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Docosahexaenoic acid supplementation induces dose and time dependent oxidative changes in C6 glioma cells

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Abstract

In view of the promising use of n-3 polyunsaturated fatty acids (PUFAs) in the prevention and treatment of neurological diseases, it is necessary to ascertain the lack of detrimental oxidative effects. We evaluated short- and long-term effects of 25, 50 and 75 μM docosahexaenoic acid (DHA) supplementation on the oxidative status of C6 glial cells. DHA was incorporated into cells dose and time dependently without any cytotoxic effect. Reactive oxygen species (ROS) level was related to DHA dose and supplementation time. At the lowest dose no significant increase in ROS values was observed at hour 24. Low doses of DHA strengthened the cellular antioxidant defence system as highlighted by a raise in both GPX and catalase activity, and the decreased levels of lipid peroxidation. This effect was pronounced at 24 h of supplementation, almost disappeared at hour 48, while after 72 h an opposite effect was observed: lipid peroxidation increased concomitantly with DHA doses. Therefore, the final effect of DHA on cellular redox status is dependent on dose and time supplementation.

Keywords: *Docosahexaenoic acid, reactive oxygen species, thiobarbituric acid-reactive substances, catalase, glutathione peroxidase, glioma cell*

Abbreviations: *CAT, catalase; DCF, 2′-7′-dichlorofluorescein; DHA, docosahexaenoic acid; G6PDH, glucose-6-phosphate dehydrogenase; GPX, glutathione peroxidase; GSH, reduced glutathione; LPO, lipid peroxides; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PLA_2, phospholipase A_2; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARs, thiobarbituric acid-reactive substances*

Introduction

Over the last years interest has been growing in the dietary use of the n-3 polyunsaturated-fatty acids (PUFAs) found in fish oils because of their beneficial effects on health. Dietary supplementation with n-3 PUFAs, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is associated with a decrease in the incidence of cardiovascular disease [1]. Several human studies [2–4] have recently reported the positive effect of n-3 PUFA supplementation against various brain dysfunctions such as epileptic seizure, depression or bipolar diseases. Furthermore, it has been also observed a beneficial effect of n-3 PUFA in the prevention of ischemic damage in rat brain [5–6]. Despite the prospect that n-3 PUFAs may be part of interesting therapeutic approach for the prevention and treatment of some neurological diseases, there is the potential that EPA and DHA intake may increase the susceptibility of membranes

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to form lipid peroxide because of the multiplicity of their double bonds [7]. Moreover, brain PUFAs are particularly sensitive to oxidation since the brain has a very active aerobic metabolism, but relatively poor antioxidant defences [8].

To date, contradictory results have been obtained about the effect of the intake of highly unsaturated fatty acids on the oxidative state of brain. Song et al. [9] demonstrated that the incorporation of high doses of n-3 fatty acids in rat tissues through the ingestion of DHA containing oil, increased tissue susceptibility to free radical attack and decreased the antioxidant α-tocopherol content.

On the other hand, several studies [10–11] suggest that increased levels of DHA in the brain might confer protection against oxidative stress. DHA dietary supplementation to rats, for example, resulted in increased DHA levels in some areas of the brain such as the cerebrum and, concomitantly, decreased levels of lipid peroxides (LPO) in those same areas; in the other brain regions such as cerebellum and brain stem, where DHA levels remained unchanged, no decrease in LPO was detected [12]. The antioxidant effect of DHA has been also reported in foetal rat brain, where the intraamniotic administration of ethyl-DHA decreases the production of LPO in the brain of near-term foetuses [13–14]. Recently [15], a decrease in LPO and reactive oxygen species (ROS) was also observed in both the cerebral cortex synaptosomes and in the cortex whole homogenate of adult rats fed DHA for 12 weeks.

Experimental data from human studies are even more controversial. For example, lipid peroxidation is not enhanced in pregnant women who received daily supply of DHA and EPA [16]. Mononuclear cells from human peripheral blood enriched with DHA are less susceptible to hydrogen peroxide-induced lipid peroxidation compared to controls [17].

In other experiments, however, feeding humans n-3 PUFA results in increased lipid peroxide production in plasma [18] and enhanced *ex vivo* oxidation of low density lipoproteins [19].

In vitro studies have also reported conflicting results. Supplements of DHA prevented human retinal pigment epithelial cells from oxidative-stress-induced apoptosis [20], whereas DHA-enriched OLN 93 cells were more susceptible to oxidative stress than naïve OLN 93 cells [21]. However, the diverse responses to DHA can also be related to the varying antioxidant capacity of different brain areas [22].

Our previous data [23] suggest adverse effects of DHA on lipid peroxidation in C6 glioma cells. In fact, we found that *in vitro* supplementation of membrane lipids with large amounts of DHA affects cellular redox status in view of the significant increase in ROS and thiobarbituric-acid-reactive substance (TBARS) levels. This modification in the cellular oxidative status was also highlighted by a significant increase in catalase (CAT) activity and a decrease in glutathione (GSH) content and glutathione peroxidase (GPX) activity.

The discrepancies observed in the literature could be attributed to the experimental protocols and DHA dosage. Previous studies [24–25] have shown that the treatment of human subjects with n-3 PUFA at low doses and without significant addition of antioxidants did not produce any detrimental effect, whereas high doses resulted in harmful modifications of the oxidative metabolism, such as an increase in red blood cell susceptibility to lipid peroxidation. It seems then that DHA may alter the cell redox status positively or negatively, depending on the concentration used.

This led us to investigate *in vitro* the dose-dependent effect of DHA on the redox status of glial cells to verify whether DHA differently affects the cellular oxidative balance at low and high concentrations. C6 cells have been used as an *in vitro* model for the study of glial cells properties because they retain some functional and morphological properties of astrocytes and oligodendrocytes and for their low PUFA content [26]. This relative PUFA deficiency allowed us to manipulate their long-chain fatty acid content by exogenous supplementation in order to study the effect of DHA on cellular oxidative balance.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), phosphate buffer saline (PBS), trypsin and Triton were purchased from Gibco-Invitrogen Ltd. (Paisley, UK). Metanolic HCl was obtained from Supelco (Bellefonte, PA, USA). Isopropanol, methanol, chloroform, hexane, cytocrome *c* were purchased from Carlo Erba (Italy). 2′,7′-dichloroflluorescin-diacetate (DCFH-DA) was provided by Molecular Probes (Eugene, OR, USA). Docosahexaenate, diethylentriaminopentacetic acid (DTPA), superoxide dismutase (SOD) were purchased from Sigma (St Louis, MO, USA). Fatty acid was dissolved in DMEM and stored in nitrogen at −80°C until use. Pyruvic acid sodium salt, β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), β-nicotinamide adenine dinucleotide phosphate sodium salt ($NADP^+$) and β-D-glucose 6-phosphate sodium salt were purchased from Fluka (Buchs, Switzerland). All other reagents utilised were of the highest grade available.

Cell culture and treatment

Rat C6 glioblastoma cells was obtained from American Type Culture Collection (ATCC) and used between passages 5–20. C6 cells were grown to confluence in DMEM supplemented with 10% heat-inactivated FBS, penicillin (50 U/ml), streptomycin

(150 μg/ml) and fungizone (100 ng/ml) at 37°C in a humidified atmosphere of 5% CO_2.

Upon reaching confluence, C6 cells were plated at a density of 2.5×10^5/ml in a 6-well plate and the following day the cultures were incubated in growth medium supplemented with a sodium salt solution of DHA (10 mM) to 25, 50 and 75 μM final concentration for 24, 48 and 72 h at 37°C. Controls were treated similarly, but fatty acid was omitted.

Cell viability assay

In the beginning of this study, non-cytotoxic dose range of DHA was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, using a diagnostic kit purchased from Sigma (No.CGD1). This method is based on the ability of live cell mitochondria to reduce MTT to formazan, a product that is suitable for optical density measurement [27]. MTT (0.5 mg/ml final concentration) was added to each well and incubated for 4 h at 37°C. The medium was then removed and the formazan was dissolved under agitation for 5 min at room temperature in acidic isopropanol (0.04 N HCl in absolute isopropanol). The amount of product formed was monitored in duplicates, by absorbance measurement at 570 nm, with background subtraction at 630 nm. The results are expressed as absorbance of MTT reduction.

Lipid extraction and analysis

Cells were washed twice in DMEM and scraped into PBS. Lipids were extracted from cells by isopropanol. After centrifugation, lipids were dried under a nitrogen stream, and re-dissolved in 1 ml of chloroform:methanol (2:1 v/v). Samples were transmethylated by methanolic HCl overnight at 70°C. The fatty acids methyl esters were analysed by a gas-chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a fused silica capillary column of Supelcowax 30 m × 0.53 mm i.d. (Supelco) [28]. Peaks were identified by a commercial standard mixture (Supelco). Results are expressed as means ± SE over a minimum of three samples. The proportions of the total fatty acids were normalized to a value of 100%. The unsaturation index was calculated as the sum of the percentages of individual fatty acids per number of double bonds.

Fluorescence-activated cell sorting (FACS) analysis

The level of ROS was determined in the control and fatty acid-treated cells by labelling with cell-permeable 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) as previously described [29]. Once diffused into the cells, DCFH-DA is hydrolysed to cell-impermeable non-fluorescent 2′,7′-dichlorofluorescin (DCFH) and trapped within the cells. DCFH is oxidized by intracellular oxidants to highly fluorescent 2′,7′-dichlorofluorescein (DCF), providing a quantitative determination of the intracellular level of oxidant species [30].

In brief, the cells were incubated with 3 μM DCFH-DA (Molecular Probes) for 20 min at 37°C, then pelletted by centrifugation and washed with PBS. The cells were resuspended in PBS, pH 7.2, containing iodide propidium to exclude dead cells, and then kept on ice before measuring fluorescence levels with Epics Elite ESP Flow-Cytometer (Beckman Coulter, Miami, FL, USA). The DCF fluorescence levels were always normalized to C6 cells autofluorescence.

Thiobarbituric acid assay

Lipid peroxidation was detected by the thiobarbituric acid assay as previously described [31]. The method measures the production of malondialdehyde or other related substances, i.e. TBARS. This method was a first global measure of peroxidation and was applied because of its sensitivity and simplicity [32].

After being exposed to fatty acid solution, containing undetectable amount of TBARS, the cells were scraped and washed with PBS. Then 0.375% 2-thiobarbituric acid and 15% trichloracetic acid were added. The tubes were placed in a water bath and kept at 95°C for 45 min. After cooling, the colour developed was read in a spectrophotometer (Beckman Coulter) at 535 nm. The concentration was calculated using an extinction coefficient of $1.56 \times 10^5 M^{-1} cm^{-1}$ and expressed as nmol TBARS/mg protein. In addition, a calibration curve was established with malondialdehyde produced by hydrolysis of 1,1,3,3-tetramethoxypropane in 0.1 M HCl as standard.

Superoxide assay

Reduction of ferricytochrome *c* to ferrocytochrome *c*, with and without the addition of SOD (10 μg/ml), was used to measure the $O_2^{\cdot -}$ formation rate. Cells were washed three times with PBS containing DTPA 0.1 mM, and then 1 ml of DTPA containing 10 μM of cytochrome c was added. The reaction was monitored spectrophotometrically (Beckman Coulter) at 550 nm, using the extinction coefficient of $2.1 \times 10^4 M^{-1} cm^{-1}$, which corresponds to ΔE between ferricytochrome *c* and ferrocytochrome *c* [33].

Glutathione assay

Reduced glutathione (GSH) content was measured in cell lysate by the GSH-400 kit (Bioxytech, Oxis International, Portland, OR, USA). This method is based on a chemical reaction that involves the formation of a chromophoric thione, which has the maximum absorbance at 400 nm.

Enzymatic assay

Cells were harvested with a rubber policeman into the medium, and spun at 1200*g* in Eppendorf tubes for 10 min at 4°C. Cells were washed once with PBS and then resuspended in a solution containing 50 mM Na-phosphate and 0.5% (v/v) Triton X-100 at pH 7.5, and sonicated for 15-s bursts. Cells were then centrifugated at 15,000*g* × 10 min and the supernatant collected for enzymatic assays.

CAT activity was measured by monitoring the disappearance of hydrogen peroxide at 240 nm, following the procedure described by Aebi [34]. Catalase activity was expressed as micromoles of H_2O_2 consumed min^{-1} mg $protein^{-1}$.

GPX activity was measured with a commercial kit (Oxis International). Absorbance at 340 nm was recorded in a spectrophotometer for 5 min. One unit of enzyme was defined as nanomoles of NADPH oxidized min^{-1} mg $protein^{-1}$.

G6PDH activity was determined, as already described [35], by measuring the rate of production of micromoles of NADPH min^{-1} mg $protein^{-1}$.

Protein determination

Protein levels were measured by Bradford's method [36] with a commercial kit (BioRad Laboratories, Hercules, CA, USA), using a bovine serum albumin standard.

Statistical analysis

Results are expressed as mean ± SE. Group means were compared by one-factor ANOVA. Upon significant interactions, differences between group means were analyzed by *post hoc* testing using Student's test. Differences were considered statistically significant at $p < 0.05$.

Results

DHA incorporation

To study the incorporation of DHA, the fatty acid pattern of glial cells was analysed at different time points upon incubation with 25, 50 and 75 μM of DHA. As shown in Table I glioma cells significantly incorporated DHA from medium and its increase was dose-dependent reaching the peak at the highest DHA supplementation. The decrease of DHA percentage after 72 h of supplementation can be due to its active chain shortening and retro-conversion as indicated by a significant increase in EPA ($p < 0.0001$ vs. control).

The increase of n-3 PUFA was accompanied by a decrease of n-6 PUFA particularly of C20:4 n-6 that it is significant ($p < 0.05$) compared to controls both at hours 48 and 72 of DHA supplementation.

The exogenous DHA appears to have replaced mainly the monoenes fatty acids whose decrease was dose-dependent, while the saturated fatty acid content remained largely unchanged (Figure 1). As consequence of fatty acid modification a significant dose-dependent increase in the unsaturation index was induced by DHA treatment (Figure 1).

Cell viability

The results from MTT assay illustrated in Table II, demonstrated that DHA has non-cytotoxic effect on cells. The cell viability in treated C6 cells was not different from controls at any time and DHA concentration.

Table I. PUFAs pattern of C6 glioma cells without DHA exposure (control) and with 25, 50 and 75 μM DHA supplementation at different time points.

Fatty acid composition (%) and time incubation	Control	25 μM	50 μM	75 μM	*p*-value
20:4n-6					
24 h	6.54 ± 0.39	6.34 ± 0.36	5.85 ± 1.03	5.28 ± 0.68	0.49
48 h	6.27 ± 0.46	4.69 ± 0.49	4.3 ± 0.6	4.06 ± 0.62	0.03
72 h	4.60 ± 0.37	3.76 ± 0.22	3.52 ± 0.13	3.3 ± 0.21	0.02
20:5n-3					
24 h	1.55 ± 0.06	2.47 ± 0.30	2.82 ± 0.52	2.78 ± 0.17	0.02
48 h	1.59 ± 0.07	3.23 ± 0.51	4.87 ± 0.10	4.91 ± 0.14	5.6^{-8}
72 h	1.37 ± 0.26	3.3 ± 0.26	4.64 ± 0.22	4.30 ± 0.15	1.02^{-5}
22:5n-3					
24 h	3.60 ± 0.19	2.98 ± 0.15	3.35 ± 0.67	2.75 ± 0.27	0.32
48 h	4.05 ± 0.29	4.37 ± 0.21	4.14 ± 0.23	4.02 ± 0.25	0.78
72 h	3.63 ± 0.49	2.87 ± 0.70	3.31 ± 0.54	3.84 ± 0.73	0.72
22:6n-3					
24 h	5.52 ± 0.46	11.85 ± 0.34	18.49 ± 2.18	22.03 ± 1.93	3.79^{-5}
48 h	6.01 ± 0.37	17.42 ± 1.38	23.39 ± 2.08	27.34 ± 2.46	1.27^{-7}
72 h	4.20 ± 0.64	11.04 ± 1.56	15.79 ± 1.50	19.64 ± 3.15	0.001

Data (percentage of total fatty acids) are expressed as mean ± SE of three separated experiments performed in triplicate. Significance of difference between treatment and control was determined by ONE—factor ANOVA.

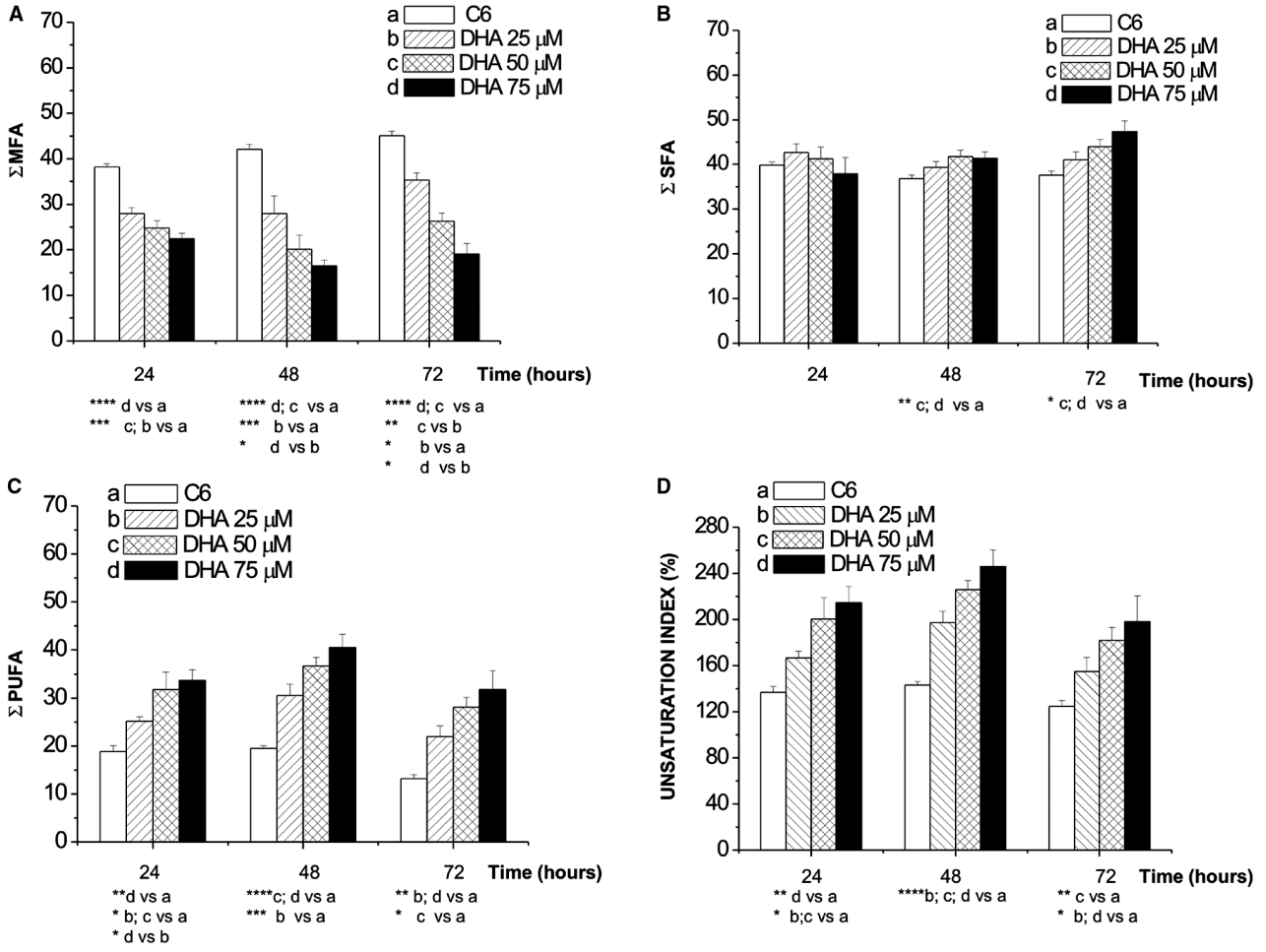

Figure 1. Fatty acids pattern (%) of C6 glioma cells untreated (control) and supplemented with DHA 25 μM (b), DHA 50 μM (c) and DHA 75 μM (d) at different time points. SFA: saturatedfatty acids; MFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; UI: unsaturation index. The values represent the mean ± SE of three independent experiments. Statistical analysis was performed by one factor ANOVA (SFA 24 h $p = 0.84$; SFA 48 h $p < 0.05$; SFA 72 h $p < 0.05$; MFA 24 h,48 h and 82 h $p < 0.0001$; PUFA 24 h $p < 0.01$; PUFA 48 h $p < 0.0001$; PUFA 72 h $p < 0.01$; UI 24 h $p < 0.01$; UI 48 h $p < 0.0001$; UI 72 h $p < 0.01$) followed with *post hoc* testing using Student's test ($p < 0.05$). ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

Effect of different concentration of DHA on cellular oxidative stress

In order to test the possible involvement of different DHA concentrations in the modification of the redox state, we determined the intracellular level of oxidant species by DCF fluorescence. The results shown in Figure 2 indicate that the production of ROS after 24 h of DHA incubation at a concentration of 25 μM was similar to controls whereas 50 and 75 μM DHA supplementation caused only a slight, but significant increase in the oxidation of 2′,7′-dichlorofluorescin (DCFH) to 2′,7′-dichlorofluorescein (DCF) as compared with untreated C6 cells. Increasing the time of DHA exposure in the medium culture, the ROS levels enhanced at any concentration used even though their production was particularly elevated after long-term supplementation of 50 and 75 μM DHA.

The possible involvement of different DHA concentrations in LPO production was investigated by measuring the intracellular TBARS content. Interestingly, C6 cells treated with DHA showed a significant decrease in TBARS levels compared with controls after 24 h incubation (Figure 3) at all concentrations used. After 48 h of DHA supplementation, the TBARS levels nearly reached the values

Table II. Effects of DHA on C6 glioma cells viability.

Time (h)	Control	25 μM	50 μM	75 μM
24	0.29 ± 0.04	0.29 ± 0.04	0.28 ± 0.03	0.29 ± 0.04
48	0.45 ± 0.08	0.45 ± 0.07	0.48 ± 0.08	0.46 ± 0.07
72	1.10 ± 0.22	1.09 ± 0.20	1.06 ± 0.15	1.10 ± 0.22

Cells were incubated without (control) and with DHA 25, 50 μM and DHA 75 μM at different time points. Viability was determined as A_{570} by colorimetric MTT assay. The values represent the mean ± SE of three independent experiments. Statistical analysis was performed by one factor ANOVA (MTT 24 h $p = 0.999$; MTT 48 h $p = 0.993$; MTT 72 h = 0.999) followed with *post hoc* testing using Student's test ($p < 0.05$).

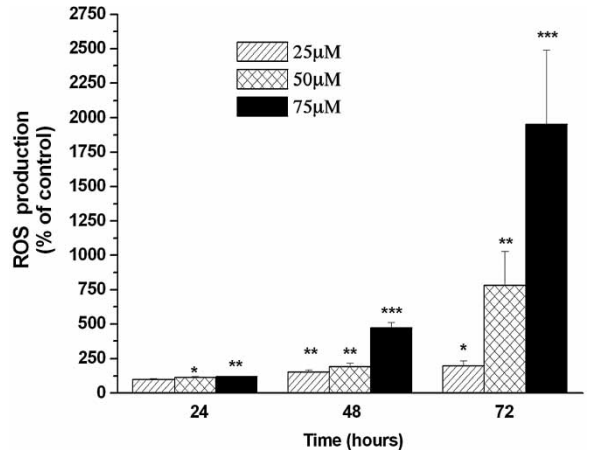

Figure 2. Intracellular oxidant species production of C6 glioma cells with 25, 50 and 75 μM DHA supplementation at different time points. ROS were determined by FACS analysis using 2′,7′-dichlorofluorescein diacetate (DCFH-DA). The values, expressed as percentage of the levels detected in controls, represent the mean ± SE of three independent experiments. Statistical analysis was performed by one factor ANOVA (ROS 24 h $p = 0.009$; ROS 48 h $p < 0.0001$; ROS 72 h $p < 0.0001$) followed with *post hoc* testing using Student's test ($p < 0.05$). $*p < 0.05$; $**p < 0.01$; $***p < 0.0001$.

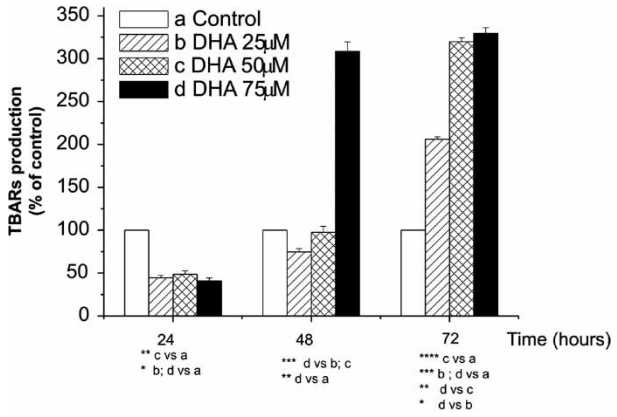

Figure 3. TBARs production in C6 glioma cells in absence (control) and in presence of DHA 25 μM, DHA 50 μM and DHA 75 μM at different time points. TBARs were measured as nmol/mg protein. The values, expressed as percentage of the levels detected in controls, represent the mean ± SE of three independent experiments. Statistical analysis was performed by one factor ANOVA (TBARs 24 h $p = 0.0079$; TBARs 48 h $p < 0.0001$; TBARs 72 h $p < 0.0001$) followed with *post hoc* testing using Student's test ($p < 0.05$). $****p < 0.0001$; $***p < 0.001$; $**p < 0.01$; $*p < 0.05$.

of controls at the lowest doses whereas they significantly increased at the highest dose. At long term incubation the TBARS levels increased significantly in DHA treated cells for all the concentrations tested compared with untreated cells and the increases were dose-dependent.

The superoxide release was similar to controls in C6 cells treated with different DHA concentrations for 24 h (Control 0.37 ± 0.04 vs. DHA 25 μM 0.35 ± 0.06; DHA 50 μM 0.33 ± 0.01; DHA 75 μM 0.32 ± 0.01).

Effects of different DHA concentrations on antioxidant defence mechanisms

As reported in Table III the supplementation of the cell culture with different DHA concentrations for 24 h did not affected the levels of intracellular reduced glutathione.

Conversely, the CAT activity significantly increased after 24 h of DHA supplementation reaching a plateau at 50 μM (Table III). After 48 h of DHA treatment the percentage increase of CAT activity was similar to that observed after short time incubation (control 12.01 ± 1,26 vs. DHA 25 μM 15.75 ± 0.41; DHA 50 μM 18.45 ± 0.80; DHA 75 μM 18.70 ± 0.97).

The inducible activity of G6PDH, the rate-limiting step in the pentose phosphate pathway, was examined in C6 supplemented with different DHA concentrations for 24 h. As illustrated in Table III not significant changes were observed in the activity at all concentrations used compared to controls.

The GPX activity was markedly affected by DHA treatment (Figure 4); at hour 24, the activity was significantly higher than control at the lowest DHA doses, whereas at 75 μM DHA the levels of GPX activity were similar to those of control. After 48 h incubation, at the lowest DHA dose the increase of GPX activity was still significant compared with untreated cells whereas at 50 μM the enzymatic activity returned at control values and decreased at the highest dose.

Discussion

In the present study, C6 cells were used both for their functional and morphological properties similar to glial cells [37–39] and for their low PUFAs content

Table III. GSH, GPX, catalase and G6PDH activities in C6 glioma cells treated with different concentration after 24 h.

	Control	DHA 25 μM	DHA 50 μM	DHA 75 μM	*p*-value
GSH (nmoli /mg protein)	1.91 ± 0.28	1,95 ± 0.26	1,95 ± 0.33	1,93 ± 0.22	0.99
Catalase (μmoli/min/mg protein)	11.75 ± 0.37[a;d]	15.98 ± 0.33[b;a]	18.28 ± 0.97[g]	18.33 ± 0.83[g;b]	<0.0001
G6PDH (μmoli NADPH/min/ mg protein	22.84 ± 1.89	24.35 ± 1.42	24.70 ± 1.15	23.39 ± 1.54	0.82

Data are expressed as mean ± SE of three separated experiments performed in triplicate. Significance of difference between treatment and control was determined by ONE -factor ANOVA with *post hoc* testing using Student's test ($p < 0.05$). Values with different superscript are significantly different[a] vs. [b] *p*, 0.05; [d] vs. [g] *p*, 0.01.

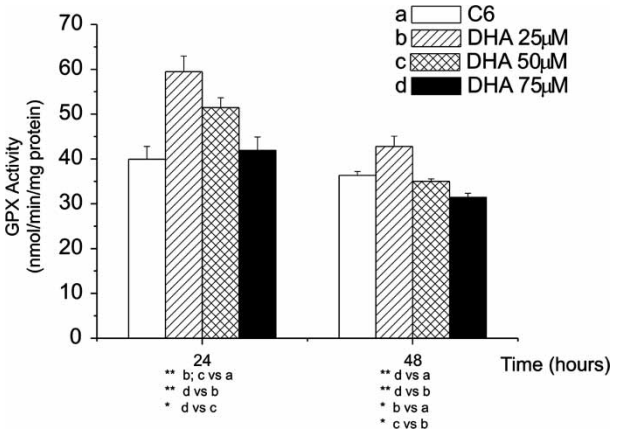

Figure 4. GPX activity in C6 glioma cells in absence (control) and in presence of DHA 25 μM, DHA 50 μM and DHA 75 μM at 24 and 48 h. GPX activity was measured as nmol/min/mg protein. Data represent the mean ± SE of three independent experiments. Statistical analysis was performed by one factor ANOVA (GPX 24 and 48 h $p < 0.0001$) followed with *post hoc* testing using Student's test ($p < 0.05$). $**p < 0.01$; $*p < 0.05$.

[26]. This relative PUFA deficiency allowed us to manipulate their DHA content by exogenous supplementation, and study the influence of different concentrations of DHA on oxidative balance.

DHA was efficiently incorporated in C6 cells and no cytotoxic effect was observed at the different DHA concentrations used in both the short and long-term treatments. On the other hand, in spite of the fact that DHA enrichment of the membrane was accompanied by a decrease in arachidonic acid (AA), AA levels did not reach values so low to be incompatible with survival, as observed in our previous study [23].

Our results have shown that the cellular redox status is differently affected by varying the concentration and time of exposure to DHA in the medium culture.

After a short incubation time, the lowest DHA dose did not affect the cellular redox status, as evaluated by ROS and superoxide production. Instead, after a short incubation period TBARs production decreased at all DHA concentrations, but increased after 48 h only at the highest DHA dose, and this increase occurred at all concentrations in the long-term treatment.

The protective effect observed after a short time could be explained by an increase in antioxidant enzyme activities such as CAT and GPX, which act as scavengers of hydrogen and LPO. Interestingly, the maximum increase in GPX activity was observed at the lowest DHA concentration, then the activity decreased to reach the control value at the highest DHA concentration. Since the glutathione levels were not affected by DHA, we can hypothesize that C22:6 ω-3 directly regulates the GPX level by modulating its gene expression. Although GPX mRNA was not determined in our study, a close correlation between activity and transcription has been reported for antioxidant enzymes including GPX [40]. On the other hand, an *in vivo* study [41] has shown that n-3 fatty acids up-regulate gene expression of antioxidant enzymes and down-regulate genes associated with the production of ROS. Recently [42], an enhanced gene expression of GPX and CAT was also observed in cardiomyocytes isolated from rats fed a diet rich in fish oil. Several mechanisms probably contribute to the increased gene expression of antioxidant enzymes after n-3 PUFA supplementation. The direct effects of n-3 PUFAs on gene expression are likely mediated by their ability to regulate the activity or abundance of transcription factors [43]. For example, DHA has been shown to activate the retinoic X receptor [44].

The findings that GPX activity exhibited an inverse dose-dependent regulation, whereas CAT activity increased concomitantly with the increase in DHA concentration, reaching a plateau at 50 μM already, support the idea that GPX is the enzyme mostly involved in the oxidative defences of glioma cells. The decrease in GPX activity would be accompanied by increased levels of oxidative stress. Both GPX and catalase are involved in the decomposition of hydrogen peroxide, catalase showing a very high Km value, while GPX a relatively low Km value [45]. It is believed that in intact cells GPX is more effective at relatively low hydrogen peroxide concentration, while catalase is more effective at high concentrations, as exemplified by the studies on erytrocytes [46–48] and hepatocytes [49]. In our previous paper [23], we demonstrated that 100 μM DHA significantly decreased GPX activity compared with controls, and this decrease was accompanied by high ROS and TBARS production, even though CAT activity increased. In this study the increase in TBARS levels after 48 h supplementation of the highest DHA dose could be due to the partial inactivation of GPX by enhanced ROS production. In this regard, GPX has been reported to undergo inactivation by nitric oxide, peroxynitrite, superoxide, hydroxyl radical, hypochlorous acid, carbonyl substances, and more generally, under various conditions of nitroxidative and glycoxidative stress [50–52]. GPX inactivation would result in the modification of cellular redox regulation and functions.

The role of DHA in inducing an antioxidative defence against active oxygen by enhancing the cerebral activity of CAT and GPX has been suggested by Hossain et al.[53]. In the cerebrum of rats fed a high cholesterol diet plus DHA, CAT and GPX activities increased with an increase in the DHA/AA ratio.

In our study, no changes in G6PDH activity were observed at hour 24 in C6 cells supplemented with different DHA concentrations. This pathway is an important source of NADPH, which plays a crucial role in the regulation of the intracellular redox state [54–55]. This result confirms the balance of the intracellular redox state under short-term supplementation since there is no request of extra reducing power.

Other potential mechanisms accounting for the decrease in lipid peroxidation observed under short term supplementation may be related to the inhibition by DHA of phospholipase A_2. An increase in the phospholipase A_2 (PLA_2, EC 3.1.1.4) activity accelerates the production of free AA and enhances the lipoxygenase and cyclooxygenase pathways which belong in the AA cascade system. Free radicals may be produced during this metabolic process that can then reactivate PLA_2. In our study DHA uptake in C6 cells is accompanied by a significant increase in EPA, whose role in the inhibition of PLA_2 is well known. [56,57]. However, this pathway would not be that important in the cellular defence against oxidative stress in long-term supplementation. In fact, even though the percentage of EPA increased with time of DHA supplementation, the redox state, which improved at hour 24, is impaired in C6 cells at hours 48 and 72 of DHA incubation. The different effect of varying DHA concentrations on oxidative state can be also explained by the fact that the fatty acid can be incorporated in different lipid classes. It has been shown [58] that at low concentrations DHA was only incorporated in PE plasmalogens whereas a high concentration of DHA was incorporated mainly in diacyl-GPE and PC. Previous studies have suggested that plasmalogens can be considered as antioxidant molecules [59], probably as a result of their vinyl ether structure [60]

Our study indicates that in C6 glioma cells DHA supplementation induces oxidative changes that are dose- and time- dependent. The balance between the oxidative stress and the antioxidant capacities of cells can minimize the oxidative perturbations caused by DHA challenge. In short-term supplementation, the slightly increased production of ROS was controlled to some extent by the induction of the antioxidant system. In long-term supplementation the oxidative stress increases with DHA concentration and cellular antioxidant capacity decreases, partially because of GPX inactivation, and then lipid peroxidation occurs. In conclusion, both time and dose of the supplement must be taken into account when using DHA as a therapeutic molecule in neurological diseases.

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