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Original Article

Effect of Repeatedly Heated Palm Olein on Blood Pressure Regulating Enzymes Activity and Lipid Peroxidation in Rats

Xin-Fang LEONG[1,2], Jumat SALIMON[3], Mohd Rais MUSTAFA[4], Kamsiah JAARIN[1]

[1] *Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia*

[2] *Department of Clinical Oral Biology (Pharmacology), Faculty of Dentistry, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia*

[3] *School of Chemical Sciences & Food Technology, Faculty of Science & Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Malaysia*

[4] *Department of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia*

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Abstract

Background: Oxidative stress is associated with the pathogenesis of cardiovascular diseases. Deep-fat frying process of dietary cooking oil plays a role in the generation of free radicals. Palm olein heated to 180°C temperature was tested for its effect on the activity of blood pressure regulating enzymes and lipid peroxidation.

Methods: Forty-two adult male Sprague-Dawley rats were equally assigned into six groups. First group was fed with normal rat chow as control group, and the subsequent groups were fed with rat chow fortified with 15% weight/weight of fresh palm olein, palm olein heated once, palm olein heated twice, palm olein heated five times or palm olein heated ten times, respectively. Feeding duration was six months. Fatty acid analyses of oil were performed using gas chromatography while peroxide values were determined using standard titration method. Plasma was collected for biochemical analyses.

Results: Repeatedly heated palm olein increased peroxide value, angiotensin-converting enzyme level, lipid peroxidation, and reduced heme oxygenase level. Fresh palm olein and palm olein heated once had lower lipid peroxidation and better outcome in blood pressure regulating enzymes activity than repeatedly heated palm olein.

Conclusion: Repeatedly heated palm olein may deteriorate the activity of blood pressure regulating enzymes and increase lipid peroxidation.

Keywords: *angiotensin-converting enzyme, heating, heme oxygenase, oxidative stress, palm oil*

Introduction

Palm oil obtained from the mesocarp *Elaeis guineensis* exhibits good frying performance and hence contributes to its widespread use in deep-frying application (1). Due to the rising demand and increase in fat intake, palm oil is major oil in the worlds' oils and fats trade with its usage to be continued through year 2016 as the most influential fat source (2). The refined, bleached and deodorized (RBD) palm olein, which is fractionated from palm oil, is commonly used as cooking oil. It offers better resistant to oxidation at high

temperature during frying with the presence of natural antioxidants from the vitamin E group, which is the tocotrienols (3). Tocotrienols, with an unsaturated side chain have been documented to have greater antioxidant properties than the saturated tocopherols against oxidative damages (4). In addition, palm olein contains almost 50 % saturated fatty acids (SFA) and 50% monounsaturated fatty acids (MUFA) with low level of polyunsaturated fatty acids (PUFA) under normal conditions (5), thus reducing susceptibility to oxidation.

The main economic factor that is put into consideration in fried food product is the cost of oil. This is because the oil forms part of the major ingredients of a fried food product. Therefore, very often the oil is repeatedly used to minimize the expenses of food preparation. During the reheating process, the oil undergoes various physical reactions such as foam formation, increases in viscosity, colour darkening and flavour deterioration which may affect the organoleptic quality such as odour, taste and nutritional value of the fried food (6). Furthermore, chemical reactions such as hydrolysis, oxidation and polymerization occur which alter the chemical structure of triacylglycerol molecule with PUFA mostly affected (7).

Thermally oxidized oils, such as those produced by repeated frying, contain a complex mixture of products such as oxidized monomers, dimers and polymers. These products have been reported to be mainly responsible for the changes in physicochemical properties of fats (8). When the frying oil is heated at high temperatures, toxic products such as hydroperoxides and aldehydes are formed, are absorbed by food, and later into gastrointestinal system and systemic circulation (9). The practice of reusing frying oil imposes harmful effects on health such as an increased risk of hypertension (10, 11), disturbance of endothelial function (12, 13) and histological abnormalities (14, 15). Free radicals generated during frying process could damage lipids to initiate lipid peroxidation. Malondialdehyde (MDA), one of the major secondary oxidation end products of peroxidized PUFA has been shown to be of biological significance (16).

Heme oxygenase (HO) is the rate-limiting factor involving in the heme catabolism, producing free ferrous ion, biliverdin and carbon monoxide (CO). Biliverdin is further converted to bilirubin, which acts as antioxidant (17). Furthermore, CO has the properties of vasodilatation, anti-proliferation, and anti-inflammation (18, 19). Among the isoforms of HO (HO-1, HO-2, HO-3), HO-1 is the isoform being suggested to contribute to the controlling of blood pressure (BP). It is inducible and highly sensitive to various stimuli which involved in oxidative and hemodynamic damage (20).

On the other hand, angiotensin-converting enzyme (ACE) plays a vital role in the regulation of BP via hydrolysis of inactive form of angiotensin I (Ang I) to the potent form of angiotensin II (Ang II). ACE is mainly located on the surface of endothelium and epithelium involving in the constriction of blood vessels that leads to elevation of BP. Effects of Ang II can be observed via two types of receptors: AT_1 and AT_2, both with different pharmacological and biochemical characteristics, respectively.

Ang II is an important factor in cardiovascular homeostasis (21). It induces oxidative stress via activation of nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) oxidase with the production of reactive oxygen species (ROS) (22) that cause hypertension and cardiac failure (23, 24). In addition, Ang II also increases lipid peroxidation (25) and stimulates production of pro-oxidant cytokines (26, 27) that eventually increase the BP.

Previous research findings in our laboratory have clearly reported that heated oils increase BP and lead to impairment in vasorelaxation (10, 12). Therefore, we would like to establish the BP raising effect of heated oils, either wholly by increasing vascular reactivity with a reduction in nitric oxide content, or via affecting BP controlling enzymes. The enzymes of interested are HO and ACE which were investigated in the present study. The current study was undertaken to evaluate the mechanism of BP raising effect by measuring activity of enzymes involving in the regulation of BP and lipid peroxidation in rats.

Materials and Methods

Palm olein and diet preparation

Palm olein was purchased from a local market. The oil was used either in fresh or heated form according to a modified method (28) which was also used in our previous studies (10, 12). Briefly, the oil was used to fry potato chips. Firstly, the sweet potatoes were peeled-off skin and cut into slices, then deep-fried using fresh palm olein for 10 min at the temperature of 180°C. The oil was then cooled to room temperature and was used to fry another batch of sweet potatoes. The frying process was performed without any addition of fresh oil. The same process was repeated to obtain palm olein heated five times and ten times. Standard rat chow (Gold Coin, Selangor, Malaysia) was grinded to coarse powder and fortified with 15% weight/weight (w/w) of the respective oils prepared. The mixture was subsequently dried in a 70°C-oven for overnight. The diet was stored in a closed cabinet and prepared weekly.

Animals and study protocol

Three months old male Sprague-Dawley rats (200 – 280 g) were used in the study. Forty-two rats were obtained from the Animal Source Unit, Universiti Kebangsaan Malaysia (UKM) with prior ethical clearance (UKMAEC: FP/FAR/2008/KAMSIAH/9-APR/220-APR-2008-FEB-2011). All animal management and handling procedures were performed according to the recommended guidelines. The animals were equally and randomly divided into six experimental groups. The experimental groups were as follows:

Group 1: (control) – normal rat chow
Group 2: fresh palm olein (FPO – 15% w/w mixed with chow)
Group 3: palm olein heated once (1HPO – 15% w/w mixed with chow)
Group 4: palm olein heated twice (2HPO – 15% w/w/ mixed with chow)
Group 5: palm olein heated five times (5HPO – 15% w/w/ mixed with chow)
Group 6: palm olein heated ten times (10HPO – 15% w/w mixed with chow)

All rats were kept in stainless-steel cages in a room maintained at 27 ± 2°C with a 12-h light-dark cycle. The animals had free access to water and food throughout the experimental study. The rats were fed daily on the oil diet based on their respective experimental groups for six months after one week of adaptation. Blood was collected through orbital sinus prior to treatment and at the end of the study. The blood was then centrifuged to obtain plasma. Aliquots of plasma were later stored at -70°C and used for the enzyme activity and lipid peroxidation studies.

Fatty acid composition

Fatty acid methyl esters were prepared from the fresh and heated palm olein by transesterification with sodium methoxide (NaOMe 1M) in hexane prior to analysis. A gas chromatography (Shimadzu GC-17A, Kyoto, Japan), equipped with a flame ionization detector (FID) was used for fatty acid profiling. Nitrogen was used as carrier gas at a flow rate of 0.40 mL/min through a BPX 70 capillary column (30 m x 0.25 mm x 0.25 m film thickness) (SGE, NJ, USA). The injector temperature was programmed at 250°C and the detector temperature was set to 280°C. Injection volume was 1 l. Retention times obtained from gas chromatography were compared with those of individually purified standards subjected to the same condition for identification of fatty acid methyl esters peaks.

Peroxide value

The peroxide content of palm olein was determined by the Official Method of American Oil Chemists' Society (Cd 8-53).

Biochemical measurements

Both HO-1 (Assay Designs, MI, USA) and ACE (USCNLife, Wuhan, China) were analyzed enzymatically on plasma samples using commercially available kits. The coloured end-product of these two enzymes was measured in a microplate reader (Molecular Devices, CA, USA) with a wavelength of 450 nm. These measurements were performed as per previous protocol (12) following manufacturers' instruction.

Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was measured in plasma in form of MDA using thiobarbituric acid. Plasma TBARS level was estimated following a method described earlier (29) with some modifications. Briefly, 2.5 ml of 1.22 M trichloroacetic acid (TCA)/ 0.6M hydrochloric acid (HCl) was used to acidify 0.5 ml of plasma sample and left to stand at room temperature for 15 min. Subsequently, 1.5 ml of 0.67% thiobarbituric acid (TBA)/0.05 M sodium hydroxide (NaOH) was added. Incubation was continued for 30 min in a 100°C water bath. Four ml of *n*-butanol was added after the mixture cooled down at room temperature. Next, the mixture was centrifuged for 10 min at 3000 rpm. The supernatant was read against *n*-butanol at Ex: 515 Em: 553 using spectroflurometer (Shimadzu RF500, Kyoto, Japan).

Protein content

The protein content in the plasma was determined using a method described by Lowry et al. (30) with some modifications. About 5 ml mixture of 2% sodium carbonate (Na_2CO_3), 2% sodium or potassium tartrate (Na/K tartrate) and 1% copper sulphate solution ($CuSO_4 \cdot 5H_2O$) with ratio 100:1:1 was added to 0.5 ml plasma sample. Next, the mixture was left to stand at room temperature for 15 min and followed by the addition of 0.5 ml of diluted Folin-Ciocalteau phenol reagent. After 35 min, the mixture was measured at 700 nm with spectrophotometer (Shimadzu UV-160A, Kyoto, Japan). Results were expressed as TBARS/protein (nmol/mg protein).

Statistical analyses

Results for BP regulating enzymes activity and lipid peroxidation were presented as percentage based on baseline values. All data analyses were conducted using SPSS version 13.0 (SPSS Inc., IL, USA). Normality of data was determined by Kolmogorov-Smirnov test. Comparison for peroxide values among the dietary groups was determined using one-way analysis of variance (ANOVA) with Tukey's Honestly Significant Differences (HSD) post-hoc test for differences between pair of means when applicable. To analyze BP regulating enzymes activity and lipid peroxidation among the experimental groups, Kruskal-Wallis and Mann-Whitney tests were performed. Statistical significance was defined as $p < 0.05$. Data were expressed as means ± SEM.

Results

Fatty acid composition of palm olein

Fatty acid analyses of fresh oil and oil subjected to different frying level were shown in Table 1. All the main compositions of fatty acid were present in the oil regardless of the frequency of oil being fried.

Peroxide content of palm olein

There was a significant increase ($p < 0.001$) in the peroxide index for both 2HPO and 5HPO (4-fold increase) while 5-fold increment for 10HPO, compared with the fresh-oil value (Table 1).

Activity of blood pressure regulating enzymes

All groups, including control exhibited a reduction in plasma HO-1 level after six months of feeding period. HO-1 activity was significantly lower ($p < 0.05$) in the reheated palm olein groups (2HPO, 5HPO and 10HPO) (Figure 1). Activity of ACE was also found to be significantly increased ($p < 0.05$) in heated palm olein groups. The percentage of increment was significantly the highest in the 10HPO group compared to the FPO and other dietary groups (Figure 2).

Lipid peroxidation

The control and the experimental groups showed an increase in the plasma TBARS level at the end of study. The percentage of increment was significantly higher ($p < 0.05$) in heated palm olein groups compared to the control and FPO groups (Figure 3).

Discussion

Palm oil is unique in terms of its ratio of SFA to unsaturated fatty acids, which is close to one. Furthermore, it is rich in antioxidant vitamin E. Due to its easy availability and affordable price, palm oil is widely accepted as dietary cooking oil in daily food preparation. In view of that, palm oil (olein) was chosen in our present study. Frying remains as one of the popular method for food preparation. The frequency of frying level in the study was to simulate the cooking condition by street vendors and in most household.

Deep-frying oil contained relatively more SFA with less unsaturated fats as observed in the present study. Even though initially PUFA composition increased and then reduced as palm olein was being repeatedly heated, we did not opine the oil was better after being heated twice in view of other findings such as peroxide values, enzymes activity and lipid peroxidation obtained from current work. Generally, heating at high temperatures has a negative effect on fatty acid composition. Presence of unsaturated bonds in the fatty acid chains render it to free radicals attack produced during frying process. Fats with higher number of unsaturated bonds are prone to oxidation. Increased in peroxide index and TBARS values in our present study may be attributed to the destruction of double bonds by oxidation and polymerization. Heat treatment causes oxidative rancidity which may increase free fatty acids (31). Hence, fatty acid composition was analyzed to observe degradation of fatty acids during frying process.

Peroxide value is used to indicate the extent of oil degradation. It measures the amount of peroxides formed in the cooking oil during oxidation process. From the results obtained, the extent of oxidation rancidity was influenced by the number of frying episode. The more frequent the oil being reheated, the higher the peroxide index. Nevertheless, compared to our previous report, soy oil had a higher peroxide value when it was being repeatedly heated under the same frying condition (12). As increased peroxide value of oil is not suitable for deep frying, our recommendation as per safety values is that the oil may not be reheated more than once.

A higher peroxide value indicates lower chemical stability of oil. Naghshineh et al. (32) postulated a high content of SFA increases the chemical stability of oil. However, peroxide value alone is not sufficient to assess the extent of oxidation and chemical stability of oil. This is because peroxides and hydroperoxides generated during frying process are unstable and easily decomposed to other compounds, reducing the peroxide index (33).

In the study, the changes in the peroxide value of the oil may be associated with a significant increase in plasma MDA. MDA is a major end product of PUFA peroxidation, which often used as an indicator of cell injury. The association may indicate that repeated heating increase oil oxidation which subsequently increases lipid peroxidation. Although lipid peroxidation may be prevented initially by antioxidants such as vitamin E in the oil, however, the repeated heating also destroyed vitamin E content (34).

HO is important in the modulation of BP and vascular tone. Biliverdin and CO, the by-products of HO, have been reported to have cytoprotective effect against oxidative damage (35). Past research study showed that high expression of HO, increased the HO enzyme activity and reduced BP (36). Due to the similarity of the effect exerted by HO-1 and its by-products to those of endothelium-derived nitric oxide (NO), there is a possible relationship between HO-1 and NO pathway. Hence, HO-1 and heme degradation products can improve vascular functions, by compensating for the loss of NO bioavailability (37). Effects of HO-1 enzyme, including depletion of pro-oxidative heme, antioxidant function of bilirubin and signalling action of CO, may also contribute to the prevention of endothelial dysfunction when peroxynitrite is forming from the reaction of superoxide with NO. Peroxynitrite is highly reactive and has negative effects on vascular function and structure (38).

Endothelial HO-1 induction by NO may act as a feedback mechanism to preserve NO-mediated endothelial regulation of vascular function. It has been reported that increasing of HO-1 *in vivo* has protective effects on NO regulation of vascular function associated with an upregulation of other important antioxidant systems protecting the vasculature, such as extracellular superoxide dismutase and plasma catalase activity (39). Thus, antioxidant effects of NO-elicited increases HO-1 expression may participate in preventing endothelial dysfunction. Endothelial dysfunction as observed in vascular disease is often associated with a loss of NO-mediated vasodilatation. Our study reported that HO-1 level was found to be decreased in the reheated oil groups, more prominent in the 10HPO group. The reason for the decrease in HO is not clear. We postulate that more peroxides are formed during frying episodes, and have a direct detrimental effect on the endothelial function. It has been suggested that hypertension is characterized by a decline in endothelial function (40). In addition, the peroxides formed may have affected the HO enzyme structure, thereby leading to the denaturation and destruction. Hence, the enzyme becomes malfunction (41).

The present results showed that ACE level was significantly elevated in the rats fed with heated palm olein. Increased in ACE level that leads to Ang II synthesis may contribute to the elevation of BP. Our finding was in contrast with Yen et al. (42) that reported no changes on the ACE level after consumption of heated vegetable oil. The discrepancy might be due to the duration of study, type of oil and animal used in the experiment.

ACE is required for the conversion of inactive Ang I to Ang II, a potent vasoconstrictor. Ang II-induced hypertension was associated with increased vascular superoxide production and impaired vasorelaxation to acetylcholine (22). Ang II exacerbated oxidative stress, and the increase in the superoxide level could results in endothelial dysfunction via scavenging NO and decreasing NO bioavailability (43). NO generated from endothelium plays a contributory role in determining the balance between relaxation and contraction of vascular smooth muscle. Hence, NO-Ang II imbalance may appear to be an important component in the vascular pathophysiology of hypertension.

Our published work has documented that intake of heated palm oil increased low density lipoprotein (LDL) cholesterol levels (44). In addition, we also reported that oxidized LDL (ox-LDL) is cytotoxic for causing ultrastructural changes in the rat aorta (45). Ang II mediates most of the biological effects of the renin-angiotensin system (RAS), such as vasoconstriction and cell proliferation, via stimulation of the Ang II type 1 (AT_1) receptor. The AT_1 receptor plays an important role in the pathogenesis of atherosclerosis and hypertension. Like oxidized LDL (ox-LDL), Ang II decreases nitric oxide synthase expression and stimulates generation of ROS (22). In addition, Ang II has been suggested to cause an increase in ox-LDL uptake, eventually causes endothelial cell injury (46). On the other hand, it was suggested that ox-LDL upregulates AT_1 receptor expression (47). Previous study showed that hypercholesterolemia is associated with enhanced AT_1 receptor expression (48). These observations may indicate the presence of a relationship between ox-LDL and RAS in hypertension. Together, the two systems may be responsible for endothelial dysfunction.

Our earlier laboratory findings showed that soy oil heated ten times had a significant percentage of increment in BP than 10HPO (unpublished data). Other parameters were also showing more severe effects using soy oil compared to palm olein such as bone histomorphometric properties (49) and lipid peroxidation (16, 50) of ovariectomized rats. From the results obtained, palm olein is more stable to repeated heating than soy oil. This might be due to the unique fatty acid composition and vitamin E content of palm olein.

Conclusion

Consumption of thermally oxidized palm olein may alter the functioning of enzymes involved in the regulation of BP, by increasing ACE level and decreasing HO activity, contributing to the development of hypertension. In addition, heated palm olein increases lipid peroxidation. Hence, palm olein should not be reheated more than once in view of its deleterious effect on health.

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Correspondence

Professor Dr Kamsiah Jaarin
MD, MMedSc Pharmacology
Department of Pharmacology
Faculty of Medicine
Universiti Kebangsaan Malaysia
Jalan Raja Muda Abdul Aziz
50300 Kuala Lumpur
Malaysia
Tel: +603-9289 5285
Fax: +603-2693 8205
Email: kamsiah@medic.ukm.my

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Table 1: Fatty acid composition and peroxide value of fresh and heated palm olein

	FPO	1HPO	2HPO	5HPO	10HPO
Fatty acid					
SFA (%)	42.87	42.64	43.03	43.25	43.28
MUFA (%)	48.94	49.24	47.32	48.21	50.64
PUFA (%)	8.18	8.52	8.87	7.97	6.08
Peroxide values # (mEq O_2/ kg)	2.22 ± 0.25*	6.41 ± 0.15*	8.35 ± 0.09*	9.18 ± 0.06*	11.76 ± 0.23*

FPO, fresh palm olein; 1HPO, palm olein heated once; 2HPO, palm olein heated twice; 5HPO, palm olein heated five times; 10HPO, palm olein heated ten times; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. * Statistic: Analysis of variance (ANOVA); indicates significant difference between groups ($p < 0.05$). # indicates values are average of three estimations (means ± SEM).

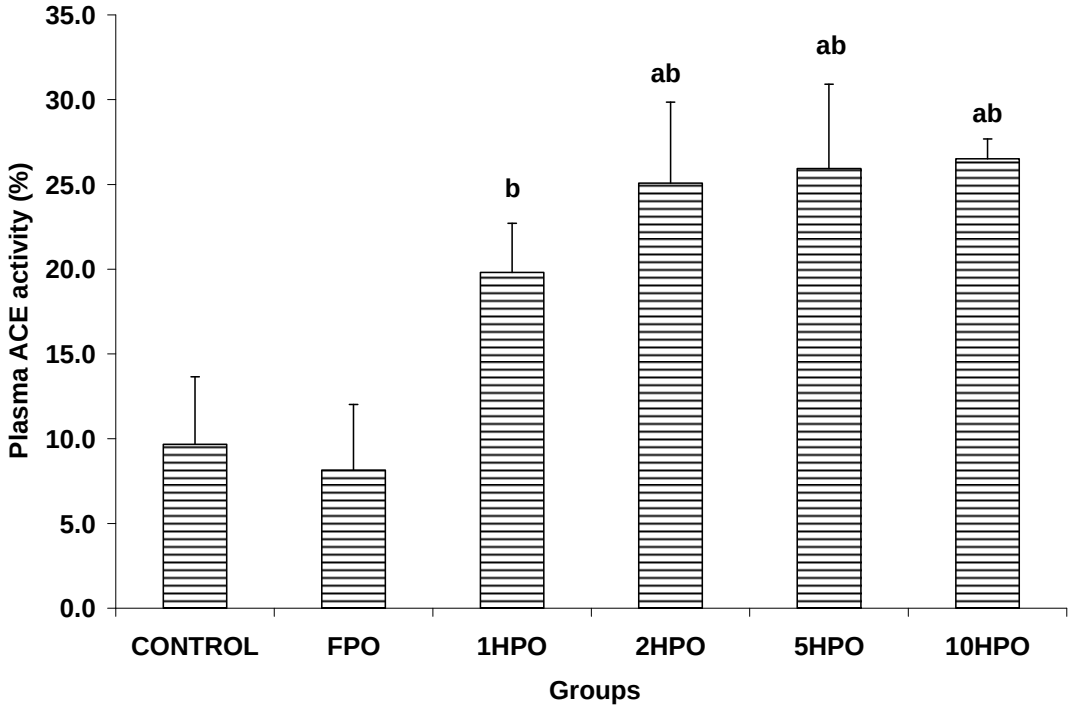

Figure 2: Percentage changes in plasma angiotensin-converting enzyme (ACE) activity after six months of feeding period with basal diet (control), fresh palm olein (FPO), palm olein heated once (1HPO), palm olein heated twice (2HPO), palm olein heated five times (5HPO) or palm olein heated ten times (10HPO). Means ± SEM, $n = 7$. Significant difference at $p < 0.05$ compared to the [a]control, [b]FPO group.

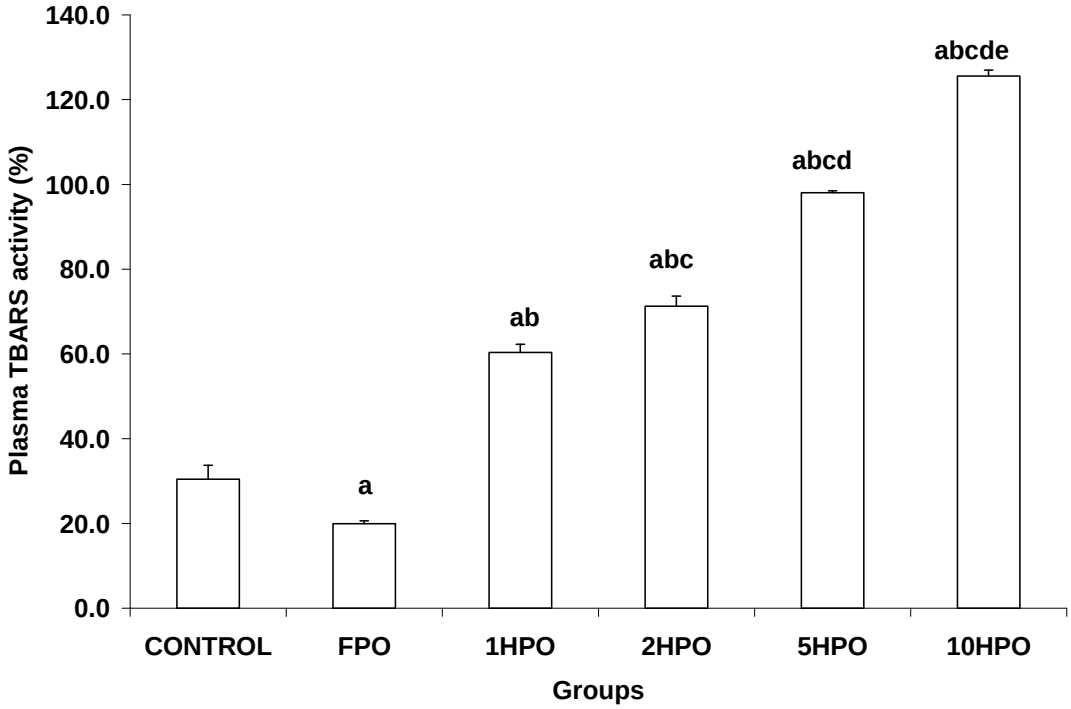

Figure 3: Percentage changes in plasma thiobarbituric acid reactive substances (TBARS) level after six months of feeding period with basal diet (control), fresh palm olein (FPO), palm olein heated once (1HPO), palm olein heated twice (2HPO), palm olein heated five times (5HPO) or palm olein heated ten times (10HPO). Means ± SEM, $n = 7$. Significant difference at $p < 0.05$ compared to the [a]control, [b]FPO group, [c]1HPO group, [d]2HPO group, [e]5HPO group.