TLR-NF-κB SIGNALING PATHWAY-BASED INVESTIGATION ON THE INFLUENCE OF ACUPUNCTURE APPLICATION OF RAW AND FRIED WHITE MUSTARD SEED PLASTERS ON NASAL MUCOSA IN RATS WITH ALLERGIC RHINITIS

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ABSTRACT

Objective: To investigate the expression of TLR-NF-κB signaling pathway in nasal mucosa of rats with allergic rhinitis (AR) and the effect of therapy with acupoint application of raw and fried white mustard seed plasters on it.

Methods: A total of 50 Wistar rats were randomly divided into the normal group, the model group, the fluticasone propionate group, the raw white mustard seed plaster group and the fried white mustard seed plaster group, with 10 rats in each group. OVA hen ovalbumin was used to prepare rat models of AR, and they were treated by intranasal administration with fluticasone propionate and acupoint application therapy respectively, and behavioral scoring was performed before and after treatment. After treatment, rats in different groups were treated with HE staining to observe the shape of nasal mucosa. The expression of TLR4 and NF-κB mRNA in the nasal mucosa was detected by the reverse transcriptase polymerase chain reaction (RT-PCR) and the expression quantities of TLR4 and NF-κB in nasal mucosa were determined by Western blotting (WB).

Results: Behavioral scores, the mRNA and protein expression of TLR4 and NF-κB in the nasal mucosa of the model group were significantly higher than those in the normal group (P< 0.05). HE staining showed that the nasal mucosa of rats in the model group was destroyed obviously, a large number of eosinophils and other inflammatory cells infiltrated. After treatment, behavioral scores, mRNA and protein expression quantities of TLR4 and NF-κB in the fluticasone propionate group, the raw white mustard seed plaster group and the fried white mustard seed plaster group were significantly lower than those in the model group (P<0.05). HE staining showed that inflammatory cell infiltration was significantly decreased, and the curative effect was more significant in the raw white mustard seed plaster group than in the fluticasone propionate group or the fried white mustard seed plaster group (P<0.05), but there was no significant difference between the fried white mustard seed plaster group and the fluticasone propionate group (P<0.05).

Conclusion: The acupoint application can reduce the expression of TLR4 and NF-κB mRNA and proteins and relieve nasal inflammatory symptoms through regulating the TLR-NF-κB signaling pathway in nasal mucosa of rats, thus treating allergic rhinitis (AR). besides, the effects of raw white mustard seed plaster are more significant than fried white mustard seed plaster.

Keywords: Acupoint application, Toll-like receptor, TLR-NF-κB pathway, Allergic rhinitis.

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Introduction

Allergic rhinitis (AR) is a non-infectious inflammatory disease of the nasal mucosa, which is involved in a variety of immunocompetent cells and cytokines. The cause of the disease is complex, and it has certain relationship with genetic, environmental, mental and other factors, and recurrent attacks seriously affect the quality of life of patients. A large number of literature reports¹-⁴ show that acupoint application, as an external medical therapy of Chinese medicine, can regulate the immune function of the body, and plays an important role in the prevention and treatment of AR.

TLR-NF-κB signaling pathway is a signaling pathway closely related to anti-inflammatory and immune mechanisms found in recent years. Studies have shown that⁵ the pathway plays an important...
role in the pathogenesis of AR. Besides, the clinical study shows that the curative effect of raw white mustard seed plaster is more significant than fried white mustard seed plaster in treatment of AR. Therefore, the rat model of AR was established with hen ovalbumin, and the expression of TLR4 and NF-κB in nasal mucosa of rats was detected by RT-PCR and Western blot in this study to explore the effect of acupoint application on the nasal mucosa of rats with AR, compare the curative effect of raw and fried white mustard seed plaster, and study the mechanism through experiments.

Materials and reagents

Experimental animals and grouping
A total of 50 Wistar rats of which the weight was 250-300g and which were 6 weeks old were provided by the animal experimental center of Zhejiang Chinese Medicine University. The number of animal license SYXX (Zhejiang) was 2013-0184. The experiment followed the experimental animal environmental installation GB14925-2001, and complied with the animal welfare and ethical principle. The experimental scheme was approved by the Animal Ethics Committee of Zhejiang Chinese Medicine University. The animals were fed in cage at room temperature of 21-25 degrees and humidity of 60-70%, and they were fed freely with normal diet. After 12 hours of day and night, and the experiment was conducted after 1 week of adaptive feeding.

Main instruments and reagents
The main instruments and reagents used in the study include fluticasone propionate nasal spray (GlaxoSmithKline, S.A), hen ovalbumin (OVA, Sigma), NF-κB p65 antibody (made by CST company, Article No. 8242S), rabbit anti-rat TLR4 polyclonal antibodies (made by Novus company, Article No. NB100-56566), raw white mustard seed plaster (raw white mustard seed, asarum, Euphorbia kansui, Corydalis yanhusuo), fried white mustard seed plaster (fried white mustard seed, asarum, Euphorbia kansui, Corydalis yanhusuo) prepared by the First Affiliated Hospital of Zhejiang Chinese Medicine University, PCR amplification instrument (Step One Plus) and gel electrophoresis imaging analysis system (Image studio).

Experimental methods

Preparation of AR model
50 rats were randomly divided into 5 groups, namely, the control group, the model group, the raw white mustard seed plaster group, the fried white mustard seed plaster group and the fluticasone propionate group, with 10 rats in each group. All rats except rats in the control group were treated with intraperitoneal injection of freshly prepared 1ml of normal saline (including OVA 0.3 mg and Al (OH)3 30 mg) solution for sensitization since the first day, once every other day, for 7 times. On the 15th day, booster immunization was conducted, and the rats were treated with nasal inhalation of 5% of OVA (50ug on each side) and inhalation of 1% of OVA spray for 10 min, and the treatment lasted for 7 days. The control group were treated with intraperitoneal injection, nasal inhalation and atomization of the same volume of normal saline, and the method was the same as above.

Standard of successful modeling
Behaviors of all the rats such as snot, sneezing and running nose were observed immediately after sensitization of the last nasal inhalation. Each rat was placed in a transparent box and were observed for 30 minutes. The scoring method is shown in Table 1, and addition result of 3 symptom scores is the total symptom score. The total score ≥ 5 points indicates successful modeling(7) (Table 1).

<table>
<thead>
<tr>
<th>Score</th>
<th>Sneezing (times)</th>
<th>Nasal scratching</th>
<th>Running nose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-3</td>
<td>Wiping nose slightly</td>
<td>To the nostrils</td>
</tr>
<tr>
<td>2</td>
<td>4-10</td>
<td>Scratching the face again and again</td>
<td>To anterior naris</td>
</tr>
<tr>
<td>3</td>
<td>&gt;11</td>
<td>Scratching nose continuously</td>
<td>Covering the face and hanging on the mustache</td>
</tr>
</tbody>
</table>

Table 1: Evaluation of symptom scores of AR.

Treatment methods
Groups were treated respectively after modeling. The control group were fed normally, and the model group were treated with nasal inhalation of 5ul normal saline in every nostril, once every other day. Fur on the back of rats in two acupoint application groups were shaved. 0.5cm*0.5cm of plasters were applied on their bilateral Feishu, Pishu and Shenshu Point (locations were chosen referring to Experimental Acupuncture mainly edited by Li Zhongren). Plasters were fixed with tapes and they were removed after 2 hours, every other day, a total of 7 times.
The fluticasone propionate group was treated by nasal inhalation, 5μl per nostril, once every two days.

**Nasal mucosa tissue sampling**

Rats were decapitated and the mandible was cut. The nasal cavities were cracked from the middle nasal suture. 5 rats were randomly selected from each group, and the nasal septum cartilage with nasal mucosa was removed. The nasal cavities of the other 5 rats were cracked by the same method as above. The nasal septum and nasal outer wall mucosa tissues were removed and placed in EP tube. After sampling, the tissues were immediately put into liquid nitrogen for preservation.

**Detection indexes**

**Behavioral score**

After treatment, each rat was put into a transparent box. Symptoms such as nasal itching, sneezing and running nose within 30 min were observed immediately and record. The coring standard of symptoms was the same as Table 1.

**Pathological observation of nasal mucosa**

The nasal septum tissues with nasal mucosa were collected by the above-mentioned method, and they were fixed in 10% neutral formaldehyde solution for 24h after being rinsed with 4 degrees of normal saline, and then they were embedded with paraffin and cut into slices. 5 slices of each specimen were selected for with HE staining to observe pathological changes in nasal mucosa tissues under light microscope.

**Detection of TLR4 and NF-κB mRNA in nasal mucosa**

Nasal mucosa tissues were collected and RNA was extracted by Trizol one-step method. For TLR4, the forward primer was 5’- CGCTCTGGCATCATCTTCCCTCAT - 3’, and the reverse primer was 5’ - AGCATATTGCTCCTCCCACTCG-3’, while for NF-κB p65, the forward primer was 5’-GGGCTGACCTGAGTCTTCTG-3’, and the reverse primer was 5’- GCTGCCTTGCTGTTCTTGAG-3’. For the internal control of β-actin, the forward primer was 5’-GGACAGTCAAGGCTGAATC-3’, and the reverse primer was 5’-ATGGGTGGTGAAGACGCCAGTA -3’. The extracted total RNA was reversely transcribed into cDNA, and the procedures were performed in accordance with the TaKaRa reverse transcription kit Prime Script TM RT reagent. The reverse transcription reaction system was added to the PCR tube without RNase, and the condition for reverse transcription reaction was 37 degrees for 15min, 95 degrees for 5s, and the cDNA was stored at 4 degree in refrigerator. Real-time quantitative PCR was conducted, and the PCR system was 20 μL. All reaction information and data were collected by Step One Plus, Ct values were measured and calculated by computer software, and the transcription level was calculated by formula 2-ΔΔCt.

**The protein expression of TLR4 and NF-κB in nasal mucosa was detected by Western Blotting**

The nasal mucosa tissues were taken and proteins were extracted according to the kit instructions. Quantification, electrophoretic separation after denaturation and PVDF transmembrane of proteins were performed, and then they were incubated with primary antibodies (TLR4 1: 200, NF-κB 1: 200), closed overnight at 4 degrees, washed with TBST for 3 times, 10 minutes each times. Mice second antibodies (TLR4 1: 10000) and rabbit anti -second antibodies (NF-κB 1:10000) were added, and then incubated at room temperature for 1.5 hours, and washed with TBST for 3 times, 10 minutes each times. Marker was calibrated and scanned by ODYSSEY scanner. Image studio image analysis software was used to determined the average gray values of TLR4, NF-κB protein and internal reference β-actin protein in the nasal mucosa of rats.

**Statistical processing**

SPSS 22 statistical software was used for statistical analysis, results of measurement data is represented by x±s, analysis of variance (ANOVA) was used for comparison of data among groups, comparison between two groups was performed by LSD method, and P<0.05 is the inspection standard of the statistically significant difference.

**Results**

**Behavioral scores of rats in different groups**

Figure 1 showed that before treatment, compared with the control group, scores of AR symptoms in the other groups were increased (P<0.05), and the total score of each group was higher than 5 points, indicating that modeling of AR was successful.
After treatment, scores of the fluticasone propionate group and the fried white mustard seed plaster group were significantly lower than those of the model group, with great significant differences (P<0.05). Compared with the fluticasone propionate group and the fried mustard seed plaster group, the decrease was more significant in the raw mustard seed plaster group (P<0.05) but there was no significant difference between the fried white mustard seed plaster group and the fluticasone propionate group (P>0.05). The results showed that fluticasone propionate nasal spray and acupoint application can effectively control the symptoms of AR, and effects in the raw white mustard seed plaster group were significantly better than those in the fried white mustard seed plaster group (Fig. 1).

HE staining results of nasal mucosa

The pathological changes of nasal mucosa and the degree of eosinophil infiltration in each group were shown in Figure 2.

![Fig. 1: The symptoms of allergic rhinitis before and after treatment in each group of rats. (x̅±s), n=10 #p<0.05 compared to control group; *p < 0.05 compared to model group](image)

**HE staining results of nasal mucosa**

The pathological changes of nasal mucosa and the degree of eosinophil infiltration in each group were shown in Figure 2.

![Fig. 2: Pathological changes of nasal mucosa in rats of each group (× 400). (A) Normal control group; (B) AR model group; (C) Fluticasone propionate group; (D) White mustard seed acupoint application group (E) Fried white mustard seed acupoint application group.](image)

It can be seen from the figure that the structure of nasal mucosa epithelium in the control group was complete, the cell arrangement was neat, and the inflammatory cell infiltration such as eosinophils was not obvious. The structure of nasal mucosa epithelium was incomplete in the AR model group, with a large number of shedding and a large number of eosinophils infiltration in the submucosa. In the fluticasone propionate group and the fried white mustard seed plaster group, the nasal mucosa was exfoliated with a small amount of eosinophilic infiltration. The structure of nasal mucosa in the raw white mustard seed plaster group was less exfoliated, and the infiltration of eosinophils in mucosa was not obvious

**mRNA expression of TLR4 and NF-κB in nasal mucosa**

The mRNA expression of TLR4 and NF-κB in the model group was significantly higher than that in the control group, with a great significant difference (P<0.05). The mRNA expression of TLR4 and NF-κB in the fluticasone propionate group and the raw and fried white mustard seed plaster groups was significantly lower than that in the model group (P<0.05), and the decrease in the raw white mustard seed plaster group was more significant (P > 0.05). See Figure 3.

![Fig. 3: mRNA expression of TLR4 and NF-κB in nasal mucosa of rats (x̅±s, n=5) #p<0.05 compared to control group; *p < 0.05 compared to model group](image)

**Protein expression of TLR4 and NF-κB protein in nasal mucosa**

The protein expressions of TLR4 and NF-κB in the nasal mucosa of rats are shown in Figure 4 and Figure 5. The results showed that the protein expressions of TLR4 and NF-κB in the model group was significantly higher than that in the control group (P<0.05). Compared with the model group, proteins of the two indexes were decreased in the raw and fried white mustard seed plaster groups and the fluticasone propionate group, and
The difference was statistically significant (P<0.05), and the decrease was more obvious in the raw white mustard seed plaster group than in the fluticasone propionate group and the fried white mustard seed plaster group (P<0.05).

Discussion and Conclusion

Most of the studies show that the main pathogenesis of allergic rhinitis is immune disorder caused by constitution, environment and other factors, resulting in increased differentiation of Th0 cells into Th2 cells, causing Th1/Th2 immune imbalance, which manifested as inflammatory reactions such as the increase of eosinophils and mast cells and over-expression of Th2 cells. In recent years, the immune inflammatory regulation mechanism of AR by TLR-NF-κB pathway has attracted much attention. Toll-like receptor (TLR) is a natural immune receptor, it is firstly found in Drosophila melanogaster, and it is widely distributed in mammalian body. It can directly identify pathogens and products, and its combination with them triggers signal transduction, which leads to the release of inflammatory mediators.

TLR4 is widely expressed in dendritic cells, macrophages and mucosal epithelial cells of the nasal mucosa of the respiratory tract(8), and it is also expressed in glandular cells and a few cellular matrix. After recognizing ligands, TLR4 can activate the signaling pathway, induce a series of gene activation, increase the co-stimulator, enhance the antigen presentation ability, and increase the secretion of inflammatory factors, thus triggering the immune response. Studies(9) have shown that the up regulation of TLR4 expression occurs in the nasal mucosa of patients with seasonal allergic rhinitis. The expression of TLR4 in the nasal mucosa of normal rats is low(10), but the expression is high in the nasal mucosa of rats with AR, with the most obvious increase in smooth muscle epithelium (P<0.01).

Sen and Baltimore(11) found nuclear transcription factor NF- κB in mature B lymphocytes and plasma cells for the first time. NF-κB usually exists in the form of p65 or P50 heterodimer, which can regulate immune related receptors, inflammatory factors, cytokines, and so on. As an inducible nuclear transcription factor, the activation of NF-κB can lead to the release of a large number of pro-inflammatory cytokines, resulting in inflammatory responses(12-13).

Research results of Zhao Jiandong, et al.(14) showed that the expression of NF-κB in nasal mucosa cells of rats with AR was significantly higher than that in the control group. Research results of Zhang Min, et al.(5) found that the expression levels of NF-κB and TNF-α in nasal mucosa tissues of AR model rats prepared by ovalbumin were significantly higher than those in the normal group (P<0.05), suggesting that NF-κB in involved in the occurrence and development of allergic rhinitis.

Researches showed that MYD 88 was raised by TLR4 combining with specific ligands, and signal IRAK was received through MAL bridging with MYD 88, which phosphorylated I-κB kinase on NF-κB after the interaction with TRAF6, making NF-κB changed into the free state to enter the nucleus, combined with targeting DNA, transcript related inflammatory cytokines, activate T cells and B cells and induce specific immune response, thus producing type I hypersensitivity (15-16). Liu Ying(17) found that the TLR4 and NF-κB mRNA and eosinophils in rats with AR were similar after stimulation with LPS, which were significantly higher than those in the control group, and there was a strong correlation between the two (r=0.799, LPS) (P<0.01).

![Fig. 4: Protein expression of TLR4 and NF-κB in nasal mucosa tissues of rats (A) Normal control group; (B) AR model group; (C) Fluticasone propionate group; (D) White mustard seed acupoint application group (E) Fried white mustard seed acupoint application group.](image1)

![Fig. 5: Protein expression of TLR4 and NF-κB in nasal mucosa tissue compared with normal group. (x̅±s, n=5) #p<0.05 compared to control group; *p < 0.05 compared to model group](image2)
These results were consistent with the results of this experiment. The expression of TLR4 and NF-κB in rats with AR after successful modeling was significantly higher than that in control group.

Clinical studies had shown that there were differences of curative effect of raw and fried white mustard seed plasters on allergic rhinitis. In the study of Yu Miao et al., it was found that the clinical efficacy of raw white mustard seed plaster on allergic rhinitis was better than that of fried mustard seed. Zhao Guojing, et al. divided drugs into the raw white mustard seed group, raw and fried white mustard seed group and the fried white mustard seed group to treat bronchial asthma, and the results showed that the long-term curative effect of raw white mustard seed group was better than the raw and fried group or the fried white mustard seed group. Therefore, in this experiment, there were raw white mustard seed plaster group and the fried white mustard seed plaster group, and the curative effect was compared between the two groups to explain the related mechanism from the perspective of immune.

The experiment showed that nasal inflammatory symptoms in rats treated with raw and fried white mustard seed plasters and fluticasone propionate were relieved significantly, compared with the model group. Besides, inflammatory changes in pathological sections of nasal mucosa were decreased, mRNA and protein expression levels of TLR4 and NF-κB were significantly decreased, and effects were more obvious in the raw white mustard seed plaster group than in the fluticasone propionate group or the fried white mustard seed plaster group. In conclusion, acupoint application therapy is effective in the treatment of allergic rhinitis (AR), and the curative effect of raw white mustard seed plaster acupoint application is obvious. The mechanism may be intervening the TLR-NF-κB pathway in nasal mucosa, effectively reducing the sensitivity of TLR4 to foreign antigens, down regulating the expression of NF-κB and reducing the release of inflammatory cytokines, thus playing an anti-inflammatory and therapeutic role.

References


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