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OXIDATIVE STRESS IN HYPERCHOLESTEROLEMIC PATIENTS AND ANTIOXIDANT STATUS OF FLUVASTATIN

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Abstract

This study was an attempt to establish the extent of increased oxidative stress in hypercholesterolemic patients and to evaluate the effect of fluvastatin on the oxidative stress and antioxidant status. The blood samples of 15 subjects (age and sex matched) each from group I (healthy subjects), group II (hypercholesterolemic patients with fluvastatin treatment) and group III (hypercholesterolemic patients without any hypolipidemic drug) were taken and centrifuged for separation of plasma. Plasma was used for the estimation of vitamin E. The separated cells were washed thrice with 0.9 % w/v cold normal saline and used for the assay of percentage hemolysis of RBCs, malondialdehyde, superoxide dismutase and hemoglobin. Levels of oxidative stress were higher in hypercholesterolemic in comparison to control and fluvastatin group. Levels of antioxidants were higher in fluvastatin group than hypercholesterolemic but were lower than controls. From these findings it was concluded that there is an increase in oxidative stress in hypercholesterolemia but it decreased significantly after 2 months of fluvastatin therapy and antioxidant status also improves in patients taking fluvastatin.

Introduction

The statins like fluvastatin significantly reduce cholesterol synthesis through inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and are widely prescribed for hyperlipidemia to reduce the risk of atherosclerotic complications[1]**.** Their efficacy in reducing cardiovascular morbidity and mortality has been demonstrated in large intervention trials[1]. However debate continues as to whether the beneficial effects of statins can be ascribed purely to their ability to reduce cholesterol or whether additional actions, independent of cholesterol lowering, play a significant role[1-3]. Studies have shown that oxidized low density lipoprotein (LDL) is a major correlate of oxidative stress in hypercholesterolemic patients and statins may reduce oxidative stress by reducing enhanced plasma levels of LDL, which are more susceptible to peroxidation in hypercholesterolemia, and change the LDL structure, making them more resistant to peroxidation[1, 4-5]. Some studies have further shown that statins may also inhibit NAD (P) H oxidase, thus decreasing the generation of reactive oxygen species (ROS), thereby adding or synergizing biological effects of antioxidants[4, 6]. Some studies have also shown that statins or their metabolites may act as antioxidants, directly or indirectly by removing "aged LDL", which is more prone to oxidation from the circulation[7]. On the basis of these findings, it is evident that among their properties statins also possess antioxidant activities[8-9]. Therefore the aim of the present investigation was to evaluate the scientific evidence of fluvastatin for such an effect and its possible clinical relevance. The antioxidant effect of statins contribute to inhibition of atherogenesis, stabilization of atherosclerotic plaque, inhibition of myocardial hypertrophy and remodeling and modulation of vascular tone[6]. Based on these arguments which formed the backbone of this study an attempt was made to find out if there really was an increased oxidative stress in hypercholesterolemics and was it relatively decreased following fluvastatin therapy when compared to normal individuals.

In this study the levels of MDA, percentage hemolysis and superoxide dismutase (SOD) in red blood cells and vitamin E in plasma were measured. Red blood cells were chosen as they are well known to be subject to increased hazards of free radical damage. Moreover, these cells have a finite life span in circulation and their sequestration and disposal by macrophages may be related to the extent of peroxidative damage caused to their membrane lipids, cytoskeletal proteins and enzymes.

Experimental

Study population

This study was conducted on 3 groups of 15 subjects each in the age group of 40-70 years. Both male and female subjects were taken in all groups.

Group I: Consisted of 15 healthy subjects (8 males and 7 females) between the age group 40-70 years having normal lipid profile (Control group).

Group II: Consisted of 15 patients (8 males and 7 females) who were already diagnosed as hypercholesterolemic and who were given treatment with HMG Co-A reductase inhibitors (fluvastatin) for minimum of 2 months with a minimum dosage of l0mg/day of fluvastatin. Treatment with simvastatin was given only in this group in order to compare the results with control and hypercholesterolemic group without any hypolipidemic drug.

Group III: Consisted of 15 hypercholesterolemic patients (8 males and 7 females) diagnosed recently but were not taking any of the lipid lowering agents (Hypercholesterolemic group).

Selection of Cases

The test group II & III for this study consisting of 15 individuals in each group were taken from the following Hospitals in Patna, India.

1. Nalanda Medical College Hospital,Patna,India.
2. Patna Medical College Hospital,Patna, India.
3. Rajeshwar Hospital,Patna, India.
4. Alam Clinic,Patna, India.
5. Care Hospital,Patna, India.

While choosing the subjects for the test and control groups, care was taken to eliminate those with habits like smoking, tobacco chewing, alcohol consumption and also those with history of chronic inflammatory diseases like tuberculosis, rheumatoid arthritis, diabetes mellitus and malignancy all of which play a vital role in contributing to oxidative stress injury. Approval to carry out these studies on human subjects was obtained from Institutional Clinical Ethics Committee of Nalanda Medical College, Patna, India and their guidelines were followed for the studies.

Sample collection

5 ml of venous blood was collected in ethylene diamine tetra-acetic acid (EDTA) containers from the median cubital vein or basilic vein of the each study subjects under strict aseptic conditions. The blood samples were centrifuged at 3000 rpm for 10 min within 3 h of collection. Plasma was separated and used for the estimation of vitamin E. The separated cells were washed thrice with 0.9 % w/v cold normal saline, after which they were suspended in an equal volume of the same saline solution. This was then stored as 50 % cell suspension at refrigerator (4-5^{0}C) and was used for the assay of:

- Percentage hemolysis of RBCs at 0 and 2 h (which represented before and after incubation with hydrogen peroxide respectively)
- Malondialdehyde (MDA)
- Superoxide dismutase (SOD)
- Hemoglobin (Hb)

Lipid peroxidation (MDA)

Red cell lipid peroxidation was studied as thiobarbituric acid (TBA) reaction products. The method of Stocks and Dormandy was followed with certain modifications[10]. 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 wavelength of 535 nm[11]. 1ml 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 of aliquot was immediately taken, to which 1ml of 28 % trichleroacetic acid (TCA) in 0.1 M sodium meta-arsenite was added. This was mixed well and allowed to stand for 10 min, after which it was centrifuged. 3 ml of the supernatant was then taken, to which 1ml of 1 % TBA in 50 mM NaOH was added. This was then kept in a boiling water bath for 15 min and later immediately cooled under tap water.

The pink chromogen was determined spectrophotometrically at the wavelength 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 [10].

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

$$MDA = \frac{A_T \times 10^9 \times 100 \times DF \times V}{\epsilon}$$

Where A_T is the absorbance of test sample, DF is dilution factor, V is volume RBC suspension and ϵ is the extension coefficient.

Oxidative hemolysis of RBCs or percentage hemolysis of RBCs

Oxidative hemolysis of erythrocytes was measured at 0 and 2 h which indicated before and after 2 h incubation with H_2O_2 respectively by the method of Kartha and Krishnamurthy[12].

Principle of this method is based on the fact that an accelerated form of non-enzymic breakdown can be induced in red cells on exposure to H_2O_2[10].

1ml of RBC 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 and incubated at 37°C.

Immediately, aliquots of 0.5 ml each were withdrawn and put into 2 different centrifuge tubes labelled as 'saline' and 'water' respectively.

To the centrifuge tube labelled '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 (NH) at 0 h or before incubation with H_2O_2.

To the centrifuge tube labelled 'water', 4.5ml of distilled H_2O was added and centrifuged. The supernatant was then separated and its optical density was determined at 520 nm in a colorimeter. This represented complete hemolysis of RBCs (CH) at 0 h or before incubation with H_2O_2.

The above procedure was again repeated after 2 h incubation with H_2O_2 at 37^0C. The centrifuge tubes labelled 'saline' and 'water' now represented non-hemolysed (NH) and completely hemolysed (CH) RBCs at 2 h or after incubation with H_2O_2.

Percentage hemolysis of RBCs at 0 h and 2 h was determined using the equation:

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

Vitamin E (α - tocopherol)

Plasma vitamin E was measured using the Emmorie Engel reaction. Emmorie Engel reaction is based on the reduction of ferric to ferrous ions by tocopherols, which then forms a red complex with α - α' dipyridyl. Tocopherol and carotenes were extracted into petroleum ether and the extinction at 450 nm was measured. A correction was made for carotenoids after adding $FeCl_3$. Reading was taken at 520 nm, exactly after 90 sec[13-14].1ml of plasma was thoroughly mixed with 1ml of redistilled 95 % ethanol in a 15 ml stoppered tube. 3ml of petroleum ether was then added and the tube was shaken vigorously for 3 min. This was then centrifuged and 2 ml of clear supernatant was transferred to a clear dry cuvette. The optical density was measured at 450 nm in a colorimeter for carotenes, taking petroleum ether as blank.

Petroleum ether was then evaporated off at room temperature and the residue was dissolved in 1ml chloroform. 1ml of 95 % ethanol was then added followed by 1ml of 0.2 % α - α' dipyridyl and 0.1 ml of 0.1 % $FeCl_3$. Exactly after 1.5 minutes, absorbance was measured at 520 nm in a colorimeter.

Concentration of vitamin E (mg/l) of plasma was determined using the equation:

$$C = \frac{A_T - A_C}{A_S}$$

Where A_T, A_C and A_S are absorbance of test, carotene and standard sample respectively.

Superoxide dismutase (SOD)

The method of Beauchamp & Fridovich was followed for measurement of SOD.

The enzyme superoxide dismutase catalyses the reaction:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{[SOD]} 2H_2O_2$$

Inhibition of the reduction of nitroblue tetrazolium (NBT) by superoxide radicals, generated by the illumination of riboflavin in the presence of oxygen. An electron donor, methionine, was used for the assay of superoxide dismutase[15].

Preparation of hemolysate

This was done by the method of McCord and Fridovich[16]. To 1ml of erythrocytes (washed with 0.9 % w/v of normal saline), 1ml of deionised water was added to lyse the cells. To this, 0.5 ml of distilled ethanol followed by 0.3 ml of chloroform was added, mixed well and allowed to stand for 15 min. Now added 0.2 ml of H_2O and centrifuged at 4°C.

The supernatant contains SOD activity and was used for the assay of SOD after dilution with potassium phosphate buffer (PH 7.8, 0.05 M). 0.1 ml of hemolysate was diluted with 1.9 ml of K-PO_4^- buffer. This was the final diluted hemolysate that was used in the procedure given below.

Four test tubes were taken and labelled as 'Test', 'Control', 'Test blank' and 'Control blank' respectively.

To the 'Test' 2.9 ml of reaction mixture with NBT containing 149 mg of methionine, 4.93 ml of NBT (1mg/ml), 0.63 ml of riboflavin (1mg/ml), and made up to 100ml with K-PO_4^- buffer (PH 7.8/0.05M) and 0.1 ml of diluted hemolysate was added.

To the 'Test Blank' 2.9 ml of same reaction mixture without NBT 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-PO_4^- buffer (PH 7.8/0.05M) was added.

To the 'Control Blank' 2.9 ml of the same reaction mixture without NBT and 0.1ml of K-PO_4^- buffer (PH7.8/0.05M) 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 a 15W fluorescent lamp for 10 minutes.

The absorbance was measured at wavelength of 560 nm in a spectrophotometer for all the four beakers.

1 unit of SOD activity was taken as that producing 50 % inhibition of NBT (nitroblue tetrazolium) reduction. The values were expressed as units /gm Hb. It was calculated using the equation:

$$\text{Unit / dl SOD (x)} = \frac{C - T}{\frac{1}{2}C} \times \frac{3}{0.5} \times \frac{2}{0.1} \times \frac{100}{0.1}$$

$$\text{SOD activity (units/g Hb)} = \frac{x}{Hb} \quad \text{[Units/g Hb of SOD]}$$

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

Estimation of hemoglobin

The hemoglobin content of erythrocytes was determined by the cyanmethaemoglobin method[17].

Hemoglobin was treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate (Drabkins Reagent). The ferricyanide oxidizes hemoglobin to methaemoglobin which is converted to cyanmethemoglobin by the cyanide. Absorbance was measured at 540 nm in a colorimeter.

20 µl of RBC suspension was added to 4 ml of ferricyanide reagent and allowed to stand for 4 min. The absorbance was measured at 540 nm against a reagent blank in a colorimeter. The absorbance of the standard solution was measured by directly taking 4 ml of the standard cyanmethaemoglobin solution (60 mg/dl)[18].

Hemoglobin (gm/dl) was determined using the equation:

$$\text{Hemoglobin (gm/dl)} = \frac{\frac{A_T}{A_S} \times \text{dilution factor} \times \text{conc. of std. (mg/dl)}}{1000}$$

Where A_T and A_S are absorbance of test and standard respectively.

Statistical analysis

All the biochemical parameters were compared using Fishers F test for analysis of variance (ANOVA) except hemolysis. Students paired t test was used for hemolysis (0h & 2h). The statistical software "SPSS Version 11" (statistical package for social sciences) was used for this purpose.

Results and discussion

In this study, the products of lipid peroxidation, hemolysis of RBCs and antioxidant levels were compared between those having high cholesterol level, those who were on treatment with fluvastatin for a minimum of 2 months (minimum dosage of 10mg/day) and those who were normal individuals taken as controls having normal lipid profile and of the same age group.

Free oxygen radicals and insufficient antioxidant enzymes have been implicated in the pathogenesis of hypercholesterolemia[2, 4, 19-20]. Statins have been used effectively for the treatment of hypercholesterolemia[2, 20-21]. These facts formed the basis of this study i.e., to investigate the antioxidant system and oxidative stress in hypercholesterolemic patients as well as in patients who were treated with fluvastatin for a minimum of 2 months.
The mean total cholesterol levels of the 3 groups are given in Table I. In patients taking fluvastatin, the mean total cholesterol reduced after 2 months of treatment (246.66 ± 8.54 mg/dl) as compared to hypercholesterolemic patients (325.33 ± 11.23 mg/dl).

RBC Malondialdehyde (MDA)

As a measure of oxidative stress, MDA the end product of lipid peroxidation was estimated by the TBA method. RBC MDA levels were highest in hypercholesterolemic group (742.67 ± 74.10 µmol/l) and lowest in control group (545.63 ± 48.03 µmol/l). The values were statistically highly significant (p=0.001) when compared hypercholesterolemic group with control group and statistically significant (p=0.034) when compared control group with fluvastatin group as indicated in Table II.

High level of MDA in hypercholesterolemic group suggested increase oxidative stress in patients of hypercholesterolemia. Its relatively lower level in fluvastatin group suggested decrease oxidative stress. These findings further favor antioxidant properties of statins like fluvastatin[2].

Percentage hemolysis of RBCs and vitamin E

Percentage hemolysis of RBCs was measured as an indicator of damage to RBC membrane as a result of oxidative stress. Amongst the antioxidants, vitamin E was chosen because, despite its low molar concentration in membranes, it effectively serves as the major lipid-soluble, chain-breaking antioxidant[24]. This study has shown increased hemolysis of RBCs in the hypercholesterolemic group (4.52 ± 1.06 %) as compared to fluvastatin (2.48 ± 0.67 %) and control groups (1.69 ± 1.04 %), but in fluvastatin group it was more than control but less than hypercholesterolemic group, both before (2.48 ± 0.67 %) and after incubation (3.52 ± 0.81 %) with hydrogen peroxide (Table III). The values were statistically highly significant both before and after incubation with hydrogen peroxide ($p<0.05$). This could be explained on the basis of increased oxidative stress in hypercholesterolemia and effect of fluvastatin in reducing oxidative stress in hypercholesterolemic patients after 2 months of treatment. Mean vitamin E levels were lower in fluvastatin group (7.74 ± 1.05 mg/dl) and lowest in hypercholesterolemic group (6.28 ± 0.68 mg/dl) as compared to control group (9.31 ± 1.36 mg/dl) as shown in Table IV. The values were statistically highly significant ($p<0.05$). These studies have shown that vitamin E plays a critical role in protecting polyunsaturated fatty acids of cell membranes against lipid peroxidation through its free-radical quenching activity

at an early stage of free-radical attack, thus suppressing hemolysis[24]. This has made it one of the important factors determining the susceptibility of red cells to auto-oxidation hemolysis[24]. Vitamin E appeared to be highly efficient as an antioxidant and is accepted as a first line of defense against lipid peroxidation[22-25].

The popular finding that vitamin E is inversely related to the respective tissue MDA level fits here as MDA in this study was found to be highest in hypercholesterolemic group (742.67 ± 74.10 µmol/l) and lowest in control group (545.63 ± 48.03 µmol/l). The increased hemolysis of RBCs in hypercholesterolemic group could be further documented by the decreased levels of vitamin E, which is a first line of defense against membrane damaging lipid peroxidation[22-25].

In fluvastatin group, the level of vitamin E was more than hypercholesterolemic group but less than control group (Table IV). The results of percentage hemolysis of RBCs were in the same order, it was least in control, more than control in fluvastatin group and highest in hypercholesterolemic group. The controls had highest level of vitamin E as they are considered to be the group with least oxidative stress due to normal lipid profile of their blood. It may be hypothesized that due to increased oxidative stress in hypercholesterolemic group, utilization of vitamin E, which is an antioxidant, might have increased. This agreed with the work of Moriel et al (2000)[19].

Superoxide dismutase (SOD)
Superoxide dismutase was chosen in this study as it plays an important role in the removal of superoxide radicals ($O_2^{\bullet-}$) formed in red cells and because hemoglobin and SOD have been shown to be in close association with red cells. In addition to this, some studies have also been suggested that SOD is one of the most important enzymes in the front line of defense against oxidative stress and is more effective in protecting the RBCs against damage by exogenous superoxide radicals ($O_2^{\bullet-}$), especially at higher concentrations[26-28]. This study showed low levels of SOD in hypercholesterolemic group (6.28 ± 0.69 units/mg) as shown in Table V. In fluvastatin group (7.74 ± 1.05 units/mg), SOD level was more than hypercholesterolemic but less than controls. The values were statistically highly significant ($p<0.05$). The low levels of SOD in the cellular and extracellular fluids reduce their oxygen derived free radical (ODFR) scavenging capacity making the tissues more vulnerable to ODFR damage[28]. The low level of SOD in hypercholesterolemic patients is indicative of increased oxidative stress in patients of hypercholesterolemia (Table V). Studies have shown that in hypercholesterolemia there was reduced activity of SOD which improved after 3 months of treatment with fluvastatin, further strengthens the idea of increased oxidative stress in hypercholesterolemics and reduction of oxidative stress after the use of fluvastatin which inhibits superoxide anion production, preserves intracellular SOD and prevents the ROS (reactive oxygen species) permeation into lipoproteins[2, 26-27]. Thus, we can conclude that the trends seen in this study definitely suggest that there is an increased oxidative stress occurring as a result of hypercholesterolemia and after the use of fluvastatin, oxidative stress decreases for which there may be two reasons. First due to decreased cholesterol levels and secondly due to anti-oxidant effect of fluvastatin as shown by many studies.

Conclusion

This study was an attempt to establish the extent of increased oxidative stress in hypercholesterolemic patients and to evaluate the effect of fluvastatin on the oxidative stress and antioxidant status after 2 months of treatment.
Levels of oxidative stress were higher in hypercholesterolemic in comparison to control and fluvastatin group. Levels of oxidative stress in fluvastatin group were lower than hypercholesterolemic but were higher than control group. Levels of antioxidants were higher in fluvastatin group than hypercholesterolemic but were lower than controls. From these findings we can conclude that there is an increase in oxidative stress in hypercholesterolemia but it decreases significantly after 2 months of fluvastatin therapy and antioxidant status also improves in patients taking fluvastatin.

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Table I Mean total cholesterol levels of the 3 groups i.e., control, fluvastatin & hypercholesterolemic

Group	N	Mean total cholesterol level (mg/dl)[a]
Control	15	161.25 ± 5.43
After minimum 2 months of Fluvastatin therapy	15	246.66 ± 8.54
Hypercholesterolemic	15	325.33 ± 11.23

N= No. of subjects, [a]mean ± SD, N = 15, SD = standard deviation

Table II Mean RBC MDA levels of the 3 groups (control, fluvastatin & hypercholesterolemic)

Group	N	Mean (µmol/l)	SD	P	Remarks
Control	15	545.63	48.03		
Fluvastatin	15	590.23	39.63	0.034	S
Hypercholesterolemic	15	742.67	74.10	0.001	HS

Name of the test used – Fishers 'F' test –for analysis of variance (ANOVA), N = No. of subjects, SD = standard deviation, p value = probability, HS=highly significant, S= significant

Table III Percentage hemolysis of RBCs before and after incubation of the 3 groups (control, fluvastatin & hypercholesterolemic)

Group	N	Mean (%)	SD	P	Remarks
Hemolysis before incubation:					
Control	15	1.69	1.04		
Fluvastatin	15	2.48	0.67	0.001	HS
Hypercholesterolemic	15	4.52	1.06	0.001	HS
Hemolysis after incubation :					
Control	15	2.78	0.88		
Fluvastatin	15	3.52	0.81	0.001	HS
Hypercholesterolemic	15	6.01	1.12	0.001	HS

Name of the test used – Fishers 'F' test

Table IV Plasma vitamin E levels of the 3 groups (control, fluvastatin & hypercholesterolemic)

Group	N	Mean (mg/dl)	SD	P	Remarks
Control	15	9.31	1.36		
Fluvastatin	15	7.74	1.05	0.001	HS
Hypercholesterolemic	15	6.28	0.68	0.001	HS

Name of the test used – Fishers 'F' test

Table V RBC superoxide dismutase (SOD) levels of the 3 groups (control, fluvastatin & hypercholesterolemic)

Group	N	Mean (units/mg)	SD	P	Remarks
Control	15	8078.63	762.50		
Fluvastatin hypercholesterolemic	15	7432.69	657.92	0.001	HS
	15	5281.79	525.19	0.001	HS

Name of the test used – Fishers 'F' test.