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In-vitro Effect of *Acanthopanax senticosus* Polysaccharide on Cultured Blood Lymphocyte Proliferation and Signal Molecules in Pigs

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Abstract. This study was designed to investigate the in-*vitro* effect of *Acanthopanax senticosus* polysaccharide (ASPS) on proliferation and signal molecules of piglet blood lymphocyte. Peripheral blood lymphocyte suspension was prepared, and T, B cells of which were separated and co-incubated with or without various concentrations of ASPS (40, 80, 160, 320 ug/ul) separately to determine lymphocyte proliferation measured by MTT assay. The supernatant concentrations of NO secreted by lymphocyte as well as intracellular cAMP and cGMP of lymphocyte were also investigated. The results showed that T cell proliferation quadraticlly changed with increased ASPS level (P<0.05). High dose of ASPS (80, 160, 320 ug/mL) significantly improved T cell proliferation (P<0.05), but low dose of ASPS (40 ug/mL) had no effect on it (P>0.05). 80, 160μg/mL ASPS significantly improved the concentration of cAMP and the ratio of cAMP/cGMP (P<0.05), meanwhile, the concentration of cGMP was significantly reduced (P<0.05) at the 20 th and 60 th min of cell culture, and the quadratic relationship were observed in these doses (P<0.05). Different levels of ASPS affected significantly on NO level (P<0.05). These results suggested that T lymphocyte possibility can be one of the direct target cell of ASPS, and ASPS acts by affecting the concentration of intracellular cAMP, cGMP and NO of lymphocyte, which may be the messenger molecule in the lymphocytes.

Keywords: herbal extract, polysaccharide, lymphocyte proliferation, signal molecules, pigs

1. Introduction

Weaning stress and immature development of immune system are considered to be the major problems to cause attenuated performance when piglets encounter environmental pathogenic microbiota. Due to concern about feed safety and the development of antibiotic-resistant pathogens, the use of most antibiotic as growth promoters has been banned in the EU since January 2006[1]. Polysaccharide as alternative to antibiotic has been proposed in previous researches. *Acanthopanax senticosus* polysaccharide (ASPS) isolated from traditional herbal medicine has been identified as one of promising macromolecule which possesses profound effects in the regulation of immune responses during the process of infectious diseases in humans and laboratory rodents *in vivo* and *in vitro*. More recently, our research concerning ASPS as feed additives of piglets have revealed that ASPS could improve growth performance of both normal piglets and challenged piglets, stimulate lymphocyte proliferation as well as affect cytokines expression and synthesis.

Immunocyte signal transduction system is generally recognized to be closely related to immunoregulatory action of polysaccharide in previous reports, which indicated that polysaccharide activating protein kinase and ion channels by combining with immunocyte membrane receptor changes the production of intracellular signal molecule encompassing cAMP, cGMP, Ca^{2+}, and nitric oxide (NO)，etc, ultimately to affect expression and production of cytokines[2], [3]. *Platycodon grandiflorum* polysaccharide -mediated induction of NO production and iNOS mRNA expression in macrophages is

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mediated, at least in part, by TLR4/NF-κB signaling pathway [4]. Han et al (2003) also demonstrated that ASPS activates B cells and macrophages by interacting with TLRs and leading to the subsequent activation of mitogen-activated protein kinases and NF-κB [5].

So far, little is known, however, about the mechanism of immunomodulatory action of ASPS in pig production. Thus, the aim of present trials was to determine the effects under in vitro conditions on blood lymphocyte proliferation, as well as intracellular signal molecules responsible for activation of lymphocyte.

2. Material and Methods

2.1. Extraction and Purification of ASPS

ASPS was isolated and characterized as described. Briefly, crushed *Acanthopanax senticosus* root was boiled in distilled water for 5 h at 100℃. After filtration to remove debris, the filtrate was concentrated in a rotary evaporator and protein of which was removed by Sevag method. Subsequently, the solution was precipitated with three volumes of ethanol and desiccation *in vacuo*. The precipitate was re-dissolved in distilled water and dialyzed for 24 h to remove the small molecular impurities, and then, the solution was concentrated again and purified by active carbon column chromatography with water and different concentration ethanol as eluent medium. The effluent was collected and the polysaccharide was quantitatively determined to be 92.74% content using a phenol sulfuric acid assay with majority of glucose residues by gas chromatography (GC) assay.

2.2. Preparation of Different Concentration ASPS Solution

Dissolved 320 mg ASPS in RPMI -1640 culture fluid with 100 ml/L fetal bovine serum (FBS, Invitrogen GIBCO) contained was adjusted pH to 7.2～7.4 by 5% $NaHCO_3$ and filtered to sterilization using 0.22μm filter to prepare 3.2 ㎎/ml ASPS culture, 1 ml of which was diluted respectively to prepare 200,400,800,1600,3200 μg/ml ASPS solutions again.

2.3. Preparation of Peripheral Blood Lymphocyte Suspension

Lymphocyte suspensions were harvested from 2 ml peripheral blood derived from piglet (Duroc ×Landrace ×Large White, 28 d of age with average BW of 8.05 kg), and blood was layered onto 4 ml lymphocyte separation medium in a 10 ml test tube and then centrifuged twice at 2000×g for 20 min. The cells were washed three times and then suspended in RPMI medium 1640 supplemented with with 10% (v/v) FBS (Invitrogen GIBCO), 100 U/ml penicillin, 100ug/ml streptomycin, and 25 mmol/l of N-(2-hydroxyethyl)-piperazine-N-2-ethane-sulphonic acid. The number of the viable cells were detected microscopically by trypan blue exclusion test and counted to adjust density to 2×10^6 cells/ml.

2.4. T, B lymphocyte Separation and Proliferation

Separated T, B lymphocyte were prepared according to the method described by Gao (2007) [6]. Proliferation assay of T, B lymphocyte respectively were measured using MTT [3-(4, 5-dimethylthiazol-2-yl) (2.5 Diphenyltetrazolium bromide; sigma)] colorimetric assay according to Choy et al (1994).

2.5. Determination of T-intracellular Activity of cAMP and cGMP

iNOS,cAMP and cGMP were measured with commercial assay kits（R&D,USA）.

2.6. Statistical Analysis

All data were processed by means of one-way ANOVA and the significance of differences between mean values was assessed using Duncan test (INC SPSS, 2008).Difference significance was taken at $p<0.05$.

3. Results

3.1. T lymphocyte Proliferation

Fig. 1 summarized the T lymphocyte proliferation results. Piglets peripheral blood T cell proliferation in vitro culture quadraticlly changed with the increased ASPS level ($P<0.05$). High dose of ASPS (80, 160, 320 ug/mL) significantly improved the T cell proliferation ($P<0.05$), but low dose of ASPS (40 ug/mL) had no effect on T cell proliferation ($P>0.05$)

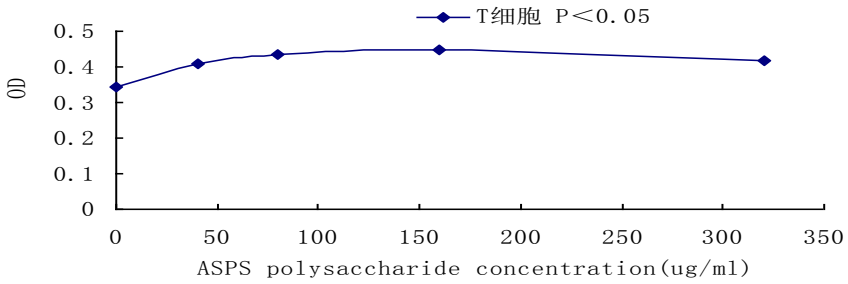

Fig. 1: Effect of ASPS on proliferation in cultured peripheral blood T-lymphocytes of weaned pigs

3.2. T-intracellular Activity of cAMP, cGMP and NO

As indicated by Table 1, 80, 160 μg/mL ASPS significantly improved the concentration of intracellular cAMP and the ratio of cAMP/cGMP of peripheral blood lymphocytes (P<0.05), meanwhile, the concentration intracellular cGMP and NO were significantly reduced (P<0.05) at the 20 th and 60 th min of cell culture, and the quadratic relationship was observed in these doses (P<0.05). In addition, 320μg/mL ASPS significantly affect on these indices at the 60th min of cell culture compared with the control group (P<0.05). Different levels of ASPS affected significantly on NO level secreted by lymphocytes (P<0.05).

Table 1. Effect of *Acanthopanax senticosus* polysaccharide (ASPS) on the concentration of cAMP, cGMP and NO in cultured peripheral blood lymphocytes of weaned pigs

Items	ASPS levels/(μg/mL)					SEM
	0	40	80	160	320	
20 min						
cAMP/(nmol/L)[1]	5.662[a]	6.487[ab]	7.042[b]	7.614[b]	6.986[ab]	0.228
cGMP[1]/(nmol/L)[1]	3.762[a]	3.381[ac]	1.619[b]	1.767[bc]	3.000[ab]	0.302
cAMP/cGMP[1]	1.505[a]	1.915[a]	4.608[bc]	4.782[b]	2.712[ac]	0.428
NO/(μg/μL)[1]	0.2617[a]	1.371[b]	1.830[c]	1.970[c]	0.952[d]	0.168
60 min						
cAMP[1]/(nmol/L)[1]	6.144[a]	7.413[ab]	8.668[b]	8.098[b]	7.732[b]	0.292
cGMP/(nmol/L)[1]	4.129[a]	3.857[ac]	1.953[b]	2.343[bc]	2.538[bc]	0.297
cAMP/cGMP[1]	1.525[a]	2.017[ac]	4.890[b]	3.640[bc]	3.302[bc]	0.383
NO/(μg/μL)[1]	0.2631[a]	1.375[b]	2.0110[c]	2.887[c]	1.709[b]	0.201

Means (n=3), in the same row, values with no letter or the same small letter superscripts mean no significant difference (P>0.05), while with different small letter superscripts mean significant difference (P<0.05); [1]means quadratic regression P - value less than 0.05; SEM: standard of error of the mean.

4. Discussion

Lymphocyte proliferation is a crucial event in the activation cascade of cellular immune responses. Proliferation reaction of T lymphocyte happened when it is stimulated by Con A. Notably, cultured T lymphocyte proliferation was markedly increased by high dose of ASPS (80, 160, 320 ug/mL). However, to our knowledge, there is little information about the cultured cell of pigs affected by ASPS. In rodents experiments, in agreement with our findings, Chen et al. (2011) showed that lymphocyte proliferation of mice spleen was enhanced by ASPS in *vitro* [7]. These findings indicated critical role in enhancing T

lymphocyte immune function of ASPS. Our results indicated T lymphocyte possiblely can be one of the direct target cell of ASPS.

cAMP and cGMP have important role in regulating immune system as the intracellular second messengers discovered earliest. Generally, they show opposite effect in which cAMP mediates immunosuppressive effect, however and cGMP promotes lymphocyte proliferation. cAMP/cGMP ratio was an important index of immune function evaluation. In our study, higer dose of ASPS significantly improved the concentration of cAMP and decreased the concentration of cGMP. The accordant change of cAMP/cGMP ratio and concentration of cAMP indicated that cAMP played the main role in cAMP/cGMP system. In addition, the effect of ASPS changed with the cultured time. Our results found that the effect of ASPS on cAMP and cGMP showed the time-dose-dependent.

NO is the broad bioactive message molecules synthetized by nitric oxide synthase (iNOS) in lymphocyte and mediates immune function by interacting with various cytokines. Bauer et al (1997) reported that the NO donor SIN-l and the SNAP can inhibit IFN, IL-2, IL-5, IL-4 secreted by T cells [8]. Bingisser et al (1998) reported that lymphocyte proliferation response was inhibited by NO induced by activiated Mφ [9]. Karama et al (1995) reported that polysaccharide improved the secretion of NO by promoting iNOS gene expression and activity of iNOS enzyme. Our results referring to the time-dose-dependent change of secretion NO of lymphocyte by ASPS indicated that NO might be the messenger molecule by which ASPS acts on lymphocyte [10].

Conclusion

We draw two major conclusions from the present study. The first relates to ASPS function and improves immune function of cultured blood T cell. The second involves possible functional pathway of ASPS and notes that ASPS acts by affecting the concentration of intracellular cAMP,cGMP and NO of lymphocyte, which may be the messenger molecule in the lymphocytes.

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