Research Paper

Effect of Total Flavones of *Astragalus* on Apoptosis and Inflammation of Alveolar Epithelial Cells Induced by Tumor Necrosis Factor Alpha through microRNA-221

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To explore the effect and molecular mechanism of total flavonoids of *Astragalus* on the apoptosis and inflammation of alveolar epithelial cells induced by tumor necrosis factor alpha. Human alveolar epithelial cells were divided into control group, tumor necrosis factor alpha group, tumor necrosis factor alpha+trifluoroacetic acid-low, tumor necrosis factor alpha+trifluoroacetic acid-medium, tumor necrosis factor alpha+trifluoroacetic acid-high group, tumor necrosis factor alpha+anti-microRNA-NC group, tumor necrosis factor alpha+microRNA-221 inhibitor group, tumor necrosis factor alpha+trifluoroacetic acid-high+microRNA-NC group, tumor necrosis factor alpha+trifluoroacetic acid-H+microRNA-221 mimic group. Real-time fluorescence quantitative polymerase chain reaction was used to detect the expression of microRNa-221; (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to detect the cell proliferation inhibition rate; flow cytometry to detect the apoptosis of alveolar epithelial cells; enzyme-linked immunosorbent assay to detect the levels of interleukin-1 beta, I interleukin-6 and interleukin-10. In tumor necrosis factor alpha-induced alveolar epithelial cells, the expression of microRNA-221 was increased, the cell proliferation inhibition rate and apoptosis rate were increased, the levels of interleukin-1 beta, interleukin-6 were increased and the level of interleukin-10 was decreased ($p<0.05$). After treatment with medium and high concentrations of total flavones of *Astragalus*, the expression of microRNA-221 in alveolar epithelial cells induced by tumor necrosis factor alpha was decreased, cell proliferation inhibition rate and apoptosis rate were decreased, interleukin-1 beta and interleukin-6 levels were decreased and interleukin-10 was increased ($p<0.05$). Inhibition of microRNA-221 expression can inhibit tumor necrosis factor alpha induced apoptosis and inflammation of alveolar epithelial cells. Overexpression of microRNA-221 can reverse the effect of total flavonoids of *Astragalus* on apoptosis and inflammation of alveolar epithelial cells induced by tumor necrosis factor alpha. Total flavones of *Astragalus* inhibited tumor necrosis factor alpha induced apoptosis and inflammation of alveolar epithelial cells by down-regulating microRNA-221.

Key words: Total flavones, *Astragalus*, microRNA-221, alveolar epithelial cells, apoptosis, inflammation

Acute lung injury is one of the clinical respiratory diseases, and its main pathophysiological characteristics are the infiltration and apoptosis of inflammatory cells, inhibiting inflammatory factor production, and reducing the apoptosis of alveolar epithelial cells are effective measures for its treatment[1]. Studies have found that Traditional Chinese Medicine (TCM) can be used to treat acute lung injury, and based on the pathogenesis of acute lung injury, TCM and monomers of TCM with significant efficacy can be selected, which can guide clinical medication[2,3]. Total flavones of *Astragalus*, a major active ingredient of antioxidation extracted from TMC *Astragalus*, have been studied and found to have immunomodulatory and anti-inflammatory effects[4]. Studies have

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reported that different concentrations of total flavones of *Astragalus* have protective effects against high glucose induced endothelial cell injury in human umbilical vein line[5]. Total flavones of *Astragalus* may play a protective role in rats with cerebral ischemia-reperfusion injury by inhibiting the level of oxidative stress, reducing inflammatory response, anti-apoptosis and other pathways[6]. In addition, total flavones of *Astragalus* can prevent the development of radiation pneumonitis in mice through antioxidant mechanisms[7]. However, the effect and mechanisms of total flavones of *Astragalus* on Tumor Necrosis Factor-Alpha (TNF-α) induced alveolar epithelial cell apoptosis and inflammation remain unclear. Studies have reported increased microRNA (miR-221) expression levels in asthmatic patients; silencing miR-221 attenuates airway inflammation in asthma models[8]. Inhibition of miR-221 can inhibit lipopolysaccharide induced secretion of inflammatory factors and inhibit cell apoptosis in alveolar epithelial cells by targeting Adiponectin Receptor 1 (AdipoR1)[9]. Inhibition of miR-221 attenuates lipopolysaccharide induced inflammation in mice with acute lung injury by enabling Suppressor of Cytokine Signaling 1 (SOCS1)/Nuclear Factor-Kappa B (NF-κB) signaling pathway inactivation[10]. Above studies demonstrated that miR-221 was involved in regulating the inflammatory response and lung injury in alveolar epithelial cells. Therefore, this experiment aimed to investigate whether total flavones of *Astragalus* affect TNF-α induced alveolar epithelial cell apoptosis and inflammation by regulating miR-221.

MATERIALS AND METHODS

Materials:

Human alveolar epithelial cells were purchased from ScienCell, United States of America (USA); Dulbecco's Modified Eagle Medium (DMEM) medium was purchased from Hyclone™, USA; recombinant human TNF-α was purchased from Invivogen, USA; total flavones of *Astragalus* were purchased from Shanghai Ziqibio Co., Ltd.; miRNA reverse transcription kit was purchased from GeneCopoeia, USA; miRNA fluorescence qualitative Polymerase Chine Reaction (qPCR) kit was purchased from Shanghai Genepharma Co., Ltd.; (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) kit, Annexin V-Fluorescein Isothiocyante (FITC)/Propidium Iodide (PI) apoptosis detection kit were purchased from Wuhan Amyjet Scientific Inc.; protein extraction kits were purchased from Wuhan Chundu Biotechnology Co., Ltd.; Interleukin-1 (IL)-1 beta (β), IL-6 and IL-10 assay kits were purchased from Beijing Baio Leibo Technology Co., Ltd.

Treatment and grouping of cells:

Human alveolar epithelial cells were cultured in DMEM supplemented with 10 μg/l of TNF-α treated human alveolar epithelial cells as TNF-α group, normal cultured cells as the control group; cells were treated with 5 μg/ml, 10 μg/ml, 50 μg/ml Trifluoroacetic Acid (TFA) and 10 μg/ml of TNF-α treated human alveolar epithelial cells, denoted TNF-α+TFA-Low (TFA-L), TNF-α+TFA-Medium (TFA-M) and TNF-α+TFA-High (TFA-H) groups; 6 h after transfection of anti-miR-NC and miR-221 inhibitor into human alveolar epithelial cells, cells were treated in 10 μg/l of TNF- α, denoted as TNF-α+anti-miR-NC group and TNF-α+miR-221 inhibitor group; 6 h after transfection of miR-NC and miR-221 mimic into human alveolar epithelial cells, cells were treated in 10 μg/ml TFA and 10 μg/l TNF-α, denoted as TNF-α+TFA-H+miR-NC, and TNF-α+TFA-H+miR-221 mimic groups.

Real time-qPCR (RT-qPCR) to detect miR-221 expression levels:

Total Ribonucleic Acid (RNA) from alveolar epithelial cells of each group was extracted and reverse transcribed into complementary Deoxyribonucleic Acid (cDNA) using miRNA reverse transcription kit, PCR amplified using U6 as internal reference, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation inhibition rate by MTT assay:

The cells in each group were cultured for 48 h, operated according to the instructions of the kit, dimethyl sulfoxide was added after the reaction of MTT solution for 4 h, and the absorbance (Optical Density (OD)) value at 490 nm was measured by a microplate reader

Cell proliferation inhibition rate=OD value of experimental group/OD value of control group.

Apoptosis by flow cytometry:

Cells cultured for 48 h were collected, manipulated according to the kit instructions, and finally the apoptosis rate was detected by upper flow cytometry.

Detection of protein expression by Western blot:

Total cellular proteins were extracted and separated by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), followed by membrane shift, blocking, addition of primary antibodies for B-cell lymphoma protein 2 (Bcl-2) and (Bcl-2)-associated X (Bax) Bax overnight at 4°, incubation with secondary antibodies for 1 h at room temperature, development, imaging and analysis of the gray levels of protein bands using image J, and calculation of protein expression levels using Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) as an internal reference.

Detection of IL-1β, IL-6 and IL-10 levels by Enzyme-Linked Immunosorbent Assay (ELISA):

The supernatants were harvested after 48 h of cell culture in each group, which was performed according to the kit operation.

Statistical analysis:

Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) 20.0 software, and the measurement data that conformed to normal distribution were expressed as mean±standard deviation ($\bar{x}\pm s$), t-test was used for comparison between two groups, one-way Analysis of Variance (ANOVA) was used for comparison between multiple groups, and Least Significant Difference (LSD)-test was used for comparison between two groups. $p<0.05$ was taken as statistically significant.

RESULTS AND DISCUSSION

Compared with the control group, the expression levels of miR-221 in TNF-α group increased, the cell proliferation inhibition rate and apoptosis rate increased, the expression level of Bax increased, and the expression level of Bcl-2 decreased in all groups ($p<0.05$); compared with TNF-α group, miR-221 expression levels in TNF-α+TFA-M, TFA-H group gradually decreased, cell proliferation inhibition rate and apoptosis rate gradually decreased, Bax expression level gradually decreased and Bcl-2 expression level gradually increased ($p<0.05$) as shown in fig. 1 and Table 1.

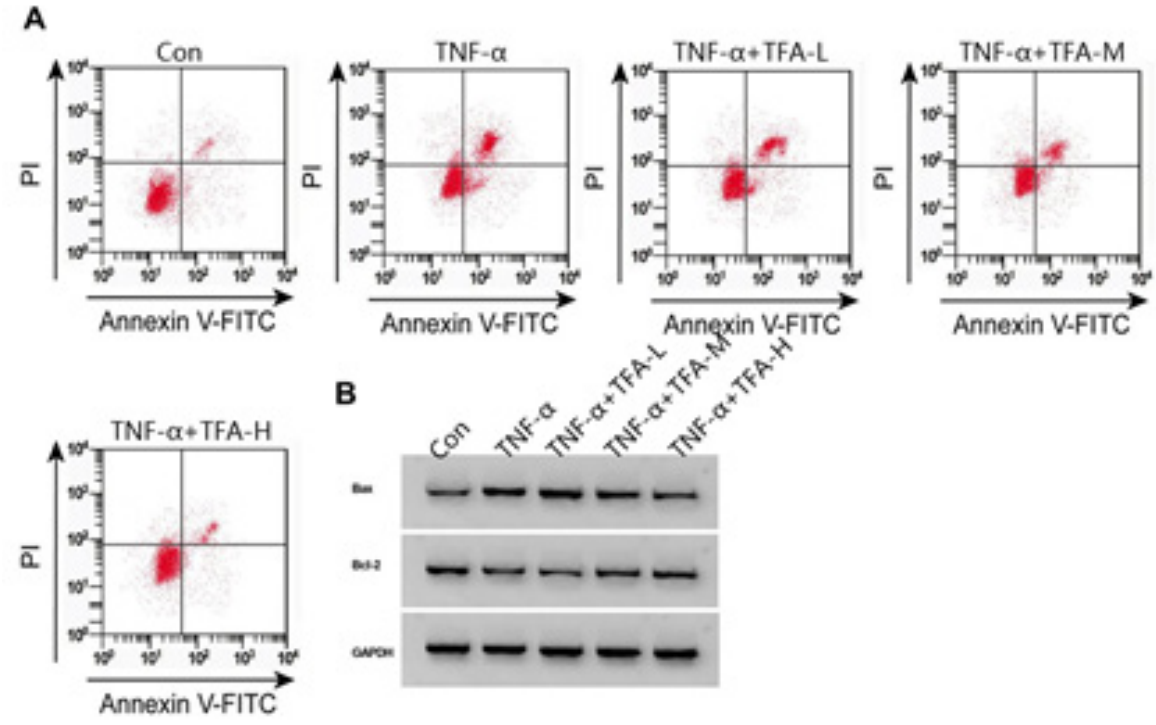

Fig. 1: The effect of total flavonoids of *Astragalus* on human alveolar epithelial cells apoptosis and Bax and Bcl-2 protein expression induced by TNF-α

TABLE 1: EFFECT OF TOTAL FLAVONES OF *Astragalus* ON CELL VIABILITY AND APOPTOSIS (x̄±s, n=3)

Group	miR-221	Inhibition rate (%)	Apoptosis rate (%)	Bax	Bcl-2
Control	1.00±0.00	0.00±0.00	6.44±0.36	0.11±0.01	0.68±0.06
TNF-α	5.65±0.16[a]	64.53±3.75[a]	25.18±1.02[a]	0.79±0.06[a]	0.15±0.01[a]
TNF-α+TFA-L	5.62±0.19	65.13±3.68	25.02±1.71	0.80±0.07	0.16±0.02
TNF-α+TFA-M	4.32±0.14[bc]	47.49±3.47[bc]	21.03±1.46[bc]	0.56±0.04[bc]	0.35±0.03[bc]
TNF-α+TFA-H	2.30±0.11[bcd]	31.27±3.10[bcd]	14.19±1.17[bcd]	0.27±0.03[bcd]	0.53±0.04[bcd]
F	386.277	224.697	127.132	129.23	121.886
p	0.000	0.000	0.000	0.000	0.000

Note: Compared with the control group, [a]p<0.05; compared with the TNF-α group, [b]p<0.05; compared with TNF-α+TFA-L group, [c]p<0.05 and compared with TNF-α+TFA-M group, [d]p<0.05

Compared with the control group, IL-1β and IL-6 levels increased, and level of IL-10 decreased in TNF-α group ($p<0.05$); compared with TNF-α group, IL-1β and IL-6 levels were gradually decreased in TNF-α+TFA-M, TFA-H group, and the levels of IL-10 were gradually increased ($p<0.05$) as shown in Table 2.

Compared with TNF-α+anti-miR-NC group, the expression levels of miR-221 were decreased in TNF-α+miR-221 inhibitor group, and the cell proliferation inhibition rate and apoptosis rate were decreased, while the expression levels of Bax were decreased, Bcl-2 were increased, and the levels of IL-1β and IL-6 were decreased, and the levels of IL-10 were increased ($p<0.05$) as shown in fig. 2 and Table 3.

Compared with TNF-α+TFA-H+miR-NC group, the miR-221 expression levels increased in TNF-α+TFA-H+miR-221 mimic group, cell proliferation inhibition and apoptosis rates increased, Bax expression levels increased, and Bcl-2 expression levels decreased ($p<0.05$) as shown in fig. 3 and Table 4.

Compared with TNF-α+TFA-H+miR-NC group, the levels of IL-1β and IL-6 increased in TNF-α+TFA-H+miR-221 mimic group, and the levels of IL-10 decreased ($p<0.05$) as shown in Table 5.

Acute lung injury is a critical illness with high lethality that is common in the clinic, and TCM has achieved significant efficacy in preventing and treating acute lung injury disease processes, and studying its pharmacological effects on acute lung injury protection may provide a reference for effective clinical treatment and drug development[11,12]. Studies have reported that total flavones of *Astragalus* may inhibit inflammatory factor secretion from osteoarthritic chondrocytes by down-regulating NF-κB signaling pathway[13]. Total flavones of *Astragalus* can reduce serum induced endothelial cell apoptosis in uremic patients[14]. Total flavones of *Astragalus* have shown protective effects in mice with viral myocarditis[15]. Total flavones of *Astragalus* alleviate experimental autoimmune encephalomyelitis through inhibition of activation of the c-Jun N-terminal kinase (JNK)/Protein kinase B (AKT)/NF-κB signaling pathway and inflammatory responses[16]. Above studies indicate that total flavones of *Astragalus* have anti-inflammatory effects. TNF-α induced alveolar epithelial cells for this experiment were used to establish the injury model, and the results showed that the TNF-α induced proliferation inhibition rate of alveolar epithelial cells increased, the apoptosis rate increased, and the levels of IL-1β and IL-6 increased and the levels of IL-10 decreased; IL-1β and IL-6 are pro-inflammatory factors, IL-10 is an anti-inflammatory factor, with high expression of IL-1β and IL-6 and low expression of IL-10 in a rat acute lung injury model[17]. Therefore, the present experimental results indicated that the model was established successfully. In this experiment, different concentrations of total flavones of *Astragalus* were used to treat TNF-α induced alveolar epithelial cells, the results showed that treatment with total flavones of *Astragalus* at medium and high concentrations reduced the cell proliferation inhibition rate and apoptosis rate, decreased the expression level of Bax, increased the expression level of Bcl-2, and decreased levels of IL-1β and IL-6 and increased levels of IL-10; studies have shown that high expression of IL-10 can inhibit the inflammatory response to

pneumonia in rats with acute lung injury[18]. These results indicate that certain concentrations of total flavones of *Astragalus* can inhibit TNF-α induced alveolar epithelial cell apoptosis and inflammatory response.

Studies have reported that miR-221 can promote apoptosis of human retinal microvascular endothelial cells under high glucose conditions[19]. miR-221 is involved in airway epithelial cell damage in asthma by targeting Sirtuin 1 (SIRT1) [20]. The results of this experiment showed that the miR-221 expression levels were increased in TNF-α induced alveolar epithelial cells; however, after inhibition of miR-221 expression, apoptosis rate of alveolar epithelial cells induced by TNF-α was decreased, the levels of IL-1β and IL-6 decreased, and the IL-10 level was increased; it showed that inhibition of miR-221 expression suppressed TNF-α induced alveolar epithelial cell apoptosis and inflammatory response. Furthermore, in this experiment, we found that treatment with total flavones of *Astragalus* decreased miR-221 expression, whereas miR-221 over-expression reversed the effects of total flavones of *Astragalus* on TNF-α induced alveolar epithelial cell apoptosis and inflammatory response.

In conclusion, total flavones of *Astragalus* inhibits TNF-α induced alveolar epithelial cell apoptosis and inflammatory response by down-regulating miR-221.

TABLE 2: THE EFFECT OF TOTAL FLAVONOIDS OF *Astragalus* ON INFLAMMATORY FACTORS

Group	IL-1β (pg/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)
Control	143.08±9.76	971.79±50.73	380.30±22.80
TNF-α	633.92±35.51[a]	143.44±14.11[a]	1339.56±57.59[a]
TNF-α+TFA-L	631.80±34.16	152.05±19.71	1319.28±67.94
TNF-α+TFA-M	504.29±22.69[bc]	366.38±24.06[bc]	979.77±42.96[bc]
TNF-α+TFA-H	316.36±20.17[bcd]	720.82±44.08[bcd]	537.16±36.55[bcd]
F	197.908	351.817	250.29
p	0.000	0.000	0.000

Note: Compared with the control group, [a]p<0.05; compared with the TNF-α group, [b]p<0.05; compared with TNF-α+TFA-L group, [c]p<0.05 and compared with TNF-α+TFA-M group, [d]p<0.05

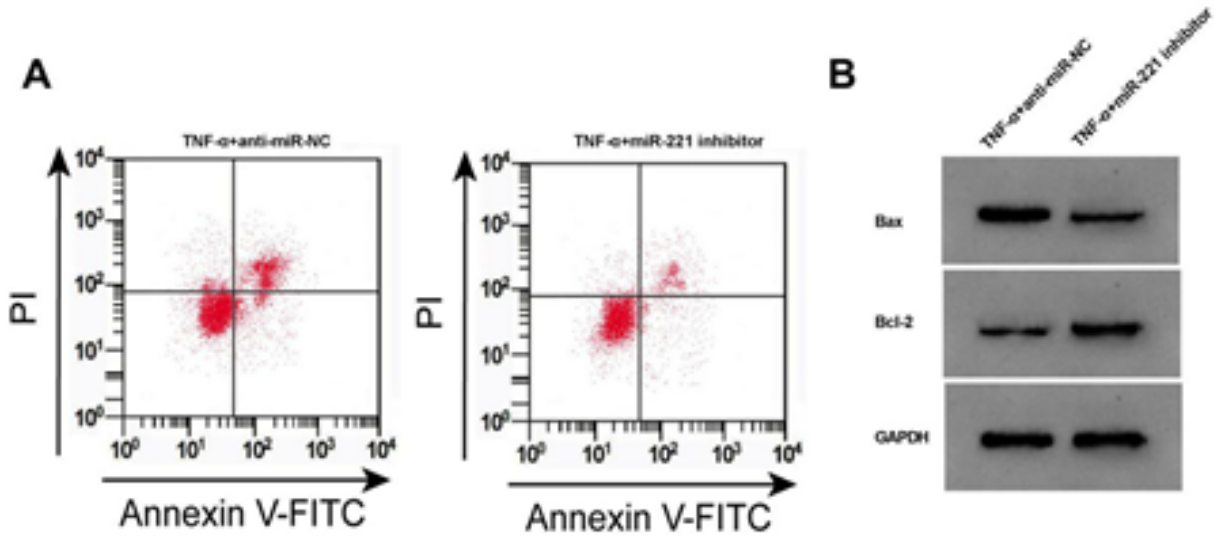

Fig. 2: The effect of inhibiting miR-221 on human alveolar epithelial cells apoptosis induced by TNF-α and the expression of Bax and Bcl-2 proteins

TABLE 3: THE EFFECT OF INHIBITING miR-221 ON CELL ACTIVITY, APOPTOSIS AND INFLAMMATORY FACTORS (x̄±s, n=3)

Group	miR-221	Inhibition rate (%)	Apoptosis rate (%)	Bax	Bcl-2	IL-1β (pg/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)
TNF-α+anti-miR-NC	5.67±0.16	64.69±4.03	25.00±1.53	0.81±0.07	0.13±0.01	640.12±39.07	150.19±19.28	1337.12±50.69
TNF-α+miR-221 inhibitor	1.88±0.09[a]	24.50±2.45[a]	10.02±0.75[a]	0.17±0.02[a]	0.58±0.05[a]	232.76±18.18[a]	854.47±48.57[a]	453.10±31.25[a]
t	35.759	14.686	15.227	15.227	15.286	16.373	23.343	25.713
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: Compared with TNF-α+anti-miR-NC, [a]p<0.05

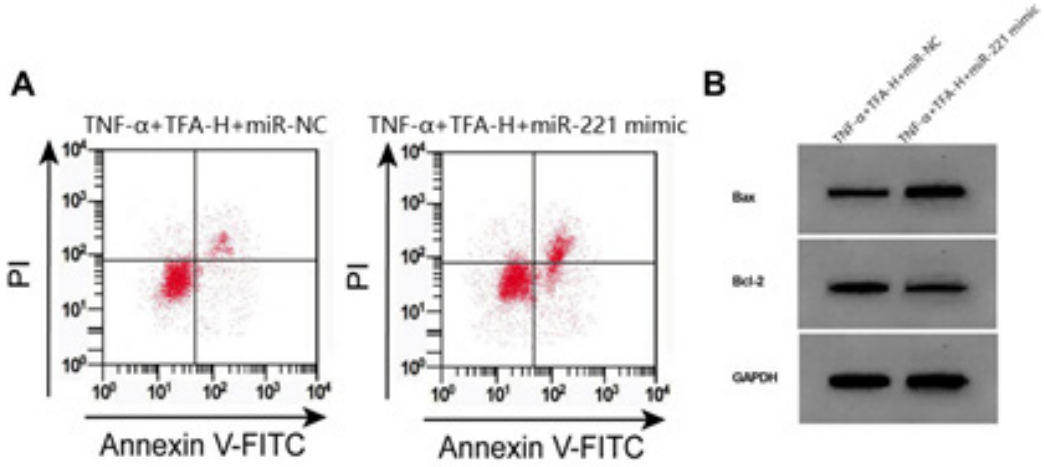

Fig. 3: miR-221 can reverse the effect of total flavonoids of *Astragalus* on human alveolar epithelial cells apoptosis, and Bax and Bcl-2 protein expression induced by TNF-α

TABLE 4: miR-221 CAN REVERSE THE EFFECT OF TOTAL FLAVONOIDS OF *Astragalus* ON CELL VIABILITY AND APOPTOSIS (x̄±s, n=3)

Group	miR-221	Inhibition rate (%)	Apoptosis rate (%)	Bax	Bcl-2
TNF-α+TFA-H+miR-NC	2.30±0.13	31.35±3.10	14.34±1.02	0.27±0.02	0.53±0.05
TNF-α+TFA-H+miR-221 mimic	4.85±0.14[a]	55.08±3.52[a]	23.73±1.25[a]	0.72±0.06[a]	0.23±0.02[a]
t	23.118	8.763	10.081	12.324	9.679
p	0.000	0.001	0.001	0.000	0.001

Note: Compared with TNF-α+TFA-H+miR-NC group, [a]p<0.05

TABLE 5: miR-221 CAN REVERSE THE EFFECT OF *Astragalus* FLAVONOIDS ON INFLAMMATORY FACTORS (x̄±s, n=3)

Group	IL-1β (pg/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)
TNF-α+TFA-H+miR-NC	316.73±22.89	725.27±49.86	542.44±31.78
TNF-α+TFA-H+miR-221 mimic	568.62±30.04a	259.49±27.83a	1191.01±55.34a
t	11.552	14.129	17.603
p	0.000	0.000	0.000

Note: Compared with TNF-α+TFA-H+miR-NC group, [a]p<0.05

Conflict of interests:

The authors declared no conflict of interests.

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