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Effects of different dietary amounts of LCPUFA n3 and vitamin B_6 on lipid composition and antioxidant defences in rat kidney

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Abstract

Our previous report demonstrated that, when vitamin deficiency is associated with high contents of long chain polyunsaturated fatty acids (LCPUFA) n3, lipid peroxidation susceptibility in rat heart and liver increases. In this paper, we evaluated the effect of the same dietary administration on lipid composition and antioxidant defenses of rat kidney. Results showed that vitamin B_6 deficiency, when associated with a fish oil diet, as compared to vegetable oil condition, increased relative kidney weight and decreased pyridoxal-5P contents. The different LCPUFA n3 dietary contents produced, on kidney phospholipids, effects interlaced with those of vitamin B_6 deficiency; in particular fish oil and vitamin B_6 deficient diet caused a significant decrease of arachidonic acid showing that the processes of elongation and desaturation of linoleic acid were slowed.

Also, peroxidation susceptibility was higher, as demonstrated both by increased TBARS formation and glutathione peroxidase activity, and by decreased vitamin E contents and reduced glutathione/oxidized glutathione ratio.

Keywords: Rat kidney; Lipid peroxidation; LCPUFA n3; Vitamin B6 deficiency

1. Introduction

Dietary intake of long chain polyunsaturated fatty acids n3 (LCPUFA n3) is known to be associated with a decrease in the incidence of cardiovascular disease [1–4].

Some positive effects of fish oil rich in LCPUFA n3 have also been demonstrated in renal pathology [5] and in the prevention of hypertensive renal damage. In these conditions, dietary polyunsaturated fatty acid supplementation increases their incorporation in glomeruli and cortical tissue, improving chronic renal injury [6].

Instead, some authors [7] have reported the deleterious effects of fish oil in animal models of renal disease – perhaps related to alterations in renal eicosanoid metabolism, since antidiuretic and antinatriuretic effects, correlated with reduced renal cortical prostaglandin E_2 are associated with increased lipid peroxidation. This last effect of dietary fish oil on renal fatty acid composition deserves particular attention, because the end-products of lipid peroxidation reactions are dangerous for the functionality of cells and tissues and are crucial factors in the pathogenesis of several diseases, including kidney damage [8].

Among factors which prevent lipid peroxidation, not only vitamin E but also vitamin B_6 seems to be involved: we have previously demonstrated that their deficiency causes an increase in the peroxidizability level in some tissues [9].

The aim of the present study was to investigate, in rat, whether diets rich in LCPUFA n3 and vitamin B_6 deficient, can affect, with synergic effects, growth parameters, vitamin B_6 status and, in particular, phospholipid fatty acid composition and susceptibility to lipid peroxidation in kidney.

2. Materials and methods

2.1. Animals and diets

This study was approved by the Animal Care Committee of the University of Bologna. The experiment was carried out on 32 male rats of Wistar strain (100 to 110 g, 1-month-old), randomly divided into 4 groups each consisting of 8

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Table 1
Composition of diets

Ingredients	Vegetable oil diets[1] (g/100 g)	Fish oil diets[1] (g/100 g)
Wheat starch	38.65	38.65
Vitamin-free casein	20.00	20.00
Dextrose	10.00	10.00
Sucrose	10.00	10.00
Soybean oil	4.00	4.00
Fish oil	—	4.00
Palm oil	3.00	—
Coconut oil	1.00	—
Cellulose	5.00	5.00
Minerals	6.15	6.15
Trace element mix[2]	1.00	1.00
Vitamin mix[3]	1.00	1.00

[1] Supplied by MUCEDOLA, Settimo Milanese (Milan) Italy.
[2] Trace element mix provided in mg/kg diet: iron, 35; zinc, 40; manganese, 55; copper, 5.5; iodine, 0.2.
[3] Vitamin mix provided in mg/kg diet: thiamin 6; riboflavin 6; pyridoxine 7 (in vitamin B6 deficient groups: 0.3); biotin 0.2; folic acid 2; niacin 30; Ca pantothenate 16; choline HCl 1000; vitamin B_{12} 0.01; (α-tocopherol 50; menadione 0.05; vitamin A 4000 U.I./kg; vitamin D_3 1000 U.I./Kg.

animals, housed in individual cages in a temperature-controlled room with a 12 h light/dark cycle.

The groups of rats were fed for six weeks on diets containing different vitamin B_6 levels and qualities of oils (Tables 1 and 2), as follows:

- vegetable oils, normal vitamin B_6 (NB_6): 4% soy-bean oil, 1% palm oil, 3% coconut oil, and 7 mg/kg of vitamin B_6;
- vegetable oils, deficient in vitamin B_6 (DB_6): 4% soy-bean oil, 1% palm oil, 3% coconut oil, and 0.3 mg/kg of vitamin B_6;
- fish oils, normal vitamin B_6 (NB_6): 4% soy-bean oil, 4% fish oil, and 7 mg/kg of vitamin B_6;
- fish oils, deficient in vitamin B_6 (DB_6): 4% soy-bean oil, 4% fish oil, and 0.3 mg/kg of vitamin B_6.

Table 2
Fatty acid composition (%) of diets

Fatty acid	Vegetable oil diets	Fish oil diets
12:0	7.68	—
14:0	3.63	—
16:0	24.28	16.62
18:0	5.32	5.32
16:1 n-7	—	3.73
18:1 n-9	27.58	23.06
18:2 n-6	26.95	26.11
18:3 n-3	4.06	3.96
18:4 n-3	—	2.46
20:4 n-6	—	1.02
20:5 n-3	—	2.06
22:4 n-6	—	0.24
22:5 n-3	—	1.27
22:5 n-6	—	1.07
22:6 n-3	—	6.17

The diets were prepared each week, stored at 4°C and given to the rats in the evening. Uneaten food was removed the next morning; food consumption and animal weights were measured daily. Because a low level of dietary vitamin B_6 decreases food intake, the same amount of food eaten ad libitum by the vitamin B_6 deficient groups was given to the normal B_6 groups. Therefore, the two groups NB_6 were pair-fed with respect to DB_6 groups. At the end of dietary treatment, the animals were anesthetized with ether, and the blood samples were collected by intracardiac withdrawal and put into heparinized tubes. Plasma was obtained by centrifugation at 2500*g* for 10 min; then the rats were sacrificed, and kidneys were quickly excised and extensively rinsed at 4°C in 9 g/L saline solution. The parts of tissues not immediately used were frozen and stored at –80°C.

3. Analytical methods

3.1. Pyridoxal-5P assay

PLP was determined in plasma and kidney homogenate by the apotryptophanase method of Furth-Walker et al. [10]. The enzyme was incubated with PLP (standard or tissue extracts) to produce the holoenzyme. The substrate, S-benzyl-L-cysteine, was hydrolyzed to benzyl mercaptan which, reacting with Elman's reagent, DTNB, produced the chromophore 2-nitro-S-thiobenzoate, quantified by spectrophotometry.

3.2. Fatty acid analysis of kidney phospholipids

Total lipids were extracted from tissues according to Folch et al. [11]. Phospholipids were separated by thin-layer chromatography using a mixture of hexane/diethyl ether (80:20 v/v) as mobile phase. Spots were made visible under UV light by spraying the dried plates with 2 g/L ethanol solution of 2′,7′ dichloro-fluorescein, and phospholipids were identified by comparison with reference standards. The silica corresponding to the band of phospholipids was scraped off and extracted by methanol. Phospholipids were methylated with methanol/hydrochloric acid (95:5 v/v) for 60 min at 70°C, according to Stoffel et al. [12] and fatty acid methyl esters were detected by gas chromatography (Hewlett-Packard model 5890) using a capillary column (HP-FFAP, 25 m x 0.32 mm i.d. x 0.52 μm film) at a programmed temperature (180°-240°C). Peaks were identified using standard fatty acid methyl esters from Supelco (Bellafonte, PA, USA).

3.3. Vitamin E assay

α-Tocopherol (α-T) was determined by HPLC. Aliquots of tissues were homogenated with 2 vol ethanol and then saponified for 30 min with an ethanol potassium hydroxide

solution in the presence of ascorbic acid under N_2 reflux in a water bath. The saponified mixture was extracted with n-hexane, the extract evaporated to dryness, and the residue diluted in appropriate volume of mobile phase. Aliquots of this solution were analyzed by an HPLC system equipped with a LiChrosorb Si 60 5 μm column (250 $\times$ 4.6 mm) (E. Merck, Darmstadt, Germany) at 30°C; the mobile phase was *n*-hexane/isopropanol (99.2:0.8 v/v) at a flow rate of 1.5 mL/min; detection was fluorometric (excitation wavelength 295 nm, emission 340 nm).

3.4. Lipid peroxidation

Rat kidneys were homogenized in a Potter Elvehjem homogenizer at 4°C with 9 vol of 0.14 mol/L NaCl, 10 mmol/L N-morpholinopropane sulfonic acid (MOPS), pH 6.5. The homogenates were then passed through two layers of cheese-cloth to remove clumps. The reaction mixture (total volume 1 mL) contained aliquots of the homogenate corresponding to 1 mg of protein and 10 μmol/L $FeCl_2$ in the same buffer used for homogenisation [13]. After 1 h of incubation at 37°C, the reaction was stopped by adding 10 μl of 20 g/L butylated hydroxytoluene (BHT) and lipid peroxidation was measured by the formation of thiobarbituric acid reactive material (TBARS) according to the procedure of Beuge and Aust [14] slightly modified: samples were added with 200 μl of 80 g/L sodium dodecyl sulfate, 1.5 mL of 200 g /L acetic acid, pH 3.5, and 1.5 mL of 8 g/L thiobarbituric acid (TBA). The mixtures were heated at 100°C for 15 min, cooled on ice and extracted with n-butanol. Organic phases were collected and absorbance was detected at 532 nm vs. appropriate blanks. The tetramethoxypropane solution yielding the TBA adduct was used for calibration of absorbance.

3.5. Glutathione reductase and glutathione peroxidase assays

Portions of kidneys were homogenized in 10 vol of 10 mmol/L potassium phosphate buffer, 30 mmol/L KCl, pH 7.4. Homogenates were sonicated three times at 40 Watt for 20 s with a break of 15 s, and then centrifuged at 2000 $\times$ g for 15 min; supernatants were used to assay enzyme activities.

Glutathione peroxidase activity (GluPx) was measured using *tert* butyl hydroperoxide as substrate [15]. This was turned into a hydroxide by GSH; the GSSG resulting from the reaction was reduced by glutathione reductase using NADPH and the decrease in absorbance was evaluated at 340 nm.

Glutathione reductase activity (GluRed) was measured following NADPH oxidation, as described by Carlberg and Mannervik [16].

Proteins were estimated according to Lowry *et al.* [17] using crystalline bovine serum albumin as standard.

3.6. Glutathione assay

Levels of reduced (GSH) and oxidized (GSSG) glutathione were evaluated by an enzymatic method [18]. Portions of tissue were homogenized with Ultra-Turrax in 1 mol/L $HClO_4$ containing 2 mmol/L EDTA. After centrifugation, supernatants were neutralized with 2 mol/L K_2CO_3 in 0.3 mol/L MOPS and total glutathione contents (GSH + GSSG) were immediately determined according to Tietze [19]. The assay mixture consisted of: 50 mmol/L potassium phosphate buffer, pH 7.4, 1 mmol/L EDTA, 0.1 mmol/L 5′5-dithio bis (2-nitrobenzoic) acid, 0.15 mmol/L NADPH, 6.10^3 U/L glutathione reductase, and a suitable volume of neutralized samples. After 1 min, the absorbance increase at 412 nm was followed for 3 min on a spectrophotometer (mod. 559 Perkin-Elmer Corp., Norwalk, CT, USA).

For GSSG determination, 50 mmol/L N-ethylmaleimide (NEM) was added to the homogenate in order to trap GSH. Neutralized samples were extracted with diethyl ether to remove excess of NEM, and GSSG concentration was determined by the above-mentioned enzyme assay. GSH concentration was calculated by subtracting GSSG from total glutathione contents.

3.7. Statistical analysis

All values were expressed as means $\pm$ standard deviations. Statistical differences were determined by one-way analysis of variance and the Student-Newman-Keuls test, and were considered significantly different at $P < 0.05$.

4. Results

The effects of different lipid quality and contents of vitamin B_6 in diet on some parameters of vitamin status and body weight are reported in Table 3.

Vitamin B_6 deficient diets caused a decrease in plasma PLP contents in comparison with normal vitamin conditions; no significant difference was observed between rats fed vegetable or fish oil diets. Kidney PLP contents were affected by dietary vitamin B_6 deficiency, which caused significant decreases with respect to normal vitamin diets, but also by the quality of dietary lipids, since rats fed fish oil showed lower PLP levels in comparison with those fed vegetable oil, in both normal and deficient vitamin conditions.

Rats fed diets containing fish oil had lower final body weight in comparison with those fed vegetable oil diets; vitamin B_6 deficiency caused a slight, not significant, decrease in growth with respect to normal vitamin conditions with vegetable oil; the association of vitamin deficiency and fish oil caused a significant growth decrease, associated with the lower feed efficiency ratio found in this group.

The relative weight of kidney was not affected by the lipid quality of diet, but vitamin B_6 deficiency caused hy-

Table 3
Growth parameters and PLP contents of kidney and plasma of rats fed diets with different lipid quality and vitamin B_6 content

	Vegetable oils		Fish oils	
	NB_6	DB_6	NB_6	DB_6
Final body weight *(g)*	311 ± 16[a]	297 ± 14[a]	293 ± 23[a]	273 ± 15[b]
Feed efficiency ratio *(growth: feed)*	0.28 ± 0.01[a]	0.27 ± 0.03[a]	0.26 ± 0.02[a]	0.23 ± 0.03[b]
Relative kidney weight *(g/100 g body weight)*	0.37 ± 0.03[a]	0.44 ± 0.06[a]	0.38 ± 0.02[a]	0.58 ± 0.13[b]
Kidney PLP *(nmol/g)*	24.06 ± 2.31[a]	13.75 ± 1.98[b]	16.78 ± 2.81[c]	6.83 ± 0.95[d]
Lipids *(g/100g tissue)*	2.20 ± 0.31[a]	1.86 ± 0.22[b]	2.55 ± 0.25[c]	2.26 ± 0.12[a,d]
Plasma PLP *(pmol/ml)*	727 ± 90[a]	102 ± 11[b]	681 ± 60[a]	103 ± 16[b]

Data are means ± SD of eight rats. Significant differences ($P < 0.05$) were determined by Student-Newman-Keuls test after ANOVA. Numbers with different superscript letters are significantly different.

pertrophic swelling of the organ; lipid contents decreased in kidneys of rats fed vitamin B_6 deficient diets in comparison with those fed normal vitamin diets. The fish oil diet seemed to favor the organ hypertrophy induced by vitamin B_6 deficiency, because the relative weight of kidney further increased in this double dietary condition: here too, lipid amounts were higher in comparison with the corresponding vitamin-deficient group fed a vegetable oil diet.

As regards the fatty acid composition of kidney phospholipids, dietary treatment affected in particular the percentages of long-chain fatty acids (Table 4). The kidneys of rats fed the fish oil diet, compared with those fed the vegetable oil diet, showed higher percentages of C18:2 n6 and lower percentages of C20:4 n6, in both normal and deficient vitamin conditions. Long-chain polyunsaturated fatty acids n3 were more frequently found in the fish oil treatment: C22:5 n3 was found only in these groups and C22:6 n3 showed higher percentages with respect to the vegetable oil diet.

Total amounts of PUFA were similar in all experimental conditions, but dietary lipid quality affected n6 and n3 total percentages, which were respectively lower and higher in rats fed fish oil in comparison with those fed vegetable oils; vitamin B_6 deficiency did not affect total n6 or n3 fatty acid contents. The unsaturation index decreased in the fish oil groups, particularly when associated with vitamin B_6 deficiency.

As shown in Table 5, TBARS production was higher in the fish oil groups, especially when associated with vitamin B_6 deficiency. Vitamin E contents were lower in both fish oil groups in comparison with the respective vegetable oil groups.

Glutathione contents and related enzyme activities showed differences connected with vitamin B_6 deficiency and diet lipid quality; vitamin B_6 deficiency caused increased GluPx activity. The highest values were found in the group fed fish oil and a vitamin B_6 deficient diet, although the increase was less evident in comparison with the respective normal vitamin group, which was also affected by dietary lipids.

GluRed activity did not follow the trend of GluPx and showed similar values in all experimental conditions. The

Table 4
Fatty acid composition (%) of kidney phospholipids from rats fed diets with different lipid quality and vitamin B_6 content

Fatty acid	Vegetable oils		Fish oils	
	NB_6	DB_6	NB_6	DB_6
16:0	26.25 ± 0.90[a]	26.87 ± 2.15[a]	26.51 ± 2.42[a]	28.11 ± 1.55[a]
18:0	17.31 ± 0.37[a]	15.32 ± 1.66[b]	18.50 ± 0.18[c]	16.49 ± 0.11[a]
16:1 n7	1.50 ± 0.08[a]	1.32 ± 0.15[b]	0.71 ± 0.14[c]	1.20 ± 0.12[b]
18:1 n9	11.39 ± 0.43[a]	12.14 ± 1.13[b]	10.16 ± 0.19[c]	11.01 ± 0.76[a]
18:2 n-6	6.89 ± 0.34[a]	9.08 ± 0.74[b]	13.47 ± 0.81[c]	13.74 ± 0.81[c]
20:4 n-6	32.91 ± 1.31[a]	30.61 ± 1.85[b]	23.97 ± 1.54[c]	22.38 ± 0.89[d]
22:5 n3	—	—	2.51 ± 0.61	2.00 ± 0.37
22:6 n-3	1.11 ± 0.03[a]	0.91 ± 0.11[a]	2.17 ± 0.48[b]	2.30 ± 0.20[b]
Total Saturated	43.56 ± 0.70[a]	42.19 ± 1.85[b]	45.01 ± 1.42[a]	44.60 ± 1.02[a]
Total PUFA	40.91 ± 1.12[a]	40.60 ± 2.69[a]	42.12 ± 1.57[a]	40.42 ± 1.01[a]
Total n-6	39.80 ± 1.09[a]	39.69 ± 2.59[a]	37.43 ± 1.05[b]	36.12 ± 0.81[b]
Total n-3	1.11 ± 0.03[a]	0.91 ± 0.11[a]	4.68 ± 0.98[b]	4.70 ± 0.25[b]
Unsaturation Index	165 ± 4.55[a]	159 ± 5.30[a,b]	157 ± 8.75[b]	151 ± 3.50[b,c]

Data are means ± SD of eight rats. Significant differences ($P < 0.05$) were determined by Student-Newman-Keuls test after ANOVA. Numbers with different superscript letters are significantly different.

Table 5
Vitamin E, lipid and TBARS contents, glutathione peroxidase and glutathione reductase activities, total glutathione contents and GSH/GSSG ratios in kidney of rats fed diets with different lipid quality and vitamin B_6 contents

	Vegetable oils		Fish oils	
	NB_6	DB_6	NB_6	DB^6
Vitamin E *(μg/mg lipid)*	0.44 ± 0.05^a	0.47 ± 0.05^a	0.30 ± 0.03^b	0.27 ± 0.03^b
TBARS *(nmol MDA/mg protein)*	17.25 ± 1.89^a	18.15 ± 0.78^a	20.00 ± 2.05^b	22.15 ± 2.15^c
GluPx *(nmol NADPH/mg protein · min)*	84.92 ± 4.72^a	128.31 ± 5.07^b	183.15 ± 18.78^c	197.23 ± 19.26^c
GluRed *(nmol NADPH/mg protein·min)*	108.66 ± 13.88^a	112.93 ± 14.64^a	106.69 ± 14.07^a	98.80 ± 12.19^a
GSH/GSSG	3.16 ± 0.45^a	2.89 ± 0.32^a	2.28 ± 0.32^b	1.92 ± 0.26^c

Data are means ± SD of eight rats. Significant differences ($P < 0.05$) were determined by Student-Newman-Keuls test after ANOVA. Numbers with different superscript letters are significantly different.

GSH/GSSG ratio decreased in rats fed fish oil, particularly in the vitamin B_6 deficient group.

5. Discussion

Besides the reported usefulness of fish oil supplementation on some renal pathologies, our data lead us to consider carefully its influence on kidney fatty acid composition and possible effects on lipid peroxidation, especially in the presence of other promoting factors, such as vitamin B_6 deficiency.

We previously reported [20] the risk of underestimating dietary conditions inducing higher susceptibility to peroxidation, such as vitamin B_6 deficiency and the presence of fish oil in diet. Moreover, we confirmed the fact that protection against lipid peroxidation differs from tissue to tissue, e.g., in liver, vitamin E action and increased glutathione peroxidase activity seem to protect this organ from oxidative stress, whereas in heart this defense mechanism appears to be less efficient.

In this work, we considered the effects of diets with a marginal content of vitamin B_6 and rich in fish oil, in order to verify in particular the behavior of rat kidney towards factors inducing peroxidation. The kidneys of rats fed vitamin B_6 deficient diets were more swollen than those of normal vitamin rats. Although these morphologic alterations were not further investigated in this framework, they are similar to those found in similar dietary conditions. The B_6 deficiency, in fact, induces tubular-interstitial nephritis, characterized by tubular atrophy, interstitial fibrosis and chronic inflammatory infiltration [21].

Different lipid quality did not cause variations in the relative weight and macroscopic aspect of kidney, but its effects on the final weight of animals were confirmed, since rats fed fish oil had lower weights. Since we didn't observe significant differences in food consumption, the lower growth could be attributed to a lower feed efficiency ratio. The deleterious effects of fish oil diet on renal function, reported by Logan *et al.* [7], may enhance the effect of dietary deficiency of vitamin B_6 as demonstrated by kidney PLP levels, which were significantly lower than those of the groups fed vegetable oil. The fatty acid composition of the different diets produced effects on renal phospholipids which were interlaced with those of vitamin B_6 deficiency, often in synergy. With regard to fatty acid metabolism, the processes of elongation and desaturation of linoleic acid for synthesizing arachidonic acid, by both LCPUFA n3 of fish oil and vitamin B_6 deficiency turned out to be slowed down: the synergic effect was revealed by the lowest amounts of arachidonic acid in rats fed fish oil and a vitamin B_6 deficient diet.

The different percentages of PUFA n6 and n3 in the kidney of rats fed vegetable or fish oil also produced differences in the unsaturation index, but TBARS production did not follow the same trend. According to Kubo *et al.* [22], this emphasizes the fact that the unsaturation index and lipid peroxidation are not necessarily correlated.

Other parameters involved in peroxidative risk and defense mechanisms also highlighted differences among the experimental groups, referable partly to dietary lipids and partly to vitamin deficiency. The contemporaneous presence in the diet of low vitamin B_6 contents and fish oil emphasizes the susceptibility to peroxidation and consequent defense attempts made by kidney. In this group, in fact, vitamin E contents decreased, whereas the activity of glutathione peroxidase, an enzyme involved in cellular defense against peroxidation, increased. Instead, the activity of glutathione reductase, an enzyme which should restore the antioxidizing defenses of the cell, did not increase, with consequent low GSH/GSSG ratios and insufficient protection against peroxidative damage.

In agreement with those of other authors [22, 23], our data show that the peroxidative risk is not only linked to the unsaturation index of tissue lipids, but also to the efficiency of defense systems. An inadequate capacity for defense towards many dietary factors inducing high susceptibility to peroxidation may also compromise organ functionality. On this subject, we intend to carry out further histopathological and ultrastructural research, in order to evaluate possible

parenchymal modifications responsible for functional alterations.

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