Study of Oxidative Stress in Hypercholesterolemia

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ABSTRACT

Introduction: Oxidative stress is an imbalance between free radicals formation and their scavenging (antioxidant system) in the body. In hypercholesterolemia, there is an increase of total cholesterol pool in cells which results into altered cell membrane due to lipid peroxidation. It is due to leakage of lipid peroxides and other free radicals from cell membrane. MDA is the marker of lipid peroxidation generating peroxides and other free radicals. RBCs are most vulnerable to oxidative stress leading to their hemolysis. SOD (Superoxide dismutase) is the marker enzyme of antioxidant systems making free radicals inactive. RBCs hemolysis, SOD and MDA has been used as the study markers of oxidative stress in case of hypercholesterolemia.

Material and Methods: Both the control and hypercholesterolemic subjects (50 in each category) were chosen from medical OPD for this study. Blood sample was taken with their consent for estimation of total cholesterol, Percentage hemolysis of HBs (before and after incubation with H_2O_2), SOD and MDA.

Result: Mean cholesterol level in hypercholesterolemic subjects was 325.30±11.20 in comparison to controls(141.20±5.40). Mean values of percentage hemolysis of HBs and MDA were found increased with significantly decreased SOD in hypercholesterolemic subjects in comparison to controls.

Conclusion: In the study RBCs hemolysis and MDA (malondialdehyde) are found increased whereas SOD is found decreased which indicates increased oxidative stress. Thus the study concludes that oxidative stress is found increased in hypercholesterolemia.

Keywords: Oxidative Stress, Hypercholesterolemia, Free Radicals, RBCs Hemolysis, SOD, MDA

INTRODUCTION

Hypercholesterolemia is a clinical condition characterized by an increase of the total cholesterol and LDL (low density lipoprotein) cholesterol. Cholesterol is an important cellular structural component present in cell membrane affecting the cellular structural and functional integrity.

Hypercholesterolemia leads to an increase of cholesterol pool which results into altered physical properties of cell membrane,[1] which may facilitate the leakage of the reactive oxygen species(ROS) from the mitochondrial electron system or the activation of NADPH oxidase.[2] These reactive free radicals lead to lipid peroxidation in the cell membrane generating lipid peroxide radicals and further other free radicals.[3]

Increased lipid peroxidation is thought to be a consequence of oxidative stress which occurs when the dynamic balance between the peroxidant and oxidant mechanism is impaired.[4] MDA (malondialdehyde)) is the marker of lipid peroxidation generating peroxides and other free radicals. It is a marker of oxidative stress which results from lipid peroxidation of polysaturated fatty acids.[5] Reactive oxygen species degrade polyunsaturated lipids forming malondialdehyde (MDA).[6] It is used as a biomarker to measure the level of oxidative stress in living organisms.[7,8]

RBCs are the largest place for the generation of free radicals. Therefore, these cells are most vulnerable to the reactive oxygen species (ROS) which accounts for more hemolysis of red blood cells. SOD (Superoxide dismutase) is the marker enzyme against oxidative stress in cells like RBCs generating superoxide (O_2). It is an enzyme degrading superoxide (o_2^-) into either ordinary molecular oxygen or hydrogen peroxide. It is the precursor of free radicals as a by product of oxygen metabolism as in mitochondria. SOD serves as a key antioxidant role in living cells protecting against toxicity of superoxide free radicals. Excess generation of free radicals depletes body antioxidants leading to increased oxidative stress as seen in conditions like hypercholesterolemia.

In addition to SOD, tocopherol a lipophilic antioxidant present in cells like RBCs play a vital role in cellular integrity by preventing polyunsaturated fatty acids of cell membrane against lipid peroxidation. It has the capacity to trap free radicals making them inactive at an early stage of free radical attack, thus suppressing hemolysis.[9] This has made it one of the important factors determining the susceptibility of red cells to auto-oxidation.[9] Therefore, the aim of present study was to determine the level of MDA, SOD and percentage hemolysis in RBCs i.e. to assess oxidative stress in hypercholesterolemic persons.

MATERIAL AND METHODS

In the present study, the level of MDA, SOD and percentage hemolysis in RBCs were determined. Red blood cells were chosen as they are well known to be susceptible to increased hazards of free radical damage. Moreover, these cells had a definite life span in circulation and their sequestration and disposal by microphages might be related to the extent of peroxidative damage caused to their membrane lipids, cytoskeletal proteins and enzymes.

For the study, permission was taken from the ethical committee of Major S.D.Singh Medical College and hospital. 50 controls as normolipidemic persons and 50 hyercholesterolemic persons were randomly selected who visited medicine O.P.D. of Major S.D. Singh Medical College and hospital for general health check up. These hypercholesterolemic persons had been

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detected abnormal lipid profile at first time. While choosing the subjects for the test and control groups, care was taken to eliminate those with habits like smoking, tobacco, pan masala chewing, alcohol consumption and also with history of chronic inflammatory disease like tuberculosis, rheumatoid arthritis, diabetes mellitus and malignancy which has a vital role in contributing to oxidative stress. Blood sample were taken from these control and hypercholesterolemic persons from medical OPD of the hospital. The purpose of study was explained to the persons for study and they agreed for giving blood samples. Regarding Sample collection, 5 ml of venous blood was collected in EDTA containers from the median cubital vein of all study subjects under strict aseptic conditions. The bloods samples were centrifuged at 3000 rpm for 10 minutes within 3 hrs of sample collection. Plasma was separated from blood samples and the separated blood cells were washed thrice with 0.9 % w/v cold normal saline after which they were suspended in an equal volume in the same saline solution. This was then stored as 50 % cells suspension in refrigerator(4-5^{0}C) and was used for the assay of (1) percentage hemolysis of RBCs at 0 and 2 hours which represented before and after incubation with hydrogen peroxide respectively, (2.) MDA(Malondialdehyde), 3. SOD (Superoxide dismutase).

Percentage or Oxidative hemolysis of RBCs. It was measured at 0 and 2 h which indicated before and after 2hrs incubation with H_2O_2 respectively. By the method of kartha and Krishnamurthy.[10] Principle of this method is based on the fact that non-enzymatic rapid breakdown of RBCs can be induced by H_2O_2. 1 ml of RBCs suspension added to 8.5 ml of 0.9% w/v of normal saline and mixed well. Then 0.5 ml of 0.44M H_2O_2 was added and incubated at 37^{0}C. Immediately an exact quantity of 0.5 ml each was withdrawn and put into 2 different centrifuge tubes labeled as "saline and water" respectively.

To the centrifuge tube labeled "saline", 4.5 ml of 0.9% w/v of normal saline was added and centrifuged. The supernatant was then separated and its absorbance (optical density) was determined at 520 nm in a colorimeter. This represented non-hemolysed RBCs at o hr or before incubation with H_2O_2.

To the centrifuge tube labeled "water" 4.5 ml of distilled water was added and centrifuged. The supernatant was separated and its optical density was determined at 520 nm in a colorimeter This represented complete hemolysis of RBCs at 0 hour or before incubation with H_2O_2.

The above procedure was again repeated after 2 hours incubation with H_2O_2 at 37 ^{0}C. The centrifuge tubes labeled saline and water now represented non-hemolysed and completely hemolysed RBCs at 2 hours after incubation with H_2O_2.

Percentage Hemolysis of RBCs at 0 hour and 2 hours were determined using the equation-

$$\text{Hemolysis (\%)} = \frac{\text{O.D. of NH (Saline)}}{\text{O.D. of CH (Water)}} \times 100$$

Superoxide Dismutase (SOD)

For the estimation of SOD, the method of Beauchamp and Fridovich was followed –

$$O^-_2 + O^-_2 + 2H \xrightarrow{\text{(SOD)}} 2\,H_2O_2.$$

Inhibition of reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated by the illumination of riboflavin in the presence of oxygen and electron donor, methionine was used for the assay of superoxide dismutase.[11]

For the estimation of SOD, hemosylate is prepared by McCord and Fridowich.[12]

To 1 mi of RBCs was washed with 0.9 % normal saline .1 mi of distilled water was added to the hemolysed RBCs. To this, 5 ml of distilled ethanol followed by .3 ml of chloroform was added mixed well and allowed to stand for 15 minutes. Now added .2 ml of distilled water and centrifuged at 4^{0}c. Supernatant contains SOD activity and was used for the assay of SOD after dilution with potassium phosphate buffer (PH 7.8, 0.05 M). Thereafter 1 ml of hemolysate was diluted with 1.9 ml of K_3PO_4 buffer. This was the final diluted hemolysate. That was used in the procedure as given below-

Four test tubes labelled as Test, Control, Test blank and Control blank were taken respectively. To the test tube labeled Test, 2.9 ml of reaction mixture with NBT containing 149 mg methionine, 4.93 ml of NBT(1mg /1ml), 0.63 ml of riboflavin and made up of 200 ML with K_3PO_4 (7.8/0.05 M) and 0.1 ml of diluted hemolysate was added.

To the test blank, 2.9 ml of same reaction mixture without NBT (1mg /1ml) and 0.1 ml of diluted hemolysate was added. To the control 2.9 ml of same reaction mixture with NBT and 0.1 ml of K_3PO_4 (7.8/0.05 M) was added. Each of the above parameters was now taken in a 10 ml beaker. The beakers were kept in an aluminium foil lined box fitted with 15 W Florescent Lamp for 10 minutes.

The absorbance was measured at wavelength of 560 nm in a spectrophotometer for all the four beakers. One unit of SOD activity was taken as that producing 50% of inhibition of NBT reduction. The values were expressed as unit/gm Hb. It was calculated using the equation-

Unit /dl SOD (×) = C-T/0.5 C×3/0.5×2/0.1×100/0.1

SOD activity (units /g Hb) =×/Hb [Units/g Hb of SOD]

Where x is unit /dl SOD, C and T are absorbance of control and test respectively.

MDA (Lipid peroxidation) estimation

Red cell lipid peroxidation was studied as thiobarbituric acid (TBA) reaction product. The method of Stock and Dormandy was followed with certain modifification.[13] The sample under test was heated with TBA at low pH and a pink chromogen, allegedly a $(TBA)_2$—MDA adduct was measured spectrophotometrically at wave length of 535 nm.[14] 1 ml of erythrocyte suspension was added to 8.5 ml of 0.9 % w/v of normal saline and mixed well. Then 0.5 ml of 0.44 M H_2O_2 was added.

From this mixture, 2.5 ml aliquot was immediately taken, to which 1 ml of 28% trichloroacetic acid (TCA) in 0.1 M Sodium meta-arsenite was added. This was mixed well and allowed to stand for 10 minutes after which it was centrifuged .3 ml of the supernatant was then taken to which 1 ml of 1% TBA in 50 mM NaOH was added. This was then kept in a boiling water bath for 15 minutes and later immediately cooled under tap water. The pink chromogen was determined spectrophotometrically at the wave length of 535 nm. Values were expressed as nanomoles of MDA formed per dl of RBC taking the molar extinction co-efficient as 1.56×10^5.[13]

MDA (nanomoles/100 ml of RBC) was determined using the equation:

$MDA = A_{t\times} 10^9 \times 100 \times DF \times V/e$

Where A_t stands for absorbance of test sample, DF is dilution factor, V is volume of RBC suspension and ' e' stands for extension coefficient**.**

RESULT

Mean values and their standard deviations of the above mentioned study parameters were calculated. Mean values of total cholesterol and their standard deviations for controls and hypercholesterolemic persons were 161.20±5.40 and 325.30±11.20 respectively as shown in table-1. And that of percentage hemolysis of HBs in before incubation and after incubation with $_{H2O2}$ was calculated by students paired-t test as in table-2, 3. Mean values of percentage hemolysis of RBCs in before incubation in controls and hypercholerolemis subjects were found 1.69±1.04 and 4.52±1.06 respectively in comparison to 2.78±0.88 and 6.01±1.12 respectively in after incubation. Calculation of mean MDA and SOD in RBCs were done using Fishers F test for analysis of variance (table no. 4 and 5). Mean RBCs MDA value in control subjects was 545.63 in comparison to 742.67±74.10 in hypercholesterolemic subjects where P-value was found highly significant. Similarly, mean values of SOD were found 8078.63±762.50 and 5281.79±525.19 in controls and hypercholesterolemic subjects respectively with highly significant P- value.

DISCUSSION

In the study, table -3 showed increased hemolysis in RBCs after incubation with H_2O_2 in comparizon to table -2 indicated decreased antioxidant activity and increased oxidative stress in hypercholesterolemic subjects. It might be due to decreased SOD activity and increased MDA activity in hypercholesteolemic subjects and its reverse in normal subjects as seen in table no. 4 and 5. SOD is considered as a standard marker of antioxidant activity and MDA as a marker of oxidative damage or stress in the the body.[7,8] In addition, tocopherol (Vit. E) a lipophilic antioxidant present in RBCs might be responsible for making RBC cell membrane fragile for hemolysis due to lipid peroxidation. Tocopherol appeared to be highly efficient as an antioxidant and is accepted as a first line of defense against lipid peroxidation.[15-17] Increased hemolysis of RBCs in hypercholesterolemic subjects might be due to additive role of SOD and MDA in oxidative stress. SOD played an important role in the removal of superoxide radical (o_2) formed in red cells.[18-20] Increased hemolysis of RBCs in hypercholesterolemia might be due to additive role of SOD and MDA in oxidative stress. Regarding SOD and MDA, values of SOD were found decreased and that of MDA increased (table 4, 5) which supported increased induced hemolysis of RBCs due to increased oxidative stress in hypercholesterolemia.

In hypercholesterolemia, Lipid peroxidation is enhanced leading to more generation of lipid peroxide radicals and oxidizable lipid substrates.[21] Peroxide radicals are generated due to in vivo Lipid peroxidation. The lipid soluble antioxidants α-tocopherol, β- carotene and lycopene can protect lipoproteins from oxidative damage by free radical and excited oxygen species.[22] Consumption of these antioxidants is enhanced to trap lipid peroxyl radicals formed during lipid peroxidation in hypercholesterolemic state.[23] Several studies have shown that intake of saturated fatty acids may increase oxidative stress by inceasing lipid peroxidation and decreasing antioxidant enzyme system. The hypercholesterolemia increases oxidative stress by increasing lipid peroxidation and decreasing antioxidant enzyme. Moreover, hypercholesterolemia is associated with increased superoxide production and nitric oxide inactivation.[24] Thus, in accordance with table-2, 3, 4, 5 the study parameters were found significantly increased ($P < 0.001$) indicating increased oxidative stress in hypercholesterolemic condition. These findings were found in accordance with previous

Group	n=no. of subjects	Mean ± SD (mg/dl)
Controls	50	161.20 ± 5.40
Hypercholesterolemic	50	325.30 ± 11.20

Table-1: Mean total cholesterol levels of control and hypercholesterolemic subjects.

Group	(n=no. of subjects)	Mean	SD (%)	P	Remarks
Controls	50	1.69	1.04	-	-
Hypercholesterolemic	50	4.52	1.06	0.001	HS

Table-2: Percentage hemolysis of RBCS before and after incubation of the RBCs before and after incubation with H_2O_2 in control and hypercholesterolemic subjects. (Befor incubation)

Group	n=no.of subjects	Mean	SD (%)	P	Remarks
Controls	50	2.78	0.88	-	-
Hypercholesterolemic	50	6.01	`1.12	0.001	HS

Table-3: Hemolysis after incubation HS: highly significant

Group	n=no. of subjects	Mean	SD (%)	P	Remarks
Controls	50	545.63	48.03	-	-
Hypercholesterolemic	50	742.67	74.10	0.001	HS

Table-4: Mean RBC MDA levels HS: highly significant

Group	n=no. of subjects	Mean	SD (%)	P	Remarks
Controls	50	8078.63	762.50	-	-
Hypercholesterolemic	50	5281.79	525.19	0.001	HS

Table-5: Mean values of SOD HS: highly significant

findings.[25-27]

CONCLUSION

The parameters of oxidative stress in this study i.e. increased RBCs hemolysis, decreased SOD and increased MDA values indicated that there were more generation of free radicals in comparison to their scavenging activity leading to more oxidative stress in hypercholesterolemia.

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