Quyu Xiaozhong recipe exerts anti-inflammatory effect in acute mastitis by inhibiting TLR4/NF-κB signal pathway

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Abstract

Purpose: To investigate the effect of Quyu Xiaozhong recipe (QYXZR) on acute mastitis induced by Staphylococcus aureus in rats via TLR4/NF-κB signal pathway.

Methods: The rat model of acute mastitis was constructed by injecting Staphylococcus aureus into the fourth pair of nipples of rats 5-7 days after parturition. The rats were treated with drugs 12 h after injection. Pathological changes in rat mammary tissue were determined using hematoxylin-eosin (H&E) staining. Myeloperoxidase (MPO) kit was used to evaluate MPO activity. Enzyme-linked immunosorbent assay (ELISA) was employed to determine the levels of inflammatory factors, while changes in protein expression of TLR4, NF-κB-p65, and IκB-α were determined by Western blot.

Results: QYXZR intervention significantly attenuated pathological damage in rats and alleviated Staphylococcus aureus-induced mastitis. At the same time, QYXZR significantly reduced the expression of MPO and the levels of inflammatory factors (p < 0.05). Further molecular mechanistic studies revealed that QYXZR exerted anti-inflammatory effects by inhibiting TLR4/NF-κB signal pathway.

Conclusion: QYXZR alleviates acute mastitis caused by Staphylococcus aureus via the inhibition of TLR4/NF-κB pathway, thus, providing the scientific and theoretical basis for the extensive use of this traditional Chinese medicine in the treatment of acute mastitis.

Keywords: Quyu Xiaozhong recipe, Staphylococcus aureus, TLR4/NF-κB pathway, Acute mastitis

INTRODUCTION

Mastitis, an inflammation of the breast tissue, affects up to 33 % of breastfeeding women, and it poses a serious health risk to mothers and their offspring [1]. Mastitis is mainly induced by bacteria, with Staphylococcus aureus being the main organism. Staphylococcus aureus-induced mastitis is characterized by chronicity, recurrence, and low cure rate [2].

Toll-like receptors (TLRs) have a key role in the innate immune system as transmembrane receptors [3]. In particular, TLR4 can be associated with biological processes such as recognition of microbial pathogens. In addition, it plays an essential role in mastitis through the recognition of bacterial molecules [4]. It has been...
shown that when a host is infected with S. aureus, TLR4 expression activates downstream NF-κB signal pathway, leading to induction of gene expression in response to inflammation [5]. Clinically, antibiotics are widely used to treat mastitis. However, long-term use of antibiotics leads to drug resistance by Staphylococcus aureus, which undoubtedly increases the difficulty in treatment of the disease. As a result, it has become necessary to search for new drugs for treating mastitis.

It has been found that traditional Chinese medicine (TCM) effectively reduces host’s resistance to antibiotics when used to treat mastitis [6]. Quyu Xiaozhong recipe (QYXZR) consists of rhubarb powder (30 g) and mirabilite (120 g), and they were confirmed to have broad anti-inflammatory and antioxidant properties. Modern pharmacological studies have shown that external applications of rhubarb and mirabilite is effective in relieving the symptoms of acute pancreatitis [7]. The above reports suggest that QYXZR has obvious anti-inflammatory effects. However, its mechanism of action for treating mastitis remains unclear.

**EXPERIMENTAL**

**Animals**

Healthy adult SD pregnant SPF rats aged 80-110 days, and weighing 220-260 g were used for establishing a rat model of acute mastitis. Permission for this research was granted by the Animal Research Ethics Committee of Zhejiang University of Traditional Chinese Medicine (approval no. ZJCLA-IACUC-20020017) and all procedures were performed on animals according to standard protocols [8].

**Drugs**

Quyu Xiaozhong's recipe contained 30 g of rhubarb and 120 g of mirabilite. The two components were thoroughly mixed with white vinegar to make a medicinal paste which was administered to rats at two doses i.e., 4.5 and 13.5 mg/kg. Amoxicillin was mixed into a suspension and given at a dose of 150 mg/kg.

*Staphylococcus aureus*

*Staphylococcus aureus* was purchased from ATCC for culture and diluted to $2 \times 10^7$ CFU/mL.

**Rat model of acute mastitis**

Lactating rats at 5 - 7 days postpartum were divided at random into sham ($n = 8$) and Model ($n = 32$). Then, to establish a rat model of acute mastitis, 100 μL of *Staphylococcus aureus* suspension was injected into the fourth pair of nipples of the Model group rats. Rats in the Sham group were injected with normal saline of the same volume.

**Grouping and treatments**

The rats were divided into 5 groups of 8 rats each randomly: sham group, model group, Amoxicillin group, Amoxicillin + low-dose QYXZR group, and amoxicillin + high-dose QYXZR group. Then, the rats were treated with drugs 12 h after the establishment of the acute mastitis model. Rats in the amoxicillin group were treated with amoxicillin suspension at a dose of 150 mg/kg, via gavage. Rats in the amoxicillin + low-dose QYXZR group were first treated with amoxicillin suspension at a rate of 150 mg/kg, via gavage, followed by treatment with low-dose QYXZR (4.5 mg/kg) made into a paste with white vinegar which was applied to their breast lumps using an ointment. Rats in the amoxicillin + high-dose QYXZR group were first treated with amoxicillin suspension as indicated in other groups, followed by application of QYXZR as a vinegar paste at a rate of 13.5 mg/kg. Drug was applied to their breast lumps using an ointment. Rats in the sham and model groups were treated with saline (1 mL). Rats in all groups were administered their various doses of drugs as aforementioned, once daily for 7 days. Finally, the rats were sacrificed via carbon dioxide inhalation, and their mammary tissues excised. One portion of each mammary gland tissue was used for histopathological examination, while the other portion was preserved at -80°C, prior to further investigations.

**Hematoxylin-eosin (H&E) staining**

Rat mammary tissue was embedded in paraffin after being fixed in paraformaldehyde. Then, sections were stained by H&E and examined under a light microscope to identify pathological changes.

**Assay of myeloperoxidase (MPO)**

Firstly, a tissue homogenate of rat mammary tissue was prepared in reaction buffer. Then, MPO activity was measured with MPO activity assay kit.

**Enzyme-linked immunosorbent assay (ELISA)**

Blood samples were collected After 24 h of drug intervention in rats, blood samples were collected, and the supernatants (sera) were
obtained after centrifugation. The cytokines TNF-α, IL-6, and IL-1β were quantified with corresponding ELISA kits.

**Western blot assay**

Lysed rat mammary tissue with RIPA lysis buffer and centrifuged for 15 min. BCA protein assay Kit was used to determine protein concentration. The proteins were transferred onto polyvinylidene fluoride membranes after being separated using 10% SDS-polyacrylamide gel. The membranes were then blocked with 5% skim milk at room temperature for 1 h. After that, the membranes were incubated with the primary antibodies overnight, and then incubated with secondary antibodies for 1 h. Finally, visualization was performed using enhanced chemiluminescence.

**Statistical analysis**

GraphPad Prism 8.0 software was used to analyze the data and t-test was used to compare data between groups. *P* < 0.05 indicated statistically significant differences.

**RESULTS**

**Effect of QYXZR on the mammary gland tissues**

H&E staining showed that the structure of the mammary lobules in the sham group was intact. In contrast, the mammary glands of the model rats showed severe lesions with a large number of exuded neutrophils in the ductal lumen. However, the number of acinaries was normal. The mammary lobules in the amoxicillin group showed more normal structure: apart from a small number of exuded neutrophils in the ducts and acinar lumens, no other obvious pathological changes were found. The mammary lobule structure of rats in the amoxicillin + low-dose QYXZR group was much better, with minimal neutrophil exudation in the duct lumen. In the Amoxicillin + high-dose QYXZR group, the rat mammary lobules had very clear structure, normal ductal epithelial cell morphology and number of acinar, and there was no neutrophil exudation in the ductal areas. The pathological findings indicated that QYXZR acted in synergy with Amoxicillin to further reduce the pathological damage in rats due to mastitis.

**Effect of QYXZR on MPO activity**

The activity of MPO may be used as an index of the degree of neutrophil infiltration, and it is one of the key indicators for the assessment of mastitis. As shown in Figure 2, MPO activity was higher in the model group than the sham (**P < 0.001**). In addition, MPO activity in the amoxicillin, amoxicillin + low-dose QYXZR, and Amoxicillin + high-dose QYXZR groups were lower than the model group. Moreover, MPO activity in the amoxicillin + high-dose QYXZR group was significantly lower than in the amoxicillin group (**P < 0.01**). Thus, QYXZR significantly mitigated mastitis.

**Effect of QYXZR on inflammation levels**

Figure 3 showed that the expression of IL-1β, IL-6 and TNF-α in the model group were higher than the sham. In addition, the levels of these inflammatory factors in the amoxicillin, Amoxicillin + low-dose QYXZR, and amoxicillin + high-dose QYXZR groups were lower than in the model group. At the same time, the levels of these inflammatory factors in the amoxicillin + high-dose QYXZR group were significantly decreased, relative to the amoxicillin group.
Thus, QYXZR reduced the levels of inflammatory cytokines.

**QYXZR inhibited TLR4/NF-κB signal pathway**

As shown in Figure 4, the expressions of TLR4, p-IκB-α, and p-NF-κB-p65 in the model group were higher than in the sham. However, the expressions of TLR4, p-IκB-α, and p-NF-κB-p65 in the QYXZR group were lower than the model group. Thus, QYXZR played an anti-mastitis role by inhibiting the TLR4/NF-κB pathway.

**DISCUSSION**

Mastitis is a breast disease that is closely associated with inflammation, and it is usually caused by infection with pathogenic microorganisms. Mastitis not only severely affects women of childbearing age, but it also occurs in women of all ages. Acute mastitis in nursing mothers is usually short-lived, and the symptoms comprise breast swelling, fever, pain, and lumps. *Staphylococcus aureus* is the most usual causative agent in mastitis [1, 2]. Therefore, this research was focused on investigation of the effect of QYXZR on acute mastitis induced by *Staphylococcus aureus* in rats.

Clinically, antibiotics are the mainstay of treatment for mastitis, but they are often associated with serious toxic side effects. The TCM formula QYXZR has been shown to be effective in the clinical management of acute mastitis. In the current work, the effect of combined use of QYXZR and Amoxicillin was studied in a rat model of *Staphylococcus aureus*-induced acute mastitis. There were severe lesions in the mammary tissue of rats injected with *Staphylococcus aureus*. However, the administration of Amoxicillin + QYXZR significantly mitigated the lesions in the mammary tissues of rats with mastitis, and the therapeutic effect was also better than those in the positive sham group and Amoxicillin group.

**Figure 3:** Effect of QYXZR on inflammation levels

![Graphs showing the effect of QYXZR on inflammation levels](image)
This study showed that QYXZR significantly mitigated mammary tissue damage in rats with *Staphylococcus aureus*-induced mastitis.

Pathogens that cause mastitis induce inflammatory responses in the mammary glands, resulting in triggering of a protective immune response in the host. Neutrophils play vital roles in the immune response, being the first phagocytes to reach the site of infection to engulf the pathogen. Neutrophils also play major defensive roles in the early stages of *Staphylococcus aureus*-induced infection [9]. Myeloperoxidase (MPO) activity in breast tissues is a biomarker of neutrophil surface activity, and the level of MPO activity indirectly reflects the degree of infiltration of neutrophils in tissues [10]. This study found that QYXZR enhanced Amoxicillin-mediated reduction in the level of MPO, indicating that QYXZR inhibited mastitis through the neutrophils.

Inflammatory cytokines are critical to the progression of mastitis. Neutrophils produce pro-inflammatory cytokines in tissues to protect the host from microbial infection [10]. It has been reported that *Staphylococcus aureus* exerts an inflammatory response by inducing the production of pro-inflammatory factors [11]. It is known that TNF-α has an essential role in inflammatory response [12], while IL-1β triggers an early inflammatory cytokine cascade [13]. In acute inflammation, the level of IL-6 is significantly increased, which further enhances inflammation [14].

In this study, there were significantly elevated levels of inflammatory cytokine in the mammary gland tissues of *Staphylococcus aureus*-infected rats. Clinical research has also shown that continued expression of inflammatory factors may lead to swelling and even rupture of mastitis cells, ultimately leading to damage to the breast tissue. *Taraxacum mongolicum* has been shown to attenuate damage in *Staphylococcus aureus*-induced mastitis by reducing levels of inflammatory cytokine [15]. This study showed that QYXZR significantly reduced the expression of inflammatory cytokines in mastitis rats, which further demonstrated that it may attenuate the inflammatory response in mastitis.

In addition, TLR4 specifically recognizes pathogens and TLR4 signal is essential in immune response. The NF-κB signal pathway has been extensively studied for its role in regulating levels of inflammatory factors [4,5]. The TLR4/NF-κB pathway is closely associated with the inflammatory response [16]. Studies have shown that nuciferine (the main bioactive compound in TCM lotus leaf) alleviated mastitis-induced damage in breast tissues by the inhibition of TLR4/NF-κB pathway [17]. In this study, QYXZR was found to significantly down-regulated the levels of TLR4, p-IκB-α, and p-NF-κB-p65 in mastitis rats. This suggests that, to some extent, QYXZR may exert anti-inflammatory effects on *S. aureus*-induced mastitis by regulating the levels of pro-inflammatory factors through inhibition of TLR4/NF-κB pathway.

CONCLUSION

This work has demonstrated that QYXZR exerts a significant anti-inflammatory effect in *S. aureus*-induced mastitis by inhibiting TLR4/NF-κB pathway. This finding provides a novel strategy for targeted treatment of acute mastitis.

DECLARATIONS

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**Ethical approval**

None provided.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Conflict of Interest**

No conflict of interest associated with this work.

**Contribution of Authors**

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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