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Original Article

Repeatedly heated cooking oils alter platelet functions in cholesterol fed Sprague dawley rats.

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ABSTRACT

Oxidized dietary lipids influence platelet activation and activated platelets play crucial role in cardiovascular events. The study was designed to investigate the comparative effects of fresh and repeatedly heated culinary oils on platelet function in rats. Coconut oil, mustard oil and sunflower oil, each representative of saturated, monounsaturated and polyunsaturated fatty acid rich oils respectively, were used for the study. Test oils were heated at 210 ± 10^{0}C for fifteen hours. Rats were divided into six groups. Fresh /heated oils (15%) and cholesterol (1%) were fed along mixed synthetic diet for eight weeks. Fatty acid analysis shows that unsaturation decreases and saturation increases as frying oil degrades. Chemical analysis revealed that the degree of deterioration is more in heated oils compared to unheated oils, but the effect was lower in heated coconut oil. In heated oil fed groups, both plasma lipids and peroxide levels were significantly elevated. HDL cholesterol levels showed a reduction in all heated oil fed groups. Relative to the fresh oil fed groups, platelet aggregating tendency was significantly increased in heated oil fed groups. Also, platelet function parameters and clotting factors were altered in heated oil fed groups in comparison with fresh oil fed groups. Heated coconut oil fed group showed lower tendency towards hyperlipidemia, peroxidation, platelet aggregation, platelet function alterations and clotting among heated oil fed groups. From these studies, it is concluded that, dietary oils heated repeatedly at elevated temperature results in significant alterations in platelet functions, compared to fresh oils in rats and the deleterious effects were lower in heated coconut oil compared to heated mustard oil and sunflower oil.

1. Introduction

Various risk factors including elevated cholesterol levels, oxidative modification of low density lipoprotein, platelet aggregation and endothelial dysfunction can contribute to cardiovascular diseases [1]. Atherosclerosis is a complex disease, characterized by severe inflammatory response towards damage of the arterial wall involving several cell types, including platelets [2]. Platelets play a major role in the hemostatic process and in thrombus formation after an endothelial injury [3].

Cooking oils function as heat transfer medium and contribute to flavor and texture of food in culinary purposes [4]. Fatty acids can affect platelet function by interacting with membrane proteins, serving as precursors for secondary messengers and thereby modify the response of platelets to aggregating agents [5]. Studies indicate that consumption of saturated fatty acids induce hyperlipidemia causing progression of atherosclerosis compared to unsaturated fatty acids [6, 7]. Based on the reports regarding the hypolipidemic effects of unsaturated oils, they are gaining popularity among consumers. Several reports indicate that saturated fatty acid percentage in oils and fats is directly related to the platelet aggregating tendency and heating increases saturated fatty acid content in oils [8, 9]. Based on these reports, coconut oil, mustard oil and sunflower oil, each representative of saturated, monounsaturated and polyunsaturated fatty acid rich oils respectively were selected for investigating platelet functions.

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deep fried foods are increasingly being consumed by public and during frying purposes cooking oils are subjected to elevated temperatures. Several chemical reactions such as hydrolysis, oxidation and polymerization takes place in oils, which produce volatile and non-volatile components having important physiological effects [10]. Thermally oxidized fat generates toxic lipid peroxidation products that would induce oxidative stress in animals [11]. Degree of saturation of oil is an important factor determining the quality of cooking oils. Unsaturated fatty acids are more susceptible to lipid oxidation than saturated fatty acids and for this reason they are good source of free radicals [12].Oxidative stress produced by free radicals is associated with the development of atherosclerosis. Oxidative stress is associated with activation of platelets and for the same reason, a role can be assigned for platelets in the cardiovascular events that are related to oxidative stress [13]. Studies regarding the effects of repeatedly heated cooking oils on platelet functions are rare and studies comparing the effects among repeatedly heated saturated, monounsaturated and polyunsaturated fatty acid rich oils are yet to come out.

In view of the above observations, the present study was designed to investigate the effects of feeding fresh and heated cooking oils on parameters such as plasma lipid levels, platelet peroxide content, hematological parameters of platelet function, coagulation parameters and platelet aggregation induced by agonists such as ADP in cholesterol fed rats.

2. Materials and Methods

2.1. Preparation of test oils

Coconut oil, mustard oil and sunflower oil were purchased locally. Heating of oils was done in gas stove that was adjusted to maintain the oil temperature at 210 ± 10^0C. Oils were heated three hours per day for five days. Samples were stored after cooling.

2.2. Animals and diet

Male Sprague–Dawley rats weighing 150–180 g were used for the study. The study was approved by institutional ethics committee for animal experiments. The animals were housed individually in polypropylene cages in a room maintained at 25 ± 10^0 C with 12 hours light and 12 hours dark cycle and provided with food and water ad libitum. Oils were fed at 15% level and cholesterol at 1% level mixed with synthetic diet for 60 days.

Composition of the experimental diet (%):
Corn starch-64%
Casein-16%
Oil*-15%
Salt mixture**- 4%
Vitamin mixture***- 1%

*The dietary oils used were: unheated and heated coconut oil, unheated and heated mustard oil & unheated and heated sunflower oil. **Salt Mixture: HMW- Hubbell et al., 1937[14].***Vitamin mixture: Retinyl palmitate (1000 IU), ergocalciferol (150 IU), α-tocopherol (12 mg), menadione (0.3 mg), thiamine(1.0 mg), riboflavin(1.0 mg), pyridoxine(0.6 mg), niacin(10.0 mg), calcium pantothenate (5.0 mg), inositol (20 mg), folic acid(0.4 mg), vitamin B12(3.0µg), biotin(20µg) and choline chloride(300 mg).

Animals were divided into six groups as follows: Group I, unheated coconut oil (UHCO); Group II, heated coconut oil (HCO); Group III, unheated mustard oil (UHMO); Group IV, heated mustard oil (HMO); Group V, unheated sunflower oil (UHSO) and Group VI, heated sunflower oil (HSO). The food intake was recorded daily and body weight of the rats recorded weekly. After completion of experimental period, rats were fasted overnight and sacrificed and blood was collected for various estimations.

2.3. Physico chemical characteristics of oil samples

2.3.1. Fatty acid analysis and calculated oxidisability value

The fatty acid composition of oil samples were analyzed in duplicates by gas chromatography. Fatty acids were converted to their methyl esters (FAME) prior to analysis [15]. Shimadzu GC-2010 Gas Chromatograph equipped with Flame Ionization Detector (Shimadzu Corporation, Kyoto, Japan) was used to determine the FAMEs composition of the tested oils. The column used was capillary column (DB-23,30m length, 0.32mm id wide bore, 0.25µm film thickness, Agilent technologies USA). Nitrogen gas was used as carrier gas with flow rate 30 ml/min and pressure 180 psi. Fatty acids were identified by comparing their retention time with authentic standards (Sigma, Sydney, Australia). The calculated oxidisability value (Cox) value of the oils was calculated by applying the formula proposed by Fatemi and Hammond [16].

Cox = [1(16:1 % + 18:1% + 20:1% + 22:1%) +10.3 (18:2 %) + 21.6 (18:3 %)] /100

2.3.2. Peroxide value

Peroxide value (PV) was determined according to AOCS Official method Cd 8-53 [17].

2.4. Plasma Lipid Profile

Plasma samples were analyzed for total cholesterol [18], triglycerides [19] and high density lipoprotein cholesterol [20].

2.5. Platelet Function

2.5.1. Platelet Preparation and Platelet aggregation test

The platelet aggregating activity was measured by spectrophotometric method as described by Sophia et al. [21]. 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 minutes to sediment the platelets [22].The platelet sediment was dispersed in washing buffer composed of 113 mM NaCl, 4.3 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 24.44 mM NaH_2PO_4 and 5.5 mM dextrose, pH 6.5 and the platelets were collected after centrifugation at 900 g for 10 minutes. The platelets were then suspended in a buffer composed of 109 mM NaCl, 4.3 mM K_2HPO_4, 16 mM Na_2HPO^4, 8.3 mM NaH_2PO_4 and 5.5 mM dextrose, pH 7.5 [23]. The suspension was adjusted to give a final optical density of approximately 0.5 at 600 nm. To 1 ml of platelet suspension, 20 µl of agonist ADP (1 mM) was added and the OD at 600 nm was measured at 1 minute intervals up to 5 minutes in a spectrophotometer.

2.5.2. Platelet function parameters

The whole blood is collected in EDTA and Platelet count (PC), Mean Platelet volume (MPV), Platelet crit (PCT) & Platelet distribution width (PDW) were analyzed using blood cell counter.

2.5.3 Estimation of platelet lipid peroxidation

Platelet TBARS were estimated by the method of thiobarbituric acid reaction as described by Ohkawa et al. [24].

2.5.4. Platelet in vitro lipid peroxidation

In order to test the effects of free radicals, the platelet preparation was incubated for 30 min. in the presence of an oxidant-generating system (0.5 mM H_20_2, 0.02 mM $FeCI_2$ and 5 mM ascorbate) at 37^0C [25]. At the end of 30 minutes incubation, lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) formation [24]. For TBARS determination test material was heated at acid pH with thiobarbituric acid (TBA) and the resulting chromogen extracted into butanol was measured at 532 nm.

2.7. Coagulation parameters

Fibrin was estimated as described by King and Wootton [26]. Citrated plasma was used for the determination of prothrombin time (PT) and activated partial thromboplastin time (APTT) as described by Dacie and Lewis [27].

2.8. Statistical analysis

The results are presented as mean value ± standard deviation for six rats. Results were analyzed using statistical programme SPSS version 11.5(SPSS Inc, Chicago, IL, USA).Comparisons between the six groups were performed by one-way ANOVA followed by Duncan's post-hoc multiple comparison tests. Significance was defined at $p<0.05$.

3. Results and Discussion

Repeated heating of cooking oils resulted in significant alterations in the physico-chemical characteristics of test oils compared to fresh oils [Table 1]. Repeatedly heated oils were having rancid odor and dark color compared to the fresh oils. Gas chromatographic analysis was done to determine the fatty acid composition of oil samples and oxidisability rates of oils were calculated from the fatty acid composition. The result [Table 1] shows the fatty acid composition of the test oils, which indicates that there was significant decrease in unsaturation and increase in saturation as frying oil degrades. There were reports that PUFA content of oils used for frying showed significant decrease in comparison with fatty acid composition of the same brand of oil before use [28]. Fats are oxidized by free radicals at the sites of unsaturated bonds in the fatty acid chains. Oils having higher ratios of unsaturated fatty acids are thus, more susceptible to oxidation. The calculated oxidizability index (Cox) for coconut oil, mustard oil and sunflower oil were 0.234, 4.06 and 6.55 respectively. These values revealed that sunflower oil, which is rich in polyunsaturated fatty acids, may be much more susceptible to oxidation compared with coconut oil and mustard oil. Oxidative stability data indicated that coconut oil, which is saturated fatty acid rich oil, is more resistant to the formation of lipid oxidation products, compared to mustard oil and sunflower oil.

Peroxide value (PV) [Table 1], which is used as indicator of oxidation taking place in the oils, was found to be significantly increased in heated oils. PV is a measure of the primary lipid oxidation and is a major concern from the point of view of toxicology. PV was observed to be significantly increased in heated oils, compared to fresh oils. This is in agreement with the findings of Priyanka et al. who observed that cooking practices like shallow frying, deep frying and sautéing significantly increases the peroxides in edible oils and fats [29]. Coconut oil was shown to be having lower peroxide value among the heated oils.

Table 1 Fatty acid composition, Calculated oxidizability (Cox) value & Peroxide value (PV) of oil samples

*Fatty acids (%)	UHCO	HCO	UHMO	HMO	UHSO	HSO
C8:0	8.73	9.40	------	------	------	------
C10:0	5.89	6.48	------	------	------	------
C12:0	44.17	51.95	------	------	0.050	3.50
C14:0	20.14	20.07	------	------	0.152	1.71
C16:0	9.38	7.69	2.13	7.84	6.40	12.65
C16:1	------	------	0.046	------	0.269	0.153
C18:0	3.15	2.38	1.08	4.72	3.84	6.54
C18:1	6.94	1.73	20.03	10.74	27.66	25.15
C18:2	1.60	0.30	12.61	9.56	60.84	49.57
C18:3	------	------	9.34	4.73	------	------
C20:0	------	------	0.868	5.62	------	------
C20:1	------	------	4.86	1.93	0.215	0.319
C22:1	------	------	49.03	54.85	0.576	0.413
Cox value	0.234	0.048	4.06	2.68	6.55	5.37
PV(mEq/Kg)	4.66±	8.61±	5.82±	11.43	5.10±	13.59^{cd}
	0.29	0.54^{a}	0.37	0.72	0.32	0.86^{c,b}

* Given in table is the percentage of fatty acids and expressed as mean of three estimations. Peroxide value is expressed as mean ± SD of four estimations. 'a' indicates values are significantly different from UHCO. 'b' indicates values are significantly different from UHMO. 'c' indicates values are significantly different from UHSO. 'd' indicates values are significantly different from HCO. Significance accepted at $p<0.05$.

Plasma total cholesterol, triglycerides, LDL-cholesterol and VLDL-cholesterol [Table 2] were found to be significantly increased in the heated oil fed groups, compared to corresponding fresh oil fed groups. Earlier studies reported that serum lipids levels of rats fed repeatedly heated oil were significantly increased [30]. Coconut oil fed group showed elevated plasma lipids among fresh oil fed groups [31], but least among heated oil fed groups. HDL-cholesterol concentration was found to be significantly decreased in heated oil fed groups compared to fresh oil fed groups, but coconut oil fed groups showed elevated levels. Previous studies conducted have reported that heated oil fed group showed decrease in HDL-cholesterol content when compared to fresh oil fed group [30]. Since LDL particles have atherogenic properties, the presence of elevated levels of these particles may have role in hyperlipidemia and hemostatic changes associated with atherosclerosis [32].

Table 2 Plasma Lipids

Group I	Group I	Group II	Group III	Group IV	Group V	Group VI
Total cholesterol (mg/dl)	178.48±15.53	201.35±17.52[a]	158.15±14.43	224.29±18.65[b,d]	151.96±13.87	233.57±19.42[c,d]
HDLc (mg/dl)	51.77±4.51	42.87±3.56[a]	45.44±4.36	37.98±4.06[b,d]	45.01±4.11	36.21±3.30[c,d]
Triglycerides(mg/dl)	9.57±0.87	10.93±0.997[a]	7.33±0.67	12.78±1.06[b,d]	8.05±0.73	13.31±1.11[c,d]
LDLc + VLDLc (mg/dl)	126.71±11.03	145.46±13.96[a]	110.38±10.07	167.67± 4.59[b,d]	106.95±9.76	176.60±16.12[c,d]

Values expressed as mean ± SD of six rats. 'a' indicates values are significantly different from group I. 'b' indicates values are significantly different from group III. 'c' indicates values are significantly different from group V. 'd' indicates values are significantly different from group II. Significance accepted at $p<0.05$.

Platelet counts, platelet crit, mean platelet volume and platelet distribution width [Table 3] showed significant alterations in heated oil fed groups. Abnormalities in platelet number are an indication of a defect in primary hemostasis. Platelet count (PC) was significantly elevated in all heated oil fed groups, but least in HCO fed groups. An increase in platelet number above normal serves as a marker of vascular disease [33].Mohammad Anwar et al., reported significant increase in platelet count in high fat diet fed rabbits in comparison to control rabbits [34]. In our study, the platelet count in rats fed heated coconut oil was lower than that of rats receiving respective sunflower oil and mustard oil. Platelet crit (PCT), which measures total volume of platelets in a given volume of blood, was significantly increased in heated oil fed groups when compared to fresh oil fed groups. Mean platelet volume (MPV) is an indicator of platelet function and increased MPV is one of the risk factors for myocardial infarction, cerebral ischemia and transient ischemic attacks [35]. Mean platelet volume (MPV) and Platelet distribution width (PDW) was significantly increased in heated oil fed groups in comparison to fresh oil fed groups, the increase being lower in coconut oil fed group. Shobha and Arun reported significant increase in platelet count, mean platelet volume, platelet crit and platelet distribution width in high fat diet group compared to normal rats [36]. Nandakumaran et al., showed that oral administration of coconut oil for a period of thirty days did not cause any increase in the various hematological and metabolic parameters investigated in adult female rats [37].

Table 3 Platelet function parameters

Group I	Group I	Group II	Group III	Group IV	Group V	Group VI
Platelet Count ($\times10^5/mm^3$)	8.23±0.75	9.47±0.82[a]	7.09±0.72	10.52±0.87[b,d]	6.29±0.67	10.86±0.90[c,d]
Platelet crit (%)	0.718±0.07	0.817±0.07[a]	0.63±0.07	1.13±0.09[b,d]	0.625±0.06	0.934±0.08[c,d]
Meanplateletolume (fL)	7.90±0.76	9.14±0.80[a]	6.83±0.73	10.58±0.88[b,d]	7.44±0.80	10.46±0.87[c,d]
Platelet distribution width (fL)	8.61±0.79	10.24±0.85[a]	7.18±0.77	11.59±0.96[b,d]	8.49±0.91	12.15±1.01[c,d]
Platelet TBARs (μM/mg protein)	0.226±0.02	0.330±0.03[a]	0.352±0.03	0.509±0.05[b,d]	0.393± 0.04	0.601±0.05[c,d]

Values expressed as mean ± SD of six rats. 'a' indicates values are significantly different from group I. 'b' indicates values are significantly different from group III. 'c' indicates values are significantly different from group V. 'd' indicates values are significantly different from group II. Significance accepted at $p<0.05$.

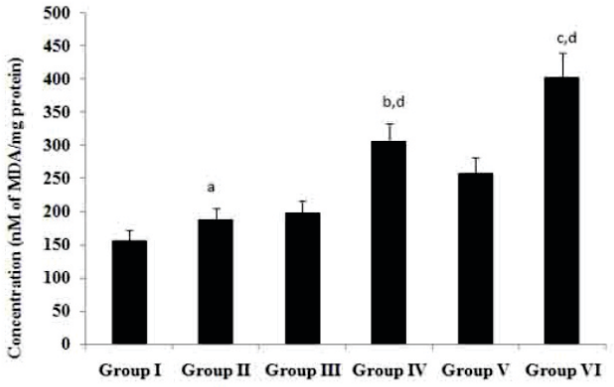
Platelet peroxide content [Table 3] was found to be significantly increased in the platelets of all the heated oil fed groups in comparison to fresh oil fed groups, but least in coconut oil fed group. The presence of lipid peroxide in food is undesirable because nutritional value decreases with the destruction of unsaturated fatty acids and other essential food constituents such as vitamins [38, 39]. The elevated concentrations of plasma TC, TG, LDL and VLDL levels in heated oil fed groups presumably could increase the susceptibility of platelet lipid peroxidation. Lipid peroxidation was lower in platelets of rats fed both fresh and heated coconut oil compared to respective mustard and sunflower oils.

Table 4 Coagulation parameters

Group I	Group I	Group II	Group III	Group IV	Group V	Group VI
Fibrin(mg/dl)	20.96±1.91	24.71±2.26[a]	17.81±1.80	28.64±2.61[b,d]	16.43±1.76	31.53±2.88[c,d]
PT (sec)	24.94±2.17	22.15±2.13[a]	28.07±2.33	19.10±2.04[b,d]	30.12±2.50	14.01±1.50[c,d]
APTT (sec)	33.57±2.92	28.75±2.76[a]	38.30±3.18	24.88±2.66[b,d]	40.03±3.33	25.23±2.42[c,d]

Values expressed as mean ± SD of six rats. 'a' indicates values are significantly different from group I. 'b' indicates values are significantly different from group III. 'c' indicates values are significantly different from group V. 'd' indicates values are significantly different from group II. Significance accepted at $p<0.05$.

Figure 1. Platelet *in vitro* lipid peroxide content



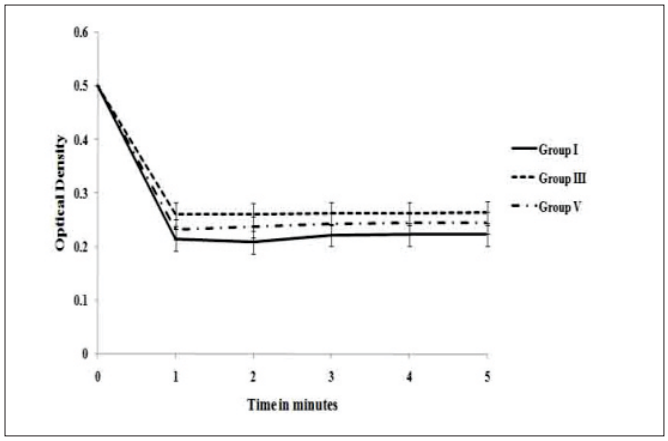
Values expressed as mean ± SD of six rats. 'a' indicates values are significantly different from group I. 'b' indicates values are significantly different from group III. 'c' indicates values are significantly different from group V. 'd' indicates values are significantly different from group II. Significance accepted at $p<0.05$.

Since membranes are rich in PUFA, they are more prone to lipid peroxidation. Additionally, consumption of oxidized dietary lipid is likely to increase the chances of peroxidation of the cell membranes. This could result in structural and hence functional changes in membranes. Niranjan and Krishnakantha reported that oxidized ghee consumption increased lipid peroxidation in rats compared to control as measured *in vitro* [40]. Platelet *in vitro* lipid peroxide content [Figure 1] was found to be significantly increased in all the heated oil fed groups, but lower in heated coconut oil fed group. Prothrombin time (PT), activated partial thromboplastin time (APTT) and concentration of fibrin [Table 4] were determined to evaluate clotting tendency. PT, which measures the clotting time of plasma in the presence of thromboplastin, was determined to assess the efficiency of extrinsic system [41]. APTT depends on substances normally present in blood for its activity and to assess the intrinsic pathway, APTT was estimated [41].PT and APTT were decreased in heated oil fed groups. Narasimhamurthy et al., observed that heated/fried oil groups of rats showed reduced prothrombin time when oils were fed in the diet at 5% and 20% levels [42]. Studies reported that prothrombin time and activated partial thromboplastin time were significantly lowered in frying oil compared to fresh oil [43]. Fibrin, the end product of blood clotting, was significantly increased in heated oil fed groups. 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 [44]. Elevated fibrin concentration, lowered prothrombin time and acitivated partial thromboplastin time are an indication of hypercoagulability associated with hyperlipidemia. The alterations in clotting factors are less in heated coconut oil fed group when compared to heated mustard and sunflower oil fed groups.

Platelet aggregating tendency, recorded as decrease in OD at 600 nm after one minute of addition of agonist[Figure 2-3], was significantly higher in heated oil fed groups compared to fresh oil fed groups. Comparing the platelet aggregation induced by ADP among fresh oil fed groups, coconut oil fed group showed significant aggregation (57.42%), followed by sunflower oil fed group (53.6%) and mustard oil group (47.86%). There are reports that platelet aggregation tended to increase with increased saturated fat consumption [45]. Among heated oil fed groups, ADP induced platelet aggregation was highest in mustard oil group (80.34%) followed by sunflower oil fed group (68.36%) and lowest in coconut oil fed group (62.26%). When platelet aggregation between fresh and heated oils are compared, coconut oil fed group showed 8.43 % increase in aggregation, while it is 67.86% and 27.54% respectively for mustard oil and sunflower oil. The oxidation of oil produces lipid peroxides, which may be one of the reasons for the elevation of total cholesterol and enhanced platelet aggregation levels [46]. In the present study, elevated cholesterol levels observed in heated oil fed groups may be inducing platelet aggregation by making platelets more sensitive to aggregatory agents [47]. Heated coconut oil fed group shows lower platelet aggregation among heated oil fed groups, which may be due to the decreased lipid peroxide levels observed in the

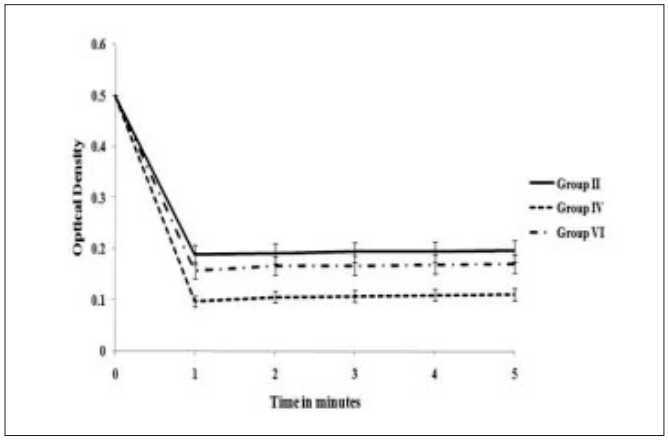
platelets. This is in agreement with the findings of Niranjan and Krishnakantha who reported that feeding high amounts of oxidized ghee resulted in increased platelet aggregation and they reasoned that the effect may be due to the oxides generated in ghee on thermal oxidation rather than due to the saturated fat present [40].

Figure 2. Platelet aggregation induced by ADP in unheated oils and fed groups



Values expressed as mean ± SD of six rats. Significance accepted at $p<0.05$. Group I- Unheated coconut oil, Group III- Unheated mustard oil,Group V- Unheated sunflower oil.

Figure 3. Platelet aggregation induced by ADP in heated oils and fed groups



Values expressed as mean ± SD of six rats. Significance accepted at $p<0.05$. Group II- Heated coconut oil, Group IV- Heated mustard oil, Group VI- Heated sunflower oil

4. Conclusion

From the above observations, the present study indicates that thermally stressed culinary oils result in significant alterations in platelet functions in rats and coconut oil fed group showed least alterations. Results suggest that fried food items prepared in unsaturated oils are more deleterious compared to those prepared in saturated cooking oils such as coconut oil.

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