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Influence of Fresh and Thermoxidized Carotino Oil on Cyclic Guanosine Monophosphate (cGMP) in Erythrocytes from *Sprague Dawley* Rats

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ABSTRACT

Cyclic guanosine monophosphate (cGMP) is a second messenger molecule involved in the intracellular signalling mechanism which is important in a wide range of cellular process including metabolism, gene expression, cell proliferation and cell death. This study was conducted to determine the effect of fresh (FCO) and thermoxidized carotino oil (TCO) on erythrocyte cGMP levels from Sprague dawley rats. A total of 30 Sprague dawley rats were randomly segregated into three groups: the first of which was placed on a Fresh Carotino Oil (FCO) diet, the second on a Thermoxidized Carotino Oil (TCO) diet and the control group on commercial rat chow only for a period of 6 and 9 weeks. The two oil diets comprised of 20% (w:w) of each oil mixed with commercial rat feed. The enzyme immunoassays, performed in week 6, revealed that the erythrocytes cGMP levels for the FCO and TCO groups were 66.198±3.193 pmol/mL and 61.990±6.318 pmol/mL respectively, and were significantly ($p<0.05$) lower than the value for the control group, 77.978±10.479 pmol/mL. The assays performed in week 9 revealed the erythrocytes cGMP levels for the FCO and TCO groups to be 66.522±8.194 pmol/mL and 56.842±8.546 pmol/mL respectively which were also significantly ($p<0.05$) lower than that for the control group, 82.817±6.677 pmol/mL. The results indicate that the presence of antioxidants, such as beta-carotene and tocols in carotino oil may modulate cGMP levels in rats.

Keywords: *Carotino oil, thermoxidation, cyclic guanosine monophosphate, cellular signalling.*

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Introduction

Cellular signalling refers to any process by which a kind of extracellular signal or a first messenger molecule activates a cell surface receptor which in turn activates a second messenger molecule. There are various environmental stimuli able to induce signalling mechanism in living organisms including photons [1] and odorants [2]. The second messenger creates intracellular changes as a result of a series of reactions. Cyclic guanosine monophosphate (cGMP) which is derived from guanosine triphosphate (GTP), acts as a second messenger much like cyclic AMP, by activating intracellular protein kinases when membrane-impermeable peptide hormones bind to the cell surface receptor [3]. It is well recognized that human red blood cells can produce cGMP [4] and that intracellular levels of this cyclic nucleotide may be important in the regulation of red cell membrane viscosity [5], cell deformability [6] and under some circumstances may directly affect ion transport [4-9]. cGMP may also affect the breakdown of cAMP which has in turn been shown to affect red cell ion transport activities [10-13]. Intracellular cGMP can be broken down by phosphodiesterases [4], [7] and [14] or it can be actively effluxed.

Carotino oil is a fully refined cooking oil produced from natural palm fruits, *Elaeis guineensis*, which retains all its phytonutrients. Carotino oil is rich in beta-carotene (approx. 600 ppm) and vitamin E (approx. 800 ppm) in the form of tocopherols and tocotrienols which have been proven to exhibit free radical scavenging activities [16] and improve immune response [17]. In the fresh form, palm oil has been shown to posses low oxidation values [18] and is less susceptible to oxidation [19]. To render oils more palatable, they are repeatedly heated or thermally oxidized. However, the thermoxidation can alter the quality of oils. In addition, ingestion of thermally oxidized oil may result in deleterious effects to cells tissues and organs due to its cytotoxic and destructive by products [20]. To date, it is still unknown whether consumption of fresh and thermoxidized carotino oil alters the intracellular signaling. Thus, the present study was performed to investigate this phenomenon with respect to the level of erythrocyte cGMP in *Sprague dawley* rats placed on normal, fresh (FCO) and thermoxidized carotino oil (TCO) diets determined using enzyme immunoassay. The finding of this study act as a means to update present information pertaining to the signal transduction mechanism with respect to carotino oil supplementation.

Materials and Methods

Materials

Carotino cooking oil was purchased from a local market in Shah Alam, Selangor and divided into two equal parts- the first to be administered in fresh form and the second after undergoing thermal oxidization. Fresh palm oil was heated at 150°C in a stainless steel pot intermittently five times, with each round lasting 20 mins with a 5 hour cooling period between each heating in order to obtain thermally oxidized palm oil with an oxidation number of 15.1. The two test diets: FCO and TCO, were formulated by mixing 20% (w:w) of each oil with commercial rat feed.

Experimental Design

A total of 30 *Sprague dawley* rats aged 8 weeks were randomly segregated into three groups: one control and two test groups. Each group consisting of 10 rats was then divided into two subgroups, 6 weeks (n=5) and 9 weeks (n=5). The first group of 10 rats was placed on a fresh carotino oil diet (first test diet), the second test group was placed on the thermally oxidized carotino oil diet (second test diet) and the third group (control) was placed on a commercial rat chow diet. The animals were housed in stainless steel cages at a room temperature of 27±2°C and quarantined for two weeks prior to being placed on the test diets. All the test and control animals had free access to food (180g/week) and distilled water for the duration of the experimental work: 6 and 9 weeks, after which the rats were dissected and blood samples collected for assays. Permission and approval for the animal studies performed were obtained from the Research Committee on the Ethical Use in Research (UiTM Care).

Enzyme Immunoassay

All blood samples were washed three times using an ice-cold HEPES washing buffer solution and lysed using an ice-cold HEPES lysis buffer solution. Erythrocyte cytosolic fractions used for immunodetection of cGMP molecules were obtained by centrifuging at 2500 g. All samples, cGMP standards, cGMP-alkaline phosphatase conjugate and primary antibody (polyclonal anti-cGMP) were simultaneously incubated at room temperature in a secondary antibody (goat anti-rabbit IgG) coated multiwell

plate. The enzyme reaction was stopped after 2 hours and changes in colour were recorded and the intensity was determined using a multiwall plate reader at a wavelength of 405 nm. The intensity of the colour is inversely proportional to the concentration of cGMP because the primary antibody competitively binds to the cGMP in the samples or cGMP-alkaline phosphatase. The measured optical density (OD) was used to calculate the cGMP concentration (pmol/mL).

Statistical Analysis

The data for the blood parameters were expressed as the mean±S.E.M. and T-test was performed to determine degree of significance between the groups where $p<0.05$ was considered to indicate significance.

Results

Figures 1 and 2 present the body weight gain of the rats placed on fresh carotino oil (FCO), thermoxidized carotino oil (TCO) and control diets for 6 and 9 weeks respectively. In week 6, the body weight gain of the FCO group was 127.8±15.4 g/rat which is significantly ($p<0.05$) higher than the control group (94±11.23 g/rat) and the TCO group (99.6± 5.94 g/rat). The body weight gain of the TCO group is higher than that of the control group, but not significantly. In week 9, the body weight gain of the FCO and TCO groups were 138.2±16.3 g/rat and 144.8±14.4 g/rat which are significantly ($p<0.05$) higher than the control group (106.4±11.7 g/rat). However, the body weight gain of the TCO group is insignificantly ($p>0.05$) greater than the body weight gain of the FCO group.

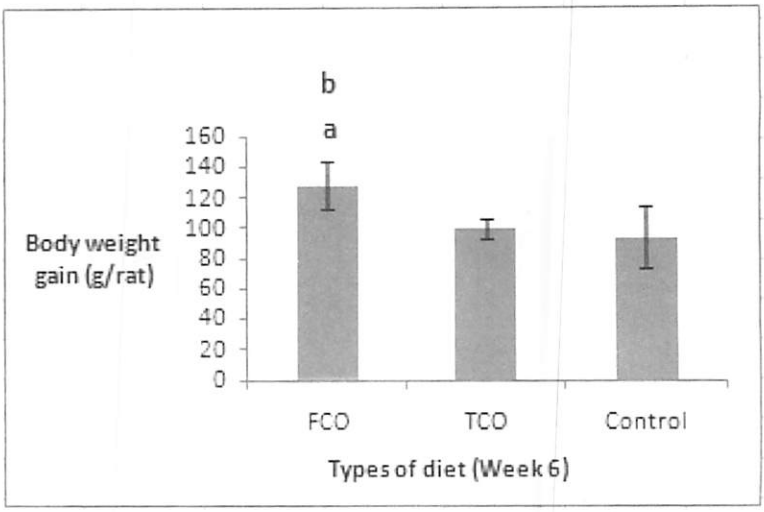

Figure 1: Body Weight Gain of *Sprague Dawley* Rats Placed on Control, Fresh (FCO) and Thermoxidized Carotino Oil (TCO) Diets. The Data Presented Corresponds to a 6-Week Feeding Period whereby Values are in the Form Mean±S.E.M. (n=5). a Indicates a Significant ($p<0.05$) Difference between the Control and Test Diet Groups and b Indicates a Significant ($p<0.05$) Difference between the Two Test Diets

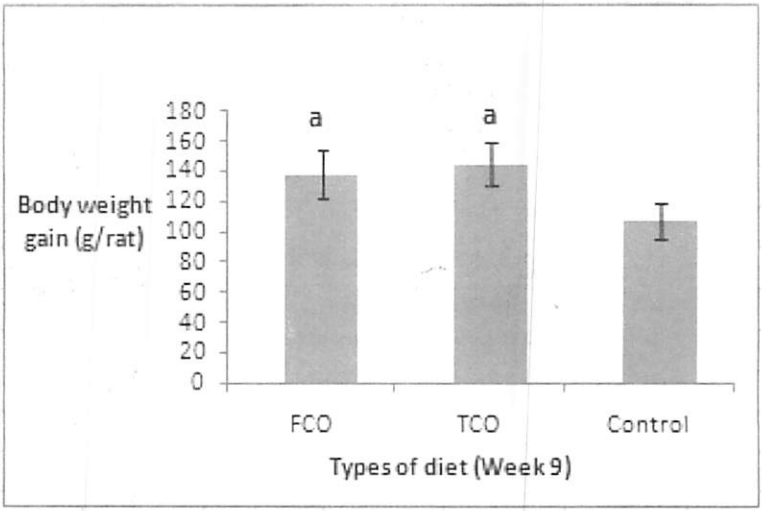

Figure 2: Body Weight Gain of *Sprague Dawley* Rats Placed on Control, Fresh (FCO) and Thermoxidized Carotino Oil (TCO) Diets. The Data Presented Corresponds to a 9-Week Feeding Period whereby Values are in the Form Mean±S.E.M. (n=5). a Indicates a Significant ($p<0.05$) Difference between the Control and Test Diet Groups

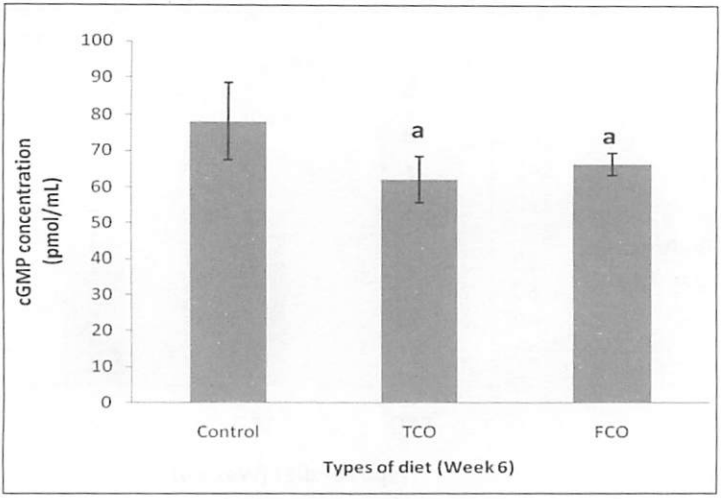

Figure 3: Erythrocyte cGMP Concentration in *Sprague Dawley* Rats Placed on Control, Fresh (FCO) and Thermoxidized Carotino Oil (TCO) Diets. The Data Presented Corresponds to a 6- week Feeding Period whereby Values are in the Form Mean±S.E.M. (n=5). A Indicates Significant ($p<0.05$) Difference between the Control and Test Diet Groups

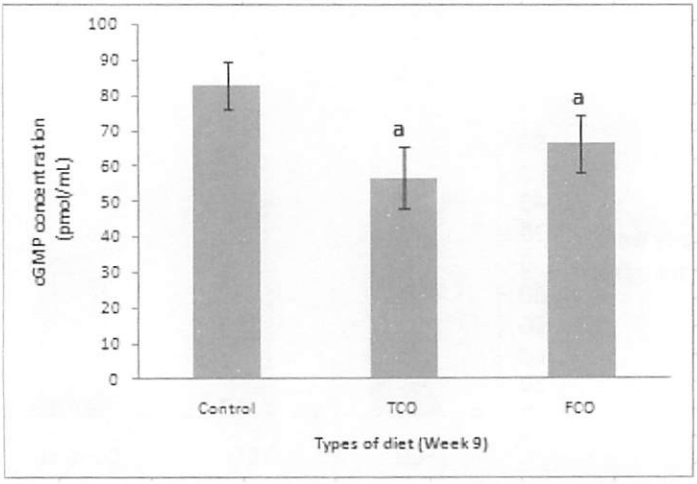

Figure 4: Erythrocyte cGMP Concentration in *Sprague Dawley* RatsPlaced on Control, Fresh (FCO) and Thermoxidized Carotino Oil (TCO) diets. The Data Presented Corresponds to a 9- Week Feeding Period whereby Values are in the Form Mean±S.E.M. (n=5). a Indicates Significant ($p<0.05$) Difference between the Control and Test Diet Groups

Figures 3 and 4 show the level of erythrocyte cGMP in rats placed on FCO, TCO and control diets for 6 and 9 weeks respectively. In week 6, the erythrocyte cGMP levels for the FCO and TCO groups were 66.198±3.193 pmol/mL and 61.990±6.318 pmol/mL respectively which are significantly ($p<0.05$) lower than control group, 77.978±10.479 pmol/mL. In week 9 where the erythrocyte cGMP level for the FCO (66.522±8.194 pmol/mL) and TCO groups (56.842±8.546 pmol/mL) were significantly ($p<0.05$) lower than that for the control group (82.817±6.677 pmol/mL). In both cases, week 6 and 9, the erythrocyte cGMP level for the FCO group is not significantly ($p<0.05$) higher than that for the TCO group.

Discussions

The body weight gain data indicates accelerated growth in the rats placed on test diets with respect to those on the control diet. Palatable foods which are rich in fat and sugar tend increase food intake, hence the activity and expression of signals controlling appetite will be balanced in favour of prolonged eating. From an evolutionary point of view it is logical that such foods are attractive, because it can be rapidly converted into energy [21]. It is therefore plausible that the palatability of rat feed mixed with FCO and TCO has an influence on rat appetite and consequent food intake, which resulted in greater body weight gain. Furthermore, the greater body weight gain for the rats fed on FCO and TCO may be due to fat accumulation in the major organs such as liver, stomach and kidney which was observed during animal dissection and blood sampling especially in week 9.

Preliminary studies were conducted to validate that the determined cGMP levels reflected different diet consumption. A preliminary study indicated that the mean of rat diet intake was 180g±1.02 g/week hence each group as stated in Section 2.2 was provided with a feed of 180g/week and the diet was administered *ad-libitum*, not through force-feeding, in order to allow the animals to self-regulate their intake according to their biological needs. Force-feeding may stress the animals and consequently affect the level of cGMP. The cGMP breakdown by cyclic nucleotide phosphodiesterases can occur during blood sampling, but can be prevented by rinsing the blood sample immediately with ice-cold HEPES buffer solution and placing and maintaining samples in an ice container. It is of note that the isolation and extraction procedure described in Section 2.3 yields the consistent cGMP values of 74.821±7.3 pmol/mL for 8-week rats.

The erythrocyte cGMP level results for rats placed on the FCO and TCO diets are significantly lower than those for rats placed on the control diet, which indicates changes in intracellular signalling may be triggered by FCO and TCO. Such evidence is in agreement with the work of Erickson *et al.* [22] who reported that macrophage protein kinase C (PKC) activity was modulated by dietary fat, containing 10% safflower oil (SAP) and a diet containing 10% menhden fish oil (MFO).). You *et al.* [23] reported the presence of red palm oil in the blood plasma 2-8.5 hours after consumption which means that since the rats had been on a 6 or 9 week diet, it is conceivable that red palm oil would have reached the blood system and been able to affect intracellular signalling inside the erythrocytes.

The cGMP concentrations achieved in red blood cells are a consequence of a balance between synthesis and removal; hence the rate of removal is equally as important as the rate of production. It has been reported that cGMP may play several important roles in erythrocytes including regulation of cellular properties [4-9] and cAMP levels [10-13]. According to Mesembe *et al.* [24] haematological parameters, such as red blood cell (RBC) count and packed cell volume (PCV) in rats placed on a thermoxidized palm oil diet were significantly lower than those placed on fresh palm oil and control diets which may be attributed to impairment of bone marrow and kidney organ with regard to suppressive effects of thermally oxidized palm oil. In the context of the work presented it is believed that the difference in cGMP concentrations between the TCO and control diet groups is related to these suppressive effects and that carotino oil alters erythrocyte properties, through the modulation of cGMP levels, with respect to changes in viscosity and deformability.

Red palm oil is a potent, anti-oxidant rich oil comprising of carotenoids, tocopherols, tocotrienols and lycopenes , which is also high in palmitic (44%) and oleic acid (40%) [25]. Red Palm Oil (RPO) supplementation has been known to cause differential phosphorylation of the mitogen-activated protein kinases (MAPKs), which are associated with improved functional recovery and reduced apoptosis [26]; further to which it has been established that the presence of antioxidants affect cellular signalling [27-28] specifically in relation to protection and better recovery from ischaemia-reperfusion [29-30]. It is therefore fair to postulate that alterations in the erythrocyte cGMP levels are a consequence of the antioxidant properties of carotino oil.

Conclusion

Chronic consumption of fresh and thermoxidized carotino oil influenced the body weight gain of experimental rats predominantly due to fat accumulation in the animal organs. Both the FCO and TCO diets were found to lower the erythrocyte cGMP levels in the rats which may be attributed to the large quantity of antioxidants, in particular beta-carotene and tocols, in carotino oil, which are known to modulate cGMP levels. The findings of this provide further evidence in relation to the effects of a red palm oil diet on upstream intracellular signalling molecules.

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