

Effect of *Gypsum fibrosum* and *Rhizoma atractylodis* on *In Vitro* Proliferation of Mouse Splenic Lymphocyte

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Abstract: Qingliang electuary plays important roles in speeding up heat dissipation, enhancing blood circulation and improving the body's immune system. The main ingredients of a qingliang electuary are *Gypsum fibrosum* (GF) and *Rhizoma atractylodis* (RA). Here, researchers report the effect of these two ingredients on *in vitro* proliferation of mouse splenic lymphocyte. The extracts of GF and RA along with ConA were tested under high temperatures (37, 39 and 42°C) in combination with different incubation times (1 or 4 h) using the MTT (3-(4, 5)-dimethylthiazol-2-yl)-5-(2, 4, 6-trimethylphenyl)-2-tetrazolium bromide) Method. The results showed that ConA significantly stimulated lymphocyte proliferation at 37°C. Under 39 and 42°C, the stimulation diminished as incubation time prolonged. High dose GF (500 µg mL⁻¹) significantly inhibited proliferation under all tested conditions but low dose GF (0.05 µg mL⁻¹) significantly increased lymphocyte proliferation at 42°C after 1 h incubation. RA water extract at 500 µg mL⁻¹ significantly increased lymphocyte proliferation under all tested conditions. The effect of RA oil was weaker compared to RA water extract and not consistent under different incubation conditions.

Key words: Qingliang electuary, Shi gao, Bai zhu, *in vitro* proliferation, mouse splenic lymphocyte, incubation

INTRODUCTION

Heat is a common environmental factor that stresses farm animals (Finch, 1984). The adverse effect of high ambient temperature on productive and reproductive efficiency of livestock is well documented. All major farm animals that experience high environmental temperature have lower feed intake, lower growth and production rate (e.g., meat, milk, eggs) and lower pregnancy (Fuquay, 1981; Habeeb *et al.*, 1992; Jordan, 2003; St-Pierre *et al.*, 2003). In addition, heat stress can directly compromise animal health and immune systems as the incidence of health problems in livestock increases during warm Summer months (Collins and Weiner, 1968; Niwano *et al.*, 1990).

The common methods to combat heat problem of farm animals are to provide shade shelters, water and forced ventilation. Additionally, literatures reported that the adverse effect of heat stress also can be mitigated through manipulation of physiological and cellular function. There is some evidence that antioxidant administration and other pharmacological treatments can reduce the degree of hyperthermia experience by animals

exposed to heat stress (Hansen *et al.*, 2001; Xue-Li *et al.*, 2011). A Qingliang electuary based on Traditional Chinese Medicine (TCM) has been developed to alleviate heat stress in farm animals in China. When the Qingliang electuary was administrated as a dietary supplement it improved heat dissipation, blood circulation, immune response and production performance in chicken and pigs (Liu *et al.*, 2005; Zhu *et al.*, 2006).

Gypsum fibrosum (GF), *Rhizoma atractylodis* (RA), *Cortex phellodendri* and *Herba agastachis* extracts are major ingredients of this electuary. All of them are commonly used in TCM for humans and animals for thousands of years. Specifically, GF is a sulfate mineral and is used to treat heat related fever and irritability such as thirst and sweat. RA is a plant material with the main clinical function of anti-diarrhea and anti-fatigue and it has been showed to increase immune response under *in vivo* and *in vitro* conditions (Liu *et al.*, 2005; Zhu *et al.*, 2007). However, not much is known about the therapeutic effects of GF and RA and their effects on *in vitro* proliferation of lymphocyte.

Lymphocytes are an important part of animal immune systems. Its amount in cells or body fluids is directly

correlated with immune response and the protective ability against diseases. Using mouse splenic lymphocytes as a model researchers characterized the effect of GF and RA on *in vitro* lymphocyte proliferation under different drug concentrations and high temperature incubation as part of the effort to understand the working mechanisms of the cooling agent.

MATERIALS AND METHODS

Animal source and spleen lymphocyte preparation: The mouse spleen lymphocyte used in the experiment was prepared from Kunming strain SPF mice provided by Vital River Lab Animal Technology Co., Ltd. (Beijing, China). Five males and five females with body weights ranging from 18-22 g were used.

The mice were sacrificed by cervical dislocation and soaked in 75% ethanol for 5 min. Under aseptic condition, spleens free of connective tissues and fat were excised and rinsed with RPMI 1640 medium (HyClone Laboratories, Logan, UT). The clean spleens were placed on a sterile nylon mesh, broken into pieces with a pair of surgical scissors and minced with the glass insert of a syringe. Homogeneous cell suspensions were collected into a sterile centrifuge tube and centrifuged for 10 min at 1500 rpm. The cell pellet was resuspended in 4 mL RPMI 1640 medium and then slowly added to a 15 mL centrifuge tube against the wall to lie on top of 6 mL LTS 1077 lymphocyte separation medium (Hao Yang Biological Products Co., Ltd., Tianjin, China) that was already in the tube. After centrifuged at 2000 rpm for 20 min, the cloudy low density cell layer (second layer) was pipetted into another centrifuge tube and washed with RPMI 1640 medium twice. The final cell pellet was resuspended in RPMI 1640 medium and cell viability (>95%) was checked by thiazolyl blue viability assay. The final cell suspension was adjusted to 4.0×10^6 cells mL^{-1} .

Drug preparation: Raw materials of *Gypsum fibrosum* (GF) (Lot No: 1040333-1, source location: Hebei province) and dry *Rhizoma atractylodis* (RA) (Lot No.: 1031260-2, source location: Jiangsu province) were purchased from Yanjing Chinese Medicinal Herb Factory (Beijing, China). Dry powder (drug) was obtained from *Gypsum fibrosum* raw material using a traditional stew-concentrate-dry (under low pressure) procedure. Essential oil of *Atractylodis* was obtained from the dry RA material and the concentration of the oil was made to 0.5 mL oil in 8 g β -Cyclodextrin (β -CD). Dry powder of RA (water soluble portion) was prepared from the oil by the same method used for GF.

Table 1: Treatment groups and their incubation regimens

Groups	Treatment	Incubation regimen
A	Drug only	37°C, 38 h
B	Drug+ConA	37°C, 38 h
C	Drug+ConA	37°C, 37 h then 39°C, 1 h
D	Drug+ConA	37°C, 34 h then 39°C, 4 h
E	Drug+ConA	37°C, 37 h then 42°C, 1 h
F	Drug+ConA	37°C, 34 h then 42°C, 4 h

Lymphocyte proliferation and incubation regimes:

Lymphocytes were proliferated in 96 well tissue culture plates. Each well contained 2.0×10^5 cells and drugs of different concentrations alone (at 37°C only) or with ConA (Sigma®, repackaged by Beijing Kehaoze Biologic Technology Co., Ltd.). The final volume of 100 μL cell $^{-1}$ was adjusted with RPMI 1640 medium that contained 0.05, 5 and 500 $\mu\text{g mL}^{-1}$ of the two powder drugs or 0.01, 1 and 100 $\mu\text{g mL}^{-1}$ of RA oil. The final concentration of ConA was 3 $\mu\text{g mL}^{-1}$. Blank controls (RPMI 1640 only) and lymphocyte (+ ConA if necessary) controls were setup for each treatment group shown in Table 1. The experiment was conducted with 3 replications. The plates were incubated in a 5% CO_2 incubator (MCO-18AIC, Sanyo Corporate, Japan) under temperature and incubation period regimes shown in Table 1. At the end of incubation, 10 μL of 5 mg mL^{-1} MTT (ultra pure grade, Amresco®, repackaged by Beijing Kehaoze Biologic Technology Co., Ltd.) was added into each well. After another 4 h of incubation at 37°C, DMSO (ACS grade, Amresco®, repackaged by Beijing Kehaoze Biologic Technology Co., Ltd.) was added to stop the reaction. The plates were read at 570 nm with a microplate reader (Model 680, Bio-Rad Laboratories, CA, USA) and OD values were recorded. Higher OD_{570} values represent higher levels of lymphocyte proliferation (Mosmann, 1983).

Statistical analysis: Data analysis was performed using DPS Statistical Software (Tang, 2010) Data were expressed as mean \pm Standard Deviation (SD). A t-test was used to evaluate the difference between blank and lymphocyte controls within each treatment group and mean comparisons among treatment groups.

RESULTS AND DISCUSSION

Lymphocyte proliferation in absence of the drugs: Within each treatment group, the average OD_{570} value for lymphocyte control was significantly higher than that of blank control ($p < 0.01$). As expected, the treatment regime had no effect on the OD_{570} of blank controls. Temperature and incubation time effect on lymphocyte proliferation was observed in the lymphocyte controls (drug free) (Table 2). Group B had significantly higher OD_{570} values

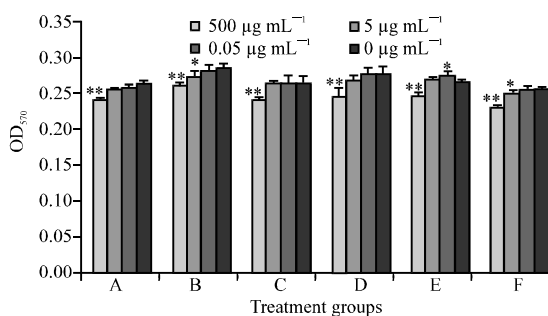


Fig. 1: Effect of *Gypsum fibrosum* water extract on the proliferation of lymphocytes. **Highly significant compared to the control ($0 \mu\text{g mL}^{-1}$) ($p<0.01$); *Significant compared to the control ($p<0.05$)

Table 2: The OD_{570} readings (proliferation of lymphocytes) of the blank and lymphocyte controls*

Groups	Blank control	Drug free lymphocyte control
A	0.2284 ± 0.0034	0.2632 ± 0.0041
B	0.2198 ± 0.0373	0.2848 ± 0.0066^A
C	0.2252 ± 0.0040	0.2640 ± 0.0086^B
D	0.2294 ± 0.0035	0.2766 ± 0.0109
E	0.2202 ± 0.0059	0.2648 ± 0.0029^B
F	0.2263 ± 0.0033	0.2564 ± 0.0019^B

*Mean \pm SD (n = 3) followed by A = $p<0.01$ when compared with group A; B = $p<0.01$ when compared with group B

compared to group A ($p<0.01$) suggesting that ConA stimulated lymphocyte proliferation at 37°C . Among groups with ConA (B to F), lymphocyte proliferation decreased as the temperature and incubating duration increased. Highly significant differences ($p<0.01$) were observed between group B and C, E and F (Table 2).

GF effect on lymphocyte proliferation: A general negative dose-response relationship between concentrations of GF and lymphocyte proliferation was observed (Fig. 1). As GF concentrations decreased, lymphocyte proliferation increased numerically with some statistical differences. GF of $500 \mu\text{g mL}^{-1}$ significantly reduced the level of proliferation ($p<0.01$) under all tested incubation regimes. At 37°C when ConA was added (Group B), $5 \mu\text{g mL}^{-1}$ was enough for a significant reduction ($p<0.05$) when compared with the drug free lymphocyte control. The trend was very different under 42°C and 1 h incubation (Group E) where $0.05 \mu\text{g mL}^{-1}$ significantly increased the proliferation ($p<0.05$).

The 42°C and 4 h incubation treatment (Group F) significantly reduced proliferation at $5 \mu\text{g mL}^{-1}$ or greater (Fig. 1).

RA effect on lymphocyte proliferation: RA at $500 \mu\text{g mL}^{-1}$ significantly increased lymphocyte proliferation compared to drug-free controls under all incubation regimes ($p<0.01$) (Fig. 2). At 5 and $0.05 \mu\text{g mL}^{-1}$ no significant effect was

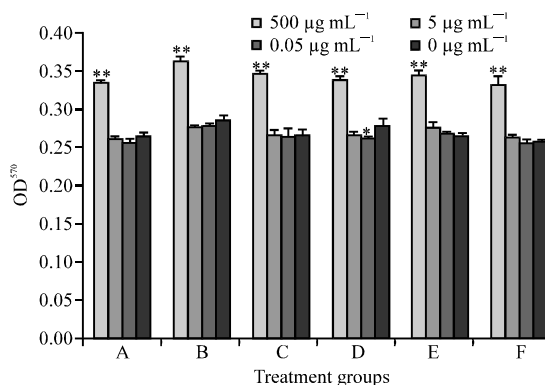


Fig. 2: Effect of *Rhizoma atractylodis* water extract on the proliferation of lymphocytes. **Highly significant compared to the control ($0 \mu\text{g mL}^{-1}$) ($p<0.01$); *Significant compared to the control ($p<0.05$)

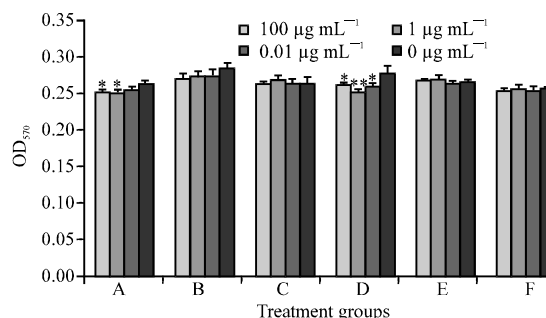


Fig. 3: Effect of *Rhizoma atractylodis* oil on the proliferation of lymphocytes. **Highly significant compared to the control ($0 \mu\text{g mL}^{-1}$) ($p<0.01$); *Significant compared to the control ($p<0.05$)

observed except for group D where $0.05 \mu\text{g mL}^{-1}$ of RA significantly decreased the level of proliferation ($p<0.05$). A weak positive dose-response relationship between concentrations of RA and lymphocyte proliferation was present under 41°C (Group E and F), i.e., as the RA concentrations increased, the level of proliferation increased. But this trend was absent for all other groups.

RA oil effect on lymphocyte proliferation: There was no apparent trend of dose-response relationship between RA oil concentration and the level of lymphocyte proliferation. All three concentrations of RA oil had no effect on cell proliferation for group B, C, E and F. However, for group D, all concentrations of AR oil significantly reduced the level of proliferation compared with controls ($p<0.05$).

Under 37°C with no Con A (Group A), 100 and $1 \mu\text{g mL}^{-1}$ treatments significantly reduced lymphocyte proliferation (Fig. 3).

To understand the positive effect of the Qingliang electuary on animals under heat stress, the current study focused on its potential immunological effect-lymphocyte *in vitro* proliferation in responding to TCM drug and ConA stimulation under different temperature and incubation time regimes. The proliferation of lymphocytes is an important immune response thus it is a common test used in cell and clinical immunity studies. ConA is the first discovered mitogen that can stimulate T-cell giving rise to different functionally distinct T-cell populations (T-cell activator) (Gonzalez *et al.*, 1994). The current study showed that under 37°C ConA significantly increased lymphocyte proliferation. But under 39°C, the stimulation effect of ConA was minimized and required long incubation time. Under 42°C, longer incubation time started to kill lymphocytes.

Researchers found that high dose of GF (500 µg mL⁻¹) significantly inhibited lymphocyte proliferation. The medium dose (5 µg mL⁻¹) also showed inhibition effect except for under 42°C incubating 1 h where proliferation was numerically higher compared with controls while the low dose GF (0.05 µg mL⁻¹) significantly increased cell proliferation. The results revealed that low dose GF promote lymphocyte proliferation under high temperatures. GF contains >95% of CaSO₄·2H₂O (PCMH, 1990) and releases calcium ions to blood streams to reduce vascular permeability and inhibit hyper-active thermoregulatory system under heat conditions. This mechanism explains the antipyretic function of GF. Cheng (2001) reported that GF increased the development and phagocytic ability of rabbit alveolar macrophage cells against staphylococcus albus and colloid gold, thus strengthened the immune response. Combined with current results we could conclude that GF (at low dose) could collaborate with ConA to stimulate lymphocyte proliferation under high temperature conditions and the mechanism could be related to Ca²⁺ regulation.

Modern pharmacological researches were conducted to understand the therapeutic mechanism of RA. For example, studies showed that RA had diuretic effect through suppression of Na⁺ and K⁺-ATP enzymes (Sato *et al.*, 1991). The drug also has inhibition effect on the formation of gastric ulcer under stress conditions, stimulation effect on bile and luteolysin secretions, anticoagulant effect and effect on bone marrow cell proliferation (Usuki 1988; Yu *et al.*, 1998; Zhang *et al.*, 1999). Yamada *et al.* (1992) reported that RA hot water extract was a mitogen. Researchers found that high dose of RA water extract (500 µg mL⁻¹) significantly stimulated lymphocyte proliferation under high temperature conditions. The active ingredients of RA are its volatile oil that is mainly consisted of hinesol and β-eudesmol as

these compounds possess important therapeutic functions (Hang, 1994). Studies also showed that both water extract and oil have activities but with different strength. The current study indicated that RA oil showed a less dose-response on stimulation of lymphocyte proliferations compared to its water extracted powder suggesting that the more active compounds are water soluble.

CONCLUSION

Low dose GF and high dose RA are found to stimulate mouse spleen lymphocyte proliferation under high temperatures. The information obtained laid a ground work for understanding of the working mechanisms of the Qingliang electuary. Future studies will be concentrating on their interaction effect when administrated simultaneously. The ultimate goal is to maximize the benefit of their use in farm animal production.

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