Effects of anisodamine on the expressions of tumor necrosis factor-α and cycloxygenase 2 in experimental infusion phlebitis

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Abstract: Objective This subject was designed to investigate effects of anisodamine on the expressions of tumor necrosis factor-α (TNF-α) and cycloxygenase 2 (COX-2) in a rabbit model of infusion phlebitis and to analyze the preventative and treatment mechanisms of anisodamine in experimental infusion phlebitis. **Method** The rabbits were randomly assigned to the control group, the model group, the magnesium sulfate group and the anisodamine group. Expressions of TNF-α and COX-2 were determined and contrasted with the control group treated with normal saline by histopathology, immunohistochemistry, reverse transcription polymerase chain reaction, and western blotting assay, respectively. **Results** Obvious pathohistological changes were observed and the model group showed the highest expressions of TNF-α and COX-2 in the four groups (P<0.01). On the contrary, anisodamine alleviated the inflammatory damage by significantly reducing the expressions of TNF-α and COX-2 compared with the model group (P<0.01). There was no difference in the expressions of TNF-α and COX-2 between the magnesium sulfate group and the anisodamine group (P>0.05). **Conclusion** Anisodamine alleviates the inflammatory damages by significantly reducing the expressions of TNF-α and COX-2, and shows significant protective effects in the animal model of infusion phlebitis.
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Keywords: anisodamine; infusion phlebitis; tumor necrosis factor-α; cycloxygenase 2

1. Introduction

Infusion phlebitis is the most common side effect of clinical intravenous drug therapy [1], and many drugs increase the risk for infusion phlebitis. However pathogenesis of infusion phlebitis has not been fully clarified and infusion phlebitis has not been effectively solved until now. Previously studies on the models of inflammation have implicated that tumor necrosis factor-α (TNF-α) and cycloxygenase 2 (COX-2) -induced prostanoids as the key factors in controlling the production of early inflammatory mediators [2].

Anisodamine is efficacious for the treatment of experimental and clinical bacterium shock [3-5]. Several clinical studies have demonstrated that anisodamine effectively prevents the occurrence of infusion phlebitis, but most of current studies of infusion phlebitis are clinical observations and influential factor analysis, lacking systematic mechanism study and animal-oriented model.

Based on these studies, this subject was designed to investigate the effects of anisodamine on the expressions of TNF-α and COX-2 in a rabbit model of infusion phlebitis and to analyze the prevention and treatment mechanisms of anisodamine for infusion phlebitis caused by intravenous administration.

2. Material and Methods

Rabbits

Specific pathogen-free male Japanese white rabbits (No. SCXK 2011-0001, age, 2 to 3 months; weight, 2.5 to 3.5 kg) were obtained from Experimental Animal Center of Henan Province, China. The rabbits were fed with standard diets in pathogen-free conditions and allowed free access to food and water. Animals were treated according to good laboratory practice (GLP).

Experimental infusion phlebitis was established as previously described [6]. Briefly, eighteen healthy rabbits were randomly assigned to three groups of the model group, the anisodamine group, and the magnesium sulfate group. All three groups continuously received intravenous injection of 2.5 ml/kg of 20% mannitol (Jiangsu Chia Tai Fenghai Pharmaceutical Co., Ltd.) 2.5 ml/kg via a peripheral ear vein twice a day for two days. The flow rate for each infusion was approximately 0.5 ml/min. After administration of mannitol, the ear vein was infused with 5 ml 0.9% NaCl solution (Ningbo Changfu Pharmaceutical Co., Ltd.) at the same flow rate, and then the infusion needle was removed. Moreover, the anisodamine group was smeared by 2% anisodamine (Beijing Double-Crane pharmaceutical Co., Ltd.) before intravenous injection, and the magnesium sulfate group was smeared by 25%

magnesium sulfate (Sanjing Pharmaceutical Co., Ltd.) after intravenous injection.

Meanwhile, the six normal rabbits of the control group received intravenous injection of pyrogen-free physiological saline via an ear vein in the same way as the control group.

Twenty-four hours after the fourth infusion, the appearances of vasculature and tissues were observed, and ears specimens around the vasculature (length 3 cm, width 1cm) were harvested and collected. All of the ears specimens were divided to two parts, one part of them was fixed in 10% neutrally buffered formalin, and the other was stored in liquid nitrogen.

Histopathological examination

Ears specimens were fixed in 10% buffered formalin and embedded in paraffin following routine methods. Deparaffinized thin sections from each paraffin block were stained with hematoxylin and eosin for histologic examination.

Immunohistochemistry

Immunohistochemistry was performed as previously described [7]. Briefly, after blocking for nonspecific sites, slides were incubated in a humid chamber overnight at 4°C with the primary antibodies, anti- TNF- α antibody or anti- COX-2 antibody, respectively. After washing with PBS, slides were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for two hours. Diaminobenzidine (DAB) was used as the chromogen, and the slides were counterstained with hematoxylin. Finally, the slides were analyzed with a spectral imaging system of Nikon NIS-Elements. Immunohistochemical expressions of TNF- α and COX-2 were represented by average optical density (OD) value of the staining positive cells.

Reverse transcription polymerase chain reaction (RT-PCR) assay

Grinded 100 mg frozen ears specimens in liquid nitrogen and the extractions of total RNA by Trizol were performed according to the manufacturer's instructions. RNA was extracted by using chloroform and precipitated by ethanol; the pellets were washed with 70% ethanol and dried. The dried RNA was suspended in 20 μl of sterile water and sent for RT-PCR analysis. 2 μg Total RNA was reverse-transcribed into cDNA in a 20 μl of reaction mixture containing: 0.5 μg Anchored Oligo (dT)18, 0.1 μg random Primers (N9), 25 U of ribonuclease inhibitor, 200 U of EasyScript RT reverse transcriptase and 4 μl of 5×RT Buffer, and 1 μl dNTPs (10 mmol/L). Samples were incubated at 25°C for 10 minutes, 42°C for 30 minutes and denatured at 85°C for 5 minutes in a S1000 PCR cycler. cDNA samples were subsequently amplified for the target sequences in a S1000 PCR cycler using a following reaction mixture contains: 5 μl cDNA, 5 μl 10×EasyTaq Buffer, 2.5 U EasyTaq DNA polymerase, 1 μl dNTPs (10 mmol/L) and 20 pmol of each primer in 50 μl final volume.

To prevent false positive results and contamination, RNA extraction, preparation of reaction mixtures and amplifications were carried out in separate rooms and each piece of RT-PCR experiment included a sample with no RNA as the negative control. The primers for GAPDH were added to each run as the positive control to assess RNA integrity and to confirm the absence of DNA polymerase inhibitors.

TNF- α primers were: 5′- GACAAGCCTCTAG CCCACG-3′, antisense 5′- GGCAAGGTCCAGGTA CTCA-3′, generating 405 bp. COX-2 primers were: sense 5′- TTGCTGAAGCCCTATGA -3′, antisense 5′- TGGGACGTTGAATGAAG-3′, yielding a 339 bp. GAPDH primers were: sense 5′-GGTCGGAGTG AACGGATTT-3′, antisense 5′-CTCGCTCCTGGAA GATGG-3′, generating 227 bp. The amplification cycling profile was as follows: initial denaturation 95°C for 2 minutes; cycling: 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute (35 cycles); final extension at 72°C for 10 minutes. After reaction completion, 5 μl of each PCR product was run on 1% agarose gel electrophoresis stained with ethidium bromide. Images were scanned by a Transilluminator JY04S Gel analysis system under UV light and analyzed by the Gel-PRO analyzer system. TNF- α mRNA and COX-2mRNA were assessed by comparing with GAPDH mRNA to investigate the mechanism of anisodamine on the expressions of TNF- α and COX-2.

Western blotting analysis

Expressions of TNF- α and COX-2 proteins were analyzed by Western blotting analysis. Briefly, the total proteins were extracted from frozen ears specimens and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to separate the proteins. At the end of the run, polypeptide bands in the gel were electrophoretically transferred to a PVDF membrane (Bio-Rad Laboratories, Inc., USA). The membrane was incubated for an hour at room temperature with anti- TNF- α antibody, anti- COX-2 antibody or anti-GAPDH antibody (Bioss Inc.), respectively. On the membrane, the binding of antibody to the specific protein band was detected with horseradish peroxidase-conjugated secondary antibody (Bioss Inc., China) and an analyzed by ECL Western blotting detection system (Beyotime Institute of Biotechnology, China).

Statistical analysis

Data were expressed as means± standard deviation (SD). All statistics were analyzed by SPSS

11.0 software (SPSS Inc., USA). The significance of differences in outcomes was determined by using oneway analysis of variance (ANOVA) followed by LSD t-test. Statistical significance was accepted at P <0.05.

3. Results

Histopathological examination

Pathohistological examination was performed by microscope. There were no abnormal changes in the control group (Figure 1A), but pathohistological changes of the model group were obviously observed such as loss of venous endothelial cells, inflammatory cell infiltration, edema and thrombus (Figure 1D). On the contrary, the magnesium sulfate group and the anisodamine group showed a significantly protective effect on vascular congestion, inflammatory cell infiltration, and proliferation, swelling of endothelium and perivascular hemorrhage (Figure 1B, 1C).

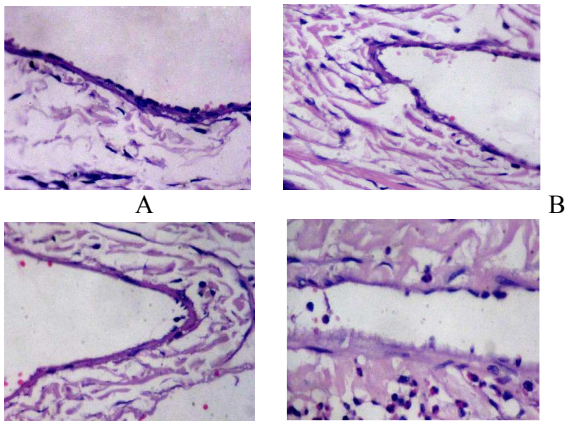

Figure 1. Histopathological examination of ears specimens in experimental infusion phlebitis (Hematoxylin and eosin staining, original magnification ×200). **A:** the control group. **B:** the magnesium sulfate group. **C:** the anisodamine group. **D:** the model group.

Immunohistochemistry

Expressions of TNF- α and COX-2 in ears specimens from the rabbit model were measured by immunohistochemistry. Specific staining of cells in the slides was obtained by using antibodies against TNF- α and COX-2 and lots of brown-yellow particles aggregated in the cytoplasm (Figure.2). Expressions of TNF- α and COX-2 were represented by OD values of the positive staining cells.

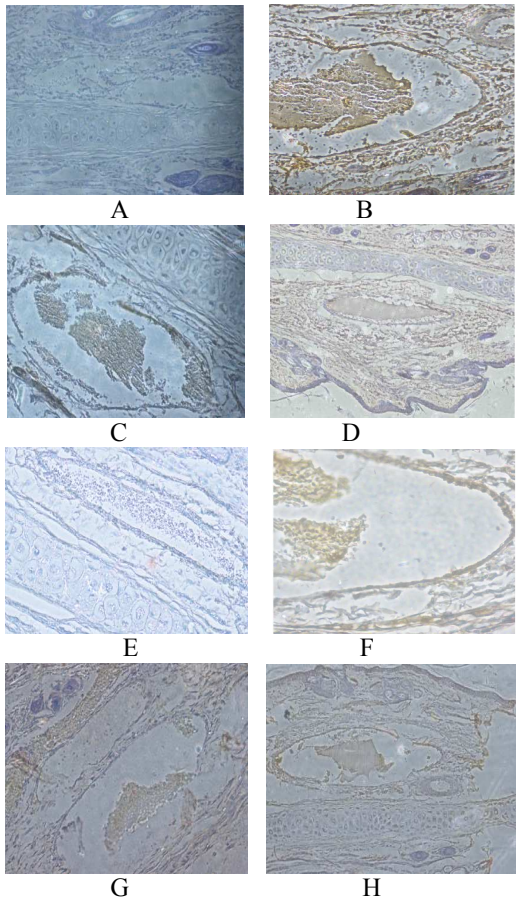

Figure 2. Immunohistochemistry examination of ears specimens in experimental infusion phlebitis (SP staining, original magnification ×100). **A:** the control group (anti-TNF- α antibody incubation). B: the magnesium sulfate group (anti-TNF- α antibody incubation). C: the anisodamine group (anti-TNF- α antibody incubation). D: the model group (anti-TNF- α antibody incubation). E: the control group (anti-COX-2 incubation). F: the magnesium sulfate group (anti-COX-2 incubation). G: the anisodamine group (anti-COX-2 incubation). H: the model group (anti-COX-2 incubation).

The results showed that the model group showed the highest expressions of TNF- α (0.6028±0.0515) of the four groups (P<0.01). The magnesium sulfate group and the anisodamine group displayed significantly higher levels of TNF- α protein expression (0.3690±0.0479 and 0.3925±0.0317) than the control group (0.1379±0.0309) (P<0.01), and there was no difference in the expressions of TNF- α between the magnesium sulfate group and the anisodamine group (P>0.05). Similarly, the model group

showed the strongest expressions of COX-2 (0.5268±0.0499) in the four groups (P<0.01), and the magnesium sulfate group and the anisodamine group displayed significantly higher levels (P<0.01) of COX-2 protein expression (0.4377±0.0609 and 0.4576±0.0394) than the control group (0.1239±0.0191). There was no difference in the expressions of COX-2 between the magnesium sulfate group and the anisodamine group (P>0.05). (Figure 3).

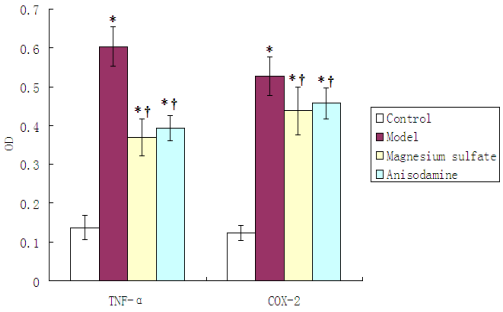

Figure 3. Semi-quantitative analysis of ***TNF- α*** and ***COX-2*** by immunohistochemistry. $^{*}P$<0.01 vs. the control group. $^{\dagger}P$<0.05 vs. the model group.

Semi-quantitative analysis of TNF- α and COX-2 by RT-PCR

The extracted total RNA had three ribosomal RNA bands, 5S, 18S and 28S. The densitometries at 260 and 280 nm were measured and all of the ratios of OD_{260}/OD_{280} were between 1.8 and 2.0. The results confirmed that extracted total RNA was stable and pure.

The expressions of TNF- α and COX-2 were positive in the four groups (Figure 4). The ratios of TNF- α mRNA/GAPDH mRNA in the model group (0.6789±0.0487) was significantly higher than those in the other groups (P <0.01), and the ratios of TNF- α mRNA/GAPDH mRNA in the magnesium sulfate group and the anisodamine group were 0.4970± 0.0513 and 0.5461±0.0501, significantly higher than those in the control group, 0.1388±0.0247, (P <0.01). There was no difference in the expressions of TNF- α mRNA between the magnesium sulfate group and the anisodamine group (P >0.05). Similarly, the ratio of COX-2 mRNA/GAPDH mRNA in the model group (0.6161±0.0504) was significantly higher than those in the other groups (P <0.05), and the ratios of COX-2 mRNA/GAPDH mRNA in the magnesium sulfate group and the anisodamine group, 0.4126± 0.0448 and 0.4219±0.0502, were significantly higher than those in the control group (0.1045±0.0183). There was no difference in the expressions of COX-2 mRNA between the magnesium sulfate group and the anisodamine group (P >0.05). (Figure 5).

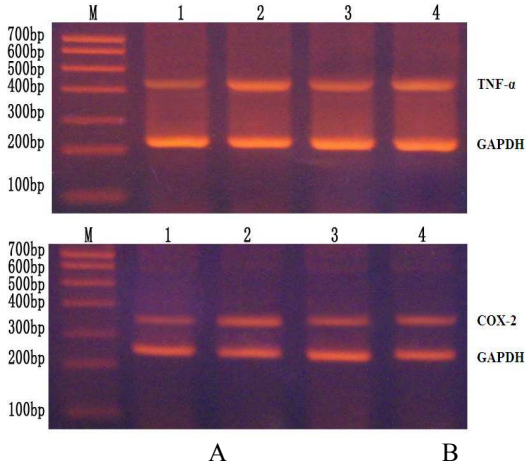

Figure 4. The expressions of TNF- α mRNA and COX-2 mRNA by RT-PCR. Lane 1: the control group; Lane 2: the magnesium sulfate group; Lane 3: the anisodamine group; Lane 4: the model group; M: Marker.

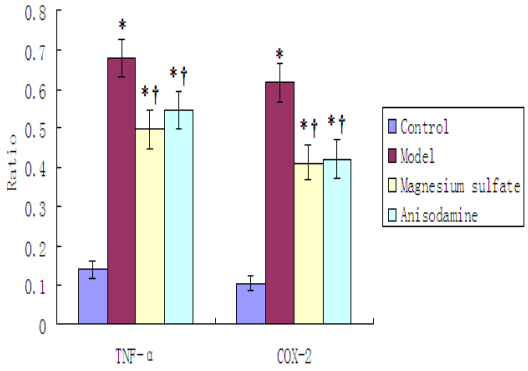

Figure 5. Semi-quantitative analysis of TNF- α mRNA and COX-2 mRNA by RT-PCR. $^{*}P$<0.01 vs. the control group. $^{\dagger}P$<0.01 vs the model group.

Western blotting analysis

Proteins extracted from frozen ears specimens were confirmed by Western blotting analysis. In the blots from extracts of specimens were probed with anti-GAPDH antibody, anti-TNF- α antibody, and anti- COX-2 antibodies, respectively (Figure 6). Blots were then scanned by a digital scanner, and the scans were examined by computerized densitometry. The expressions of TNF- α and COX-2 were assessed by comparing with GAPDH.

The ratio of TNF- α /GAPDH in the model group (0.8197±0.0647) was significantly higher than in the other groups (P <0.01), and the ratios of TNF- α /GAPDH in the magnesium sulfate group and the

anisodamine group (0.5425±0.0282 and 0.5865±0.0357) were significantly higher than in the control group (0.1688±0.0212) (P<0.01). There was no difference in the expressions of TNF-α between the magnesium sulfate group and the anisodamine group (P>0.05). Similarly, the ratios of COX-2/GAPDH in the model group (0.7925±0.0378) were significantly higher than in the other groups (P<0.05), and the ratios of COX-2/GAPDH in the magnesium sulfate group and the anisodamine group (0.4280±0.0484 and 0.4619±0.0474) were significantly higher than in the control group (0.1234±0.0186). There was no difference in the expressions of COX-2 between the magnesium sulfate group and the anisodamine group (P>0.05). (Figure 7).

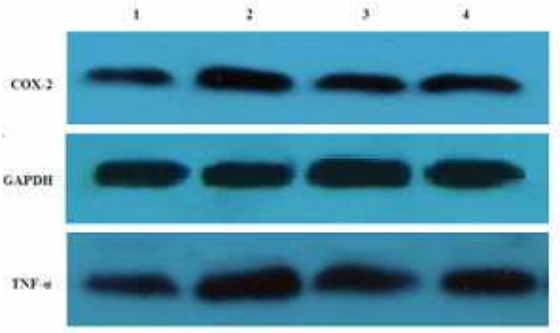

Figure 6. The expressions of TNF-α and COX-2 by Western blotting assay. Lane 1: the control group; Lane 2: the magnesium sulfate group; Lane 3: the anisodamine group; Lane 4: the model group.

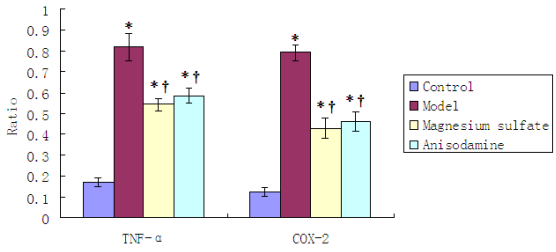

Figure 7. Semi-quantitative analysis of TNF-α and COX-2 by western blot assay. *P<0.01 vs. the control group. †P<0.01 vs. the model group.

4. Discussions

Chemical, physical, and microbial phlebitis have been described [8] and infusion phlebitis is the most common side effect of clinical intravenous drug therapy [1]. It is reported that infusion phlebitis occurs in 25% or more of hospitalized patients and its local symptoms are painful iatrogenic disorders with possible severe outcomes lasting from one week to several months; such as pain, swelling, and the appearance of tender cords. In most cases, infusion phlebitis induces to patients with more extra pain, additional medical cost, and greatly interferes with medication compliance and life qualities of patients [9].

Many drugs increase the risk for infusion phlebitis and antibiotics and cytotoxic drugs are the most common classes of drugs to increase risk [10]. It is reported that hyperosmolar solutions, drugs with high or low pH, and undissolved particles ranging from 1 to >25 μm in size are the most common classes of drugs to increase risk [11].

Frequent and intolerant phlebitis limited the usage of several effective agents, but pathogenesis of infusion phlebitis is not fully clarified and it has not been effectively solved until now. The most prevalent opinion is that the endothelium is intermittently exposed to high concentrations of the drug given intravenously, and chemical irritation of the endothelium leads to a sterile inflammation, endothelium sheds by toxicity, with granulocyte migration and subsequent induction of thrombosis[1, 12], in some cases followed by thrombosis[13].

It is generally accepted that acute inflammation starts a cascade of cytokines and chemokines that attract immune and nonimmune cells to infiltrate disrupted and damaged tissue [14]. TNF-α is a powerful and pleiotropic pro-inflammatory cytokine primarily produced by activated macrophages[15] and plays a critical role in inflammatory process and tissue destruction through the nuclear factor-κB (NF-κB) signaling pathway[16] and may initiate an inflammatory cascade consisting of other inflammatory cytokines[17]. Similarly, COX-2 induced production of prostanoids is often implicated in inflammatory diseases, characterized by edema, production of chemotactic factors, and infiltration of inflammatory cells [2]. Unlike COX-1, which is a constitutive enzyme expressed in most tissues and is essential for various physiological functions, COX-2 is an inducible enzyme that is induced in a variety of cell types by inflammatory stimuli, including lipopolysaccharide (LPS), phorbol ester, and cytokines such as interleukin (IL) -1β, and TNF-α [17].

Multiple cytokines, chemokines, and angiogenic factors have been identified as early participants and previous studies have shown that the models of inflammation have implicated TNF-α and COX-2-induced prostanoids as the key factors in controlling the production of early inflammatory mediators and expressions of both TNF-α and COX-2 are up-regulated during inflammation[2]. Those revelations intensify interests in controlling inflammation for disease prevention and treatment.

Anisodamine, a peripheral muscarinic receptor antagonist, is an alkaloid extracted from a Chinese herb. It is a naturally occurring atropine derivative

and a potent vasoactive drug and has been widely used for many years to improve microvascular perfusion and to treat acute disseminated intravascular coagulation in patients in bacteremic shock. It has been credited with contributing to improving the survival rate in the treatment of acute epidemic meningococcal meningitis and decreasing the mortality of meningococcemia from 67% to 12.4% [18]. Previous studies demonstrate that anisodamine has an anti shock effect contributing to prolong the survival of mice injected with Shiga toxin (Stx) and inhibit the production of TNF-α, IL-1β, and IL-8 from human peripheral blood monocytes stimulated with Stx[19]. Moreover, anisodamine is also an inhibitor of platelet aggregation, granulocyte aggregation, and thromboxane synthesis. Further study showed that anisodamine significantly inhibited the A-23187-induced release of PG and LT from mouse macrophages in a dose-dependent manner [20] and the TNF-alpha inhibitory effect was via a prostaglandin E2-dependent mechanism [19].

Several clinical studies have demonstrated that anisodamine effectively prevent the occurrence of infusion phlebitis, but little is known about its mechanism and most of current studies of infusion phlebitis are clinical observations and influential factor analysis, lacking systematic mechanism study and animal-oriented model.

Based on these reports, this study was designed to investigate the effects of anisodamine on the expressions of TNF-α and COX-2 in a rabbit model of infusion phlebitis comparing with magnesium sulfate as the positive control. The present study found obviously pathohistological changes, such as loss of venous endothelial cells, inflammatory cell infiltration, edema and thrombus. The model group showed the strongest expressions of TNF-α and COX-2 of the four groups (P<0.05). On the contrary, anisodamine alleviated the inflammatory damages by significantly reducing the expressions of TNF-α and COX-2 compared with the model group (P<0.05), and there was no difference in the expressions of TNF-α and COX-2 between the magnesium sulfate group and the anisodamine group (P>0.05). The results showed the significant protective effects of anisodamine on relieving vascular congestion, inflammatory cell infiltration, and proliferation, swelling of endothelium and perivascular hemorrhage in the animal model of infusion phlebitis with significantly reducing the expressions of TNF-α and COX-2.

In conclusion, the present study has established a method for preventing phlebitis caused by intravenous administration and analyzed the preventing mechanisms of anisodamine for infusion phlebitis. It has demonstrated that activations of TNF-α and COX-2 could be the important mechanisms of pathogenesis of infusion phlebitis and it gives us a theoretical basis of systematic mechanism study on the usage of anisodamine, which effectively prevents the occurrence of infusion phlebitis in clinical practice. This finding is a new strategy in the prevention and treatment of infusion phlebitis, and it should enrich the methods and theories of preventing and treating infusion phlebitis, and expand the use of anisodamine in clinical practices.

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