

Does Astragalus Membranaceus Polysaccharides in Astragalus Mongholicus as One of Six Herbs in Bushen Formula Can Be Effective to Decrease the Hepatitis B Viral Load and Alanine Aminotransferase of CHB Patients?

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Abstract

Hepatitis B was the most widespread viral disease and had a very high infection in China during the last 40 years of the 20th to 21st century. The traditional Chinese medicine Bushen formula can treat hepatitis B by lower the mildly elevated alanine aminotransferase and HBV DNA levels for patients. Previous research has shown that the organic compound Astragalus polysaccharide can increase the percentage of CD4+ and CD8+ lymphocyte subsets and reduce hepatic damage caused by chronic hepatitis B. This study investigates APS in treating mice's hepatic injury by building a CCl4-induced liver failure model and CD4+ CD8+ lymphocyte subsets in peripheral blood analysis models.

Keywords: Astragalus membranaceus polysaccharides, (APS), Hepatitis B, Hepatitis B Virus (HBV), Carbon tetrachloride (CCl4) induced-liver failure models, hydrodynamic injection, Ziziphus jujube fruit (FZJ)

1. Introduction

Hepatitis B, which is caused by HBV (hepatitis B virus) was first discovered in 1966. chronic liver disease, such as chronic hepatitis has widely infected more than 350 million people since the twenty-first century [2]. In some regions that have more developing countries, such as Africa and Southeast Asia, the prevalence is extremely high, with more than fifty per cent of the population getting infected by HBV (hepatitis B virus) [2].

In China, traditional Chinese medicine has been widely used for many decades to treat hepatitis B virus patients. One of the TCM Bushen formulas, composed of six herbs, had been proven to show positive effects on activating the CD4+CD25+ Tregs [5] and expression of Foxp3 in peripheral blood, so CD4+ and CD8+ cells can be more effectively activated and it also successfully decrease the mildly elevated alanine aminotransferase (ALT) and HBV, which are two important indicators of the hepatitis B, in blood serum of patients' body. Also, the result can be repeat CD4+ Tells which are stimulated by hepatitis B core antigen (HBcAg), which is essential for triggering a cellular response to kill infected cells as its derived peptides are expressed on the cell surface [1][2]. However, a single type of herb formula has not been shown an exact role in curing hepatitis B. Due to the uncertainty of the Bushen formula, exactly which components have the most potent immunomodulatory effect is still questionable.

As the Bushen formula is composed of six different

herbs: *Astragalus mongholicus*, *Fructus Ligustri Lucidi*, *Longspur epimedium*, *ClawVine*, *Rhizoma Picrorhizae*, and *Pericarpium citri reticulatae viride*. The *Astragalus mongholicus* get one type of effective compound from its roots, called Astragalus membranaceus polysaccharides (APS) shown to have the biological ability to increase the count of macrophage [4] through activating the nuclear factor- κ B/Rel (NF- κ B/Rel), then subsequently, the granulocyte-macrophage colony-stimulating factor(GM-CSF), nitrogen monoxide, and inducible NO synthase. Since now there are twenty-four different types of A. membranaceus polysaccharides found from the root extract of A. membranaceus and twenty of them have shown different pharmacological properties of antioxidant, antitumor, immunomodulation, antiviral and anti-inflammation, etc. [5]. In those different types of A. membranaceus polysaccharides, the type 10 and type 11 APS show pharmacological functions of antiviral and immunomodulation. It can show exactly the APS (types 10 and 11) can provide a new therapeutic modality in HBV treatment.

T-lymphocytes have vital effects on immunomodulatory mechanisms And the A. membranaceus polysaccharides have an effect on suppressing the CD4+CD25+ Tregs effects and activating CD4+ cells to transfer more Th2 from Th1, showing a possible way to boost adaptive immunity as it can induce the activation and differentiation of DCs.[5] Also, it shows can promote lymphocyte

proliferation and promote the IFN-gamma, IL-2, IL-4 and IL-10 secretion in chicken with the Newcastle vaccine. [5]. After applying six groups of diets supplemented with A. membranaceous polysaccharides, analysing with peripheral leukocyte counts and CD4+CD8+ lymphocyte subsets analysis and lymphocyte proliferation assay, shows an increasing trend of white blood cells and lymphocytes as the amount of dietary APS increases. Also, the percentage of CD4+ lymphocyte subsets in peripheral blood has increased [7], which offers a potential pathway for adjusting immunomodulatory mechanisms in the human body.

The A. membranaceous polysaccharides are expected to have some immunomodulatory effects on the treatment of hepatitis B, by decreasing the mildly elevated alanine aminotransferase (ALT) and HBV. It has been shown that after the active viral replication of HBV DNA in serum. In the following stages of hepatitis B infection, the HBV DNA level slightly decreases but the antibody to HBcAg is positive. However, the alanine aminotransferase (ALT) level has elevated mildly.[2] In this stage. This is corresponding with the treatment time that the claimed effect of the Bushen formula to decrease the ALT level. That might show a potential stage that could maximise the effectiveness of A. membranaceous polysaccharides to decrease the ALT level by promoting the effect of CD4+ and CD8+ T cells.

So that, in order to show the potential effects of APS on decreasing the HBV Viral DNA loading, and activating CD4+ and CD8+ T cells and Treg. This paper investigates the effect of setting different doses of APS on experimental animals (mice) to demonstrate the potential effectiveness of the pathway of APS, with different doses in the human body.

Hypothesis predict that one of the compound called *Astragalus polysaccharides* (APS) in herb *Astragalus Mongholicus*, an ingredient in TCM Bushen formula, is an

effective component to reduce alanine aminotransferase and hepatitis B virus DNA levels in patients diagnosed with hepatitis B, which can be demonstrated by establishing liver failure models based on using CCl4 (Carbon tetrachloride) and mice hepatic B infection and the cell subsets analysis of peripheral leukocytes counts and CD4+ and CD8+ lymphocytes. The treatments can be applied on clinical samples as well.

2. Method and material

2.1 Experiment 1: The establishment of hepatic B injury models and the grouped treatments

The establishment of hepatic results are given with injection of CCl4 solution to damage the hepatic function of rats, then divided into 3 groups to investigated different possible results given with different concentration of APS in the rats' dietary supplement. All the rats, except the normal group, were given with simultaneously injection with CCl4 in olive oil at a dose of 6 mL/kg at the beginning of the experiments. Then at the following stages, at every half week for 8 weeks. During that time, all mice were fed simultaneously with isovolumetric amount of dietary supplement with high fat content and low protein content (81.5 % of cornmeal, 18 % of fat and 0.5% cholesterol) [2]. The rats in normal group and control group were fed with normal dietary supplement including wheats and rice, and water. The six groups of mice are given simultaneously with different treatments, which are shown on the graph. with 1.0 ml/300g, with 100mg/kg, 150mg/kg, and 250 mg/kg of APS and in their dietary supplements, and also, and the all those different groups of treatment, including one normal group, one control group, three different groups of *Astragalus Polysaccharides* (APS), and one group of *Zizyphus jujube fruit* (FZJ) once a day. Simultaneously, set the injection of CCl4 once a day for 8 weeks. After the 6 weeks all the rats were euthanized.

**Table 1 Different treatment Used to build the CCl4-induced hepatic injury mouse model
Sample collection (ALT analysis of blood sample)**

Groups	Gavage	Dosage
Normal	Distilled water	1.0 mL/300g [1] (no CCl4 injection, fed with high fat and low protein before)
Negative Control	Distilled water	1.0 mL/300g [1] (have CCl4 injection, fed with high fat and low protein before)
Low dose of <i>Astragalus Polysaccharides</i> (APS)	5% APS solution	1.0 mL/300g (100mg/kg) [1]
Medium dose of <i>Astragalus Polysaccharides</i> (APS)	5% APS solution	1.0 mL/300g (200mg/kg) [1]

Groups	Gavage	Dosage
High dose <i>Astragalus Polysaccharides (APS)</i>	5% APS solution	1.0 mL/300g (300mg/kg) [1]
Positive Control: <i>Zizyphus jujube fruit (FZJ)</i>	70% ethanol extract of FZJ	1.0 mL/300g (7.5mg/kg) [5]

All rats except the dead, would be anaesthetized with urethane which had a dose of 5mL/kg, abdominal injection, and blood sample of 5mL will be gotten from its hepatic veins of each mice in six groups. Then, the blood samples from different mice would be configured at a rate of 5000r/minute for 15 minutes at 2 Celsius degrees, all samples being kept at 20 Celsius degrees in the freezer. All samples are used for following steps. The serum sample are used to detect a set of parameters, such as the alanine aminotransferase (ALT) by using the Hitachi 7150 automatic biochemistry analyzer. (Hitachi, Japan) [6]. Histological examination of CCl4-induced liver damages of mice: The hepatic tissues of six groups different mice include liver and spleen were rinsed with phosphate buffered saline solution (PBS), and then, dissect into 6 different portions with the same size of 8 micrometer. Then, choose one of each of six groups of mice to be fixed in 5% paraformadehadye. Then, the method of hemotoxylin & eosin (H&E) and Van Geison's stain (VG) are used to stain those sections [9]. The content of collagen standard of condition of liver detected by Van Geison's stain method is referred to the standard set by China Medical Association in 2001 [1], which will be classified into 5 degrees: 0, normal(no fibrous tissue); 1+, fibrosis present (collagen has extended form central vein to peripheral region of liver section); 2+, mild fibrosis (having more extended collagen but without compartmental formation); 3+ (moderate amounts of collagen fibres present with some pseudolobe formation) and the most serious type: 4+ (the majority of fibres presents with a thickening of the partial compartments and frequent pseudolobe formation, totally lose hepatic function).

2.2 Experiment 2: the cell subsets analysis of peripheral leukocytes counts and CD⁴⁺ and CD⁸⁺ lymphocytes

The flow cytometry was used to analyzed the proportion of CD4+ and CD8+ T lymphocytes in all these six groups. CD4+ and CD8+ lymphocyte subsets in the blood of mice treat with different treatments were analyzed by three colors flow cytometry. Before that another six groups of mice are used to infected with the HBV virus by setting up the mice infection model. The hydrodynamic transfection method as a widely used way to establish the infection model of mice. The plasmid which has entire HBV DNA will be diluted into the buffer Phosphate-buffered saline (PBS) and have rapidly tail intravenous injection (IOCV), so that the pressure plasmids made will be transfected into the hepatic cell of mice [7]. Based on that traditional method, the HBsAg viral reading frame was inserted into pAAV-HAV1.2 plasmid by point mutation technique based on the existing high pressure hydrodynamic transfection method of pAAV-HBV1.2 plasmid. The six groups of mice all infected with HBV in this method and set one group of mice as control group. Each group of mice are fed with this treatment once a day for three weeks. After three weeks, all rats are anaesthetized with 5mL/urethane and taking its peripheral 1mL blood sample from the vena cava vein of mice for determining the percentage of subset of CD4+ and CD8+ in the blood sample, because the HBsAg can induce the myeloid-derived repressor cells (MDSCs) and regulatory T-cells, which will then repress the activation of CD4+ and CD8+ cell in hepatitis B patient's body. Therefore, the percentage of subsets of CD4+ and CD8+ cells can be used as an indicator of the activity of Hepatitis B virus in mice's body. [7].

Table 2: Different treatments used to build the hepatitis B infection and treatment mouse model

Groups	Gavage	Dosage
Normal HBV affected group (injected pAAV-HAV1.2 plasmid)	Distilled water	1.0 mL/300g [1]
<i>Astragalus Polysaccharides (APS)</i>	5% APS solution	1.0 mL/300g (100mg/kg)[1]
<i>Astragalus Polysaccharides (APS)</i>	5% APS solution	1.0 mL/300g (200mg/kg)[1]
<i>Astragalus Polysaccharides (APS)</i>	5% APS solution	1.0 mL/300g (250mg/kg)[1]
Positive Control <i>Zizyphus jujube fruit (FZJ)</i>	70% ethanol extract of FZJ	1.0 mL/300g (7.5mg/kg) [5]

As a result, the mutant plasmid of HBsAg-deficient hepatitis B mouse model will be established. Then with the same treatment as the CCl4-induced hepatic injury model, using six groups of mice to give different sets of three groups of dosages of APS, and one group of FZJ, and one control group. With comparing in order the blood samples are washed with PBS for three times and it take 250 µL of blood samples in six different tubes. Then, each of it will be added into an Eppendorf tube (Hamburg, Germany) along with a kind of diluted primary antibodies mouse IgG2b-FITC (Sigma, USA) which is used for anti-CD4 and the secondary antibodies mouse IgG2a-Quantum Red (Sigma, USA), that is applied to anti-CD8. Incubating in the incubator for one hour at room temperature. After that, the tubes for twice with PBS (1:20) solution used to dilute the 400ml of hemolysin solution. The last stage is to wash the cells for twice and brought to a final volume of 500ml. Two-color flow cytometric analysis was conducted with a Coulter XL (Beckman Coulter Corp., USA) [6].

3. Statistical Analysis

The statistical significance of all these various of data collected from building CCl4-induced liver failure model, the hepatitis B infection and treatment mouse model, flow cytometry used to analyze the CD4+ and CD8+ lymphocyte subsets, and the liver samples' images of mice used for histological examinations of fibrosis in liver, all of the methods above use the student's T-Test on GraphPad Prism® at (p <0.05). The analysis of ALT in blood samples of mice uses Hitachi 7150 automatic biochemistry analyzer (Hitachi Japan)

4. Results

4.1 Possible results for experiment 1: ALT levels and hepatic damage (hepatic fibrosis level examined by standard formula of Chinese Medical Association in 2001, and these levels of hepatic damage are recorded in Table 3)

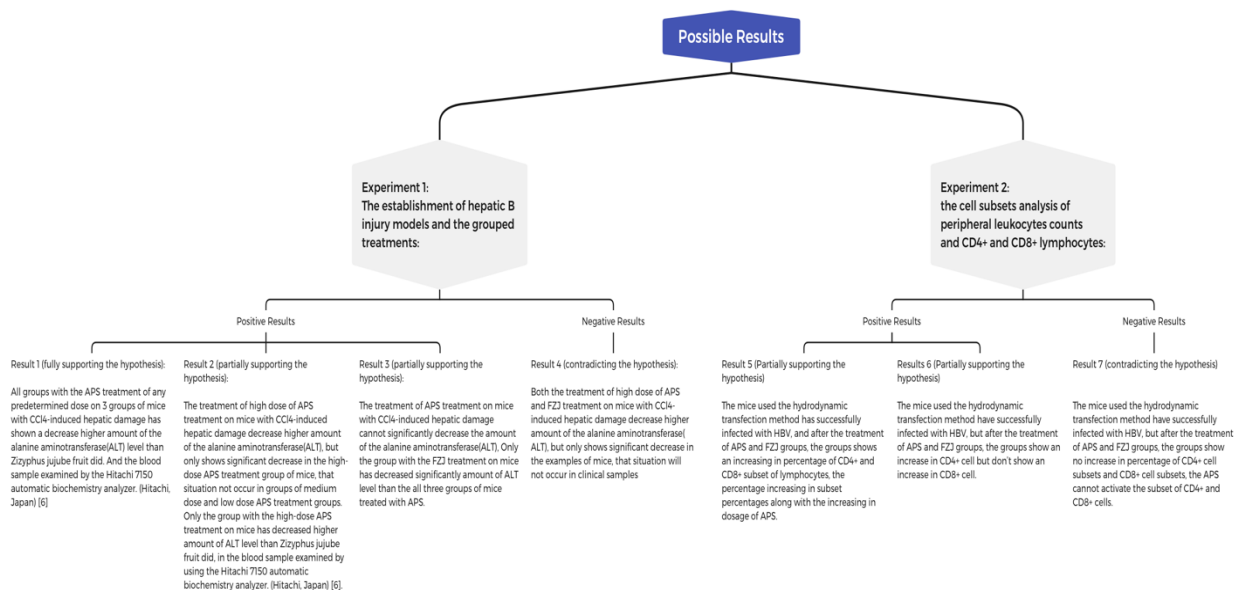


Figure 1 the possible results of experiment 1 and 2.

Possible Result 1: All groups with the APS treatment of any predetermined dose on 3 groups of mice with CCl4-induced hepatic damage has shown a decrease higher amount of the alanine aminotransferase (ALT) level than Zizyphus jujube fruit did. And the blood sample examined by the Hitachi 7150 automatic biochemistry analyzer. (Hitachi, Japan) [6].

In the any dosage of *Astragalus Polysaccharides* (APS) treatment, APS shows a significant lower alanine aminotransferase (ALT) level in the blood sample, the another positive result, which is the group treated with

Zizyphus jujube fruit (FZJ) (1.0 mL/300g (7.5mg/kg) also shows a certain decrease of ALT level in all six groups of mice, but it is still lower than the highest dose of APS treatment. Also, the liver section of mice treat with APS, comparing to control group, which will have the most serious damage situation of liver section (4+) [1], will shows have relatively less detriments, as an level 1+ in high dosage, 2+ in medium and low dosage, but the group used with FZJ will have relatively higher level of liver damage, which has more abundant amount of collagen fibres and some formation of pseudolobe (3+).

Possible Result 2: The treatment of high dose of APS treatment on mice with CCl₄-induced hepatic damage decrease higher amount of the alanine aminotransferase(ALT), but only shows significant decrease in the high-dose APS treatment group of mice, that situation not occur in groups of medium dose and low dose APS treatment groups. Only the group with the high-dose APS treatment on mice has decreased higher amount of ALT level than *Zizyphus jujube fruit* did, in the blood sample examined by using the Hitachi 7150 automatic biochemistry analyzer. (Hitachi, Japan) [6].

In the high dosage of *Astragalus Polysaccharides* (APS) treatment, APS shows a significant lower alanine aminotransferase (ALT) level in the blood sample Like the possible result 1 the group of mice treated with FZJ also shows a certain decrease of ALT level in all six groups of mice, but it is still lower than the highest dose of APS treatment. But, only the liver section from mice in high dosage treatment group, comparing to control group, which will have the most serious damage situation of liver section (4+) [1], will shows have relatively less detriments, as a level 1+. FZJ will have relatively higher level of liver damage (3+), which has more abundant amount of collagen fibres and some formation of pseudolobe [1].

Possible Result 3: The treatment of APS treatment on mice with CCl₄-induced hepatic damage cannot significantly decrease the amount of the alanine aminotransferase(ALT), Only the group with the FZJ treatment on mice has decreased significantly amount of ALT level than the all three groups of mice treated with APS. The dosage of any groups of mice cannot get significantly decrease in the ALT level in the blood sample, except the FZJ, which has been proved to have anti-oxidative effect of hepatic injury function[5]. The situation shows that APS might have potential to lower the hepatic damage, but it cannot help to take away most of the fibrosis of liver.

Possible result 4: Both the treatment of high dose of APS and FZJ treatment on mice with CCl₄-induced hepatic damage decrease higher amount of the alanine aminotransferase(ALT), but only shows significant decrease in the examples of mice, that situation will not occur in clinical samples. That example shows the contrast between clinical infection period might be shorter than four weeks or as long as 10 years in real example of liver damage caused by hepatotoxic substances and virus (HBV) [2] and the APC might only use to reduce the fibrous proliferation caused by chronic hepatitis B in specific development stage of virus.

Possible results for experiment 2: the cell subsets analysis of peripheral leukocytes counts and CD4⁺ and CD8⁺

lymphocytes;

Possible Result 5: The mice used the hydrodynamic transfection method has successfully infected with HBV, and after the treatment of APS and FZJ groups, the groups show an increasing in percentage of CD4⁺ and CD8⁺ subset of lymphocytes, the percentage increasing in subset percentages along with the increasing in dosage of APS. That result shows the APS have the ability to activate the CD4⁺ and CD8⁺ and let them have more active cellular immunity. It has shown that the activated CD4⁺ can derive to T-helper cell 1 and T-helper cell 2. And The T-helper cell 2 can secrete cytokines such as IL-4 and IL-10 to regulate humoral immunity and B-cell proliferation. Possible Result 6: The mice used the hydrodynamic transfection method have successfully infected with HBV, but after the treatment of APS and FZJ groups, the groups show an increase in CD4⁺ cell but don't show an increase in CD8⁺ cell. The result shows the possible of APS increasing the amount of CD4⁺ in the mice which has infected with HBV by using the hydrodynamic transfection method but the APS cannot significantly increase the proliferation and expression of CD8⁺ cells, in this situation, the cell subsets analysis of peripheral leukocytes counts and CD4⁺ and CD8⁺ lymphocytes will not be successful.

Possible result 7: The mice used the hydrodynamic transfection method have successfully infected with HBV, but after the treatment of APS and FZJ groups, the groups show no increase in percentage of CD4⁺ cell subsets and CD8⁺ cell subsets, the APS cannot activate the subset of CD4⁺ and CD8⁺ cells. That result shows the APS and FZJ cannot improve the proliferation of CD4⁺ and CD8⁺, and that might show APS and FZJ has no positive effect on activate the expression of CD4⁺ and CD8⁺ cells

5. Discussion

In the previous studying, the traditional Chinese medicine Bushen formula can effectively decrease the mildly elevated alanine aminotransferase (ALT) and HBV DNA expression in the blood sample of patients. In order to studying further about exactly one of the component *Astragalus polysaccharides* (APS) in *Astragalus Mongholicus*, this studying use two sets of experiments to investigate the potential of *Astragalus polysaccharides* (APS)'s effects on lowering the mildly elevated ALT and activate the subset of CD4⁺ and CD8⁺ lymphocytes in the immune system to boost active immunity, with a positive comparison of *Zizyphus jujube fruit* (FZJ), which has been proved to be effective to provide protective effect to carbon tetrachloride-induced (CCl₄-induced) hepatic injury [5].

The possible result 1, 2, 3 have the consistent result of how APS protect the liver from CCl₄ induced hepatic damage, these are positive results, to prevent more damage to liver from hepatotoxic materials, like CCl₄ solution. Possible result 1 is fully supporting the hypothesis by the increasingly using dosage of higher concentration of APS in dietary supplement of the three groups of mice, can reduce the hepatic damage caused by CCl₄ solution injection. The possible results of 2 and 3 are both partially support the hypothesis. They show the potential of experimental difference of setting difference by setting different concentration of solution. As a result, the CCl₄-induced hepatic injury can be fixed in different certain, within the level between 1+ to 3+ comparing to model group's liver section (4+, the most serious damaged level caused by CCl₄, highest proportion of fibrosis and pseudo lobe) The But some of concentration of APS at certain high levels has the possibility of acting as an anti-nutritional factor in experimental animals. [6], that could also be potential for hepatitis B patients to be suffered from malnutrition if the APS is used as an effective treatment method. It might affect the effects of APS treatments. So before the transition to clinical testing on patients, the more representative experimental animals should be used to do more complex experiments, such as finding the optimal dosage of the concentration of APS and to the maximum extent to minimize the side effects such as malnutrition.

The possible result 4 and 7 both show the contradiction to the hypothesis of applying APS treatment for hepatic injury and decreasing the concentration of HBV DNA, increasing the expression of subset CD4⁺ and CD8⁺ cells. This main contrast is caused by the time lag of APS having effect on model of mimicking the hepatic damage on mice.

That tell that the time period of designing experiment should be more precise. Especially about the result 7 in the experiment 2. The experiment can design two different models to making predicted results of two set of virus model having different effects of acute and chronic infection of hepatitis B, to elaborate more details, the possible result of CD4⁺ and CD8⁺ activation caused by virus, because the diminished HBV DNA levels always happened within six weeks of acute infection of HBV in mice. But in chronic infection of HBV in mice, the HBsAg can contain viral DNA in plasma for more than half a year [8]. The situation is way more different in patients. In some examples, the patients with more acute HBV infection, the diminished HBV DNA level will last from six weeks to more than 10 years in chronic HBV infection, causing cirrhosis or other hepatic injury, and the HBeAg will still exist in the blood sample, making CD4⁺

CD8⁺ regulation has different effect. [2] So different sets of modelling will be more accurate to get optimal dosage of APS in decreasing the ALT level and coordinate the CD4⁺ and CD8⁺ increasing in appropriate time period, such as the stage 2 of diminish.

The result 5 and 6 are positive results, but only partially supporting the hypothesis. About result 6, it shows the using of APS to activate the CD4⁺ and CD8⁺ show the potential of using APS as a way to activate CD4⁺ and CD8⁺ cells have different relationship. As the dietary supplementation of PAS increased only the CD4⁺ subsets, such as Th1 and Th2, but not the CD8⁺ cells' subsets, like the cytotoxic cell. So, it needs more indication factors of mice to get sufficient evidence to prove the function of CD4⁺ and CD8⁺ has been successfully activated by APS. Some other viral protein expressed by HBV, like HBsAg, which can directly regulate those antigen presenting cells and induce the myeloid-derived repressor cells (MDSCs) and regulatory T-cells, which will then repress the activation of CD4⁺ and CD8⁺ cell in hepatitis B. So in the future research of using corresponding organic component like APS to treat hepatic chronic disease, the experiments should be re-design and looks more for the other dependent variables, such as HBsAg and some cytokines produced by subset of CD4⁺ and CD8⁺ cells, like IL-2 and IFN- γ when hepatic injury modeling built and be treated with APS.

6. Conclusion

Generally, this study explores the therapeutic effect of *Astragalus polysaccharides* (APS) in activating the subset of CD4⁺ and CD8⁺ cells and decreasing in alanine aminotransferase (ALT) in mice. It shows that the have set two experiments. The first one is to investigate the effectiveness of APS on treating hepatic injury, mimicking the damaged liver tissue induced by Carbon tetrachloride to, and the level of ALT is recorded. The result shows that APS can have effectiveness to decrease the ALT but it has potential side effect to be an anti-nutritional factor in excessive amount, so more experiments are needed to shows the potential of transition to clinical samples. The second experiment using the hydrodynamic transfection method to transmitted the viral plasmid into the virus. And the results show the APS can increase the expression of subsets of CD4⁺ and CD8⁺ cells, but due the difference between acute and chronic hepatitis B infection in mice and people, we need do more experiments and re-design the experiment to detect other cytokines and cells that have big effects on regulating the cells' immunity response. And also have more different representative animals.

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