ORIGINAL ARTICLE

Effects of Phytosterol Supplementation on Lipid Peroxidation Induced by Carbon Tetrachloride in a Rat Model

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ABSTRAK

Fitosterol adalah sterol tumbuhan yang mempunyai struktur kimia menyerupai kolesterol. Ia mempunyai kesan anti-kolesterol, anti-kanser dan anti-oksida yang mungkin disumbang oleh perencatan peroksidasi lipid. Bagaimanapun, kajian mengenai kesan fitosterol ke atas peroksidasi lipid adalah terhad. Tujuan kajian ialah untuk menentukan kesan fitosterol ke atas malondialdehid (MDA) plasma dan organ pada model tikus yang terdedah kepada karbon tetraklorida. Tikus kajian dibahagikan kepada empat kumpulan iaitu Kawalan normal (NC), Karbon tetraklorida (CCl_4), Fitosterol (P) dan Fitosterol + karbon tetraklorida (P+CCl_4). Kumpulan P dan P+CCl_4 menerima pra-rawatan fitosterol secara subkutaneous pada dos 140 mg/kg sekali seminggu selama 5 minggu, sementara kumpulan NC and CCl_4 hanya menerima minyak zaitun (vehikel). Satu dos oral karbon tetraklorida diberikan kepada tikus kumpulan CCl_4 dan P+CCl_4 untuk mengaruh peroksidasi lipid. Selepas 24 jam, kesemua tikus dikorbankan dan MDA plasma dan organ-organ diukur. Seperti jangkaan, karbon tetraklorida telah meningkatkan aras MDA plasma dan hepar kumpulan CCl_4 berbanding kumpulan kawalan normal. Pra-rawatan fitosterol (kumpulan P+CCl_4) berjaya menghalang peningkatan MDA tersebut. Rawatan fitosterol pada tikus normal (kumpulan P) telah menurunkan aras MDA hepar. Rumusan kajian ialah fitosterol adalah efektif sebagai perencat peroksidasi lipid. Ia berpotensi sebagai suplemen untuk mengurangkan peroksidasi lipid pada individu yang sihat.

Kata kunci: fitosterol, karbon tetraklorida, peroksidasi lipid, malondialdehid, hepar

ABSTRACT

Phytosterols are plant sterols with a chemical structure similar to cholesterol. It has anti-cholesterol, anti-cancer and anti-oxidant properties which are probably mediated by suppression of lipid peroxidation. However, there are limited studies on the effects of phytosterols on lipid peroxidation. The aim of this study is to determine the effects of

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phytosterols on plasma and tissue malondialdehyde (MDA) of rats exposed to carbon tetrachloride. The rats were divided into four groups of normal control (NC), carbon tetrachloride (CCl_4), phytosterol (P) and phytosterol+carbon tetrachloride (P+CCl_4). The P and P+CCl_4 groups were pretreated with subcutaneous phytosterol at 140 mg/kg once weekly for 5 weeks while the NC and CCl_4 groups only received olive oil (vehicle). A single oral dose of carbon tetrachloride was then given to rats in the CCl_4 and P+CCl_4 groups to induce lipid peroxidation. After 24 hours, all the rats were sacrificed and the plasma and tissue MDA were measured. Our results showed carbon tetrachloride had caused significant elevations of the plasma and hepatic MDA of the CCl_4 group compared to the NC group. Phytosterol pretreatment (P+CCl_4 group) were able to prevent the MDA elevations. Phytosterols treatments in normal rats (P group) were found to reduce the hepatic MDA level. The conclusion of this study was that phytosterols are effective suppressor of plasma and hepatic lipid peroxidation. They have potential as supplements to further reduce lipid peroxidation in healthy individuals.

Key words: phytosterols, carbon tetrachloride, lipid peroxidation, malondialdehyde, liver

INTRODUCTION

Phytosterols or plant sterols are a group of steroid alcohols, phytochemicals naturally occuring in plants. They have a chemical structure which is similar to cholesterol (Weihrauch & Gardner 1978) and exist in several forms in plants (Law 2000; Katan et al. 2003; Abumweis et al. 2007) including β-sitosterol, campesterol, stigmasterol and cycloartenol (Ostlund 2002). Phytosterols are natural components found in the human diet. It made up 0.1 to 0.5% w/w of vegetable oils or margerine (Kochlar 1983) and are also found in corn, wheat and rice. Phytosterol intake varies according to the type of diet taken. Europeans consume about 200-300 mg/day of phytosterol (Morton et al. 1995) while the vegetarian Japanese have a higher intake of phytosterol (Nair et al. 1984).

Phytosterols are well known for their ability to lower plasma cholesterol level (Klingberg et al. 2008) by interfering with the absorption of cholesterol from the gastrointestinal system (Jones 1999; Hayes et al. 2004; Jia et al. 2007). Phytosterols have also been shown to possess anti-cancer properties against gastric, colon, ovary and breast cancers (Choi et al. 2007; Mendilaharsu et al. 1998; De Stefani et al. 2000; McCann et al. 2003; Ju et al. 2004). These studies suggested that phytosterols may be useful in the prevention of cardiovascular diseases and cancers.

Oxidative stress is thought to be involved in the pathogenesis of coronary and peripheral arterial diseases (Smith et al. 1993). During lipid peroxidation, lipids are oxidized to form free radicals which can cause extensive tissue damage. Several cardiovascular risk factors have been identified that promote cardiovascular disease via lipid peroxidation (Rumley et al. 2004). Therefore, reducing lipid peroxidation is important in prevention of cardiovascular disease. This may be achieved through the anti-oxidant properties of phytosterols. It is also believed that oxidative stress may contribute to carcinogenesis by DNA. However, there is a growing body of evidence showing that lipid peroxidation do not promote carcinogenesis but may actually

inhibit most cancer cells (Zanetti et al. 2003; Gago-Dominguez et al. 2005).

Phytosterols were found to exert anti-oxidant effects on the oxidation of methyl linoleate in solution. They also suppressed the oxidation and consumption of α-tocopherol in β-linoleoyl-γ-palmitoyl phosphatidylcholine (PLPC) liposomal membranes (Yoshida & Niki 2003). Vivancos and Moreno (2005) reported that phytosterols were able to increase the anti-oxidant enzyme activities of superoxide dismutase and glutathione peroxidase in cultured macrophage cells exposed to oxidative stress by phorbol 12-myristate 13-acetate. Therefore, an alternative mechanism of protection from oxidative stress by phytosterols is by increasing the antioxidant enzyme activities.

There were limited published reports on the effects of phytosterol on lipid peroxidation. The aim of our study is to determine the effects of pretreatment with phytosterol on lipid peroxidation in plasma and organs of rats exposed to carbon tetrachloride.

MATERIALS AND METHODS

A preliminary study was conducted to determine the suitable dose of carbon tetrachloride which can induce lipid peroxidation in various rat organs. Hafsah (2005) found that the dose of 1.0 ml/kg of carbon tetrachloride overwhelmed the effects of phytosterols which were given at weekly doses of 140 mg/kg (unpublished).

In this study, 24 male Sprague-Dawley rats weighing between 175 to 200 grams were obtained from the UKM Animal House. The rats were housed in plastic cages at room temperature (29±3ºC) and daily dark/light cycle. The rats were fed standard rat pellets (Gold Coin, Malaysia) and distilled water *ad libitum*. The rats were allowed to adjust to the new environment for a week before the study was started. The study was approved by the UKM Animal Ethics Committee.

The rats were randomly divided into 4 groups of normal control (NC), carbon tetrachloride (CCl_4), phytosterol (P) and phytosterol+carbon tetrachloride (P+CCl_4). The latter two groups were pretreated with a subcutaneous injection of phytosterol (MPOB, Malaysia) at a dose of 140 mg/kg, once a week for five weeks (Yoshida & Niki 2003). These phytosterols were extracted from palm oil and are composed of 60% β-sitosterol and 40% stigmasterol and campesterol. The NC and CCl_4 groups received 2 ml/kg olive oil (vehicle) (Oomerbhoy Ltd, Mumbai) subcutaneously once a week for the same duration. Rats in the CCl_4 and P + CCl_4 groups were then given a single dose of 0.5 ml/kg carbon tetrachloride (BDH Chemicals, England) diluted in olive oil via oral gavage to induce lipid peroxidation. Rats in the NC and P groups received equivalent amounts of olive oil (vehicle). After 24 hours, blood samples were taken via the orbital sinus under anesthesia. The blood was left for 30 minutes and centrifuged at 3000 rpm for 5 minutes to separate the plasma. The plasma was stored at -20°C. The rats were then sacrificed and the liver, heart, kidneys and lungs were dissected out. The plasma and tissue malondialdehyde (MDA) levels were measured according to the method of Ledwozyw et al. (1986). According to this method, malondialdehyde is formed as an end product of lipid peroxidation which reacts with TBA (Thiobarbutaric acid) reagent under acidic condition to generate a pink-coloured product. Plasma (0.1 ml) was added to 0.4 ml of distilled water, followed by the addition of 2.5 ml of trichloroacetic acid (TCA). The mixture was left at room temperature for 15 minutes. TBA (1.5 ml) was then added and heated in a water bath at 100°C for 30 minutes until a faint pink colour was obtained. After cooling, the colour was ex-

tracted in 1 ml of buthanol and the intensity was measured using the spectrophotometer at EX 515nm and EM 553nm. 1,1,1,3-tetraethoxypropane (Sigma, USA) was used as the standard.

The results are expressed as mean±SEM. The statistical significance of the data was determined using one-way analysis of variance (ANOVA) and post hoc Tukey test. The level of significance was set at $p<0.05$.

RESULTS

In order to determine the suitable dose of carbon tetrachloride for induction of lipid peroxidation, we have carried out a preliminary study. In the study, rats were given either 0.5 ml/kg or 0.75 ml/kg of carbon tetrachloride and sacrificed after 24 hours. The plasma and liver melondialdehyde (MDA) were then measured. We found from our preliminary study that the lowest dose of carbon tetrachloride that would produce significant lipid peroxidation in plasma and liver was 0.5 ml/kg (Figures 1 and 2). Results of the present study showed that the plasma MDA of carbon tetrachloride (CCl_4) group was significantly higher than the normal control group. The plasma MDA levels of the P and P+CCl_4 groups were significantly lower than the CCl_4 group (Figure 3). The hepatic MDA level for the CCl_4 group was significantly higher than the normal control group. The hepatic MDA levels of the P and P+CCl_4 groups were significantly lower than the CCl_4 groups. In addition, the hepatic MDA level of P group was significantly lower than the normal control group (Figure 4). There was no significant change in renal MDA level in the CCl_4 group compared to the normal control group. The renal MDA levels for the P and P+CCl_4 groups were significantly lower compared to the CCl_4 group (Figure 5). There was no significant change in the cardiac MDA level in the CCl_4 group compared to normal control group. The cardiac MDA level for the P group was significantly lower than P and P+CCl_4 groups (Figure 6). The MDA levels in the lungs showed no significant findings in all the groups (Figure 7).

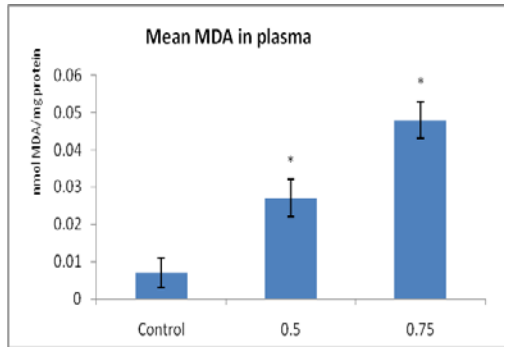

Figure 1: Mean MDA in plasma in the preliminary study to determine suitable dose of carbon tetrachloride. *indicate significant difference compared to control group ($p<0.05$). Data is expressed as mean ± SD.

Control: control group
0.5 : 0.5 ml/kg of CCl_4 group
0.75 : 0.75 ml/kg of CCl_4 group

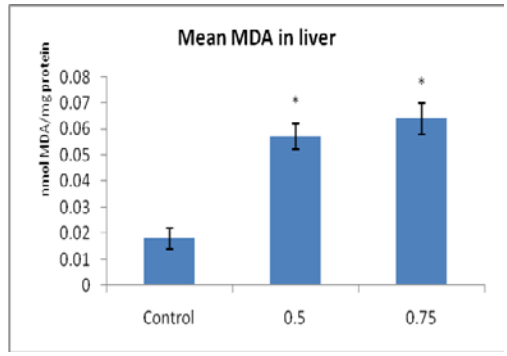

Figure 2: Mean MDA in liver in a preliminary study to determine suitable dose of carbon tetrachloride. * indicate significant difference compared to control group ($p<0.05$). Data is expressed as mean ± SD.

Control: control group
0.5 : 0.5 ml/kg of CCl_4 group
0.75 : 0.75 ml/kg of CCl_4 group

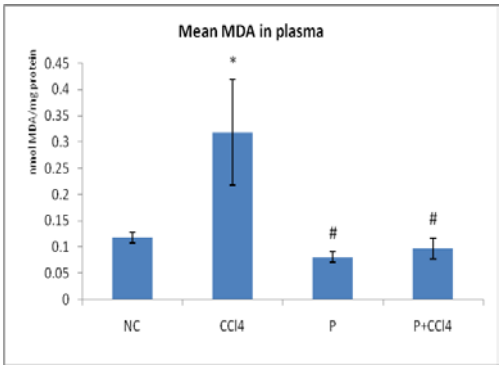

Figure 3: Mean MDA in plasma.* indicate significant difference compared to control group. # indicate significant difference compared to CCl_4 group ($p<0.05$). Data is expressed as mean ± SD.

NC : Normal control group
CCl_4 : Carbon tetrachloride group
P : Phytosterol group
$P+CCl_4$: Phytosterol + Carbon tetrachloride group

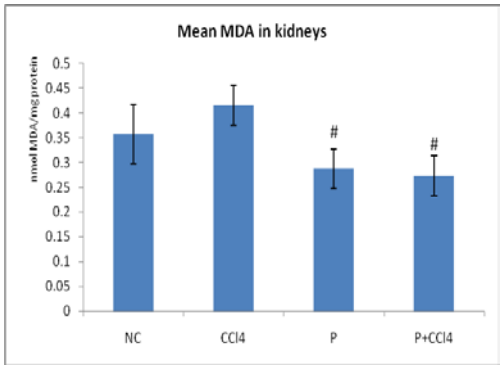

Figure 5: Mean MDA in kidneys. #indicate significant difference compared to CCl_4 group ($p<0.05$). Data is expressed as mean ± SD.

NC : Normal control group
CCl_4 : Carbon tetrachloride group
P : Phytosterol group
$P+CCl_4$: Phytosterol + Carbon tetrachloride group

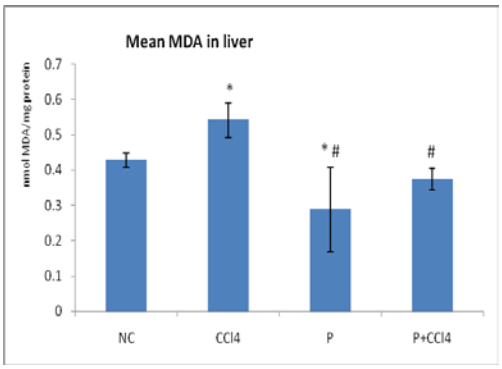

Figure 4: Mean MDA in liver. *indicate significant difference compared to control group. # indicate significant difference compared to CCl_4 group ($p<0.05$). Data is expressed as mean ± SD.

NC : Normal control group
CCl_4 : Carbon tetrachloride group
P : Phytosterol group
$P+CCl_4$: Phytosterol + Carbon tetrachloride group

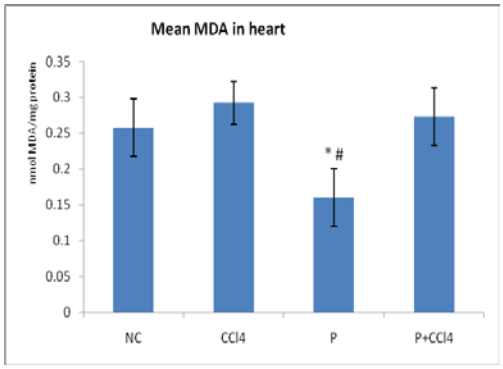

Figure 6: Mean MDA in heart.* indicate significant difference compared to control group. # indicate significant difference compared to CCl_4 group ($p<0.05$). Data is expressed as mean ± SD.

NC : Normal control group
CCl_4 : Carbon tetrachloride group
P : Phytosterol group
$P+CCl_4$: Phytosterol + Carbon tetrachloride group

DISCUSSION

Carbon tetrachloride is metabolised by cytochrome P-450 enzymes in the liver to the reactive trichloromethyl radical. The radical is oxidized further, forming the even more reactive trichloromethylperoxyl radical (McGregor & Lang 1996;

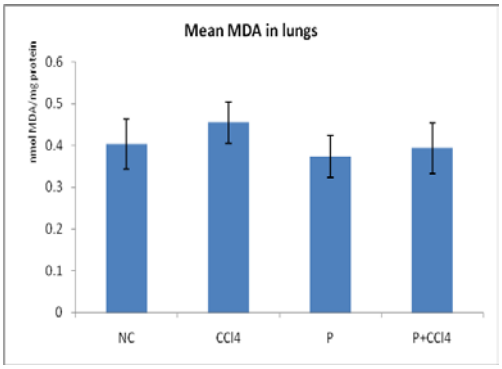

Figure 7: Mean MDA in lungs. There is no significant findings for either group ($p<0.05$). Data is expressed as mean ± SD.

NC : Normal control group
CCl_4 : Carbon tetrachloride group
P : Phytosterol group
$P+CCl_4$: Phytosterol + Carbon tetrachloride group

IPCS 1999). These reactive metabolic intermediates of carbon tetrachloride, particularly trichloromethylperoxyl radical, can cause lipid peroxidation (IPCS 1999). In our study, carbon tetrachloride has induced lipid peroxidation as shown by elevations of MDA levels in the rat's plasma, liver, kidneys, heart and lungs. However, only the plasma and hepatic MDA levels were significantly raised. Carbon tetrachloride is known to cause injuries to various organs of the body (Reynolds et al. 1984) including the liver, kidneys, heart, lungs, gastrointestinal tract and central nervous system (ATSDR 2005). The primary targets for carbon tetrachloride toxicity are the liver and kidneys (IPCS 1999).

Phytosterols taken into the body are incorporated into cell membranes (Awad et al. 2004) and are highly concentrated in the lungs, adrenal cortex, intestinal epithelia and ovaries (Sanders et al. 2000). In our study, we find that phytosterol pretreatment was able to prevent elevation of MDA levels in the plasma, liver and kidneys but was unable to do the same for the heart and lungs. Therefore, pretreatment with phytosterol was able to reduce lipid peroxidation in the plasma and protect the liver and kidneys against lipid peroxidation damage. These findings were consistent with other findings that showed that pretreatment with other antioxidants, such as vitamin E reduced the hepatotoxic action of carbon tetrachloride (IPCS 1999).

In a study by Mora-Ranjeva et al. (2006), phytosterol in the form of sitosterol and stigmasterol were incorporated into human keratinocytes (SVK14 line) and exposed to ultraviolet light. It was found that sitosterol induced significant decrease (-30%) in lipid peroxidation whereas stigmasterol markedly increased lipid peroxidation (+70%). Results of this study also showed that the effects of phytosterol on lipid peroxidation also depended on the form of the phytosterol. In this study, we have used phytosterols derived from palm oil which was made up of 60% β-sitosterol and 40% campesterol and stigmasterol.

We found that there were measurable MDA levels in the plasma and organs of rats in the normal control group. This may be contributed by the free radicals being produced even under normal conditions by either leakage of activated oxygen from mitochondria during oxidative phosphorylation or by the multiple redox-active flavoproteins (Messner & Imlay 2002; Imlay 2003; Seaver & Imlay 2004). Phytosterol supplementation was able to reduce the MDA levels of the liver and heart of normal rats. Based on this result, phytosterol may be useful as supplements for healthy individuals to maintain their oxidative status but this requires further studies.

The phytosterol dosage used in this study is equivalent to the human intake of 20 mg/kg body weight/day. This is high considering that the daily intake of phytosterols in the human diet is about 2.5 to 4.0 mg/kg bodyweight (Morton et al. 1995). With regards to its toxicity, ad-

ministration of phytosterols at the daily doses of 6.6 g/kg bodyweight in rats did not show any subchronic toxicity or teratogenic effects (Hepburn et al. 1999). In a human study, the daily intake of 9.0 grams of phytosterols for eight weeks did not cause any adverse effects (Katan et al. 2003). However, it was shown to reduce absorption of lipid soluble antioxidants such as α-carotene, β-carotene and vitamin E (Law 2000).

In conclusion, phytosterols may be used to reduce lipid peroxidation in the plasma, liver and kidneys. Phytosterol supplementation has a good safety profile and may further reduce lipid peroxidation in healthy individuals.

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