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Impact of Smoking on Platelet, Coagulation and Lipid Profile in Young Male Subjects

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Abstract: This study aims to investigate the effects of smoking on platelets function, coagulation and lipid profiles in young males. Sixty young males (20 Cigarette smokers, 20 Shisha smokers and 20 nonsmokers) were enrolled in this study. Blood pressure, body mass index, serum lipid profile, means platelet volume, platelet count, clusters of differentiation and coaggulability were investigated. Results: There is no significant difference between the three groups as regard platelet count, mean platelet volume, Hemoglobin concentration, White Blood Cells count and Red Blood Cells count. High density lipoprotein cholesterol levels were lower in Shisha smokers group compared with nonsmokers group. Triglyceride levels were higher in Shisha smokers group compared with nonsmokers group. Fibrinogen was significantly increased for the Cigarette smokers group and Shisha group compared to non-smokers (P<.01). However, the Prothrombin Time was not changed significantly in the smokers groups. There was a significant difference in alteration of CDs markers expression in both Cigarette and Shisha smokers. The results of the current study revealed higher triglyceride levels and lower high density lipids-cholesterol levels in Shisha smokers group and significant alteration of CDs expression in both Cigarette and Shisha smokers compared to nonsmokers healthy male subjects.

Key words: Smoking · Platelet Volume · Platelet Count · Coagulability and Lipid Profile

INTRODUCTION

Cigarette smoking is a serious health problem and most important avoidable causes of death in worldwide. Today at least 20% of all cancers are estimated to be attributable to smoking, but this figure is expected to increase because of the uptake of tobacco use in low-income countries. Tobacco products contain more than 50 established or identified carcinogens and these may increase risk of cancer by causing mutations that disrupt cell cycle regulation, or through their effect on the immune or endocrine systems [1].

Shisha is familiar to about 1 billion people throughout the world and is daily used by more than 100 million men and women in Africa, Asia and several Mediterranean countries. The Hooka, Water Pipes, or Narghile, which are names of Shisha known in different parts of the world, had been smoked for at least 400 years [2]. Despite the fact that civilization has progressed into the third millennium, its popularity has not decreased and in fact it is experiencing a great revival. Different progenitors and types of the water pipe have been described, varying in material, size and shape. What remain unaltered are the consisting parts, which include the body, bowl and tube [3].

Smoking is associated with the pathogenesis of pulmonary and cardiovascular disease, such as atherosclerosis and coronary artery disease. Several further reports suggest that there is also an association with thrombosis. Leukocytes, platelets and predominantly activated subgroups of these cells are involved in this process. However, there have been only a few studies addressing the effect of smoking on platelets. In addition, many of the studies have not compared the data with those of nonsmoking control groups [1- 3].

Several laboratory approaches have been made to measure platelet function in smokers. All of them include test systems that focus on the whole population of platelets in given samples. In these studies, methods like aggregation assays, pro-thrombin time, fibrinogen and platelet markers. Later on, platelet analyzer (PFA-100) has been introduced for the evaluation of platelet function in

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whole blood samples [4]. This system measures platelet reactivity in collagen-epinephrine or collagen-ADP-coated membranes under computer-controlled, constant shear flow conditions. This study try assess the effect of Cigarette smoking on platelet function by using in vitro techniques such as flow Cytometric analysis, lipid, coagulation profiles and platelet function analyzer (PFA 100).

MATERIALS AND METHODS

Enrollment of Subjects: Prior to inclusion, a complete medical history was taken. Intended enrollment comprised of 60 healthy male subjects age ranged from 20 to 40 years. For the initial evaluation procedure each subject was examined medically by a physician at King Abdulaziz University out patient clinics in order to exclude any abnormal medical problems. Then medical history was taken carefully to collect information about his general condition, physical activity and current medication. Subjects were excluded from the study in any case of history of platelet abnormalities, bleeding or vascular disorders, liver or renal disease, ingestion of any kind of drug affecting platelet function for at least 12 days prior to sample collection. Procedures used in this study were approved by the Ethics Committee of the Faculty of Applied Medical Sciences at King Abdulaziz University, Saudi Arabia. Written informed consent was obtained from each subject prior to the start of the study.

Analytical Methods

Blood Sampling and Laboratory Methods: Blood samples were collected from antecubital veins with a 20G butterfly without the use of a tourniquet. All samples were collected and properly mixed with the anticoagulant by gently inverting the tubes three to four times. For sample collection, EDTA as anticoagulant was used for complete blood counts (CBC) including platelet count and hematocrit, were measured in automated cell analyzer. For analyses with the platelet function analyzer PFA-100 blood was collected in Sodium Citrate tubes. Those samples were stored at room temperature (+20±2°C) until measurement. For PFA-100 method, analyses were done within 4 hours after sample collection.

Complete Blood Count: Complete blood count (CBC) was measured in Beckman Coulter AC-T (Beckman Coulter, Fullerton, CA, USA) at king Abdulaziz University Hospital. Complete blood count provided white blood cells count (WBC), red cells count (RBC), platelets count (Pct), mean platelets volume (MPV) measurement of hematocrit (Hct) and Hemoglobin (Hgb).

Biochemical Parameters: Biochemical parameters including serum for total Cholesterol, Triglycerides (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL) all were measured at the same time when the medical pre-check up was performed. All of the biochemical assessments were performed using a fully automated analyzer (Dimenition RxL, Diagnostics) machine at king Abdulaziz University Hospital.

Hemostaseological Analyses: Normal platelet function was confirmed by a platelet function test panel, which was performed on each blood sample and a non-indicative clinical history for platelet dysfunction. The samples were tested for activated partial thromboplastin time (aPTT), Prothrombin time (PT), fibrinogen on a fully automated coagulation analyzer (Dade Behring) at king Abdulaziz University Hospital.

Platelet Function Analyzer (Pfa-100 Analyzer): In each sample, two different cartridges (Dade Behring) were used. They consisted of a sample pouch and a capillary with a membrane coated with collagen (2 µg equine type I) and additionally either epinephrine (10 µg epinephrine bitartrate; Col/EPI) or ADP (50 µg adenosine 5-diphosphate; Col/ADP). The sample was aspirated with a constant negative pressure (40 mbar) through the aperture and capillary of the test cartridges. Contact of platelets with the coated membrane resulted in platelet activation. A platelet plug was formed that finally occluded the aperture of the high shear flow system. Time from the first contact of the sample with the membrane until occlusion of the membranes aperture was measured in seconds (closure time [CT]) and reflected the PHC of the blood sample.

Until analysis, the disposables that originated all from the same lot were stored at +2 to 8°C. Prior to analysis, the cartridges were warmed up to room temperature for 15 minutes. Eight hundred micro-liters of citrated blood were pipetted into each cartridge for *in vitro* analysis. Measurements were repeated when duplicate determinations were obtained, in which one analysis was not measurable (maximum test time) and the other analysis was =150 s with Col/Epi, or =100 s with the Col/ADP cartridge, or duplicate determinations in which one was two times the value of the other result.

Flow Cytometric Analysis: Direct immuno-fluorescence technique was used for the determination of changes in platelet activity and response to Cigarette smoking. Fluorescein-isothiocyanat (FITC), anti-CD62P-FITC and Phycoerythrin (PE)-conjugated anti-CD61-PE (Becton-Dickinson, San Jose, CA, USA), were used in this study. Platelet rich plasma (PRP) was prepared by centrifugation of whole blood at 900 rpm for 10 minutes. An aliquot of 100µl platelet-rich-plasma was directly labeled with monoclonal antibodies.

For analyses of, CD40, CD41, CD62p (P-selectin), IgG isotype controls were applied to detect non-specific staining (Becton Dickinson, Mountain View, USA). After incubation (30 minutes at 20±2°C) in a dark place, the samples were re-suspended in 1 ml of Phosphate buffer solution (PBS) and stored at + 4 °C for a maximum of 120 minutes prior to flow cytometric analysis. CD61 was used as tagging antibody to detect CD62P positive platelets. Samples were analyzed on a (FACScan®) cytometer (Becton Dickinson). Fluorescent beads were applied daily to ensure the stability of the system (CaliBRITE™, Becton Dickinson). Following the setting of the appropriate threshold in the forward side scatter (FSC), 10,000 events were acquired in a life-gate. List mode data were acquired and analyzed using (CELLQuest®) software (Becton Dickinson). The results were expressed percentage of antibody-positive cells. Percentage of antibody-positive cells was defined as cells with specific fluorescence higher than the isotype and autofluorescence samples.

Statistical Analysis: Statistical analyses were performed with SPSS PC software package. Differences between the three study groups were based on student -*t*- test (*P* value of <0.05).

RESULTS

A total of 60 subjects were included in this study. As shown in Table (1), nonsmoker aged 23.0 ±1.4 years with an average body weight of 77.5 ± 15.2 kg, whereas, Cigarette smokers between 23±2.2 years old weighted of 79.4 ± 22.7 kg with an average consumption of 16.2 ± 8.6 Cigarette/day over 5 ± 3 years. Shisha smokers with an average body weight 87.9 ± 23.0 kg aged 24.3 ± 2.7 and consumed 10.4 ± 7.0 Shisha/week. Shisha smokers had a significantly higher BMI than Cigarette smokers. Both Cigarette and Shisha smokers had significantly higher mean arterial blood pressure compared to the control subjects.

Table (2), shows no significant difference of the hematological results indicated between three groups as regard to platelet count, mean platelet volume, RBCs count, however, hemoglobin and total white blood cells count, were higher in smokers than in nonsmoking subjects (*$P < 0.05$).

Table (3): Demonstrates that, there was no significant difference between the three groups as regard to cholesterol level and LDL concentration. However, Triglyceride was significantly higher in Shisha compared to non-smoker and Cigarette smokers. In the other hand,

Table 1: Demonstrates the effect of smoking on different physical and vital Parameters for control, Cigarette and Shisha smokers groups.

Subjects	Age (Year)	Smoking Period (years)	Number of Shisha/week	Weight (Kg)	Height (Cm)	BMI (Kg/m^2)	Blood Pressure (mmHg)
Cigarette Smoking	23±2.2	5±3.0	16.2±8.6	79.4±22.7	175.3±5.67	25.7±6.8	137/86±12.2/8.7
Shisha Smoking	24.3±2.7	5.9±2.9	10.4±7.0	87.9±23.0	171.78±7.55	29.6±7.6	140/90±12.2/8.7
Control	23.0±1.4	-	-	77.5±15.2	174.55±7.2	25.5±5.0	120/77.05±12.2/8.7

Table 2: Illustrates the Effect of smoking on complete blood picture in control, Cigarette and Shisha smoking groups.

Subjects	Pct (X10^9/L)	MPV (fl)	Hgb conc. (g/dl)	WBC count (10^9/l)	RBC count (x 10^6/l)
Cigarette Smoking	264.2±64.3	8.2±1.0	17.6*±0.9	8.2*±1.8	5.5±0.5
Shisha Smoking	306±79.7	8.3±0.9	16.6*±1.5	7.5*±1.6	5.3±0.9
Control	278.0±50.4	8.4±0.8	14.2±1.1	6.1±2.0	5.4±0.6

Pct = Platelet count; Hgb conc. = hemoglobin *Significance (P<0.05)

Table 3: Demonstrated the Effect of smoking on the concentration of the Lipid profile among the three different groups.

Subjects	Cholesterol	Triglyceride	HDL	LDL
Cigarette Smoking	3.6±0.6	0.9±0.5	1.0±0.2	2.4±0.5
Shisha Smoking	4.1±1.0	1.2±0.9	0.9±0.4	2.8±1.0
Control	3.7±1.0	0.7±0.3	1.1±0.3	2.5±0.9

Table 4: Shows the effect of smoking on concentration of fibrinogen, PT and aPTT in control, Cigarette and Shisha smoking groups.

Subjects	PT / (sec)	aPTT/sec	Fib. (mg/dl)
Cigarette Smoking	14.8± 2.2	55.7*±18.9	228.6*±116.3
Shisha Smoking	13.7± 3.6	52.4*±10.5	319.1*±20.5
Control	14.3±2.0	34.6±2.9	194.3±55.7

Results are presented as mean ± SD N=20 *Significance (P<0.05)
PT= Prothrombin time. Fib= Fibrinogen concentration
aPTT= activated Partial Thromboplastin time

Table 5: Effects of Shisha smoking on platelet function was measured by using PFA (100), (Clouse time/ seconds), in compression with normal control.

	(Col/ADP)	(Col/EpI)
Shisha Smoking	100.7± 30.3*	151.2±5.2*
Control	85±12.3*	135±9.5*

Results are presented as mean ± SD N=20 *Significance (P<0.05)
Col/ADP CT (s) =closure time using a collagen- and ADP-coated test tube membrane in seconds;
Col/EPI CT (s) =closure time using a collagen- and epinephrine-coated test tube membrane in seconds.

Table 6: Demonstrates the effect of smoking on Platelets (CDs) Markers in control, Cigarette and Shisha smoking groups.

Subjects	CD 40	CD 41	CD 62
Cigarette Smoking	11.64*± 8.36	79.01*± 32.43	52.58*± 24.99
Shisha Smoking	8.64*#± 6.77	78.52*± 14.61	39.62*#± 15.04
Control	4.11± 2.32	96.03± 4.05	25.58± 9.20

CD= Cluster of Differentiation # Significance between Cigarette and Shisha *Significance (P<0.05)

HDL concentration was significantly lower in Shisha group than non smoker. Total cholesterol and LDL were higher in Shisha smokers but did not reach significance compared to the non-smokers.

As shown in Table (4): Fibrinogen was significantly increased for the smoking group and Shisha group compared to non-smokers (P<.05). However, the PT was not changed significantly in the smoking group. At the same time, a PTT was prolonged significantly in Cigarette and Shisha smoking groups in comparison to non-smokers subjects (P<.01).

Table (5), shows closure time (CT) after stimulation with epinephrine or ADP. The results represent only Shisha smokers in compression with normal healthy subjects. There was a normal closure time (CT) in non-smokers groups (control) in contrast to, Shisha smokers group which had shown a prolonged closure time.

Table (6), demonstrates a significant increase in CD40 in Cigarette and Shisha smokers compared to non-smokers. Cigarette smokers showed a significant higher value than Shisha smokers. At the same time, there was a significant decrease of CD41 in Cigarette and Shisha smokers compared to control with no significant difference between Cigarette and Shisha smokers. There was a significant increase in CD62 in both Cigarette and Shisha smokers with Cigarette smokers showed significantly higher values than Shisha.

DISCUSSION

Platelets are un-nucleated fragments of bone marrow megakaryocytes. They contain few viable mitochondria, glycogen, at least three types of morphologically different granules (α-granules, dense core granules, lysosomes) and a complex membranous system α-granules contain adhesion molecules important for platelet-platelet interactions and platelet interactions with other blood cells, mitogenic factors, plasma proteins and factors relevant for coagulation and fibrinolysis. Platelets are involved in hemostasis, wound healing and inflammation [21].

For decades, epidemiological data have demonstrated the association of smoking with the incidence of coronary heart disease, myocardial infarction and stroke. In many of the acute clinical events, thrombotic occlusion of the vessel occurs, a process that is often associated with platelets. Therefore, the definition of the relation between platelets and smoking seems important [22]. Hung *et al.* [23] demonstrated that smoking-stimulated platelet aggregate formation in habitual smokers. Another effect of smoking found by different groups is an increase in serum fibrinogen levels.

Cigarette smoking is associated with higher serum levels of cholesterol [11] and lower plasma concentrations of high-density lipoprotein (HDL) cholesterol; also, smokers have higher plasma triglyceride concentrations than nonsmokers [8] and smoking enhances platelet aggregation [12, 13]. In this study it has been shown that, there was no significant differences between the three groups, except for Triglyceride was significantly higher in the Shish smokers in comparison to the other groups. For the HDL it was in consistence with the finding of [12, 13], that there was significant decreased in the concentration of HDL in Shisha group than non smokers. This mechanism may be due to impairment of lipoprotein metabolism, reduces the distensibility of blood vessel walls [9, 14] and induces a prothrombotic and pro-inflammatory state [15]. These novel risk factors for

atherosclerosis all increase dose-dependently with the number of Cigarettes smoked per day, the number of pack-years of Cigarettes smoked and serum nicotine concentrations [16, 17].

In general, the study of platelets *in vitro* is virtually limited to highly artificial situations. Therefore, it must be accepted that no technique provides a true measure or model of thrombus formation. However, this study presents data obtained with whole blood in an *in vitro* system that measures platelet function in a reproducible manner. Using the PFA-100 method in this study there was increased platelet reactivity to Col/ADP and (Col/EPI) in Shisha smokers whereas no changes were observed in non-smokers controls, which is in accordance with the results of the other investigator [11]. This study has shown that the increase in platelet reactivity was specifically associated with an increased platelets expression of CD41 after smoking Shisha, thus suggesting that this change likely plays a major role that smoking may induce increase in platelets reactivity.

Flow Cytometric findings may also explain the differences in platelet function found between Smokers and nonsmokers. Surface expression of the integrins CD 41b, part of the highly expressed GpIIb/IIIa receptor responsible for fibrinogen binding and platelet aggregation, part of the collagen receptor important for platelet adhesion, was higher in control group compared to smokers. Recent evidence implicates platelet activation and the consequent increased expression of platelet CD62 as direct mediators of vascular inflammation and atherosclerosis. Experimental data suggests that platelet CD62 is an important link between thrombosis and vascular injury. Platelet CD62 is rapidly expressed on the surface of activated platelets and mediates adhesion of platelets to neutrophils and monocytes. Therefore activated platelets can directly trigger an inflammatory reaction in endothelial cells by promoting endothelial expression of adhesion molecules that may mediates the acute target organ damage that occurs in severe hypertension. Also, platelet cytokine CD40 ligand (CD40L), a transmembrane protein, was originally described on stimulated CD4+ T cells and was found on stimulated mast cells as well as basophils [30]. The results in this study have shown that, an increased expression of CD40 markers in both smoker groups in compression to non smokers control group which may indicate induction of chemokine secretion and upregulation of adhesion molecules. [31], this process leads to recruitment to and extravasation of leukocytes at the site of injury and thereby immediately links hemostasis to the inflammatory system.

CONCLUSION

In general, the study of platelets *in vitro* is limited to highly artificial situations. Therefore, it must be accepted that there is no technique will provides a true measurements or model of thrombus formation. The increased cardiovascular morbidity and mortality among Cigarette smokers is also mediated in part by enhanced platelet reactivity and activation. Therefore, investigations of the influence of smoking on platelets remain an important field in research of the pathogenesis of acute thrombotic occlusions in the smoking population.

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