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Assessment of nitric oxide and oxidized LDL associated with low grade inflammation: A non-invasive risk predictor of coronary heart disease

Thirnavukkarasu Jaishankar, Meera Shivasekar*, V.M. Vinodhini

[1]Department of Biochemistry, SRM Medical College Hospital & Research Centre, Kattankulathur, Kancheepuram - 603203, Tamil Nadu, India

***Corresponding author:** Dr. Meera Shivasekar, Department of Biochemistry, SRM Medical College Hospital & Research Centre, Kattankulathur, Kancheepuram - 603203, Tamil Nadu, India. Email: mshivasekar@gmail.com

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Abstract

Coronary heart disease (CHD) is caused when cholesterol carried in blood form plaque in the covering of coronary arteries leading to atherosclerosis. The diminished nitric oxide (NO) production and inflammation are involved in the development of atherosclerosis. The pathogenesis of atherosclerosis is implicated by decreased availability of NO. Reduced NO level in the endothelium makes it vulnerable and increases the passage of leukocyte and LDL oxidation in the sub endothelial space which ultimately leads to CHD**.** The objective of this study is to evaluate the association of NO and ox-LDL along with hs-CRP in subjects with CHD. This cross-sectional study showed that the mean NO levels were decreased significantly in CHD group compared with that in the controls. A significant increase in Oxidized LDL and hs-CRP was observed in CHD group when compared with that of controls. Increased oxidative stress associated with diminished bioavailability of nitric oxide undergoes oxidative modification of LDL leading to low grade inflammation.

Keywords: coronary heart disease; C-reactive protein; high density lipoprotein; low density lipoprotein; nitric oxide; oxidized low density lipoprotein

Introduction

Nitric oxide (NO) is a diatomic free radical formed by many cells in the body and it is short lived and degraded in few seconds which are composed of one atom of nitrogen and one atom of oxygen. NO acts as a biological messenger and it is termed as a potent relaxant of peripheral vascular smooth muscle.[1] In blood flow regulation, production of nitric oxide by vascular endothelium is primarily important. Unusual production of NO occurs in altered disease states, adversely affecting vascular functions and blood flow.[2] Amino acid L-arginine produces NO with the help of the enzyme endothelial nitric oxide synthase (eNOS). Endothelial NOS (eNOS) enzyme produces NO in blood vessels and epithelial cells.[3] Relaxing vascular smooth muscle endothelium-derived relaxing factor causes vasodilation by generating nitric oxide. NO prevents platelet aggregation and acts as a neurotransmitter. NO plays a vital role in macrophage function. Vasodilators are important in regulation of blood pressure.[4] Nitric oxide is a significant biomarker of inflammation and oxidative stress.[5]

Reduction in bioactivity of eNOS due to the damage in endothelium is caused by excess of lipoproteins persuaded by atherosclerosis.[6] The normal endothelial cell phenotype is anti-atherogenic. A decrease in NO bioavailability is the essential factor common to coronary

heart disease (CHD).[7] Availability of substrate L-arginine along with increased concentration of circulating inhibitor ADMA including changed levels of expression in eNOS, signaled transduction reducing eNOS activation, decreased tetrahydrobiopterin (BH4) availability are some of the variation of factors which reduce the availability of NO.[8] When endothelium becomes more permeable to lipoproteins, they move below the endothelial layer and lose their quality of being cell-repellent. Inflammatory cells move into the vascular wall resulting in retention of LDL in intima which undergoes oxidative modification.[9] Lipid entry into the arterial wall is a key process in atherogenesis.[10] LDL is one of the key lipid protein–complex in blood which is responsible for the transport and delivery of lipids, including cholesterol, triglycerides, and phospholipids throughout the body via receptor-mediated endocytosis.[11] Modified LDLs no longer bind to the LDL receptor and they are typically cleared through "scavenger" receptors on macrophages and endothelial cells.[12] Low-density lipoproteins formed by accumulation of fatty substances, cholesterol, cellular waste products, calcium, and fibrin in the inner lining of the arterial wall are promoted by atherosclerotic lesions.[13] Accumulation of LDL-C along with inflammation in intimal wall is an initial process in CHD.[14] Circulating LDL infiltrate into the endothelial cell, later oxidized by action of oxygen free radicals. Accumulation of ox-LDL elicits an immune response. It induces activation of macrophages; ox-LDL up taken by macrophages via phagocytosis result in formation of foam cells. Increased endothelial permeability and increased LDL intimal retention play a major role in CHD.[15]

The endothelial dysfunction associated with oxidized LDL and CRP leads to the development of atherosclerosis.[16] C-reactive protein is a pentraxin family of protein, an acute phase reactant with molecular weight of 23kDa and act as a high sensitive marker for inflammation.[17] CRP plays a major role in immunity and prevent autoimmunity.[18] Aggregated CRP activates complement system with pro-inflammatory effect. The level of CRP rise drastically during inflammatory process.[19] The elevated concentration of hs-CRP directly implies sub clinical inflammation in individuals. The elevated concentration of CRP is released by liver through interleukin-6 stimulation and they are also produced in atheromatous lesions.[20] American Heart Association identified hs-CRP as optimal inflammatory biomarker to estimate risk of CHD. CRP plays an important role in pathogenesis of atherosclerosis. Any inflammatory changes stimulate an acute phase response by macrophage, endothelial cells, adipocyte to secrete cytokines and chemokines. The cytokines regulate the production of CRP. The pathological process of atherosclerosis is the prominent cause of cardiovascular disease.[21]

Life style factors like smoking, obesity, diet, and exercise can also have a greater impact on CHD risk.[22] Production of CRP results in synthesis of interleukin-1 and interleukin-6 up-regulated by increased oxidative stress. Proliferation of endothelial cell matrix enhances thickening of the basement membrane leading to atherosclerosis. It also increases enzymes involved in collagen synthesis and enhance endothelial cell collagen IV and fibronectin synthesis. Resulting in excess cross linking of collagen and increased production of extracellular matrix protein in vessel walls.[23] In turn, there is an accumulation of LDL-C particle which are more prone to get oxidized. As a result endothelial cell damage occurs that stimulate inflammatory change and adhesion and proliferation of vascular smooth cells.[24] Endothelial cell dysfunction and CHD play a major part in the pathogenesis of end organ damage. Endothelial dysfunction is well-defined as failure of vascular endothelium to perform its normal role in vasodilation. Inflammatory response plays a significant role in the functioning of endothelium. Because of endogenous and exogenous affecters, the balance between endothelium-derived contracting and relaxing factor get disturbed in endothelial dysfunction.[25] Higher concentration of high sensitive C-reactive protein can predict vascular inflammation and it is also associated with an increased risk of CHD; hs-CRP biomarkers have been developed to obtain more knowledge about the role of the vessel wall. Higher production of reactive oxygen species may impair nitric oxide (NO) bioavailability and inhibit endothelial NO synthase activity causing dysfunction in endothelium thereby promoting coronary heart disease.

Aim and objective

The objective of the study is to evaluate the association of NO, ox-LDL along with high sensitive C-reactive protein in subjects with CHD.

Materials and methods

This cross-sectional study was conducted among 194 subjects from June 2019 to Dec 2019. The sample size for the study was 194 subjects at SRM Medical College Hospital and Research Centre, Chennai, Tamil Nadu, India. A total of 97 CHD subjects attending the Cardiology Department and 97 normal healthy subjects were selected as control are taken from master health check-up programme and medicine OP who were age and sex matched in the age group 25–55 years. By the institutional ethical committee (ECN: 1513/ICE/2018) the study protocol was approved.

Inclusion Criteria

Clinically diagnosed both male and female patients with chest pain, ECG changes, and increased cardiac marker.

Exclusion Criteria

The subjects who were on treatment for diabetes, hypertension, thyroid, arthritis, rheumatoid arthritis, and acute/chronic infection patients.

The details of study were explained to all the patients whoever registered themselves for the program and a written informed consent was taken. After overnight fasting, blood sample (5ml) was collected in sodium citrate and plain vacationer under aseptic precaution. For the measurement of Lipid profile, the following were measured: total cholesterol by **cholesterol oxidase** method, triglycerides by **glycerol peroxidase** method along with HDL-C and LDL-C by **direct method** using Beckman Coulter Auto analyzer (AU480); 2ml of blood sample was used for the former processes and the remaining 3ml of blood was allowed to clot for 30 min and then centrifuged at 2500 RPM for 10 min for the quantification of NO by UV Spectrophotometer, ox-LDL, and hs-CRP in Marketable ELISA Kit.

Statistical analysis

By using Statistical Package for Scientific Studies (SPSS) version 16, all the data were analyzed. The results were symbolized as mean ± standard deviation. For analyzing the difference between the mean levels of various parameters Student's t-test was used. By using Pearson's correlation equation the correlation between various variables were assessed. For statistical significance, the p-value <0.05 was considered.

Result

Totally 194 age and sex matched subjects in the age group 25–55 years were included. Among the 97 CHD subjects, 45 were male subjects and 52 were female subjects with average age 42.93 ± 3.39 years and among 97 healthy controls, 43 were male and 54 were female with average age of 41.30 ± 3.82 (Table 1). The Mean levels of total cholesterol, triglyceride, LDL-C, ox-LDL, and hs-CRP are increased in patients with CHD compared with the controls, whereas the mean levels of HDL-C and NO didn't vary significantly among the groups (Table 2).

Pearson correlation analysis with **NO** showed (i) a significant negative correlation with total cholesterol, triglycerides, LDL-C, TC/HDL Ratio, LDL/HDL Ratio, ox-LDL, and hs-CRP (ii) positive correlation with HDL-C.

Pearson correlation analysis with **ox-LDL** showed (i) a significant positive correlation with total cholesterol, triglycerides, TC/HDL Ratio, LDL/HDL Ratio, LDL-C and hs-CRP (Figure 2A); (ii) a negative correlation with FBG, HDL-C, and nitric oxide (Table 3).

Discussion

Nitric oxide is a pleiotropic factor which act as a free radical regulator containing several biological functions including vasodilation, neurotransmission, and inflammation along with

Table 1 Demographic features of the study subjects.

Parameters	Controls (n = 97)	Non diabetic CHD patient (n = 97)	p-Value
Mean age (years, mean ± S.E.M.)	41.8 ± 9.7	42.3 ± 10.5	NS
Male (%)	40 (41.2%)	45 (46.3%)	—
Female (%)	57 (58.7%)	52 (53.6%)	—

macrophage-mediated immunity.[26] Nitric oxide also acts as an inflammatory mediator. Nitric oxide has both pro-and anti-inflammatory effects because of its versatility. There are several factors that affect the effectiveness of NO. Elevated level of LDL suggests the increased inflammatory events in patients who are positively correlated with the progression of CHD. Uptake and accumulation of oxidatively modified LDL by macrophages in the vessel wall initiate a wide range of bioactivities followed by migration into the intima leading to formation foam cells.[27] Endothelial cells undergo oxidative modification, which causes overproduction of the reactive oxygen species which are crucial for the progression of CHD such as endothelial dysfunction and plaque disruption.[28] The events of CHD begin with the LDL-C deposition in the arterial wall.[29] LDL-Cs are bound by endothelial cells which are activated (by injury) and these cells are attached to macrophages which promote free radicles that oxidize LDL. By endothelial dysfunction, oxidized LDL stimulates the release of growth factors and it is also cytotoxic to endothelial and smooth muscle cells which induce endothelial cell dysfunction. Studies stated that increasing the production of oxygen free radical causes hyperlipidemia which directly impairs the function of endothelial cells; oxygen free radicals can injure tissues and accelerate nitric oxide decay, reducing its vasodilator activity.[30] Increased vascular permeability, leukocyte adhesion, and thrombosis cause endothelial injury.[31] Studies reported that NO has a dual effect on LDL oxidation. In macrophage-dependent functions, oxidized LDL is increased by stimulating platelet adhesion and aggregation by decreasing endothelial production of nitric oxide.[32]

Table 2 Biochemical parameters of coronary heart disease subject and normal individuals.

Parameters	Controls (n = 97)	CHD Subjects (n = 97)	p-Value
FBG (mg/dl)	90.24 ± 4.18	94.29 ± 6.98	NS
Total cholesterol (mg/dl)	168.8 ± 16.3	239 ± 41.42	<0.001
Triglyceride (mg/dl)	84.6 ± 30.5	159.7 ± 69	<0.001
HDL (mg/dl)	46 ± 9	34 ± 7	<0.001
LDL (mg/dl)	106 ± 12.59	161.9 ± 27.46	<0.001
TC/HDL Ratio	3.71 ± 0.70	6.17 ± 1.14	0.0982
LDL/HDL Ratio	2.35 ± 0.53	4.22 ± 0.75	0.1023
Nitric Oxide (µmol/L)	19.08 ± 4.74	12.97 ± 1.20	<0.001
ox-LDL (U/L)	16.73 ± 3.55	41.53 ± 8.72	< 0.0001
hs-CRP (mg/L)	1.92 ± 0.47	3.80 ± 1.35	0.0784

FBG- Fasting Blood Glucose, TC- Total Cholesterol, TG- Triglyceride, HDL- High Density Lipoprotein, LDL- Low Density Lipoprotein, NO- Nitric Oxide, ox-LDL- Oxidized Low Density Lipoprotein, hs-CRP- High sensitive C - reactive protein
Values expressed as Mean±SD.
*p-Value < 0.05 is considered to be significant; NS-Not significant; **Highly Significant; ***Very Highly significant.

Table 3 The Pearson correlations analysis between nitric oxide and oxidized LDL with other biochemical parameters in subjects with CHD.

Variables	NO	P	Ox-LDL	P
FBG	-0.150[b]	<0.0001***	–0.106[b]	<0.0001***
Total cholesterol	-0.812[b]	<0.0001***	0.920[a]	<0.0001***
Triglyceride	0.036[a]	<0.0001***	0.145[a]	<0.0001***
HDL-C	0.046[a]	<0.0001***	–0.148[b]	<0.002**
LDL-C	–0.999[b]	<0.0001***	0.996[a]	<0.0001***
VLDL-C	–0.113[b]	<0.0001***	0.144[a]	<0.0001***
TC/HDL Ratio	–0.059[b]	<0.0001***	0.634[a]	<0.0001***
LDL/HDL Ratio	–0.066[b]	<0.0001***	0.743[a]	<0.0001***
NO	-	<0.0001***	–0.995[b]	<0.0001***
ox-LDL	–0.995[b]	<0.0001***	—	<0.0001***
Hs-CRP	–0.976[b]	<0.0001***	0.821[a]	<0.0001***

*P value < 0.05 is considered to be significant; NS-Not significant; **Highly Significant; ***Very Highly significant.
[a]Positive correlation
[b]Negative correlation

Impairing NO production by class B scavenger receptor, CD36 are the another mechanism of ox-LDL. Excitingly, the complex can return to the caveolae to restore eNOS function when ox-LDL is removed.[33] Ox-LDL reduces the availability of NO due to excess production of free radical superoxide (O_2-). Superoxide inactivates NO producing peroxynitrite (OONO-) causing injury to the coronary endothelium. Free radicals which are derived from oxygen along with NO induce lipid peroxidation in LDL-C which contribute to the pathogenesis of CHD.[34] More studies stated that Ox-LDL is the major cause for hyperlipidemia which triggers CHD.[35] In monocytes, macrophages as well as in neutrophils, the pro-inflammatory cytokines lead to the expression of NO during the inflammation process leading to produce large amounts than normal physiological concentrations.[35] The hs-CRP is an acute-phase protein released into the blood by liver during inflammation associated with occurrence of heart disease and assessment is highly sensitive to the quantification of CRP. Higher level of hs-CRP is accepted as a risk aspect for cardiovascular disease.[36] Ridker et al., found that it is four times possible for healthy individuals with elevated hs-CRP values to have CHD.[37] Ndrepepa stated that upcoming adverse cardiovascular events are associated with elevated hs-CRP level in healthy persons and in subjects with CHD. Fall level in progression of atherosclerosis and recovering the risk of CHD are associated with decreased levels of hs-CRP and LDL-C cholesterol.[38] Inflammation promotes endothelial dysfunction and atherogenesis. It is a significant risk aspect in CHD. The oxidation of LDL-C is induced by free radical from the macrophage. Oxidized LDL enhance the formation of foam cells and atherosclerotic plaque involved in progression of atherosclerosis. Scavenger receptors are responsible for the uptake of oxidized LDL-C particle into the sub intimal region of the arteries causing atherosclerotic lesion which in turn result in raised levels of hs-CRP which are useful in assessing cardiovascular risk.[39] Sproston et al., stated that expression of adhesion molecules are up-regulated by CRP and inhibited eNOS.[40] Jean Davignon et al., also reported that eNOS in cardiovascular endothelial cells obstructing angiogenesis are down regulated through CRP which are repressed by NO production influencing the pathogenesis of atherosclerotic vascular disease stimulated along with leukocyte adherence and inflammation.[41]

Conclusion

This study concludes that the levels of CRP are increased due to inflammation. The level of serum hs-CRP, a low grade inflammatory marker, is significantly high in CHD subjects. Subjects with low grade systemic inflammation and coronary heart disease are related with increased oxidative stress leading to impaired NO bioavailability. Reduced NO and increased oxidized LDL in patients may be a strong predictor of CHD. In addition with LDL-C, the assessment of oxidized LDL and NO may contribute to an early finding of CHD which reduces the morbidity and the mortality risk.

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