

Immunoenhancement effect of rehmannia glutinosa polysaccharide on lymphocyte proliferation and dendritic cell

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ABSTRACT

The aim of this study is to investigate immunomodulatory effect of rehmannia glutinosa polysaccharide (RGP) on murine splenic lymphocyte and bone marrow derived dendritic cells (DCs). Splenic lymphocytes obtained from mice were co-cultured with RGP for 48 h and then harvested for analyzing with MTT method. The cytokine production of T lymphocytes was measured by ELISA. Effects of RGP treatment on DCs were investigated and assessed by MTT method. The results showed RGP significantly stimulated lymphocyte proliferation and the growth rate of T cell was more significant. The IL-2 and IFN- γ production of T lymphocyte were significantly upregulated after being stimulated with RGP. DCs stimulating on proliferation of T cells and the ability of antigen presenting of DCs have been enhanced under the stimulation of RGP. In conclusion, these findings provided valuable information that RGP possessed strong immunoenhancement activity, which provided the theoretical basis for the further experiment.

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1. Introduction

Rehmannia glutinosa (RG) has been used as traditional Chinese herbal medicine for thousands of years. Rehmannia refers to the root of RG, one herb belonging to the Scrophulariaceae family. It was recorded in Chinese medical classics “Shennong’s Herba” and was thought as a “top grade” herb in China (Zhang et al., 1993). Many clinical and experimental studies show that RG possesses various pharmacological properties.

RG was associated with ameliorate of progressive renal failure and diabetic nephropathy (Harari, Valleljian, Meylan, & Pantaleo, 2005; Kang, Sohn, Moon, Lee, & Lee, 2005; Lee, Choi, Cho, & Kim, 2009; Yokozawa, Kim, & Yamabe, 2004). And it could be used as a hypoglycemic and the treatment of various diabetic disorders (Huang, Niu, Lin, Cheng, & Hsu, 2010; Zhang, Zhou, Jia, Zhang, & Gu, 2004). It was also reported that RG extract could enhance the bone metabolism in osteoporosis (Oh et al., 2003), inhibit liver

inflammation and fibrosis (Wu, Wu, Tsai, Lin, & Chao, 2011). Besides, it has anti-fatigue (Tan et al., 2012), anti-depressant (Zhang, Wen, Wang, Shi, & Zhao, 2009), neuroprotective effect (Zhang et al., 2008) and so on. Furthermore, RG could inhibit inflammatory responses (Baek et al., 2012), reduce the syndromes of inflammation (Lau et al., 2009; Liu, Tang, Xu, Xia, & Xie, 2007; Sung, Yoon, Jang, Park, & Kim, 2011; Waisundara, Huang, Hsu, Huang, & Tan, 2008) as well as inhibit nitric oxide production (Lau et al., 2012) and protect against cell damage via scavenging free radicals (Yu et al., 2006). Aqueous extract from a steamed root of RG was reported to suppress the production of TNF- α and IL-1 in mouse astrocytes (Kim et al., 1999).

About 70 monomeric compounds have been separated from *Rehmannia glutinosa*, including iridoids, saccharides, phenol glycoside ionone, flavonoid, amino acid, inorganic ions, as well as other trace elements (Oshio & Inouye, 1982; Tomoda, Kato, & Onuma, 1971). Researches have also indicated that polysaccharides are the main chemical components related to the bioactivities and pharmacological properties of the herb (Li, Yu, & Wang, 2004).

In the past few years, pharmacological researches on RG and its active principles mainly focused on wide actions on the blood system, endocrine system, cardiovascular system and the nervous system. This study focused on *Rehmannia glutinosa* polysaccharide (RGP) function on immune system. The aim of this research was to investigate the effect of RGP on the proliferation of mouse lymphocyte from spleen, and the activity of bone marrow derived

Abbreviations: RG, *Rehmannia glutinosa*; RGP, rehmannia glutinosa polysaccharides; IL-2, interleukin-2; IFN- γ , interferon- γ ; DCs, dendritic cells; PHA, phytohemagglutinin; LPS, lipopolysaccharide; ELISA, enzyme linked immunosorbent assay; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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dendritic cells (DCs), which provide the theoretical basis for the further experiment in vivo to study whether RGP could promote immune response.

2. Materials and methods

2.1. Reagents

The purified RGP ($\geq 98\%$ purity) was obtained from Ci Yuan Biotechnology Co. Ltd., Shanxi, China. RPMI-1640 (Gibco) supplemented with benzylpenicillin 100 IU mL^{-1} , streptomycin 100 IU mL^{-1} and 10% fetal bovine serum (FCS, Hyclone), was used for re-suspending the lymphocytes, diluting the mitogen and culturing the lymphocytes. RPMI-1640 supplemented with recombinant murine granulocyte macrophage colony stimulating factor (GM-CSF, Amgen, Thousand Oaks, CA) (20 ng/mL), rmlL-4 (R&D Systems, Minneapolis, MN) (20 ng/mL), 10% FCS, benzylpenicillin 100 IU mL^{-1} , streptomycin 100 IU mL^{-1} , NaHCO_3 2.0 g, HEPES 2.38 g, was used for re-suspending and culturing DCs. Phytohemagglutinin (PHA, Sigma), as the T-cell mitogen, was dissolved into 0.1 mg mL^{-1} with RPMI-1640. Lipopolysaccharide (LPS, Sigma), as the B-cell and DCs mitogen, was dissolved into 0.05 mg mL^{-1} with RPMI-1640. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco Co.) was dissolved into 5 mg mL^{-1} with calcium and magnesium-free (CMF) phosphate-buffered saline (PBS, pH 7.4). These reagents were filtered through a $0.22 \mu\text{m}$ syringe filter. PHA and LPS solution was stored at -20°C , MTT solution at 4°C in dark bottles. Dimethyl sulfoxide (DMSO) was produced by Zhengxing Institute of Chemical Engineering in Suzhou, China.

2.2. Splenic lymphocyte proliferation assay

Following the dissection of the abdomens, the spleens were harvested, and spleens from ICR mouse were gently mashed by pressing with the flat surface of a syringe plunger against a stainless steel sieve (200 mesh). After the red blood cells were disrupted, the splenocytes were washed twice. The resulting pellet was re-suspended and diluted to $2.5 \times 10^6 \text{ mL}^{-1}$ with RPMI-1640 with fetal bovine serum after the cell viability was assessed by trypan blue exclusion. The solution was divided into two parts: one part was added with PHA or LPS, and respectively incubated into 96-well culture plates, $100 \mu\text{L}$ per well. Then, RGP at series of concentrations were added, in cell control group and PHA or LPS control group, RPMI-1640 medium and PHA or LPS respectively, $100 \mu\text{L}$ per well, four wells each concentration. The final concentration of PHA or LPS reached to $10 \mu\text{g mL}^{-1}$ or $5 \mu\text{g mL}^{-1}$. The plates were respectively incubated in a humid atmosphere with $5\% \text{ CO}_2$ (Thermo) at 37°C for 48 h. Briefly, $30 \mu\text{L}$ of MTT (5 mg mL^{-1}) was added into each well at 4 h before the end of incubation. Then the plates were centrifuged at $1000 \times g$ for 10 min at room temperature. The supernatant was removed carefully and $100 \mu\text{L}$ of DMSO was added into each well. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance of cells in each well was measured by microliter enzyme-linked immunosorbent assay reader (Model RT-6000, Leidu Co., Ltd., Shenzhen City) at a wave length of 570 nm (A_{570} value) as the index of lymphocytes proliferation (Fan et al., 2012). Meanwhile, the lymphocytes proliferation rate was calculated as follows:

$$\text{Proliferation rate (\%)} = \frac{\bar{A}_{(\text{test group})} - \bar{A}_{(\text{control group})}}{\bar{A}_{(\text{control group})}} \times 100\%$$

2.3. Measurement of IL-2 and IFN- γ

Lymphocytes were collected as in Section 2.2. Cells were incubated for 48 h. Culture supernatants were collected and the concentrations of interferon γ (IFN- γ) and interleukin 2 (IL-2) were assayed by ELISA kit (R&D, Co., USA).

2.4. Analysis of a range of RGP doses on DCs stimulating proliferation of T cells

2.4.1. Generation of immature DCs

The femurs and tibiae of ICR mouse (4–6 weeks old) were removed and isolated from surrounding muscle tissue. Intact bones were washed twice in PBS. Bone marrow cells were flushed from the femur and tibiae of ICR mouse and treated with ACK lysis buffer (Sigma) to lyse erythrocytes. DCs were cultured at a starting concentration of $2.0 \times 10^6/\text{mL}$ in round-bottomed 12-well plates with RPMI-1640 supplemented with GM-CSF, rmlL-4, 10% FCS, 2 mL per well. Cells were cultured in a humidified chamber at 37°C and 5% atmospheric CO_2 . After incubation for 24 h, the medium with non-adherent cells was replaced with fresh medium. The culture medium was removed and replenished with an equal volume of fresh medium every two days. On the 7th day, full matured DCs were harvested for stimulation of T cell proliferation assays.

2.4.2. RGP on mature DCs

On the 7th day of incubation, different concentrations (200, 100, 50, 25 and $12.5 \mu\text{g mL}^{-1}$) of RGP, serum-free RPMI-1640 or $5 \mu\text{g mL}^{-1}$ LPS were added into DCs incubated for another 48 h. After 30 min treatment with $50 \mu\text{g mL}^{-1}$ mitomycin C at 37°C , cells were washed with PBS twice, and then resuspended in complete RPMI-1640 at a concentration of $5 \times 10^5/\text{mL}$.

2.4.3. Allogeneic mixed lymphocyte reaction

Spleens from ICR mouse were harvested sterility and gently mashed by pressing with the flat surface of a syringe plunger against a stainless steel sieve (200 mesh). After the red blood cells were lysed, the splenocytes were washed twice and resuspended in complete RPMI-1640. Splenocytes ($1.0 \times 10^6/\text{mL}$) were cultured in 96-well plates in a volume of $100 \mu\text{L}/\text{well}$. The mature DCs were added into each well, four wells each group.

2.4.4. Analysis of T cell proliferation

The cultures were incubated at 37°C and $5\% \text{ CO}_2$ for 72 h. In the last 4 h of incubation, MTT (5 mg mL^{-1} , $30 \mu\text{L well}^{-1}$) was added into each well. Then supernatant were discarded before DMSO ($100 \mu\text{L well}^{-1}$) were added. Finally, A_{570} was tested as the index of BMDCs stimulating the proliferation of T cells.

2.5. Ability of antigen presentation assessment of RGP on DCs

2.5.1. Generation of immature DCs

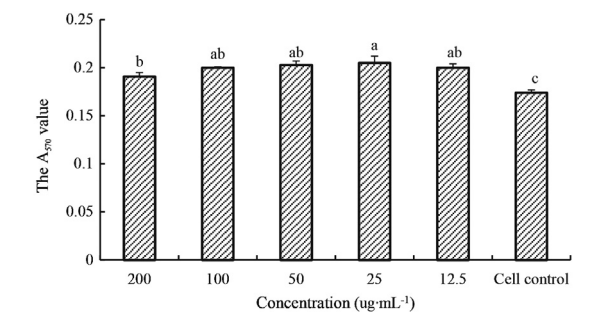
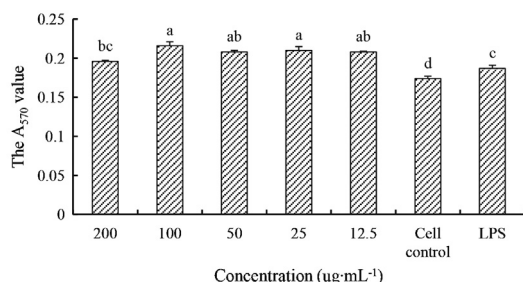
The immature DCs were collected as Section 2.4.1.

2.5.2. Preparation of allogeneic lymphocyte

OVA solution mixed with aluminium adjuvant, each mouse was immunized subcutaneous with $100 \mu\text{g}$ OVA. On the 7th, 14th day, the mice were separately boosted. Three days after the last immunization, the responding cells were collected as in Section 2.4.3. Lymphocytes were resuspended in complete RPMI-1640 at a concentration of $1.0 \times 10^6/\text{mL}$, supplemented with OVA ($100 \mu\text{g mL}^{-1}$), in round-bottomed 96-well plates.

2.5.3. Analysis of antigen presentation ability

DCs having been treated with mitomycin C were added into lymphocytes, four wells each group. The cultures were incubated

(a) Effects of B lymphocyte proliferation in single stimulation of RGP (A_{570} values).^{a-c} Bars in the figure without the same superscripts differ significantly ($P < 0.05$).(b) Effects of B lymphocyte proliferation in synergistical stimulation of RGP with LPS (A_{570} values).^{a-d} Bars in the figure without the same superscripts differ significantly ($P < 0.05$).**Fig. 1.** Effects of RGP on B lymphocyte proliferation.

at 37 °C and 5% CO₂ for 72 h. In the last 4 h of incubation, MTI (5 mg mL⁻¹, 30 μL well⁻¹) was added into each well. Thereafter, supernatant was discarded before DMSO (100 μL well⁻¹) were added. Finally, A_{570} was tested as the index of ability of antigen presentation of DCs.

2.6. Statistical analysis

Data are expressed as mean ± standard errors (S.E.). Duncan and LSD's multiple range test were used to determine the difference among groups. P -values of less than 0.05 were considered to be statistically significant.

3. Results

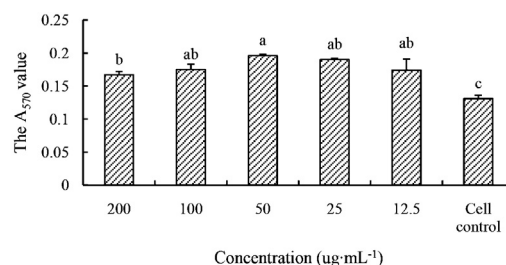
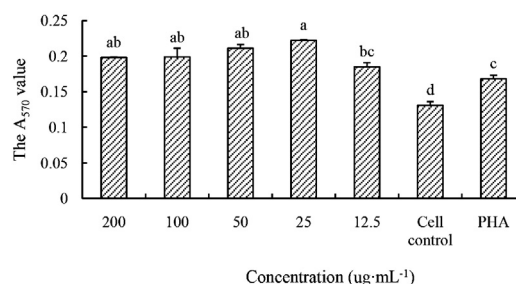
3.1. Effects of RGP on splenic lymphocyte proliferation

In single stimulation of RGP on B lymphocyte proliferation, the A_{570} values of each concentration group were significantly higher than those of the control group ($P < 0.05$) (Fig. 1(a)). In synergistical stimulation with LPS, the A_{570} values of concentration between 100 and 12.5 μg mL⁻¹ were significantly higher than those of the LPS group ($P < 0.05$) (Fig. 1(b)).

Similar result was also found in single stimulation of RGP on T lymphocyte proliferation (Fig. 2(a)). While in synergistical stimulation with PHA, the A_{570} values of concentration between 200 and 25 μg mL⁻¹ were significantly higher than those of the PHA group ($P < 0.05$) (Fig. 2(b)).

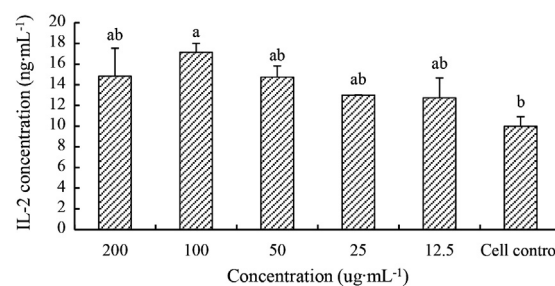
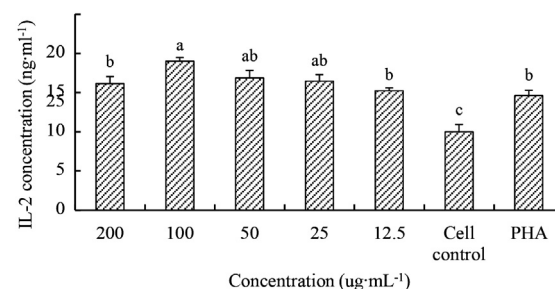
3.2. Effect of RGP stimulating cytokine production of T lymphocyte

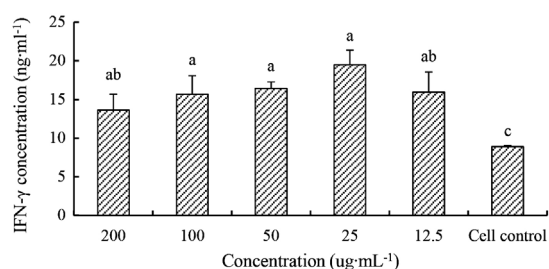
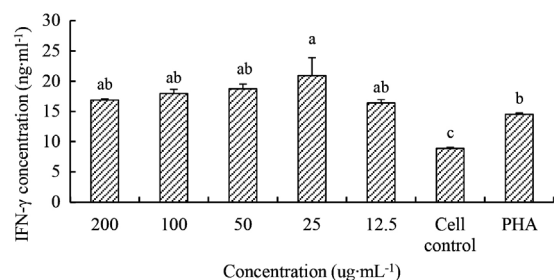
In single stimulation with RGP, the concentrations of IL-2 of RGP group were higher than that of cell control group ($P < 0.05$), especially at 100 μg mL⁻¹ RGP group (Fig. 3(a)). In synergistical

(a) Effects of T lymphocyte proliferation in single stimulation of RGP (A_{570} values).^{a-c} Bars in the figure without the same superscripts differ significantly ($P < 0.05$).(b) Effects of T lymphocyte proliferation in synergistical stimulation of RGP with PHA (A_{570} values).^{a-d} Bars in the figure without the same superscripts differ significantly ($P < 0.05$).**Fig. 2.** Effects of RGP on T lymphocyte proliferation.

stimulation of RGP with PHA, IL-2 of RGP group at 100 μg mL⁻¹ was significantly higher than that of PHA group ($P < 0.05$) (Fig. 3(b)).

Whereas in single stimulation with RGP, the concentrations of IFN-γ of RGP groups were all significantly higher than that of cell control group ($P < 0.05$) (Fig. 4(a)). And under the same condition,

(a) Effects of IL-2 secretion of T cells in single stimulation of RGP (ng·mL⁻¹).^{a-b} Bars in the figure without the same superscripts differ significantly ($P < 0.05$).(b) Effects of IL-2 secretion of T cells in synergistical stimulation of RGP with PHA (ng·mL⁻¹).^{a-c} Bars in the figure without the same superscripts differ significantly ($P < 0.05$).**Fig. 3.** Effects of RGP on IL-2 secretion of T cells.

(a) Effects of IFN-γ secretion of T cells in single stimulation of RGP (ng·mL⁻¹).^{a-c} Bars in the figure without the same superscripts differ significantly ($P < 0.05$).(b) Effects of IFN-γ secretion of T cells in synergistical stimulation of RGP with PHA (ng·mL⁻¹).^{a-c} Bars in the figure without the same superscripts differ significantly ($P < 0.05$).**Fig. 4.** Effects of RGP on IFN-γ secretion of T cells.

the production of IFN-γ of RGP group at 25 μg mL⁻¹ was significantly higher than that of PHA group ($P < 0.05$) (Fig. 4(b)).

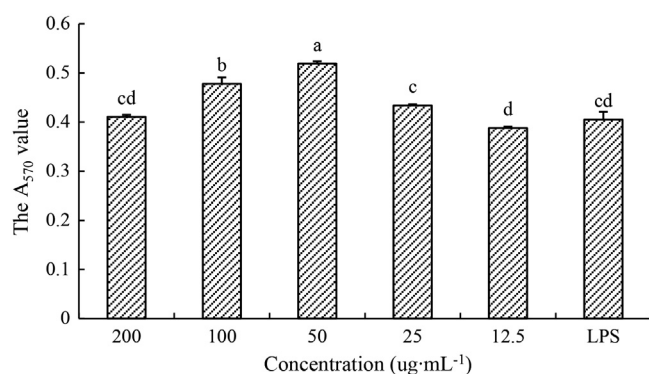
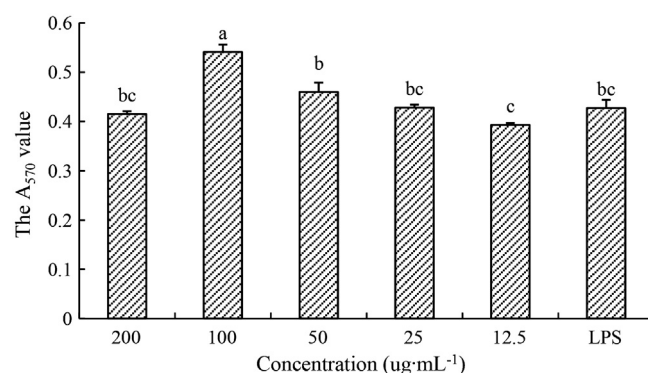
3.3. Effect of RGP on immunocompetence of DCs

3.3.1. RGP enhanced the stimulation of DCs on T lymphocytes proliferation

At 25–200 μg mL⁻¹, the A_{570} values of RGP group were higher than that of LPS group and at 50 and 100 μg mL⁻¹, they were significantly higher than that of LPS group (Fig. 5). Therefore, RGP could enhance the ability of DCs stimulating on T lymphocytes proliferation in different degree.

3.3.2. RGP improved the antigen presenting ability of DCs

Compared with LPS group, the A_{570} values were higher between 25 and 200 μg mL⁻¹ RGP group. The 100 μg mL⁻¹ RGP group was the highest and significantly higher than that of LPS group ($P < 0.05$). Therefore, RGP at 100 μg mL⁻¹ could improve antigen presenting ability of DCs (Fig. 6).

**Fig. 5.** Effects of RGP on stimulation of DCs on T cells proliferation (A_{570} values).**Fig. 6.** Effects of RGP on ability of antigen presenting of DCs (A_{570} values).

4. Discussion

RG is a therapeutic Scrophulariaceae herb. Its root has been used for different medical purposes as traditional Chinese medicine (TCM) for thousands of years. Polysaccharides from many different kinds of TCM have been widely studied. They are proved to have various activities, such as antitumor (Huang, Jin, Zhang, Cheung, & Kennedy, 2007; Zhao, Dong, Chen, & Hu, 2009), anticancer (Zong, Cao, & Wang, 2012), antioxidant (Mateo-Aparicio, Mateos-Peinado, Jimenez-Escrig, & Ruperez, 2010), immunomodulation (Yi et al, 2012; Luo, Sun, Wu, & Yang, 2012), and so on. RGP is one of the major active components of RG. As far as being reported, RGP has many pharmacological functions. This research focused on its immunological function.

B and T lymphocyte are both important for immunologic response in organism. T lymphocyte possesses mediating cellular immunity as well as adjusting immunity. B lymphocyte mainly participates in the humoral immunity. Proliferation of lymphocyte is the most immediate index of reflecting organic immunity (Pan, Liu, & Wang, 2011).

In order to investigate the role of RGP on immunological enhancement, the effect on lymphocyte proliferation was examined. According to this research, RGP (12.5–200 μg mL⁻¹) significantly stimulated both B and T lymphocyte proliferation (Figs. 1a and 2a). In coordination with PHA or LPS administrating drug, the similar results were found (Figs. 1b and 2b). These indicated that the proliferation ability of B and T lymphocyte have been enhanced after being treated with RGP, and RGP also could strengthen enhancement of PHA and LPS on lymphocyte proliferation. Information was provided that RGP could be an effective immunological adjuvants.

As shown in Fig. 7, the lymphocytes proliferation rates were various with different stimulation methods. When in single stimulation of RGP, the B lymphocyte proliferation rate was 16.09%, whereas the T lymphocyte proliferation rate was 40.07%. Under the condition of being in synergistical stimulation of RGP with PHA, we got the highest lymphocyte proliferation rate (58.40%), and in case of being in single stimulation of RGP, the T lymphocyte proliferation rate was 40.27%, which is the second highest. Furthermore, RGP's stimulation on T lymphocyte was significantly better than that of B lymphocyte (Fig. 7). It might come to the conclusion that potential of RGP on cellular immunity was better than that on humoral immunity.

Cytokine production by CD4⁺ helper T lymphocytes during immune response plays an important role in regulating the nature of the response. For example, IFN-γ and IL-2 are secreted by type 1 helper T cells (Th1 cells) and mediate cellular immunity (Letsch & Scheibenbogen, 2003; Pan, Liu, & Wang, 2011). They both can promote proliferation and differentiation of Th1 cells.

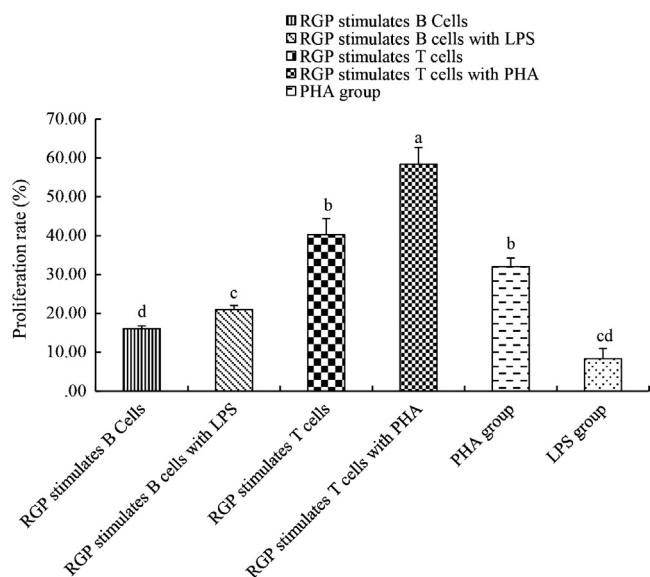


Fig. 7. Effects of RGP on lymphocyte proliferation rate.

IFN- γ is a pleiotropic cytokine with immunomodulatory effects on different kinds of immune cells. In mammals and poultry, IFN- γ has been an indicator for cell-mediated immunity of infected organism (Ottenhoff & Mutis, 1995; Zheng, Zhao, & Cui, 2007). Therefore, preliminary assessment for activation extent of T cells could be made by detecting the content of IFN- γ (Cheeseman, Lillehoj, & Lamont, 2008; Park, Lillehoj, & Allen, 2008). IL-2 is one of the cytokines that plays an important role in immunomodulation. IL-2 is a critical component in activation and differentiation of T lymphocyte as well as the regulation of the immune system (Zhou, 2010). Some studies suggested that increasing IL-2 secretion was more effective control of viremia in infected individuals, especially associated with IFN- γ (Harari, Vallelan, Meylan, & Pantaleo, 2005; Lichterfeld et al., 2004; Migueles et al., 2002; Younes et al., 2003). Therefore, the concentration changes of IFN- γ , IL-2 were important to immune response.

In this research, the concentrations of IL-2 in RGP groups were higher than that of cell control group. Especially in $100 \mu\text{g mL}^{-1}$, the concentration of IL-2 was significantly higher (Fig. 3(a)). In coordination with PHA administrating drug, the similar results were found (Fig. 3(b)). The concentrations of IFN- γ in RGP groups were all significantly higher than that of cell control group (Fig. 4(a)). When in synergistical stimulation with PHA, the concentrations of IFN- γ in RGP groups were all higher than that of PHA group, and in $25 \mu\text{g mL}^{-1}$ was significantly higher (Fig. 4(b)). It indicated that RGP could promote T cells' secretion of both IFN- γ and IL-2. These provided the information that RGP could promote proliferation and differentiation of Th1 cell, and promote the Th1-mediated cellular immunity. It might also come to the conclusion that RGP enhancing on Th1-mediated cellular immunity made it the immunoenhancement of adaptive immunity.

DCs are the most potent professional antigen presenting cell as far as we know. DCs are the master regulators of the adaptive immune response (Banchereau & Palucka, 2005; Banchereau et al., 2003; Hartgers, Figdor, & Adema, 2000; Steinman, Hawiger, & Nussenzweig, 2003). They participate in differentiation of T cells subset and inherent immunity response (Lin & Wang, 2011). It is the only kind of cell that can immediate contact with T cells and promote proliferation of T cells (Banchereau & Steinman, 1998). DCs function as an important bridge cell between inherent immunity and adaptive immunity (Guernonprez, Valladeau, Zitvogel, Thery, & Amigorena, 2002; Thery & Amigorena, 2001). In brief, DC locates

in the center of the process of inducing, maintaining and regulating immune response (Janeway, 2008).

In this study, we made DCs as our research model for further investigating the immunological function of RGP. According to the result, after being treated with DCs, which were stimulated with RGP (50 and $100 \mu\text{g mL}^{-1}$), the proliferations of T lymphocyte were significantly higher compared with that of LPS group (Fig. 5). Furthermore, in this experiment, DCs and T cells both come from ICR mice, thus they have the same MHC-II molecules, which makes testing on its ability of antigen presenting possible. The result indicated that antigen presenting ability of DC had been significantly enhanced after being incubated with RGP ($100 \mu\text{g mL}^{-1}$) (Fig. 6).

In conclusion, in vitro, RGP not only could significantly promote T and B lymphocyte proliferation singly or synergistically with PHA and LPS, but also could increase the concentration of IFN- γ and IL-2 secreted by T lymphocyte. Besides, RGP promoted DCs stimulation on T lymphocyte proliferation and its ability on antigen presentation. RGP demonstrated strong immunoenhancement activity, which provides the theoretical basis for the further experiment.

Acknowledgments

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