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Spectrophotometeric and Titrimetric Analysis of Lipid Peroxidation Products in Living Tissues

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A number of methods are available to measure lipid peroxidation, but no single assay is an accurate measure of the whole oxidation process. Present study therefore an attempt to analyze the different lipid peroxidation products viz. Malondialdehyde, Hydroperoxide and Diene conjugates by simple spectrophotometric and titrimetric techniques. To justify the accuracy and reproducibility of results, I compared the values of lipid peroxidation products of old animal tissues with the young animals.

Key words: Lipid peroxidation, Free radicals, Oxidative stress, Malondialdehyde, Hydroperoxide and Diene conjugates.

Introduction :

Oxidation is a part of the normal metabolism of the human body. In these metabolic processes highly reactive molecules, free radicals, are processed. Free radicals are atoms or molecules that contain one or more unpaired electrons. These compounds have important physiologic functions e.g. for the function of phagocytes, but they are also capable to induce oxidative damage to essential biomolecules such as nucleic acids, lipids and proteins. Free radicals may play a role in the development of atherosclerosis and other degenerative diseases primarily by oxidizing polyunsaturated fatty acids in lipoproteins, especially low-density lipoprotein (LDL). These oxidation reactions can be prevented by a complex antioxidant defense system in the human body, which includes enzymes such as paraoxonase (PON), superoxide dismutase (SOD), catalase, glutathione peroxidase, and antioxidants such as vitamin E and C, β-carotene, urate and thiols. Normally in the body exists a balance between the formation of radicals and defense, but “oxidative stress” may result when these systems fail to cope with the production of radicals. Disturbance in the balance may, according to current

knowledge, contribute to the development of atherosclerosis and other vascular dysfunction.

Lipid peroxidation is one of the important outcome of the oxidative stress. Lipid peroxidation *in vi*vo is a fundamentally deteriorative reaction that is involved in aging processes, atherosclerosis and cancer (Muscari *et al*. 1990; Hatoroft, 1965; Cerutti, 1985). Lipid Peroxidation brings about several changes in biological membrane (Leyko and Bartosz, 1986). It is a highly destructive process in cellular organelles and whole organism. The loss of biochemical function and/or structural architecture may lead to damage or death of cell (Kale and Sitaswad, 1990). The lipid peroxidation is also a key indicative of oxidative stress induced by aging. Present study, therefore, an attempt to analyse the lipid peroxidation products in the terms of conjugated dienes, hydroperoxides and malondialdehyde in the mice tissues by spectrophotometric and titrimetric techniques.

Material and Methods :

Male Swiss albino mice were selected from an inbred colony and maintained on standard mice feed (Hindustan Lever Ltd., New Delhi) and water *ad libitum*. Mice were maintained at constant temperature (22±1°C) and light (12L: 12D). Thiobarbituric acid were purchased from Sigma, USA. All other chemicals used were of analytical grade. Mice were divided into two groups (6 animals in each group) : The first group was of 6-8 week old mice and second group was of 19 month old. Mice were autopsied by cervical dislocation and various organs viz. brain, spleen, liver and kidney were removed for biochemical estimation of lipid peroxidation.

Lipid peroxidation products were assayed on the basis of following principals –

Thiobarbituric Acid Assay :

Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids,serves as aconvenient index for determining the extent of the peroxidation reaction. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a light pink color species absorbing at 535 nm.

Diene Conjugate Assay :

Lipid peroxidation is accompanied by a rearrangement of the polyunsaturated fatty acid double bonds, leading to the formation of conjugated dienes, which absorb at 233 nm. Therefore, lipid peroxidation can be assayed by recording the increase in absorbance of extracted membrane lipids at 233 nm.

Hydroperoxide Assay (Titrimetric analysis)

Hydrogen peroxide is usually encountered in the form of an aqueous solution containing about 6 percent or 30 percent hydrogen peroxide, and frequently referred to as '20-volume','40-volume', and 100-volume hydrogen peroxide respectively; this terminology is based upon the volume of oxygen liberated when the solution is decomposed by boiling. Thus 1 mL of '100-volume' hydrogen peroxide will yield 100 ml of oxygen measured at standard temperature and pressure.

The following reaction occurs when potassium permanganate solution is added to hydrogen peroxide solution acidified with dilute sulphuric acid :

$$2MnO_4^- + 5H_2O_2 + 6H^+ = 2Mn^{2+} + 5O_2 + 8H_2O$$

This forms the basis of the method of analysis given below.

Statistical analysis :

Statistical analyses were carried out by Microsoft World Excel – 2000. The values are expressed as mean ± S.E.M of 6 animals. The difference between two groups was analyzed by student's t-test.

Results :

It is evident from table that, 19-month-old mice (control) have a considerably higher value of lipid peroxidation in comparison to normal group (6-8 week old). Magnitudes of lipid peroxidation products were found as per following order –

Melondialdehides : Spleen > Liver > Kidney > Brain

Diene Conjugates : Liver > Brain > Spleen > Kidney

Hydroperoxides : Brain > Spleen > Kidney > Liver

Values of SEM have been remained in between 1 to 3%, which indicate the sensitivity of the techniques.

Table-1 : The amount of MDA (nM/g wet tissue), Diene conjugates (mM/100g wet tissue) and Hydroproxides (mM/100g wet tissue) in mice tissue. Values ± S.E.M. of six mice in each group.

	Parameters	Young (6-8 week old)		Old (19 month old)	
		Values	SEM	Values	SEM
Brain	**MDA**	138.114	1.36	162.347*	1.21
	Dc	40.361	1.61	59.235 *	1.87
	HP	18.251	0.84	26.131*	1.12
Liver	**MDA**	312.457	2.16	347.752*	2.25
	Dc	76.283	2.65	105.661*	3.13
	HP	11.356	0.41	19.711*	0.61
Spleen	**MDA**	341.353	2.44	381.412 *	2.81
	Dc	23.731	0.91	32.561 *	1.09
	HP	17.183	0.72	24.915 *	0.94
Kidney	**MDA**	215.512	2.19	284.201 *	2.21
	Dc	17.182	0.88	31.823 *	1.22
	HP	12.561	0.46	21.416 *	0.83
*Statistically significant diffrence with young mice at P< 0.001					

Discussion :

A variety of methods are available to measure lipid peroxidation, but no single assay is an accurate measure of the whole oxidation process. Direct evaluation of LDL oxidation is difficult, because lipoprotein oxidation is likely to occur in the mileum of the arterial wall. The extent of lipid oxidation can be measured by measuring the primary and secondary peroxidation products. In our epidemiological studies and human supplementation trials we have used a wide variety of assays to evaluate lipid peroxidation. In most of the trials we have measured oxidation using plasma total peroxyl radical trapping antioxidant parameter (TRAP), the oxidation susceptibility of VLDL+LDL or LDL to oxidation (after induction of Cu2+), F2-isoprostanes, baseline diene conjugates in LDL (LDL-BCD) and hydroxyl fatty acids.

Out of these different methods, isoprostanes are currently thought to be the most valuable biomarkers of lipid peroxidation. F2-isoprostanes are produced in vivo from the peroxidation of unsaturated fatty acids. F2-isoprostanes have been shown to be present in increased amounts in human atherosclerotic lesions. Also plasma, serum or urinary levels of F2-isoprostanes have been shown to be increased in subjects with hypercholesterolemia, liver cirrhosis and asthma.

The F2-isoprostane concentrations can be determined from serum, plasma or urine samples by GC/MS with negative-ion chemical ionization using a deuterium-labeled F2-isoprostane as an internal standard. F2-isoprostane levels are presented against creatinine concentration. Another method to measure lipid peroxidation in vivo is plasma C18 hydroxy fatty acid concentration. In this method lipids are extracted from plasma by the method of Folch. Extracted samples are swiftly hydrogenated by using platinum as a catalyst, following saponification and conversion to methyl esters. Silica SPE columns are used to purify the formed monohydroxy fatty acid methyl esters. A tetramethylammonium hydroxide derivatization is carried out for all the hydroxy groups. Finally, the concentrations of monohydroxy fatty acids (OH groups at positions 8, 9, 10, 11, 12, 13, 15 and 16 of the C18 chain) are determined by GC/ MS with electron impact mass spectroscopy. Total concentration and

concentrations of single acids are determined. C19 hydroxy fatty acid is used as an internal standard.

In addition to F2-isoprostanes and C18 hydroxy fatty acids, the lipid peroxidation in vivo can also be evaluated by measuring baseline (uninduced) diene concentration. Baseline diene concentrations are considered to be an early stage marker of polyunsaturated fatty acid oxidation. In this method LDL is precipitated with heparin/citrate and in the following extraction, diene concentration is measured photometrically against hexane. Final results are presented against cholesterol concentration of precipitated LDL. This analysis method has been used, for example, in different supplementation studies and studies studying the effects of physical exercise on lipid peroxidation in humans. One of the most commonly used *ex vivo* methods in our clinical trials is copper-induced serum oxidation. In this method serum is diluted with phosphate buffered saline (PBS) and oxidation is initiated by the addition of copper. The formation of conjugated dienes is followed by monitoring the change in absorbance at 234 nm and lag-time to the maximum oxidation rate (lag-time) is determined.

Another commonly used ex vivo method in our trials is plasma/LDL total peroxyl radical trapping antioxidant parameter (TRAP). Plasma total peroxyl radical trapping potential (TRAP) is determined with a modification of the method by Metsä-Ketelä et al (1991).

Results of present investigation corroborates the findings of Manda and Bhatia (2003 a, b, c and d). Results evaluated from present therefore clearly suggest the reproducibility of the assay methods applied for the estimation of different lipid peroxidation products. Oxidation is a part of the normal metabolism of the human body. In these metabolic processes highly reactive molecules, free radicals, are processed. Free radicals are atoms or molecules that contain one or more unpaired electrons. These compounds have important physiologic functions e.g. for the function of phagocytes, but they are also capable to induce oxidative damage to essential biomolecules such as nucleic acids, lipids and proteins. Free radicals may play a role in the development of atherosclerosis and other degenerative diseases primarily by oxidizing polyunsaturated fatty acids in lipoproteins, especially low-density lipoprotein (LDL). These oxidation reactions can be

prevented by a complex antioxidant defense system in the human body, which includes enzymes such as paraoxonase (PON), superoxide dismutase (SOD), catalase, glutathione peroxidase, and antioxidants such as vitamin E and C, β-carotene, urate and thiols. Normally in the body exists a balance between the formation of radicals and defense, but "oxidative stress" may result when these systems fail to cope with the production of radicals. Disturbance in the balance may, according to current knowledge, contribute to the development of atherosclerosis and other vascular dysfunction.

Measurement of lipid peroxidation in our nutritional studies
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