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ANTIATHEROGENIC AND ANTIOXIDANT IMPACT OF OMEGA-3 FATTY ACIDS AND NATURAL NANOPARTICLE HDL ON COPPER MEDIATED OXIDATIVE MODIFICATION OF LDL IN NORMAL AND DIABETIC WITH HYPERLIPIDEMIA SUBJECTS

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ABSTRACT

Chronic diseases are now the dominant contributors to the global burden of disease, and CVD is the largest contributor to the chronic disease cluster. Although CVD death rates are declining in most high income countries, trends are increasing in most low and middle income countries. The response-to-injury hypothesis explains atherosclerosis as a chronic inflammatory response to injury of the endothelium, which leads to complex cellular and molecular interactions among cells derived from the endothelium, smooth muscle and several blood cell components. Inflammatory and other stimuli trigger an overproduction of free radicals, which promote peroxidation of lipids in LDL trapped in the subendothelial space. Evidence is presented that natural nano-particle HDL and Omega-3 fatty acids have an antioxidant impact and prevent the peroxidation of lipids and thus are protective against the development of atherosclerosis. Paraoxonase (PON) or arylesterase is a natural nanoparticle HDL associated enzyme that protects LDL as well as HDL from oxidative stress. The present study has shown that the formation of conjugated dienes was significantly decreased by 21.5% and 17.87% with respect to control after adding Omega-3 fatty acids and HDL respectively when LDL isolated from normal subjects was subjected to Cu^{++} mediated *in vitro* oxidative modification whereas this decrease was found to be 13.25 % and 12.82 % respectively when LDL isolated from hyperlipidemic subjects was subjected to oxidation. On the other hand the antioxidant power of plasma isolated from normal subjects increased by 54.28% and of plasma isolated from hyperlipidemic subjects increased by 69.81% after treatment with Omega-3 fatty acids. This increase was found to be 53.57% and 68.68% in plasma isolated from normal and hyperlipidemic subjects respectively, after treatment with HDL .A significant increase was observed in the antioxidant power of LDL also after *in vitro* treatment with Omega-3 fatty acids being 15.58% in normal subjects and 32.46% in hyperlipidemic subjects. Treatment with HDL also had a similar effect, increasing the antioxidant power of LDL isolated from normal subjects by 13.72% and that from hyperlipidemic subjects by 32.84%. The present study proves both of them effective antioxidants, Therefore, Omega-3 fatty acids can be used as a nutritional supplement to avoid the risk of cardiovascular diseases and natural nano-particle HDL can replace harmful drugs in the treatment of CVD.

KEYWORDS: CVD, LDL Oxidation, HDL, Omega-3 fatty acids.

INTRODUCTION

Cardiovascular disease is the leading cause of deaths worldwide, though since the 1970s, cardiovascular mortality rates have declined in many high-income countries (Mendis *et al.*, 2011). At the same time, cardiovascular deaths and disease have increased at a fast rate in low- and middle-income countries (Finegold *et al*., 2012). Although cardiovascular disease usually affects older adults, the antecedents of cardiovascular disease, notably atherosclerosis, begin in early life, making primary prevention efforts necessary from childhood (McGill *et al.*, 2008).

Atherosclerosis and its complications continue to be the major cause of premature death in the developed world. Centuries of debate over the origin of this condition were finally resolved by the unifying "response-to-injury"

hypothesis (Ross 1986). This hypothesis explains atherosclerosis as a process of chronic inflammatory response to injury of the endothelium, which leads to complex molecular and cellular interactions between cells derived from the endothelium, smooth muscle and several blood cell components. Inflammatory stimuli trigger the release of cytokines, growth factors and the generation of free radicals with their metabolic consequences manifested in transmigration and proliferation of vascular smooth muscle cells. The process also triggers the oxidation of LDL, which further potentiates the adhesion and migration of blood monocytes into the vessel walls where they differentiate to macrophages. The rapid uptake of oxidized LDL (ox-LDL) via the macrophage scavenger receptor causes the transformation of macrophages into the lipid-laden foam cells, characteristic of a fatty streak, an early sign of atherosclerosis (Ross 1993 and 1995).

Epidemiological studies and prospective randomized trials have consistently shown a powerful inverse association between the magnitude of HDL-C and CVD (Movva and Rader, 2008). HDL prevents atherosclerosis by reverting the stimulatory effect of oxidized LDL on monocyte infiltration. The HDL-associated enzyme paraoxonase inhibits the oxidation of LDL. PAF-acetyl hydrolase, which circulates in association with HDL and is produced in the arterial wall by macrophages and degrades bioactive oxidized phospholipids. Oxidized LDL inhibits these enzymes. Thus, oxidized LDL and HDL are indeed antagonists in the development of cardiovascular disease (Mertens and Hobvoet, 2001).

HDL is highly heterogeneous, with subfractions that can be identified on the basis of density, size, charge, and protein composition. The concept that certain subfractions of HDL may be better predictors of CVD risk is of great concern (Kones, 2011) .HDL is the smallest of the lipoproteins particles with a size of 6-12.5 nanometers. HDL is composed of approximately: 55% protein, 3-15% triglycerides (TG), 26-46% phospholipids (PL), 15-30 cholesterol esters (CE), 2-10% cholesterol (Arsenault *et al*., 2010).

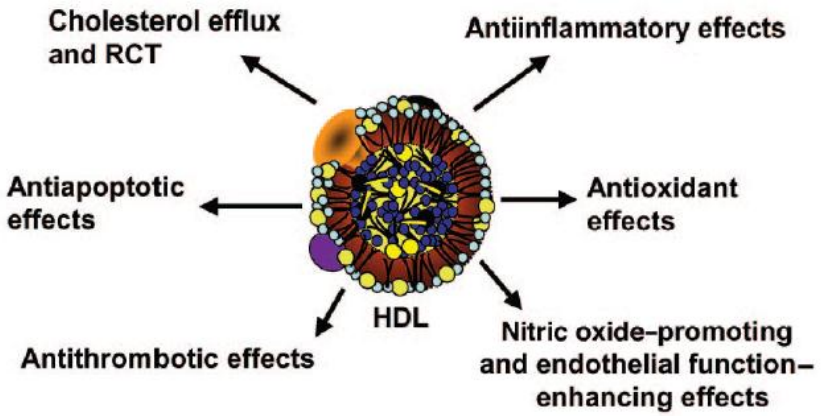

Figure 1: Several anti-atherogenic properties of HDL (Eren *et al*., 2012)

Another important part of this study, Omega-3 fatty acids (OFA) present in fish oils have been shown to lower triglyceride (blood fat) levels, minimize inflammation and clotting, and increase HDL ("good") cholesterol. Research indicates that Omega-3 fatty acids may help reduce the risk and symptoms of a variety of disorders influenced by inflammation, including heart attack and stroke. Present in cold water fish such as wild Alaskan salmon, sardines, herring, mackerel and black cod. Taking two grams daily of a fish oil supplement that contains both essential Omega-3 fatty acids, EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) is recommended. (Weil, 2012) . The purpose of this study was to examine the antioxidant impact of Omega-3 fatty acids and HDL on *in vitro* Copper mediated oxidative modification of LDL.

MATERIALS AND METHODS

Chemicals

Softgel capsules manufactured by Dr. Reddy's Laboratories containing a high-potency pharmaceutical grade marine lipid concentrate that provides a perfect balance of EPA and DHA were bought from medical store.

Estimation

Plasma cholesterol, LDL, HDL and its subfractions cholesterol (Annino and Giese, 1976), Isolation of HDL and its fractions-HDL_2, HDL_3 (Patsch *et al*., 1989), isolation of LDL (Weiland and Siedel, 1989), *ex vivo* and *in vitro* Cu^{++} -mediated LDL oxidation (Esterbauer *et al*., 1989,1992). The method of Benzie and Strain (1996) was used for measuring the ferric reducing ability of plasma, the FRAP assay, which estimates the "total antioxidant power", with minor modification.

Experimental design

Fresh blood samples of normal and diabetic with hyperlipidemia subjects were collected from Pathology Lab. of Sardar Bhagwan Singh Post Graduate Institute, Balawala, Dehradun. Plasma obtained from them was pooled and LDL and its sub-fractions were isolated.

Table 1: Average Values of Age, Weight, Height, Male, Female of Normal and diabetic with hyperlipidemia subjects.

S. No.	Parameter	Normal (n= 45)	Diabetic With Hyperlipidemia (n= 60)
1.	Age (years)	32 ± 1.86	58 ± 3.56
2.	Body weight (kgs)	59.42 ± 8.39	64 ± 9.41
3.	Male	26	29
4.	Female	19	31
5.	Drugs used by the patients	-	Human insulin, Dayonil, Metformin, Pioglitazone, Glynase, Glyciphase etc

RESULTS

Table 2: Average value of TC, Total protein, LDL-C,LDL protein, HDL-C, HDL_2-C, HDL_3-C ,their protein and non – HDL cholesterol protein in normal and diabetic with hyperlipidemia subjects.

S.NO	PARAMETERS	Normal Value (µg/ml)	Diabetic With Hyperlipidemia Value (µg/ml)
1.	Total cholesterol in plasma	42.80 ± 2.39	114.66 ± 2.24
2	Low Density Lipoprotien – Cholesterol (LDL- C)	14.9 ± 2.09	60.89 ± 0.53
3.	Total protein in plasma	1.212 ±0 .066	1.190 ± 0.015
4.	Total protein in LDL	1.179 ± 0.348	1.046 ± 0.28
5.	High Density Lipoprotein cholesterol (HDL)	5.36 ± 0.46	7.025 ± 0.63
6.	High Density Lipoprotein-2 cholesterol(HDL_2-C)	1.816 ± 0.34	1.514 ± 0.51
7.	High Density Lipoprotein-3 Cholesterol(HDL_3-C)	3.172 ± 0.53	4.33 ± 0.247
8.	High Density Lipoprotein-protein(HDL)	0.301 ± 0.111	0.332 ± 0.0016
9.	High density Lipoprotein –2 protein(HDL_2)	0.164 ± 0.00014	0.183 ± 0.036
10.	High Density Lipoprotien –3 protein(HDL_3)	0.601 ± 0.018	0.304 ± 0.0007
11.	Non- HDL – cholesterol	37.44 ± 1.93	107.63 ± 1.61 *

*****Indirectly calculated values

All values are mean ± S.D from pooled serum of normal subjects (n=45)

Table 3: Cu^{2+} mediated oxidation of LDL isolated from normal subjects in the absence and presence of Omega-3 fatty acids (OFA) and HDL.

Time in minutes	Conjugate diene formation (µM/ml) Without OFA/HDL	Conjugate diene formation (µM/ml) With OFA	Conjugate diene formation (µM/ml) With HDL
0’	286.23	286.23	286.23
10’	378.45	316.46	321.68
20’	372.56	324.85	336.72
40’	376.67	312.35	354.57
60’	384.14	332.63	367.64
80’	386.30	336.42	348.21
100’	393.44	324.74	375.59
120’	403.22	387.68	385.39
140’	429.82	375.45	402.67
160’	444.56	332.76	339.57
180’	446.58	339.62	353.84
200’	434.42	340.82	356.77
Fold/ %	34.11%	16.02 %	19.77 %

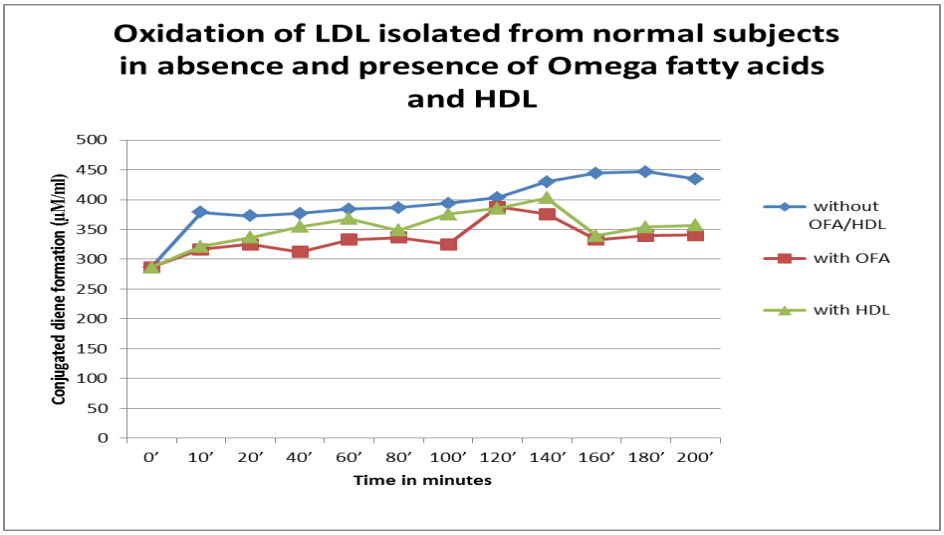

Figure 2:

Table 4: Cu^{2+} mediated oxidation of LDL isolated from diabetic with hyperlipidemia subjects in the absence and presence of Omega-3 fatty acids and HDL.

Time in minutes	Conjugate diene formation (µM/ml) Without OFA/HDL	Conjugate diene formation (µM/ml) With OFA	Conjugate diene formation (µM/ml) With HDL
0’	506.45	506.45	506.45
10’	480.56	434.39	456.34
20’	504.67	463.39	467.79
40’	514.83	479.59	484.72
60’	494.56	468.57	432.64
80’	551.22	453.83	478.42
100’	506.43	472.59	479.53
120’	508.91	485.39	482.47
140’	568.22	497.47	493.86
160’	579.48	513.45	506.67
180’	592.46	502.35	511.85
200’	598.43	519.16	521.70
Fold/ %	15.37%	2.44%	2.92%

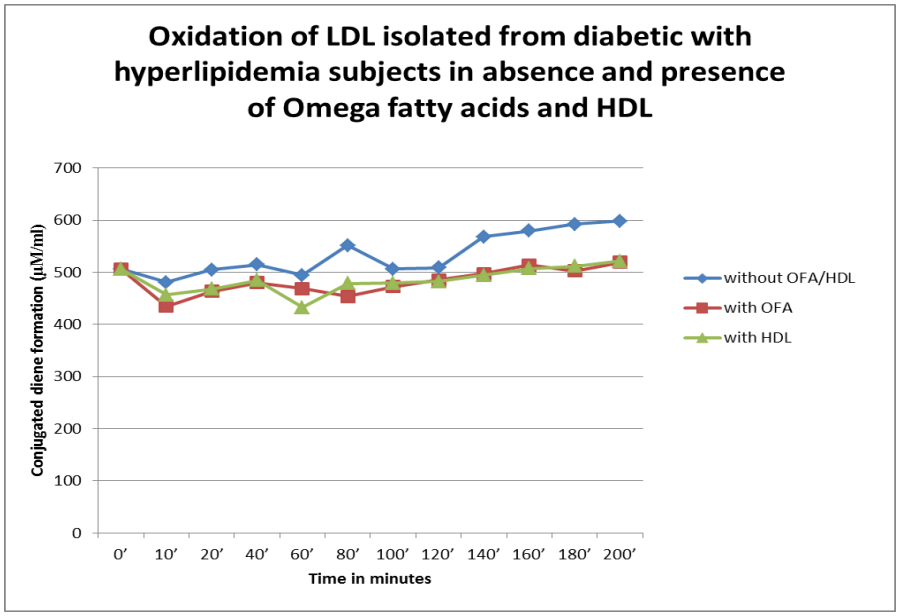

Figure 3:

Table 5: Total free radical scavenging property at different time intervals of plasma isolated from normal and diabetic with hyperlipidemia subjects in absence and presence of Omega-3 fatty acids and HDL.

Time in minutes	Total antioxidant power of plasma (µM/ml)					
	NORMAL SUBJECTS			HYPERLIPIDEMIC SUBJECTS		
	Without OFA/HDL	With OFA	With HDL	Without OFA/HDL	With OFA	With OFA
0’	274.73	413.46	410.58	182.95	260.37	257.82
1’	432.46	647.63	642.94	172.35	347.82	324.93
2’	442.73	767.84	762.82	166.84	390.79	373.92
3’	445.37	856.72	846.93	159.29	416.83	405.82
4’	454.73	922.62	901.73	151.65	440.72	410.95
5’	443.36	969.68	954.82	139.72	462.86	446.20
Fold change	1.56 ↑	2.34↑	2.32↑	1.30↓	1.78↑	1.73↑

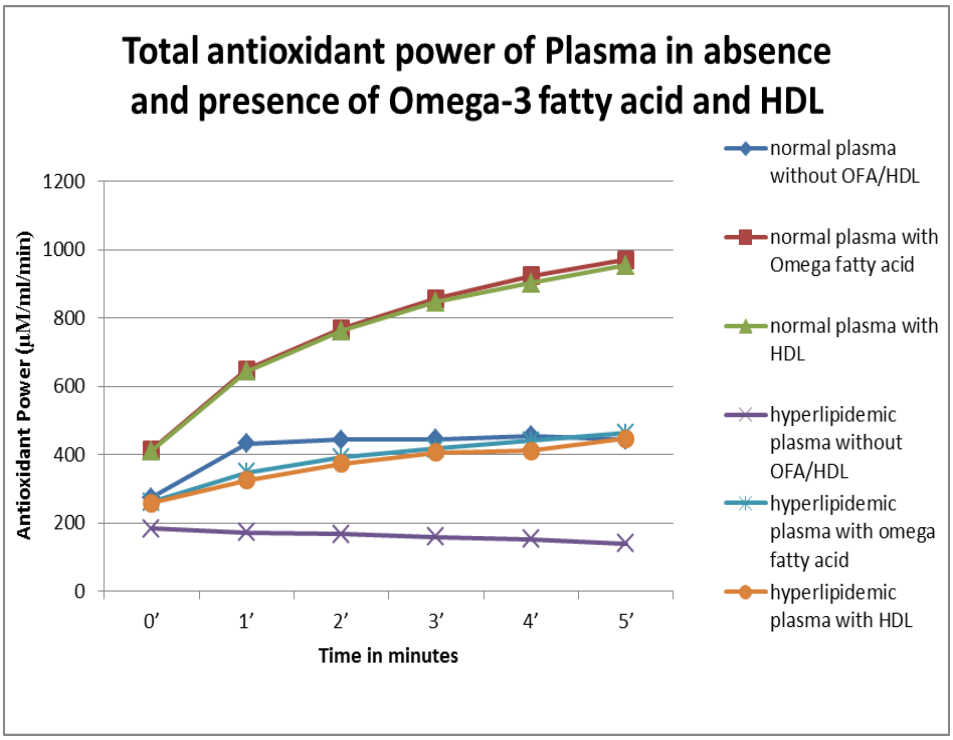

Figure 4:

Table 6: Total free radical scavenging property at different time intervals of LDL isolated from normal and diabetic with hyperlipidemia subjects in absence and presence of omega fatty acids and HDL.

Time in minutes	Total antioxidant power of LDL (µM/ml)					
	NORMAL SUBJECTS			HYPERLIPIDEMIC SUBJECTS		
	Without OFA/HDL	With OFA	With HDL	Without OFA/HDL	With OFA	With HDL
0’	403.83	450.38	444.39	294.39	397.39	387.35
1’	487.37	511.83	497.29	332.28	412.48	411.68
2’	524.59	522.81	512.29	344.39	444.37	432.67
3’	551.29	546.92	587.57	347.53	446.92	442.43
4’	563.49	589.72	601.72	351.92	482.39	495.45
5’	601.68	712.73	697.43	360.62	533.97	536.97
Fold change	1.48↑	1.58↑	1.56↑	1.22↑	1.34↑	1.38↑

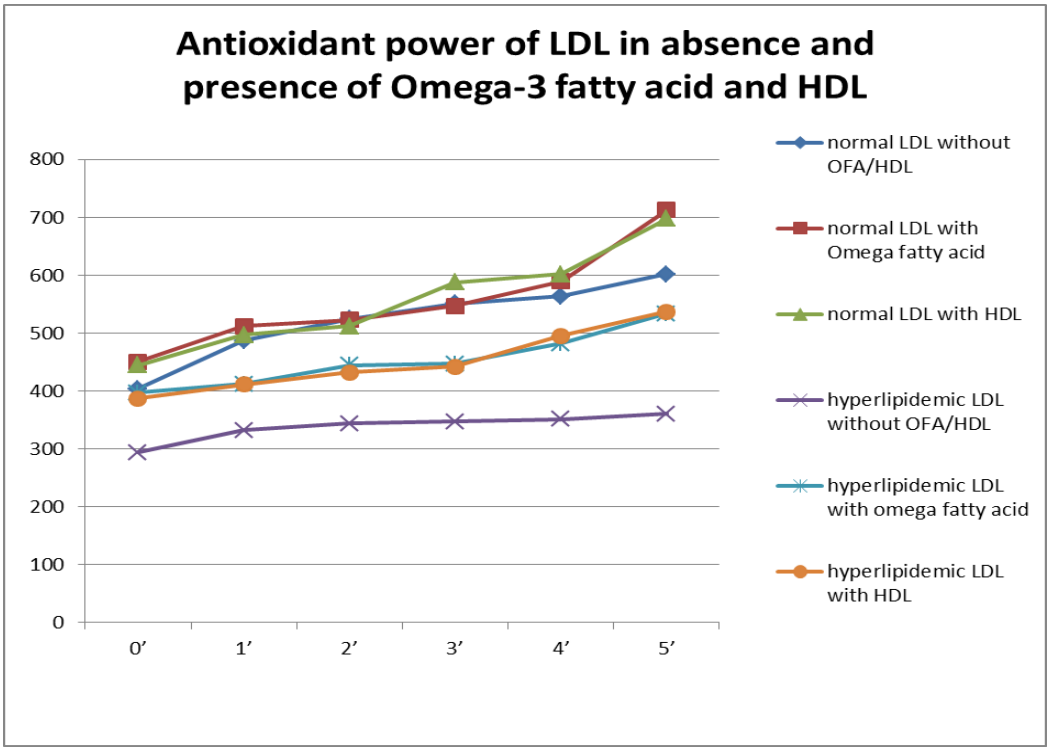

Figure 5:

RESULTS AND DISCUSSION

At high concentrations, free radicals can cause structural damage to cells, proteins, nucleic acid, membranes and lipids, which exhibits a displacement towards the oxidative burst from phagocytes in which foreign components like proteins are denatured and destroyed. Many diseases such as rheumatid arthritis, pulmonary abnormalities, cardiovascular diseases, reproductive disorders, infertility, retinopathy, diabetes, neurodegenerative diseases, nephropathy and cancer. One of the major cause of these diseases is the oxidative stress within the cells which arises due to an imbalance between the level of antioxidants produced and its scavengers. It has been shown that dietary factors such as saturated and trans fatty acids, cholesterol, some coffee lipids and sodium, in addition to lack of Omega-3 fatty acids may promote development of atherosclerosis (Keys 1970). Beneficial effects of supplementation with very long-chain Omega-3 fatty acids have been shown on development of atherosclerosis in pigs (Weiner *et al.*, 1986 and Barbeau *et al.*, 1997) as well as monkeys (Parks *et al.*, 1990 and Supari *et al.*, 1995) Paraoxonase, a HDL-associated enzyme, prevents LDL oxidation by hydrolyzing lipid peroxides, cholesterol linoleate hydroperoxides, and hydrogen peroxide (Mackness *et al.*, 1993 and Aviram *et al.*, 1998).

The present study on Omega-3 fatty acids and natural nanoparticle HDL shows that these substances have a strong antioxidant property and they can be used in the prevention of *in vivo* oxidative modification of LDL and thus lower the risk of developing lipid profile abnormality leading to various diseases.

As shown in Table no.3 when LDL isolated from normal subjects was subjected to Cu^{++} mediated oxidative modification in presence of Omega-3 fatty acid the increase from basal to maximal amount of conjugated diene formation which was achieved after 12 hours of incubation was reduced to just 16.02% as compared to control where this increase was 34.11%. The presence of HDL also had a significant impact on Cu^{++} mediated in vitro oxidative modification of LDL reducing the increase from basal to maximal CD values to just 19.77%. In case of LDL isolated from diabetic with hyperlipidemia subjects (Table no. 4), this increase was 2.44% in presence of Omega-3 fatty acid and 2.92% in presence of HDL which were greatly reduced as compared to control where this increase was 15.37%.

FRAP is a novel method for assessing "antioxidant power" in which Ferric ion is reduced to ferrous ion at low pH and lead to formation of coloured ferrous tripyridyltriazine complex**.**

Total antioxidant power of normal plasma, without any drug increased from 274.73 µM/ml to 428.58 µM/ml(1.56 fold increase) with time (from 0' to 5') whereas in hyperlipidemic patients it decreased from 182.95 µM/ml to 140.84 µM/ml (1.30 fold decrease) with time (from 0' to 5'). After in vitro treatment of normal plasma with omega fatty acids and HDL, total antioxidant power increased by 2.34 fold and 2.32 fold respectively whereas in hyperlipidemic plasma it increased by 1.72 fold and 1.73 fold respectively (Table no 5).

Similarly when total antioxidant power of LDL was measured, without any drug it increased from 403.83 µM/ml to 601.68 µM/ml (1.48 fold increase) with time (from 0'to 5') whereas in hyperlipidemic patients the increase was just 1.22 fold. After in vitro treatment of normal LDL with omega fatty acids and HDL, total antioxidant power increased by 1.58 fold and 1.56 fold respectively compared to control where this increase was 1.48 fold only, whereas in hyperlipidemic patients it increased by 1.34 and 1.38 fold respectively (1.22 fold increase in control) Table no 6.

Our results were consistent to previous findings which says, Normal functional HDL has high levels of anti-oxidants and active anti-oxidant proteins and enzymes with high anti-oxidant potential and has anti-inflammatory activity (Rao *et al*., 2011). HDL can directly inhibit oxidation of LDL; or other targets containing phospholipids. In addition, inhibition of oxidative events and oxidative stress *in vivo* may be achieved indirectly *via* other functions of HDL, such as induction of cholesterol efflux and, in general, *via* 'anti-inflammatory' functions of HDL (Podrez 2010).

In case of Omega-3 fatty acids it has been demonstrated that dietary intake of long-chain Omega-3 fatty acids reduces the risk of cardiac arrest (Yamada *et al*., 1997). Feeding experiments in animals (Kang *et al*., 1996 and Nair *et al*., 1996) support this observation, whereas one report showed increased risk of coronary heart diseases with high intake of omega-3 fatty acids (Pietinen *et al*., 1997). Some other studies show neither beneficial nor harmful effects of Omega-3 fatty acids/fish intake in relation to cardiovascular diseases (Morris *et al*., 1995 and Ascherio *et al*., 1995). Overall, fish consumption seems to be beneficial, and a systematic review of 11 prospective cohort studies concluded that fish intake notably reduced mortality due to coronary heart disease in populations at increased risk (Marckmann *et al*., 2000). Moreover, another review of several epidemiological and intervention studies concludes that Omega-3 fatty acids protect against cardiovascular disease (Milner *et al*., 1981). Our results also prove Omega-3 fatty acids effective against atherosclerosis being strong antioxidants capable of inhibiting LDL oxidation upto an extent.

CONCLUSION

The present study proves Omega-3 fatty acids and HDL effective antioxidants. Therefore Omega-3 fatty acids can be used as nutrional supplements to avoid the risk of cardiovascular diseases and HDL can replace harmful drugs in the treatment of CVD.

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