Analysis of the improvement effect of Astragalus extract on oxidative stress injury in viral myocarditis through STAT3/IL-6

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ABSTRACT

The objective of this study was to investigate the improvement effect of Astragalus (AS) extract on oxidative stress (OS) and inflammatory response of myocarditis (MYO) cells through the STAT3/IL-6 axis. For this purpose, The MYO model cells prepared by intervening cardiomyocyte HL-1 with Coxsackievirus B3 (CVB3) were divided into four groups: model group, as well as high- (H-), medium- (M-) and low-dose (L-) AS groups treated by 80, 40, and 20 µg/mL AS, respectively. Conventionally cultured cells were set as the normal group. Cell multiplication and apoptosis, as well as levels of Myocardial injury markers (cTnT, BNP and CK), inflammatory cytokines (ICs; TNF-α, IL-1β and IL-6) and OS indices (SOD, GSH-Px and MDA), were measured. STAT3/IL-6 pathway expression was also observed. Results showed that the model group presented decreased cell multiplication than the normal group, but with increased myocardial injury, apoptosis rate, Caspase3 protein, ICs and OS reaction ($P < 0.05$); In the three AS-intervened groups, enhanced cell multiplication, while reduced myocardial injury, apoptosis rate, ICs and OS response were observed, especially in H-AS group ($P < 0.05$). Besides, STAT3 and IL-6 concentrations, statistically increased in the model group, were reduced by AS intervention ($P < 0.05$). Colivelin, a specific activator of STAT3, further aggravated the apoptosis, inflammatory reaction and OS response of MYO cells ($P < 0.05$), but its impacts on MYO cells could be reversed by AS. In conclusion, AS can ameliorate MYO, and its mechanism is related to the inhibition of cellular inflammatory response and OS response through the STAT3/IL-6 axis.

Introduction

Myocarditis (MYO), a chronic disease of the heart characterized by localized or diffuse inflammatory lesions of the myocardium, usually presents with inflammatory cell infiltration accompanied by degeneration and necrosis of adjacent cardiomyocytes (CMs). According to statistics, the global incidence of MYO has reached 10-22/100,000, among which viral MYO is the most prevalent(1). Most MYO is stable, with the general clinical manifestations of fever, muscle aches, and palpitations, as well as a certain degree of cardiac dysfunction and dyspnea in severe cases. Nonetheless, 4-5% of patients have fulminant onset, which is usually serious and has a high risk of sudden death(2). In a survey conducted by Ammirati, it was found that more than 200,000 patients worldwide died of fulminant MYO in 2019, ranking second only to myocardial infarction and coronary heart disease among cardiovascular diseases(3). Instead of a unified clinical guideline for MYO treatment due to the great differences among patients, the best therapeutic drug is usually selected according to the patient's condition(4). In recent years, traditional Chinese medicine, with higher safety, stability and economic effects compared with Western medicine, is especially suitable and has gradually gained clinical recognition in treating chronic diseases with a long treatment cycle. Among them, we found that Astragalus (AS) is a perennial herb that has the functions of enhancing immune function, liver protection, diuresis, anti-aging, anti-stress, antihypertensive and extensive antibacterial effects. At the same time, it can be used to dilate blood vessels and improve blood circulation and metabolic environment, allowing for its wide application in the treatment of heart disease, hypertension, diabetes and other diseases(5, 6). As far as MYO is concerned, some studies have also pointed out that AS has excellent application results, but its specific mechanism has not been fully defined(7, 8).

As we all know, the destruction of immune function and aggravation of inflammatory reaction in heart tissue under bacterial infection are the main pathological processes of MYO(9), so the treatment of MYO should be based on inhibiting the inflammatory response. STAT3/IL-6 axis, with obvious abnormal expression under hepatitis B virus infection, is a classic inflammatory signaling pathway in clinical practice(10). Moreover, we found that this axis can inhibit Th17 cell differentiation and participate in MYO progression(11). AS, on the other hand, can affect breast cancer cell growth and metastasis via STAT3/IL-6 transduction(12). Thus, we speculate that the therapeutic mechanism of AS for MYO may be related to the STAT3/IL-6 axis, but there is no clinical study to confirm our view.

Accordingly, this study aims to render more reliable reference and guidance for future clinical treatment of MYO by exploring the mechanism of AS extract's impact on MYO cells and its relationship with the STAT3/IL-6 axis.

Materials and Methods

Study area

The study was carried out at the Eighth Medical Center of the General Hospital of the People's Liberation Army from June 2021 to October 2021.

Cell data

Supplied by ATCC and isolated from SD rats aged 1-3 days, CMs HL-1 were cultured in a high-glucose DMEM comprising 1% antibiotics and 10% fetal bovine serum (FBS).

MYO model building

A MYO model was established by intervening CMs with Coxsackievirus B3 (CVB3)(13). Cells were inoculated on a 6-well plate when 80-90% growth density was observed. Conventional cultured cells were used as a normal group, while cells infected with 1×10^2 PFU CVB3 and cultured for 48h were used as MYO model cells.

Cell multiplication assay

AS extract was manufactured by Shaanxi Wanyuan Biotech. MYO model cells were assigned to 4 groups: model group, as well as high- (H-), medium- (M-) and low-dose (L-) AS groups. Normal group cells were inoculated in 96-well plates (5×10^3 cells/well) for culture. 24 hours later, 0.1% dimethyl sulfoxide (DMSO) was added to normal and model groups, while AS extracts of 80, 40, and 20 μg/ml were added to H-, M- and L-AS groups, respectively. After a 48-hour incubation, 1.2 mg/ml MTT solution was added for 4 h of incubation (37 °C). Cell absorbance$_{490nm}$ in each group was determined with the use of an Absorbance Microplate Reader.

Apoptosis assay

Cells were inoculated into a 6-well plate with 2×10^6 cells/well, and grouped according to the above methods for 24 hours of intervention following a 24-hour culture. They were then digested with 0.25% trypsin and immersed in a binding buffer. After washing, Annexin V and PI, each with 5 μL, were used for staining. Flow cytometry (FCM) determined the cell apoptosis rate.

qRT-PCR experiment

The Trizol-extracted total RNA was determined by a UV spectrophotometer for purity, after which it was synthesized into cDNA. The quantification of STAT3 and IL-6 levels was carried out following the supplier's recommendations. See Table 1 for primer sequences. Gene expression relative to GAPDH was calculated using the formula $2^{-\Delta\Delta Ct}$.

Western blot (WB) test

SDS-PAGE was performed on RIPA buffer-lysed cells after the verification of concentration by BCA. Then the cells were transferred to PVDF membranes, sealed with 5% skim milk for 1 hour, and incubated overnight at 4 °C. The second antibody labeled with horseradish peroxidase was added to the membranes the next day, and incubated at room temperature for 2 hours. ECL development was then performed and the relative expression of proteins was calculated by scanning the membrane with Quantity One.

ELISA

Cell supernatant was obtained by centrifugation (12000 x g) for 15min, and the concentrations of cTnT, BNP, CK, TNF-α, IL-1β, IL-6, SOD, GSH-Px and MDA in cells were detected according to the recommendations of ELISA kits all supplied by Beijing Transgen Biotech.

Pathway intervention

MYO cells obtained during the logarithmic phase of growth were pretreated with 0.5 μmol/L Colivelin (CLN; a STAT3 specific activator) for 1h to establish MYO cells capable of activating STAT3/IL-6 pathway expression.

Statistical processing

Data processing and graphic plotting were performed by SPSS *v*22.0 and GraphPad *v*8, respectively. All experiments were repeated three times in this study, and the results were expressed as (x̄±s). Repeated measures analysis of variance and LSD post-hoc test were used for comparison among multiple groups, with the significance level set as $P < 0.05$.

Results

Effects of AS on MYO cell damage

First, we examined markers of myocardial injury in cells under AS intervention. The results showed significantly higher cTnT, BNP and CK in the model group compared with the normal group, indicating the presence of serious myocardial injury ($P < 0.05$). cTnT, BNP and CK were found to be statistically decreased in AS-treated cells, among which the H-AS group had the lowest levels, which were still higher when compared to the normal group ($P < 0.05$, Figure 1A-C). It can be seen that the myocardial in-
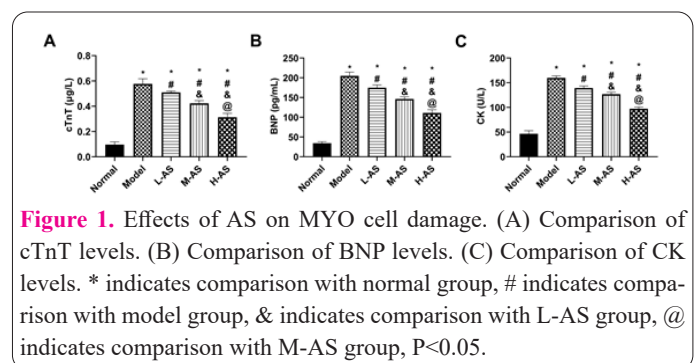

Figure 1. Effects of AS on MYO cell damage. (A) Comparison of cTnT levels. (B) Comparison of BNP levels. (C) Comparison of CK levels. * indicates comparison with normal group, # indicates comparison with model group, & indicates comparison with L-AS group, @ indicates comparison with M-AS group, P<0.05.

Table 1. Primer sequences.

	F (5'-3')	R (5'-3')
STAT3	ACAACTTTGGTATCGTGGAAG G	ATGAGCCCCAGCCTTCTCCAT
IL-6	CTTCGGTCCAGTTGCCTTC	TGAAGAGGTGAGTGGCTGTCT
GAPDH	CCAGCCGAGCCACATCGCTC	ATGAGCCCCAGCCTTCTCCAT

jury has been alleviated to some extent after AS treatment.

Influence of AS on MYO cell activity

To confirm AS's influence on MYO cell activity, we detected MYO cell proliferation and apoptosis via MTT (Figure 2A) and FCM assays (Figure 2B, C). First, we found that cell proliferation in the model group was reduced compared with the normal group ($P < 0.05$), while the apoptosis rate was increased ($P < 0.05$), indicating significantly reduced activity of MYO model cells established by CVB3 intervention. Second, higher cell proliferation capacity was determined in the three AS-intervened groups compared with the model group, but was still lower when compared to the normal group ($P < 0.05$); Among them, the H-AS group had the highest while the L-AS group had the lowest proliferative capacity ($P < 0.05$). In terms of apoptosis, the three AS-treated groups showed a lower apoptosis rate than the model group, but a higher one when compared to the normal group ($P < 0.05$); Among them, the lower apoptosis rate was determined in H-AS while highest in L-AS group ($P < 0.05$).

Influence of AS on inflammatory response and oxidative stress response (OSR) of MYO cells

Subsequently, we detected inflammatory cytokines (ICs) TNF-α, IL-1β and IL-6 (Figure 3A), as well as SOD, GSH-Px and MDA (Figure 3B) levels to confirm the influence of AS on inflammatory response and OSR of MYO cells. The results identified elevated ICs TNF-α and IL-1β and IL-6 in the model group compared with the normal group ($P < 0.05$), suggesting obvious inflammatory reaction in MYO cells. In the three AS-intervened groups, notably reduced TNF-α and IL-1β and IL-6 were observed, with the lowest levels found in the H-AS group and the highest in the L-AS group ($P < 0.05$), which indicated that AS could inhibit MYO cell inflammation. Besides, we found reduced SOD, GSH-Px and elevated MDA in the model group compared with the normal group ($P < 0.05$), suggesting that MYO cells also had obvious OSR. However, after AS intervention, SOD, GSH-Px increased and MDA decreased ($P < 0.05$), indicating that AS could inhibit the OSR in MYO cells.
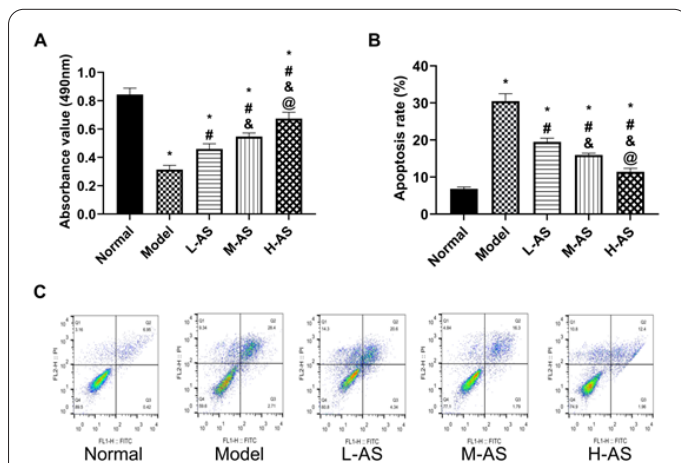

Figure 3. Influence of AS on inflammatory response and oxidative stress response of MYO cells. (A) Levels of ICs TNF-α, IL-1β and IL-6. (B) Levels of oxidative stress indexes SOD, GSH-Px and MDA. * indicates comparison with normal group, # indicates comparison with model group, & indicates comparison with L-AS group, @ indicates comparison with M-AS group, P<0.05.
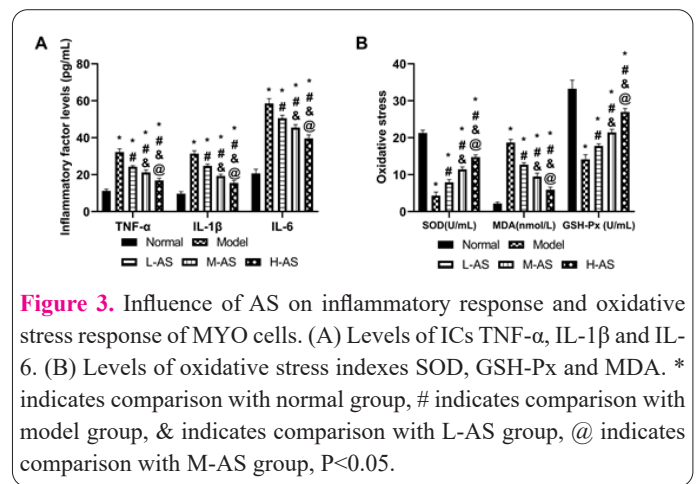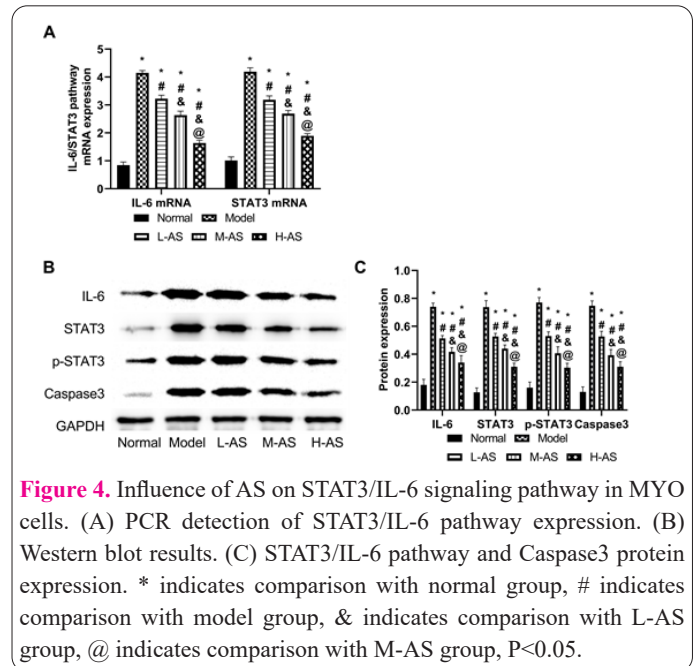

Figure 4. Influence of AS on STAT3/IL-6 signaling pathway in MYO cells. (A) PCR detection of STAT3/IL-6 pathway expression. (B) Western blot results. (C) STAT3/IL-6 pathway and Caspase3 protein expression. * indicates comparison with normal group, # indicates comparison with model group, & indicates comparison with L-AS group, @ indicates comparison with M-AS group, P<0.05.

Influence of AS on STAT3/IL-6 pathway and protein expression in MYO cells

Then, we detected the expression of the STAT3/IL-6 pathway and Caspase3 protein by PCR (Figure 4A) and WB (Figure 4B, C). The results showed elevated STAT3 mRNA and IL-6 mRNA in the model group compared with the normal group ($P < 0.05$); while STAT3 mRNA and IL-6 mRNA in AS intervention groups were lower than those in the model group and higher than those in the normal group ($P < 0.05$). And similarly, the levels were the lowest in the H-AS group and the highest in the L-AS group ($P < 0.05$). According to WB results, STAT3, p-STAT3, IL-6, and Caspase3 protein levels in the normal group were the lowest among the five groups, followed by H-AS, M-AS, and L-AS groups, with those in the model group being the highest ($P < 0.05$). Thus, the STAT3/IL-6 pathway is obviously activated in MYO cells, and the use of AS can inhibit STAT3/IL-6 to a certain extent; The expression of the Caspase3 protein is also consistent with the apoptosis rate of cells, which can validate our results.

AS ameliorates myocardial injury through STAT3/IL-6 axis

Through the above experiments, we can see that the H-AS group had the most significant improvement effect on MYO cells, so we chose this dose for follow-up ana-

Figure 2. Influence of AS on MYO cell activity. (A) MTT assay results. (B) Apoptosis rate. (C) FCM assay results. * indicates comparison with normal group, # indicates comparison with model group, & indicates comparison with L-AS group, @ indicates comparison with M-AS group, P<0.05.

lysis. We set MYO cells intervened by 80 μg/ml AS and CLN as group A, MYO cells intervened with CLN alone as group B, and MYO model cells as the model group. The experimental results revealed no significant difference in cTnT, BNP and CK levels between group A and model group ($P > 0.05$), higher than those in group B ($P < 0.05$, Figure 5A-C). It suggested that the improvement of myocardial cell injury by AS was completely reversed by CLN.

Influence of AS on MYO cell activity through STAT3/IL-6 axis

Detecting the cell activity again, the results showed equivalent cell proliferation between group A and model group ($P > 0.05$), higher than that of group B ($P < 0.05$, Figure 6A). A similar apoptosis rate was also determined in group A and the model group by FCM (Figure 6B) ($P > 0.05$), which was lower than that of group B ($P < 0.05$, Figure 6C). Finally, Caspase3 protein detection results were also consistent as described above, that is, Caspase3 protein expression differed insignificantly between group A and the model group, significantly lower than those in group B ($P < 0.05$). Furthermore, we found that STAT3/IL-6 pathway expression in group A was no different from that in the model group ($P > 0.05$), while STAT3, p-STAT3 and IL-6 levels in group B were significantly increased ($P < 0.05$, Figure 6D, E), which indicates the success of CLN intervention on the one hand, and that CLN's activation and induction of STAT3/IL-6 pathway can be inhibited by AS on the other.

Influences of AS on inflammatory response and OSR through STAT3/IL-6 axis

The inflammatory reaction (Figure 7A) and OSR (Figure 7B) were also detected in the above three groups of cells. The results showed that the TNF-α and IL-1β and IL-6 levels in group A were (30.94±2.71 pg/mL), (31.29±0.80 pg/mL) and (60.72±3.26 pg/mL), respectively, which had no difference from those in the model
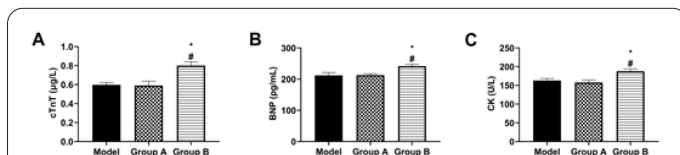
Figure 5. AS ameliorates myocardial injury through STAT3/IL-6 axis. (A) Comparison of cTnT levels. (B) Comparison of BNP levels. (C) Comparison of CK levels. * indicates comparison with model group, # indicates comparison with group A, P<0.05.
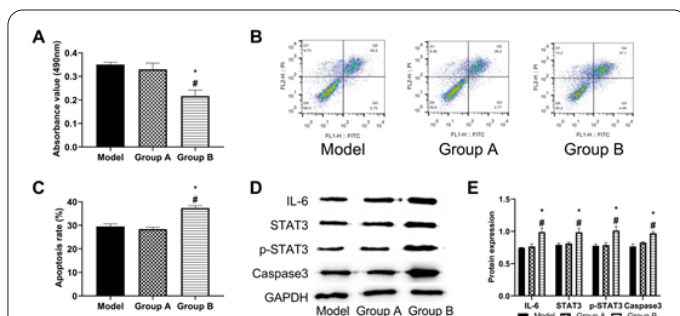
Figure 6. Influence of AS on MYO cell activity through STAT3/IL-6 axis. (A) MTT assay results. (B) FCM assay results. (C) Apoptosis rate. (D) Western blot results. (E) STAT3/IL-6 pathway and Caspase3 protein expression. * indicates comparison with model group, # indicates comparison with group A, P<0.05.
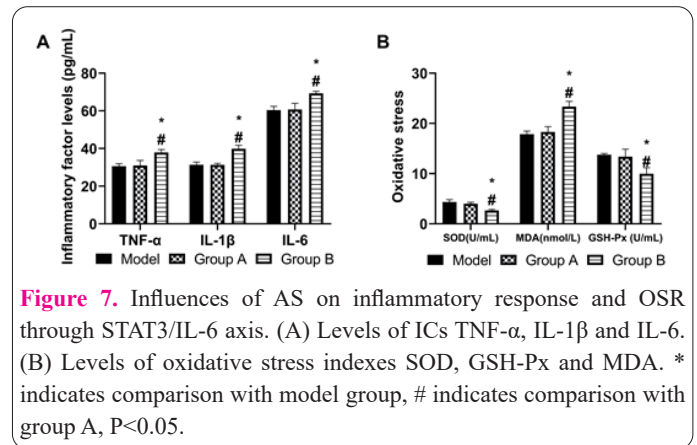
Figure 7. Influences of AS on inflammatory response and OSR through STAT3/IL-6 axis. (A) Levels of ICs TNF-α, IL-1β and IL-6. (B) Levels of oxidative stress indexes SOD, GSH-Px and MDA. * indicates comparison with model group, # indicates comparison with group A, P<0.05.

group ($P > 0.05$); while the TNF-α and IL-1β and IL-6 levels in group B were (37.90±1.56 pg/mL), (39.81±1.87 pg/mL) and (69.31±1.05 pg/mL), respectively, higher than those in group A and model group ($P > 0.05$). Similarly, SOD, GSH-Px and MDA levels in group A and the model group showed no significant differences ($P > 0.05$), the SOD, GSH-Px of which was higher and the MDA was lower compared with Group B ($P < 0.05$).

Discussion

As a prevalent chronic heart disease, MYO has shown an increasing incidence in recent years, the potential threat of which warrants clinical attention(14). CVB3, as an unenveloped single-stranded RNA enterovirus, is also the major cause of viral MYO. Therefore, CVB3 is commonly used to induce MYO cell models in clinical trials, with well-documented effects(15, 16). However, despite increasing cognition of MYO in clinical practice by relying on this method, there is still no unified clinical treatment guide for MYO treatment. Hence, the exploration of diagnosis and treatment strategies for MYO is still a hot spot in clinical research. In recent years, the excellent application results of AS in many chronic diseases make it a new choice for future treatment of MYO. However, we still need to fully grasp the exact mechanism of action of AS before its specific application, so as to provide more effective reference opinions for clinical practice.

In this study, we detected MYO injury, cell activity, ICs and OSR under AS intervention. First, obviously reduced proliferation ability was observed in the model group compared with the normal group, while the myocardial injury markers, apoptosis rate, Caspase3 protein, ICs and OSR were notably increased, indicating that MYO could accelerate CM apoptosis and inflammatory injury. In previous studies, we also found statistically increased apoptosis rate and intensified inflammatory reactions of CMs in MYO model rats(17, 18), which can not only verify the pathological effect of MYO but also confirm the success of our modeling. Subsequently, in the three groups of cells intervened by AS, the proliferation ability of cells was significantly enhanced, while the apoptosis rate, ICs and OSR were significantly reduced, which indicated that AS could effectively alleviate the pathological process of MYO and inhibit MYO progression. In the study of Shao et al., we found that AS can also improve the myocardial remodeling function of ischemic cardiomyopathy rats(19), which once again emphasizes the improvement effect of AS on the pathological process of chronic diseases. Among them,

the H-AS group has the most significant improvement in cell activity and inflammatory reaction, which demonstrated better efficacy of AS when used in large dosages as well as the high safety of AS. However, follow-up human experiments are needed to confirm the optimal dosage in clinical practice.

Based on the above results, we can initially confirm that AS has effective anti-inflammatory, anti-oxidative and anti-apoptotic effects on MYO cells, but the specific pathway of its action is still unclear. As aforementioned, the effect of AS on MYO may be related to the STAT3/IL-6 axis, so we also detected the expression of this pathway in MYO cells. The results identified notably enhanced STAT3 and IL-6 levels in the model group, which indicated that the STAT3/IL-6 pathway was activated in MYO; while the application of AS reduced STAT3 and IL-6 expression and inhibited this signal pathway, which was consistent with our expectation. STAT3/IL-6 pathway, as a classic pro-inflammatory pathway, is known to be able to accelerate the process of inflammatory injury and oxidation reaction by activating the release of inflammatory mediators in many diseases(20-22), and in MYO, the activation state of STAT3/IL-6 pathway has also been confirmed(23). Then, MYO was further interfered with AS and CLN, the STAT3/IL-6 pathway activator. There are many reports (24-36) about the effect of medicinal plants in the treatment of various diseases.

The results showed that under the action of CLN, the apoptosis, inflammatory reaction and OSR of MYO cells were further intensified, and the malignant pathological changes were more significant than those of model group cells. However, cells treated with AS+CLN showed no difference in activity and pathological manifestations compared with the model group, indicating that the effects of either AS or CLN on MYO cells could be reversed with the co-use of AS and CLN, with a synergistic and complementary relationship between the two, which confirmed our views that AS affects MYO development through the STAT3/IL-6 pathway.

AS reduces inflammation and oxidative stress in MYO cells and inhibits apoptosis via inhibiting the STAT3/IL-6 axis, and thus halts the development of MYO, which has important application value in future clinical treatment of MYO.

Conflicting interests
The authors declared no potential conflicts of interest with respect to the research.

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Not applicable.

Availability of data and materials
The datasets used and analyzed in the current study will be available from the corresponding author upon request.

Ethics approval and consent to participate
The study protocol was approved by the Ethics Committee of The 8th Medical Center of Chinese PLA General Hospital.

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