



Induced oxidative stress and cell death in the A549 lung adenocarcinoma cell line by ionizing radiation is enhanced by supplementation with docosahexaenoic acid

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Title:

Induced oxidative stress and cell death in the A549 lung adenocarcinoma cell line by ionizing radiation is enhanced by supplementation with docosahexaenoic acid

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Abstract:

Both ionizing radiation and docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid (PUFA), have been shown to inhibit tumor cell growth at least in part by increasing oxidative stress. In the current study, the effects of ionizing radiation, DHA, or a combination of the two on cell proliferation, anchorage-independent growth, apoptosis and lipid peroxidation in A549 lung adenocarcinoma cells were examined. In the current study, significant decreases in cell proliferation and colony formation were noted for ionizing radiation or DHA treatments, while a combination of the two showed significant reductions over either treatment alone. Conversely, lipid peroxidation and apoptotic cell death showed significant increases with ionizing radiation and DHA treatments, while cells receiving both treatments demonstrated further significant increases. Moreover, addition of vitamin E, an antioxidant, was able to

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3 completely reverse lipid peroxidation and cell death due to ionizing radiation and partially reverse these
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5 changes in DHA treatments. Finally, the preferential incorporation of DHA into lung and xenograft
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7 compared to liver tissue is demonstrated in an *in vivo* model. These findings confirm the potential of
8
9 DHA supplementation to enhance the treatment of lung cancer using ionizing radiation by increasing
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11 oxidative stress and enhancing tumor cell death.
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14 15 16 17 18 19 **Introduction:**

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22 The standard of treatment for the most aggressive forms of lung cancer, the foremost cause of
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24 cancer-related death worldwide, involves radiation therapy [1]. While radiation treatment is
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26 widespread in the management of cancers and new delivery systems and technologies are constantly
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28 being developed to enhance its efficacy and reduce damage to surrounding tissues [2], effectively
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30 overcoming radioresistance in tumor cells remains a problem [3].
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34 It is well established that ionizing radiation induces oxidative stress to facilitate cell killing [4].
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36 The same mechanism has been postulated to account for some of the anti-cancer properties of omega-3
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38 polyunsaturated fatty acids (PUFAs) [5]. Indeed, omega-3 PUFAs have also been shown to increase the
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40 effectiveness of drugs that work through the generation of reactive oxygen species (ROS) in models of
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42 mammary cancer [6,7]. Of particular interest is docosahexaenoic acid (DHA), a 22 carbon omega-3 PUFA
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44 with 6 *cis* double bonds that create a number of methylene carbon bridges that are highly susceptible to
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46 oxidative stress [8]. Furthermore, addition of antioxidants has been shown to reverse the enhanced
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48 response of DHA-supplemented cancer cells to ROS-generating compounds [7,9], including an *in vivo*
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50 study on lung adenocarcinoma cells showing vitamin E was capable of counteracting omega-3 PUFA-
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52 induced growth inhibition [10].
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3 While two previous studies did not find any significant inhibition on breast cancer xenograft or
4 leukemia growth when combining radiation with an omega-3 PUFA diet over the omega-3 PUFA
5 supplementation alone [11,12], other studies have shown combination treatment with ionizing radiation
6 and omega-3 PUFAs results in enhanced cancer cell death [13-15]. Interestingly, omega-3 PUFAs have
7 also been associated with protective effects on normal tissues during ionizing radiation treatments
8 [16,17].

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11 In the current study, the effects of combination treatment with DHA and ionizing radiation on
12 cell proliferation, anchorage-independent cell growth, lipid peroxidation, and apoptosis in the A549 lung
13 adenocarcinoma cell line was examined. Significant decreases were noted in cell proliferation and soft
14 agar growth *in vitro*, which corresponded to increased in lipid peroxidation and apoptosis. These effects
15 were partially reversed by the addition of Vitamin E treatments. Moreover, concentrations of DHA that
16 did not show significant levels of toxicity on their own were able to enhance radiosensitivity. Finally,
17 omega-3 PUFAs are shown to have a greater than 2-fold increase in incorporation into xenograft tissue
18 compared to control liver tissues in an *in vivo* model of lung cancer, providing a potential explanation
19 for how omega-3 PUFAs might enhance tumor response to radiation while alleviating adverse effects in
20 normal tissue.

21 22 23 **Materials & Methods:**

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26 ***Cell Lines & Reagents-*** A549 lung adenocarcinoma cells were purchased from ATCC (Manasses,
27 VA). Fatty acid methyl esters (FAME) and Vitamin E (Sigma, St. Louis, MO) were dissolved in ethanol
28 (EtOH), flushed with nitrogen gas, protected from light and stored at -20°C for no more than 60 days.
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Cell Culture- A549 cells were maintained in RPMI-1640 supplemented with 10% FBS (Hyclone, Logan, UT). Cells were grown as monolayers at 37°C in a humidified environment with 5% CO₂. 24 hours after plating, cultures were supplemented with either 100 uM or 25 uM FAME of DHA and linoleic acid (LA), or an equal volume of ethanol (EtOH) was added. Vitamin E treatments were done at a concentration of 50 uM.

Ionizing Radiation Treatments- 24 hours after FAME treatment, cells in 6 cm dishes were given a 1 Gy dose of superficial X-ray radiation using a Picker Zephyr superficial X-ray machine with a 4mm Al half value layer (HVL) and a 7 cm cone (Picker, Boston, MA).

Cell Viability Assays- Cells were trypsinized and counted using trypan blue staining and a hemacytometer. Unstained cells were counted as viable.

Anchorage-Independent Cell Growth Assays- A 0.4% solution of noble agar (Sigma, St Louis, MO) was combined with RPMI-1640 media containing 25 uM or 5 uM concentrations of FAME or EtOH. The cells were seeded over a 0.8% noble agar base layer in triplicate for each independent experiment. Cells were rehydrated every 3 days with the appropriate medium. After 3 weeks, cells were stained with crystal violet (Sigma, St. Louis, MO) and colonies numbers were determined.

Annexin V-FITC/PI Analysis- Analysis was performed as described previously [18]. Briefly, cells were trypsinized, counted, and 1.0×10^6 cells were assayed using an Annexin V-FITC Apoptosis Detection Kit (Calbiochem, San Diego, CA). Briefly, cells were added to binding buffer and incubated with Annexin V-FITC, washed, resuspended and had propidium iodide added before being analyzed by flow cytometry.

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4 **Thiobarbituric Acid Reactive Substances (TBARs) Assay-** Cells were trypsinized, and counted,
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6 and equal cell numbers were pelleted and lysed in GTP-lysis buffer [50mM HEPES (pH 7.5), 15mM NaCl,
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8 6mM sodium deoxycholate, 1% NP-40, 10% glycerol, 10mM MgCl₂, 1 mM EDTA] containing freshly added
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10 protease and phosphatase inhibitors by passing the pellet through a 26⁵/₈ gauge needle 20 times. Equal
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12 volumes of cell lysates were added to a 20% trichloroacetic acid (TCA)/ 0.67% thiobarbituric acid (TBA)
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14 solution and heated at 100° C for 20 minutes before being cooled on ice and centrifuged to remove
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16 precipitates. Supernatants were then read at 538 nm to determine levels of malondialdehyde (MDA)
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18 formed.
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23 **Animal Study-** The feeding study was performed as described previously [3]. Briefly, adult
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25 female NCr homozygous (nu/nu) athymic nude mice were maintained and bred under aseptic conditions
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27 with constant temperature and humidity. Animals were implanted with A549 xenografts in the right
28
29 flank and randomly assigned to experimental treatment groups, which were fed diets composed of the
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31 AIN-93M casein based diet containing defined amounts of essential fatty acids starting the day of
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33 implantation (Table 1). The experimental diets consisted of (1) a control omega-6, 8% corn oil and (2) a
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35 high fat omega-6, 24% corn oil and (3) a high fat omega-3, 8% corn oil and 16% Dhasco™ oil (Martek,
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37 Columbia, MD) diet. Animals were pre-fed diets 8 weeks prior to xenograft implantation and for the
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39 duration of the feeding study (23 days). Food intake was monitored daily by weighing uneaten portions
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41 and isocalorically controlled by proportionally adjusting the amount given to all groups. At the
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43 conclusion of the study, paired xenografts, lungs and livers were dissected from animals, weighed and
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45 analyzed for fatty acid content.
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53 **Fatty Acid Analysis-** Matched A549 xenografts, livers and lungs from 5 mice from each diet
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55 were homogenized in 2.0 mL of GTP-lysis buffer containing 0.02% BHT [50mM HEPES (pH 7.5), 15mM
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3 NaCl, 6mM sodium deoxycholate, 1% NP-40, 10% glycerol, 10mM MgCl₂, 1 mM EDTA, 0.02% butylated
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5 hydroxytoluene (BHT)] containing freshly added protease and phosphatase inhibitors. Samples were
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7 analyzed for protein concentration using BIO RAD's DC assay (Hercules, CA). Whole cell homogenate was
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9 fatty acid extracted using a 2:1 chloroform:methanol mixture. The samples were vortexed and
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11 centrifuged at 1,000 x g for 5 minutes. The chloroform layer was extracted and a 19:0 internal standard
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13 was added and dried under nitrogen. 14% boron trifluoride (BF₃) / methanol was added to dried sample
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15 and incubated at 110°C for 15 minutes. Petroleum ether containing 0.02% BHT was added to the
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17 samples. The petroleum ether fatty acid containing fraction was dried in anhydrous Na₂SO₄ / NaHCO₃
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19 (2:1, w,w). The samples were flushed with nitrogen and stored at -20°C until use. An Agilent
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21 Technologies 6890N Chromatograph, which contained an SP2340 capillary column (Supelco, St. Louis,
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23 MO), was used. A temperature program of a total time of 20 minutes with a starting temperature of
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25 120°C was formulated. The injector was set at 245°C and flame-ionization detector (FID) at 250°C. The
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27 program was modified so that rate 1 was set at 5°C / min to 160°C and rate 2 was set at 10°C / min to
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29 240°C and held at 240°C for four minutes. Values for each fatty acid are reported as percentage of total
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31 fatty acids.
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Statistical Analysis- With the exception of the animal study, all experimental results were independently repeated at least three times. All quantitative data shown represent the compiled data as percentages versus control treatments with error bars representing standard deviation, except in the case of the final xenografts weights where they represent standard error of the mean, and statistical analyses were performed using the Student's *t* test and/or ANOVA with the Tukey method for pairwise comparison on SAS® software, with values of at least $p \leq 0.05$ being considered significant.

Results:

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3 ***A Combination of Ionizing Radiation and DHA Treatments Enhance Inhibition of Cell Proliferation and***
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5 ***Anchorage-Independent Growth Compared to Either Treatment Alone-***
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8 While both DHA ($57 \pm 7\%$ decrease vs. EtOH control) and a 1 Gy dose of ionizing radiation ($17 \pm$
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10 11% decrease vs. EtOH control) significantly inhibited the degree of cell proliferation of the
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12 radioresistant A549 human lung adenocarcinoma cell line, a combination of the two resulted in a
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14 different, synergistic enhancement ($83 \pm 8\%$ decrease vs. EtOH control) compared to either treatment
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16 alone (Figure 1A). Interestingly, unpublished data from our laboratory has shown IC50 concentrations of
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18 DHA are less than $\frac{1}{4}$ th the amount when examining colony formation and anchorage-independent
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20 growth in soft agar compared to standard tissue culture models. Ionizing radiation treatments (1 Gy
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22 dose) also showed greatly enhanced inhibition of colony formation ($46 \pm 8\%$ decrease vs. EtOH control),
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24 while DHA treatment alone also significantly decreased colony formation ($60 \pm 7\%$ decrease vs. EtOH
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26 control), and a combination of the two acted synergistically to almost completely negate growth in soft
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28 agar ($95 \pm 4\%$ decrease vs. EtOH control) (Figure 1B). These *in vitro* data show good potential for
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30 promise in more clinically relevant models, as anchorage-independent growth in soft agar is generally
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32 indicative of how *in vivo* xenografts in athymic mice will respond to similar treatments [19].
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40 ***Decreases in Cell Proliferation upon DHA and/or Ionizing Radiation Treatment Correlate with***
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42 ***Increased Apoptosis and Lipid Peroxidation***
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45 The noted decreases in cell proliferation are proportional to increases in apoptosis noted in
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47 A549 cells treated with DHA ($27 \pm 3\%$ Annexin V-FITC positive cells), ionizing radiation ($18 \pm 4\%$ Annexin
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49 V-FITC positive cells), or a combination of the two ($52 \pm 13\%$ Annexin V-FITC positive cells) (Figure 2A).
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51 Moreover, oxidative stress plays a large role in cell death associated with these treatments as significant
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53 increases in the formation of MDA, a major product of lipid peroxidation [20], are noted upon
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55 supplementation with DHA (MDA formed was $286 \pm 16\%$ vs. EtOH control), treatment with ionizing
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3 radiation (MDA formed was $192 \pm 49\%$), or both (MDA formed was $490 \pm 90\%$ vs. EtOH control) (Figure
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6 2B).

10 ***Non-toxic Concentrations of DHA Enhance Radiosensitivity***

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12 While IC50 concentrations of DHA have proven to work well in combination with ionizing
13 radiation, examining the effect of lower doses of DHA that do not display significant levels of growth
14 inhibition on their own, could better demonstrate a radiosensitizing effect. When doses of DHA that do
15 not significantly inhibit cell growth or colony formation in soft agar on their own were used, both cell
16 proliferation (Figure 3A) and soft agar growth (Figure 3B) showed a greatly enhanced response to
17 ionizing radiation treatment. These data further demonstrate DHA's ability to enhance response to
18 ionizing radiation treatment.
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31 ***Addition of Antioxidants is Capable of Decreasing Lipid Peroxidation Levels by DHA and Ionizing*** 32 ***Radiation Treatments Resulting in Partial Reversal of Growth Inhibition***

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35 To confirm decreases in cell proliferation were associated with increased oxidative stress,
36 Vitamin E, an antioxidant, was added to cell culture treatments. Vitamin E treatment was capable of
37 undoing the noted increase in lipid peroxidation by ionizing radiation in EtOH treatments (MDA formed
38 was reduced from $203.2 \pm 31\%$ to $114 \pm 14\%$ vs. EtOH control), while vitamin E treatment only partially
39 reduced MDA formation resulting from DHA treatment (MDA formed was reduced from $268.41 \pm 16\%$
40 to $171 \pm 33\%$ vs. EtOH control) (Figure 4A). A combination of ionizing radiation and DHA also showed
41 only a partial decrease in lipid peroxidation levels with the Vitamin E supplement (MDA formed was
42 reduced from $403 \pm 56\%$ to $187 \pm 24\%$ vs. EtOH control) (Figure 4A). Interestingly, the addition of
43 antioxidant to DHA and DHA with ionizing radiation treatments resulted in levels of lipid peroxidation
44 that were statistically the same, suggesting Vitamin E is capable of reversing the effects of lipid
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3 peroxidation due to ionizing radiation completely, while only having a limited effect on DHA-induced
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5 lipid peroxidation at the given dose.
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9 The decreases in lipid peroxidation with vitamin E treatment did not significantly reverse
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11 inhibition of cell proliferation rates in ionizing radiation treatments ($80 \pm 13\%$ cell number to $93 \pm 6\%$ vs.
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13 EtOH control) (Figure 4A). However, significant increases were noted in the cell numbers of DHA treated
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15 cells supplemented with Vitamin E ($46 \pm 6\%$ cell number to $70 \pm 11\%$ vs. EtOH control), while the
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17 combination of ionizing radiation with DHA also showed significant increases in cell number upon
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19 antioxidant treatment ($17 \pm 8\%$ cell number to $43 \pm 18\%$ vs. EtOH control) (Figure 4B).
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27 ***DHA Preferentially Incorporates into Lung and Xenograft Tissue In Vivo***

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29 To examine how dietary DHA incorporates into tissues *in vivo*, liver, lung and A549 xenograft
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31 specimens from a nutritional study on athymic mice fed a low fat diet of omega-6 PUFAs, a high fat
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33 omega-6 PUFA diet, or a high fat diet rich in DHA were analyzed for fatty acid content. Final weights of
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35 A549 xenografts in the study showed significantly smaller tumors in animals fed the low fat omega-6
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37 and DHA diets compared to the high fat omega-6 PUFA diet (Figure 5A). Changes to the average
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39 percentage of DHA in the livers of mice fed diets high in DHA resulted in a roughly 4.5-fold increase
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41 compared to animals fed a control amount of omega-6 PUFA ($461.83 \pm 24.39\%$), while DHA levels in lung
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43 ($675.87 \pm 42.00\%$) and A549 xenograft tissue ($827.5 \pm 127.68\%$) showed greater increases in DHA
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45 composition, and were significantly higher than the noted upregulation into liver tissue (Figure 5B).
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47 Moreover, not only was the relative increase of DHA in xenograft higher than what was noted in liver
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49 tissue, it was also significantly higher ($p = 0.0473$) than the increase noted in lung tissue of animals fed a
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51 diet high in DHA (Figure 5B). A summary of percent total fatty acids is also provided (Table 2). These
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53 findings indicate a potential use of DHA nutritionally in sensitizing lung cancers to radiation therapy.
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Discussion:

This study demonstrates the efficacy of combining nutritional supplementation of DHA with ionizing radiation to inhibit the growth of an *in vitro* model of lung cancer in standard tissue culture and in soft agar. Furthermore, it associates this inhibition with increases in apoptosis and oxidative stress, as vitamin E was capable of abrogating the degree of proliferative inhibition in DHA treatments with or without the addition of ionizing radiation, while the effect on ionizing radiation alone shows the same trend, but is not statistically significant from the control. Additionally, we show DHA may be a promising radiosensitizer in a cell line noted to have radioresistance, as non-toxic doses were still capable of enhancing response of the lung adenocarcinoma cells to ionizing radiation. Finally, we present data that suggests dietary DHA may be preferentially incorporated into lung tissues, including lung cancers, by showing higher increases in DHA composition into A549 xenograft and lung compared to liver tissue in an *in vivo* athymic mouse model where animals were fed diets high in DHA.

Much work remains to be done before the mechanisms behind DHA's anti-cancer properties are fully elucidated, and while it seems likely they are many, DHA represents an extreme as the longest and most unsaturated fatty acid found in most biological membranes, and thus the most highly susceptible to oxidation [21]. These properties would seem to lend DHA well to enhancing treatments like ionizing radiation that function largely through targeted increases in oxidative stress [22], unlike other PUFAs like oleic acid (OA), which lack the abundant methylene carbon bridges and do not show enhanced response to radiation [23]. While previous work looking at combining DHA and ionizing radiation treatment has been done in transformed models of colon, breast, astrocytoma and lymphoma [10-15,23], this is the first report examining a model of lung cancer.

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3 Other reports have noted DHA preferentially incorporates into heart, liver and lung tissues [24],
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5 but to our knowledge, this is the first report to suggest that DHA incorporation into lung tissue may be
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7 especially high. Because lung cancer tends to be difficult to treat, DHA's promise as a radiosensitizer for
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9 lung cancer could greatly enhance a common treatment regimen, especially given the observation that
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11 xenograft tissue from a lung cancer model showed relative increases in DHA even higher than control
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13 lung tissue.
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18 According to the National Cancer Institute's Fact Sheet, roughly half of all people treated for
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20 cancer receive radiation therapy. New agents are constantly being developed to enhance
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22 radiosensitivity [25], and while DHA may not present the same commercial incentives as more
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24 traditional therapeutic agents, further research into applications combining DHA with radiation
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26 treatments is warranted. The anti-cancer properties of nutritional supplements like DHA present a
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28 unique opportunity to inexpensively advance clinical outcomes while decreasing toxicity to patients,
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30 unlike most other conventional treatments.
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Figure/Table Legends:

Figure 1- A. A549 lung adenocarcinoma cells were supplemented with 100 μ M concentrations of docosahexaenoic acid (DHA), linoleic acid (LA) or a control volume of ethanol (EtOH) for 24 hours before being treated with a 1 Gy dose of superficial X-ray radiation. After another 24 hours, cells were trypsinized, stained with trypan blue, and quantified using a hemacytometer. Graphs represent the percentage of unstained cells compared to EtOH treatment alone. **B.** A549 cells were plated in a 0.4% noble agar/RPMI-1640 solution containing 25 μ M concentrations of DHA, LA or a control volume of EtOH, on top of a 0.8% basement layer. 24 hours after plating, cells were treated with a 1 Gy dose of

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3 superficial X-ray radiation and allowed to grow for 3 weeks before being stained with crystal violet and
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5 having colony numbers quantified. Graphs represent the percentage of colonies formed compared to
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7 EtOH treatment alone. Statistical significance ($p \leq 0.05$) between treatments was determined using
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9 ANOVA and is indicated as followed: * (versus EtOH); # (versus DHA); & (versus EtOH + 1 Gy).
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12 Redundant symbols were not shown.
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15 **Figure 2-** A549 lung adenocarcinoma cells were supplemented with 100 μM concentrations of DHA, LA
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17 or a control volume of EtOH for 24 hours before being treated with a 1 Gy dose of superficial X-ray
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19 radiation. After another 24 hours, cells were trypsinized, and assayed as followed: **A.** Cell pellets were
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21 stained with Annexin V-FITC followed by washing and propidium iodide and analyzed by flow cytometry.
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23 Graphs represent the percentage of cells stained positively by Annexin V-FITC. **B.** Cells were counted
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25 and equal numbers were lysed using a 26 $^{5/8}$ gauge needle, before malondialdehyde (MDA) formation
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27 was analyzed by thiobarbituric acid reactive substances (TBARs) assay. Graphs represent the percentage
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29 of MDA formed in treatments compared to EtOH alone. Statistical significance ($p \leq 0.05$) between
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31 treatments was determined using ANOVA and is indicated as followed: * (versus EtOH); # (versus DHA);
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33 & (versus EtOH + 1 Gy). Redundant symbols were not shown.
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40 **Figure 3- A.** A549 lung adenocarcinoma cells were supplemented with 25 μM concentrations of
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42 docosahexaenoic acid (DHA), linoleic acid (LA) or a control volume of ethanol (EtOH) for 24 hours before
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44 being treated with a 1 Gy dose of superficial X-ray radiation. After another 24 hours, cells were
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46 trypsinized, stained with trypan blue, and quantified using a hemacytometer. Graphs represent the
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48 percentage of unstained cells compared to EtOH treatment alone. **B.** A549 cells were plated in a 0.4%
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50 noble agar/RPMI-1640 solution containing 5 μM concentrations of DHA, LA or a control volume of EtOH,
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52 on top of a 0.8% basement layer. 24 hours after plating, cells were treated with a 1 Gy dose of
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54 superficial X-ray radiation and allowed to grow for 3 weeks before being stained with crystal violet and
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4 EtOH treatment alone. Statistical significance ($p \leq 0.05$) between treatments was determined using
5 ANOVA and is indicated as followed: * (versus EtOH); # (versus DHA); & (versus EtOH + 1 Gy).
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10 Redundant symbols were not shown.
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17 **Figure 4-** A549 lung adenocarcinoma cells were supplemented with 100 uM concentrations of DHA, LA
18 or a control volume of EtOH, and a 50 uM treatment of Vitamin E for 24 hours before being treated with
19 a 1 Gy dose of superficial X-ray radiation. Non-treated (NT) cells received neither Vitamin E nor ionizing
20 radiation treatments. After another 24 hours, cells were trypsinized, and assayed as followed: **A.** Cells
21 were stained with trypan blue and quantified using a hemacytometer. Graphs represent the percentage
22 of unstained cells compared to EtOH treatment alone. Addition of Vitamin E resulted in significant
23 differences ($p \leq 0.05$) due to 1 Gy radiation, but not DHA, to be reversed. **B.** Cells were counted and
24 equal numbers were lysed using a 26 ⁵/₈ gauge needle, before MDA formation was analyzed by TBARs
25 assay. Graphs represent the percentage of MDA formed in treatments compared to EtOH alone.
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Statistical significance ($p \leq 0.05$) between treatments was determined using ANOVA and is indicated as
followed: * (versus EtOH); # (versus DHA); & (versus EtOH + 1 Gy); ! (versus DHA + 1 Gy). Redundant
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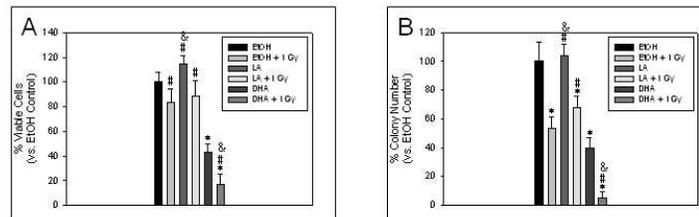
Figure 5 - Athymic mice were implanted with A549 xenografts and fed a control omega-6 PUFA diet
containing 8% corn oil (CO), 24% CO or a 8% CO/16% Dhasco oil (DO) diet high in the omega-3 PUFA
DHA for 4 weeks. **A.** Final xenografts weights are shown as a percentage of the average 8% corn oil
xenografts. At the conclusion of the study, xenografts and livers were dissected, and homogenized, and
fatty acid methyl esters were prepared for analysis by gas chromatography. Statistical significance ($p \leq$

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3 0.05) between treatments was determined using the student's t-test and is indicated as followed: *
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5 (versus 8% Corn Oil); # (versus 24% Corn Oil). Redundant symbols were not shown. **B.** Results indicate
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7 the percentage of DHA incorporation for liver, lung and xenograft tissue in the 8% CO, 24% CO and
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9 8%CO/16%DO diet relative to the 8% CO diet (n=5). Statistical significance ($p \leq 0.05$) between DHA
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11 content in the tissues from the 16% Dhasco + 8% Corn Oil diet was determined using the student's t-test
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13 and is indicated as followed: * (versus Liver); # (versus Lung). Redundant symbols were not shown.
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21 **Table 1-** Composition of animal diets in %component/kcal/diet/day.
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27 **Table 2-** Results are presented as percentage total fatty acid content for liver, lung and xenograft tissue
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29 in the 8% CO, 24% CO and 8% CO/16% DO diets. All values are represented as the percentage of each
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31 fatty acid relative to total fatty acid content \pm standard deviation, n=5. Additionally, total n-6 and n-3
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33 PUFA content is listed for each tissue and diet.
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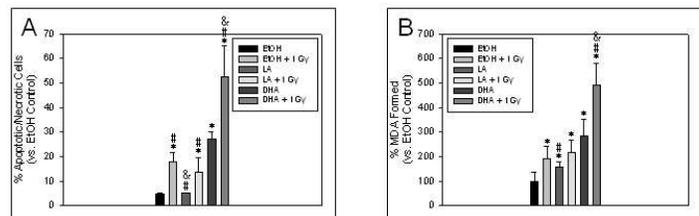
Figure 1



A549 lung adenocarcinoma cells were supplemented with 100 μ M concentrations of docosahexaenoic acid (DHA), linoleic acid (LA) or a control volume of ethanol (EtOH) for 24 hours before being treated with a 1 Gy dose of superficial X-ray radiation. After another 24 hours, cells were trypsinized, stained with trypan blue, and quantified using a hemacytometer. Graphs represent the percentage of unstained cells compared to EtOH treatment alone. B. A549 cells were plated in a 0.4% noble agar/RPMI-1640 solution containing 25 μ M concentrations of DHA, LA or a control volume of EtOH, on top of a 0.8% basement layer. 24 hours after plating, cells were treated with a 1 Gy dose of superficial X-ray radiation and allowed to grow for 3 weeks before being stained with crystal violet and having colony numbers quantified. Graphs represent the percentage of colonies formed compared to EtOH treatment alone. Statistical significance ($p \leq 0.05$) between treatments was determined using ANOVA and is indicated as followed: * (versus EtOH); # (versus DHA); & (versus EtOH + 1 Gy). Redundant symbols were not shown.

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Figure 2

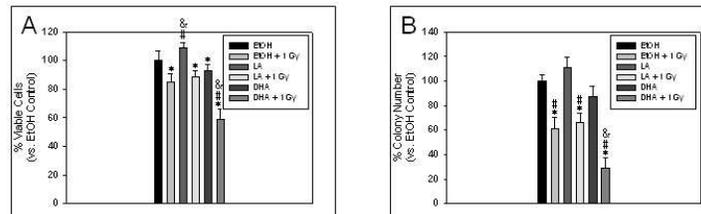


A549 lung adenocarcinoma cells were supplemented with 100 μ M concentrations of DHA, LA or a control volume of EtOH for 24 hours before being treated with a 1 Gy dose of superficial X-ray radiation. After another 24 hours, cells were trypsinized, and assayed as followed: A. Cell pellets were stained with Annexin V-FITC followed by washing and propidium iodide and analyzed by flow cytometry. Graphs represent the percentage of cells stained positively by Annexin V-FITC. B. Cells were counted and equal numbers were lysed using a 26 5/8 gauge needle, before malondialdehyde (MDA) formation was analyzed by thiobarbituric acid reactive substances (TBARs) assay. Graphs represent the percentage of MDA formed in treatments compared to EtOH alone. Statistical significance ($p \leq 0.05$) between treatments was determined using ANOVA and is indicated as followed: * (versus EtOH); # (versus DHA); & (versus EtOH + 1 Gy). Redundant symbols were not shown.

254x190mm (96 x 96 DPI)



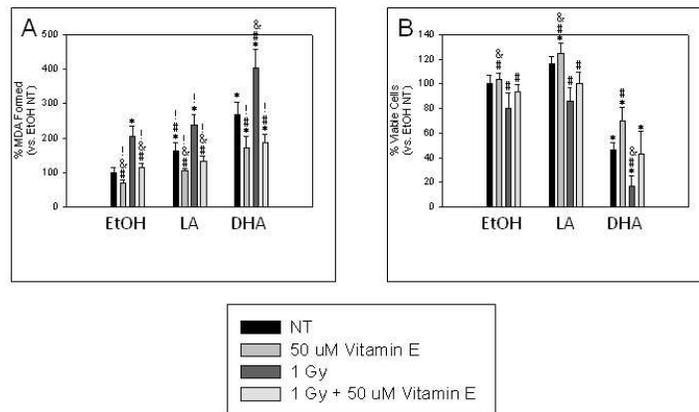
Figure 3



A. A549 lung adenocarcinoma cells were supplemented with 25 μ M concentrations of docosahexaenoic acid (DHA), linoleic acid (LA) or a control volume of ethanol (EtOH) for 24 hours before being treated with a 1 Gy dose of superficial X-ray radiation. After another 24 hours, cells were trypsinized, stained with trypan blue, and quantified using a hemacytometer. Graphs represent the percentage of unstained cells compared to EtOH treatment alone. B. A549 cells were plated in a 0.4% noble agar/RPMI-1640 solution containing 5 μ M concentrations of DHA, LA or a control volume of EtOH, on top of a 0.8% basement layer. 24 hours after plating, cells were treated with a 1 Gy dose of superficial X-ray radiation and allowed to grow for 3 weeks before being stained with crystal violet and having colony numbers quantified. Graphs represent the percentage of colonies formed compared to EtOH treatment alone. Statistical significance ($p \leq 0.05$) between treatments was determined using ANOVA and is indicated as followed: * (versus EtOH); # (versus DHA); & (versus EtOH + 1 Gy). Redundant symbols were not shown.

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Figure 4

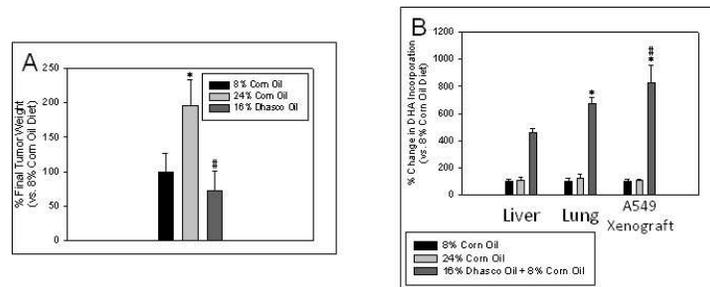


A549 lung adenocarcinoma cells were supplemented with 100 μ M concentrations of DHA, LA or a control volume of EtOH, and a 50 μ M treatment of Vitamin E for 24 hours before being treated with a 1 Gy dose of superficial X-ray radiation. Non-treated (NT) cells received neither Vitamin E nor ionizing radiation treatments. After another 24 hours, cells were trypsinized, and assayed as followed: A. Cells were stained with trypan blue and quantified using a hemacytometer. Graphs represent the percentage of unstained cells compared to EtOH treatment alone. Addition of Vitamin E resulted in significant differences ($p \leq 0.05$) due to 1 Gy radiation, but not DHA, to be reversed.

B. Cells were counted and equal numbers were lysed using a 26 5/8 gauge needle, before MDA formation was analyzed by TBARS assay. Graphs represent the percentage of MDA formed in treatments compared to EtOH alone. Statistical significance ($p \leq 0.05$) between treatments was determined using ANOVA and is indicated as followed: * (versus EtOH); # (versus DHA); & (versus EtOH + 1 Gy); ! (versus DHA + 1 Gy). Redundant symbols were not shown.

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Figure 5



Athymic mice were implanted with A549 xenografts and fed a control omega-6 PUFA diet containing 8% corn oil (CO), 24% CO or a 8% CO/16% Dhasco oil (DO) diet high in the omega-3 PUFA DHA for 4 weeks. A. Final xenografts weights are shown as a percentage of the average 8% corn oil xenografts. At the conclusion of the study, xenografts and livers were dissected, and homogenized, and fatty acid methyl esters were prepared for analysis by gas chromatography. Statistical significance ($p \leq 0.05$) between treatments was determined using the student's t-test and is indicated as followed: * (versus 8% Corn Oil); # (versus 24% Corn Oil). Redundant symbols were not shown. B. Results indicate the percentage of DHA incorporation for liver, lung and xenograft tissue in the 8% CO, 24% CO and 8%CO/16%DO diet relative to the 8% CO diet ($n=5$). Statistical significance ($p \leq 0.05$) between DHA content in the tissues from the 16% Dhasco + 8% Corn Oil diet was determined using the student's t-test and is indicated as followed: * (versus Liver); # (versus Lung). Redundant symbols were not shown.

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Table 1

| Component | 8%CO (g) | 24%CO (g) | 8%CO+16%D0 (g) |
|---------------------|----------|-----------|----------------|
| Corn oil | 29.60 | 73.85 | 24.62 |
| Dhasco oil | 0.00 | 0.00 | 49.23 |
| AIN-93M mineral mix | 13.65 | 13.69 | 13.69 |
| L-cystine | 0.70 | 0.70 | 0.70 |
| choline | 0.98 | 0.98 | 0.98 |
| AIN-93 vitamin mix | 3.90 | 3.91 | 3.91 |
| casein | 54.61 | 54.76 | 54.76 |
| cellulose | 19.50 | 19.56 | 19.56 |
| cornstarch | 159.64 | 90.63 | 90.63 |
| dyetrose | 53.13 | 30.17 | 30.17 |
| sucrose | 34.28 | 19.46 | 19.46 |
| TotalDiet | 369.99 | 307.71 | 307.71 |

Values are in %component/kcal/mouse/day. CO: Corn Oil, D0: Dhasco™ Oil.

Table 1- Composition of animal diets in %component/kcal/diet/day.
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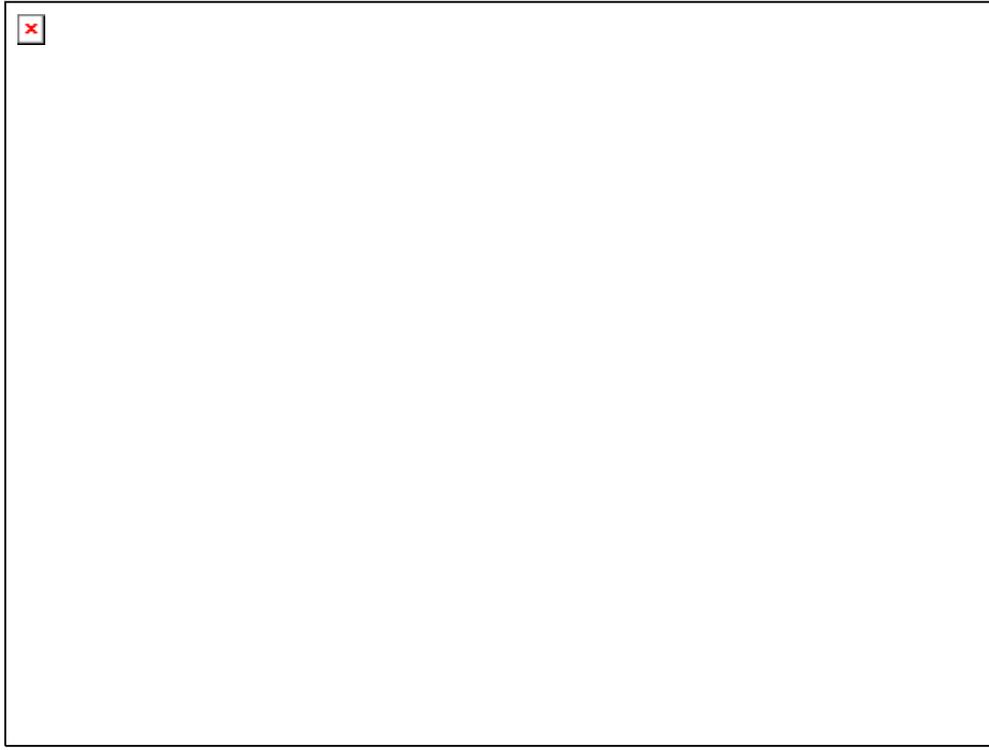


Table 2- Results are presented as percentage total fatty acid content for liver, lung and xenograft tissue in the 8% CO, 24% CO and 8% CO/16% DO diets. All values are represented as the percentage of each fatty acid relative to total fatty acid content \pm standard deviation, n=5. Additionally, total n-6 and n-3 PUFA content is listed for each tissue and diet.
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