The Effect of Vitamin E on Splenocytes Apoptosis of Gamma-Irradiated BALB/c Mice

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ABSTRACT

Apoptosis, a physiologic mechanism to eliminate unwanted cells, is also induced by ionizing irradiation, through production of free radicals. It has been demonstrated that antioxidants such as vitamin E are able to protect cells from damage caused by free radicals. Taken together we found it reasonable to make an attempt to evaluate the protective effect of vitamin E against apoptosis. The irradiated mice received 1 Gy/day gamma radiation for one day (low dose) or for three successive days (high dose, 3Gy). The splenocytes were then isolated at 6, 14 and 24 h after exposure. DNA gel electrophoresis and DNA fragmentation assay were done in addition to the evaluation of splenocytes cytology.

Our results showed that Vitamin E can reduce apoptosis against low dose irradiation. However it is not able to completely block programmed cell death in high dose irradition.

Keywords: Apoptosis; Free Radicals; Gamma Radiation; Vitamin E

INTRODUCTION

Apoptosis is a physiological form of cell death, it occurs in response to a variety of physical stresses including hyperthermia,[1] ionizing irradiation[2] and agents such as glucocorticoid hormones,[3] calcium ionophores,[4] and environmental contaminant such as 2, 3, 7, 8- tetrachloro dibenzo - γ - dioxin.[5]

It has been shown that many kinds of cells including thymocytes, circulating lymphocytes,[6] and resident peritoneal macrophages[7] undergo apoptosis after exposure to clinically relevant doses of ionizing radiation.

Apoptosis could be characterized by several biochemical and morphological changes, including DNA fragmentation, impairment of ATP synthesis, shrinkage of cytoplasm, condensation of nuclear chromatin, endoplasmic reticulum-derived vacuoles and the "bubbling" of cytoplasm.[8,9] The condensed nucleus is fragmented into membrane–enclosed "apoptotic bodies". The final biochemical characteristic of apoptosis is nuclear DNA fragmentation into oligonucleosomal subunits,[10,11] that can be recognized from random cleavage observed in cells undergoing necrosis.

There are several defense mechanisms that protect living organisms against free radicals. Vitamin E, a fat soluble vitamin, is one of the biological antioxidants, which protects cells from oxidative damage. It scavenges free radicals, terminating chain reaction of lipid peroxidation.[12]

Taken together, we aimed to study the effect of vitamin E on low and high doses of γ-irradiation induced apoptosis. In this study BALB/c mice were subjected to γ-irradiation with or without vit. E supplementation. The number of splenocytes were determined, DNA gel electrophoresis and DNA

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fragmentation assay by diphenylamine method were done and cytology of splenocytes were evaluated with Wright-Giemsa staining at 2, 6, 14 and 24 h after completion of irradiation.

MATERIALS AND METHODS

All materials were purchased from Sigma Co. (St. Louis, MO, USA) unless otherwise stated.

Vitamin E

Vit. E was purchased from sigma Co. cat. no. T 3251.

Animals and Diet

Male BALB/c mice (16 – 18 g) at 6-8 weeks old obtained from Razi Institute (Karaj, Iran), were maintained on a regular mice chow diet for a week. The mice were housed, five animals per cage, in transparent plastic box with chip bedding and a stainless steel wire lid. The room temperature was kept at 20 – 22^{o}C with a constant humidity and a 12: 12 h light – dark cycle. Following this adjustment period, mice were divided into two groups (36 mice in each group). Vit. E group, took vit. E 1 g/kg of diet. The diet was prepared manually 1 g vit. E was added to 1 Kg powder diet. Another group took regular chow diet. Vit.E was added to the mice chow 4 weeks before they were exposed to radiation.

Ionizing Irradiation

Whole body irradiation was performed on 12 mice from each group (vit. E or regular chow diet) by ionizing radiation using ^{60}Co-γ-rays from a Gamma cell 220 Machine (Nordian Co., Canada) with a dose rate of 0.1 Gy/s in Iranian energy atomic organization, Tehran.

Splenocyte Isolation

The mice were killed with ether after appropriate incubation time. Their spleens were gently removed and suspended in ice–cold culture medium (RPMI-1640 medium containing 5% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycine). The spleens were then meshed with tweezers to release the splenocytes. The spleen cell suspensions were washed once in culture medium and resuspended in warm isotonic ammonium chloride to lyse the red cells. After centrifugation, the cells were resuspended again in culture medium.

Viable cells number was determined by trypan blue dye exclusion method.[13]

DNA Electrophoresis

Splenocytes were lysed in lysis buffer and incubated with proteinase K (50 µg/ml at 37 ^{o}C for 45-60 min).

The DNA sequentially extracted with phenol/ chloroform/isoamyl alcohol (25:24:1). The aqueous phase was precipitated with two volumes of 100% ethanol at–20 ^{o}C overnight. Pellets were air dried and resuspended in Tris-EDTA buffer (10 mM Tris - HCl, pH 7.8, and 1mM EDTA).[14]

Horizontal electrophoresis of DNA was performed for 2 h at 4 V/cm in 1% agarose gel with 90 mM Tris, 90 mM boric acid, and 2mM EDTA, pH 8.0, as running buffer, DNA was visualized after electrophoresis by ethidium bromide staining.[15]

DNA Fragmentation Assay

A total of 2×10^{6} cells was transferred to a microcentrifuge tube. The cells were lysed with 0.5ml ice-cold lysis buffer (10mM Tris- HCl, PH 7.5, containing 1 mM EDTA and 0.2% TritonX-100). Fragmentated DNA was separated from intact chromatin by centrifugation for 10 min at 13000 ×g, 4^{o}C (preparation B). The supernatant was carefully transferred to a tube (preparation A). 0.5 ml lysing buffer was added to pellet containing preparation B. 0.5 ml of 25% trichloroacetic acid (TCA) was added to the A and B preparations and vortex vigorouly. The tubes were placed at 4^{o}C and left the precipitate over night. The precipitates were centrifuged for 10 min at 13000 ×g. The supernatants were aspirated and discarded. 80µl of 5% TCA was added to each pellet, the DNA was hydrolyzed by heating for 20 min at 83^{o}C in a water bath. 160 µl of diphenylamine solution was added to the test tubes and to a blank containing 80µl 5% TCA. All tubes were vortexed and then left overnight at room temperature. In order to read the optical density (OD), the collected supernatants were transferred to 96 well plate and optical densities were read at 620 nm by ELISA reader. The percentage of fragmentated DNA was calculated according to the following formula:

$$\% \text{ fragmented DNA} = \frac{OD\ 620\ tubeA}{OD\ 620\ tubeA\ + OD\ 620\ tubeB}$$

Data Analysis

Two way analysis of variance (ANOVA) were used in statistical calculation as appropriate. The results are presented as the mean ± S.D. P-value<0.05 was considered significant.

RESULTS

Vitamin E Suppressed Apoptosis in the Mice Received 1 Gy γ-Irradiation for One Day

Mice were killed with ether and splenocytes were isolated 2,6,24 hr. after irradiation.

Cell Counting

As seen in table 1, at 2 h after radiation we did not find any significant change in the cell numbers of irradiated groups with or without vit E supplementation compared with control, but the results of cell counting 6 h. after irradiation showed that in group without vit E supplementation the cell number significantly decreased compared to their control ($1.8x10^7$ decreased to $0.56x10^7$) and 24h ($1.87x10^7$ decreased to $0.7x10^7$). Also, in group without vit E, there was significant change in cell number after 6 and 12 h. irradiation as compared to the respective controls. However, in group with vit E, there was no significant change in cell number as compared to the respective controls (Table 1).

DNA Fragmentation Assay

As seen in table 2, significant increase was observed for the DNA fragmentation in the splenocytes isolated at 2 and 6 h after irradiation for both supplemented and non-supplemented groups compared to the controls. However, the results of DNA fragmentation at 24 h. after irradiation showed no significant change in groups with and without vit E, as compared to the controls.

DNA Electrophoresis

Electrophoretic analysis of DNA at 2 h. after irradiation showed typical ladder pattern in groups with and without vit E (Figure 1, Lane 3,4) but there was no ladder pattern in unirradiated splenocytes .

Electrophoretic analysis of DNA after 24 h in one day irradiation showed no ladder in each group (Figure 2). These results confirm the results of DNA fragmentation assay.

Vitamin E was not Able to Suppress Splenocytes Apoptosis of Mice Received γ-Irradiation 1 Gy/Day for 3 Successive Days

After the first step, the mice subjected to 1 Gy/day radiation with the same dose rate but for 3 successive days in order to observe the effect of vit E on higher doses of γ-irradiation.

Cell Counting

The results presented in table 3 shows that irradiation caused a significant decrease in cell

Table 1. The splenocytes were isolated at 2,6 and 24 hr after the completion of irradiation and viable cells were determined by trypan blue exclusion ($x10^7$).

Group \ Cell viability / spleen		Hours after one day irradiation		
		2	6	24
Without vit. E	Irradiated	1.132±0.265	0.56±0.262*	0.7±0.175*
	Unirradiated	1.015±0.049	3.745±1.377	2.228±0.534
With Vit. E	Irradiated	2.345±0.94	1.8±0.69	1.871±0.33
	Unirradiated	2.555±0.494	2.45±0.643	2.003±0.136

* Significant difference between irradiated and unirradiated groups p<0.05

Table 2. The splenocytes were isolated at 2,6,24 h after 1Gy /day irradiation in one day and percentage of DNA fragmentation assayed by diphenylamine method.

Group \ % DNA fragmentation		Hours after one day irradiation		
		2	6	24
Without vit. E	Irradiated	7.045±1.308*	8.92±0.622*	3.48±0.24
	Unirradiated	2.605±0.516	3.12±0.484	3.44±1.28
With Vit. E	Irradiated	6.721±0.721*	6.85±0.694*	4.64±0.0424
	Unirradiated	3.88±0.777	3.68±0.695	4.506±1.265

* Significant difference between irradiated and unirradiated groups p<0.05

Table 3. The splenocytes were isolated at 6, 14, 24 h after 3 successive days irradiation and viable cells were determined by trypan blue exclusion(x10^7).

Group	Cell viability / spleen	Hours after 3 days irradiation 6	14	24
Without vit. E	**Irradiated**	0.228±0.017*	0.241±0.098*	0.1*
	Unirradiated	1.596±0.526	1.193±0.111	1.208±0.226
With Vit. E	**Irradiated**	0.702±0.286	0.99±0.5	0.584±0.151*
	Unirradiated	1.781±0.459	1.66±1.09	1.383±0.48

* Significantly difference between irradiated and unirradiated groups p<0.05

numbers in the group without vit E. It was also demonstrated that vit E did not inhibit the decrease of number of splenocytes when the sampling was done 24 h after radiation. However, vit E supplemented groups had more viable cells than non-supplemented groups.

DNA Fragmentation Assay

Table 4 shows the results of DNA fragmentation assay in which groups without vit E, did not show any significant changes in DNA fragmentation, because the damage caused cell death and we did not observe

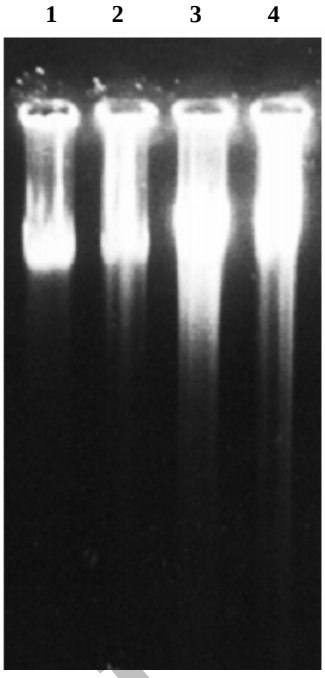

Figure 1. Electrophoretic analysis of DNA 2 hours after one day irradiation, lane 1. group without vit E, without irradiation, lane 2. group with vit E, without irradiation, lane 3. group without vit E, with irradiation, lane 4. group with vit. E, with irradiation.

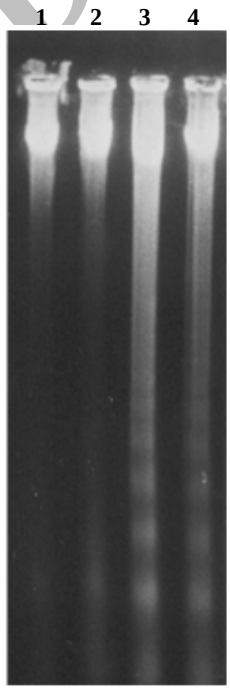

Figure 2. Electrophoretic analysis of DNA 24 hours after one day irradiation, lane 1. group without vit E, without irradiation, lane 2. group with vit E, without irradiation, lane 3. group without vit E, with irradiation, lane 4. group with vit. E, with irradiation.

Table 4. The splenocytes isolated at 6, 14, 24 h after 3 successive days irradiation and the percentage of DNA fragmentation assayed by diphenylamine method.

Group	% DNA fragmentation	Hours after 3 days irradiation 6	14	24
Without vit. E	**Irradiated**	2.983±0.028	2.7±0.626	5.25
	Unirradiated	2.715±1.149	2.216±0.317	5.33±0.3
With Vit. E	**Irradiated**	4.943±0.695*	2.626±0.0374*	10.07±2.207*
	Unirradiated	3.133±0.988	1.916±0.274	4.54±0.635

* Significant difference between irradiated and unirradiated groups p<0.05

any DNA fragmentation even after 6 hours. But in groups with vit E, we observed a significant increase in DNA fragmentation as compared to controls.

Wright–Giemsa Staining

The splenocyte showed disruption of nucleus under the microscope. There was a fragmentated nucleus in figure 3a and condensed chromatin in figure 3b and also vacuoles in cytoplasm of apoptotic cell in figure 3c. These results confirm the other findings presented in this study (Figure 3).

DISCUSSION

The results at 2 h. after 1 Gy gamma irradiation showed an increase of DNA fragmentation without decreasing the cell numbers. It would suggest that splenocytes underwent apoptosis; however, the cells were still alive and they were not completely eliminated. The data obtained from 6 h. period after irradiation showed an increase in the DNA fragmentation but the group received vit. E supplementation, demonstrated no reduction in viable cell numbers. These results would propose the radioprotective effect of vit. E in low dose of γ-irradiation.

However, a considerable disruption of apoptotic cells was occurred at 24 h. after 1 Gy irradiation in group irradiated without vit. E supplement. Thus DNA fragmentation was not seen. In parallel, the results of cell numbers showed protective effect of vit. E at 24 h. after radiation. The results of DNA gel electrophoresis were in consonance of the above data.

An in vitro study on human lymphocytes had documented the radioprotective effect of antioxidant.[16] Biochemical evaluation had shown that membranes are the sensitive targets in gamma radiation.[17] Also, Petryna has shown in rats that vit E level decreased after exposure to small and high doses of gamma irradiation.[18] Since vit E incorporates into membranes where it plays its antioxidant role we used it as radioprotective supplementation in mice. Based on our data it could completely inhibit apoptosis induced by 1 Gy irradiation.

Mcclain, et al, have shown that levels of DNA fragmentation in MOLT-4 cell line not treated with Trolox were directly related to both radiation dose and time of post irradition.[19] Also, Nair CK, et al, have shown that alpha-tocopherol monoglucoside (TMG), a water soluble derivative of vit E offers protection against deleterious effect of ionizing radiation, both under in vitro and in vivo conditions.[20]

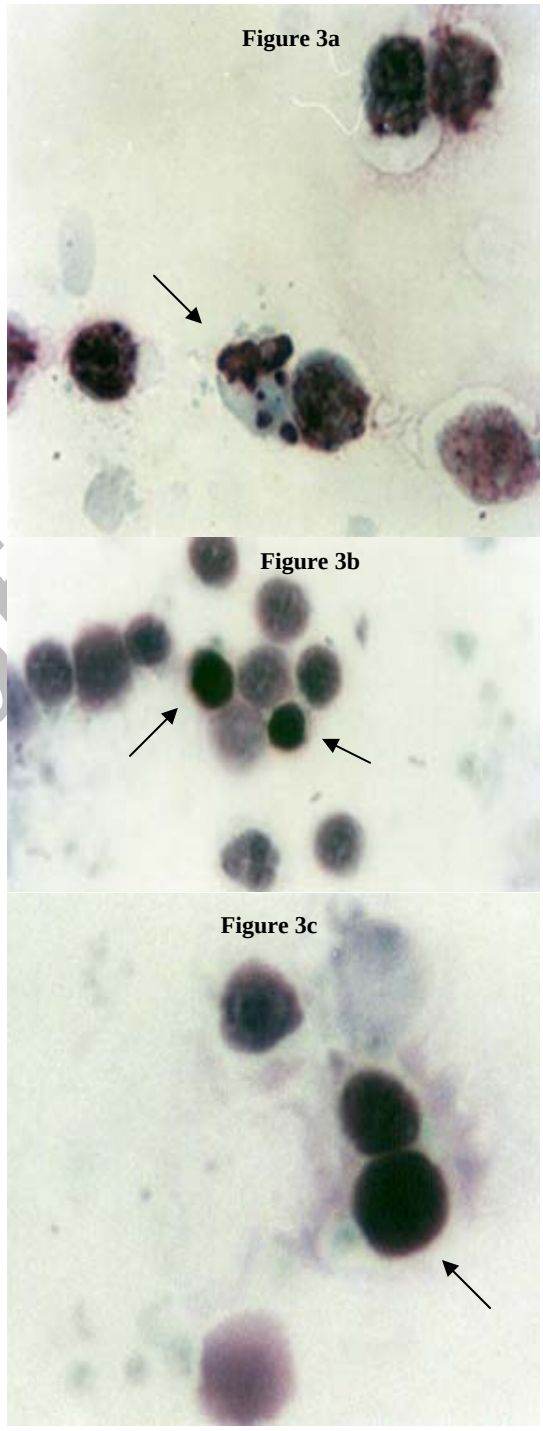

Figure 3. Wright–Giemsa staning of spleen cells after one day irradiation; 3a. fragmented nucleus; 3b. condensed chromatin; 3c. vacuoles in cytoplasm, of apoptotic cells.

In order to observe whether vit. E can also exert its protection in higher dose of γ-irradiation, the second set of experiments were designed. Dose of vit. E was kept as the previous section but the duration of γ-irradiation was increased up to 3 days. 6 and 14 h. after completion of radiation the sampling were done. It was found that vit E supplemented group showed a slight decrease in the cell number ($p>0.05$).

In contrast, in the group which did not take vit. E, it was found a dramatic decrease in the number of splenocytes. The results of DNA fragmentation assay showed a significant increase in DNA fragmentation in vit E supplemented groups as compared to the control but there was no significant change in DNA fragmentation assay in group without vit. E. It indicates that all sensitive cells eliminated in group without vit E after 3 days irradiation. However when the sampling was done 24 h. after irradiation, it was demonstrated that vit E can only delay the programmed cell death and can not completely protect splenocytes from the cell death caused by higher dose of γ-irradiation.

In conclusion, we showed that radioprotective effect of vitamin E is dependent on the dose–rate of γ-irradiation, but vit. E does not block splenocytes apoptosis when the cells exposed to the higher dose of γ-irradiation, which could be due to partial ability of vit. E to eliminate free radicals from micro environment.

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