Effect of Steeped Black Tea (*Camellia sinensis* (L.) var. assamica) on Immunoglobulin Titer and Lymphocyte Proliferation in Responses to Hepatitis B Vaccine in Mice

Jason Merari Peranginangin*, Opstaria Saptarinia, Erly Ismawati, Rizal Aan Wihardy, Rizky Fadlya

Faculty of Pharmacy, Setia Budi University, Surakarta 57127, Indonesia

Abstract

Effect of steeped black tea (*Camellia sinensis* (L.) var. assamica) on immunoglobulin titer (IgM and IgG) and lymphocyte proliferation in responses to hepatitis B vaccine in BALB/c mice has been investigated. The study was divided into two stages i.e. the determination of immunoglobulin titer and the test of lymphocytes proliferation. In the first stage mice were divided into 5 groups each consisting of 5 mice. Group I, II and III was given steeped black tea respectively with a dose of 600 mg/kg bw; 1.2 g/kg bw and 2.4 g/kg bw. Group IV was given Stimuno® with a dose of 6.5 mg/kg bw, and group V was given aquadest as negative control. All groups were induced by hepatitis B vaccine on day -0 (after 7 days of acclimatization). Serum was taken on day-14 and 21 for measurement of IgM titer and IgG, respectively. In the second stage, the mice were grouped as in the first stage, then all groups were induced by hepatitis B vaccine at day-0 and day-7. On day-27 lymphocyte was isolated and then tested for the growth and proliferation of lymphocytes. The results of this study showed that the steeped black tea has an effect in increasing IgM and IgG titer of BALB/c mice induced by hepatitis B vaccine, where the most effective dose was 1.2 g/kg bw. Steeped black tea also could increase lymphocytes proliferation in mice BALB/c induced by hepatitis B vaccine, where the most effective dose was 1.2 g/kg bw.

© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

Peer-review under responsibility of the School of Pharmacy, Bandung Institute of Technology

**Keywords:** *Camellia sinensis* L. var. assamica; immunoglobulin M and G; hepatitis B vaccine; proliferation; lien lymphocyte

* Corresponding author. Tel.: +62-8122643987; fax: _62-271-853275.  
E-mail address: jason.merari@yahoo.com
1. Introduction

Hepatitis B is a very dangerous disease which is transmitted by parenteral or sexual, ranked nine causes of morbidity and mortality in the world. Infection with hepatitis B virus (HBV) indicate some sort of clinical manifestation: fulminant, acute, chronic, and not real. Fulminant and acute form is the cause of high mortality. Chronic hepatitis is a form that is responsible for the spread of the virus and potentially develop into cirrhosis and hepatocellular carcinoma. When hepatitis B virus enters the body, it will stimulate an immune response by forming antibodies so that it can resist the entry of virus into other liver cells that have not been infected. If our body healthy and have strong body defense system, then the new viruses can be destroyed by the immune system and the ailment is not prolonged. However, if the immune system fails, the virus will get into liver cells and insert their genetic material (DNA) into the cell nucleus and then multiplying. One of the mechanisms in the body that have the ability against the hepatitis B virus, is through the immune system. This system can inhibit replication and reduce the activity of virus, and the changes can be measured through the immune response like antibody level and lymphocytes proliferation.

Tea is one of the most heavily consumed beverages in the world. The relationship between tea drinking and human health is becoming a subject of intense study by scientists throughout the world. Most of teas are derived from the leaf of the *Camellia sinensis* plant. Tea can be group into three types including black tea, oolong tea, and green tea in result manipulations of leaf. Green tea is not fermented and is a major beverage consumed in Asian countries which contain water, proteins, carbohydrates, minerals, vitamins and polyphenols of the flavonoid type. Dry green tea leaves content 6–16% catechins, and catechins are a group of natural polyphenols. Thus, black tea generally refers to the fermented tea that is more popular in Iran, North America and Euro especially U.K. During fermentation a major part of the catechins is transformed into theaflavins and thearubigens. The ratio of catechin content in green tea to black tea is 3.1 to 1. Black tea extracts have been found to exhibit antioxidative, anticarcinogenic and antimutagenic activities. These activities are assumed to result from the polyphenols present in black tea, such as theaflavin and thearubigins. Theaflavins and thearubigins are the products of oxidation and condensation reactions during fermentation of tea leaves. Several methods have been developed to extract the bioactive materials from black tea. The most commonly used solvents for initial extraction step are boiling water and ethanol.

Tea contains many compounds, especially polyphenols, a heterogeneous group of chemicals characterized by hydroxylated aromatic rings. Polyphenols contained in teas are classified as catechins, and are collectively referred to as catechins. Tea contains six primary catechins compounds: catechin, gallocatechin, epicatechin, epigallocatechin gallate. These constituents possess antioxidant action, although to vary degree and are considered as potent scavengers of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxy radicals and nitric oxide produced radicals and nitric oxide produced by various chemicals. Catechins and their derivatives are shown to contribute beneficial health effects ascribed to tea by their antioxidant, antimutagenic and anticarcinogenic properties. Black tea consumption has been linked to lowering of various forms of cancer. The constituents also have been shown to have cardioprotective, neuroprotective, antidiabetic, and antimicrobial properties. In addition, black tea has been found to be useful in the treatment of arthritis, high cholesterol levels, infection, and impaired immune function. The present study is an effort to investigate the role of steeped black tea in overcoming the hepatitis B induction of mice BALB/c by determination of immunoglobulin titer and the test of lymphocytes proliferation.

**Nomenclature**

| Acronym | Definition          |
|---------|---------------------|
| HBV     | Hepatitis B Virus   |
| ROS     | Radical Oxygen Species |
2. Experiments

2.1. Material

2.1.1. Materials

Materials used for the isolation and culture of lymphocytes are chloroform, ethanol, RPMI-1640 (Sigma-Aldrich), HEPES buffer solution (Sigma-Aldrich), fetal bovine serum (Gibco), penicillin-streptomisin 1%v/v (Gibco), fungizon 0.5% v/v, ammonium klorida, aquabidest, MTT Formazan (Sigma-Aldrich). Materials used for measurement of immunoglobulin are bovine serum albumin, phosphate buffer saline, Tween 20, phosphate buffer saline, Tween20, alkaline phosphatase conjugate antimouseIgM and IgG, nitrophenil phosphate. Black tea powder was obtained from West Java.

2.1.2. Instrument

Instruments used for the isolation of lymphocytes are sterile tweezers, scissors, balance, petri dishes, syringe, centrifuge tube, Sorvall MC-12V microcentrifuge (Dupont), refrigerator, micropipette (Socorex), laminar air flow, hemocytometer (Neubauer). Instruments used for lymphocyte proliferation test and measurement of immunoglobulin are microcentrifuge tubes (Eppendorf), centrifuge tubes (Nunc), vortex mixer, incubator (Thermo Scientific), cooling centrifuge (Sigma 3K12), microplate 96 well (Nunc), blue tip, yellow tip, ELISA reader (Bio-Rad).

2.1.3. Animal

Male BALB/c mice (20-25 g) obtained from laboratory of Pharmacology Setia Budi University and adapted to laboratory conditions for 7 days with free access to food and water. After adaptation animal weighted and were randomly divided into 5 following groups (5 animal in each groups):

- Group I: received steeped black tea (600 mg/kg bw orally)
- Group II: received steeped black tea (1.2 g/kg bw orally)
- Group III: received steeped black tea (2.4 g/kg bw orally)
- Group IV: received Stimuno® (6.5 mg/kg bw orally)
- Group V: control animals received aqua destorally.

2.2. Methods

2.2.1. Determination of immunoglobulin titer

Test materials were given once a day during the study. On day-0 after each acclimatization of mice,hepatitis B vaccine was administered intra peritonially into mice. At day-7 after administration of hepatitis B vaccine, blood was collected from retro orbital plexus for measurement of immunoglobulin M titer using ELISA. Furthermore, at day-21 after administration of hepatitis B vaccine, blood was drawn for measurement of immunoglobulin G titers by ELISA.

2.2.2. Test of lymphocytes proliferation

Test materials were given once a day during the study. On day-0 and day-7 after each acclimatization of mice, hepatitis B vaccine was administered into mice intraperitoneally. On day-27, lymphocytes were isolated and then tested for the growth and proliferation of lymphocytes.

3. Results and Discussion
IgM and IgG levels after administration of hepatitis B vaccine have been determined by indirect ELISA. Blood sample was taken from the retro-orbital plexus of mice at day-14 (seven days after first vaccine induction for IgM) and day-21 (fourteen days after first vaccine induction for IgG). Blood sampling was adjusted to the production curve of antibodies in Sheehan (1997)\textsuperscript{15} (Fig. 1).

![Figure 1: Antibody production curve primary and secondary immune responses\textsuperscript{15}](image)

**Table 1. Average optical density (OD) of IgM and IgG**

| Group | Treatment                        | IgM level (OD ± SD) | IgG level (OD ± SD) |
|-------|----------------------------------|---------------------|---------------------|
| I     | 600 mg/kg bw steeped black tea  | 0.81 ± 0.06         | 0.89 ± 0.10         |
| II    | 1.2 g/kg bw steeped black tea   | 0.98 ± 0.07*        | 1.22 ± 0.10*        |
| III   | 2.4 g/kg bw steeped black tea   | 1.06 ± 0.07*        | 1.31 ± 0.12*        |
| IV    | 6.5 mg/kg bw Stimuno\textsuperscript{®} | 1.42 ± 0.08*    | 1.87 ± 0.13*        |
| V     | Aquadest                        | 0.73 ± 0.05         | 0.73 ± 0.09         |

Data are expressed as average±SD. Significant difference was calculated by using one-way ANOVA followed by Tukey post-test. \(P<0.05\) was considered as statistically significant. \(n=5\) in each group. *Values are significantly different from aquadest group at \(P<0.05\)

![Figure 2: Graphic of IgM level at day-14 after administration of hepatitis B vaccine](image)

The effect of steeped black tea on IgM and IgG levels can be seen in figure 2 and 3. Steeped black tea with dose 2.4 g/kg bw (group 3) showed the highest increased of IgM and IgG level among than other dosages, and significantly different from control group. However, the increase was still lower compared to Stimuno\textsuperscript{®} group.

The IgG level obtained in this study was higher than IgM level. This is in accordance with the curve in figure 1 (Sheehan, 1997), that IgM will be produced earlier after antigen exposure, then within the next 3-4 days to be followed by the production of IgG. The IgG are higher in number and reached a peak at day 14.
Table 2. Average optical density (OD) of lymphocyte proliferation

| Group | Treatment                               | (OD ± SD)   |
|-------|-----------------------------------------|-------------|
| 1     | 600 mg/kg bw steeped black tea          | 0.27 ± 0.02 |
| 2     | 1.2 g/kg bw steeped black tea           | 0.37 ± 0.06*|
| 3     | 2.4 g/kg bw steeped black tea           | 0.29 ± 0.04 |
| 4     | 6.5 mg/kg bw Stimuno®                   | 0.36 ± 0.05*|
| 5     | Aquadest                                | 0.25 ± 0.02 |

Data are expressed as average±SD. Significant difference was calculated by using Kruskal–Wallis followed by Mann–Whitney post-test. 
P<0.05 was considered as statistically significant. n=5 in each group.*Values are significantly different from aquadest group at p<0.05

Figure 4 showed the effect of steeped tea proliferation of lymphocyte cells. An increase of OD average means an increase of lymphocytes proliferation. Steeped black tea at dose of 1.2 g/kg bw (Group II) showed the highest lymphocytes proliferation. Higher dosage of steeped black tea did not followed with the higher lymphocytes proliferation.

Black tea is a drink that are familiar to Indonesia people. The main contents of black tea included theaflavine, thearubigin, flavonoid and cathecin that is a derivative of polyphenols. Polyphenols and derivatives are an antioxidant compound that is shown to improve the immune system and improve cytotoxic t cells\textsuperscript{16}. These various compounds suspected can stimulate the lymphocytes proliferation. According to Middleton \textit{et al.}\textsuperscript{17}, flavonoid in
addition as immunomodulator can also be the immunosuppressant, where until the certain dosages flavonoid can increase the immune system. Because black tea contains quite a lot of flavonoids then ability of cell lymphocytes proliferation decrease after giving steeped black tea at dose 2.4 g/kg bw.

4. Conclusion

Steeped black tea has an effect in increasing immunoglobulin titer (IgM and IgG) and in lymphocyte proliferation in mice BALB/c administered hepatitis B vaccine, where the most effective dose was 1.2 g/kg bw.

References

1. Ioshimoto LM, Rissato ML, Bonilha VSJ, Miyaki C, Raw I, Granovski N. Safety and Immunoencicity of Hepatitis B Vaccine Butang in adults. Rev. Inst. Med. trop. S. Paulo 1999; 41: 191-3.
2. Wijayakusuma H. Tumpas Hepatitis dengan Ramuan Herbal. Pustaka Bunda. Jakarta; 2008.
3. Zhu Y, Huang H, Tu Y. A review of recent studies in China on the possible beneficial health effects of tea. Int J Food Sci Tech 2006; 41: 333-40.
4. Leung LK, Su Y, Chen R, Zhang Z, Huang Y, Chen ZY. Theaflavins in black tea and catechins in green tea are equally effective antioxidants. J Nutr 2001; 131: 2248-51.
5. Hernández FTT, Rodríguez-Rodríguez E, Sánchez-Muniz FJ. The green tea, a good choice for cardiovascular disease prevention? Arch Latinoam Nutr 2004; 54: 380-94.
6. Yoshino K, Hara Y, Sano M, Tomita S. Antioxidative effects of black tea theaflavins and thearubigin on lipid peroxidation of rat liver homogenates induced by tert-butyl hydroperoxide. Biol Pharm Bull 1994; 17: 146-9.
7. Sesso HD, Gaziano JM, Buring JE, Hennekens CH. Coffee and tea intake and the risk of myocardial infarction. Am J Epidemiol 1999; 149: 162-7.
8. Graham HN. Green tea composition, consumption, and polyphenol chemistry. Prev Med 1992; 21: 334-50.
9. Van het Hof KH, Kivits GAA, Weststrate JA, Tijburg LBM. Bioavailability of catechins from tea: the effect of milk. Eur J Clin Nut 1992; 52: 356-9.
10. Isemura M, Saeki K, Kimura T, Kayakawa S, Minami T, Sakuza M. Tea catechins and related polyphenols as anticancer agents. Biofactors 2000; 13, 81-5.
11. Lambert JD, Hong J, Yang GY, Liao J, Yang CS. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. Am J Clin Nutr 2005; 81: 284S-91S.
12. Lin YS, Tsai YJ, Tsay JS, Lin JK. Factors affecting the levels of tea polyphenols and caffeine in tea leaves. J Agric Food Chem 2003; 51: 1864-73.
13. Khan SM, Kour G. Subacute oral toxicity of chlorpyriphos and protective effect of green tea extract. Pesticide Biochem and Physiol 2007; 89: 118-23.
14. Yokozawa T, Nakagawa T, Kitani K. Antioxidative activity of green tea polyphenol in cholesterol-fed rats. J Agric Food Chem 2002; 50: 3549-52.
15. Sheehan C. Clinical Immunology, Principles and Laboratory Diagnosis, 2nd Ed. New York: Lippincott; 1997. P. 26.
16. Barataydijaja KG. Imunologi Dasar. Edisi VIII. Jakarta: Balai Penerbit Fakultas Kedokteran Universitas Indonesia; 2009.
17. Middleton E, Kandaswami C, Theoharides TC. The Effect of Plant Flavonoids on Mammalian Cells: Implication for Inflammation, Heart Disease, and Cancer. Pharmacol Rev 2000; 52: 673-751