dorsal horn is an important reason for the abnormal nerve conduction and regulation mechanism dominating the prostate.[32]

Acupuncture has unique advantages in analgesic treatment. Research showed that acupuncture can regulate the function of immune system and has bidirectional benign adjustment effect on the number, activity and function of different immune cells. It has different regulatory effects on immune molecules such as immunoglobulin, complement system and cytokines. It can also participate in the regulation of immune response through the influence on immune cells and immune molecules, so as to enhance the immune function of the body.[17] Acupuncture signals can also be transmitted into the spinal cord through the excitation of deep receptors and nerve endings at the acupoints. On the one hand, after the afferent impulse enters the spinal dorsal horn, it affects visceral activity and pain in adjacent segments through segmental connections in the spinal cord. More importantly, it can achieve analgesic effect by activating the high center to release downward inhibition of impulse.[17] EA has a great advantage in the analgesic treatment of clinical pain diseases. However, the efficacy of EA analgesia is also affected by many factors, especially the choice of EA frequency. Han Jisheng's research showed that the different frequencies of EA can activate different neurotransmitters and neuropeptides in the brain. Applying 2 Hz electrical stimulation on acupoints can cause a large number of enkephalins and endorphins to be released from the brain and spinal cord, while 100 Hz electrical stimulation can cause a large number of dynorphins to be released from the spinal cord. Both have analgesic effects, but they have their own characteristics. If alternating dense waves of 2 Hz and 100 Hz are used, the above three peptides can be released at the same time to play a synergistic analgesic effect. Therefore, 2/100Hz dense waves were used in this study.[33]

In the present study, we measured the plantar mechanical pain threshold of CPPS rats in different intervention periods of EA. The results showed that the pain threshold of rats in the EA group showed a continuous increasing trend, and the pain threshold significantly increased after the third course of acupuncture treatment, which proved that EA at Guanyuan (CV4), Zhongji (CV3), Sanyinjiao (SP6) and Huiyang (BL35) did have analgesic effect on CPPS. EA could also improve the pathological morphology of prostate tissue in CPPS rats, restore the structural damage and reduce the infiltration of inflammatory cells in interstitial tissue. EA can also reduce the expression of PGE2 and SP in the serum of CPPS rats. As a major pro-inflammatory PGE, PGE2 is a highly active inflammatory mediator in the inflammatory response and can be involved in the production and regulation of inflammatory pain, and can regulate pain in both peripheral system and nervous system. PGE2 plays an important role in pain by directly stimulating nerve endings, increasing the sensitivity of nociceptors to pain-causing substances, or initiating pain-mediated signal transduction pathways to cause pain.[34] SP is an important transmitter of pain information and consists of the release of damaged C and δ fibers, which can transmit pain signals from the primary nerve to the higher nerve. In addition to transmitting pain information, SP can also stimulate lymphocytes and macrophages to release more inflammatory mediators, further aggravate inflammatory response and pain, and form a vicious circle.[35] Therefore, EA at Guanyuan (CV4), Zhongji (CV3), Sanyinjiao (SP6) and Huiyang (BL35) can play an analgesic role by participating in the body's inflammatory reaction and immune response, restoring the pathological state of diseased tissues, repairing damaged structures and tissues, and inhibiting the expression of inflammatory factors and pain-causing substances.

RNA-Seq can study gene function and gene structure from a holistic level, and reveal specific biological processes and molecular mechanisms in the process of disease occurrence.[36] In this study, a total of 559 differentially expressed genes were detected after comparison between the EA group and the model group, among which 250 genes were significantly up-regulated and 309 genes were significantly down-regulated. Then, the model group was compared with the sham operation group. After excluding the interference of operation, a total of 46 up-regulated genes and 65 down-regulated genes were screened out, which may be most relevant to the molecular mechanism of EA analgesia.

GO analyses revealed that the most significantly enriched biological processes of genes in SCDH after EA were related with granulocyte chemotaxis, migration and cytokine metabolic. This result implied that EA may be the predominant mechanism involved in the pathophysiology of CPPS by regulating immune response and inflammatory response. KEGG analysis revealed that the most abundant pathways involved in EA are related to "signal transduction pathways" and "immune system". The "PI3K−Akt signaling pathway" was significantly upregulated, which was consistent with previous studies.[37] Phosphatidylinositol-3-kinase (PI3K) is an intracellular phosphatidylinositol kinase, and Akt is a direct downstream effector of PI3K. PI3K−AKT pathway is an important intracellular signal pathway, which regulates the cell cycle by transmitting various upstream signals.[38] PI3K−AKT signaling pathway is an important signal pathway of cellular anti-inflammatory response and also an important target signaling pathway of pain regulation. Previous studies have shown that the PI3K−AKT signaling pathway can be involved in the spinal central mechanism of neuropathic pain, inducing the activation of spinal microglia and the release of inflammatory factors, and promoting the production of pain behaviors to a greater extent.[39,40] PI3K−AKT signal can be activated by a series of signal transduction pathways including "Toll-like receptor", "B-cell receptor" and "JAK-STAT", among which JAK-STAT, as an important biological process involved in cell proliferation, differentiation, apoptosis and immune regulation, is activated by inflammatory cytokines after spinal cord neuron injury. Studies have shown that STAT3 phosphorylation can induce an immune response, activate microglia, astrocytes and other glial cells, and promote the occurrence of neuropathic pain.[41] We further verified by qRT-PCR, that the expression levels of Pik3r2, Akt1 and Casp9 genes in the L5-S2 spinal dorsal horn of CPPS rats were significantly up-regulated, and the expression levels of Jak2 and Stat3 genes were significantly down-regulated. Therefore, our data suggest that the PI3K−AKT/JAK-STAT signaling pathway in the L5-S2 spinal dorsal horn may play a role in mediating CPPS pain symptoms and neuroinflammation, and EA can intervene in this process through acupoint-nerve conduction.

In the present study, cluster analysis indicated a high level of concordance of datasets within sham operation, model and EA group samples and a clear segregation between the three groups. However, we noticed that the correlation coefficient between sample 2 in the sham operation group and other samples in the group was low. Considering the number of samples, we did not eliminate these data, so our present RNA-Seq dataset may possess certain weakness. However, RNA-Seq is a preliminary data screening method, qRT-PCR assay should be followed for further validation of targets. In this study, only genes and signaling pathways with significant differential expression were verified and it was concluded that EA of Guanyuan (CV4), Zhongji (CV3), Sanyinjiao (SP6) and Huiyang (BL35) may alleviate CPPS inflammatory response and reduce pain sensitivity by activating PI3K−AKT/JAK-STAT signaling pathway in the L5-S2 spinal dorsal horn of CPPS rats, regulating the expression levels of Pik3r2, Akt1, Casp9, Jak2 and Stat3 genes, and regulating the expression levels of pain-related factors PGE2 and SP. So, this interpretation has some limitations. Namely, other mechanisms may be also involved in the anti-inflammatory and analgesic effects of EA that were not investigated in the present study.

Chronic pelvic pain is the most typical symptom of CPPS, it has a wide range of pain sites, a long course of disease and the lingering is difficult to heal. In addition to chronic pelvic pain, CPPS usually accompanied by psychosocial disorders, like depression, anxiety and mood alterations. The study also found that the pain symptoms of CPPS were also associated with brain-related complications, increased susceptibility to seizures and displayed higher stress levels.[42,43] Therefore, in the treatment of CPPS, it is necessary to relieve pain symptoms, EA just provides a new idea and method for the treatment of CPPS.

Conclusion

Inflammation of local prostate tissue is an important factor inducing pain. Our previous studies showed that EA at Guanyuan (CV4), Zhongji (CV3), Sanyinjiao (SP6) and Huiyang (BL35) points can significantly increase the pain threshold of heat stimulation in CPPS rats, inhibit the expression of P2X7R, NLRP3, caspase-1 and IL-18 mRNA in prostate tissue, and reduce the expression level of TNF-α, IL-1β and PGE2 by activating P2X7R/NLRP3 pathway. This research through transcriptome analysis also showed that EA could activate PI3K-Akt/JAK-STAT pathway in L5-S2 spinal dorsal horn, reduce the levels of pain-related factors PGE2 and SP in serum by regulating the expression of key

genes Pik3r2, Akt1, Casp9, Jak2 and Stat3 in this pathway to reduce the pain sensitivity of CPPS rats. This may be the central therapeutic targets of EA in the treatment of pain of CPPS.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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