Effect of Bioactive Peptides from the Oviductus Ranae of Rana Temporaria Chensinensis on Immune Function in Mice

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Abstract: Objective To study the effect of bioactive peptides from the oviductus ranae of rana temporaria chensinensis on immune function in normal mice.

Method

The mice were randomly divided into normal saline control group, low-dose group,middle-dose group and high-dose group.

Results

ConA was used to induce spleen lymphocytes of mice. T and B lymphocytes proliferation was detected. Serum hemolysin test and neutral red phagocytosis test were to detect the effect of bioactive peptides from the oviductus ranae of rana temporaria chensinensis on humoral immunity in mice.

Conclusion

The results showed that bioactive peptide from the oviductus ranae could obviously promote lymphocyte proliferation induced by ConA, increased serum hemolysin antibody titer, and enhanced allergic reactions and thymus index. Bioactive peptide from the oviductus ranae of Rana chensinensis could enhance immunity function in mice .

Oviductus Ranae is the dry fallopian tube of female rana temporaria chensinensis, with the efficacy of tonifying the kidney to arrest spontaneous emission, nourishing yin of the lung, bringing down a fever, and diminishing inflammation(Zhang, M.,2013). The mainly effective ingredients are protein (about 60%), amino acid,aliphatic acid,lipid-soluble vitamin,carbohydrate,phospholipid, estradiol,pregnendione,and microelement etc, full of nutriments(Sun,J.Z.,2008). Protein is difficult to get digestion and absorption by human body, thus technical study that macromolecular substance is hydrolyzed into small molecular peptide has deeply concerned. The innate immunity of vertebrates is mediated by a network of host-defense mechanisms involving in part a nonspecific chemical defense system that includes broad-spectrum antimicrobial peptides (Bals, 2000; Aov et al.,1991; Jonathan et al.,2007). These peptides inhibit growth of numerous pathogenic microorganisms,including enveloped viruses,Gram-positive and Gram-negative bacteria, protozoa, and fungi (Nicolas and Mor, 1995; Baba and Schneewind,1998; Hancock, 2001; Zasloff, 2002).

Materials

Objects

ICR mouse inbred lines, male, 10-12 weeks old, 20±2g, BAKB/c mice (H-2a) , 10-12 weeks old , 20±2g, cavy, male, 300±50g, and leghorn, male, 2kg±500g were all purchased from basic medical experimental animal research center in Jilin University.

Drug to be assayed and assay reagent

Drugs to be tested: bioactive peptides from the oviductus ranae of rana temporaria chensinensis

Oviductus Ranae of Rana temporaria chensinensis David was hydrolyzed by the combination of caroid and alkaline proteinase. The optimal hydrolysis conditions by caroid are: substrate concentration 1%, enzyme dosage 1200 U/g, temperature 60 ℃, time 3.5 h and pH 6.0. By alkaline proteinase they are enzyme dosage 500 U/g, temperature 50 ℃, time 3.0 h, and pH 8.0.

Common chemical reagents

Sodium thioglycolate was from Sinopharm Chemical Reagent Co., LTD (Shanghai, China)

Neutral red was from Shanghai Reagent Factory III.

Primary instruments

Constant temperature incubation of HERA cell CO_2; Clean bench of SW-CJ-1F ; Microplate reader of Model 680;Reversed biological microscope of XSB-1A;Electronic scales of METTLER TOLEDO;Sigma centrifuge table with high speed and low temperature ; Memmert thermostatic water bath ; -80℃Cryogenic refrigerator of Haier ; Autoclave sterilizer of SANYO ; Beckman PH meter ; Microscope of Olympus; Electric heating Incubators ; JN-A precision torsion balance.

Methods

Animal grouping

ICR mice were randomly divided into 4 groups, 10 ones in each group.In the control group, the mice were injected with saline solution. In the drug group of low dose, mid dose and high dose ,the drug was given by intragastric gavage at the rate of 100mg/kg/d,200mg/kg/d and 400mg/kg/d for 15 days.

Methods

Activity determination of mice NK cell

Mice spleen cells and Yac-1 cells suspension were prepared with the concentration of 4×10^6/mL and 2×10^5/mL respectively. Spleen cells in the cell culture bottle were cultured in 5% CO_2 incubator at 37℃for 2h. 100μL target cells and culture solution were added to the target control well respectively. 100μL effector cells and culture solution were added to the effector control well respectively. The plate was continuously cultured in the 5%CO_2incubator at 37℃for 48h. 10μL of MTT was added to each well and cultured in the 5%CO_2incubator at 37℃ for 4h. The plate was centrifuged at 1000 rpm / min for 10min. The supernatant was discarded for 150μL. 150μL dimethyl sulfoxide and 20μL Glycine buffer(PH10.5) were added per well to dissolve armour ,the reduction products of MTT. OD was measured at 570nm by microtiter plate reader.

NK activity (%) = ｛ 1- [Experimental hole (Titer+Target cells) OD-Effector cells group OD] /Target cells group OD×100 ｝

Determination on proliferation effect of medicated serum on T and B lymphocyte of mice spleen

The prepared medicated serums with the concentration of 20%, 10%, 5% were obtained by 56% water bath, 30min inactivation and 0.22μL microfiltration membrane sterilization.

Cells were obtained with 3×10^6/mL concentration by 10% calf serum of RPMI-1640 culture solution. 140μL cell suspension was added to each well of the 96-well plate.The plate was continuously cultured in the 5% CO_2 incubator at 37℃for 68h. 10μL of MTT (5mg/mL) was added to each well and cultured in the 5%CO_2incubator at 37℃for 4h. The plate was centrifuged at 1000 rpm / min for 10min. 150μL dimethyl sulfoxide and 20μL Glycine buffer(PH10.5) were added per well to dissolve armour ,the reduction products of MTT. OD was measured at 570nm by microtiter plate reader and used to show degree of cell proliferation.

Determination on serum hemolysin antibody titer induced by red blood cell of leghorn

On the 8^{th} day of mice given drugs to be tested, it is after 7 days of abdominal cavity immunization on 0.2mL red blood cell of leghorn. After the last dosage for 12 hours, the mouse was killed by damage cervical. Blood samples were drawn from orbit and centrifuged at the rate of 2000rpm/min for 10min to separate the serum. 1mL serum, 1mL red blood cell diluent of leghorn, and 1mL complement serum diluents of guinea pig were preserved in 37℃ water for 30min and were centrifuged with 2000rpm/min for 10min after ice bath terminated. 1mL liquid supernatant were obtained and put in the Dushi reagent 3 test tube. 0.25 mL red blood cell diluent of leghorn and 4 mL Dushi solution were put in another test tube and were half hemolysis standard solution of leghorn red blood cell. The serum samples were shaked, posput for 10min and determined the OD value in the 540nm wave-length.

Serum samples HC_{50} = serum samples OD value/ half hemolysis OD value of leghorn red blood cell

Determination on activation function of macrophages

Assay of devour neutral red was carried out to determine. 1×10^6/mL abdominal macrophage suspension of mice was prepared. 100μL suspension was put into the 96 holes plate of round bottom. Culture medium was shaked off slightly. 100μL neutral red solution was put into each hole and continuously cultivated in 37℃ incubator for 30min. The solution was shaked off the neutral red. OD value was measured on the 540nm enzyme-linked immunosorbent assay.

Statistical treatment

Data were figured by mean ±SD. The results were statistically analyzed with the methods of variance analysis and t-test. $P<0.05$ was considered to have statistical significance.

Results

The effect of drugs to be tested on mice NK cell activity

The mice were continuously administrated with three concentrations of test drugs for 15 days. Cell line Yac-1of C57BL/6 mice specific lymphoma were taken as a target cell. NK cell activity was determined by MTT method. Results indicated that the cell killing rate of normal saline control on Yac-1 was 50.83% in100 mg/kg/d. Killing rate of low dose drug group was 78.53% and showed significant differences ($P < 0.05$) in comparison with the saline control. Cell activity of NK was remarkably enhanced in the low dose group.

Moreover, NK cell activity was primarily functioned by cell toxicity and side effects. Killing ability of spleen NK cell significantly enhanced by drugs to be tested on cell line Yac-1 was further showed..

Table1 Effect of drugs to be tested on mice NK cell activity

Groups	n	NK cell activity
normal saline control group	8	50.83±21.60
100 mg/kg drug group	8	78.53±16.86*
200 mg/kg drug group	7	61.57±21.63
400mg/kg drug group	9	49.39±21.11

Compared with normal saline control *P < 0.05.

Direct effect of medicated serum on mice spleen T and B lymphocytest

Results showed that spleen lymphocyte proliferation can be directly or indirectly promoted by varying concentration of medicated serum. 20%, 10%, 5% medicated serum can only promote the proliferation trend of spleen B lymphocyte. With the induction of ConA, T cell proliferation capability of the three medicated serum groups was markedly enhanced in comparison with the normal saline control (P < 0.01). B lymphocyte proliferation can obviously be promoted by LPS inducer. The effects of serum induced group on spleen lymphocytes was significant (P < 0.01). The proliferation was greatly promoted.

Table 2 Effect of medicated serum on B lymphocyte proliferation (X±SD) of LPS-induced mice

	OD values			
Groups/concentration	normal saline control group	100 mg/kg drug group	200 mg/kg drug group	400mg/kg drug group
20%	0.607±0.198	0.506±0.094	0.730±0.052	0.474±0.049
10%	0.450±0.042	0.491±0.092	0.578±0.094	0.419±0.079
5%	0.448±0.034	0.504±0.087	0.522±0.067	0.403±0.088

Table 3 Effect of medicated serum on T lymphocyte proliferation (X±SD) of ConA-induced mice

	OD values			
Groups/concentration	normal saline control group	100 mg/kg drug group	200 mg/kg drug group	400mg/kg drug group
20%	0.495±0.226	0.535±0.101	0.746±0.087**	0.539±0.124
10%	0.486±0.080	0.534±0.094	0.768±0.078**	0.550±0.166
5%	0.494±0.080	0.550±0.102	0.753±0.085**	0.597±0.165

Compared with normal saline control group **P < 0.01.

Table 4 Effect of medicated serum on spleen lymphocyte proliferation (X±SD) in mice

	OD values			
Groups/concentrations	normal saline control group	100 mg/kg drug group	200 mg/kg drug group	400mg/kg drug group
20%	0.652±0.124	0.557±0.107	0.858±0.110**	0.799±0.155
10%	0.432±0.110	0.555±0.103	0.728±0.121**	0.756±0.152
5%	0.496±0.115	0.554±0.100	0.662±0.116**	0.526±0.103

Compared with normal saline control **P < 0.01.

Effect of red blood cell of leghorn on the formation of serum hemolysin antibody titer in mice

Different concentrations of test drugs were given by intragastric gavage for 15 days. On the eighth day, the mice were immune with 5% CRBC. The spleen IgM、IgG antibody secretion were inspected after 7 days. The amount of IgM、IgG antibody secretion was increased significantly from the dose of 100 mg/kg. There was significant differences compared with saline control, which indicated that the drugs to be tested had certain promoting role in the function of B lymphocyte antibody-secreting.

Table5 Effect of drugs on the mice serum hemolysin antibody titer caused by red blood cell of leghorn(X±SD)

Groups	n	HC50
normal saline control group	9	0.464±0.108
100 mg/kg drug group	9	0.368±0.088**
200 mg/kg drug group	9	0.474±0.082
400mg/kg drug group	10	0.450±0.124

Compared with normal saline control *P < 0.05.

Effect of drugs on phagocytic function of mice peritoneal macrophages

Phagocytic function of mice peritoneal macrophages was detected by neutral red phagocytosis. The results showed that the experimental group treated with drug dose at 400 mg/kg showed superior phagocytic ability compared to that of normal saline control group ($p<0.05$), which indicated that the test drugs enabled the peritoneal macrophages to perform better phagocytic function, and the specific immune function of mice could be enhanced by this means.

Table 6 Effect of drugs on phagocytic function of mice peritoneal macrophages (X±SD)

Groups	n	OD values
Normal saline control group	7	0.261±0.032
100 mg/kg drug group	10	0.304±0.048
200 mg/kg drug group	8	0.253±0.117
400 mg/ drug group	9	0.339±0.089*

Compared with normal saline control group *P < 0.05.

Conclusion

In this assay, normal ICR mice were taken as objects. Different concentrations of Oviductus Ranae Bioactive Peptides were given by intragastric gavage for 15 days. The activity of mice NK cells could be enhanced; The proliferative potential of splenocytes in mice could be promoted; Besides, the phagocytosis of peritoneal macrophage in mice was reinforced; The number of IgM and IgG antibodies in mice, specific to the leghorn red blood cell, could be enhanced. The results showed that implementing of the immune function in mice was relevant to the enhancement of cellular immunity. The function of humoral immunity was essential.

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