The proliferative effects of alfalfa polysaccharides on the mouse immune cells

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Abstract: In order to investigate the proliferative effects of alfalfa polysaccharides on mouse immune cells, and to explore the proliferative effects mechanisms of alfalfa polysaccharides on immune cells, mouse immune cells and alfalfa polysaccharides was used as research material. The effects of alfalfa polysaccharides on the mouse immune cells were detected using MTT colorimetric assay, which proliferative effects of mouse spleen lymphocyte and mouse bone marrow dendritic cells, cytotoxic activity of NK cells against K562 cells, energy metabolism of mouse peritoneal macrophages. The results show that alfalfa polysaccharides can promote the proliferation of mouse spleen lymphocytes and bone marrow dendritic cells，and it has the function of regulate the body's cellular and humoral immune; Alfalfa polysaccharides can enhance the killing activity of NK cells against K562 target cells, and it has the function of enhance the body's non-specific immune; Alfalfa Polysaccharides can enhance the energy metabolism of mouse peritoneal macrophages, which can enhance antigen presentation accessory cells.
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Keywords: Alfalfa polysaccharides; immune cells; immunomodulatory; cell proliferation

1. Introduction

Alfalfa is a very high-quality perennial leguminous grass and has a long history of cultivation in China. Alfalfa dry matter up to 10%-15%, alfalfa grass has the characteristic of high output, good palatability, nutrient-rich, livestock easy to digest, so regarded it as "the king of the grass" (Wang et al., 2007). Alfalfa was a traditional Chinese medicine, it has the characteristic of heat-clearing and detoxifying, cooling blood rain, replenishing qi, invigorating spleen and kidney etc (Ma et al., 2000). Alfalfa polysaccharide is extracted from alfalfa polysaccharide and it is the main bioactive components. Most of the polysaccharide can enhance the body's immune activity and it is one of the main pharmacological effects, which makes polysaccharide immune pharmacological a hot topic. Alfalfa polysaccharide can promote the growth of immune organs, improve the T cell conversion rate significantly, improve the degree of new castle disease antibody in serum of drops and index of the macrophage phagocytosis and enhance the immune effect of vaccine so on (Wang et al., 2008). Alfalfa polysaccharide can promote the proliferation of B cells in porcine peripheral blood and improve the level of Immunoglobulin G (IgG) (Zhao et al., 2005). Alfalfa Polysaccharides can collaborate within a certain range of concentration of Concanavalin A (Con A) or lipopolysaccharide (LPS) promote the proliferation of Peripheral blood, spleen T and B cells, T cells in the thymus, bursa B cells of chicken (Liu et al., 2010a; Wang et al., 2003; Tang et al., 2007).

At present, the studies of alfalfa polysaccharides on immunomodulatory activity mainly focusd on T, B cell proliferation (Liu et al., 2010b; Zhang et al., 2010). In this paper, mouse immune cells was used as the research object, to investigate the proliferative effects of alfalfa polysaccharides on mouse spleen lymphocyte and mouse bone marrow dendritic cells, cytotoxic activity of natural killer cell (NK cell) gainst K562 cells, energy metabolism of mouse peritoneal macrophages. Therefore it providing important insights for the studies of alfalfa polysaccharides on immunomodulatory activity.

2. Materials and Methods

2.1 Materials

Balb/c mouse was provided by experimental animal center of Liaoning Medical University. Con A, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) was purchased from Sigma Inc. Roswell Park Memorial Institute-1640 (RPMI-1640) and fetal bovine serum (FBS) was purchased from Hyclone Inc.

2.2 Extraction and preparation of alfalfa polysaccharides

Take the budding stage of alfalfa stems and leaves of part made of air-dried samples, crushed into powder, and passed through a No. 40 mesh sieve. Alfalfa powder mixed with distilled water in proportion to 1:25, 95℃ water bath extraction 2h, repeat 2 times. Evaporation of the water bath to 1/3, adding 2 times the volumes of 95% ethanol solution,

full oscillation, placed for 6 h. Centrifugation, the precipitate was collected, to remove protein by sevag method. Chloroform and n-butanol (5:1) and sample was mixed in equal volumes, oscillation separate with separatory funnel and get purification alfalfa polysaccharide (Su et al., 2007; Yang et al., 2002; Yang et al., 2004).

Alfalfa polysaccharides was dissolved with DMSO, diluted with RPMI-1640 medium, filtered for sterilization, aliquoted. It should be diluted to the required concentration with RPMI-1640 medium prior to treatment, and the final concentration of DMSO should no more than 0.1% in the experiments, for control specimens, the same volume of 0.1% DMSO without alfalfa polysaccharides was added.

2.3 The proliferative effects of alfalfa polysaccharides on the mouse spleen lymphocytes

Mouse were killed with a broken neck cord, disinfect with 75% alcohol, spleens were removed and rinsed with RPMI-1640 medium. Break up the spleen cells with RPMI-1640 medium and collect it, plus Tris-NH4Cl remove the red blood cells, the precipitate was collected by centrifugation (Yu et al., 2012). The cells were cultured in RPMI-1640 medium containing 10% FBS in a 37 ℃ incubator with 5% CO2 (Freshney, 2000; Guan, 2005; Zhou, 2004). The medium was refreshed when it became yellow.

The MTT assay was used to evaluate proliferative effects as described by Ho et al. (2005). Cell suspension of 200μl was plated on each well of 96-well microplates at the concentration of 1.0×10^5 cells/well. Cells in logarithmic growth phase, with alfalfa polysaccharides containing complete RPMI-1640 media and corresponding controls were set simultaneously. Four replicates were prepared for each treatment and cultured. After the addition of MTT 20μl (5mg/ml phosphate buffered saline (PBS)) each well, the cells were cultured for another 4h. The supernatant was discard. After the addition of 200μl DMSO in each well, the samples were incubated in the dark for 30min, and then swirled for mixing. Absorbance A at 570nm was measured using enzymatic reader. Experiments were repeated three times.

The stimulation index (SI) reveal cell proliferation, SI=Processing well A value/Control wells A value.

2.4 Cell activity effects of alfalfa polysaccharides on NK cells

Spleen lymphocytes suspension of 100μl was plated on each well of 96-well microplates at the concentration of 1.0×105 cells/well. Add alfalfa polysaccharides to each well and the final concentration of alfalfa polysaccharides should be 5mg/L, 25mg/L, 125mg/L, the cells were cultured in 37 ℃ incubator with 5% CO_2 24h.

Take K562 cells in the logarithmic growth phase made of a cell suspension of 2.0×10^5 cells/ml, adjustment of effector cells to target cells ratio was 50:1 (He et al., 1996). Set the control group and the experimental group, cell suspension of 200μl was plated on each well of 96-well microplates. The MTT assay was used to evaluate cell activity effects as described by Ho et al. (2005). Absorbance A at 570nm was measured using enzymatic reader. Experiments were repeated three times.

Cell activity= 1-(experimental cells well A value -NK cells control well A value)/K562 cells control well A value

2.5 The proliferative effects of alfalfa polysaccharides on the mouse bone marrow dendritic cells

Mouse were killed with a broken neck cord, disinfect with 75% alcohol, bone marrow cavity were rinsed with RPMI-1640 medium, and bone marrow Cells were collected. In order to lysis of red blood cells, adding pre-warmed 0.83% Tris-NH4Cl lysis solution. Then adding an equal volume of RPMI-1640 complete medium, mix well, to terminate the cracking reaction, the cells precipitate was collected by centrifugation.

The cells were cultured using RPMI-1640 medium that contains 10% fetal calf serum in a humidified atmosphere at 37℃, 5% CO_2 (Freshney, 2000; Guan et al., 2005; Zhou et al., 2004). The culture medium was refreshed when its color become yellow; and the cells were passaged when 70%-80% confluent. The cells are semi-adherent and semi-suspended without the need of trypsinization when subcultured (Wang et al., 2012).

bone marrow dendritic cells suspension of 100μl was plated on each well of 96-well microplates at the concentration of 1.0×10^5 cells/well. Add alfalfa polysaccharides to each well and the final concentration of alfalfa polysaccharides should be 5mg/L, 25mg/L, 125mg/L, the cells were cultured in 37 ℃ incubator with 5% CO_2 24h. The MTT assay was used to evaluate cell activity effects as described by Ho et al. (2005). Absorbance A at 570nm was measured using enzymatic reader. Experiments were repeated three times.

2.6 The effects of energy metabolism of alfalfa polysaccharides on mouse peritoneal macrophages

Mouse were killed with a broken neck cord, disinfect with 75% alcohol, abdominal cavity were rinsed with RPMI-1640 medium, abdominal cavity

washings were collected, the cells precipitate was collected by centrifugation. The cells were cultured using RPMI-1640 medium that contains 10% fetal calf serum in a humidified atmosphere at 37℃, 5% CO_2 (Freshney, 2000; Guan et al., 2005; Zhou et al., 2004).

Peritoneal macrophages suspension of 100μl was plated on each well of 96-well microplates at the concentration of 1.0×10^5 cells/well. Add alfalfa polysaccharides to each well and the final concentration of alfalfa polysaccharides should be 5mg/L, 25mg/L, 125mg/L, the cells were cultured in 37 ℃ incubator with 5% CO_2 24h. The MTT assay was used to evaluate cell activity effects as described by Ho et al. (2005). Absorbance A at 570nm was measured using enzymatic reader. Experiments were repeated three times.

2.7 Statistical analysis

Data were analyzed using the GLM procedure in Statistical Analysis System (SAS Inc., Cary, NC, USA) and compared with a multiple comparison test (DUNCAN). A value of $P<0.05$ and $P<0.01$ was thought of as statistically significant.

3. Results

3.1 The proliferative effects of alfalfa polysaccharides on the mouse spleen lymphocytes

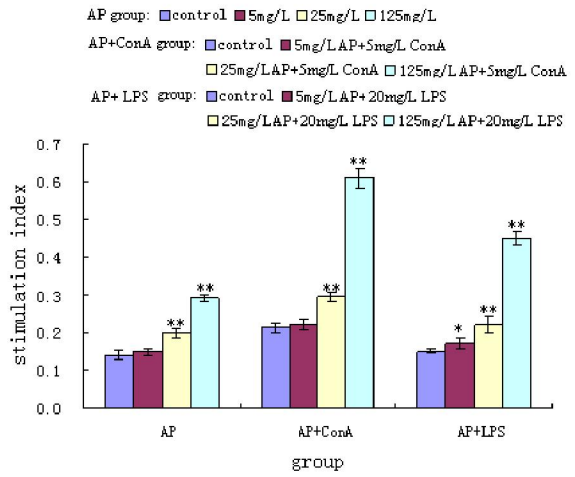

Figure 1. The proliferative effects of mouse spleen lymphocytes. Statistical significance to corresponding controls is marked with (*) ($P<0.05$) and (**) ($P<0.01$) (n=3).

MTT assay showed that, alfalfa polysaccharides, alfalfa polysaccharides collaborative ConA, alfalfa polysaccharides collaborative LPS can promote the proliferation of spleen lymphocytes (Figure 1). Alfalfa polysaccharides (AP) group, the stimulation index of experimental samples treated with alfalfa polysaccharides of 25 mg/L, 125 mg/L displayed significant differences compared with the controls ($P<0.01$). AP and ConA group, the stimulation index of experimental samples treated with AP of 25 mg/L and ConA of 5 mg/L, AP of 125 mg/L and ConA of 5 mg/L displayed significant differences compared with the controls ($P<0.01$). AP and LPS group, the stimulation index of experimental samples treated with AP of 5 mg/L and LPS of 20mg/L, AP of 25 mg/L and LPS of 20 mg/L, AP of 125 mg/L and LPS of 20 mg/L displayed significant differences compared with the controls ($P<0.05$ and $P<0.01$).

3.2 Cell activity effects of alfalfa polysaccharides on NK cells

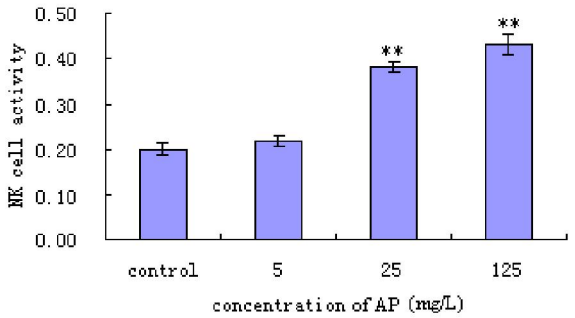

Figure 2. Cell activity effects of alfalfa polysaccharides on NK cells. Statistical significance to control is marked with (*) ($P<0.05$) and (**) ($P<0.01$) (n=3).

MTT assay showed that, alfalfa polysaccharides can promote cell activity of NK cells (Figure 2). The NK cell activity of experimental samples treated with alfalfa polysaccharides of 25 mg/L, 125 mg/L displayed significant differences compared with the controls ($P<0.01$).

3.3 The proliferative effects of alfalfa polysaccharides on the mouse bone marrow dendritic cells

The MTT assay determines the activity of mitochondrial succinate dehydrogenase and is therefore able to detect alterations of mitochondrial function. This is used as a measurement of cell viability, and hence an indicator of cell death. But this test does not distinguish between apoptosis and necrosis as well as the inhibition of cell growth (Mosmann, 1983). MTT assay showed that, for alfalfa polysaccharides treated mouse bone marrow dendritic cells, the viable cell population decreased significantly with elevated alfalfa polysaccharides concentration, appearing dose-dependent (Figure 3).

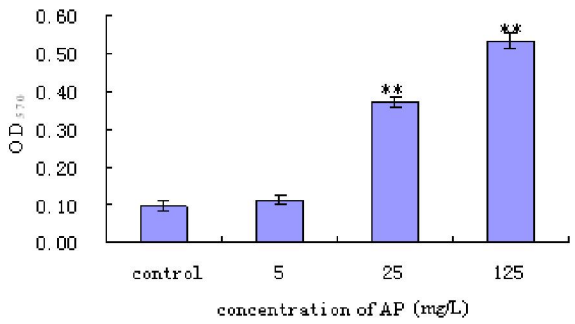

Figure 3. Cell proliferation analysis of the mouse bone marrow dendritic cells were treated with AP, OD values reflect viable cell population size. Statistical significance to control is marked with (*) (P<0.05) and (**) (P<0.01) (n=3).

3.4 The effects of energy metabolism of alfalfa polysaccharides on mouse peritoneal macrophages

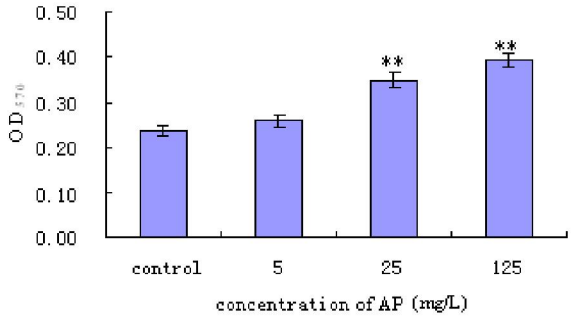

Figure 4. The effects of energy metabolism of alfalfa polysaccharides on mouse peritoneal macrophages. OD values reflect viable cell population size. Statistical significance to control is marked with (*) (P<0.05) and (**) (P<0.01) (n=3).

MTT assay showed that, alfalfa polysaccharides can promote energy metabolism of mouse peritoneal macrophages (Figure 4). The energy metabolism of experimental samples treated with alfalfa polysaccharides of 25 mg/L, 125 mg/L displayed significant differences compared with the controls (P<0.01).

4. Discussion

Alfalfa polysaccharide can stimulation of mice spleen T and B cell proliferation singly. Alfalfa polysaccharide can synergy ConA stimulation of T cell proliferation. Alfalfa polysaccharide can be coordinated LPS stimulated B cell proliferation. Alfalfa polysaccharide can enhance NK cell killing activity to K562 target cells in mouse. That is to say alfalfa polysaccharide can by promoting the specific immune and nonspecific immune function to enhance the body's immune function. Alfalfa polysaccharide can stimulate the proliferation of bone marrow dendritic cells of mouse, can stimulate mouse abdominal cavity macrophage the improvement of energy metabolism. So alfalfa polysaccharide can improve advising cell antigen presented by role to improve the activity of immune cell function.

All directly or indirectly involved in the cells of the immune response are called immune cells and their wide variety, different functions, interaction and interdependence. According to their function and its mechanism in the immune response divide into two major categories of lymphoid cells and adjuvant. There are some other cells, such as all sorts of granulocyte and mast cells are involved in the immune response of a particular segment. Lymphocytes, including T cells, B cells and natural killer cells (NK cells) and killer cell (K), after the antigen stimulation in lymphocyte proliferation to differentiation, to produce specific immune response of cells, it was called immune active cells, is mainly refers to the T and B cells, plays a central role in the process of immune response. T cells is stationary normally, once was further proliferation when activation antigen stimulation finally divided into effect T cells and has cellular immune function. B cell antigen became activation, proliferation and differentiation after stimulation, and become plasma cells ultimately, plasma cells produce antibodies that form the body's humoral immune. NK cells mainly exists in peripheral blood and spleen, doesn't depend on antibody and antigenic stimulation and sensitization can kill target cells. On the surface of cell has interferon and IL-2 receptor. Interferon act on on the NK cell and can make the NK cells enhance recognition structure and dissolve target cells and the activity of the target cells. IL-2 can stimulate NK cells proliferate and produce interferon, play a strong role. Accessory cells including mononuclear phagocytes and dendritic cells (D cells). They can capture and process antigens and antigen presented to immune active cells. Mononuclear phagocytes include blood monocytes and macrophages in the organization. Mononuclear cells in the bone marrow into the blood after the mature and stay for a few hours or a few months later, then in the bloodstream by the circulation of the blood distribution around the body in a variety of tissues and organs, finally mature into macrophages. Macrophage surface have MHC Ⅱ molecules and MHC Ⅰ molecules. Dendritic cells derived from bone marrow and spleen of the red pulp, then distributed in the spleen and lymph nodes and connective tissue when it matured. Most of the

dendritic cells have much MHC Ⅰ and Ⅱ molecules,and a small number of dendritic cell surface receptors have a Fe and C3b receptor, they can combine with antibodies to antigens presented to lymphocytes (Yang et al., 2003). Dendritic cells is the most powerful antigen presenting cells in the body and it can start the initial T cell mediated immune response of cells (Steinman, 19991).

The alfalfa polysaccharide which extracted from alfalfa showed has strong stimulation of mouse spleen lymphocyte proliferation. Within the scope of the test dose, alfalfa polysaccharide can be a single activation of mouse spleen lymphocyte proliferation, and the spleen and lymph nodes is mature the main room, so alfalfa polysaccharide can promote T, B cell proliferation. ConA main rolewas mediated the specific cellular immune response in T cell. LPS main role was mediating specific humoral immune response in B cell of the body. Alfalfa polysaccharide can synergy ConA stimulation of T cell proliferation, or collaborative LPS stimulated B cell proliferation. That alfalfa polysaccharide on the body of T and B cells of the immune. So alfalfa polysaccharide on the cellular and humoral immune responses are the specificity of a certain degree of positive adjustment, so as to enhance the body's nonspecific immune function. Alfalfa polysaccharide can enhance mouse NK cell killing activity to K562 target cells, which prompt alfalfa polysaccharide can activate NK cells in mice. Within the scope of the test dose, alfalfa polysaccharides separate effect on the body's nonspecific immune function also has a certain role in promoting. Within the scope of the test dose, alfalfa polysaccharide can stimulate the proliferation of bone marrow dendritic cells, and can stimulate abdominal cavity macrophage the improvement of energy metabolism. That alfalfa polysaccharide can improve advising cell antigen presented by improve the activity of immune cell function.

Traditional Chinese medicine has certain concentration-response relationship (Li et al., 2001), the study of traditional Chinese medicine monomer are generally recognized as the concentration is too high (150 mg/L), even effective traditional Chinese medicine monomer is meaningless. Group alfalfa polysaccharide concentration of only 5 mg/L, 25 mg/L, 125 mg/L, alfalfa polysaccharide has the highest concentration of 125 mg/L, immune enhancement is best, whether the concentration as the best dose, the group will further research.

Mature dendritic cells go through four stages: Precursor dendritic cells in the bone marrow and blood, peripheral blame drenchs immature dendritic cells in the tissue, lymph and blood mature dendritic cells in the process of secondary lymphoid tissue in the mature dendritic cells. GM-CSF is an essential part of dendritic cells in vitro culture cell factor, it can promote the differentiation of dendritic cells, enhance the function of a variety of immune molecules on the surface, GM-CSF and IL-4 combined together when inducing differentiate into dendritic cells and augmentation (Wang et al., 2005).

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