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Effect of esculentoside A on autoimmunity in mice and its possible mechanisms[1]

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KEY WORDS esculentoside A; autoimmunity; inflammation; intercellular adhesion molecule-1; apoptosis; rheumatoid arthritis

ABSTRACT

AIM: To investigate the influence of esculentoside A (EsA) on autoimmunity in mice and its possible mechanisms. **METHODS**: The level of anti-ds DNA antibody, proliferation of lymphoid cells, and inflammation by pathologic section of joint in mice were examined. The autoimmunity model is made through immunizing mice with formaldehyde treated *Campylobacter jejuni* strain CJ-S_{131} and Freund's complete adjuvant. The apoptosis of T cell was analyzed through morphology and flow cytometry (FACS). The expression of ICAM-1 mRNA in human umbilical vein endothelial cell line (ECV304) was determined by coupled reverse transcription and PCR amplification (RT-PCR). **RESULTS**: EsA could potently lower the level of anti-ds DNA antibody, inhibit the proliferation of lymphoid cells, and ameliorate inflammation in the joint of model mouse. The apoptosis of thymocyte activated by ConA was markedly accelerated while the expression of ICAM-1 mRNA in ECV304 was decreased by EsA. **CONCLUSION**: EsA has the positive curative effect on autoimmunity in a mouse model, which may function through inhibition of expression of ICAM-1 mRNA in ECV304 and acceleration of thymocyte apoptosis.

INTRODUCTION

Esculentoside A (EsA), a kind of saponin isolated from the Chinese herb *Phytolacca esculenta*, is reported to possess several pharmacological effects including anti-inflammation and immunoregulation. The Chinese herb *Phytolacca esculenta* has been proved to have striking therapeutic effects on a number of diseases such as rheumatoid arthritis, edema, and tumor. EsA is the saponin which has the strong effects on acute and chronic inflammation in several kinds of models[1]. Moreover, EsA markedly inhibits humoral and cellular immunity such as diminishing production of antibody and lowering secretion of tumor necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6) by several kinds of cells, and diminishes function of over-activated macrophages such as phagorytosis and secretion of cytokines[2-7]. It is generally accepted that many immune cells and cytokines are involved in the occurrence and progress of autoimmune diseases. The previous results showed that EsA possessed therapeutic effect on an autoimmune disease, passive Heymann nephritis[8]. We supposed that EsA might have favorable effect in the treatment of autoimmunity.

Autoimmune diseases represent an immune attack on self-tissue characterized with the production of auto-antibody, inflammation, proliferation of lymphoid tissues, and glomerulonephritis caused by immune complexes. To gain a better understanding of the pharmacological effects of EsA, a model of autoimmunity was established through immunizing mice with formaldehyde treated *Campylobacter jejuni* strain CJ-S_{131} and Freund's complete adjuvant. After the positive results had been found, the possible mechanisms were studied from two aspects including inhibiting the expression of ICAM-1 mRNA in ECV304 and accelerating apoptosis of thymocyte activated by ConA.

MATERIALS AND METHODS

Reagents Inactived strain CJ-S_{131} was purchased from Shanghai Health and Epidemic Prevention Station.

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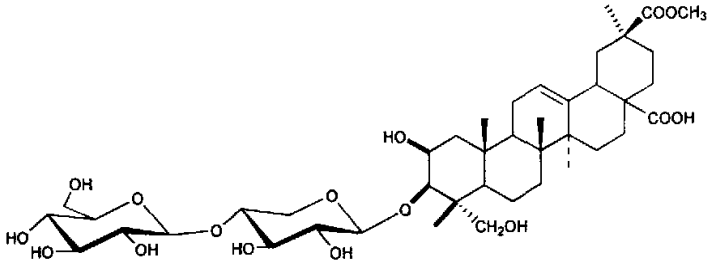

Esculentoside A

Freund's complete adjuvant and PCR marker were obtained from Sino-American Biotechnology Company. ^{3}H-TdR was obtained from Institute of Atomic Nucleus (Chinese Academy of Medical Sciences, Shanghai). Spin or vacuum (SV) total RNA isolation system kit and access RT-PCR system kit was purchased from Promega Corporation. Primers of ICAM-1 and β-actin were synthesized by Shanghai Sangon Corporation. Esculentoside A (purity > 99 %) was provided by Prof YI Yang-Hua (Second Medical Miliuary University, Shanghai). Annexin v-fitc kit was purchased from Pharmingen Corporation.

Animal and cell line BABL/C mice, weighing 18 – 22 g, were from the Animal Center of Second Military Medical University (Grade Ⅱ, Certificate № 02-25-7).

Human umbilical vein endothelial cell line ECV304 was obtained from Kenen Company, Beijing. The ECV304 cells were cultured in modified Eagle's medium (MEM; Gibco) containing 20 % fetal bovine serum (FBS; Gibco).

Animal model and drug treatment Murine model was established as follows: CJ-S_{131} suspension [3×10^{12} CFU (colony forming unit)/L] was mixed with equivalent Freund's adjuvant complete. After full emulsifiifcation, this emulsion (50 μL) was injected into murine footplate. Two weeks later, the second injection was made in murine tail vein with 0.2 mL CJ-S_{131} suspension (3×10^{12} CFU/L).

Thirty BALB/c mice were divided five groups (six mice in each group): the first group was normal mice, which were treated equal volume of normal saline instead of CJ-S_{131} suspension in two injections. The control group was model mice, which were given equal volume of normal saline instead of EsA; the model mice in the rest three groups were given ip EsA at a dose of 5, 10, or 20 mg/kg respectively, once a day till four weeks after the second injection.

At the end of the experiment, blood samples were centrifuged and plasma was kept at −20 ℃ until the assays were performed. The ankle joint was collected after the spleen was obtained in the aseptic condition.

Anti-ds DNA antibody The detection of anti-ds DNA antibody in the murine serum was performed as previously described[9]. In brief, ds DNA (0.25 μg/well) was put into 96-well microplate for 24 h at 4 ℃, and washed three times by PBS. The serum was diluted with PBS at 1∶100 dilution, and this diluted serum (100 μL) was added into the well. Reaction was performed for 2 h at 37 ℃. Coat anti-mouse IgG-HRP (1∶2000 dilution) was added and kept for 2 h at 37 ℃. Then 100 μL *o*-phenylenediamine solution was added and kept for 30 min at 37 ℃, and 50 μL H_2SO_4 solution was added to stop the reaction. The control well was added with PBS instead of the serum. The value of absorbance at 490 nm (A_{490}) was examined. The experimental result was represented by enzyme index (EI). EI = [A_{490} in experimental mouse/(mean A_{490} in normal mouse + 3 SD) × 100].

Splenocyte proliferation Single cell suspension was prepared from murine spleen of each group. Spleen cells (1×10^6/well) were seeded in 96-well microplate for 72 h. Cells from each group were stimulated with ConA, LPS or without mitogen respectively. ^{3}H-TdR (18 500 Bq/well) was added at 20 h before the end of culture. Cells were harvested after cultivation. The incorporation of ^{3}H-TdR was determined and expressed as Bq.

Histological examination Ankle joints were obtained from killed mice and fixed with 10 % formalin solution for 24 h. The joints were decalcified with 10 % decalcifying fluid for 21 d. After full decalcification, joints were longitudinally cut open and were made into tissue sections. For histological examination, those sections were deparaffinized, hydrated, and stained with

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hematoxylin and eosin according to standard procedures, then examined under the microscope.

Expression of ICAM-1 mRNA ECV304 cells at confluence were pretreated with serum-free MEM for 24 h. The medium was then replaced with fresh medium containing 1 mg/L lipopolysaccharide (LPS, Sigma), and cells were incubated for 6 h. Then EsA was added at concentrations of 2.5, 5, or 10 mg/L respectively. The cells were incubated for 24 h at 37 ℃, and the total RNA was extracted according to SV total RNA isolation system kit. RT-PCR was performed according to access RT-PCR system kit. The sense primer sequence of ICAM-1 was 5′-TCT CGT GCC GCA CTG AAC TGG AC-3′, and antisense primer was 5′-CCT TCT GAG ACC TCT GGC TTC GT-3′; the sense primer sequence of β-actin was 5′-GTG GGG CGC CCC AGG CAC CA-3′, and antisense primer was 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′[10]. The PCR was performed using the following parameters: 94 ℃ for 30 s, annealing at 58 ℃ for 1 min, and extension at 72 ℃ for 1.5 min. The cycle was repeated at 28 times. ICAM-1 mRNA was amplified and analyzed by electrophoresis in 1.5 % agarose gel. ICAM-1 mRNA was quantitated by computer digital scanning density image system (Shanghai Sixing Biological Technology Co Ltd).

Thymocyte apoptosis Single thymocyte suspension was prepared from murine thymus and thymocytes (1×10^6 cells/flask) were added to five culture flasks respectively. A control group was treated with pure mediums. ConA (25 mg/L) was added into the rest groups, three groups of which were given simultaneously EsA at concentrations of 2.5, 5 or 10 mg/L respectively. They were incubated at 37 ℃ and harvested after 1.5 h.

Morphology analysis of thymocyte apoptosis was performed according to standard procedure. In brief, harvested thymocytes were fixed with 4 % para-formaldehyde solution overnight. After potch with PBS, cells were fixed for 2 h. Thymocytes were washed with PBS and were after fixing with 1 % osmic acid for 2 h. Thymocytes were embeded with Epon812, polymerized, and sliced. The slices were stained with acetic uranium for 30 min and then with lead citrate for 30 min. These cells were observed under a Hitachi H-800 transmission electron microscope and photographed.

Apoptosis analysis by flow cytometry was performed according to Annexin v-fitc kit. In brief, harvested thymocytes were washed twice with PBS and diluted with binding buffer. Dilution 100 μL (1×10^9 cells/L) were added 5 μL Annexin v-fitc and 2 μL propidium iodide. The reaction went on 15 min in the darkness at room temperature (20 – 25 ℃). Binding buffer 400 μL was supplied into the solution. Thymocyte apoptosis was determined by flow cytometry.

Statistics Data were expressed as $\bar{x} \pm s$. Each experiment was repeated at least three times. Differences were considered statistically significant when $P < 0.05$ as analyzed by ANOVA and unpaired t-test.

RESULTS

Effects of EsA on the level of anti-ds DNA antibody The anti-ds DNA antibody increased markedly in the serum of model mice ($P < 0.01$). EsA effectively lowered the level of anti-ds DNA antibody in the serum of model mice in a dose-dependent manner. At the dose of EsA 20 mg/kg, the level of anti-ds DNA antibody was close to the normal (Tab 1).

Tab 1. Effect of EsA on the level of anti-ds DNA antibody. $n = 6$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* **normal group.** $^fP < 0.01$ *vs* **control group.**

Group		A_{490}	EI
Normal		0.70 ± 0.04	85 ± 5
Control		1.03 ± 0.15^c	125 ± 18^c
EsA/mg·kg^{-1}	5	0.91 ± 0.06^f	110 ± 7^f
	10	0.80 ± 0.06^f	97 ± 7^f
	20	0.71 ± 0.11^f	86 ± 13^f

Effects of EsA on splenocyte proliferation In model mice, the proliferation of splenocyte cells was distinct from that in normal mice whether stimulated with ConA, LPS or without mitogen. Cell proliferation was decreased dose-dependently by EsA. When no mitogen, the inhibitory rate of EsA at the dose of 5, 10, and 20 mg/kg was 26 %, 33 %, and 42 % respectively ($P < 0.01$). When splenocytes were stimulated with ConA, cell proliferation was close to the normal at the dose of EsA 10 mg/kg. However, when mitogen was LPS, cell proliferation was close to the normal at the dose of EsA 20 mg/kg (Tab 2).

Effects of EsA on inflammation of joint Synovial pannus appeared in the ankle joint of model mouse. Synovium was heavily infiltrated with inflammatory cells and cartilage adjacent to synovial pannus

Tab 2. Effects of EsA on splenocyte proliferation. $n = 6$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* **normal group.** $^fP < 0.01$ *vs* **control group.**

Group		Splenocyte proliferation/Bq		
		No mitogen	ConA	LPS
Normal		9.4 ± 2.5	182 ± 46	28 ± 4
Control		66 ± 7^c	405 ± 65^c	86 ± 11^c
EsA/mg·kg^{-1}	5	27 ± 6^f	281 ± 40^f	67 ± 8^f
	10	21.6 ± 2.6^f	179 ± 6^f	53 ± 9^f
	20	17 ± 4^f	76 ± 12^f	28 ± 3^f

was degraded. The bigger the dose of EsA was, the more remittent the inflammation of ankle joint became. When the dose of EsA was minor, synovial pannus decreased and cartilage could be seen. When the dose of EsA reached 20 mg/kg, the formation of synovial pannus was inhibited significantly and the intact cartilage could be seen. The result showed the inflammation was ameliorated markedly by EsA (Fig 1).

Effects of EsA on expression of ICAM-1 mRNA When the ECV304 cell was stimulated by LPS, the expression of ICAM-1 mRNA increased approximately two times. This increased expression was remarkably attenuated by the addition of EsA. The ratio of ICAM-1/β-actin mRNA from lane 1 to lane 5 was 0.472, 0.814, 0.693, 0.615, 0.563, respectively (Fig 2). The result indicated that the enhanced expression of ICAM-1 mRNA by LPS in the ECV304 cell was decreased by EsA.

Effects of EsA on thymocyte apoptosis Thymocytes activated by ConA showed characteristic alteration of cell morphology of apoptosis, namely cell shrinkage, plasma membrane blebbing, and nuclear condensation. After these cells were given EsA, apoptosis of activated thymocyte had an accelerated trend. Thymocytes were treated with EsA at the concentration of 10 mg/L and then apoptosis body appeared. This result preliminarily indicated that EsA could accelerate apoptosis of thymocyte activated by ConA (Fig 3). To conform this effect of EsA, we analyzed apoptosis of thymocyte by flow cytometry. It is found that the ratio of apoptosis of thymocyte activated by ConA was approximately 4 times higher than that in the normal thymocyte. When the activated thymocytes were given EsA at the concentration of 10 mg/L, the ratio of apoptosis of thymocyte was approximately two times higher than that in the activated thymocytes. EsA markedly accelerated apoptosis of thymocyte activated by ConA (Fig 4).

DISCUSSION

Chinese people have utilized the herb *Phytolacca esculenta* for the therapy of various diseases for more than a thousand year. Some of these diseases belong to autoimmune disease. The previous report showed that

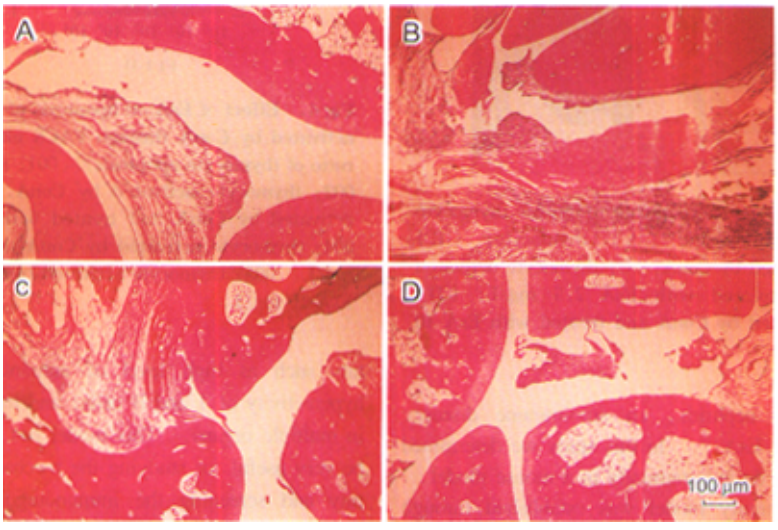

Fig 1. Effects of EsA on inflammation of joint through analysis of pathologic section. (A) normal; (B) model; (C) model mice treated with EsA 10 mg/ kg; (D) model mice treated with EsA 20 mg/kg. ×100.

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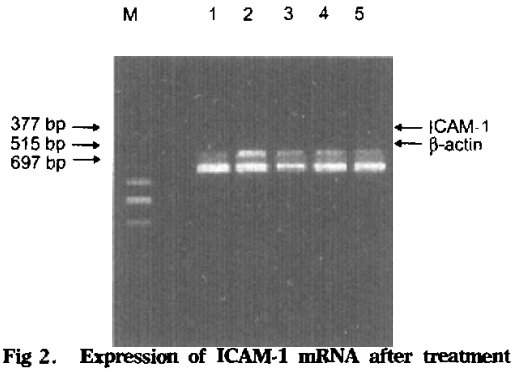

Fig 2. Expression of ICAM-1 mRNA after treatment with EsA. Lane M: DNA marker; Lane 1: control; Lane 2: EVC304 stimulated by LPS; Lane 3: EVC304 stimulated by LPS and treated with EsA 2.5 mg/L; Lane 4: EVC304 stimulated by LPS and treated with EsA 5 mg/L; Lane 5: EVC304 stimulated by LPS and treated with EsA 10 mg/L.

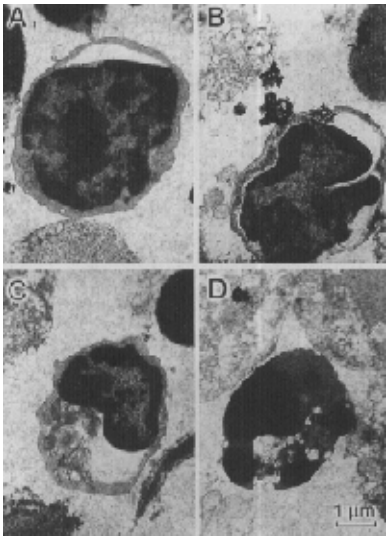

Fig 3. Effect of EsA on apoptosis on murine thymocyte by electronic microscope (A) thymocyte; (B) thymocyte activated by ConA; (C) thymocyte activated by ConA and treated with EsA 2.5 mg/L; (D) thymocyte activated by ConA and treated with EsA 10 mg/L. ×10 000.

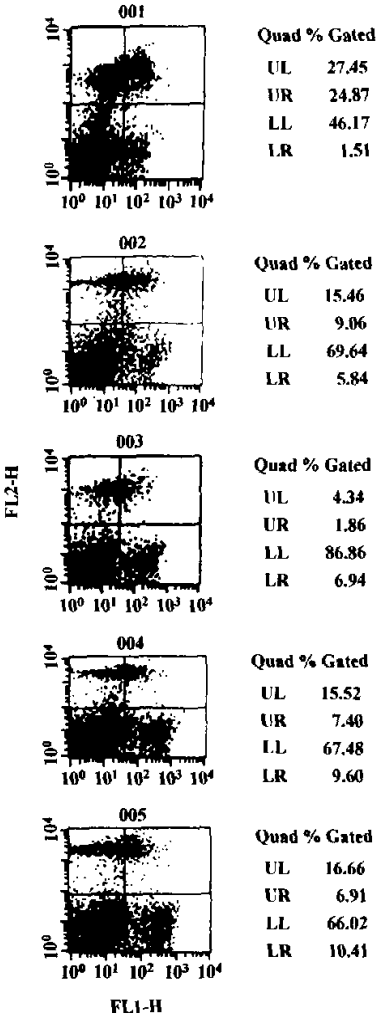

Fig 4. Effect of EsA on apoptosis of murine thymocyte activated by ConA through FACS analysis. (LR was ratio of thymocyte apoptosis). 001: normal thymocyte. 002: thymocyte activated by ConA. 003: thymocyte activated by ConA and treated with EsA 2.5 mg/L. 004: thymocyte activated by ConA treated with EsA 5 mg/L. 005: thymocyte activated by ConA treated with EsA 10 mg/L.

passive Heymann nephritis, an autoimmune disease, could be cured by EsA[8]. In this study we determined the effects of EsA on autoimmunity in a mouse model.

The autoimmunity model was established through immunizing mice with formaldehyde treated *Campylobacter jejuni* strain CJ-S_{131} and Freund's complete adjuvant, which have some characteristics of autoimmunity such as high level of autoantibodies in serum, hyperactivity of T and B cells, and inflammatory injuries in multiple organs. This model has been recognized to be very useful for studying the mechanism of autoimmunity and screening the immunoregulators[11]. To an autoimmune disease, the importance of autoantibodies as diagnostic markers has been demonstrated by the pioneering studies of Eng Tan[12]. The major cellular

antigens recognized by autoantibodies in the systemic autoimmune diseases have been identified and characterized over the past 30 years. Anti-ds DNA antibody is an autoantibodies directed to intracellular antigens, which can be detected in many systemic rheumatic diseases[13]. It is involved in the etiopathogenesis of autoimmune rheumatic diseases such as rheumatoid arthritis. Another typical character of autoimmune disease is hyperactivity of T and B cells. It can lead to the massive lymphoproliferation and the disorder balance of immune system in autoimmune diseases. In rheumatoid arthritis, cellular interactions between activated synovial T cell and peripheral blood T cells may involve in the evolution of the chronic synovial inflammatory process[14]. The hyperactivity of T and B cells can cause the attack to self-tissue and the damage of joint.

The present study showed that EsA could lower the high level of anti-ds DNA antibody and inhibit the hyperactivity of T and B cells in a dose-dependent manner. It suggested that EsA might ameliorate symptom of autoimmune rheumatic disease such as rheumatoid arthritis through the inhibition of autoantibody production and lymphocyte proliferation. In the experiment of splenocyte proliferation, we found that EsA 10 mg/kg decreased splenocyte proliferation to the normal when splenocytes were stimulated with ConA. However, EsA 20 mg/kg could get to the same effect when mitogen was LPS. It suggested that EsA might have stronger effect on T cells than B cells.

In the joint of model mouse appeared the trilogy of rheumatoid arthritis: proliferation of synovialis, inflammation, and destruction of cartilage. Those symptoms had been strongly ameliorated after treatment with EsA. T cells can produce many pro-inflammatory cytokines such as TNF, IL-1, and IL-6. IL-1 and TNF are major cytokines which can lead to the inflammation of synovitis and destruction of cartilage[15]. Recombinant antagonist of IL-1 receptor and monoclonal antibody of TNF had shown good effect in the therapy of rheumatoid arthritis[16,17]. EsA can inhibit the production of IL-1 and TNF from synovial cell[4]. Therefore, EsA might ameliorate the trilogy of rheumatoid arthritis through influencing the production of cytokines. The present study testified that EsA could have good effects on autoimmune disease such as rheumatoid arthritis.

To further investigate the mechanism of EsA on autoimmunity, we examined the apoptosis of activated thymocyte and expression of ICAM-1 mRNA by EVC304. It is well known that activated T cells play a very important role in the pathology of autoimmune disease[18]. T cells existed widely in the pannus of rheumatoid arthritis. In the clinic practise, this disease can be cured through decreasing the quantity of T cells such as drainage through alimentary duct or irradiation of lymphoid tissue. EsA has been showed to markedly inhibit humoral immunity[2]. The present result suggested that EsA might inhibit cellular immunity since it could accelerate the apoptosis of activated thymocyte. Therefore, EsA may be favorable for autoimmune disease such as rheumatoid arthritis through the inhibition of immunity.

Adhesion molecules are very important because adhesion molecule expression and interactions are probably involved in initiation and propagation of autoimmune diseases including rheumatoid arthritis (RA), systemic lupus erythematosus, Sjogren's syndrome, autoimmune thyroid disease, multiple sclerosis, and diabetes mellitus. In rheumatoid arthritis, adhesion molecules can lead to lymphocyte infiltration of joint and inflammation. ICAM-1 plays a pivotal role in the activation and close adhesion of leucocyte, which is the most important step in the exudation of inflammatory cell[19]. EsA decreased expression of ICAM-1 mRNA, which suggested that the inhibition of adhesion molecules might be one of the mechanisms of anti-inflammation of EsA.

In summary, our study clearly demonstrated that EsA had the positive curative effect on the autoimmunity in a mouse model, which may function through anti-inflammation and inhibitory effect on immune system. These pharmacological effects of EsA strongly suggested its potential therapeutic role to the autoimmune disease such as rheumatoid arthritis. Therefore, it is deserved that we pay close attention to EsA, a hopeful prodrug to autoimmune disease.

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商陆皂苷甲对自身免疫性模型小鼠的影响及其作用机制[1]

R96 A

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关键词 商陆皂苷甲；自身免疫；炎症；细胞间粘附分子-1；细胞凋亡；类风湿性关节炎

目的：考察商陆皂苷甲对自身免疫性模型小鼠的影响及其可能的机制。**方法**：自身免疫性小鼠模型通过甲醛化空弯菌 CJ-S_{131}辅以佐剂免疫小鼠获得。检测正常小鼠、模型小鼠及商陆皂苷甲处理的模型小鼠血清中的抗 ds DNA 抗体水平、细胞增殖及骨关节病理切片。通过形态学和流式细胞仪检测胸腺细胞凋亡。通过逆转录 PCR 的方法检测 ECV304 细胞中 ICAM-1 的 mRNA 表达。**结果**：商陆皂苷甲能有效地降低模型小鼠的抗 ds DNA 抗体水平、抑制淋巴细胞增殖并能缓解其骨关节炎症。而且，商陆皂苷甲能显著促进 ConA 活化的胸腺细胞的凋亡并能降低 ECV304 细胞中 ICAM-1 的 mRNA 表达。**结论**：商陆皂苷甲对自身免疫性模型小鼠有治疗作用；促进胸腺细胞凋亡和降低 ECV304 细胞中 ICAM-1 的 mRNA 表达可能是其作用的重要机制。

(责任编辑 韩向晖)