Original article

N-3 fatty acids ameliorate radiation-induced liver injury in the rat

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SUMMARY

Irradiation causes veno-occlusive liver disease and radiation hepatitis. N-3 fatty acids are rapidly incorporated in the cellular membranes and modulate anti-inflammatory cytokines production, thus pretreatment with them could be an effective prevention modality to the effects of ionizing radiation. The purpose of this study was to investigate the effect of n-3 fatty acids on liver hemodynamics and microscopic appearance after ionizing radiation treatment. Three groups of rats were exposed to a single dose of 11 Gy of gamma radiation or sham radiation. Group control [C] was treated for 7d before radiation with standard rat chow, group fasted [F] with rat chow but fasted for the last 12 hours, and group n-3 with rat chow plus n-3 fatty acids. At days (d) 1, 3, 7, 15 and 30 post-radiation 2 rats per study-group were subjected to liver microcirculation assessment by means of laser-Doppler flowmetry and the livers were then processed for histology and immunohistochemistry. Liver microcirculation was decreased progressively from d1 to d30 in groups F and C in relation to n-3. Histological examination revealed dilatation of central veins and congestion of portal sinusoids on d1, hepatocyte degeneration on d3 and progressive hepatocyte necrosis from d7 up to d30. These findings were less prominent in group n-3 in relation to F and C. Apoptosis induction, assessed by means of Bax, was found to be less prominent in F and C groups in relation to n-3, while, when assessed by means of Bacl-2 and Bcl-xL, it was found to be expressed in F and C rather than in n-3 group, throughout the whole study period. It is concluded that n-3 fatty acids protect the rat liver from radiation-induced injury and thus patients eligible for abdominal radiotherapy could theoretically get the benefit of liver protection, by receiving an oral supplementary nutrition containing n-3 fatty acids for at least seven days prior to treatment. However, further investigation is needed.

Key words: radiation hepatitis, n-3 fatty acids, microcirculation, apoptosis, radiation

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INTRODUCTION

Radiotherapy, although a theoretically directed against malignant tissue, also affects the adjacent healthy tissue, i.e. the small bowel and the liver.[1-3] The tissue radiation tolerance depends on the type of tissue cells, since each type of cell has a different sensitivity index, mainly depending on its mitotic activity.[3,4] The cytotoxic effect of ionized radiation is mediated by the free radical generation, through a process that involves radiolysis of the intracellular water to yield superoxide, hydrogen peroxide and hydroxyl radicals.[3] In clinical practice, the commonest injury after abdominal irradiation is intestinal mucosal damage.[3,5,6] Although less clinically prominent, a similar injury occurs in the liver with clinical and morphological characteristics described as a veno-occlusive disease.[2,4-9] Pathogenesis remains unclear; however the initial injury is likely to occur in the endothelial cells of central veins and sinusoids.[1,4]

Experimental evidence, primarily focused on the small bowel injury, demonstrates that formulas that either interfere with the production of oxidants, or scavenge oxygen radicals or simply protect the cell by reducing the inflammatory process, seem to prevent or reduce tissue cell damage, even in cases where they are administered after exposure to radiation.[5,7,8,10-12] However, the effect of such modalities in reducing liver damage needs further research.

Dietary n-3 fatty acids, which are rapidly incorporated within the cellular membranes, are considered to work through the modulation of the tissue inflammatory reaction by means of anti-inflammatory cytokines production. The purpose of this study was to investigate whether n-3 fatty acids, given as pretreatment, could exert any beneficial effect by preventing or reducing radiation-induced liver injury.

MATERIAL AND METHOD

Animals

Sixty male Wistar rats, weighing 200-220g were employed. The animals were housed in stable laboratory conditions for at least one week. The experimental protocol was approved by the Department of Animal Care And Use Committee of the Greek Ministry of Agriculture and adhered to the European Community Guiding Principles for the Care and Use of Animals. Food was given as per protocol before the experiment [time 0], and all animals had free access to water.

Study Design

Rats were divided into three groups of 20 rats each: Rats of Group C [control] were fed for 7 consecutive days before the experiment with standard rat chow; Rats of Group F [fasted] were fed for 7 consecutive days with rat chow but remain fasted for the last 12 hours; Rats of Group n-3 [n-3 treatment] were fed for 7 days with rat chow and additionally received n-3 fatty acids.

Ten rats from each group were subjected to irradiation [R] at time 0, creating thus sub-groups of CR [Control+Radiation], FR [Fasted+Radiation], and n-3R [n-3+Radiation], while the remaining ten rats of each group served as controls, i.e. without exposure to irradiation: sub-groups CP [Control+Placebo], FP [Fasted+ Placebo], and n-3P [n-3+Placebo].

On days 1, 3, 7, 15 and 30 post-irradiation or placebo irradiation, 2 animals from each sub-group were anaesthetized and subjected to laparotomy for liver microcirculation assessment by means of laser-Doppler flowmetry. The livers were then received for histology and immunohistochemistry.

Treatment

As a source of n-3 fatty acids the commercially available emulsion of Supportan Drink [Fresenius Kabi, Hellas] was used. This regimen, used as dietary supplement has a ratio of n-6: n-3 fatty acids 2.5:1. A total volume of 25ml [37.5 Kcal] was given to each animal every day, by immersing some rat chow pellets in it, in order to secure total receive of the nutrient.

Radiation technique

Mild hypnosis for immobilization of the animals was achieved by intramuscular injection of 0.005 mg/100g fentanyl hydrochloride [Fentanyl, Janssen Belgium] and 0.5mg/100g midazolam [Dormicum, Roche Hellas], 5min before time 0, ensuring spontaneous respiration throughout the procedure.

The animals of Radiation sub-groups were placed in supine position on a Plexiglas board, two animals at a time and were exposed to a single dose of 11Gy of gamma radiation from a ^{60}Co source, administered at 1.5cm depth below the skin, the source-skin distance being 80cm. The rectangular radiation area was extended from 1cm above the xiphoid to 1cm below the anus and 0.5cm laterally on each side of the animal. The Placebo treated sub-groups were subjected to similar anaesthesia procedure but without exposure to irradiation and served as inter-group controls.

After irradiation lasting 5min, the rats were placed in cages and had free access to water until handling as per study protocol.

Assessment of liver microcirculation

On days 1, 3, 7, 15 and 30 post-irradiation liver microcirculation measurements were performed in 2 rats of each sub-group. Rats were anaesthetized and laparotomized. Liver microcirculation was assessed by means of the laser-Doppler flowmetry technique.

The laser-Doppler probe used was a self-adhesive single fibre probe [PF319:2L, Perimed, Sweden] connected to the Periflux PF2B [Perimed, Sweden] flowmeter. It is constituted of one optical fibre with a diameter 0.5 mm and with a small latex sheet attached to its angular tip. This latex sheet adheres stable to moist surfaces, permitting a very stable laser-Doppler signal to be obtained. All measurements were performed with a signal processing Periflux filter at 4 kHz and with time constant of the output amplifier at 0.2 sec. The laser-Doppler flowmeter readings, in arbitrary units of flux, were continuously transferred and stored in a serially connected IBM-PS2 PC, by the use of the Perisoft software [Perimed, Sweden], for further analysis.

Liver histology

After the microcirculation assessments were completed, the rats were sacrificed and the livers were processed for histology and immunohistochemistry.

The liver samples were examined for Bax indicator

[which promotes apoptosis] and for Bcl-2 and Bcl-xl indicators [which inhibit apoptosis].

Statistical analysis

All values were expressed as the means ± SD. Statistical analysis was made by Statview [Brain Power Inc, Calabalas, CA], in a Macintosh computer. ANOVA-test for repeated measurements was used. Differences were considered significant at the level $p<0.05$.

RESULTS

Liver microcirculation in radiated rats was found to be dramatically decreased progressively [p=0.01], from the 1st day to the 30th day in Groups F and C in relation to n-3 [Diagram 1]. Rats subjected to sham-radiation exhibited no change in microcirculation values throughout the study period.

Histology reveals -as early as 24 hours post-irradiation- congestion in portal tract vessels and dilatation in central veins, but there were no significant differences between groups [Fig. 1]. On day 3, except the congestion in sinusoids, a mild degeneration of hepatocytes was prominent equally in all groups [Fig. 2]. On day 7 hepatocytes started to degenerate with greater extend in groups C and F. Apoptotic nuclei, focal intralobular necrosis and cytoplasmic degenaration was found in group C and F, while mild focal steatosis but without foci of necrosis in n-3 group [Fig. 3]. Further on, from 15th up to 30th day hepatocyte necrosis was progressive in F and C groups, and mild steatosis of hepatocytes, Kupffer cells hyperplasia and focal nitralobular necrosis of hepatocytes was less prominent in group n-3. [Fig.4, Fig 5]

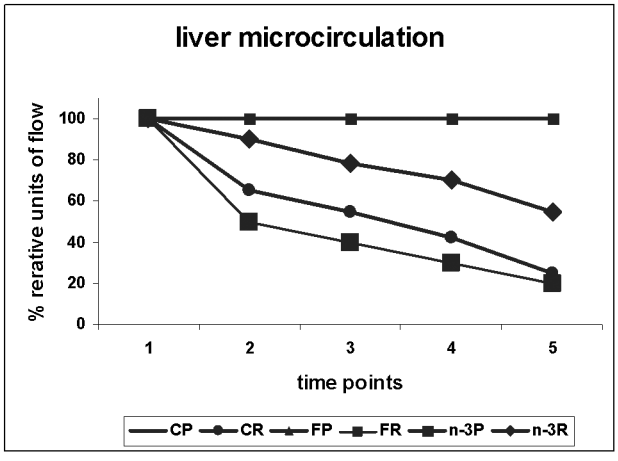

Diagram 1: Liver microcirculation changes [expressed as % of initial measurement received as 100%] during the study period, i.e. at the 1st, 3rd, 7th, 15th and 30th day of study.

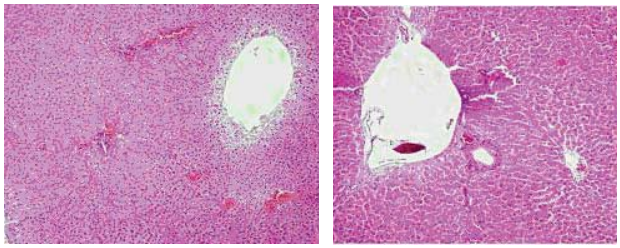

Figure 1. Day 1: Congestion in vessels in portal tract. Distention in central veins. No differences between C and n-3 groups. HE X40

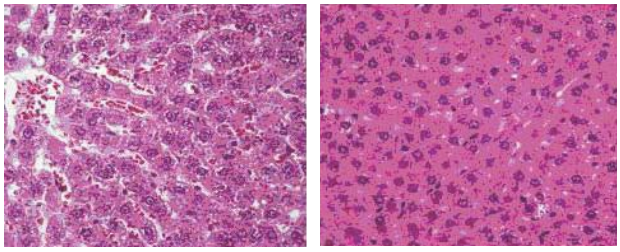

Figure 2. Day 3: Congestion in sinusoids, mild degeneration of hepatocytes [left, group C]. Hyperplasia of Kupffer cells, mild degeneration of hepatocytes [right, group n-3]. HE X40

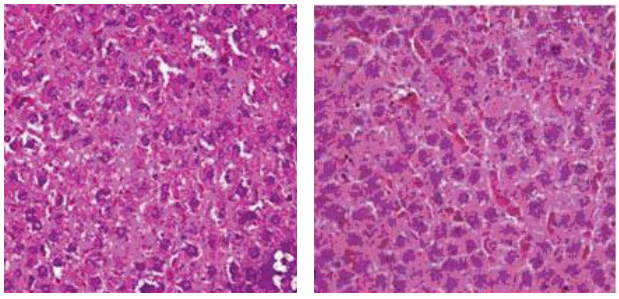

Figure 3. Day 7: Apoptotic nuclei, focal intralobular necrosis, degenaration of cytoplasm [left, group C]. Mild focal steatosis, without necrosis [right, group n-3]. HE X40

When the apoptosis was assessed by means of Bax, it was found to be less prominent in F and C in relation to n-3 group. When the apoptosis was assessed by means of the Bcl-2 and Bcl-xL, it was found to be expressed in F and C rather than in n-3, throughout the whole study period.

Regarding the apoptotic process, the immunohistochemical examination revealed that it was more prominent in the group n-3 than in the other two groups. More

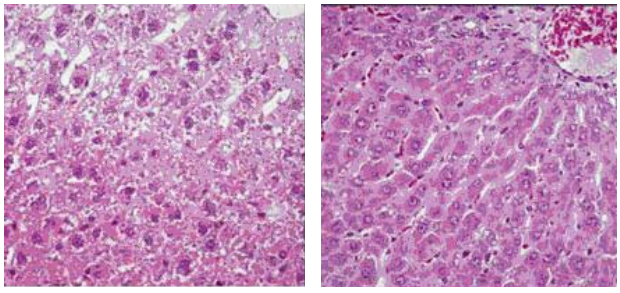

Figure 4. Day 15: Necrosis of hepatocytes. [left, group C]. Mild steatosis of hepatocytes, vacuolated nuclei, Kupffer cells hyperplasia [right, group n-3]. HE X40

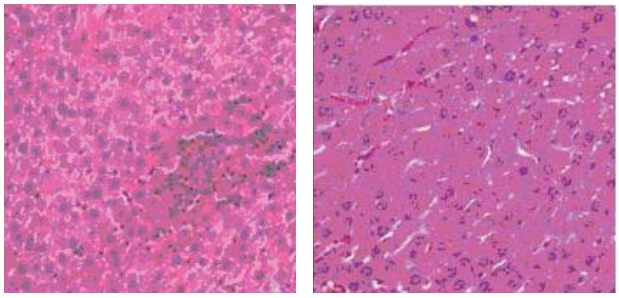

Figure 5. Day 30: Necrosis of hepatocytes with inflammatory infiltration. Apoptotic nuclei. [left, group C]. Mild steatosis of hepatocytes. [right, group n-3]. HE X40

specifically, when the apoptosis was assessed by means of Bax-indicator [apoptosis promoted], it was found to be less prominent in F and C in relation to n-3 group. When the apoptosis was assessed by means of Bcl-2- and Bcl-xL-indicators [apoptosis inhibited] it was found to be expressed in F and C rather than in n-3, at all study periods (Fig. 6-11). The interaction of n-3 PUFAs with the apoptosis procedure consists of promotive effects on apoptosis, which results in faster detachment of damaged cell from the irradiated tissue.

DISCUSSION

In this study we demonstrate the protective effect of n-3 fatty acids in liver microcirculation against irradiation damage, by decelerating the degree of degeneration of the liver vascular bed, and by ameliorating the dilation and congestion of central veins in the liver.

Polyunsaturated fatty acids [PUFAs] are considered as essential for the structure of cell membrane, since their central role is to maintain the structure, fluidity and function of the membrane. Both n-3 and n-6 fatty acids use for their metabolism the same enzymes, and this fact leads

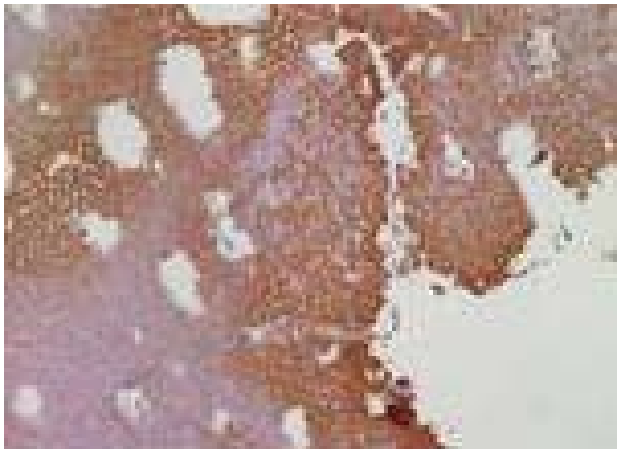

Figure 6. Day 3: Intense membranous as well as cytoplasmic Bax expression of the apoptotic hepatocytes in the centrilobular zone 3 extending to the zone 2 – [group n-3].

Figure 7. Day 3: Absent Bax expression – [group C]

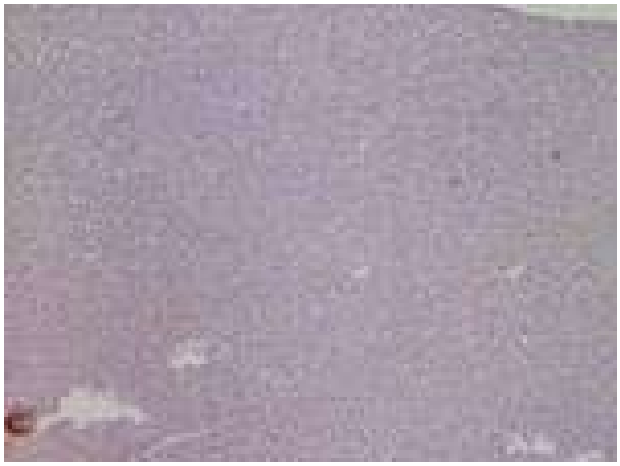

Figure 8 Day 30: Mild cytoplasmic Bcl-2 expression of the apoptotic hepatocytes in the centrilobular zone 3 – [group n-3]

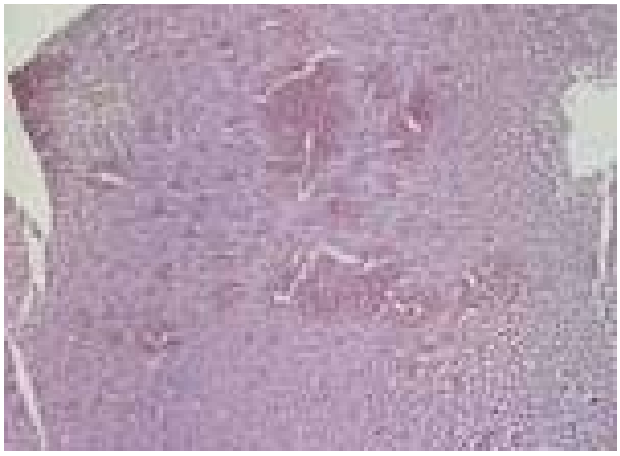

Figure 9. Day 30: Moderate membranous Bcl-2 expression of the apoptotic hepatocytes in the centrilobular zone 3 – [group n-3].

Figure 10. Day 30: Absent Bcl-xL expression – [group n-3]

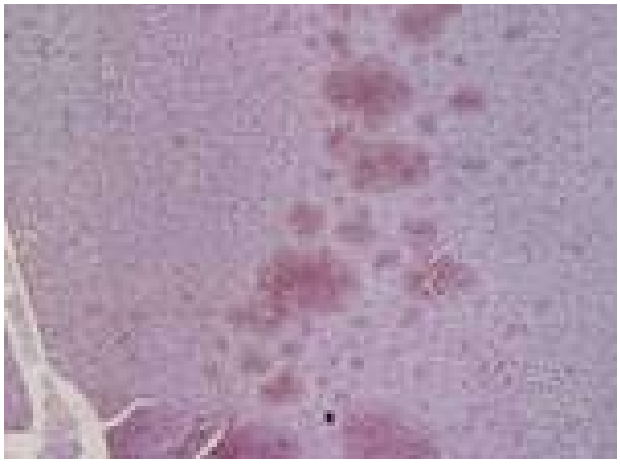

Figure 11. Day 30: Mild cytoplasmic Bcl-xL expression of the apoptotic hepatocytes in the centrilobular zone 3 – [group C]

to competition between the two families. That is why the rate of n-6:n-3 PUFAs plays a central role in the diet. If the amount of dietary consumption of n-3 fatty acids is increased, then the degree of incorporation of n-3 fatty acids in tissue lipids will be greater. And as it is well known that n-3 PUFAs are implicated in the anti-inflammatory process, in this case the amount of proinflammatory substances will be decreased. On the other hand, increased delivery of n-6 improves or even releases the inflammatory chain reaction within the cell.[3]

Many experimental and clinical studies have been conducted exploring the clinical significance of an increased ratio of *n*-6:n-3 in artificial nutrition. In other words, whether substitution of n-6 PUFAs or increase of dietary emulsion with n-3 could intervene therapeutically or at least minimize the inflammatory process. Thus, in colorectal cancer patients a ratio of less than 2·5:1 was found to reduce rectal cell proliferation,[4] while in cardiovascular disease patients food consumption with a ratio of less that 4:1 led to a decrease in total mortality. A ratio of 2:1 or 3:1 suppressed inflammation in rheumatoid arthritis patients and a ratio of 5:1 benefited asthma sufferers.[5] Increasing dietary n-3 fatty acids has also been shown to decrease the incidence of malignancy in experimental animal with mammary, pancreatic, prostatic and gastrointestinal neoplasms.[6-9] This preparation contains PUFAs in a proportion of n-6:n-3, 2.5:1, this rate being within the protective ranges according to recent bibliography.[6-9]

The mechanism through which n-3 PUFAs play a protective role in cellular integrity remains unclear. The most probable answer may lie in the fact, that PUFAs play a critical role in the modulation from eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA] of the production of eicosanoids, which in their turn regulate the production of cytokines and nitric oxide [NO].[10-13] Eicosanoids can be produced through two pathways; either from arachidonic acid [AA] or from EPA and DHA. The eicosanoids deriving from AA play a stronger proinflammatory role than those deriving from EPA or DHA. In other words, eicosanoids deriving from n-6 fatty acids stimulate the inflammation process to a greater degree than those deriving from n-3 fatty acids.[3]

Additionally, human and animal studies demonstrated that, dietary supplementation with fish oils results in a decrease in membrane levels of AA, as it is replaced by the n-3 PUFA, and an associated decrease in its capacity to produce AA-derived eicosanoids, as a result of competitive inhibition of oxygenation of AA.[10-13] There is a consequent increase in the level of EPA derived eicosanoids, which have a significant effect on platelet aggrega-

tion, vasoconstriction, neutrophil function, inflammation and immunity.[14] Moreover, n-3 fatty acids decrease the expression of certain adhesion molecules on the surface of endothelial cells,[15,16] monocytes[17] and lymphocytes.[18] Rats receiving a diet rich in fish oil or supplementing the diet of healthy humans with n-3 PUFAs decrease adhesion molecule expression on the surfaces of lymphocytes[19] and monocytes,[20] which was accompanied by functional changes [i.e. decreased adhesion of lymphocytes].[19] Eicosanoids clearly have a direct effect on the function of immune cells and as they themselves can be modulated by n-3 PUFAs, this provides a putative mechanism for their effects. In the present study, rats treated with rat chow enriched with the preparation Supportan Drink showed a different response than those with placebo. Probably the n-3 PUFAs are responsible, since it is the only changed parameter between the groups, that plays a role in the cellular and tissue reaction.

Radiation exposure induces growth factors and cytokine production, such as tumor necrosis factor [TNF-a], and interleukin [IL-1]. TNF-a in its turn, stimulates fibroblast proliferation and enhance inflammatory response. Other factors implicated in the radiation response are basic fibroblast growth factor and platelet-derived growth factor β, which may be associated with late effects of radiation on vessels.[21] Furthermore, radiation generates free radicals, mostly hydroxyl-, responsible for extensive cell damage. N-3 PUFAs and radiation have opposite actions; n-3 PUFAs are able to control, at least to some degree, the adverse effects of irradiation related to the inflammatory process produced. Moreover, n-3 PUFAs have also been found to inhibit interleukin [IL]-1 b, TNF production and act as free radical scavengers.[22,23] Those interactions are insinuated by this study, which showed a dramatically decreased liver microcirculation in groups without n-3 fatty acids, in comparison with the results in the group which was fed with rat chow enriched with n-3 fatty acids. However the multifactorial involvement of n-3 PUFAs does not allow us to elucidate the pathway through which their protective action is promoted.

There is considerable evidence that radiation is implicated in tissue damage. The radiation-induced apoptotic pathways are tightly regulated by the balance between pro-apoptotic and anti-apoptotic molecules.[24] Among them, modulations of Bcl2 family proteins are important to decide the fate of irradiated cells. If the apoptotic pathway does not work properly, the damaged cells have a chance to transform into a carcinoma. The decreased expression of Bcl-XL, which is involved in protection of apoptosis, was also clearly observed in irradiated cells.[25,26] The decreased expression of Bcl-XL protein after radiation was even more prominent in the presence of n-3 PUFAs, implying that the down-regulation of Bcl-XL is increased via a PUFA mediated pathway. Moreover the Bax-indicators were at all moments increased in the n-3 PUFAs group. The interaction of n-3 PUFAs with the apoptosis procedure consists of promotive effects on apoptosis, which results in faster detachment of damaged cell from the irradiated tissue.

To summarize, it seams that enriching the diet with n-3 fatty acids can protect tissues which are adjacent to or for away from irradiated regions from the severity of the inflammatory process due to the adverse effect of irradiation. Further investigation will help to confirm the protective effects of omega 3 fatty acids.

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