An experiment of the combined treatment of traditional Lei-huo-jiu therapy with Chinese medicine for the lacrimal gland of Sjögren's syndrome

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The following article was mistakenly run in volume 2, issue 2 (June 2009) instead of in the present special issue, for which it was intended. The publisher regrets the error.

Abstract This experiment chooses nonobese diabetic (NOD) mouse as the animal model of Sjögren's syndrome and investigates the morphologic changes, the expression of inflammatory factors and growth factors of this mouse's lacrimal gland in response to a combined treatment of traditional Lei-huo-jiu therapy alone and in combination with Chinese medicine. The methods were to (1) use a morphological approach to directly observe pathological changes of the lacrimal gland in response to combined treatment and (2) to detect the level of tumor necrosis factor (TNF)- α , interleukin (IL)-1, and nuclear factor kappa B (NF- κ B) in lacrimal gland tissue caused by the combined treatments using an immunohistochemical approach. There is a reduction of the mast cell's degranulation and modulation of the level of cytokines in TNF- α , IL-1, and NF- κ B in the combined therapy group. The combined treatment of traditional Lei-huo-jiu therapy with Chinese medicine can improve the pathological changes of the

lacrimal gland tissue of the NOD mouse through modulating the level of TNF- α , IL-1, and NF- κ B which results in improved tear secretion and function of the lacrimal gland.

Keywords Sjögren's syndrome · Lacrimal gland · Lei-huo-jiu · Nonobese diabetic (NOD) mouse

Introduction

Sjögren's syndrome (SS) is a tear deficiency which includes both primary and secondary dry eye. Primary dry eye has the main symptom of dryness of the mouth and eye, while secondary dry eye is caused by autoimmune phenomena. Epidemiology indicates that the rate of dry eye is 0.5% and 3–5% following the San Diego Standard [1] and the Europe Standard [2], respectively. The main pathological course of SS is leukocytic infiltration of the lacrimal and salivary glands, which release a series of cytokines leading to the inflammation and destruction of glandular tissues, with resultant tear and salivary deficiency. The etiological agent for SS is not known. Many studies have investigated the protein content of tears of Sjögren's syndrome patients, which includes the autoimmune cytokines, inflammatory cytokines, growth factors, and so on.

While the treatment for SS has been limited to topical lubricants and cyclosporine, traditional Chinese medicine (TCM) may hold promise as a complementary measure to existing therapy. Our clinical observations have indicated that using Lei-huo-jiu in a moxibustion technique to infiltrate the area around both eyes of SS has yielded positive effects. To test our clinical observation, this experiment investigates the mechanisms of the combined treatment of traditional Lei-huo-jiu therapy alone and in combination with Chinese herbal medicines using a murine

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model for SS. To test the response to treatment, we will examine the lacrimal gland's secretory capacity by morphological methods and will measure inflammatory cytokines and growth factors in lacrimal gland tissue using an immunohistochemical approach.

Materials and methods

Experiment materials

Animal model selection There were thirty female nonobese diabetic (NOD) mice with SPF grade, aged 5 weeks old, and body weight 17.0–20.2 g and ten female KunMing mice with SPF grade aged 5 weeks old. Mice were provided by Chinese Academy of Medical Sciences.

Experimental Chinese medicines (1) Zhao shi Lei-huo-jiu, ChongQing Zhao Shi Lei-huo-jiu was provided by the Traditional Medicine Institute and (2) Chinese medicine (Yang Yin Sheng Jin fang) was from Hong shen, Mai dong, Tian hua fen, Wu wei zi, Bo he, and so on.

Main reagents These are 4% paraformaldehyde, Bouin solution, bitter acid, gradient alcohol (70–100%), xylene, hematoxylin–eosin stain, acetone, tumor necrosis factor (TNF) antibody, interleukin (IL)-1 antibody, nuclear factor kappa B (NF- κ B) antibody, ChemMateTMEnVision+/HRP immunohistochemistry kit (DAKO Co.), and DAB developer (DAKO Co.).

Custom-designed smoking box for mice undergoing Lei-huo-jiu therapy This box was 70×40×50 cm³, transparent top with six round air holes (each 1 cm²) convenient to observe the animal's condition in the box during treatment, and one treatment hole (diameter 4 cm) on each side for the Lei-huo-jiu stick.

Groups

NOD rats, whose exocrine glands demonstrate lymphocytic infiltration, have been certified as the ideal animal model of SS.

1. Control group: ten KunMing mice
2. Model group: ten NOD mice
3. Lei-huo-jiu therapy group: ten NOD mice
4. The combined therapy of Lei-huo-jiu therapy and Chinese medicine group: ten NOD mice

Methods

Administration methods Neither the control group nor the model group underwent treatment. In Lei-huo-jiu therapy

group, Lei-huo-jiu treatment duration was 20 min and bid for 4 weeks. In the combined therapy group, Lei-huo-jiu treatment duration was 20 min and bid, plus Chinese herbal medicinal broth with intragastric administration bid. The dose is calculated according to body weight of the mouse. Duration of treatment is 4 weeks.

Lei-huo-jiu therapy Place the two treatment groups of mice into a smoking box and light two Lei-huo-jiu sticks, then insert them into the box through the side holes. Sticks were kept lit throughout 30 min of treatment. Mice were observed during treatment to ensure normal activity during smoking treatment session.

Specimen and stain Mice were euthanized and both lacrimal glands were fixed in 4% formalin solution for 24 h, then placed into gradient alcohol for dehydration, and finally embedded into paraffin and cut into 3- μ m sections and stained with hematoxylin and eosin.

Morphological observation Morphological changes of the mice's lacrimal tissues were observed using a light microscope.

Immunohistochemical detection Once dewaxed, paraffin sections are put into 0.3% H₂O₂ solution for blocking endogenous peroxidase, specimens were then doused with lotic water, then into phosphate-buffered saline (PBS) for 5 min, and this was repeated three times before placing into the humid box. Dilute the TNF antibody (1:100), IL-1 antibody (1:50), and NF- κ B antibody (1:100) with antibody dilution as first antibody, dropwise apply 50 μ L of first antibody fluid onto each section, and 4°C refrigerate for 24 h, laying negative control at the same time. Dropwise

Fig. 1 Normal control Kun Ming mice's lacrimal gland tissue, HE ×400

Fig. 2 In model group, endothecium secretory cells of gland alveoli flatten and the acinar lumina expand. Some cavities are full of deep-brown globular structures, HE $\times 400$

apply 50 μ L of second antibody fluid onto each section after 24 h, incubate for 1.5 h at room temperature, then place into PBS buffer, soak for 5 min, and repeat three times, followed by coloration, dehydration, transparency, and mounting.

Outcome analysis Picking the upper, middle, and lower three optical fields, we took photographs in bright field for the immunohistochemical specimens using a light microscope.

Image analysis Measure the immunohistochemical staining intensity by using the Image Plus Pro image analysis system and undertake quantitative analysis. Calculate the integrated optical density (IOD): $\text{IOD} = \text{area} \times \text{density}$.

Statistic analysis Use SPSS software for statistical analysis.

Fig. 3 In model group, some endothecium secretory cells of gland alveoli accumulate great of secretory granules, so the acinar lumina disappear or collapse, HE $\times 1,000$

Fig. 4 In model group, grain-doff mast cells are observed in connective tissue surrounding the lacrimal gland tissue, toluidine blue stain $\times 400$

Result

Light microscopic observation

Lacrimal gland tissue in control group Lacrimal gland tissue consists of glandular acini, which are round or irregular cellular islands, with a central air space. Surrounding glandular acini are endothelial secretory cells which are lined up in order, with an intermittent arrangement of myoepithelial cells. Acini of the lacrimal gland are grouped in lobules which are separated by fibrous tissue (Fig. 1).

Lacrimal gland tissue in model group Endothelial secretory cells of the acini line up in a disorderly fashion, and their secretory granules are lost, so the cells flatten and the acinar

Fig. 5 Pathological changes in the combined therapy group are less pronounced compared to the model group, HE $\times 400$

Fig. 6 The lacrimal gland tissue's pathological changes in Lei-huo-jiu therapy group, HE $\times 400$

lumina expand (Fig. 2). While some endothelial secretory cells accumulate great number of secretory granules, the acinar lumina disappear or collapse (Fig. 3). Some cavities are full of deep brown globular structures. To add to the abnormal appearance, grain-doff mast cells are observed in the connective tissue surrounding the lacrimal gland tissue (Fig. 4).

Lacrimal gland tissue in treated group Compared with the model group, pathological changes of the lacrimal gland tissue are less pronounced in the combined therapy group. There is less disorder of the endothelial secretory cells of the acini, with less distention or collapse of acinar lumina (Figs. 5 and 6). Pathological changes of lacrimal gland tissue in Lei-huo-jiu therapy group is slightly improved when compared with the combined therapy group.

Fig. 7 TNF- α shows diminished expression in the interstitium surrounding acinar cells in the control group, showing up buff, $\times 200$

Fig. 8 In the model group there is greater expression of TNF- α with increased stain intensity compared to the control group, $\times 200$

Expression of three inflammatory factors in lacrimal gland tissue

Expression of TNF- α in lacrimal tissue

TNF- α is present in the interstitium surrounding acinous cells in control group, showing up buff (Fig. 7). In the model group, there is greater expression of TNF- α , with both area and stain intensity increased compared to that of the control group (Fig. 8). In both treatment groups, TNF- α is observed to less than in the model group, with a comparatively reduced area and stain intensity (Figs. 9 and 10).

Expression of IL-1 in lacrimal tissue

IL-1 is present in the interstitium surrounding acinous cells in the control group, with slight staining (Fig. 11). In the

Fig. 9 TNF- α in the Lei-huo-jiu treatment group shows less expression with reduced stain intensity compared to the model group, $\times 200$

Fig. 10 TNF- α in the combined therapy group shows less expression with reduced stain intensity compared to the model group, $\times 200$

model group, there is relatively increased expression, with both area and stain intensity greater than that seen in the control group (Fig. 12). In the combined therapy group, IL-1 is present to a lesser degree than in model group, both in area and stain intensity (Fig. 13), while in Lei-huo-jiu alone treatment group, there is no apparent difference compared to the model group (Fig. 14).

Expression of NF- κ B in lacrimal tissue

NF- κ B is present in the interstitium surrounding acinous cells in the control group, showing up buff (Fig. 15). In the model group, there is relatively increase expression, both area and stain intensity greater than that seen in the control group (Fig. 16). In both treatment groups, NF- κ B is reduced compared to the model group, both in area and stain intensity (Figs. 17 and 18).

Fig. 11 IL-1 is expressed in the interstitium surrounding acinar cells in the control group with slight staining, $\times 200$

Fig. 12 In the model group, IL-1 shows relatively increased expression with greater staining intensity compared to the control group, $\times 200$

Computer image analysis

Compared with the control group, the IODs of TNF- α , IL-1, and NF- κ B are larger in model group ($P < 0.05$ and $P < 0.01$, respectively). Compared with the model group, the IOD of all three cytokines is lower in the combined therapy group ($P < 0.05$), and the IOD of TNF- α and NF- κ B, but not IL-1, is lower in the Lei-huo-jiu therapy group ($P < 0.05$). Table 1

Discussion

Animal model

SS is an autoimmune disease with lymphocytic infiltration of the exocrine glands as a distinct pathologic feature of the

Fig. 13 IL-1 shows relatively decreased expression in the combined therapy group compared to the model group, $\times 200$

Fig. 14 In Lei-huo-jiu therapy group, no difference was observed as compared with the model group, $\times 200$

Fig. 16 In the model group, there is relatively increased expression of NF- κ B with increased staining compared to the control group, $\times 200$

disease. NOD mice, whose exocrine glands show lymphocytic infiltration, have been generally accepted as an ideal animal model for SS. Researches of NOD mice have shown that lymphocytic infiltration is observed in the pancreas, lacrimal gland, submaxillary gland, and other organs of these animals [3]. Humphreys Beher [4] confirmed that for NOD mice, the lacrimal gland's function is reduced. We have observed pathological changes in 5-week-old NOD mice, when infiltration of inflammatory cells begins to appear in their lacrimal glands. This inflammation in lacrimal gland is consistent with the distinctive pathological change of SS.

When compared with control group, the lacrimal gland of the untreated NOD model group has several differences: (1) There are groups of infiltrating inflammatory cells. (2) Endothelial secretory cells of glandular acini line up in an

obviously disorderly manner and their secretory granules are lost, so that the cells flatten and the acinar lumina appear to be distended (Fig. 2). (3) Other endothelial secretory cells accumulate a great number of secretory granules, to the degree that the acinar lumina disappear or collapse (Fig. 3). This indicates that those secretory granules are difficult to discard, and such inspissation of these granules may aggravate local inflammation. Some cavities are full of deep brown globular structures. (5) Grain-doff mast cells are observed in connective tissue surrounding the lacrimal gland tissues (Fig. 4).

Activated mast cells produce remarkable effects which include the infiltration of lymphocytes to participate in the release of various mediators and cytokines [5]. Since grain-doff mast cells cannot be found in the control group, we speculate that some of the inflammatory cytokines may

Fig. 15 NF- κ B is minimally expressed in the interstitium surrounding the acinar cells in the control group, showing up buff, $\times 200$

Fig. 17 In the Lei-huo-jiu therapy group, NF- κ B expression is reduced compared to the model group, $\times 200$

Fig. 18 In the combined therapy group, NF-κB expression is reduced compared to the model group, $\times 200$

arise from the grain-doff mast cells, but this requires confirmation. These three morphological changes which we have observed in the NOD mouse's lacrimal gland are in keeping with early pathological changes of SS in other related research [6–13], so it is here further supported as an ideal animal model of SS.

The traditional therapy for the NOD mouse's lacrimal gland

In TCM terms, “Zhao Shi Lei-huo-jiu” is a moxibustion treatment that delivers Chinese herbs in a strong smoke which is thought to adjust the Qi–blood of an organism's meridians. This effect is very similar to acupuncture. Related empirical study has confirmed that it can advance local blood circulation rapidly and promote topical metabolism and function. According to TCM theory, we chose Hong shen, Mai dong, Wu weizi, Tian huafen, and Bo he to compose our formula. In the formula, Hong shen benefits vital energy and promotes the production of body fluid; Mai dong, Wu weizi nourishes yin; Tian huafen removes heat to promote salivation; and Bo he is cool.

After 4 weeks of treatment, the results show that (1) in both the Lei-huo-jiu therapy group and the combined

therapy group, pathological changes of NOD mice's lacrimal gland are improved compared with the model group; (2) deep brown globular structures are observed in all the NOD mice groups, while in Lei-huo-jiu therapy and the combined therapy groups, this phenomenon is reduced; and (3) grain-doff mast cells are observed in connective tissue surrounding the lacrimal gland tissue in all the untreated NOD mice, while in the two treated groups, there is a reduction of the population of grain-doff mast cells. We presume that the curative effect following traditional treatments is that they may reduce local inflammation and promote the lacrimal gland's secretory function.

The traditional therapy for NOD mice's inflammatory cytokines in lacrimal gland

Some researches indicated that there is an elevated level of TNF and IL-1 in SS patients' blood, and the level of these two cytokines is related to pathological grading. Researchers consider that these two cytokines play an important role in autoimmune diseases. In the clinical setting, it is difficult to harvest the lacrimal gland. Thus, research to investigate inflammatory cytokines in lacrimal tissue has been rarely conducted.

We detected levels of TNF- α , IL-1, and NF- κ B in murine lacrimal gland tissue using an immunohistochemical approach. The results show that the levels of TNF- α , IL-1, and NF- κ B are higher in the model group when compared to the control group. On the other hand, when compared with the model group, the levels of all these three cytokines are lower in the combined therapy group. Levels of TNF- α and NF- κ B were lower in the Lei-huo-jiu therapy alone group, but the level of IL-1 was not decreased as it was in the combined therapy group. To some degree, the addition of the Chinese herbal therapy in the gastric lavage potentiated the therapy to reduce all three inflammatory cytokines in lacrimal gland. It appears that inflammatory mechanisms are at play, and therapy to reduce these inflammatory cytokines can improve the pathological changes of the lacrimal gland of NOD mice. Further studies are needed to understand the relatively higher levels of NF- κ B in relation to the elevated levels of the other

Table 1 Computer image analysis for IOD of three inflammatory cytokines (IOD = area $\times$ density)

Group	TNF- α (IOD)	IL-1 (IOD)	NF- κ B (IOD)
Control group	206,811.8 $\pm$ 333.87	1,861,260 $\pm$ 371.32	1,949,437 $\pm$ 426.72
Model group	1,813,602 $\pm$ 200.92**	2,325,086 $\pm$ 510.41*	2,411,554 $\pm$ 355.95*
Lei-huo-jiu therapy group	1,449,907 $\pm$ 518.56***	2,412,896 $\pm$ 421.73	1,894,509 $\pm$ 392.81***
Combined therapy group	1,478,449 $\pm$ 232.39****	1,949,694 $\pm$ 316.44****	2,165,945 $\pm$ 386.39****

*P<0.05 (model group is significantly different as compared with control group); **P<0.01 (model group is significantly different as compared with control group); ***P<0.05 (Lei-huo-jiu therapy group is observed to be different as compared with model group); ****P<0.05 (the combined therapy group is significantly different as compared with model group)

inflammatory cytokines in the model group and what role it may play in pathogenesis of SS.

Conclusion

Lei-huo-jiu therapy alone and combined therapy can modulate the level of inflammatory markers in lacrimal gland in an animal model of Sjögren's syndrome. We speculate that inflammatory mechanisms may be at play in the pathological changes of lacrimal tissues since these changes improve with treatment. Further studies are needed to confirm this pathophysiological mechanism of disease.

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