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LOSS OF PROTECTIVE FUNCTION OF PARAOXONASE ASSOCIATED WITH CARDIOVASCULAR DISEASES IN BANGLADESHI ORIGIN

Md Amir Hossain[1*], Israth Jahan Tuhin[1], Md. Mahfuzur Rahman[2], Muhammad Saiedullah[2]

1. Manarat International University, Dhaka, Bangladesh.
2. Bangladesh University of Health Sciences (BUHS), Dhaka, Bangladesh

Correspondence
Md. Amir Hossain*
Department of Pharmacy, Manarat International University, Dhaka, Bangladesh.
✉ mamirhr@gmail.com

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ABSTRACT

Cardiovascular diseases (CVDs) are the major health problem globally and considered to be the leading causes of death. The prevalence of CVDs is also rising among the adults in Bangladesh with the highest mortality rates. This study aimed to determine the serum Paraoxonase activity, lipid peroxidation product malon dialdehyde (MDA), and vitamin C status in subjects with cardiovascular diseases and to compare with apparently healthy subjects of Bangladeshi origin. In this case-control study, 34 male subjects from cardiac intervention and surgery (CIS) with an indication of angina, myocardial infarction, stroke and age, and body mass index-matched 24 apparently healthy males (controls) were included. Clinical and anthropometric data were recorded, and biochemical parameters were measured from serum samples. Serum Paraoxonase activity, MDA, and vitamin C concentrations were measured by standard spectrophotometric method. The mean age and body mass index (BMI) of the total subjects were 49±13 years and 22.6 ± 2.9 kg/m^2, respectively. Serum Paraoxonase activity was significantly lower in subjects with CIS compared to control (113.7 ± 54 vs. 194.9±95 U/L, p=0.005). Serum MDA in CIS group was 7.7 ± 0.3 µmol/L and 6.6 ± 0.4 µmol/L in control group (p=0.0467). The mean values of serum vitamin C in CIS and control were 22.3 ± 4.4 and 24.8 ± 5.1 µmol/L, respectively. Vitamin C level was significantly lower in the CIS group compared to control (p = 0.057). Results of this study indicated that oxidative stress and loss of anti-atherogenic function of high-density lipoprotein particles Paraoxonase might be associated with cardiovascular diseases in subjects of Bangladeshi origin.

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INTRODUCTION

Cardiovascular diseases are the leading cause of death in the world, which include cardiac arrhythmias, stroke, coronary artery disease, and heart failure, and the incidence of this disease is increasing alarmingly in Bangladesh as well, but its determinants are not fully resolved [1, 2]. In 2016, the World Health Organization (WHO) reported about 18 million people had died from cardiovascular diseases (CVDs), which represented 31% of global death in this particular year [3]. In past years, the prevalence of CVDs among Bangladeshi peoples also significantly raised and the death associated with CVDs alarmingly increased [4]. Moreover, the expected mortality projection rate related to CVDs in Bangladesh will be 21 times higher in 2025 compared to the rate was in 2003 [5].

Oxidative stress is one of the reasons behind CVDs; it mainly happens due to the imbalance between the generations of reactive oxygen species (ROS) and scavenging capacity of antioxidants. An increasing number of studies have found, ROS has a role in the pathogenesis of premature aging as well as numerous diseases like CVDs [6-8]. Although the mechanism of oxidative stress contributing to CVDs remain obscure. However, a growing body of shreds of evidence observed the increased formation of ROS contributed oxidative stress [9, 10]. Besides, Vitamin C has received considerable attention due to its powerful antioxidant properties [11, 12]. It is well established that vitamin C inhibits oxidation of low-density lipoprotein, thereby reducing atherosclerosis [13]. Also, the levels of Vitamin C related to CVDs-related mortality, patients having less serum vitamin C are more prone to mortality from stroke [14, 15]. On the other hand, dietary supplementation of Vitamin C reduced CVDs-related difficulties and increased in serum vitamin C functioned as cardio protective [15, 16].

Moreover, Paraoxonase (PON) is a group of enzymes present in three forms (PON1, PON2, PON3) encoded by genes *PON1*, *PON2*, and *PON3* [17]. These enzymes are homologous and HDL related proteins produced by the liver. Each of them has a vital role in human physiology, and they act as anti-inflammatory, antioxidant, and a thermo protective [18]. For example, PON1 can reduce oxidative stress in lipoprotein, PON2 function as a cellular antioxidant, and PON prevents LDL oxidation [19, 20].

Thus, this phenomenon could be related to their anti-oxidative properties, and PON may have essential roles in protecting CVDs.

Therefore, emerging evidence suggests that oxidative stress, antioxidant, and paraoxonase may have a role in cardiovascular diseases. To understand the relationship, we determined the serum Lipid peroxidation by measuring Malon dialdehyde (MDA), which is an important product of oxidative stress and widely recognized as a biomarker of oxidative related damage [21], antioxidant (vitamin C) level, and serum paraoxonase activity of patients and healthy controls. Our data suggest that oxidative stress and loss of protective function of HDL particles may be associated with cardiovascular diseases in subjects of Bangladeshi origin.

METHODS AND MATERIALS

Subjects and Study Design

The case-control study was conducted on 34 male subjects from cardiac intervention and surgery (CIS) with an indication of angina, myocardial infarction, and stroke, and 24 number of healthy control groups. The case subjects were randomly recruited with consents from the National Institute of Cardiovascular Diseases (NICVD), Sher-e-Bangla Nagar, Dhaka under a protocol jointly approved by the participating institutions. Healthy controls were recruited randomly matched with age, sex, and BMI.

Samples Collection and Storage

About 5 mL of venous blood was collected from each of the CIS patients and the control subjects by following all aseptic precautions. Immediately, the drawn blood sample was transferred to a vacuum tube. The collected specimens were kept at 25°C for clotting, followed centrifuged for 10 minutes at 3000 RPM. Serum was then transferred with micropipette taking care of not taking any red cell in micro centrifuge tubes and was stored at -20°C until analysis.

Determination of serum PON activity

Paraoxonase activity was measured according to the modified method by Eckerson *et al.* [22] as aryl esterase activity and expressed in international units (U) per milliliter of serum and 1 U will correspond to the quantity of enzyme that hydrolyzes 1 μmol of 4-nitrophenyl acetate to 4-nitrophenol per minute, at pH 7.4 and 37°C. Briefly, 2 μL of serum was added to 248 μL of 25 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM $CaCl_2$, 2.5% methanol and 0.625 mM 4-nitrophenyl acetate. The rate of generation of 4-nitrophenol was

determined at 405 nm using micro plate ELISA reader in kinetic mode. Results were calculated using molar absorptivity at pH 7.4, ε_{405} = 14000 $M^{-1}cm^{-1}$ [22].

Determination of Lipid peroxidation of serum

Lipid peroxidation was estimated by the method of Siddique *et al.* [23]. Eleven (11) µL of 500 mM butylated hydroxyl toluene (BHT) was added in each micro centrifuge tube in which 210 µL serum or standard was taken, then nine µL of 35% HCl was added and mixed and incubated at 60 °C for 80 minutes. After incubation, all tubes were cooled at room temperature, and 680 µL of N, N-dimethyl -phenyl indole (NMPI) was added and mixed. After centrifugation at 12,000 rpm for 5 minutes, 660 µL of clear supernatant was transferred to new tubes, and 115 µL of concentrated HCl was added, mixed, and incubated at 45°C for 1 hour. After incubation, centrifuged at 12,000 rpm for 5 minutes, and the absorbance of clear supernatant was recorded at 590 nm. Results were calculated against the standard curve [23].

Determination of Serum Vitamin-C

Vitamin-C concentration in the serum was estimated by the phenyl-hydrazine spectrophotometry method of Lowry et al. [24]. For measurement purposes, 300 µl serum and 1.2 ml TCA (Trichloroacetic Acid Solution) solution were taken in a test tube, mixed gently with a pipette, and centrifuged at 3000 rpm for 10 minutes. After centrifugation, clear 0.96 ml supernatant was taken and treated with 0.4 ml DTC (Dinitrophenylhydrazine-Thiourea-Copper Sulphate) solution and incubated at 60°C for 60 minutes in a water bath. Followed, immediately sample was placed in ice-cold water for chilling. The whole procedure was repeated for 0.3 ml of the standard solution of ascorbic acid, and for reagent blank and absorbance was taken against reagent blank at 520 nm [24].

Statistical Analysis

Continuous variables were presented as mean and standard deviation, and the results of categorical measurements were presented in number (%). Significance was assessed at a 5 % level of significance. Student's' test (two-tailed, independent) was used to find the significance of the study parameter using MedCalc® 11.2, and graphical presentations were made by GraphPad Prism 6.01.

RESULTS

Demographic Characteristics

The demographic characteristics and clinical examination information of participants are

given in Table 1. All the participants were males. 34 male case subject's mean age was 51 ± 12 years, and the mean age of twenty 24 controls group were 48 ± 14 years. The mean BMI of the case and control group was 22.3 ± 2.9 Kg/m^2 and 23.1± 2.9 Kg/m^2, respectively. Fisher exact test showed that hypertension is strongly associated with CVD events [OR=8.3 (95% CI: 2.5 - 27.6) with relative risk of 2.4 (95% CI: 1.4 - 4.2); p<0.001] but diabetes mellitus is not associated with CVD events [OR=1.1 (95% CI: 0.3 - 3.6); p =1.00]. Smoking tends to be associated with CVD events [OR=3.1 (95% CI: 1.0 - 9.3) with relative risk of 1.6 (95% CI: 1.0 - 2.4); p = 0.0617] (Table: 1)

Table 1: Demographic data as well as clinical and laboratory findings in patients with AS and healthy controls

Variables	**Case (*n*=34)**	**Control (*n*=24)**	**P-value**
Age (years)	51 ± 12	48 ± 14	0.4324
Gender (Male)	34	24	-
BMI (kg/m^2)	22.3 ± 2.9	23.1± 2.9	0.3611
Unstable angina (Yes/No)	29/5	0/24	-
Myocardial Infarction (Yes/No)	20/14	0/24	-
Stroke (Yes/No)	27/7	0/24	-
Hypertension (Yes/No)	25/9	6/18	<0.001[§]
Diabetes Mellitus (Yes/No)	9/25	6/18	1.00[§]
Smoker (Yes/No)	19/15	7/17	0.0617[§]

BMI, Body Mass Index; Data presented as Mean±SD; Variables between case and control were compared by unpaired t-test; p<0.05 was considered as statistically significant. [§,] Fisher exact test.

Serum MDA Level

In this study, serum MDA was considerably higher in the CIS group compared to the control group, and it was statistically significant (Figure: 1A). The serum MDA level was in CIS group was (7.7 ± 0.3 µmol/L) and (6.6 ± 0.4 µmol/L) in control group, respectively with the significant value of (P=0.0467).

Serum Vitamin-C

The serum vitamin C level was significantly lower in the CIS group compared to control, but it was not statistically significant (Figure : 1B). We found the serum Vitamin-C levels were 22.3 ± 4.4µmol/L in the case group and 24.8 ± 5.1 µmol/L in control healthy group. However, it was not statistically significant (p = 0.057).

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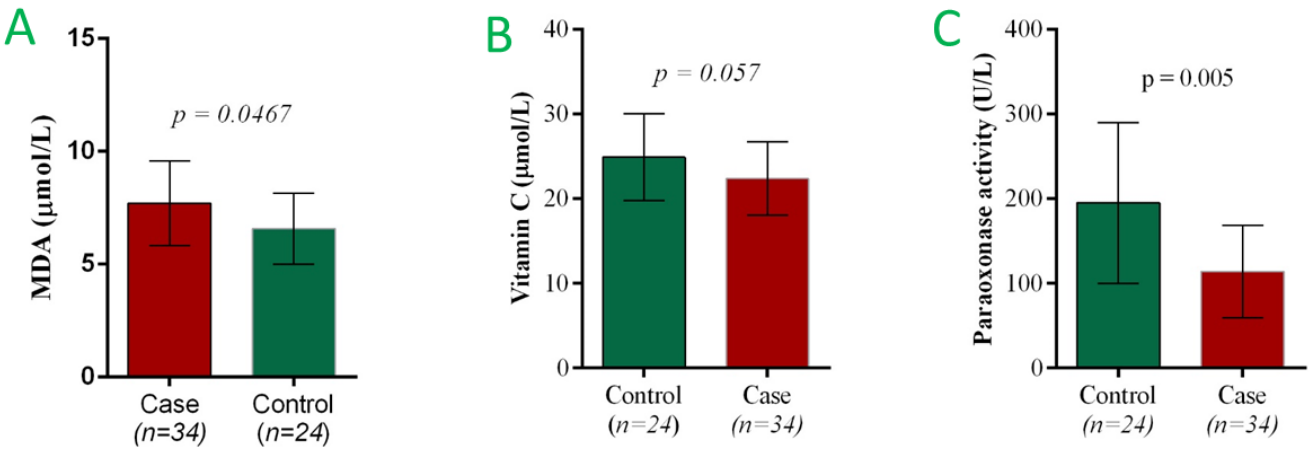

Figure 1: Mean serum Vitamin C, MDA, paraoxonase level in patients, and control subjects. A. Comparison of lipid peroxidation products MDA between case and control; B. Comparison of Vitamin C status between Case and control; C. Comparison of paraoxonase activity between case and control; MDA: Malondialdehyde, Data presented as Mean±SD; Variables between case and control were compared by unpaired t-test; $p<0.05$ was considered as statistically significant. MDA, Malondialdehyde.

Our results indicate that serum Paraoxonase activity and serum antioxidant (Vitamin-c) level are inversely related to CVDs, the person with more serum antioxidant (Vitamin-c) and serum Paraoxonase activity are less prone CVDs. However, serum MDA level tends to be associated with CVDs. Thus, PON enzyme activity is negatively associated with CVDs.

Serum PON Activity

The serum paraoxonase activity was significantly higher in the control group in compared with the case group (Figure : 1C). The significant value was (p=0.005). The serum PON activity was 113.7 ± 54 U/L in the patient group, and it was 194.9±95 U/L in the control group.

Furthermore, an analysis of the results indicates that paraoxonase activity is highly related to various cardiac events (Table-2).

Table 2: Relationship paraoxonase activity with hypertension and CVDs

Variables	Spearman's Rank the correlation coefficient (ρ)	p-value
CVDs vs. PON activity		
Unstable angina	-0.312	0.0170
Myocardial Infarction	-0.376	0.0036
Stroke	-0.355	0.0062
Hypertension vs PON activity	-0.285	0.0302

PON, Paraoxonase; CVDs, Cardiovascular diseases

DISCUSSION

This is the first prospective study of serum paraoxonase activity and CVDs in Bangladesh. These findings indicate that low serum paraoxonase activity is a predictive risk factor for CVDs with other established risk factors. It has been proved beyond doubt that oxidative stress is responsible for a number of diseases, including cardiovascular diseases, neurodegenerative diseases, cancer, and **chronic obstructive pulmonary disease** [25]. Antioxidants play a role in cardiovascular diseases, but the mechanism of antioxidant action has not yet been fully elucidated. Furthermore, several studies suggested, antioxidant therapy and high serum antioxidant level has beneficial effects on CVDs [26, 27]. It's believed that oxidative stress mainly induces CVDs due to its product ROS can cause vascular damage [28]. In our study, the relationship among antioxidants, oxidative stress, and paraoxonase activity in Bangladeshi cardiovascular patients were investigated. We found that subjects with CVDs correlated with serum antioxidants, oxidative stress, and paraoxonase activity. Intriguingly, the men have deficient serum vitamin-C levels prone to oxidative stress, and high vitamin-c may be beneficial. In different Randomized controlled trials also revealed vitamin-c could improve endothelial function, which is protective in CVDs [29].In this study, CVDs have higher MDA levels in compared to the control group, which was similar to other studies, like Nakkeeran et al. also found CVDs patients have a higher MDA level than healthy controls [30]. We also found a substantial difference in serum paraoxonase activity between CVDs group and control group. The subjects with CIS had less

paraoxonase activity in comparison with the control healthy group. Notably, Notably, patients with a preexisting disease or other CVDs risk factors have less paraoxonase activity [31]. Another study has found paraoxonase is a strong prognostic factor among patients with arteriosclerosis; patients having favorable paraoxonase activity had a better prognosis [32]. Studies in mice also suggested that PON1 knockout mice show a high level of oxidative stress compared to normal, and their HDL not able to protect LDL from oxidation [33, 34]. Besides, paraoxonase also has beneficial effects as an antioxidant in cancer [35]. Therefore, our finding indicated that paraoxonase activity was a more potent prognostic factor in the men at risk of cardiovascular diseases in Bangladesh.

CONCLUSION

Oxidative stress and low serum paraoxonase activity are associated with cardiovascular diseases in subjects of Bangladeshi origin. Paraoxonase activity may serve as a new prognostic biomarker for patients with cardiovascular diseases, and a high level of paraoxonase activity may serve as protective.

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