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Orginal Article

A COMPARATIVE STUDY OF LIPID PROFILE AND ANTIOXIDENTS BETWEEN INDIANS AND IRANIANS IN MYSORE,INDIA

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ABSTRACT

Objective: High Density Lipoprotein (HDL) particles are distinguished by their capacity to exert a wide spectrum of antiatherogenic biological activities, including their ability to protect Low Density Lipoprotein (LDL) against oxidation, and their potent anti-inflammatory actions on cells of the arterial wall. Since now cardiovascular disease (CVD) is beginning to be recognised as a consequence of inflammation, attention is focussed not only on oxidation but also on inflammation which may be measured as circulating levels of an acute-phase protein, such as C-reactive protein (CRP).

Methods: 5ml of blood was drawn from subjects (Iranians and Indians) in fasting. Lipid profile and lipid per -oxidation and anti-oxidant activities were measured.

Results: Our result showed that the serum lipoproteins of Indians readily get oxidized by Cu^{++} or benzoyl peroxide. Interestingly, the serum lipoprotein of Iranians living in Mysore also showed a similar profile of Cu^{++} and Benzoyl peroxide catalysed oxidation.

Conclusion: Life style and classical risk factors yet higher HDL and higher Paraoxonase (PON) activity in the blood of the Iranians suggests that diet can make a different to the conventional risk factors.

Keywords: Lipid profile, CVD, Inflammation, PON,CRP

INTRODUCTION

It is expected that CVD will overtake infectious disease as the world's leading cause of death and disability by 2020 [1]. It has been shown that cardiovascular disease (CVD) associated with different risk factors [2]. The burden of CVD is increasing roughly in developing Countries [3]. The occurrence of CVD in Iran population is not only high but also the major causes of death in Iran [2,3]. Large numbers of Iranians have one or more major risk factors for CVD. Coronary artery disease, the most common form of CVD, is the major cause of death in Iran today [4].In a study conducted in 45 rural villages in India, 32 percent of all deaths were appropriate to CVD, outranking infectious diseases, which were dependable for 13 per cent giving clear data that the epidemic has reached its advanced stage even in rural India [5].This chronic inflammation may be a part of the diet and lifestyle as well as environment in which Indians live. To investigate this hypothesis, we wanted to choose a genetically distinct group but living in the same environment and having access to the same food materials as Indians. The Iranian community living in Mysore provided a convenient model to test these hypotheses. Since the Iranians are genetically distinct from the Indian population but living in the same environment and exposed to the same risk factors as the other Indians, a comparison of the HDL and PON of the Iranians with that of the Indians would give a comparative picture of the susceptibility of the two to dietary and other modifications. This comparative study will give insights into the contribution of environmental factors and lifestyle on the risk of cardiovascular diseases.

MATERIALS AND METHODS

Materials

Serum samples were collected from Volunteers. Reduced glutathione (GSH), 5, 5'-Dithiobis2-nitrobenzoic acid (DTNB), Ethylene demine tetra acetic acid (EDTA),Ribo flavin,Niro Blue Tetrazolium (NBT), Tris buffer, Bovin Serum, Ascorbic acid were from SRL. Vitemin E was from MERCK. Phenyl acetate was from Sigma Aldrich Company USA. Sulfuric Acid and all other chemicals were purchased from Ranbaxy chemicals (New Delhi). Acids, bases, solvents, and salts used for the purification were of analytical grade.

Subjects

In this study we used two groups of volunteers. Indians and Iranians living in Mysore, India. We compared serum of 30Indians and 30 Iranians. Subjects were between 22 and 50 years of age.

Blood analysis

5ml of blood was drawn from subjects (Iranians and Indians) in fasting and aliquoted in two tubes without anticoagulant at Biochem Diagnostic and Research Lab, Mysore, India. One of that was used for lipid profile in Biochem Lab by enzymetic method using commercial kit. another tube was used in the determination of antioxidant statuses (GSH, Total antioxidant capacity by ferricyanide reduction and phosphomolybdate reduction, and Reduced Glutathione) and susceptibility of LDL to oxidation. Serum was separated by centrifugation (REMI, R8C LABORATORY CENTRIFUGE, India) at 3000 rpm for 10 min and serum was aspirated and stored at -20 degrees until used.

Biochemical analysis

Serum was analyzed for various biochemical parameters by enzymatic methods using commercial kit [TG & Cholesterol (ANAMOL LABORATORIES PVT. LTD. Maharashtra, India), LDL-C &HDL-C (Accurex Biomedical Pvt.Ltd. Mumbai, India), C - reactive protein (HS-CRP) (FUTURA SYSTEM S.R.L.India.], MINDRAY CHEMISTRY ANALYZER, BS-200(CHINA). Total Cholesterol (TC), Triglyceride (TG) and HDL – Cholesterol were assayed using commercial kits. TG and Cholesterol kits were from ANAMOL LABORATORIES PVT. LTD. Maharashtra, India, HDL-C kit was from

Accurex Biomedical Pvt. Ltd. Mumbai, India. C- reactive protein (HS-CRP) (FUTURA SYSTEM S.R.L.India.], MINDRAY CHEMISTRY ANALYZER, BS-200(CHINA).

Determination of total antioxidant activity

For total antioxidant assay various concentrations (5, 10 and 20 µg in 1 ml) of Standard Ascorbic acid were mixed with 1 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated in a water bath at 95°C for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm.900 µl of serum was used as unknown in the above assay and the total antioxidant capacity was calculated in comparison with ascorbic acid.

Determination of Reduced Glutathione (GSH)

Reduced glutathione in the serum was assayed using the reaction with DTNB and compared with a standard solution of reduced glutathione.Serum (0.2ml) was mixed with EDTA solution (1.8 ml, 0.1%w/v) and centrifuged to remove any precipitate. To the supernatant, DTNB reagent (1 ml, 40 mg, 5, 5' dithhiobis-2 nitro benzoic acid in 100 ml of 1% (w/v) sodium citrate) was added and the absorbance was measured at 420 nm. A calibration curve was prepared using reduced glutathione (0 to 60 µg).

Isolation of Human LDL

LDL and HDL were prepared from either pooled serum or individual serum samples by density gradient centrifugation carried out according to the method of Redgrave et al. (1975). A stepwise gradient was prepared as follows: 10mlof serum was mixed with solid KBr such that the density was about 1.3 g/ml.30 ml of saline, PH=7, was layered on top of the serum sample. The tubes were centrifuged in a Sorvall ultra centrifuge in F-50L fixed angle rotor for 3hours, at 40000 rpm (200 000 g) at 4°C.After centrifugation, the tubes were carefully removed from the rotor and placed in the vertical position. The fractions were aspirated from the top. The LDL fraction appears as a yellow layer in the center of the tube, and HDL appears as an orange-layer at the bottom of the tube. The LDL and HDL fraction were collected separately.LDL and HDL containing fractions were dialyzed in the dark for 6 and 8hours against double distilled water.

Determination of Lipoprotein Oxidation

Lipid oxidation in lipoproteins was assessed by spectrophotometric monitoring of conjugated diene formation, serum (5ul) was diluted with phosphate buffered saline (PBS, 995µl) (5 mM phosphate buffer, 125mM NaCl, pH=7.4). LDL and HDL were diluted 1:50 with PBS. 100 µl of diluted serum sample, LDL or HDL was mixed with 850µl PBS and 50µl of 5µ M copper sulfate ($CuSo_4$). The OD of the mixture was monitored at 234 nm every 10 min for up to 90min. The optical density was plotted against time. From this plot the duration of the lag time, time of completion of oxidation and time of decline phase were determined.

The serum, LDL and HDL were also oxidized by Benzoyl peroxide. Benzoyl peroxide (0.1 gr) was dissolved in 1ml Chloroform. Then 5 µl of this was transferred into test tubes and the solvent was removed. To the tubes 895µl PBS was added and vigorously vortexed to suspend the benzoyl peroxide.Diluted serum sample LDL or HDL (100µl) were then added and the absorbance of the mixture was measured at 234 nm every 10 min for up to 90min.The optical density was plotted against time. From this plot the duration of the lag time, time of completion of oxidation and time of decline phase were measured.

Determination of protection of lipoprotein from oxidation by Ascorbic acid:

Protection of serum lipoproteins from oxidation by Ascorbic acid was assessed by spectrophotometric monitoring of the formation of conjugated dienes by copper induced oxidation as described above. However prior to inducing oxidation by copper sulphate, the serum sample was mixed with increasing concentration of ascorbic acid (0.6 to 5.7 µM) The reaction was monitored by for up to 90min at 234nm.

Determination of protection of lipoprotein from oxidation by Vitamin E:

Protection of serum lipoproteins from oxidation by Vitamin E was assessed by spectrophotometric monitoring of the formation of conjugated dienes by copper induced oxidation as described above. However prior to inducing oxidation by copper sulphate, the serum sample was mixed with increasing concentration of Vitamin E (0.2 to 2 µM). The reaction was monitored by for up to 90 min at 234nm.

Protection of PON activity from oxidation by Ascorbic acid and Vitamin E:

Protection of serum PON activity from oxidation by Ascorbic acid or Vitamin E was assessed by spectrophotometric monitoring of the PON activity using phenyl acetate as substrate by copper induced oxidation as described above. However prior to inducing oxidation by copper sulphate the serum sample was mixed with increasing concentration of ascorbic acid (0.6 to 5.7 µM) or vitamin E(0.2 to 2 µM) 234 nm absorbance was monitored for 90 min. The PON activity was determined at the end of 90 min as described above.

Estimation of protein: The protein content of the samples was assessed based on [6].

Statistical analysis:

Mean and standard deviation were calculated for the samples. Comparison between two groups was done using Students t test. Correlation coefficients were calculated by Pearsons Product-Moment correlation.

RESULTS

Table1.Comparison of antioxidant and anti-inflammatory status.

	Indians in Mysore	Iranians in Mysore	P value
Hs-CRP(mg/lt)	2.66 + 3.69	1.80 + 2.85	0.160
Total antioxidant (µg of Ascorbic acid/ml)	256.8 + 233.3	333.4 + 218.2	0.097
Reduced glutathione(mg/lt)	5.51 + 2.84	14.7 + 3.2	0.000
234nm absorbing substances (OD at 234nm)	0.15 + 0.05	0.18 + 0.04	0.010
PON activity(x10^5u/lt)	1.90 + 0.60	2.69 + 0.63	0.000

Although the Hs-CRP was not significantly different between two groups, the Iranians had fewer members of individuals with Hs-CRP > 3 mg/dl (13.3% Vs 26.6% of in Indians).

The total antioxidant capacity was not significantly different in the two groups. The reduced glutathione was significantly higher among Iranians than Indians. The 234 nm absorbing substances among Iranians were also significantly higher than that of the Indians. PON activity was significantly higher in Iranians than Indians.

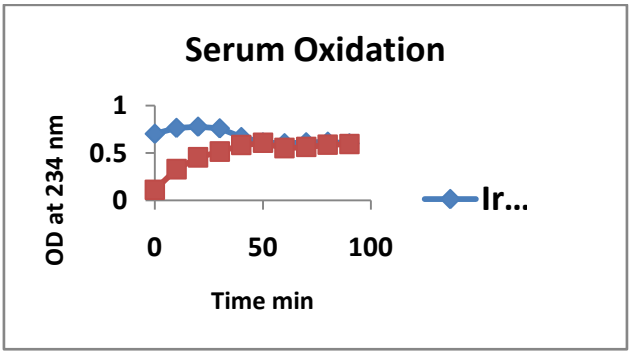

Fig.1: The oxidation of serum lipoproteins by Cu^{++}.

Serum was subjected to oxidation using Cu^{++}. Absorbance at 234 nm was monitored for up to 90 min. Serum lipoproteins of Iranians did not show any increase in the diene formation. Whereas the Indians showed a rapid decrease significantly ($p<0.05$) when their Cu^{++} were used for oxidation which is shown in figure 1.

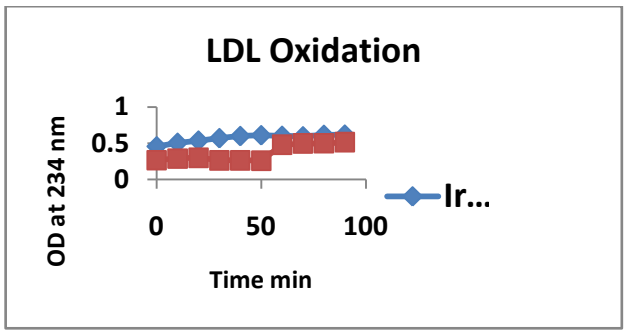

Fig.2: LDL oxidation of Indians and Iranians in India by Cu^{++}.

LDL oxidation of Indian and Iranian samples by Cu^{++}. Results are mean of 4 diterminations.

The LDL of Indians showed a lag phase followed by the propagation phase whereas the Iranian LDL did not show a lag phase which is shown in figure 2.

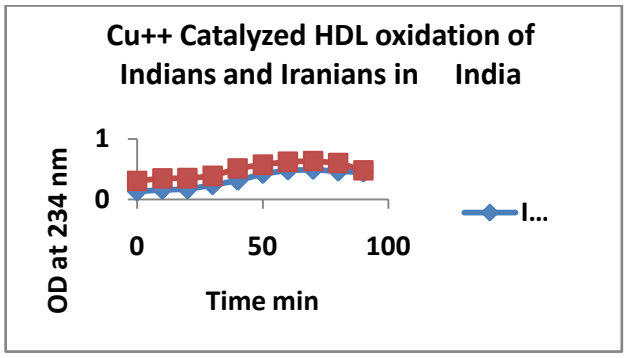

Fig.3: HDL oxidation of Indian and Iranian sample by Cu^{++}.

Iranians showed similar kinetics of HDL oxidation as Indians but the 234 nm absorbing substances were significantly ($p< 0.05$) higher in Iranians. Results are mean of 4 determinations.

Protection of lipoprotein oxidation by exogenous antioxidant

Ascorbic acid was used as a water soluble antioxidant and vitamin E as a lipid soluble antioxidant. The effect of exogenously added ascorbic acid on serum lipoprotein oxidation is shown in figure 4.

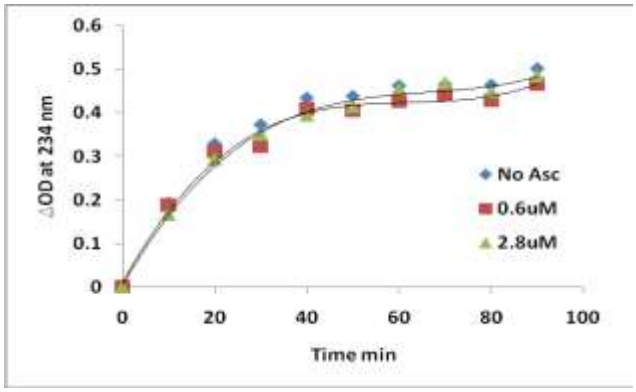

Fig 4. Effect of Exogenous Ascorbic acid on serum lipoprotein oxidation

Oxidation of serum of Indians in the absence or present of increasing conditions of Ascorbic acid as discribed in methods.Results are mean of 5 determinatint.Ascorbic acid did not protect the lipoproteins from by Cu^{++} catalyzed oxidation.

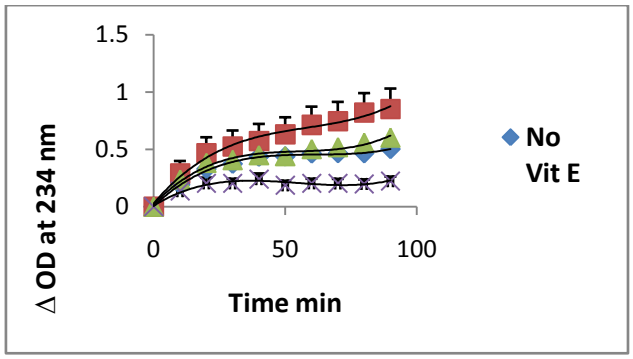

Fig.5: Effect of Exogenous Vitamin E on serum lipoprotein oxidation by Cu^{++}

Oxidaion of serum of Indians in the absence or present increasing concentration of Vitamin E. The Results are mean + SD (n=5).

At lower concentration vitamin E acted as a pro oxidant and increased LDL oxidation. It was inhibitory only at higher concentration. The effect of Ascorbic acid and vitamin E on the prevention of loss of PON under *in vitro* oxidation.

Table 2: Subjective characteristics and lipid profile of Indians and Iranians are living in Mysore.

	Indians in Mysore	Iranians in Mysore
n	**30**	**30**
Age (years)	36.1 + 10.3	35.9 + 9.5
Gender Male	80 %	80 %
female	20 %	20 %
Lipid profile		
Total Cholesterol	179 + 22.1	179.2 + 25.4
Total Triglyceride	159.3 + 75.8	121.3 + 50.4
HDL-C	44.1 + 4.2	48.5 + 5.5
LDL-C	121.2 + 22.8	124.1 + 36.6

Results are Mean + SD (n=30)

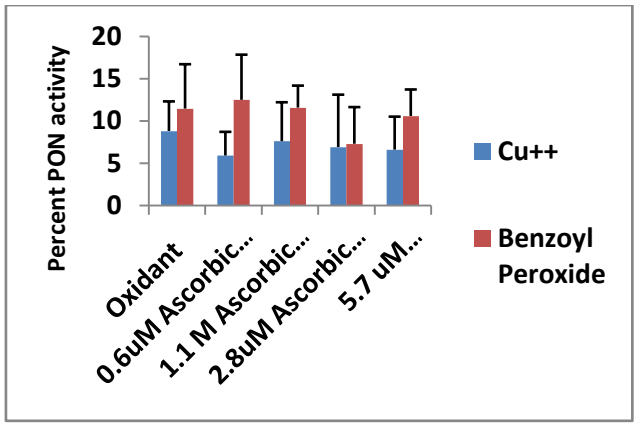

Fig 6: Effect of Exogenous Ascorbic acid on Cu^{++} and Benzoyl peroxide catalyzed inactivation of serum PON activity

Effect of oxidation of serum on PON activity by Cu^{++} and Benzoyl Peroxide in the absence or presence of different amounts Ascorbic acid.Results are mean + SD (n=5).

Effect of oxidation of serum on PON activity by Cu^{++} and Benzoyl Peroxide in the absence or presence of different amounts of Vitamin E as in Figure 7. Results are mean + SD(n=5).

Both by Cu^{++} and Benzoyl peroxide over 80% of the PON activity.Both Ascorbic acid and vitamin E were unable to prevent the loss of inactivation of PON activity. There was no dose dependency of the Ascorbic acid or vitamin E on PON activity.

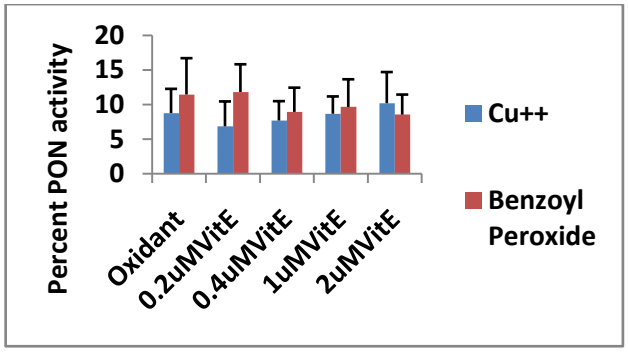

Fig 7.Effect of Exogenous Vitamin E on the Cu^{++} and Benzoyl peroxide catalyzed inactivation of PON

DISCUSSION

The oxidation hypothesis of atherosclerosis implies that if oxidation of LDL can cause atherosclerosis [7], then antioxidants should prevent it. We are protected against oxidative stress by a variety of antioxidants with different functions. For example, Superoxide dismutase quenches superoxide, which results in the inhibition of peroxynitrite and inhibition of transition metal ion formation. Catalase protects from H_2O_2 formed by the dissimulation peroxides reduces lipid by peroxides to their corresponding alcohols. Vitamin E and Vitamin C are the most abundant and reactive radical scavenging antioxidants [8]. Bowry et al., (1992) [9] discovered that, under certain cases Vitamin E actually acts as a pro oxidant. In fact this has been observed in the case of isolated LDL *in vitro.* Ascorbic acid acts as a strong reducing agent and act reduce Fe^{+++} and Cu^{++} to Fe^{++} and Cu^{+} respecting the Cu^{+} is a more potent oxidant than Cu^{++}. *In vivo* experiment and chemical trials unfortunately did not show expected beneficial effects [9, 10].Our results on the protection of lipoprotein oxidation by adding exogenesis Vitamin E and Ascorbic acid also did not show any beneficial effects. It is possible that the antioxidants need to get associated with the lipoprotein for them to protect the lipoprotein from oxidation. Our results are consistent with the literature reports that show little or no benefit of using antioxidants. The serum lipoproteins of Indians were highly susceptible to oxidation whereas the lipoproteins of Iranians were resistant to oxidation. Indians had a shorter lag phase of oxidation, they reached the maximum in shorter time and the oxidation products decomposed within the duration of the experiment. Whereas the serum of the Iranians did not reach the maximum within the duration of the experiment. When Iranians living in Mysore were compared with Indians or Iranians, they showed remarkable differences. Their serum lipid profile was similar to that of the Indians than Iranians. There was no difference in the total cholesterol and LDL-C. However, the total triglycerides were lower than the Indians. The total antioxidant capacity was the same as that of the Indians. But they had higher reduced glutathione. Surprisingly the 234nm absorbing substances in their blood resembled that of the Indians and not Iranians. Their HDL-C was lower than that of Iranians but the PON activity was the same. The serum lipoproteins, HDL and LDL of Iranians in India were more susceptible to oxidation than Indians.

CONCLUSION

Our results suggest that diet can play a major role in modifying the risk factors of CVD. Iranian population residing in Mysore would represent a genetically homogenous group living in a different environment. The environmental influences would reflect on their risk factors for CVD. Interestingly the HDL-C of the migrant Iranians decreased and the susceptibility of serum lipoproteins to oxidation increased. This is a direct reflection of the antioxidant status of their lipoproteins.

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