List of Publications
INTRODUCTION

Cardiovascular diseases remain the leading cause of morbidity and mortality in developed as well as developing nations in spite of great advances made in understanding mechanisms underlying the diseases. Since High Density Lipoprotein (HDL) has been the only negative risk factor identified so far, the focus is shifting towards this molecule as a diagnostic and therapeutic target[8]. HDL is a circulating non covalent assembly of proteins and lipids. The Framingham study showed that plasma levels of HDL-Cholesterol (HDL-C). HDL-C estimates the reverse cholesterol transport component (RCT) of HDL. Since RCT is not the only antiatherogenic mechanism of HDL, it is important to assay other functions like antioxidant and anti-inflammatory activity. Studies have shown the susceptibility of High Density Lipoprotein (HDL) molecule to modification invivo. Presence of modified HDL in atherosclerotic tissue has confirmed its physiological relevance. In this study we have subjected serum to copper catalysed oxidation and then estimated the HDL-C and PON1 activity using phenyl acetate and paraoxon as substrates. On oxidation PON1 activity was reduced by over 80 % where as HDL-C was not significantly different. IgY was prepared against oxidised HDL. When IgY was added to the oxidised serum a precipitate was obtained which had less than 5 % of the PON1 activity. When this was mixed with unoxidised HDL the PON1 activity of the latter was competitively inhibited. Our results suggest that the oxidised HDL is detected quantitatively as HDL-C but its functionality is reduced by over 80 % giving a false sense of security to patients at risk for Cardio Vascular Disease (CVD).

KEYWORDS: PON1, Oxidized HDL, IgY, Competitive inhibition.

Oxidized HDL inhibits PON1 activity associated with native HDL.

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ABSTRACT

HDL is clinically determined as HDL-Cholesterol (HDL-C). HDL-C estimates the reverse cholesterol transport component (RCT) of HDL. Since RCT is not the only antiatherogenic mechanism of HDL, it is important to assay other functions like antioxidant and anti-inflammatory activity. Studies have shown the susceptibility of High Density Lipoprotein (HDL) molecule to modification invivo. Presence of modified HDL in atherosclerotic tissue has confirmed its physiological relevance. In this study we have subjected serum to copper catalysed oxidation and then estimated the HDL-C and PON1 activity using phenyl acetate and paraoxon as substrates. On oxidation PON1 activity was reduced by over 80 % where as HDL-C was not significantly different. IgY was prepared against oxidised HDL. When IgY was added to the oxidised serum a precipitate was obtained which had less than 5 % of the PON1 activity. When this was mixed with unoxidised HDL the PON1 activity of the latter was competitively inhibited. Our results suggest that the oxidised HDL is detected quantitatively as HDL-C but its functionality is reduced by over 80 % giving a false sense of security to patients at risk for Cardio Vascular Disease (CVD).

KEY WORDS: PON1, Oxidized HDL, IgY, Competitive inhibition.

Preparation of lipoproteins by ultracentrifugation method

Human serum HDL and LDL were isolated by density gradient ultracentrifugation using a Sorvall UC, ultracentrifuge. The centrifugation was carried out in a vertical rotor, T-1250 with vertical tubes of 30 ml capacity. Appropriate amount of Potassium bromide (KBr) was added to the serum to get a density of 1.3 g/ml. Normal saline of density 1.006g/ml was taken in the centrifuge tubes and the KBr added serum sample was layered below the saline layer without disturbing the two layers Centrifugation was carried out at 45,000 rpm for 3 hrs at 4°C in a vertical tube rotor. The fractions were collected from the top by aspiration. Fractions were dialyzed to remove KBr[11]. Protein was estimated by Lowry’s method[11].

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Determination of HDL-C in serum
HDL-C was determined in serum after precipitation with phosphotungstic acid in presence of magnesium chloride.

Serum oxidation
Serum was subjected to copper catalysed oxidation by mixing 990 µl of serum with 10 µl of 5 mM CuSO₄ for 1 hr prior to determination of HDL-C and paraoxonase activity.

HDL oxidation
HDL (200µg protein) was oxidised using copper sulphate (10µM) at room temperature for 1 hr in Phosphate buffer saline (PBS) pH 7.4.

Paraoxonase assay with phenyl acetate as substrate
PON1 activity towards phenyl acetate (PA) was measured spectrophotometrically at 270nm in an automated Shimadzu UV-1601, UV-Visible spectrophotometer. The assay mixture included 2mM phenylacetate, and 10mM Tris HCl buffer, pH 8.3 containing 2mM CaCl₂. The reaction was initiated by adding serum or HDL. Absorbance was monitored continuously for 3 minutes. Nonenzymatic hydrolysis of phenylacetate was subtracted from the total rate of hydrolysis. The molar extinction coefficient, ε270, for the reaction was 1.310 M⁻¹cm⁻¹. One unit of paraoxonase activity is defined as 1µmol of phenylacetate hydrolyzed per minute under the assay conditions described above.

Paraoxonase assay with paraoxon as substrate
PON1 activity towards paraoxon (PON) was quantified spectrophotometrically using 10 mM Tris-HCl buffer, pH 8.3 containing 2mM CaCl₂, and 2mM paraoxon. The reaction was initiated by adding serum or HDL and monitored continuously for 3 minutes at 25°C by measuring the appearance of p-nitro phenol at 405 nm in an automated recording Schimadzu UV-1601, UV-Visible spectrophotometer. The blank (incubation mixture without serum) was run simultaneously to correct for spontaneous breakdown of substrate. One unit of PON1 activity is defined as 1µmol of p-nitro phenol formed per minute. The amount of p-nitro phenol was calculated from the molar extinction coefficient at pH 8.3 which was 18.05x10⁶ M⁻¹cm⁻¹.

Immunization
Immunization of chickens for polyclonal antibody production is comparable to that of rabbits with respect to route of injection, the amount of antigen used and the kinetics of specific antibody generation. Laying hens were injected subcutaneously at multiple sites in the wing with 50µg antigen in complete Freund’s adjuvant. The eggs were collected after the 7th day and were stored at 4°C prior to IgY purification. Specific antibody titers were boosted by two or three additional injections at two week intervals.

Preparation of IgY against oxidized HDL
Egg yolk was taken and diluted 5 times with 0.1 M phosphate buffer pH 7.6, 3.5 % W/V of polyethylene glycol was added, centrifuged at 5000 g for 20 min. The clear slight yellowish supernatant was taken and the percentage of Polyethylene glycol (PEG) in the supernatant was made up to 12 %, followed with centrifuge at 5000 g for 25 min. The precipitate obtained was diluted in phosphate buffer and it was again precipitated with 12 % PEG. Centrifuged at 5000 g for 25 min. The precipitate obtained was diluted in minimum amount of buffer and used.

Immunodiffusion
0.8 % Agar in phosphate–buffered saline pH 7.2 was autoclaved, cooled slightly and was poured in 60-mm petri dishes. The gel was allowed to harden. Gel was cut out to form a series of wells. The central well was filled with the antibody and the peripheral wells were filled with the antigen. The plates were incubated for 48 hrs at 37°C. Precipitin bands were observed and photographed.

Precipitation of oxidized HDL using IgY
Oxidized HDL was precipitated by adding 100 µl of IgY to 400µg of oxidised HDL and incubated at room temperature (RT) for one hour. Centrifugation was carried out at 6000 rpm at RT to separate the precipitate and the supernatant. Precipitate was dissolved in 100 µl of PBS buffer and supernatant were used for arylesterase and paraoxonase activity.

Effect of oxidized HDL on PON1 activity
HDL (400µg) was incubated with increasing concentration of solubilized precipitate (25, 50 and 100 µl) at RT for 1 hr prior to determining the PON1 activity of HDL using PA and PON as substrates.

Determination of nature of inhibition
PON1 activity was measured using Phenyl acetate as substrate as described above using different concentrations of substrate from 0.25mM to 2.0 mM in the absence or presence of the oxidised HDL. Plot of 1/V vs 1/S was plotted.

RESULTS:
The HDL-C measured in random samples of serum was found to be between 35-60 mg/dl. When the serum was subjected to oxidation by Copper ions, followed by the determination of HDL-C, there was a small decrease in the HDL-C but the difference was not statistically significant (Fig 1). However the PON1 activity decreased by about 82 % when Phenyl acetate was used as a substrate and 90 % when Paraoxon was used as substrate (Fig 2).

Figure 1 Determination of HDL-C before and after oxidation of serum

<table>
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<th>Oxidised</th>
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Serum was oxidised with copper ions prior to determining HDL-C. Results are mean±SD (n=5).

Figure 2 PON1 Activity after copper catalysed oxidation

<table>
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<tr>
<th>PON1 Activity</th>
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<th>PA</th>
<th>PON</th>
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<td>Percent PON Activity</td>
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<td>Serum was oxidised with copper ions prior to determining PON1 activity using PA and PON as substrates</td>
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HDL prepared by Ultracentrifugation was oxidised by copper ions and was used to raise IgY in hens. The IgY reacted with oxidised HDL but did not react with unoxidised HDL forming a precipitin band (Fig 3).

**Figure 3 Precipitin bands by immunodiffusion in oxidised HDL and oxidised serum**

Treatment of oxidised serum with antibody gave a visible precipitate. When the precipitate was removed from the supernatant, the PON1 activity of the supernatant increased. The precipitate however had a small amount of PON1 activity (<8%) when Phenylacetate was used as a substrate, but with Paraoxon as substrate there was no detectable activity. When the precipitate was added to unoxidised HDL, the activity of PON1 decreased in a dose dependent manner (Fig 4), whereas when the supernatant was added the PON1 activity increased. This observation was made with both Phenylacetate as substrate and Paraoxon as substrate (Fig 5).

**Figure 4 PON1 activity in the presence of increasing concentration of oxidised HDL**

Serum was oxidised and oxidised HDL was precipitated using IgY. The precipitate or the supernatant were mixed with HDL prior to determining its PON1 activity with PA and PON as substrates. Results are mean±SD (n=3)

**Figure 5 PON1 activity with PA and PON as substrates in the presence of oxidised HDL**

Lineweaver-Berk plot for the inhibition of PON1 by oxidised HDL is shown in Fig 6. The nature of inhibition appeared to be competitive.

**Figure 6 Lineweaver-Berk plot for the inhibition of PON1 by oxidised HDL**

DISCUSSION:

HDL particle consists of about 25% cholesterol and 5% Triglyceride (TG). Most of the cholesterol is in the esterified form and the rest is unesterified. Measurement of HDL-C is the most accessible laboratory measurement of HDL lipoprotein. The primary mechanism by which HDL is believed to be antiatherogenic is through the reverse cholesterol transport which involves eight steps, four organs/tissues and six enzymes. Since reverse cholesterol transport is believed to removes peripheral cholesterol and transports it to the liver for conversion to bile acids and excretion, cholesterol associated with HDL was considered “good”. Even the Framingham study has shown a correlation of increased HDL-C with reduction in risk of heart diseases.

It is now realized that the non-RCT function of HDL may be as important as RCT if not more, in antiatherogenic effects of HDL. These functions of HDL are mediated through the enzymes/proteins associated with HDL par-
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ticle like PON1, LCAT, Glutathione peroxidase and Apo A1. HDL is also known for its anti-inflammatory activity. This is brought about through interaction with the vascular endothelium as well as circulating pro inflammatory cells. HDL has been shown to reduce the expression of adhesion molecules by endothelial cells[19], inhibit the expression of MCP-1[18] and can modulate vascular tone by affecting Nitric oxide production[20]. HDL can also interact with circulating WBCs and limit inflammation.

Nicholls et al[21] showed that myeloperoxidase preferentially associates with HDL, and is responsible for the oxidation of Apo A1. The oxidized Apo A1 is unable to participate in RCT.

HDL oxidation was shown to proceed without a lag phase since HDL particles have very little antioxidants in them[22]. Thus HDL is more susceptible to oxidation than LDL which has substantially more antioxidants associated with it. Evidence for oxidized HDL in humans has not been vigorously pursued. There are several reports in literature for the oxidative modification of HDL and its subsequent loss of function. HDL has been oxidized by metal ions, peroxyl and hydroxyl radicals, hypochlorous acid and cigarette smoke[8]. These invitro agents also have an in vivo relevance. Oxidized HDL has been detected in atherogenic plaques[23]. Since blood has high concentration of antioxidants, it may be an unlikely location for invitro oxidation of HDL. It is estimated that about half of all the HDL is in the interstitial fluid compartment. It is likely that HDL is oxidized in this compartment during inflammation. The oxidized HDL molecules could then reenter circulation. Because of their small size, they become readily diffusible through the endothelium. Modification of HDL by oxidation or non enzymatic glycation has been shown to reduce the anti inflammatory properties as well as RCT. Our results show that when whole serum was subjected to oxidation in vitro, followed by the determination of HDL-C there was no significant difference (p > 0.05) in the amount of HDL-C. These results suggest that oxidative modification of serum proteins does not affect HDL-C measurement. However, the oxidation of serum caused 82-90 % loss of PON1 activity associated with HDL, suggesting that the proteins/enzymes associated with HDL can undergo modification resulting in a loss of function. Hence HDL-C alone is inadequate to explain the cardio protection offered by HDL.

When the oxidised HDL was precipitated using an antibody (IgY) the PON1 activity of the serum increased suggesting that the oxidized HDL was acting as an inhibitor of native HDL. This was confirmed when the oxidized HDL was added back to the serum, the PON1 activity of the serum decreased in a dose dependent manner. Our results show that the nature of inhibition was competitive. Our results also show that serum from patients having heart diseases have oxidized HDL as seen from the increase in PON1 activity after precipitation of oxidized HDL with IgY.

Taking into account the diversity of functions of HDL, it is difficult to imagine that all these functions are mediated by a single HDL species. Several sub species of HDL are identified on the basis of their density, size or electrophoretic mobility. Thus the term ‘HDL’ may refer to distinct and varied functionalities. These particles might play important physiological roles that have little to do with RCT. While some of these species’ are highly susceptible to oxidation others may be quite resistant. Thus whether oxidation of HDL is a cause of atherosclerosis or a consequent of it needs to be investigated.

ACKNOWLEDGEMENTS

REFERENCES:
4. Tsugikazu Komoda The HDL handbook: biological functions and clinical implications

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Polyclonal antibodies to oxidized apolipoprotein A-I: quantitative detection of oxidized HDL

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Abstract

Cells of the immune system recognize Damage Associated Molecular Patterns (DAMP) through specific receptors and react by producing specific antibodies. Oxidized lipoproteins are recognised by the immune system through the DAMP receptors. The oxidized lipids and proteins both act as epitopes. The objective of this study was to raise antibodies against oxidized Apo A-I and to test whether they can recognize oxidized High Density Lipoprotein (Ox-HDL). Apo A-I was purified from HDL prepared from human serum, by preparative electrophoresis. It was oxidized using 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH). Oxidized Apo A-I was used to immunize egg laying hens. The post immunized eggs were used to isolate Immunoglobulin Y (IgY). The IgY was used in developing an Enzyme Linked Immunosorbant Assay (ELISA) for ox-HDL. Cross reactivity to other oxidized and glycated molecules like Low Density Lipoprotein (LDL) and Bovine Serum Albumin (BSA) were also tested. The IgY to ox-Apo A-I reacted equally with unoxidized, oxidized and glycated HDL and maximally with unoxidized, oxidized and glycated LDL. Glycated BSA reacted with the antibody more than the oxidized BSA. Our results suggest that since AAPH can oxidize both lipids and proteins, the oxidation specific epitopes on lipids as well as proteins are recognized by the immune system producing antibodies that cross react with all oxidized molecules. Hence antibodies to oxidized Apo A-I cannot be used to quantitatively estimate ox-HDL in human serum and tissue samples.

Keywords: Apo A-I, Ox-Apo A-I Ox-HDL, glycated HDL, Ox-LDL, Glycated LDL, IgY

Introduction

Oxygen and oxidation are of vital important to our existence. Oxidative phosphorylation and non respiratory oxygenation are essential components of our metabolism, signal transduction, and protection against invading pathogens. (Miller et al 2011). However, this vital process can also have its side effects: oxidation
reactions can lead to the formation of reactive oxygen species which in turn can oxidize lipids and proteins.

The oxidation of lipoproteins, particularly of Low Density Lipoprotein (LDL) has been shown to the cause of atherosclerosis. However, High Density Lipoprotein (HDL) can prevent LDL oxidation by several mechanisms. HDL is itself an antioxidant particle and can reduce the level of oxidized LDL by accepting the lipid hydroperoxides of oxidized LDL. The lipid hydroperoxides can break down forming malondialdehyde, ketones, free radicals and other degradation products which can react with proteins and modify them. Modified proteins often lose their properties when they are sufficiently modified. Amino acid residues in the HDL-associated protein, apo A-I, such as methionine, cysteine, tyrosine, and lysine residues, can be selectively modified under the action of these oxidants. Oxidized amino acid residues, including chlorotyrosines, nitrotyrosines and oxidized lysine and methionine residues, are present in apo A-I isolated from plasma and from human atherosclerotic lesions. Moreover, von Eckardstein et al (1991) reported that the oxidized form of apo A-I was found in freshly prepared HDL particles from healthy human plasma.

The modified proteins have to be cleared from circulation since they can cause further damage. Oxidized lipoproteins in circulation are removed by phagocytic cells. Phagocytic cells have a complex mechanism to recognize these oxidized lipoproteins and distinguish them from the unmodified lipoproteins. This is done through pattern recognition of the molecules by the specific receptors which can recognize a wide variety of patterns.

The class B scavenger receptor family has many receptors, but CD36 is a predominant receptor associated with pattern recognition particularly oxidized lipoproteins (Endemann et al 1993). Over past several years attempts have been made to understand the chemical and structural nature of the oxidized phospholipid ligands recognized by the pattern recognition receptors. Bird et al (1999) showed that the receptors for the ox-LDL on mouse peritoneal macrophages recognized both the oxidized lipids and the oxidized protein moieties. Boullier et al (2000) showed that the copper oxidized LDL bound to CD36 and 50% of the CD36 binding activity was because of the oxidized lipid fraction and the other 50% resided in the protein fraction.

Natural antibodies are also an important part of the innate immunity. Unlike the highly specific humoral immune response of the adaptive immunity which secretes predominantly IgG, special subsets of B cells called B-1 cells secrete predominantly IgM and IgA; these are the natural antibodies (Miller et al 2011). The natural antibodies bind to oxidation specific epitopes. In normal mice 20-30% of the all IgM derived from B-1 cells bound to oxidation specific epitopes (Chou et al 2009). Thus the overall pattern of responses and the types of antibodies elicited by an
oxidized lipoproteins particle can be complex involving different receptors and signalling pathways.

In this study we have purified Apo A-I and oxidized it using AAPH. The oxidized Apo A-I was used as an antigen to raise antibodies in the egg to provide a specific antigenic determinant for oxidized HDL.

Materials and Methods

Gelatin, Acrylamide, N,N’ methylene bisacrylamide, Sodium dodecyl sulphate, Ammonium per sulphate, TEMED, Bromo Phenol Blue, and Potassium Bromide were purchased from SRL, Mumbai. 2,2’-Azobis(2-amidinopropane) dihydrochloride (AAPH) from Sigma Aldrich, USA. Freund’s Complete Adjuvant, Freund’s Incomplete Adjuvant and Polyethylene glycol (PEG) were from HiMedia, We used Rabbit anti Chicken IgY Antibody-HRP conjugate from Merck as a secondary antibody and o-Phenylenediamine (OPD) as a substrate for HRP and Bovine Serum albumin (BSA) from Sigma Aldrich, USA.

Preparation of HDL

Human serum HDL was isolated by density gradient ultracentrifugation using a Sorvall WX100 Ultracentrifuge. The centrifugation was carried out in a fixed angle rotor T-1250 (SL 6102823) with tubes of 36 ml capacity. Appropriate amount of KBr was added to the serum to get a density of 1.3 g/ml. Normal saline of density 1.006g/ml was taken in the centrifuge tubes and the KBr added serum sample was layered below the saline layer without disturbing the two layers. Centrifugation was carried out at 45,000 rpm for 3 hrs at 4°C. The fractions were collected from the top by aspiration. Fractions were pooled and dialyzed to remove KBr (Naidu A K et al 2002). Protein was estimated by Lowry method (Lowry OH et al, 1951).

SDS-PAGE

Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (Laemmli 1970) using 10% cross linked gel as the resolving gel and 2.5% cross linked stacking gel. The resolving gel contained 30% Acrylamide, 0.8% N,N’ methylene bisacrylamide, 10% SDS and 1.5 M Tris-HCl buffer pH 8.8. The various components were mixed, denatured and polymerization was initiated by adding ammonium persulphate and 20% of TEMED. Stacking gel contained 30% Acrylamide, 0.8% N, N’ methylene bisacrylamide, 10% SDS and 0.5M Tris – HCl buffer pH 6.8, ammonium persulphate and TEMED. Tris (1.5M), glycine (0.192M) buffer pH 8.3 containing 0.1% SDS was used as the reservoir buffer.

The sample buffer comprised of 1.5 M Tris, 10ml of glycerol and 50mg bromophenol blue made up to 100 ml in distilled water. Samples were mixed with
sample buffer and immersed for 2 minutes in boiling water, cooled and loaded onto the gel. Standard protein markers were also run each time along with the sample. Electrophoresis was carried out at 90 volts for two and a half hours. The gels were stained using coomassie brilliant blue R-0.25% set in methanol, glacial acetic acid and water 45: 5: 50 v/v and destained with methanol, glacial acetic acid and water 50: 7: 43 v/v.

**Purification of ApoA1 by electro elution**

ApoA1 from human serum HDL (prepared by ultracentrifugation) was purified by SDS–PAGE (Laemmli 1970), followed by electro elution. One dimensional preparative and analytical SDS-PAGE were performed in a vertical slab gel unit using 10% separating gels and 2.5% stacking gels. The gel was stained with Coomassie Brilliant Blue solution. Apo A1 (28 KD) protein from polyacrylamide gel was extracted by the electrophoretic elution method using dialysis membrane for protein retention. Protein band with 28 KD size was excised from the polyacrylamide gel and cut into small pieces. For electrophoretic elution, gel fragments were homogenized and equilibrated with reservoir buffer (1.5 M Tris-HCl buffer (pH 8.3) with glycine (0.192M) containing 0.1% SDS). The equilibrated gel fragments were then placed in a dialysis tube with 12 KD cut off. The dialysis tube was placed in a Horizontal electrophoresis apparatus. The electro elution of Apo A1 present in dialysis tubes was performed at 50 V for 8h at 4°C in Tris glycine buffer containing 0.1% SDS (pH 8.3). After the electro elution the free ApoA1 protein from the dialysis bag was transferred to a centrifuge tube. Centrifugation was carried out at 10,000 rpm for 10 mins. ApoA1 present in the supernatant was carefully separated from the gel residues. SDS from Apo A1 sample was removed by extensive dialysis of ApoA1 against water. The diluted ApoA1 was lyophilized and protein content was estimated by Bradford method (Bradford M M, 1976) and used for antigen preparation.

**Preparation of Antigen**

Purified antigen was prepared by incubating 1mg/ml of Apo A1 in 40mmol/L AAPH in PBS 37°C for 3 hrs. After incubating the sample was dialysed against 10mM NaCl and used as antigen (Takanari Nakano and Atsuo Nagata 2003, Brett Garner 1998).

**Raising antibodies (IgY) to oxidized Apo A-I in Chickens**

Immunization of chickens for polyclonal antibody production is comparable to that of rabbits with respect to route of injection, the amount of antigen used and the kinetics of specific antibody generation. Laying hens were injected subcutaneously at multiple sites in the wing with primer dose of 200µg/200µl oxidised Apo A-I emulsified in complete Freund’s adjuvant. Specific antibody titres were boosted by two or three additional injections with 100µg/200µl emulsified with incomplete Freund’s adjuvant at two week intervals. The eggs were collected daily after the 35th day and were stored
at 4°C prior to IgY purification. The collection of eggs were ended on 56th day or continued if antibody is required followed by additional booster doses (Bizanov G and Jonauskiene 2003). Antibody titres were determined by checker board analysis using Indirect ELISA.

**Purification of IgY**
Egg yolk was taken and diluted 5 times with 0.1 M phosphate buffer pH 7.6, 3.5% w/v of polyethylene glycol was added, centrifuged at 5000 g for 20 min. The clear slight yellowish supernatant was taken and the percentage of Polyethylene glycol (PEG) in the supernatant was made up to 12%, followed with centrifugation at 5000g for 25 min. The precipitate obtained was diluted in phosphate buffer and it was again precipitated with 12% PEG, centrifuged at 5000g for 25 min. The precipitate obtained was diluted in minimum amount of buffer (1-2ml) and used (Polson A 1980).

**Development of Indirect Elisa for Anti oxidized HDL**
Ninety-six-well microplates were coated overnight at 4°C with antigen (oxidized HDL or other proteins, and serum) in 50µl coating buffer, plates washed with 200µl of PBS, followed by incubation for 2 hrs at 37°C with 100µl of 1% Gelatin in PBST as blocking buffer. Plates were washed twice in PBST, 50µl primary antibody (Antioxidized Apo A-I IgY) 1:8000 dilution added, and plates incubated for 2 h at 37°C. Following washing with PBST thrice, 50µl of secondary Antibody Rabbit IgG anti IgY-HRP conjugate (1:15000 dilution) and incubated for 1 and half hrs at 37°C. Following three washes with PBST, 100 µl of OPD was added and color developed for 5–10 min at RT. The reaction was stopped by addition of 50 µl 1N H₂SO₄, and the absorbance read at 490 nm in a plate reader.

Oxidization of protein samples to check the Cross Reactivity of anti ApoA1 antibody.
2mg HDL/LDL/BSA was incubated in 4mmol/L of AAPH for 3 hrs at 37°C. After incubation, the samples were dialyzed against 10mM NaCl and used as antigen.

Glycation of protein samples to check the Cross Reactivity of anti ApoA1 antibody
Glycated Apo A-I was prepared by incubating 1mg Apo A-I in 1ml of 5mM glyoxal in PBS containing 0.05% sodium azide at 4°C for ten days. The glycated ApoA1 was dialysed against water to remove free glyoxal and used as antigen. 0.5 ml of (4.68mg) of HDL/LDL/BSA were non-enzymatically glycated by incubating with 1M glyoxal. After one week of incubation at 4°C the samples were dialyzed against 10mM NaCl and used as antigen.
**Results**

The electrophoretic patterns of serum protein and Apo A-I are shown in Figure 1.

HDL was subjected to SDS-PAGE. The band corresponding to ApoA1 was cut, electroeluted and concentrated. Lane 1. Molecular weight markers, Lane 2. HDL, Lane 3. Purified ApoA1 (CBB Stain), Lane 4. Purified ApoA1 (silver stain)

The isolated band corresponded to 28 KD and gave a single band by coomassie blue staining as well as silver staining.

When the purified Apo A-I was oxidized with cupric ions and subjected to Mass spectral analysis, very small fragments were obtained which could not be used for raising antibodies. When the Apo A-I was then oxidised with AAPH, the protein gave a 20 KD fragment (Figure 2).
Figure 2. Mass spectrum of Oxidised Apo A-I. Smaller fragments if any could not be detected.
The antibody obtained in the egg (IgY) in the pre induction and post induction eggs under different dilution is shown in Figure 3.

![Graph showing antibody titre in pre induction and post induction eggs](image)

**Figure 3. Antibody Titre in pre induction and Post induction eggs**

Antibodies against Oxidised Apo A1 were raised in laying hen. The pre induction IgY and post induction IgY was tested for antigen-antibody reactions by ELISA as described in methods. With increasing dilution, the difference between the pre induction and post induction IgY decreased.

The checker board analysis of antigen-antibody reaction with different dilutions of antibody and different concentration of antigens is shown in Figure 4A and Figure 4B.

![Checker-board analysis](image)

**Figure 4 A. Checker- Board analysis for antigen-antibody reaction.**
The Antibody (IgY) to oxidised Apo A1 was diluted to 1:100 and 1:1000 and tested against oxidised Apo A1 at concentrations 1ng to 1000ng as described in methods.

**Figure 4B. Checker-Board analysis for antigen–Antibody reaction.**

The Antibody (IgY) to oxidised Apo A1 was diluted to 1:5000 and 1:100000 and tested against oxidised Apo A1 at concentrations 1ng to 1000ng as described in methods.

Antibody diluted 100 and 1000 times gave a linear increase in optical density with increasing antigen concentration. However when the antibody was diluted 5000 to 100000 times did not show an increase in optical density with increasing antigen concentration. In fact above 100ng of antigen there was a decrease in optical density with increasing antigen concentration.

The antibody reactivity against oxidized proteins and lipoproteins is shown in Figure 5.
HDL, ApoA1, LDL and BSA were oxidized or glycated as described in the methods and compared with unmodified molecules by ELISA.

HDL and LDL showed the same reactivity with anti Apo A1 antibody whether it was control, oxidized or glycated. Cross reactivity was highest with LDL. Only the oxidized and glycated Apo A1 showed modification specific antigenic response. BSA showed a reduced level of cross reactivity when oxidized or unmodified. However when it was glycated it showed increased cross reactivity.

**Discussion**

Our objective was to raise antibodies against oxidized HDL using its different oxidation specific epitopes. The HDL was isolated and oxidized with copper which would generate essentially lipid oxidation products as epitopes. In order to have specific protein oxidation products, we purified Apo A-I from HDL and subjected it to copper catalyzed oxidation. However the copper catalysed oxidation generated extensive oxidation, resulting in protein fragmentation, as seen from mass spectrum of Apo A-I. Hence we used AAPH to oxidize the Apo A-I.

With increasing activity antigen concentration there was an increase in the optical density at 490nm only when the antibody was diluted up to 1000 times. Beyond this dilution, there was no concentration dependent increase in optical density, in fact beyond 100ng of antigen there was decrease in optical density. This suggests that with increasing antigen concentration there was a decrease in the antigen-antibody reaction. The reason for this decrease is not known. In this ELISA technique we depend on the attachment of the antigen to the ELISA plate and assume that at higher concentration of the antigen, more antigens would be binding, but in this assay with increasing antigen concentration, lesser amount of antigen appears to be binding to the ELISA plate.

Being a small antigen, with probably limited number of epitopes, it is possible that as the concentration of antigen is increased, there could be protein-protein interaction in the antigen, masking the epitopes. This is consistent with the observation that Apo A-I is a hydrophobic peptide (Sorci-Thomas et al 1998, Lyssenko et al 2012).

The cross reactivity of anti ox-Apo A-I antibody against all the tested epitopes suggest that the antibody is raised against the oxidation epitope. Studies from our lab have shown that HDL and LDL of Indian subjects is modified. Hence it is possible that the oxidized, glycated and unoxidized molecules all gave the same reactivity with the anti ox-apo A-I antibody. Interestingly LDL gave the highest cross reactivity suggesting that the epitopes generated in oxidised Apo A-I are also present on LDL molecule. It is unlikely that Apo A-I is found on LDL, but since Apo A-I is highly
hydrophobic it may be associated with phospholipids which in turn could be oxidized. Hence the oxidation specific epitopes could include both protein and lipid oxidation products.

Thus it appears that the oxidized Apo A-I has been antigenic not only because of the peptide, but also because of the oxidation specific epitopes.

**Conclusion**

Our results suggest that the oxidation specific epitopes can recognize a variety of oxidized molecules, with a wide range of cross reactivities. Hence the antibody to oxidized Apo A-I will not only react with oxidized HDL but also with other oxidized molecules particularly LDL.

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