

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

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Abstract

Background: Oxidative stress is associated with the pathogenesis of cardiovascular diseases. The process of deep-fat frying in dietary cooking oil plays a role in the generation of free radicals. In this study, palm olein heated to 180 °C 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 6 groups. The first group was fed with normal rat chow as the control group, and the subsequent groups were fed with rat chow fortified with 15% weight/weight of the following: fresh palm olein, palm olein heated once, palm olein heated twice, palm olein heated 5 times, or palm olein heated 10 times. The duration of feeding was 6 months. Fatty acid analyses of oil were performed using gas chromatography. Peroxide values were determined using standard titration. Plasma was collected for biochemical analyses.

Results: Repeatedly heated palm olein increased the levels of peroxide, angiotensin-converting enzyme, and lipid peroxidation as well as reduced the level of heme oxygenase. Fresh palm olein and palm olein heated once had lesser effects on lipid peroxidation and a better effect on the activity of blood pressure–regulating enzymes than repeatedly heated palm olein.

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

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

Introduction

Palm oil obtained from the *Elaeis guineensis* mesocarp exhibits good frying performance, which contributes to its widespread use in deep-frying applications (1). Due to the rising demand and increase in fat intake, palm oil is the major oil in the world's oil and fat market, and palm oil is projected to remain the most influential fat source through 2016 (2). The refined, bleached, and deodorised palm olein, which is fractionated from palm oil, is commonly used as cooking oil. It offers better resistance to oxidation at high temperature during frying as well as natural antioxidants

from the vitamin E group, the tocotrienols (3). Tocotrienols, which have an unsaturated side chain, have greater antioxidant properties than the saturated tocopherols (4). In addition, palm olein contains almost 50% saturated fatty acids (SFA), 50% monounsaturated fatty acids (MUFA), and low levels of polyunsaturated fatty acids (PUFA) under normal conditions (5), which reduce susceptibility to oxidation.

The main economic factor considered in fried food products is the cost of oil because oil is one of the major ingredients in these products. Therefore, very often the oil is repeatedly used to minimise the expense of food preparation.

During the reheating process, the oil undergoes various physical reactions, such as formation of foam, increases in viscosity, darkening of colour, and deterioration of flavour. These changes may affect the organoleptic qualities, such as the odour and taste, and the nutritional value of the fried food (6). Furthermore, repeated heating causes chemical reactions, such as hydrolysis, oxidation, and polymerisation, that alter the chemical structure of triacylglycerol molecules, of which PUFA molecules are affected the most (7).

Thermally oxidised oils, such as those produced by repeated frying, contain a complex mixture of products, such as oxidised monomers, dimers, and polymers. These products have been reported to be the substances mainly responsible for changes in the physicochemical properties of fats (8). When frying oil is heated at high temperatures, toxic products, such as hydroperoxides and aldehydes, are formed, absorbed by the food, and subsequently absorbed into the gastrointestinal system and introduced into systemic circulation after consumption (9). The practice of reusing frying oil causes harmful health effects, 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 by initiating lipid peroxidation. Malondialdehyde (MDA), one of the major secondary oxidation end products of peroxidised PUFA, has been shown to be of biological significance (16).

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

On the other hand, angiotensin-converting enzyme (ACE) plays a vital role in the regulation of BP via hydrolysis of the inactive form of angiotensin I (Ang I) to the active form, angiotensin II (Ang II). ACE is mainly located on the surface of endothelium and epithelium involved in the constriction of blood vessels, which leads to elevation of BP. Effects of Ang II can be observed via 2 types of receptors, AT₁ and AT₂, which both have different pharmacological and biochemical characteristics.

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) oxidases and the production of reactive oxygen species (ROS) (22), which cause hypertension and cardiac failure (23,24). In addition, Ang II increases lipid peroxidation (25) and stimulates the production of pro-oxidant cytokines (26,27) that eventually increase BP.

Our laboratory has previously shown that heated oils increase BP and lead to an impairment in vasorelaxation (10,12). Therefore, in this study, we determined whether heated oils affect BP by increasing vascular reactivity due to a reduction in nitric oxide (NO) content or by affecting the BP-regulating enzymes, ACE and HO. The effect of heated oils on the activity of BP-regulating enzymes and lipid peroxidation was studied in rats.

Materials and Methods

Palm olein and diet preparation

Palm olein was purchased from a local market. The oil was used as either the 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 as follows. First, sweet potatoes were peeled and cut into slices. Next, the slices were deep-fried using fresh palm olein (FPO) for 10 min at 180 °C. The oil was then cooled to room temperature and used to fry another batch of sweet potatoes. The frying process was performed without the addition of fresh oil. The same process was repeated to obtain palm olein heated 5 times and 10 times. Standard rat chow (Gold Coin, Selangor, MY) was ground to coarse powder and fortified with 15% weight/weight (w/w) of the prepared oils. The mixture was subsequently dried in a 70 °C oven overnight. The diet was stored in a closed cabinet and prepared weekly.

Animals and study protocol

Three-month-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, 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 6 experimental groups. The experimental groups were as follows:

- a. Group 1 (control): normal rat chow
- b. Group 2: 15% w/w FPO mixed with chow
- c. Group 3: 15% w/w palm olein heated once (1HPO) mixed with chow
- d. Group 4: 15% w/w palm olein heated twice (2HPO) mixed with chow
- e. Group 5: 15% w/w palm olein heated 5 times (5HPO) mixed with chow
- f. Group 6: 15% w/w palm olein heated 10 times (10HPO) mixed with chow

All rats were kept in stainless steel cages in a room maintained at 27 °C (SD 2) with a 12-hour light-dark cycle. The animals had free access to water and food throughout the study. The rats were fed daily with the oil diet based on their respective groups for 6 months after 1 week of adaptation. Blood was collected through the orbital sinus prior to treatment and at the end of the study. The blood was then centrifuged to obtain plasma. Aliquots of the plasma were stored at -70 °C and used later for the enzyme activity and lipid peroxidation studies.

Fatty acid composition

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

Peroxide measurement

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

Biochemical measurements

The HO-1 (Assay Designs, Michigan, US) and ACE (USCNLife, Wuhan, CN) activities were analysed in plasma samples using commercially available kits. The coloured end products of these 2 enzymes were measured in a microplate reader (Molecular Devices, California, US) at 450 nm. These measurements were performed according

to a previous protocol (12) and following the manufacturers' instructions.

Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation in the plasma samples, as determined by the MDA levels, was measured using thiobarbituric acid. The plasma TBARS levels were estimated following a previously described method (29) with some modifications. Briefly, 2.5 mL of 1.22 M trichloroacetic acid/0.6 M hydrochloric acid was used to acidify 0.5 mL of the plasma sample, which was incubated at room temperature for 15 min. Next, 1.5 mL of 0.67% thiobarbituric acid/0.05 M sodium hydroxide was added, and the samples were incubated for 30 min in a 100 °C water bath. After the mixture had cooled to room temperature, 4 mL of *n*-butanol was added. The mixture was then centrifuged for 10 min at 1500g. The supernatant was analysed against *n*-butanol (Ex: 515, Em: 553) using a spectrofluorometer (Shimadzu RF500, Kyoto, JP).

Protein content

The protein content of the plasma was determined using a method described in Lowry et al. (30) with some modifications. About 5 mL of a 100:1:1 mixture of 2% sodium carbonate, 2% sodium or potassium tartrate, and 1% copper sulphate solution was added to 0.5 mL plasma. Next, the mixture was incubated at room temperature for 15 min, and 0.5 mL of diluted Folin-Ciocalteu phenol reagent was then added. After 35 min, the absorbance of the mixture was measured at 700 nm with a spectrophotometer (Shimadzu UV-160A, Kyoto, JP). The results are expressed as TBARS/protein (nmol/mg protein).

Statistical analyses

The results for the BP-regulating enzymes activities and lipid peroxidation levels were presented as percentages of the baseline values. All data analyses were conducted using SPSS version 13.0 (SPSS Inc., Chicago, Illinois, US). Normality of the data was determined by Kolmogorov-Smirnov test. The peroxide values among the dietary groups were compared using one-way analysis of variance (ANOVA) with Tukey's Honestly Significant Differences post-hoc test for differences between pairs of means when applicable. To analyse the differences in the BP-regulating enzyme activities and levels of lipid peroxidation among the experimental groups, the Kruskal-Wallis and Mann-Whitney tests were performed. Statistical significance was defined as $P < 0.05$. Data are expressed as means (SD).

Results

Fatty acid composition of palm olein

Fatty acid analyses of fresh oil and oil subjected to different frying levels are shown in Table 1. All of the main fatty acids were present in the oil regardless of the frequency of frying.

Peroxide content of palm olein

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

Activity of blood pressure-regulating enzymes

All groups, including the control, exhibited a reduction in plasma HO-1 levels after 6 months of feeding. HO-1 activity was significantly lower ($P < 0.05$) in the heated palm olein groups (Figure 1). The activity of ACE was also found to be significantly increased ($P < 0.05$) in the heated palm olein groups. The percentage increase was significantly higher in the 10HPO group compared with the FPO and other dietary groups (Figure 2).

Lipid peroxidation

The control and the experimental groups showed increases in the plasma TBARS level at the end of study. The percentage increase was significantly higher ($P < 0.05$) in heated palm olein groups compared with 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 the antioxidant vitamin E. Due to its availability and affordable price, palm oil is widely used as a dietary cooking oil in daily

food preparation. Therefore, palm oil (olein) was chosen for our present study. Frying remains one of the most popular methods for food preparation. The various frequencies of frying were used in this study to simulate the cooking conditions used by street vendors and most households.

Deep-frying oil contained relatively more SFA with less unsaturated fats, as observed in the present study. Initially, the PUFA composition increased and then decreased as palm olein was repeatedly heated. However, due to the other findings shown in this work, such as the peroxide values, enzymes activities and lipid peroxidation values, we do not think that the oil was improved after being heated twice. Generally, heating at high temperatures has a negative effect on the fatty acid composition. The presence of unsaturated bonds in the fatty acid chains render it accessible to attack from the free radicals produced during frying process. Fats with higher numbers of unsaturated bonds are prone to oxidation. The increased peroxide and TBARS values shown in our present study may be attributed to the destruction of double bonds by oxidation and polymerisation. Heat treatment causes oxidative rancidity, which may increase free fatty acids (31). Hence, the fatty acid composition was analysed to observe the degradation of fatty acids during the frying process.

The peroxide value is used to indicate the extent of oil degradation. It measures the amount of peroxides formed in the cooking oil during the process of oxidation. From the results obtained, the extent of oxidation rancidity was influenced by the number of frying episodes. The more frequently the oil was reheated, the higher the peroxide index. Nevertheless, compared with our previous report, soy oil had a higher peroxide value when it was repeatedly heated under the same frying conditions (12). As an increased peroxide value indicates that oil is not suitable

Table 1: Fatty acid compositions and peroxide values of fresh and heated palm olein

| Composition | FPO | 1HPO | 2HPO | 5HPO | 10HPO |
|--|--------------------------|--------------------------|--------------------------|--------------------------|---------------------------|
| 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 (mEq O ₂ /kg) ^a | 2.22 (0.44) ^b | 6.41 (0.26) ^b | 8.35 (0.16) ^b | 9.18 (0.11) ^b | 11.76 (0.40) ^b |

^a Values are the averages of 3 estimations in mean (SD). ^b Analysis of variance indicates significant difference between groups ($P < 0.05$). Abbreviations: FPO = fresh palm olein, 1HPO = palm olein heated once, 2HPO = palm olein heated twice, 5HPO = palm olein heated 5 times, 10HPO = palm olein heated 10 times, SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

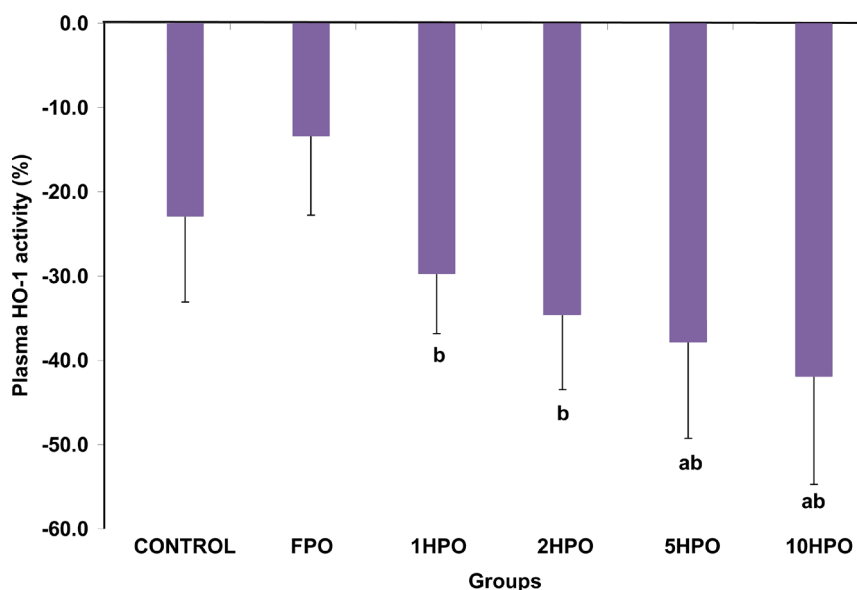


Figure 1: Percentage of changes in the plasma heme oxygenase-1 (HO-1) activity after 6 months of feeding with the basal diet (control), fresh palm olein (FPO), palm olein heated once (1HPO), palm olein heated twice (2HPO), palm olein heated 5 times (5HPO), or palm olein heated 10 times (10HPO). The bars represent mean, and the error bars, SD, with $n = 7$ in each group. Significant differences ($P < 0.05$) were observed in the heated palm olein groups compared with the ^a control or ^b FPO groups.

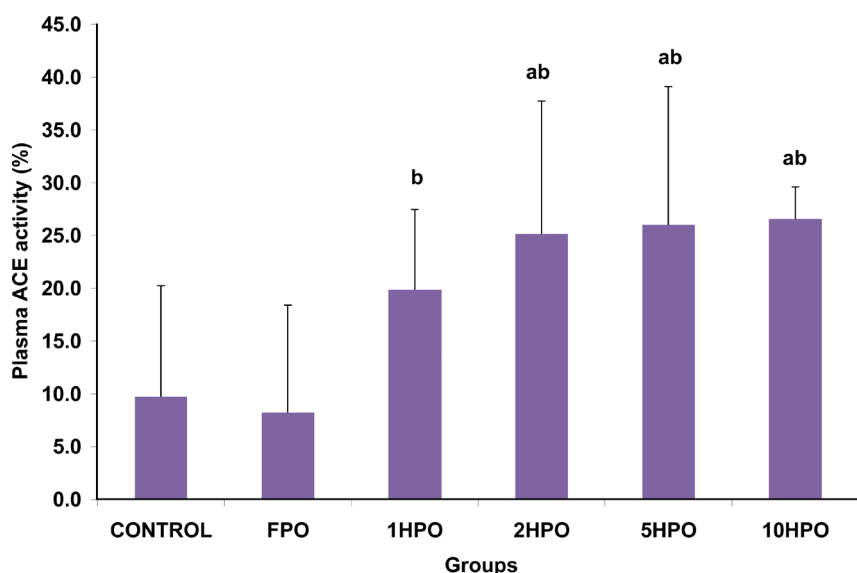


Figure 2: Percentage of changes in plasma angiotensin-converting enzyme (ACE) activity after 6 months of feeding with the basal diet (control), fresh palm olein (FPO), palm olein heated once (1HPO), palm olein heated twice (2HPO), palm olein heated 5 times (5HPO) or palm olein heated 10 times (10HPO). The bars represent mean, and the error bars, SD, with $n = 7$ in each group. Significant differences ($P < 0.05$) were observed in the heated palm olein groups compared with the ^a control or ^b FPO groups.

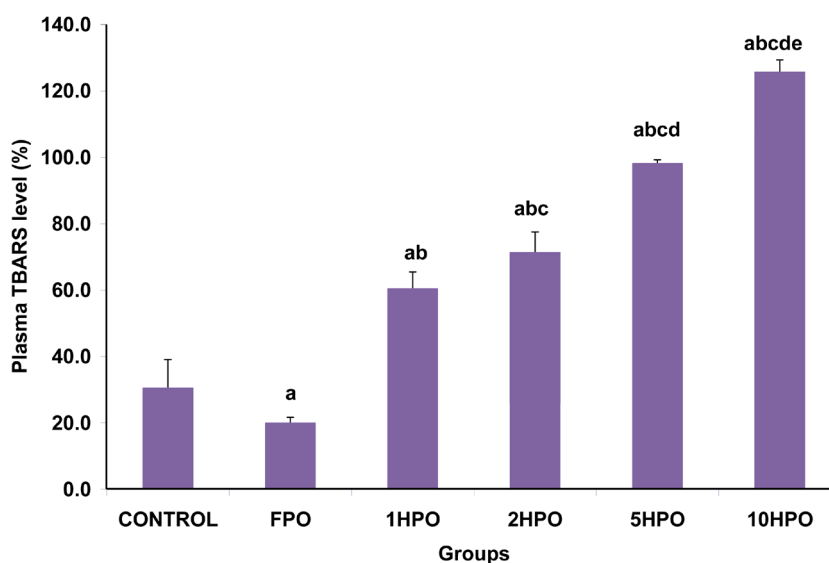


Figure 3: Percentage of changes in the plasma thiobarbituric acid reactive substances (TBARS) level after 6 months of feeding with the basal diet (control), fresh palm olein (FPO), palm olein heated once (1HPO), palm olein heated twice (2HPO), palm olein heated 5 times (5HPO) or palm olein heated 10 times (10HPO). The bars represent mean, and error bars, SD, with $n = 7$ in each group. Significant differences ($P < 0.05$) were observed in the palm olein groups compared with the ^a control, ^b FPO, ^c 1HPO, ^d 2HPO, or ^e 5HPO groups.

for deep-frying, our recommendation concerning safety is that the oil should not be reheated more than once.

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

In this 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 and is often used as an indicator of cell injury. This association may indicate that repeated heating increases oil oxidation, which subsequently increases lipid peroxidation. Although lipid peroxidation may be initially prevented by antioxidants, such as vitamin E, in the oil, repeated heating also decreases the 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 effects against oxidative damage (35). Past research has shown that high expression of HO increases the HO enzyme activity and reduces BP (36). Due to the similarity of the effect exerted by HO-1 and its by-products to those of endothelium-derived NO, there is a possible relationship between the HO-1 and NO pathways. Hence, HO-1 and heme degradation products can improve vascular function by compensating for the loss of NO bioavailability (37). The effects of the HO-1 enzyme, including the depletion of pro-oxidative heme and promotion of the antioxidant function of bilirubin and the signalling action of CO, may also contribute to the prevention of endothelial dysfunction when peroxynitrite is formed 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 HO-1 activity *in vivo* has protective effects on the NO regulation of vascular function and are associated with an upregulation of other important antioxidant systems that protect the vasculature, such as extracellular superoxide dismutase and plasma catalase activities (39). Thus, antioxidant effects of NO-elicited increases in 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 shows that the HO-1 level decreased in the animals fed the heated oil, most prominently in the 10HPO group animals. 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 characterised by a decline in endothelial function (40). In addition, the peroxides formed may have affected the HO enzyme structure, thereby leading to denaturation and destruction of the resulting malfunctioning enzyme (41).

Our study shows that the ACE level is significantly elevated in the rats fed heated palm olein. Increases in the 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), who reported no changes in the ACE level after consumption of heated vegetable oil. The discrepancy might be due to the duration of the study and the 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 is associated with increased vascular superoxide production and impaired vasorelaxation to acetylcholine (22). Ang II exacerbates oxidative stress, and the increase in the superoxide level could result in endothelial dysfunction via scavenging NO and decreasing NO bioavailability (43). NO generated from the endothelium plays a contributory role in determining the balance between relaxation and contraction of vascular smooth muscle. Hence, the NO-Ang II imbalance may be an important component in the vascular pathophysiology of hypertension.

Our previous work has shown that intake of heated palm oil increases low-density lipoprotein (LDL) cholesterol levels (44). In addition, we have also shown that oxidised LDL (ox-LDL) is cytotoxic, 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 (AT₁) receptor. The AT₁ receptor plays an important role in the pathogenesis of atherosclerosis and hypertension. Like ox-LDL, Ang II decreases NO synthase expression and stimulates the generation of ROS (22). In addition, Ang II has been suggested to cause an increase in ox-LDL uptake, eventually causing endothelial cell injury (46). On the other hand, it has also been suggested that ox-LDL upregulates AT₁ receptor expression (47). Previous work has shown that hypercholesterolemia is associated with enhanced AT₁ receptor expression (48). These observations may indicate the presence of a relationship between ox-LDL and RAS in hypertension. Together, the 2 systems may be responsible for endothelial dysfunction.

Our earlier findings showed that soy oil heated 10 times caused a more significant increase in BP compared with 10HPO (49). Other parameters also showed more severe effects using soy oil compared with palm olein, such as bone histomorphometric properties (50) and lipid peroxidation (16,51) of ovariectomised rats. In the results obtained from those studies, palm olein is more resistant 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 oxidised palm olein may affect the functions of enzymes involved in the regulation of BP by increasing the ACE level and decreasing HO activity, which contributes 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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Authors' Contributions

Conception and design, obtaining funding: KJ
 Provision of study materials: JS, KJ
 Collection and assembly of data: XFL, JS
 Analysis and interpretation of the data, drafting of the article, statistical expertise: XFL
 Critical revision and final approval of the article: XFL, JS, MRM, KJ
 Administrative, technical, or logistic support: JS, KJ

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