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 rapidly 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 attributable to CVD, outranking infectious diseases, which were dependant for 13 per cent giving clear data that the epidemic that 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 susceptibility of LDL to oxidation. Serum was separated by centrifugation (REMI, RSC LABORATORY CENTRIFUGE, India) at 3000 rpm for 10 min and serum was aspirated and stored at -20 degrees until used.

MATERIALS AND METHODS

Materials

Serum samples were collected from Volunteers. Reduced glutathione (GSH), 5, 5'-Dithiobis2-nitrobenzoic acid (DTNB), Ethylene diamine tetra acetic acid (EDTA), Ribo flavin, Niro Blue Tetrazolium (NBT), Tris buffer, Bovin Serum, Ascorbic acid were from SRL-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 enzymatic methods using commercial kits. TG and Cholesterol kits were from ANAMOL LABORATORIES PVT. LTD. Maharashtra, India, HDL-C kit was from RANBAXY MULTIVITAMIN & Heparin and Benzoyl peroxide catalysed oxidation..

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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 suggest that diet can make a different to the conventional risk factors.

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

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).

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Received: 21 April 2014, Revised and Accepted: 10 May 2014

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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 assessed using the reaction with DTNB and compared with a standard solution of reduced glutathione. Serum (0.2 ml) 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 μg, 5’ dithiobis-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 preparation was used as follows: 10 ml of serum was mixed with solid KBr such that the density was about 1.3 g/ml. 30 ml of saline, 0.1% w/v) and centrifuged to remove any precipitate. To the supernatant, DTNB reagent (1 ml, 40 μg, 5’ dithiobis-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).

Determination of Lipoprotein Oxidation

Lipid oxidation in lipoproteins was assessed by spectrophotometric monitoring of conjugated diene formation, serum (5 μl) was diluted with phosphate buffered saline (PBS, 99.5 μl) (5 mM phosphate buffer, 125 mM 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 850 μl PBS and 50 μl of 5 μM copper sulphate (CuSO4). The OD of the mixture was monitored at 234 nm every 10 min for up to 90 min. 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 1 ml Chloroform. Then 5 μl of this was transferred into test tubes and the solvent was removed. To the tubes 895 μl PBS was added and vigorously vortexed to suspend the benzoyl peroxide. Diluted serum sample LDL or HDL (100 μl) were then added and the absorbance of the mixture was measured at 234 nm every 10 min up to 90 min. 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 90 min at 234 nm.

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 234 nm.

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

Table 1. Comparison of antioxidant and anti-inflammatory status.

<table>
<thead>
<tr>
<th></th>
<th>Indians in Mysore</th>
<th>Iranians in Mysore</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs-CRP (mg/l)</td>
<td>2.66 + 3.69</td>
<td>1.80 + 2.85</td>
<td>0.160</td>
</tr>
<tr>
<td>Total antioxidant (μg of Ascorbic acid/ml)</td>
<td>233.3</td>
<td>218.2</td>
<td></td>
</tr>
<tr>
<td>Reduced glutathione (mg/l)</td>
<td>5.51 + 2.84</td>
<td>14.7 + 3.2</td>
<td>0.000</td>
</tr>
<tr>
<td>23 nm absorbing substances (OD at 234 nm)</td>
<td>0.15 + 0.05</td>
<td>0.18 + 0.04</td>
<td>0.010</td>
</tr>
<tr>
<td>PON activity (x10^3/u/l)</td>
<td>1.90 + 0.60</td>
<td>2.69 + 0.63</td>
<td>0.000</td>
</tr>
</tbody>
</table>

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.

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.

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.

Ascorbic acid did not protect the lipoproteins from by Cu++ catalyzed oxidation.

Results are Mean + SD (n=30)

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

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

Oxidation of serum of Indians in the absence or presence 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.

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.
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.

ACKNOWLEDGMENT

The author thanks Tonekabon Branch Islamic Azad University, Iran for the support.

REFERENCES