A Randomized and Controlled Experimental Study on the Effects of Epimedium-Propolis Adjuvant on Immune Function in Mice, Rabbits and Chickens

Yuanliang Hu DVM, PhD, Jiaguo Liu DVM, PhD, Dalu Song DVM, Zhigang Xu DVM

ABSTRACT

Four related randomized and controlled experiments were performed to evaluate the effects of Epimedium-Propolis adjuvant (EPA) on T lymphocyte transformation rates in mice and chicks and antibody levels in rabbits and chickens. In Experiment I, mice were divided into 4 groups and injected with the immunosuppressive agent cyclophosphamide (Cy), or EPA+Cy, or EPA only and compared to untreated saline control. Results showed that Cy down-regulated T lymphocyte transformation rate (SI), while EPA up-regulated it. In Experiment II, chicks were injected with either 0.2 ml or 0.4ml EPA and compared to untreated saline control. EPA increased SI in a dose dependent and age related manner in chicks. In experiment III, infectious canine hepatitis virus (ICHV) antibody level was evaluated in rabbits injected with virus + EPA and without EPA. Antibody levels were higher in the EPA group. Experiment IV results demonstrated that EPA and Freund’s adjuvant (FA) created nearly equal antibody increases in hens, however, EPA did not demonstrate the adverse side effects seen with FA. In conclusion, EPA improved cellular and humoral immune function of animals in a dose dependent manner with the greatest intensity of the effect in young animals. In addition, the use of EPA as an adjuvant is as effective as Freund’s adjuvant but without the deleterious side effects on health. This study adds to the body of research demonstrating the benefit of using the Chinese herbal medicine, Epimedium along with extracts from propolis as an adjuvant in vaccines for animals.

Key words: Epimedium-Propolis adjuvant, cyclophosphamide, T lymphocyte transformation rates, cellular immunity, humoral immunity

ABBREVIATIONS

EPA  Epimedium-Propolis Adjuvant
Cy  Cyclophosphamide
PS  Physiological Saline
FCS  Fetal Calf Serum
FA  Freund’s adjuvant
ICHV  Infectious Canine Hepatitis Virus
XOO  Xanthomonas Oryzae Pv. oryzae
NK  Natural Killer
PHA  Phytohemagglutinin
SI  Stimulation Index

An adjuvant is an immunological agent that can enhance the immune response to an antigen by an animal. They can be used as an immunostimulant to enhance vaccine response and can be injected prior to antigen exposure or together with the antigen. Although widely used adjuvants such as oil emulsion, aluminum and some chemicals are effective, they often have severe side effects such as local inflammation, slow biodegradation and absorption, difficult preparation and inability to effectively improve the immunogenicity of a weak antigen. Epimedium-Propolis adjuvant (EPA) is a combination of flavonoids from the Chinese herbal medicine, Epimedium, combined with extracts from propolis. It has been shown to be an effective immune enhancing agent in both clinical applications and experimental studies. In a previous experimental study conducted by the authors, it was demonstrated that EPA enhanced the activity of natural killer (NK) cells in peripheral blood, increased the phagocytic activity of macrophages and increased concentrations of cAMP and cGMP in plasma of chickens and mice.

The objective of this randomized, controlled study was to add to this body of knowledge by determining in 4 related experiments the effects of EPA on T lymphocyte transformation rates (SI) in 4 and 8 week-old mice and 3 day-old chicks when given EPA alone or with the immunosuppressive agent cyclophosphamide (Cy). In addition, antibody levels were measured in viral and
bacterial challenged rabbits and hens, respectively, with and without administration of EPA. The hypothesis of the study was that EPA would stimulate T lymphocyte transformation rates and perform as an effective adjuvant even in the face of an immunosuppressive agent and that it would have its greatest effect on young animals with an immature immune system.

MATERIALS AND METHODS

The experimental mice, chicks, hens and rabbits used in this study were housed, fed and watered conventionally. All care and experimental procedures were performed in accordance with the guidelines of the Chinese Council for Animal Care. In Experiment 1, twenty-four healthy mice (4-weeks-old) were randomly divided into 4 groups (n=6): Group 1 was dosed with 25 mg/kg (Cy), Group 2 dosed with 0.2 ml physiological saline (PS), Group 3 dosed with 0.2 ml EPA (flavones:20 mg/ml) + 25 mg/kg Cy, or Group 4 with 0.2 ml EPA. Mice were injected subcutaneously once every 48 hours (hrs) in the lumbar muscles for a total of three injections. Forty-eight hrs after the last injection, an orbital blood sample (heparin anticoagulation) was collected. T lymphocyte transformation rate was determined by micro-whole blood culture ³H-TdR (tritiated thymide) incorporation assay.³⁷ The results are recorded as Stimulation index (SI). The formula to calculate this number was as follows: 

\[ SI = \frac{PHA+ CPM \text{ (mean of 3 tubes)}}{PHA- CPM \text{ (mean of 3 tubes)}} \]

The same experiment was also conducted on 8-week-old mice (Table 1).

In Experiment II, three hundred sixty healthy Roman male chicks (3-days-old) were randomly divided into 3 groups. Chicks in Group 1 were injected with 0.2 ml PS, chicks in Group 2 were injected with 0.2 ml EPA, while chicks in Group 3 were given 0.4 ml EPA. Heart blood was collected from each group at 7, 21, 35 and 49 days (Table 2). T lymphocyte transformation rate was determined as described in Experiment I with the following exception: in Experiments I and II, the concentration of fetal calf serum (FCS) and phytohemagglutinin (PHA) had different results with CPM (RPMI 1640), so the concentration of FCS and PHA that would stimulate CPM at the highest level was selected as a culture and stimulation condition. Therefore, in Experiment I, 10% FCS and 0.02% PHA was selected; while in Experiment II, 2.5% FCS and 10% PHA was selected.

Table 1: Experimental Groups and Test Material Dose; Experiment I

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>Dose</td>
<td>Cy (25 mg)</td>
<td>PS (0.2mL)</td>
<td>EPA (0.2mL)+Cy (25 mg/kg/BW)</td>
<td>EPA (0.2 mL)</td>
</tr>
<tr>
<td>N=</td>
<td>6</td>
<td>6</td>
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<td>6</td>
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</table>

Twenty-four healthy mice (4-weeks-old) were randomly divided into 4 groups. Group 1 was dosed with 25 mg/kg (Cy), Group 2 dosed with 0.2 ml physiological saline (PS), Group 3 dosed with 0.2 ml EPA + 25 mg/kg Cy, or Group 4 with 0.2 ml EPA. Mice were injected subcutaneously once every 48 hours (hrs) in the lumbar muscles for a total of three injections. Forty-eight hrs after the last injection, a blood sample (heparin anticoagulation) was collected. The experiment was repeated in 8-week-old mice.

Table 2: Experimental Groups and Test Material Dose; Experiment II

<table>
<thead>
<tr>
<th>Group</th>
<th>I</th>
<th>II</th>
<th>III</th>
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</thead>
<tbody>
<tr>
<td>Dose</td>
<td>PS (0.2mL)</td>
<td>EPA (0.2 mL)</td>
<td>EPA (0.4 mL)</td>
</tr>
<tr>
<td>Blood Collected</td>
<td>Days 7, 21, 35, 49</td>
<td>Days 7, 21, 35, 49</td>
<td>Days 7, 21, 35, 49</td>
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In Experiment II, three hundred sixty healthy Roman male chicks (3-days-old) were randomly divided into 3 groups. Chicks in Group 1 were injected with 0.2 ml PS, chicks in Group 2 were injected with 0.2 ml EPA, while chicks in Group 3 were given 0.4 ml EPA. Heart blood was collected (n=8) from each group at 7, 21, 35 and 49 days.

Table 3: Experimental Groups and Test Material Dose; Experiment III

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<tr>
<th>Group</th>
<th>A</th>
<th>B</th>
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<tbody>
<tr>
<td>Dose</td>
<td>EPA-ICHV (1 mL)</td>
<td>ICHV (1 mL)</td>
</tr>
<tr>
<td>N=</td>
<td>4</td>
<td>4</td>
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</tbody>
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Eight adult Chinchilla rabbits were divided into two groups: the experimental group with EPA adjuvant plus Infectious Canine Hepatitis Virus (Group A) and the control group given ICHV without adjuvant (Group B). Rabbits in the 2 groups were subcutaneously injected in the neck with 1 ml of EPA-ICHV or ICHV respectively, once per week repeated 4 times. Ear vein blood was collected on the 7th day after the first vaccination and then repeated every 2 weeks for a total of 5 collections.
In Experiment III, eight adult Chinchilla rabbits were divided into two groups: the experimental group with EPA adjuvant plus Infectious Canine Hepatitis Virus (Group A) and the control group without adjuvant (Group B). Rabbits in the 2 groups were subcutaneously injected in the neck with 1 ml of EPA-ICHV or ICHV respectively, once per week repeated 4 times (Table 3). Ear vein blood was collected on the 7th day after the first vaccination and then repeated every 2 weeks for a total of 5 collections. Serum ICHV antibody OD490 was measured in each sample with indirect ELISA method. 

In Experiment IV, six healthy Roman hens which had just started to lay eggs were divided into two groups: EPA group and Freund’s adjuvant (FA) group (Table 4). Two ml of EPA + Xanthomonas oryzae pv. oryzae (XOO) was injected into the pectoralis muscle in the EPA group and 2 ml of FA + XOO was injected into the pectoralis muscle in the Freund’s Group. Xanthomonas oryzae pv. oryzae (XOO) is a bacterium which is associated with rice blight. A second injection was given to each group in the 10th week (when the antibody titer had decreased). In the FA group, Freund’s complete adjuvant was given the first time, and Freund’s incomplete adjuvant (does not contain cell wall of Mycobacterium tuberculosis to enhance immunization) was given at the second time. After the first vaccination, the effects of the two agents on the diet, life quality and egg laying of hens were observed daily. The egg yolk XOO antibody titer was determined by a double diffusion method, determined once daily at the start of egg laying by hens. Once the antibody was at measurable levels, the detection was carried out every 3 days for three months.

Statistical significance for all 4 experiments in this study was determined by using a T-test. This simple statistical method was used since comparison was aimed

| Table 4: Experimental Groups and Test Material Dose; Experiment IV |
|-----------------|-----------------|
| **Group**      | **EPA-XOO**     | **FA-XOO**     |
| Dose           | EPA-XOO (2 mL)  | FA-XOO (2 mL)  |
| N=             | 3               | 3              |

Six healthy Roman hens were divided into two groups: EPA group or Freund’s adjuvant (FA) group. Two ml of EPA + Xanthomonas oryzae pv. oryzae (XOO) or 2 ml of FA + XOO was injected into the pectoralis muscle. A second injection was given to each group in the 10th week (when the antibody titer had decreased).

| Table 5: Effect of EPA on T lymphocyte transformation rate (SI) in mice; Experiment I |
|-----------------|-----------------|-----------------|-----------------|
| **Group**       | **4-Week-old**  | **8-Week-old**  |
| 1 (Cy)          | 1.08±0.04       | 1.14±0.96       |
| 2 (PS)          | 1.32±0.05**     | 1.30±0.19       |
| 3 (EPA+Cy)      | 1.24±0.08*      | 1.24±0.05       |
| 4 (EPA)         | 1.72±0.68**     | 1.40±0.10*      |

*P<0.05, **P<0.01; mice of same age compared to control

In 4-wk old mice, SI was greatest in Group 4 (EPA), while Group 1 (Cy) had the lowest value. The level of SI in Group 4 was very significantly (P<0.01) higher than the other 3 groups, while the SI level in Group 1 was very significantly (P<0.01) lower than the other 3 groups. These results indicate that EPA adjuvant could significantly enhance T lymphocyte transformation rate while Cy could significantly inhibit it in 4-week-old mice. EPA adjuvant has an enhancing effect on the transformation of T lymphocytes in 8-week-old mice and can antagonize the immunosuppression of Cy but the effect is weaker in the older mice.

| Table 6: Effect of EPA on T lymphocyte transformation rate (SI) in chicks; Experiment II |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Group**       | **7-day-old**   | **21-day-old**  | **35-day-old**  | **49-day-old**  |
| 1 (PS)          | 3.68±0.86       | 4.94±2.13       | 5.40±1.91       | 6.64±3.20       |
| 2 (EPA 0.2ml)   | 4.23±0.68       | 10.35±5.09**    | 9.20±4.28*      | 8.10±3.95       |
| 3 (EPA 0.4ml)   | 5.30±1.57       | 13.29±5.98**    | 11.34±3.34**    | 7.16±3.38       |

*P<0.05, **P<0.01; chicks of same age compared to control

Group 3 (0.4ml EPA) had the highest SI (Day 7, 21, 35), followed by Group 2 (0.2ml EPA), and Group 1 (saline) with the lowest values. There was no significant difference among the 3 groups (P>0.05) at the age of 7 days and 49 days, while at the age of 21 days and 35 days, SI in Groups 2 and 3 were significant or very significantly higher (P<0.05 or P<0.01) than that in Group 1.
RESULTS

The lymphocyte transformation rate (SI) in 4-week-old mice is shown in Table 5. SI was greatest in Group 4 (EPA), while Group 1 (Cy) had the lowest SI. The level of SI in Group 4 was very significantly (P<0.01) higher than the other 3 groups, while the SI level in Group 1 was very significantly (P<0.01) lower than the other 3 groups. These results indicate that EPA adjuvant could significantly enhance T lymphocyte transformation rate of 4-week-old mice, and Cy could significantly inhibit T lymphocyte transformation rate of 4-week-old mice. The level of SI in Group 3 (EPA+Cy) was also significantly (P<0.05) higher than Group 1, and its value was close to that of Group 2 (saline). There was no significant difference between Groups 2 and 3 (P>0.05), which indicates that EPA adjuvant can effectively antagonize the immune suppressing effect of Cy on T lymphocyte transformation rate of 4-week-old mice and return T lymphocyte activity to normal level (Table 5).

For 8-week-old mice, the SI values in each group were consistent with those for the 4-week-old mice. There was a difference between Groups 1 (Cy) and 4 (EPA) (P<0.05) but no significant difference among the other groups (P>0.05). This indicated that although EPA adjuvant has an enhancing effect on the transformation of T lymphocytes in 8-week-old mice and can antagonize the immunosuppression of Cy, it is a weaker effect in the older mice (Table 5).

Results of Experiment II showed that Group 3 chicks (0.4ml EPA) had the highest SI, followed by Group 2 (0.2ml EPA), and Group 1 (saline) with the lowest values. There was no significant difference among the 3 groups (P>0.05) at the age of 7 days and 49 days, while at the age of 21 days and 35 days, SI in Groups 2 and 3 were significant or very significantly higher (P<0.05 or P<0.01) than that in Group 1 (Table 6).

Experiment III which evaluated the effect of EPA on ICHV titers as depicted in Figure 1, show that OD490 values in rabbit serum of Group A (experimental group with adjuvant) were higher than those of Group B (control group) without adjuvant. Significant (P<0.05) or a very significant difference (P<0.01) was observed for all days except the 7th day after the first vaccination. For Group B, the antibody level increased slightly after vaccination, and reached its peak at the 21st day and then decreased rapidly, with the 35th day containing antibody levels close to that of the 7th day. For Group A, the antibody level increased significantly after vaccination and continued to rise at the 21st day. It peaked at the 28th day, then decreased slowly through the 35th day, although, the antibody titer was still significantly higher than at 7th day (P<0.01).

In experiment IV, after the first injection, there were no significant adverse effects on hens injected with EPA. The hens had normal appetites by the 2nd day following injection and started egg laying 1 week later. The hens in the FA group had severe adverse effects during the first 4 days after injection demonstrated by loss of appetite, poor health for 1 week and a delay of egg laying for 2 weeks. After the second injection, the effects of the two different adjuvants on health and egg laying of the hens were similar to the effects observed after the first injection (FA compromised health of hens) with slightly less severity.

For both adjuvants, similar increasing antibody titers were detected 1 week after inoculation with a high titer level (1:64) still present 2 weeks post-inoculation. This high titer remained for at least two months with the second injection boosting titer levels again to 1:256 at two weeks post-inoculation (Figure 2).

DISCUSSION

T lymphocytes are the primary cell involved in cellular immune function. There are several subgroups, which jointly constitute the immune regulatory network.9 The T lymphocyte transformation rate reflects the functional activation of T cells directly. Results from Experiment I showed that EPA adjuvant can significantly improve the transformation rate of T lymphocytes in mice at the age of 4 weeks even when exposed to the deleterious effects of an immunosuppressive agent such as cyclophosphamide. Experiment II demonstrated similar effects in that EPA adjuvant significantly improved the T lymphocyte transformation rate in chicks at ages 21 and 35 days. In addition, Experiments I and II also demonstrated that the intensity of the EPA adjuvant effect was both related to the dose and the age of the animals. The effect of the EPA adjuvant in 0.4ml/bird was greater than that in 0.2ml/bird, and its effect on 4-week-old mice and 21 and 35 day-old chicks was more intense than on 8-week-old mice and 49-day-old chicks (Tables 5 and 6).

More mature animals have completed development of their immune organs while the immune organs and immune function in young animals is still immature. This juvenile immune system allows the EPA adjuvant to have a more significant positive effect on young animals as an immune enhancer alone or together with an antigen. With enhanced function, young animals can improve their early immune response ability and resistance to infectious disease. A further consideration is the use of EPA adjuvant in sick animals. It is expected that it would enable an animal that is suffering from a poorly functioning immune response the ability to more effectively confront infectious agents.

The use of EPA also significantly enhances the humoral immune function. The results from Experiment III demonstrated that the addition of EPA in a vaccine administered to study animals can significantly improve the level of serum ICHV antibody production and can maintain a higher antibody titer for a longer time (Figure
Group A titers (EPA adjuvant group) were higher than those of Group B (control group). Significant ($P<0.05$) or a very significant difference ($P<0.01$) was observed for all days except the 7th day after the first vaccination. For Group B, the antibody level increased slightly after vaccination, and reached its peak at the 21st day and then decreased rapidly, with the 35th day containing antibody levels close to that of the 7th day. For Group A, the antibody level increased significantly after vaccination and continued to rise at the 21st day. It peaked at the 28th day, then decreased slowly through the 35th day, although, the antibody titer was still significantly higher than at 7th day ($P<0.01$).

For both adjuvants, similar increasing antibody titers were detected 1 week after inoculation with a high titer level (1:64) still present 2 weeks post-inoculation. This high titer remained for at least two months with the second injection boosting titer levels again to 1:256 at two weeks post-inoculation. Results showed that EPA stimulated antibody production levels similar to those of Freund’s adjuvant, without the adverse side effects.
1). In Experiment IV, the EPA adjuvant and Freund’s adjuvant had similar egg yolk XOO antibody titers. Differences between the 2 adjuvants, however, occurred when technical production difficulty of the adjuvant and adverse health effects were examined. There is a greater delay in egg laying with FA along with greater adverse effects on life quality. In addition, the EPA adjuvant is technically simpler and faster to produce. It is an aqueous solution, which is more readily mixed with the antigen and does not have a long emulsion time like FA (0.5-1 hrs) which is associated with greater loss of the antigen. Finally Freund’s adjuvant vaccine has a higher viscosity compared to EPA, making it difficult to use for subcutaneous skin injections.

In conclusion, the EPA adjuvant improves both cellular and humoral immune functions. The hypothesis of the study was proven through demonstration of enhanced immune function through increasing T lymphocyte transformation rate even when animals were exposed to cyclophosphamide. In addition, the EPA adjuvant was associated with prolonged increases of serum antibody levels in rabbits exposed to an infectious agent and also stimulated antibody production levels similar to those of Freund’s adjuvant, without the adverse side effects. The experiments conducted in this study add to the body of research demonstrating the benefit of using Epimedium-Propolis as an adjuvant in vaccines for animals.

**FOOTNOTES**

a. Shanghai Hualian pharmaceutical company  
b. Nanjing General Hospital of Nanjing Military Command Experimental Animal Center  
c. Institute of Traditional Chinese Veterinary Medicine, College of Veterinary Medicine, Nanjing Agricultural University. It contains 1% Epimedium total flavone and 10% Propolis  
d. A state-owned chick farm  
e. Gibco products, United States  
f. Culture media developed by Roswell Park Memorial Institute; Gibco products, United States  
g. Provided by the Nanjing Police Dog Institute  
h. Gibco products, United States, including the complete adjuvant and incomplete adjuvant  
i. Provided by the Department of Plant Pathology

**REFERENCES**


