

Original Research Article

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Supplementation of Virgin Coconut Oil Compared with Copra Oil, Olive Oil and Sunflower Oil on Thrombotic Factors in Rats and *In Vitro* Platelet Aggregation

Sakunthala Arunima and Thankappan Rajamohan*

Department of Biochemistry, University of Kerala, Thiruvananthapuram-695581, Kerala, India

*Corresponding author.

Abstract

Virgin coconut oil (VCO) extracted from fresh coconut kernel is becoming very valuable because of its numerous beneficial properties. In the present study, comparative effect of VCO with copra oil (CO), olive oil (OO) and sunflower oil (SFO) on thrombotic factors and platelet aggregation were investigated. Male Sprague-Dawley rats were fed test oils at 8% level for 45 days along with the synthetic diet. Results demonstrated that compared to CO, a prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT) were observed in VCO fed rats and was comparable with OO and SFO. Supplementation of VCO reduced the coagulation factors namely factor V, fibrin, fibrinogen and thromboxane B2 levels in plasma compared to those fed CO, OO and SFO. Compared to other test oils, platelet aggregating tendency was also reduced in VCO fed rats. The polyphenolic fraction (PF) isolated from VCO inhibited *in vitro* platelet aggregation induced by ADP compared to PF from other oils. These results indicated that supplementation of VCO has significant antithrombotic effect by inhibiting the activation of platelets and coagulation factors compared to rats fed other test oils.

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Introduction

Coronary thrombosis is a major cause of sudden cardiac death, acute myocardial infarction, unstable angina pectoris and silent myocardial ischaemia (Davies and Thomas, 1984; De Wood et al., 1980; Fuster and Chesebro, 1986; Gurfinkel et al., 1994). The thrombogenic state arises when an imbalance exists between procoagulant and profibrinolytic activity (Astrup, 1958; Nossel, 1998). A significant component of thrombogenesis is contributed by platelet and its reactivity (Huo and Ley, 2004). Dietary fat appears to influence both the atherosclerotic and thrombogenic components of coronary heart disease (CHD) (Miller, 1997; Hornstra, 1980). Previous studies have shown that fatty acids affect coagulation of blood (Leray, 2001; Tholstrup, 2003;

Nelson et al., 1997), but the effects of individual fatty acids on haemostasis are still controversial (Hoak, 1997; Knapp, 1997), which depends on both the fatty acid chain length and the degree of saturation (McGregor et al., 1980; Hornstra and Starrenburg, 1973). There is evidence that dietary long-chain saturated fatty acids appear to increase platelet aggregation whereas intake of short and medium-chain fatty acids has been negatively correlated with platelet aggregation (Takachi et al., 2004). Although polyunsaturated fatty acids have been reported to reduce platelet aggregation (Phang et al., 2013; Gao et al., 2013; Moertl et al., 2011), but available evidence on this is equivocal (Mutanen and Freese, 1996; Rand et al., 1988). There are reports which suggest a possible relationship between dietary fat, lipid peroxidation and thrombus formation (Barrowcliffe et al., 1984).

Apart from fatty acids, antioxidant components present in dietary oil may indirectly inhibit platelets through scavenging of reactive oxygen species (ROS) (Petroni et al., 1994). Previous studies from our laboratory have reported that consumption of virgin coconut oil (VCO) exerts significant antithrombotic effect compared to copra oil (CO) in cholesterol fed rats. In the present study, we investigated the comparative effect of consumption of VCO with CO, monounsaturated fatty acid (MUFA) rich olive oil (OO) and polyunsaturated fatty acid (PUFA) rich sunflower oil (SFO) on thrombotic factors and platelet aggregation in rats fed normal diet.

Materials and methods

Chemicals

Adenosine 5'-diphosphate, factor II, fibrinogen, hemoglobin, and other biochemicals were purchased from Sigma Aldrich Co. (St Louis, MO, USA). All the other chemicals used were of analytical grade. 11-dehydro TX B2 immunoassay kit was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA).

Extraction of virgin coconut oil and copra oil

The solid endosperm of mature coconut (West coast tall variety) was crushed, made in to viscous slurry and squeezed through cheese cloth to obtain coconut milk, which was refrigerated for 48 hours, then subjected to mild heating (50° C) in a thermostat oven. The obtained VCO filtered through cheese cloth was used for the present study (Nevin and Rajamohan, 2004). CO was

extracted from coconut meat, which was dried in sunlight continuously for 4 days to remove moisture and the resulting copra was pressed in a mill to obtain CO (Nevin and Rajamohan, 2008).

Olive oil and sunflower oil

Olive oil and sunflower oil were purchased from the local market.

Animals and diet

Male Sprague-Dawley rats (100 –120 g body weight) bred in our department animal house were used for the study. The animals were individually housed under hygienic conditions in polypropylene cages in a room maintained at an ambient temperature of 25°± 10°C with a 12 h light and 12 h dark cycle. The rats were randomly divided into four groups of six rats each. Each rat was given a synthetic diet containing 8% dietary oils daily for 45 days (Table 1). Experimental groups were as follows: Group I rats given CO, Group II rats given VCO, Group III rats given OO and Group IV rats given SFO. All the animal cares and procedures were according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The entire experimental protocol was approved by Institutional Animal Ethics Committee (IAEC), University of Kerala. Food intake was monitored routinely and body weight was determined weekly. After 45 days, animals were fasted overnight and sacrificed by thiopentone sodium injection (>40 mg per kg body weight) and the blood was collected for various estimations.

Table 1. Formulation of synthetic diet.

Ingredients ^a	Group I	Group II	Group III	Group IV
Corn starch	71	71	71	71
Casein	16	16	16	16
Copra oil	8	--	--	--
Virgin coconut oil	--	8	--	--
Olive oil	--	--	8	--
Sunflower oil	--	--	--	8
Salt mixture	4	4	4	4
Vitamin mixture	1	1	1	1

^a g per 100 g wet weight.

Biochemical investigation

Citrated plasma was used for the determination of coagulation parameters viz., prothrombin time, activated partial thromboplastin time, fibrinogen, fibrin, factor V and

11-dehydro thromboxane B2. Prothrombin time (PT) was estimated using liquiplastin kit from Tulip Diagnostics (P) Ltd, Goa, India (Hull et al., 1982). Activated partial thromboplastin time (aPTT) was estimated using Liquicelin kit from Tulip Diagnostics (P) Ltd, Goa, India (Hoffmann

and Meulendijk, 1978). Citrated plasma was diluted with 2 mL of isotonic saline and from this fibrin was estimated according to the method described by King and Wootton (1959). Plasma fibrinogen levels were estimated as described by Clauss (1957) using fibrinogen kit from Tulip Diagnostics (P) Ltd, Goa, India. Factor V was assayed by the method of Daniel (1955). The concentration of 11-dehydro thromboxane B2 in plasma was determined by EIA kit purchased from Cayman Chemical Co.USA (Takasaki, 1991).The absorbance was read at 412 nm and concentration of each sample was obtained from the standard curve.

Platelet preparation and platelet aggregation test

Blood was collected in anticoagulant solution (2.4% sodium citrate, 1.5% citric acid and 1.8% dextrose). The ratio of the blood to anticoagulant solution was approximately 5:1 and the platelet rich plasma (PRP) was separated by centrifugation at 1850 rpm for 7 minutes. PRP was centrifuged at 4500 rpm for 18 minute to sediment the platelets (Chopra, 1999). The platelet sediment was dispersed in washing buffer composed of 113 mM NaCl, 4.3 mM KH₂PO₄, 4.3 mM Na₂HPO₄, 24.44 mM NaH₂PO₄ and 5.5 mM dextrose (pH 6.5) and the platelets were collected after centrifugation at 900 g for 10 minutes. The platelet aggregating activity was measured by spectrophotometric method as described by Joseph et al. (2005).

Platelet aggregation *in vitro*

In vitro platelet aggregation was performed by the method of Duttaroy and Jorgensen (2004). Polyphenols from test oils were extracted according to the method described previously (Arunima and Rajamohan, 2013). 250 µL of platelet suspension was incubated with 50µg of polyphenol fraction isolated from test oils and allowed to stand for 3 minutes. Absorbance of the sample and

control were measured at 600 nm immediately after the addition of agonist (10 µM ADP) and at 120 minutes in a spectrophotometer. The values were expressed as percentage aggregation.

Statistical analysis

Statistical differences were determined using one way ANOVA followed by Duncan's, post-hoc test to identify the differences using SPSS 11.5 (SPSS Inc., Chicago IL, USA). Differences of $p < 0.05$ were considered to be significant. Data are reported as mean \pm SEM unless otherwise stated.

Results

Effect of VCO on blood coagulation parameters

Fig. 1 summarizes the levels of prothrombin time (PT) and activated partial thromboplastin time (aPTT) in rats fed test oils. A prolonged PT and aPTT were observed in VCO fed rats when compared to those fed CO. But no significant difference in PT and aPTT were observed among rats fed VCO, OO and SFO. VCO supplementation significantly ($p < 0.05$) decreased the factor V levels when compared to rats fed other test oils. Factor V levels were also significantly ($p < 0.05$) decreased in CO fed rats in comparison to those fed OO and SFO. But supplementation of SFO increased the factor V levels than OO fed rats (Fig. 2). The concentration of fibrinogen was significantly ($p < 0.05$) decreased in VCO fed rats when compared to other oil fed rats. Fibrinogen levels were also found to be decreased in CO fed rats compared to those fed OO and SFO. Rats fed OO showed increased levels of fibrinogen compared to SFO fed rats. The fibrin levels were also significantly decreased in VCO fed rats compared to other oil fed rats. But there was no significant difference in fibrin levels among rats fed CO, OO and SFO (Table 2).

Table 2. Concentration of fibrinogen (mg/dL) and fibrin (mg/dL).

Groups	Fibrinogen	Fibrin
I	273.12 \pm 27.76 ^a	17.61 \pm 1.6 ^a
II	237.67 \pm 21.69 ^b	15.38 \pm 1.55 ^b
III	319.68 \pm 29.09 ^c	19.64 \pm 1.66 ^{a,c}
IV	275.26 \pm 28.38 ^d	18.32 \pm 1.79 ^{a,c}

Values are mean of six rats \pm SEM, values not sharing a common superscript differs significantly at $p < 0.05$. Group I – 8% CO fed rats; Group II – 8% VCO fed rats; Group III – 8% OO fed rats; Group IV – 8% SFO fed rats.

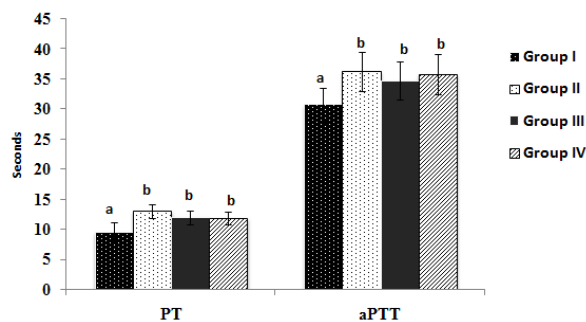


Fig. 1: Prothrombin time and activated partial thromboplastin time. Values are mean of six rats \pm SEM, values not sharing a common superscript differs significantly at $p < 0.05$. Group I – 8% CO fed rats; Group II – 8% VCO fed rats; Group III – 8% OO fed rats; Group IV – 8% SFO fed rats.

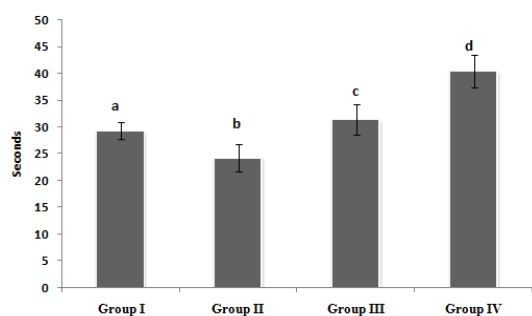


Fig. 2: Concentration of factor V. Values are mean of six rats \pm SEM, values not sharing a common superscript differs significantly at $p < 0.05$. Group I – 8% CO fed rats; Group II – 8% VCO fed rats; Group III – 8% OO fed rats; Group IV – 8% SFO fed rats.

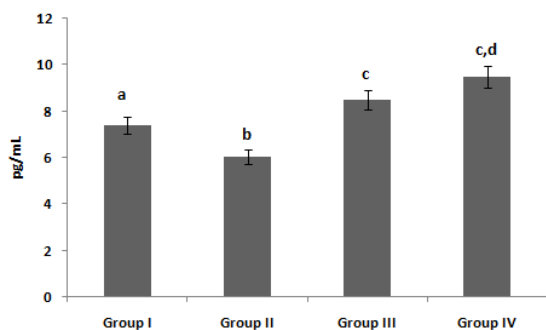


Fig. 3: Concentration of 11-dehydro TX B2 in plasma. Values are mean of six rats \pm SEM, values not sharing a common superscript differs significantly at $p < 0.05$. Group I – 8% CO fed rats; Group II – 8% VCO fed rats; Group III – 8% OO fed rats; Group IV – 8% SFO fed rats.

Effect of VCO on 11-dehydro thromboxane B2 in plasma

Concentration of 11-dehydro thromboxane B2 (11-dehydro TX B2) were significantly ($p < 0.05$) decreased in VCO fed rats compared to those fed other test oils.

Supplementation of CO decreased the 11-dehydro TX B2 levels when compared to rats fed OO and SFO. But there was no significant difference in 11-dehydro TX B2 levels among OO and SFO fed rats (Fig. 3).

Effect of VCO on platelet aggregation *in vivo* and *in vitro*

The platelet aggregating tendency was significantly lowered in VCO supplementation compared to other oil fed rats (Fig. 4). There was no significant difference in the aggregation tendency among rats fed CO and SFO. Results from *in vitro* analysis indicated that polyphenol fraction (PF) extracted from VCO significantly ($p < 0.05$) decreased ADP induced platelet aggregation than other test oils. But there was no significant difference in platelet aggregating tendency among PF isolated from CO, OO and SFO (Fig. 5).

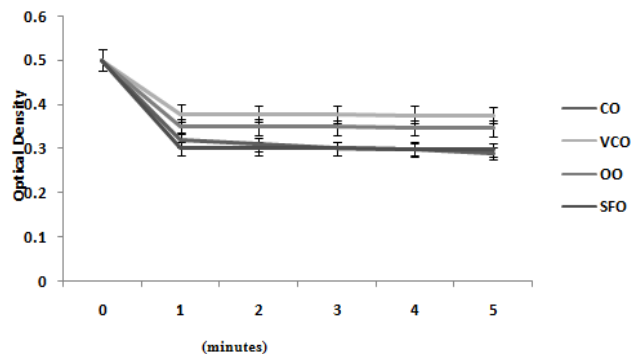


Fig. 4: Platelet aggregation induced by ADP *in vivo*. Values are mean of six rats \pm SEM, values not sharing a common superscript differs significantly at $p < 0.05$. Group I – 8% CO fed rats; Group II – 8% VCO fed rats; Group III – 8% OO fed rats; Group IV – 8% SFO fed rats.

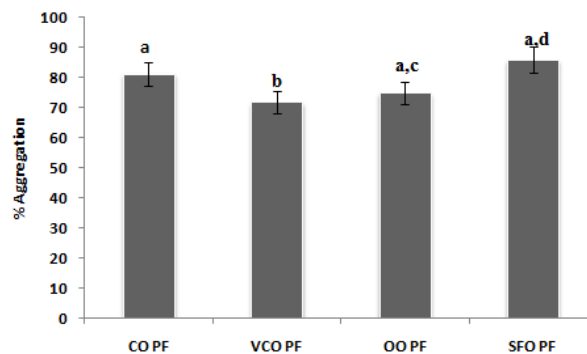


Fig. 5: Platelet aggregation induced by ADP *in vitro*. Values are mean of six experiments. Values not sharing a common superscript differs significantly at $p < 0.05$. Group I- PRP + ADP (10 μ M) + 50 μ g CO PF; Group II- PRP + ADP (10 μ M) + 50 μ g VCO PF; Group III- PRP + ADP (10 μ M) + 50 μ g OO PF; Group IV- PRP + ADP (10 μ M) + 50 μ g SFO PF.

Discussion

Results obtained indicate that supplementation of VCO had a significant beneficial effect on blood coagulation factors in rats fed normal diet. PT and the aPTT are global coagulation tests used to assess the coagulation system. PT, which measures the clotting time of plasma in the presence of thromboplastin, was determined to assess the efficiency of extrinsic system, while, aPTT depends on substances normally present in blood for its activity and to assess the intrinsic pathway. Studies suggested that short aPTT probably represent an increase in the procoagulant potential (McKenna et al., 1977) and were associated with an increased risk of thrombosis (Landi et al., 1992; Gallus et al., 1973). In the present study, a prolonged PT and aPTT were observed in VCO fed rats when compared to CO fed rats. Studies revealed that apart from fatty acids, the unsaponifiable components present in dietary oils have a role in regulating the coagulation system. There are reports that polyphenols possess antithrombotic effects, it was evidenced by a prolonged PT and aPTT (Kim et al., 2012). Chemical analysis of the test oils has revealed that VCO by wet processing retains higher amounts of polyphenols and tocopherols than other test oils (Arunima and Rajamohan, 2013). These higher amounts of polyphenols present in VCO may prolong the PT and aPTT and which may be one of the reasons for its antithrombotic potential.

The factor V levels were found to be decreased in VCO fed rats compared to other groups. Factor V is a 330-kDa glycoprotein synthesized in the liver and is released in the bloodstream as a single-chain inactive pro-cofactor. After limited proteolysis by thrombin or factor Xa, factor V is converted to its activated form, factor Va (Monkovic and Tracy, 1990). The factor Va acts as a non enzymatic cofactor of factor Xa in the conversion of prothrombin to thrombin (Nesheim et al., 1979; Rosing et al., 1980). The lesser levels of factor V observed in VCO fed rats reflects reduced thrombotic risk. Moreover, low levels of both fibrin and fibrinogen observed in VCO fed rats are associated with decreased clotting of the blood. A raised plasma fibrinogen concentration is a powerful predictor of risk of fatal CHD (Miller et al., 1996). Clotting response is enhanced by activated blood platelets and the fibrin formed reinforces the fragile platelet mass to a stabilized thrombus of great pathological significance (Mosesson, 2005). Elevated fibrin concentration, lowered prothrombin time and activated partial thromboplastin time are an indication of hypercoagulability (Curnow et al., 2007; McKenna et al., 1977).

Blood clotting is affected by many substances within our body, which depends on a balance between substances that promote coagulation and those that inhibit it (Lipe and Ornstein, 2011). Thromboxane A₂ (TX A₂) produced by activated platelets, has prothrombotic properties, stimulating activation of new platelets as well as increasing the platelet aggregation. There are reports that thromboxane-dependent platelet activation enhances cerebral thrombosis (Patrono et al., 1991). Moreover, TX A₂ acts as both a vasoconstrictor and platelet activator (Ellis et al., 1976). TX A₂ has a short half-life in the body and is rapidly hydrolyzed to thromboxane B₂ (TX B₂). 11-dehydro TX B₂ is a metabolite of TX B₂ with a circulating half-life ($t_{1/2}$) of 45 minutes; its measurement in plasma or urine will give a time-integrated indication of TX A₂ production (Catella et al., 1986). An increased production of TX B₂ might be contributory to thrombosis (Saldeen et al., 1983). Our results revealed significant decrease in 11-dehydro TX B₂ levels in VCO fed rats as compared to those fed other oils. There are reports that saturated fats have an inhibitory effect on TX B₂ production (Steel et al., 1990), while, unsaturated fatty acids *viz.*, oleic, linoleic and arachidonic acid enhances the production of TX B₂ (Ishitsuka et al., 2004; Muakkassa et al., 1991; Whelan et al., 1993). But supplementation of n-3 PUFA reduces TX B₂ production (Vilaseca et al., 1990).

Blood platelets are known to play a role in the regulation of hemostasis and thrombosis (Véricel et al., 2004) and consequently in the major cardiovascular complications (Massberg et al., 2002). Platelet-dependent thrombus formation plays an essential role in the manifestation of ischemic heart syndrome (Meade et al., 1986; Heinrich et al., 1994). Platelets are thought to initiate a series of intricate reactions by adhering to the injured arterial lining, aggregating irreversibly to form a platelet plug, and releasing vasoactive metabolites and hydrolytic enzymes that might in turn alter both the function and structure of the vessel (Colman, 1975). Platelets mediate both thrombotic occlusion of the entire epicardial coronary artery and also accumulate in the microcirculation resulting in impairment of microcirculation and provoking myocardial ischemia during reperfusion (Gawaz, 2004).

Studies suggest that individual saturated fatty acids differently affect platelet aggregation capacity (Fuhrman et al., 1986; Renaud et al., 1986). There are reports that chain length of fatty acids plays an important role in platelet aggregation and the inhibitory effect on platelet aggregation was increased with increase in chain length

up to C 14 (Kitagawa et al., 1984). Fatty acid analysis of test oils revealed that oil extracted from coconut mostly consists of short and medium chain fatty acids (Arunima and Rajamohan, 2013). *In vivo* experiments have shown that feeding medium chain triglycerides (MCT) decreases thrombosis formation in rats (Kaunitz, 1986). Intervention studies have shown that increased platelet aggregation was found in linoleic acid enriched diets (Mutanen and Freese, 1996). Apart from fatty acids, polyphenols and tocotrienols are known to inhibit platelet aggregation (De Lange et al., 2007; Qureshi et al., 2011). Chemical analysis of these test oils has revealed that VCO by wet processing contain increased polyphenolic contents (84 mg/100 g oil), which is significantly ($p < 0.05$) higher than other test oils viz., CO (64.4 mg/100 g oil), OO (75.63 mg/100 g oil) and SFO (55.26 mg/100 g oil).

HPLC analysis of the phenolic fraction of VCO has revealed the presence of caffeic acid, *p*-coumaric acid, ferulic acid, (+)-catechin hydrate and syringic acid compared with CO, OO and SFO (Arunima and Rajamohan, 2013), which may have a synergistic effect on platelet aggregation. In addition, the nonsaponifiable fraction of VCO contains appreciably higher amounts of antioxidants, namely vitamin E (33.12 mg/100 g oil) and β -carotene (196mg/100 g oil) (Arunima and Rajamohan, 2012). Increased amounts of these non-saponifiable components present in VCO may partly be responsible for the decreased platelet aggregation compared with other oils. The inhibitory effect of VCO on platelet aggregation was confirmed by the *in vitro* platelet aggregation assay using polyphenol fraction isolated from test oils. A decreased aggregation rate was observed with VCO polyphenols on ADP induced *in vitro* platelet aggregation.

These results demonstrated that supplementation of VCO have a significant antithrombotic effect compared to those fed CO, OO and SFO, which is characterized by an increased fibrinolytic activity as well as decreased rate of platelet aggregation.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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of Kerala had no role in the design, analysis or writing of this article. All authors read and approved the final manuscript. The authors declare that there are no real or perceived conflicts of interest.

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