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Do Diet Supplements and Diabetes Increase Lipid Oxidation End Produces in Urine?

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Abstract: The study aimed to investigate the effect of diabetes (IDDM) and some dietary factors such as consumption of cod liver oil on lipid peroxidation end products detected in urine. Changes in lifestyle, diets, and diseases, such as diabetes mellitus, result in a wide variety of oxidative changes in biological components, such as fluids and tissues. This paper is concerned with developing and determining thiobarbituric acid-malondialdehyde (TBA-MDA) in urine samples using an HPLC-based method. The TBA-MDA adduct excreted among healthy individuals in urine was expressed on a body-weight basis, at approximately 38 nmol/kg/24 h. A level of 128 and 149 nmol/kg/24 h was detected in insulin-dependent diabetes mellitus (IDDM) patients and cod liver oil consumers (CLOCs), respectively. Simple modifications to the Kosugi two-step method-based HPLC, UV/VIS, and fluorescence increased both the sensitivity and specificity of the assay. To develop the method based on the two-step TBA reaction depended on cooling the urine samples for 60 min and then heating the samples for 20 minutes. The Diethylthiobarbituric acid-MDA adduct was extracted by the addition of 5 ml ethylacetate and diethyl ether (3:2 ratio). DETBA was used instead of TBA and an Acrodisc 4, an HPLC–certified, 0.45-μm syringe filter, was used to purify the samples prior to injection onto an HPLC column. Different types of columns were tested, including silica and reverse phase C18 columns. Urine TBA-MDA levels from IDDM patients and CLOCs were increased 3- and 4-fold over levels from healthy subjects, respectively.

Key words: DETBA-MDA adduct · lipid peroxidation · diet · Insulin Dependent Diabetes Mellitus (IDDM) diabetes · cod liver oil consumers · TBARS · Malondialdehyde (MDA)

INTRODUCTION

The advanced development of analytical procedures has led to monitoring the products of oxidative changes and their effects in biological fluids such as urine and plasma. TBA test is one of the most common tests, which use to determine lipid oxidation whether *in vivo* or *in vitro*.

Lipid oxidation may be defined as the process of oxidative deterioration of polyunsaturated fatty acids and is initiated and propagated by free radicals [1]. A free radical can be defined as any molecular species capable of independent existence which contains an unpaired electron in an orbital, such as $CH_3^{\bullet}$ or $Cl^{\bullet}$, the dot representing a single (unpaired) electron [2, 3].

In cellular systems, lipid oxidation is of absolute importance, particularly in membranes where most of the oxygen–activating enzymes are found and there is typically a relatively high concentration of polyunsaturated fatty acids, which are more susceptible to oxidation. Lipid peroxidation in cell membranes can disintegrate the membrane structure and cause loss in the function of the cell organelles [5].

Mechanism of lipid peroxidation: Lipid peroxidation has been extensively reviewed by a number of authors [6-9]. The process of lipid peroxidation includes three main steps-initiation, propagation and termination.

The initiation reactions result in the production of a small number of highly reactive fatty acids molecules with unpaired electrons, the free radicals denoted by $R^{\bullet}$ During the propagation reactions, atmospheric oxygen reacts with these radicals to form peroxy radicals, $ROO^{\bullet}$ (Fig. 1b), which are also highly reactive and further react with other unsaturated fatty acids to generate hydroperoxides, ROOH and another free radical (Fig. 1c). The free radical can go round and repeat this process thereby forming a chain reaction

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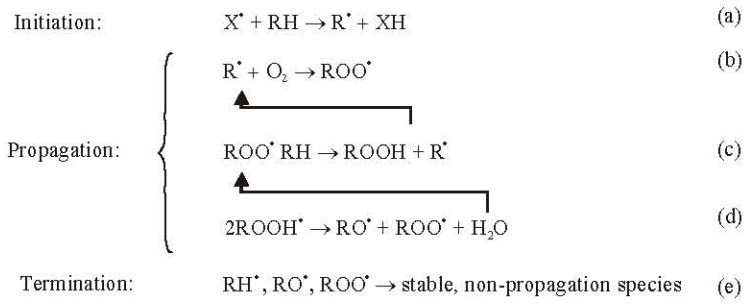

Fig. 1: Outline of the reactions involved in the auto-oxidation of unsaturated fatty acids[10]

(Fig. 1b) but the hydroperoxide can break down to give other free radicals (Fig. 1d) which can also behave much as ROO• did. Consequently, the ever increasing number of free radicals accumulates in the lipid which absorbs considerable quantities of oxygen from the air. Eventually, the free radical concentration reaches a point when they start to react with each other to produce stable end-products and these are known as the termination reactions (Fig. 1e) [10].

In 1944, Kohan and Liversedge [11] were the first scientists who described the TBA assay on the oxidation products in animal tissues. These authors suggested that the reactant substances could be a carbonyl compound [11]. Bernheim and co-workers (1947) studied the reaction between TBA and the oxidation products from certain lipidic substances [12]. According to these pioneer experiments, the coloured reaction products were alklidene thiobarbituric acids. However, the TBA test proved to be more sensitive than Kerbs test [13].

Many studies indicated to the harmful effect of lipid peroxidation *in vivo*. Fortunately with advanced technology in HPLC enabled researches to develop accurate methods of lipid peroxidation detection. Malondialdehyde (MDA) reacts with TBA to form a pink pigment MDA $(TBA)_2$ adduct which can be measured spectrophotometrically, floumertically or by HPLC. The major problem associated with this method is interfering with other compounds specifically in biological fluids, such as nucleic acids, benzoate, carbohydrates, bile pigments, amino acids that may react with TBA [14, 15]. Obviously, colorimetric methods are associated with interfering TBA with other compounds and the pigment might come from malondialdehyde or other compounds such molecules reported previously. Consequently, HPLC technique would overcome these problems specially with using HPLC facilities to confirm peak identity such as retention time and spectra studies or mass spectrometry.

The principle of the TBA test depends on the interaction between thiobarbituric acid (TBA) and malondialdhyde (MDA). Practically, the investigators in this study examined several materials to optimise the determination of TBA-MDA in urinary execrations. For example 1,1,3,3 tetraethxypropane (TEP) or 1, 1,3,3 Tetramethxypropane (TMP) as source of artificial MDA were tested. In actual TBA test the sample exposes to temperature very approach from boiling point 95°C at low pH 3 and the result is a pink colour which measures at 532 or 539nm. The chromgen can be extracted into ethylacetate, diethyl ether or chloroform [16, 17].

Malondialdehyde (MDA) $CHOCH_2CHO$ is forming naturally as a side product in the biosynthesis of prostaglandins. For example, by β- scission of peroxidised polyunsaturated fatty acids (PUFA) mainly arachidonic acid. Colorimetric measurements of the TBA adduct is simple and sensitive, but it lacks specificity. The current work overcomes this problem by using a separation technique that depends on HPLC UV-VIS and fluorescence detectors to separate MDA-TBA adduct [18].

2-Thiobarbituric acid TBA and diethylthiobarbituric acid (DETBA) are widely used compounds because their reactivity, generally with carbonyl substances (aldehydes, ketones) the wide using of TBA test, which has been used in the determination of lipid peroxidation.

Different wavelengths were used to measure TBA-MDA adduct such as 539nm, 532nm (UV/VIS detector) [19] and fluorescence at excitation 532 and emission 553nm. A 532nm absorbing red pigment has been widely used as an excellent index in medical and food science field [20].

MATERIALS AND METHODS

Subjects: Twelve healthy males and females are involved in this study, ages between 25 and 55 y. They having no diabetes or consumers of cod liver oil or having any medical history of heart disease or cancers. Twelve patients with diabetes (IDDM) duration of diabetes were

in the range of 5-10 years. All the diabetic subjects were not following healthy dietary habits and on an insulin therapy of 1-2 insulin injections per day. The insulin used was human insulin in all the cases. Six Cod Liver Oil Consumers (CLOC) duration intake one month, of clear capsule containing: of cod liver oil 800 mg, with multivitamins and garlic. Informed consent was obtained from all the participants. Twenty four hours urine was collected and each aliquot was clearly time-labelled on each sample and stored in 2ml aliquots, at -70°C until required.

Methods

Reagents and chemicals: Methanol, ethyl acetate, ethanol were HPLC grade. HPLC grade water and methanol (mobile phase) was prepared by passing through a Millipore membrane (0.45µm). 1,3 diethyl-2thiobarbituric acid (DETBA) 99% (Aldrich), Ethanolamine and 1,1,3,3 tetraethxypropane (TEP) 97%, were purchased from Sigma. Butylated hydroxy toluene (BHT 5 mM) was added to the samples during preparation to reduce lipid peroxides during hydrolysis and acetic acid used to enhance the hydrolysis process.

Analytical and extraction procedure for standards: The concentration of 1, 3 diethyl-2-thiobarbituric (DETBA) was 0.5% for standard and urine samples. DETBA was dissolved in phosphate buffer (0.1M) adjusted to pH 3 with phosphoric acid. A 0.055g of 1,1,3,3 tetraethxypropane TEP (malondialdhyde bis) was dissolved in one litre of water to prepare stock solution of 250 µM. This stock was kept at 4°C and was stable for several months. TEP standard solution and reagent recovery were prepared freshly every day according to Table 1. Five different standards of TEP in a concentration diluted in a range from 0 to 400 µl were converted to DETBA-MDA adduct in a ratio of 2 molecules of TBA to one molecule of MDA [16]. The standards were analysed by HPLC and the data was used to prepare a calibration curve.

Analytical and extraction procedure for urine samples: To examine and develop TBA test for urine samples several experiments have been conducted. For example, different amounts (urine, water, BHT and acetic acid), concentrations and company chemicals brands such as DETBA, TEP and organic solvents were experimented. Different columns such as Silica and C-18 columns were also experimented. Urine samples from different categories such as healthy, IDDM and CLOC were analysed by two step method. One step method [17] was experimented but the results were not satisfactory. Different amounts of urine (0 to 2400 µl) were transferred to a screw capped tube according to table 2 and 3. Each urine sample was injected three times and analysed by HPLC UV/VIS and fluorescence detectors.

Developing two-step method: Different mobile phases were investigated in this work such as 20, 40, 50, 60 and 80% methanol to water (v/v). The best results were obtained from (50:50 v/v) methanol-water (v/v) with 0.1 ethanolamine. The total running time was 30 minutes. Two wavelength were studied 532 and 539 nm therefore the results indicated to better coefficient of variation (C.V) for wavelength 532 nm, thus this wavelength was used to determine TBA-MDA adduct in urine samples.

Table 1: Reactive Oxygen Species (ROS) of physiological interest[4]

Radical	Name	Typical biological target
$O_2^{-\bullet}$	Superoxide	Enzymes
H_2O_2	Hydrogen peroxide	Unsaturated fatty acids
$OH^\bullet$	Hydroxyl	All biomolecules
$R^\bullet$	R-yl	Oxygen
$RO^\bullet$	R-oxyl	Unsaturated fatty acids
$ROO^\bullet$	R-dioxyl (R-peroxyl)	Unsaturated fatty acids
ROOH	Hydroperoxide	Unsaturated fatty acids
$^1O_2$	Singlet molecular oxygen	H_2O
$NO^\bullet$	Nitroxyl	Several

Table 2: Developed Two-Step method for standard (Modified than Kosugi *et al.* [16])

Tube contents (µl)	Tube 0 (µl)	Tube 1 (µl)	Tube 2 (µl)	Tube 3 (µl)	Tube 4 (µl)	Tube 5 (µl)
DETBA 0.5%	3000	3000	3000	3000	3000	3000
BHT 0.5%	25	25	25	25	25	25
Acetic acid 2%	100	100	100	100	100	100
TEP 250µM	0	25	50	100	200	400
Water	400	375	350	300	200	0
Total	3525	3525	3525	3525	3525	3525

Table 3: First serial trials to develop two-step method for urine samples

Tube contents (l)	1st trial* (l)	2nd trial (l)	3rd trial (l)	4th trial (l)	5th trial (l)	6th trial (l)
Urine	0	25	50	100	200	400
Acetic acid 2%	100	100	100	100	100	100
Water	400	375	350	300	200	0
BHT 0.5%	40	40	40	40	40	40
DETBA 0.5%	200	200	200	200	200	200
Total	740	740	740	740	740	740
Extraction	3ml ethyl acetate-2ml diethyl ether	3ml ethyl acetate-2ml diethyl ether	3ml ethyl acetate-2ml diethyl ether	3ml ethyl acetate-2ml diethyl ether	3ml ethyl acetate 2ml diethyl ether	3ml ethyl acetate-2ml diethyl ether
Re-dissolving	1500 l water: methanol (1:1)	1500 l water: methanol (1:1)	1500 l water: methanol (1:1)	1500 l water: methanol (1:1)	1500 l water: methanol (1:1)	1500 l water: methanol (1:1)

The method was developed basically from Kosugi *et al.* [16]. The developed method was based on cooling urine samples for 60 minutes at 5°C that enhances the formation of aldehyde to TBA 1:1 ratio following that the samples were heated for 20 minutes that enhance the formation of DETBA to malondialdhyde in 2:1 ratio (producing red pigment). Amounts of 2 ml urine with 3ml of 0.5% DETBA, 50µl of 0.5% BHT and 200µl 2% of acetic acid were mixed in a screw capped tube for 25 seconds on vortex. Tubes (closed firmly) were cooled down on ice for 60 minutes and incubated in water bath for 20 minutes 95°C. Tubes were acidified to pH 1 with hydrochloric acid 6M (approx.10 µl). Extraction was executed with 3ml ethyl acetate and 2 ml diethyl ether. Tubes were centrifuged for 10 minutes at 3000 rpm and transferred the upper layer in small tubes. Under stream of nitrogen the samples were dried at 40°C.

The dried pigment was re-dissolved with 200 µl of methanol and water (1:1). Samples were filtered before injection, 20 µl of each sample was injected onto HPLC.

TBA residues: Presence of residues has been found in urine samples and standards due to cooling on ice before injection onto HPLC. There was no data reported in the literature about the presence of residues before injection onto HPLC consequently, different experiments have been conducted to resolve this problem. To purify the samples different syringe filters were used such as Whatman, Gelman filter syringes and even used filter paper which removed the red colour of DETBA-MDA adduct. Successfully, Acrodisc 4 HPLC certified 0.45 µm syringe filter was used to remove the residues.

Quality control: The procedure was tested each time by carrying out a reagent recovery using only reagents and also a spiked sample at the second concentration that treated exactly like a sample following hydrolysis process.

Chromatographic separations: Chromatographic separations were carried out by using HPLC system, consisted of apparatus solvent delivery module and injection system with 20 µl sample loop, Reversed Phase Liquid Chromatography (RP-LC) column C-18, S50D52-9337. The apparatus attached with UV visible detector Spectroflow 757 (absorbance detector) Kratos Analytical, set at 532 nm and 0.1 sensitivity absorbance unit and an integrator (Hewelett-Packared) HP 3395. Mobile phase consisted of 50% methanol to 50% water and 0.1% ethanolamine, flow rate 0.7 ml/minute and running time 30 min. Quantification was based on peak area and component Identification using UV/VIS spectra.

RESULTS

A linear distribution plot representing area against concentration has been achieved for standards of TEP. A good linear correlation was achieved with visible and fluorescence detectors r^2>0.9895 over a range of concentrations. The results of a comparison study between the two detectors; UV/VIS at 533nm and fluorescence at 515nm (excitation) and 553nm (emission) gave SEM ±0.182 and ±8.01 respectively.

The study of using different wavelengths 532nm *vs* 539nm, UV/VIS detector in urine samples for healthy volunteer, two-step (50:50 v/v) water methanol concluded that the results of 532nm is better (SEM±.32) than 539nm (SEM±4.74) therefore 532nm was selected to be used for all experiments.

Standards of 1,1,3,3-tetraethoxypropane were included in parallel with all samples The average retention time in the current work was 12 minutes for urine samples, peak areas were converted to /nmol/kg/24h TBA-MDA adduct by comparison to this standard. The within-day coefficient of variation was 5.0% or less.

Table 4: Second non-serial trials to develop two-step method for urine samples

Tube contents (l)	1st trial* (l)	2nd trial (l)	3rd trial (l)	4th trial (l)	5th trial (l)	6th trial (l)	7th trial (l)
Urine	2000	2000	2000	1200	600	2400	400
Acetic acid 2%	200	200	100	300	300	300	100
Water	0	200	0	0	0	0	0
BHT 0.5%	50	50	25	120	120	120	40
DETBA 0.5%	3000	3000	3000	600	600	600	3000
TEP (spiking)	0	0	0	0	600	0	
Extraction	3ml ethyl acetate-2ml diethyl ether	3ml ethyl acetate-2ml diethyl ether	3ml ethyl acetate-2ml diethyl ether	3ml ethyl acetate-2 ml diethyl ether	3ml ethyl acetate-2 ml diethyl ether	3ml ethyl acetate-2ml diethyl ether	3ml ethyl acetate-2 ml diethyl ether
Re-dissolving	200 µl water methanol(1:1)	200 µl water methanol (1:1)	200 µl water methanol(1:1)	200 µl water methanol(1:1)	200 µl water methanol (1:1)	200 µl water methanol (1:1)	200 µl water methanol(1:1)

*This amounts were used in actual developed method

Table 5: MDA levels in standard, HPLC UV/VIS detector, two-step method 50%-50% water-methanol, 532nm

Concentration	MDA M	Coefficient of Variation (CV)
1st	0.54	4.37±1.68
2nd	1.71	19.64±3.91
3rd	3.42	9.17±0.90
4th	6.85	0.09±4.13
5th	13.70	0.25±5.43

All values are the mean of 3 measurements; values are Means±SD

Table 6: TBA-MDA in urine samples for different volunteers, HPLC UV/VIS detector, two-step method 50%-50% water-methanol, 532nm

Category	TBA-MDA nmol/kg.24h	SD±SEM
Healthy volunteers	38	9.5±4.89
Diabetes IDDM	128	25.8±4.69
Cod liver consumers	149	10.7±1.68

3 repeated measurements of each injection

The TBA-MDA levels were found to be in average of 38 nmol/(kg.24h) urine among healthy individuals Table 6. Level of 128 nmol/ (kg.24h) was detected in the urine of patients with insulin dependent diabetes mellitus (IDDM). High amounts of TBA-MDA adduct have been found to be 149 nmol/ (kg.24h) in urine for cod liver oil consumers Table 6 and Fig. 3.

It has been noticed that an increase in TBA-MDA pigment among patients with Insulin Dependent Diabetes Mellitus (IDDM). The results from the above chromatogram indicate to the increase peak area that approached the third concentration of standard.

DISCUSSION

It is difficult to generalise one method to be used in different fluids and tissues such as plasma and urine. The general aim of developing TBA test in urine samples was to be more sensitive, accurate and less complicated and costs. Some considerations had to be taken into account when working with two-step method. For example, in 1993, Kosugi *et al.* [16] reported that yellow colour is due to the reaction between TBA with sugars or alkalans but not related malondialdehyde or lipid peroxidation end products. Bile salts produce a different chromgen and this interference can be overcome by separations out the authentic TBA-MDA adduct by using HPLC technique. For these reasons the investigators used HPLC for obtaining accurate results than colorimetric determinations.

In the current work DETBA-MDA adduct was extracted by 5ml of ethylacetate and diethyl ether (3:2 ratio) while Kosugi and co-workers extracted by 3ml chloroform. At the same time he used TBA instead of DETBA which was used in the current method.

Different wavelengths were examined to measure TBA-MDA adduct such as UV visible detectors at 539nm, 532nm and fluorescence excitation at 515nm and emission at 553nm. In 1978, Buege and Aust [21] reported that 532nm absorbing red pigment that has been widely used as an excellent index in medical and food science field.

Our results were in agreement with [16-22-23] the quantity of the TBA-MDA adduct excreted in the urine expressed on a body weight basis, ~38 nmol/(kg.24h), are similar to the 26-95 nmol/ (kg.d) range reported by Kosugi *et al.* 1993. A ~50 nmol/ (kg.d) of TBA-MDA were reported by Nelson *et al.* [22] and Wander *et al.* [23].

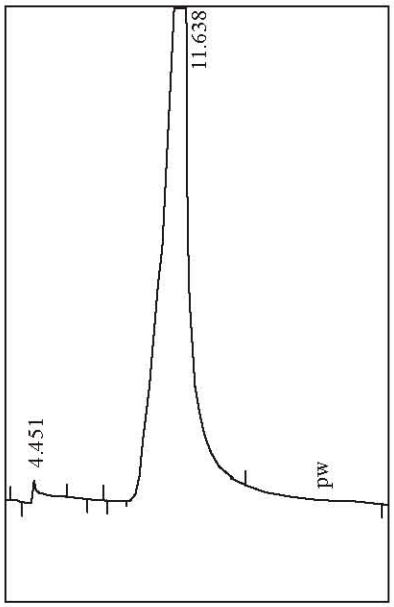

Fig. 2: Example of typical HPLC UV visible detector chromatogram obtained from external standard, third concentration, two-step method 532nm

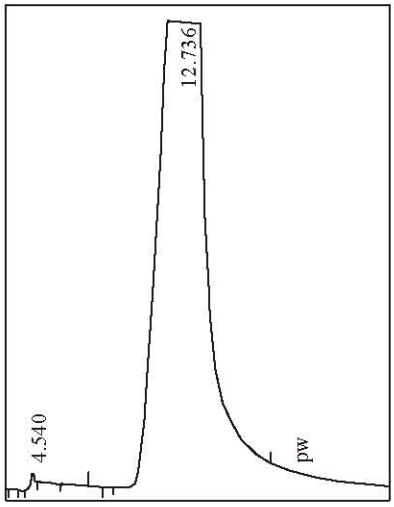

Fig. 3: Example of typical HPLC UV visible detector, chromatogram obtained from cod liver oil consumers (CLOC) two-step method 532nm

Volunteers who ingested cod liver oil and multivitamins with garlic were situated between third and fourth concentration; 149nmol/(kg/24h) (Fig. 2 and 3). It is worth mentioning that a decline level of TBA-MDA had noticed when CLOC stopped taking CLO for one week (Fig. 4).

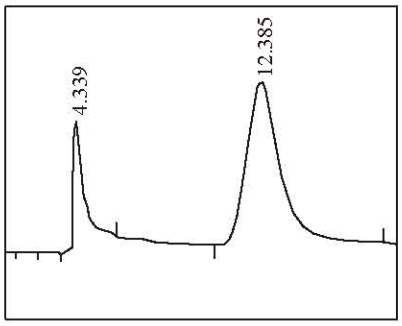

Fig. 4: Example of typical HPLC UV visible detector chromatogram obtained from CLOC (above) after one week gave up taking CLO, two-step method 532 nm

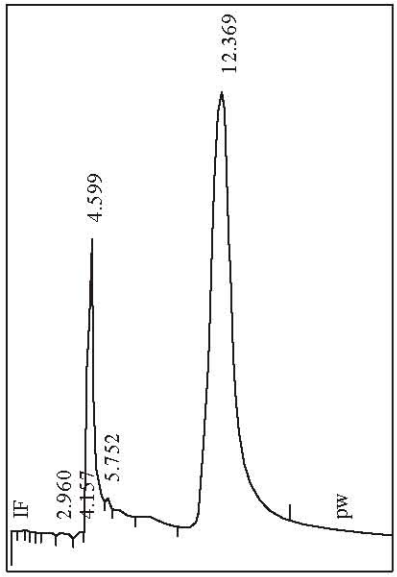

Fig. 5: Example of typical HPLC UV Visible detector chromatogram obtained from IDDM urine, two step-method and 532nm

In 1991, Meydani *et al.* [24] found increased plasma lipid peroxide levels measured as thiobarbituric reactive substances (TBARS) and in 1988, Piche *et al.* [25]. reported increased urinary malondialdehyde analyzed by HPLC. Therefore, to measure the more definitive thiobarbituric acid-malondialdhyde (TBA-MDA) adduct after consumption of a fish oil supplement. However, in this latter study, the authors concluded that the increase was the result of oxidation products in the supplement rather than an increased production of these substances *in vivo*. They suggested that fish oil supplements that were not previously autoxidized did not necessarily increase the amount of MDA produced *in vivo*. In current

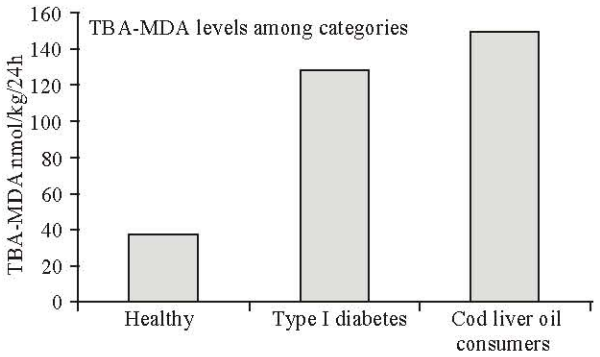

Fig. 6: TBA-MDA levels in urine samples among healthy, type I diabetes and cod liver oil consumers

work, the dose of fish oil was a pharmaceutical pack of clear capsule containing 800 mg cod liver oil and the validation date was approved by a printed date on the supplement. Consequently, there is no doubt that cod liver oil in this study was not oxidized before administration.

In men between the ages of 30 and 65 y, Nelson *et al.* [22] found that both the urinary TBARS and the TBA-MDA adduct increased after 40 d of consuming a salmon-containing diet but they made no measurements of the MDA content of the cooked fish consequently that the origin of the TBARS and MDA excreted in the urine could not be established. The increased *in vivo* peroxidation that occurs when fish or fish oils are consumed could be prevented by a sufficient intake of dietary vitamin E. The RDA of 10 mg a-tocopherol equivalents may be inadequate [24-25]. Meydani and Packer [24-26] reported a 35% increase in a group of similarly aged women after 1 month supplementation with 1.7 g eicosapentaenoic acid (EPA) and 0.7 g docosahexaenoic acid (DHA). In 1991, Haglund *et al.* [27] found that increase values of TBA-MDA adducts in plasma in volunteers in age from 41 to 60 y after supplementation with fish oil. The use of the fish oil supplement significantly increased the urinary content of TBARS when expressed as nmol/(kg/24h).

In diabetes, constant hyperglycemia may cause high production of free radical attributed to protein glycation or glucose auto-oxidation [28].MDA levels were found higher in type II diabetes mellitus patients than in healthy control in study carried out by Kesavulu *et al.* [29]. The research group also observed that NIDDM patients with coronary heart disease had higher level of MDA than those diabetic without this disease. It shows that cardiovascular disease have also been related to free radical mediated mechanisms and to lipid peroxidation and they are the major causes of mortality and morbidity in haemodialysis patient. Our results indicated to the increase of urinary excretion of TBA-MDA in insulin dependent diabetes mellitus IDDM 3-fold over healthy volunteers. In 1997, Szaleczky *et al.* [30] assumed that type I diabetes, when blood glucose is strictly controlled by intensive insulin therapy, is not accompanied by changes in the prooxidant-antioxidant balance. Clearly in this study blood glucose was not controlled compared to Szaleczky *et al.* [30] study. In 1992, Lee and co-workers [31] noticed that a high level of oxidative stress is induced by uncontrolled diabetes in rats.

CONCLUSION

Our data suggest that the changes in lifestyle, diet supplements such as taking cod liver oil and diseases such as diabetes mellitus, have a wide variety of oxidative changes in biological components in physiological fluids. In our study, specific TBA-MDA test was developed to determine thiobarbituric reactive substances-malondialdhyde (TBA-MDA) HPLC based method in urine samples. The quantity of the TBA-MDA adduct excreted among healthy individuals in urine was 38 nmol/(kg.24h). Levels of 128 and 149 nmol/(kg.24h) was detected in urine of patients with insulin dependent diabetes mellitus (IDDM) and cod liver oil consumers. Different modifications to Kosugi Two-step method based HPLC UV/VIS and fluorescence would increase both the sensitivity and the specificity of the assay. The proposed technique is easier, faster and more sensitive to use in normal and pathological urine samples.

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