Biosaintifika 9 (2) (2017) 201-208

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

Capability of Vitamin E as a Radioprotector in Suppressing DNA Damage Determined with Comet Assay

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DOI: 10.15294/biosaintifika.v9i2.8716

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History Article

Received 28 December 2016
Approved 14 May 2017
Published 17 August 2017

Keywords

DNA damage; vitamin E; radioprotector; comet assay

Abstract

Radiation has a potent to damage cells. Radiation may act directly or indirectly on deoxyribonucleic acid (DNA) that results in the degeneration of tissues and necrotic, and thereby it needs a potent radioprotector to prevent these damages. Vitamin E is natural product known as an antioxidant which has potential as radioprotector. This research aimed to determine the capability of vitamin E with emphasized on the searching for its optimal concentration as radioprotector of DNA damage. This study used blood samples of healthy person irradiated with gamma rays at a dose of 6 Gy as the lethal dose to lymphocytes. The cocentrations of vitamin E from 0 to 0.8 mM was added into blood 15 minutes before irradiation. Isolation of lymphocytes was done using gradient centrifugation method. Evaluation on the capability of this compound in suppressing DNA damage was done by using alkaline Comet assay and data analysis was done using CaspLab program. The results show that addition of vitamin E could suppres these DNA damages and 0.8 mM of vitamin could reduce DNA damage up to 94.2%. We conclude that vitamin E effectively suppresed DNA damages induced by radiation. This information may benefit to the patient from negative impacts of radiotherapy.

How to Cite

Darlina, A, L. D., Alatas, Z., Kisnanto, T. & Syaifudin, M. (2017). Capability of Vitamin E as a Radioprotector in Suppressing DNA Damage Determined with Comet Assay. *Biosaintifika: Journal of Biology & Biology Education*, 9(2), 201-208.

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p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

When a track of ionising radiation passes through a cell it will deposit energy which can disrupt the organic molecules mainly that most sensitive molecule within the cell i.e. deoxyribonucleic acid (DNA) which is the most target and termed as the blueprint of life. It is well known that free radicals formed by the radiolysis of cellular aqueous milieu, and their interaction with one another and with oxygen are primary mediators of radiation injury (Hall & Giaccia, 2006; Tetriana et al. 2015). Ionizing radiation induce the production of Reactive Oxygen Species (ROS) through hydrolysis of water. Including superoxide, hydrogen peroxide, and hydroxyl radicals as the most reactive radicals. Such ROS can initiate oxidative cellular injury as well as activated intracellular signaling pathways and stimulated cytochrome c release from mitochondria leading to apoptosis (programmed cell death) (Azzam et al., 2012).

Radioprotectors are compounds that are designed to reduce damage occured in normal tissues caused by ionizing radiation. These compounds are mostly antioxidants and must be exist before or at the time of radiation for effectiveness as protector (Citrin et al., 2010). This radiation modifier or protectors is expected to alter the response of normal tissues to irradiation via free radical scavenging and/or H atom donation (Rahman et al., 2015). Radioprotectants are also important in suppressing the accumulation of genetic mutations, cell death or tissue disorganisation in patients undergoing radiotherapy or individuals exposed to non-lethal, but higher than normal, levels of radiation in accidental event (Liu, 2010).

Although endogenous antioxidant systems such as glutathione, thioredoxin, superoxide dismutase, and catalase normally inhibit the deleterious effects of ROS, these systems may be overwhelmed in irradiated cells. Exogenously supplemented antioxidants, or agents that stimulate endogenous antioxidant systems within cells, have shown promise in terms of suppressing the harmful effects of irradiation. A variety of reducing agents, such as vitamin E analogs, polyphenols, thiols and superoxide dismutase mimetics have been described as potential radiation countermeasures in the recent past (Singh et al., 2012; Weiss et al., 2009; Dumont et al., 2010).

Natural products which benefit to human health have been attractive targets for research (Singh et al., 2012). For both prevention and therapy of human diseases, these compounds are common in our diets and are often perceived as being more 'natural' and better suited for medicinal purposes due to being well tolerated and minimally toxic even at the upper ranges of dietary intake. Vitamins are prominent among natural compounds considered beneficial for human health (Satyamitra et al., 20111). Vitamin E is well known as antioxidant, neuroprotector, and also anti-inflammatory properties (Singh et al., 2013). It is essential because body cannot produce vitamin E so it should be obtanied from food supplements. Vitamin E represents a generic term for all tocopherols and their derivatives with naturally occurring and biologically active stereoisomeric compounds of α-tocopherol (AT) (Palozza et al., 2008).

Comet assay, also known as single-cell gel electrophoresis, is a simple method for measuring DNA strand breaks in eukaryotic cells. The technique includes cells embedding in agarose on a microscope slide, lysis with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix, electrophoresis at high pH results in structures resembling comets, and observation with fluorescence microscopy. Finally the analysis is that the intensity of the comet tail relative to the head reflects the number of DNA breaks (Olive et al., 2006; Speit & Hartmann, 2005; Singh et al., 1988). The purpose of this study was to examine the capability of vitamin E as radioprotector in suppressing the radiation induced DNA damage The benefits of this research are to protect normal tissue from radiation damage due to radiotherapy in cancer patients..

METHODS

Vitamin E was provided as dl-α-tocopherol soft capsule at a concentration of 250 IU (Catalent, Australia). A 1 mM stock solution of vitamin E was prepared in polyethilene glicol (PEG) mixed wtih Tween 20 solution and serial dilutions with PBS were made in order to achieve a working concentration of 0.2 mM; 0.4 mM; 0.6 mM dan 0.8 mM of vitamin E .

The study was performed on peripheral blood samples obtained from two healthy males, non-smoking, non-alcoholic donors with ages of 30 and 50 years. The donors are never exposed to ionizing radiation. Three mL venous blood were collected under sterile conditions in vacutainer tubes (Becton Dickinson, NJ, USA) containing lithium heparin as anticoagulant. After collection, the blood was divided into 6 tubes of samples.

The present study was carried out in 6

groups, group 1: control (cells without radiation), group 2 : radiation control (RC: cells were exposed to 6 Gy of gamma radiation), group 3: cells treated with 0.2 mM vitamin E before radiation exposure, group 4: cells treated with 0.4 mM vitamin E before radiation exposure, group 5; cells treated with 0.6 mM vitamin E before radiation exposure, and group 6: cells treated with 0.8 mM vitamin E before radiation exposure.

Before radiation, blood samples were incubated for 15 minutes with the serial concentration of vitamin E. The blood samples were then irradiated with gamma radiation in the ice. The source of gamma radiation used was Cobalt-60 (IRPASENA, PATIR, BATAN). All of the blood samples were irridiated with dose of 6 Gy, at dose rate of 1 Gy/minutes as a lethal dose of gamma radiation for lymphocytes cells. Lymphocyte cells were isolated 5–15 minutes after radiation and examined for induced DNA damages using comet assay. One blood sample without antioxidant served as control in both series, and these samples were also irradiated with 6 Gy.

After radiation, lymphocyte were isolated from the blood samples using Histopaque (Sigma) according to standard method (Panda et al., 2012). Three mL of fresh heparinized blood was mixed with the equal volume of phosphate buffered saline (PBS, Merck), which was overlaid on 3 mL of Histopaque. Lymphocytes were separated using density gradient centrifugation. Then the separated lymphocytes were washed twice with PBS by centrifugation for 15 min at 1000 rpm and cells were suspended in minimum volume of RPMI -1640 (Gibco).

Cell viability was measured using trypan blue dye exclusion method (Chung et al., 2015). The lymphocytes were mixed with equal volume of 0.4% trypan blue dye for 3 minutes and counted using haemocytometer. Viable and dead cells were scored under the microscope.

About 10^4 cells per 100 µL of medium was taken from each dose treatment for Comet assay by following the standard procedure with slightly modification (Singh et al., 1988). Fully-frosted microscopic slides were prepared. Each slide was covered with 1% Normal Melting Point (NMP) agarose (Sigma). After solidification, the slides were then coated with 0.6% NMP agarose. A Low Melting Point (LMP) agarose was melted and stabilized in a waterbath (RTE10) at 37°C. For each sample and control, 5 µL of cell homogenate was mixed with 100 µl of 1% LMP agarose and placed on the slides. After 10 minutes of solidification on ice, the slides were covered with 0.5% LMP agarose. The slides were then immersed in a pre-chilled lysis solution ((2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris–HCl, adjust until pH 10 with NaOH (Sigma) and added 1% Triton X-100 (Sigma) and 10% dimethyl sulfoxide (Sigma) and kept in at 4°C for 60 minutes. The slides were placed horizontally in a humidity chamber at 37 °C for 30 minutes. All slides were then immersed in an alkali solution (0.3 M NaOH, 1 mM Na2EDTA; pH 12.1) for 40 minutes. Electrophoresis in a pre-chilled alkali solution (0.3 M NaOH, 1 mM Na2EDTA; pH 13) at 1 V/cm was done for 20 minutes in refrigerator (4°C). After electrophoresis, the slides were rinsed gently three times with neutralization buffer (0.4 M Tris–HCl, pH 7.5) to remove excess alkali and detergents. Each slide was fixed with methanol, stained with ethidium bromide (20 µg/ml) and covered with a coverslip. Slides were stored at 4°C in sealed boxes until analysis.

The stained samples with ethidium bromide were observed using a Nikon flourescence microscope. A total of 50 randomly captured comets from each slide were examined at 250x magnification using an epifluorescence microscope that connected through computerized to an image analysis. Cells were piled not counted. The image of comet was digitally analyzed using *CASPLab comet assay software.*

Each experimental set consists of duplicated slides. The various parameters measured in the exposed and control groups were evaluated using Excell program (StaSoft, Tulsa, USA). Each sample was characterized for the extent of DNA damage by considering the mean, SE (standard error of the mean), median and range of the comet parameters.

RESULTS AND DISCUSSION

The viability cells in this research was observed under microscope. The results showed that the number of cells in all groups was in enough number (around 10^7 cells/mL) and suitable for Comet assay. In this research, the DNA damage of irradiated lymphocytes was assessed by comet assay by staining the cells with ethidium bromide and the comet that mainly consist of single stranded DNA can be seen with a fluorescence microscope as presented in Figure 1.

Results of visualization showed that lymphocyte cells irradiated with gamma ray formed tail of comet due to DNA damage in the form of breaking one DNA strand (single strand break/SSB) and the rupture of both strands of DNA at the opposite position (double strand breaks/DSB). The DNA damage can be estimated

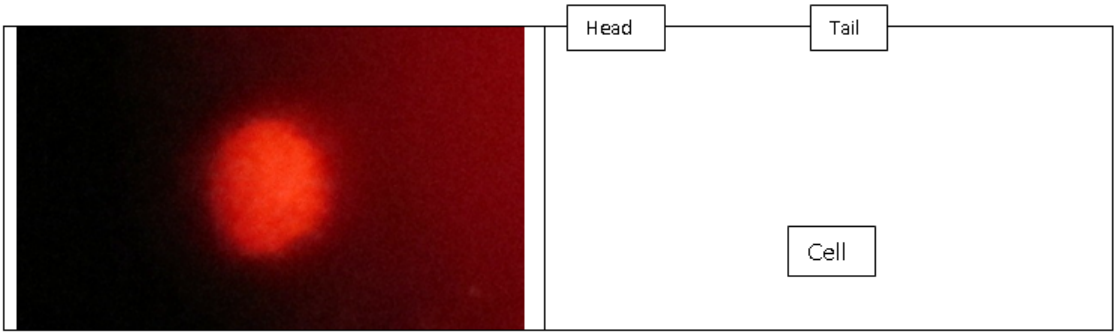

Figure 1. Results of visualisation of DNA stained with EtBr in Comet assay of the lymphocytes of control blood sample (left) and that exposed to 6 Gy dose of gamma radiation with tail of comet (right).

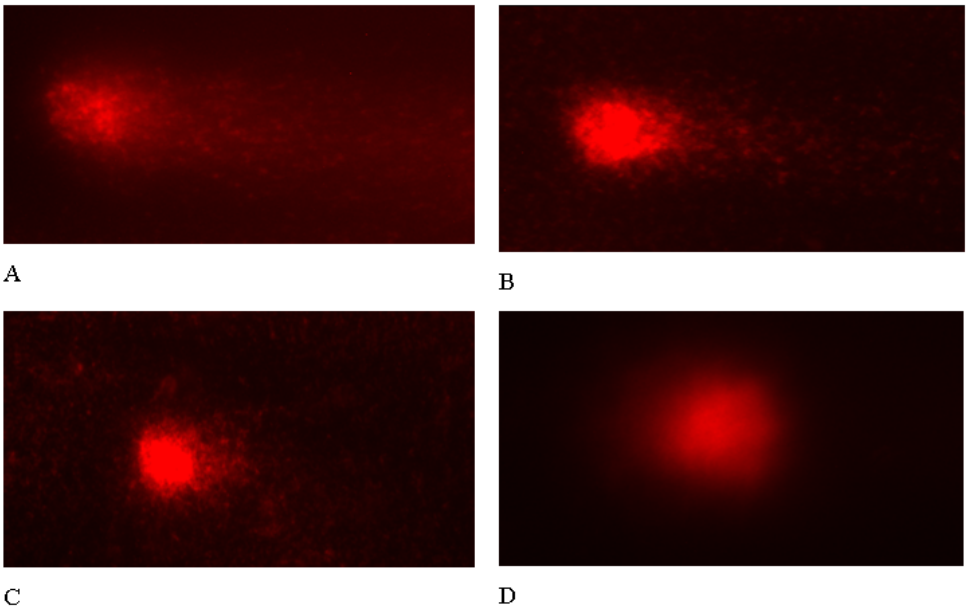

Figure 2. Visualization of comet of lymphocyte irradiated with 6 Gy and treated with vitamin E at consentrations of 0.2 mM (A), 0.4 mM (B), 0,6 mM (C), and 0.8 mM (D).

by measuring the length of the comet tail using an ocular scale fitted in the eyepiece of the microscope or by visual scoring of degree of damage from 0 to 4 according to comet appearance [Figure 1]. Alternatively, there are numerous image analysis software to quantitate additional DNA damage parameters such as percentage of DNA in head, percentage of DNA in tail, tail moment (product of tail length and percentage of DNA in tail), and tail area.

In order to avoid or inhibit these DNA damages we treated the blood with an antioxidant vitamin E before irradiation. In this study four concentrations of vitamin E (0.2; 0.4; 0.6; and 0.8 mM) were added to the blood 15 minutes before irradiation. All of the results are presented in Figure 2.

From the visualization presented in Figure 2, it can be seen that there is a shortening the tail of comet of lymphocytes treated with vitamin E. Higher concentration of vitamin E resulted in shorter tail of comet which means that there is a reduction of DNA damages in the presence of vitamin E and this treatment effectively reduce the deleterous effects of gamma radiation.

The comet itself can be analysed by visual scoring or computerised image analysis. In this research the comet were analyzed using CASPLab Comet Assay software which reported using a range of different endpoints. This comet assay sofware can measure 8 parameters of the digital image of comet value : Long-tailed comet (TL), DNA Tail, Tail Moment (TM), Olive tail moment (OTM), DNA head, DNA Percentage in head, and the length of comet (L.comet). Head DNA that indicating the number of DNA in head of comet is an additional parameter in computerized program of CASP Lab.

The graph in Figure 3 demonstrate the relationship between vitamin E concentration

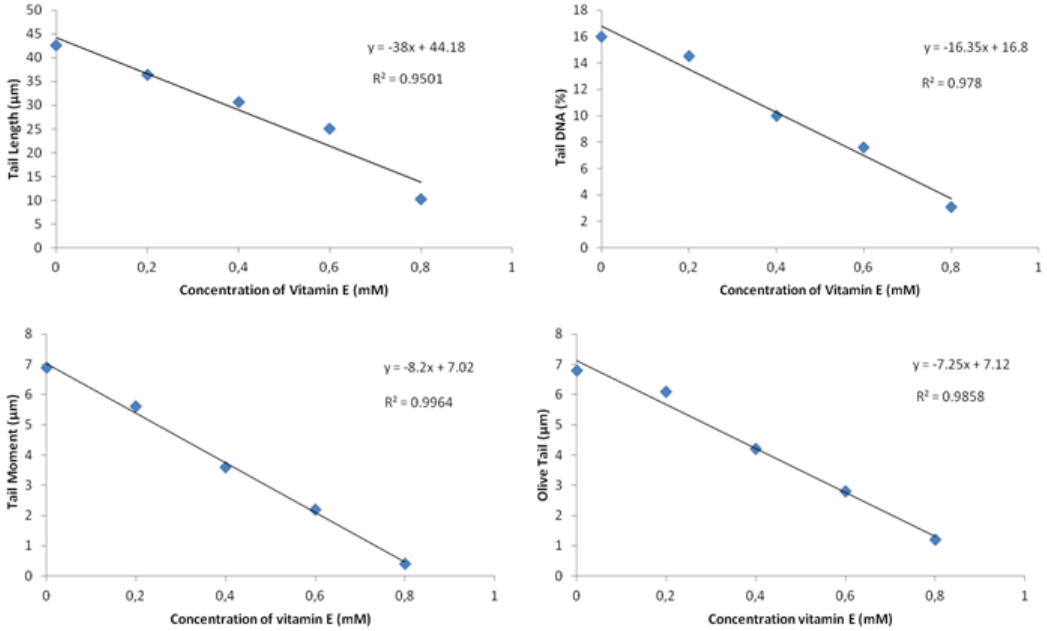

Figure 3. The effect of vitamin E addition on the four parameters of Comet assay (DNA fragment) where DNA damage was reduced by vitamin E with each correlation coefficient (R^2).

and DNA damages induced by 6 Gy gamma radiation. Here we found that each parameter has tendention to decrease by increasing the concentration of vitamin E added (Figure 3). The correlation coefficient (R2) for every parameter : (a) tail moment (TM), (b) olive tail moment (OTM), (c) tail DNA, and (d) tail length (TL) more than 0,9 indicating a very good relationship.

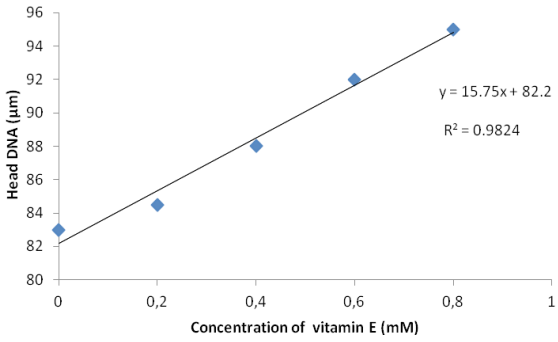

Figure 4. Relationship between *head DNA* with the increasing of vitamin E concentration

Figure 4 shows that head DNA and the concentration of vitamin E have a positive correlation meaning that higher concentration of vitamin E higher number of DNA in head. This finding indicating that vitamin E effectively reduce DNA damage due to ionizing irradiation. Among comet tail parameters, TM gave the highest percentage of reducing damage given with 0.8 mM of vitamin E. Based on 5 parameters in comet test it is known that vitamin E is a good radioprotector in suppressing DNA damage post irradiation with R^2 more than 0.9.

The percentage reduction in DNA damage to increase the concentration of vitamin E in the fourth comet tail parameters are shown in Table 1. Seen the higher the concentration of vitamin E is added before irradiation can reduce radiation-induced DNA damage. The value of TM provides the highest percentage decrease DNA damage the four parameters of comet tails. The decreasing of DNA damage percentage shown in the highest value of TM at a concentration of 0.8 mM vitamin.

The cell-type-of-choice in biomonitoring research activities is mostly the lymphocyte because blood is easily collected and lymphocytes have proved to be good surrogate cells. In Comet assay the process of electrophoresis is done under the alkaline conditions where the strand breaks through their ability to relax DNA supercoiling, allow the negatively charged DNA loops to extend towards the positively charged anode (Singh et al., 1988).

Similar research was performed by Singh et al. (2013) who has done an *in vivo* study to determine the effect of tocopherol succinate (TS) treatment against DNA damage caused by ionizing radiation in peripheral blood mononuclear cells, splenocytes and thymocytes of mouse. The mice were treated with vehicle or TS (400 mg/kg) and exposed to high dose (9.2 Gy that is the $LD_{90/30}$ dose that causes hematopoietic injury) of ^{60}Co γ-radiation 24 h after drug injection. Peripheral blood, spleen and thymus were collected 30

Table 1. Percentage of decreasing in DNA damage due to the administration of vitamin E± SD.

Concentration of Vitamin E (mM)	Decreasing in DNA damage (%)			
	Tail length	Tail DNA	Tail moment	Olive tail moment
0	0	0	0	0
0.2	14.6±8.3	9.3±6.5	18.8±4.0	10.3±2.0
0.4	28.0±6.6	37.5±4.4	47.8±2.4	39.1±8.0
0.6	41.4±13.0	52.5±5.7	68.1±2.5	58.8±2.3
0.8	75.8±5.0	80.6±2.0	94.2±0.4	82.3±0.9

minutes and 4 h after irradiation, and used for alkaline comet assay. The administration of TS significantly inhibited DNA damage in peripheral blood cells and thymocytes compared with vehicle-treated mice, evidenced by the shorter tail length and smaller percentage of DNA in the comet tail. Another study conducted by Yassa et al. (2011) aimed to investigate potential protective vitamin E against pesticide diazinon (DZN) in murine. The results showed that mice treated with vitamin E reduced DZN- induced DNA damage where the length TL shortened by up to 50%. These results suggest that vitamin E has a protective effect on DZN-induced DNA and showing that vitamin E prevent genotoxicity induced by DZN. It indicates that vitamin E is also effectively suppressing the harmfull effects induced by other chemical.

Since many types of radiation are now being frequently used in clinical treatment of patients with cancer and in exprimental research, it is essential that more detailed information on the chemical capabilities in minimizing the effect of irradiation be obtained. In this research, like other studies, we are searching for the efficacy of natural chemical in suppressing the negative effects of ionizing radiation. And many studies had shown that vitamin E can scavenge molecular oxygen, peroxide and hydroxyl radicals and atomic oxygen radicals induced by ionizing radiation.

The understanding in radiation effects has placed emphasis on the search for antioxidant agents that are suitable as radiation countermeasures (Sing et al., 2012; Weiss & Landauer, 2009; Dumont et al., 2010). Although endogenous antioxidant systems (glutathione, thioredoxin, superoxide dismutase, and catalase) normally inhibit the deleterious effects of ROS, these systems may be overwhelmed in irradiated cells. Exogenously supplemented antioxidants, or agents that stimulate endogenous antioxidant systems within cells, have shown promise in terms of suppressing the harmful effects of irradiation. If present in the cells at the time of radiation exposure, such antioxidants may protect cells from radiation damage by scavenging ROS before they act on cellular components. A variety of reducing agents, such as vitamin E analogs, polyphenols, thiols and superoxide dismutase mimetics have been described as potential radiation countermeasures in the recent time.

Here we tested the potentail of vitamin E as a Radioprotector to lethal dose of gamma ray. It was approved that administration of vitamin E modulated the expression of antioxidant enzymes and inhibited expression of oncogenes in irradiated cells (Singh et al., 2013). Vitamin E is a group of eight structurally related fat-soluble vitamins, four tocopherols (α, β, γ, and δ), and in more detail, acting as antioxidants by preventing the propagation of free radical reactions by donating hydrogen from their phenolic group to stabilise the radicals, and thereby break the chain of events leading to oxidative damage. The tocopherols protect the structure and function of human cell membranes (Vasilyeval & Bespalov, 2015; Niki, 2014; Satyamitra et al., 2011). And here we treat the blood with vitamin E 15 minutes before irradiation. This is due to the fact from some other published results (Singh et al., 2013; Maurya et al., 2006; Ghosh et al., 2009; Citrin et al., 2010) that vitamin E did not protect mice when administered as a mitigator after irradiation and it can be used only as a radio-protector of DNA which is a target for mutagens and carcinogens, and induce changes in DNA structure giving rise to mutations and/ or cell death.

Nair et al. (2003) had been done a very comprehensive study on the efficacy of tocopherol monoglucoside (TMG) as radioprotector and they found that, this chemical, which is a water soluble derivative of vitamin E, offers protection against the deleterious effects of ionizing radiation, either in vivo or in vitro studies, to biological systems. It has a potent antioxidant and an effective free radical scavenger, so that it protects DNA from radiation-induced strand breaks formation. It also protected thymine glycol formation induced by gamma-radiation. It prevents gamma-radiation-induced loss of viability of EL-

tumor cells and peroxidation of lipids in microsomal and mitochondrial membranes. It reduce upto 75% embryonic mortality resulting from exposure of pregnant mice to ionizing radiation (2 Gy) by ip administration (0.6 g/kg, body wt) prior to irradiation. This chemical TMG also offered protection to mice against whole body gamma-radiation-induced lethality and weight loss. Study also showed that the LD50(30) of mice increased from 6 to 6.72 Gy upon post irradiation administration of a single dose of TMG (0.6 g/kg, body weight) by intraperotoneal route.

Vitamin E can alleviate radiation induced decrement in delayed-type hypersensitivity and as adjuvant to other radioprotectant. A very old and simple research conducted by Mahdy (1991) from Middle Eastern Regional Radioisotope Centre for the Arab Countries, Cairo, in Egypt found that intraperitoneally injection of vitamin E before whole body gamma irradiation at the dose of 7 Gy to female albino rats remarkably recovered in the serum protein content at all post-irradiation days, while it slightly recovered in the level of serum urea.

Basically there are some mechanisms of this chemical in its action. One investigator revealed that vitamin E act by preventing lipid peroxidation which does not generally play a major role in cell killing by ionizing radiation. Other suggested the chemical by scavenging of secondary radicals but it needs a very high concentration to effectively prevent DNA damage which is responsible for classical reproductive cell death, or by suppressing the protracted oxidative stress which is difficult to be distingusihed from its role as scavenging radicals, decreasing oxygen concentration which is due to the fact that hypoxic cells are radioresistant so that treatments that decerase microenvironmental oxygen can be radioprotective, enhancing the DNA repair which is a major factor in determining the radiosensitivity (Bump, 1998).

Borek (2008) proposed that vitamin E is an antioxidant and its radiprotective action is depend on the oxygen partial pressure in tissue and it was shown that this chemical effectively protect tissue from deletirius effects of ionizing radiation in high oxyen pressure such as lung. Recent studies have suggested several proposed alternative mechanisms: most notably, an indirect effect of tocopherols in eliciting specific species of radioprotective growth factors or cytokines such as granulocyte colony-stimulating factor (G-CSF). The vitamin E treatment in irradiated group of rat presented more acinar cells than the irradiated group, but no statistically significant difference was observed ($p>0.05$). They conclude that vitamin E seems to have failed as a radioprotective agent on acinar cells in rat parotid glands (Gomes et al., 2013).

Our results demonstrate that vitamin E has the potential to protect DNA damage lynphocyte cell from radiation injury . As is known radiotherapy given to patients often results in immunologic cell damage resulting in decreased immunity. Lymphocyte cells have immune cells an important role in immunity. This study are useful to enrich radioprotectant information that can protect radiotherapy patients from the effects of radiation

CONCLUSIONS

Ionizing radiation at a dose of 6 Gy, which is equivalent to highly lethal doses for humans, effectively cause the DNA damage. This study shows that the addition of vitamin E is significantly suppressed the DNA damages at all concentration tested, concentration of 0.8 mM of vitamin E could reduce DNA damage up to 94.2%. It was given just before the irradiating gamma rays

ACKNOWLEDGEMENTS

This work in part was financially supported by annual project of the Center for Technology of Radiation Safety and Metrology, National Nuclear Energy Agency (project year of 2014).

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