Concerted Effects of L-carnitine and Vitamin E on Cardiopulmonary Apoptosis Induced by Gamma Irradiation in Rats

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**Introduction**

Radiotherapy is a key modality in the treatment for a wide range of malignant tumors (Kang et al., 2010; Zagar et al., 2010; Biagioli & Hoffe, 2010 and Khana & Alhomidaa, 2011). It destroys cancer cells, but it also affects some of the normal cells nearby, induce severe acute and chronic side effects (Armstrong et al., 2010). Ionizing radiation has been shown to generate reactive oxygen species (ROS) in a variety of cells. These ROS have the potential to damage critical cellular components such as DNA, proteins, and lipids and eventually results in physical and chemical damage to tissues that may lead to cell death or neoplastic transformation (Pang et al., 2011; Buonanno et al., 2011 and Hua et al., 2015). Oxidative stress caused by overproduced ROS has been known to be an important mechanism involved in inflammation and cardiac disease, such as ROS-induced JNK pathway and apoptotic signaling activation (Chen et al., 2013). As ROS appear to be mediators of the apoptosis by ionizing radiation, factors, that regulate the fate of such ROS, may be of a great importance in the protection of cells against ionizing radiation-induced cell death.

L-carnitine (β-hydroxy-γ-N-trimethylammonium butyric acid, L-car) is a naturally occurring quaternary ammonium compound biosynthesized from amino acids lysine and methionine (Chao et al., 2011). L-carnitine plays an essential role, as a cofactor, in the transport of long-chain fatty acids from the cytosol to the mitochondrial matrix as acylcarnitine esters where β-oxidation takes place (Şıktar et al., 2011 and Khana & Alhomidaa, 2011). L-car has a high antioxidant potential, shows

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free radical scavenging activity and prevents the accumulation of free fatty acids and their toxic intermediates, thus preventing their harmful effects on mitochondrial and cell membranes (Dunning & Robker, 2012 and Li et al., 2012). L-car is used in the prevention and treatment of oxidative stress and related health problems (Ahmed et al., 2014 and El-sherbini et al., 2017). L-car has a protective effect against the toxic actions of different drugs, which induces oxidative stress and/or carnitine deficiency. It is used to treat cyclophosphamide and ifosfamide-induced cardiotoxicity (Sayed-Ahmed et al., 2014) and amiodarone-induced pulmonary toxicity (Gado & Aldahmash, 2013) in addition to neurodegenerative disorders (Jones et al., 2010).

Vitamin E (Vit E) is a physiologic component of cellular membranes, a potent lipid-soluble, chain-breaking antioxidant that plays a crucial role in the protection of cellular membranes from damage caused by ROS (Durmus et al., 2011). The most active form of Vit E in humans is α-tocopherol. Vit E is a free radical scavenger, thus preventing damage to cell structures and physically stabilizes membrane permeability and fluidity (Üçüncü et al., 2006).

Therefore, this study has been initiated to study the protective potential of Vit E and/or L-car against gamma irradiation-induced oxidative injury via apoptotic pathway.

Materials and Methods

Drugs and chemicals
Vitamin E and L-carnitine, were obtained from Sigma Chem. Co (Sigma-Aldrich Chemie GmbH, Germany). All other chemicals used were of analytical grade and were obtained from either Sigma-Aldrich or Merck (Merck, Germany). L-car was freshly dissolved in a distilled water immediately before administration. Vitamin E was dissolved in corn oil just before administration.

Animals
Thirty-six Sprague Dawley male rats (150-200gm) were provided from the National Center for Radiation Research and Technology Animal House. Animals were kept under standard conditions and were allowed free access to a standard requirement diet and water ad. Libitum. Animals were kept under a controlled lighting condition (light:dark, 13h:11h), 25±2°C, relative humidity 50%. Animals were acclimatized to laboratory conditions before the test. The animals’ treatment protocol has been approved by the Animal Care Committee of the National Center for Radiation Research and Technology, Cairo, Egypt.

Irradiation
Whole-body γ-irradiation was performed at the National Centre for Radiation Research and Technology (NCRRRT), Cairo, Egypt, using an AECL Gamma Cell-40 biological irradiator. Animals were irradiated at an acute single dose level of 5Gy delivered at a dose rate of 0.012Gy/s.

Experimental design
In this experiment, the animals were divided into 6 groups (6 rats in each group). Group 1: Normal control rats were injected with normal saline (0.5ml/150g, intraperitoneal (i.p.)) and treated with corn oil (0.5ml/150g, orally) for 5 consecutive days. Group 2 (L-car): Rats were injected with L-car (300mg/kg, i.p.) for 5 consecutive days (Yurut-Caloglu et al., 2010). Group 3 (L-car+Vit E): Rats were injected with L-car (300mg/kg), 30min later; rats were treated with Vit E (50mg/kg, orally) for 5 days (Bozaykut et al., 2014). Group 4 (IRR): Rats were injected with normal saline (0.5ml/150g, i.p.) and treated with corn oil (0.5ml/150g, orally) for 5 consecutive days. One hour later; rats were exposed to whole body gamma-irradiation (5 Gy). Group 5 (L-car+ IRR): Rats were injected with L-car (300mg/kg, i.p.) for 5 consecutive days, 1h later; rats were exposed to whole body gamma-irradiation (5Gy). Group 6 (L-car+Vit E+ IRR): Rats were injected with L-car (300mg/kg), 30min later; rats were treated with Vit E (50mg/kg, orally) for 5 days, 1h later; rats were exposed to whole body gamma-irradiation (5Gy).

Groups of Vit E and Vit E prior gamma-irradiation were done in many previous studies (Üçüncü et al., 2006; Hadi et al., 2012; Yildiz et al., 2011 and Razavi et al., 2013). Seven days after the last treatment, the blood was collected by cardiac puncture from the ether anesthetized rats. Serum was separated by centrifugation at 3000rpm for 15min and utilized for the estimation of cardiac marker enzymes, lipid profiles, caspase-3 activity, C-reactive protein (CRP), potassium level and Bax. After collection of blood samples, the rats in different groups were sacrificed and their hearts and lungs were excised.
immediately and were fixed in neutral formalin for 24h for Bcl-2 immunohistochemical assay.

Assessment of serum creatine kinase and lactate dehydrogenase activity

The activities of creatine kinase (CK-MB) and lactate dehydrogenase (LDH) were assayed, in serum, using commercial kits purchased from spectrum diagnostic (Cairo, Egypt) following the methods of Swanson & Wilkinson (1972) and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (1980), respectively.

Serum lipid profiles and potassium

Serum low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), cholesterol, and triglycerides were determined using the corresponding test reagent kit (Bio diagnostic, Egypt) (Wieland & Seidel, 1983; Lopes-Virella et al., 1977; Richmond, 1973 and Fossati & Prencipe, 1982, respectively). The serum potassium level was obtained by an emission flame photometer.

Caspase enzyme activity, Bax and C-reactive protein assays

Caspase-3 activity and Bax were assayed according to the manufacturer’s instructions using the ELISA Kit (Cusabio Biotech Co., China) and C-reactive protein was determined using immunoturbidimetric assay (Sigma diagnostics Ltd).

Immunohistochemical Detection of Bcl-2

Expression of Bcl-2 immunoreactivities (Bcl2-ir) was detected using avidin Biotin Complex (ABC) method (Tousson et al., 2014 a) Paraffin sections (5μm thick) of fixed rat heart and lung that mounted on gelatin chromalum–coated glass slides were dewaxed and rehydrated sections were washed in distilled water for 5 min, rinsed in Phosphate buffered saline with Tween-20 (PBST) for 10min and incubated with 10% normal goat serum for 15min to reduce non-specific background staining. Then, the sections were incubated with anti-rabbit Bcl-2 (monoclonal antibody (Dako, 1:80 and 1:2000, respectively) for 1-2h at room temperature. The sections after 5 baths in PBST were incubated with biotinylated goat anti-rabbit immunoglobulin (Nichirei, Tokyo, Japan). The sections after 5 baths, in PBST, were further incubated with Avidin Biotin Complex (ABC:Nichirei, Tokyo, Japan) for 1h at RT. The reaction was developed by using 20mg 3-3’-diaminobenzidine tetrahydrochloride (DAB, Wako pure chemical industries, Ltd) in 40ml PBST, pH 7.2 containing 10ml of hydrogen peroxide (H$_2$O$_2$) for 7-9min at a dark room followed by distilled water then dehydrated and mounted. The criterion for a positive reaction confirming the presence of Bcl-2 is a dark, brownish, intra cytoplasmic precipitate. For the negative control, the primary antibody was omitted to guard against any false positive results which might be developed from a non-specific reaction. Brightness, contrast were adjusted using Adobe Photoshop software. Image analysis was adjusted using PAX-it image analysis software.

Statistical analysis

Data are expressed as (mean±SEM, n= 6). Statistical differences between groups were carried out using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. A P value of 0.05 or less was taken as a criterion for a statistically significant difference.

Results

Biochemical parameters

Administration of Vit E (50mg/kg) and/or L-car (300mg/kg) for 5 consecutive days showed no significant changes in the levels of serum lipid profiles, C-reactive protein (CRP), potassium (K), and activities of CK-MB and caspase-3 compared to the control group.

Exposure to gamma radiation significantly increased serum levels of glucose, cholesterol, triglycerides and LDL-C and decreased levels of HDL-C as compared to control group (Fig. 1). The pre-administration of Vit E and/or L-car for 5 consecutive days prior to irradiation resulted in significant decreases in cholesterol, triglyceride and LDL-C levels and increased HDL-C levels, compared to irradiated group (Fig. 1).

Figure 2 shows that gamma irradiation increased serum levels of potassium and C-reactive protein and the activities of cardiac markers enzymes LDH and CK-MB compared to control group. The pre-administration of Vit E and/or L-car for 5 consecutive days prior to irradiation resulted in significant decreases in potassium and CRP levels, LDH, CK-MB activities compared to the irradiated group (Fig. 2).
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Fig. 1. Effect of vitamin E (Vit E) and/or L-carnitine (L-car), gamma irradiation (IRR) and their combination on cholesterol, triglycerides, high density lipoprotein (HDL) and low density lipoprotein (LDL) levels (Data are presented as mean±S.E., n= 6, a and b indicate a significant change from control, gamma irradiation respectively at P≤0.05 using ANOVA followed by Tukey-Kramer as a post ANOVA test).

Fig. 2. Effect of vitamin E (Vit E) and/or L-carnitine (L-car), gamma irradiation (IRR) and their combination on creatine kinase (CK-MB) and lactate dehydrogenase (LDH) activities, C-reactive protein (CRP) and potassium (K) levels (Data are presented as mean±S.E., n= 6, a and b indicate a significant change from control, gamma irradiation respectively at P≤0.05 using ANOVA followed by Tukey-Kramer as a post ANOVA test).
Exposure to gamma radiation significantly increased serum Bax level and caspase-3 activity as compared to the control group (Fig. 3). The pre-administration of Vit E and/or L-car for 5 consecutive days prior to irradiation resulted in significant decreases in Bax level and caspase-3 activity compared to the irradiated group (Fig. 3).

**Bcl-2 determination by immunohistochemical staining of heart sections**

The effect of L-car and Vit E on myocardial tissue following gamma irradiation inducing a heart damage was examined by immunohistochemical analysis of Bcl-2 expression in the myocardial tissue (Fig. 4). Myocardial tissue of the normal control group (a) showed a strong positive expression of Bcl-2 (negative immunohistochemical reaction) indicating normal biological processes (Fig. 4 a). In contrast, the tissue of the irradiated group showed a weak positive expression of Bcl-2 (immunopositivity indicated by brown color) indicating abnormal nuclear and myofibrillar ultra structural damage (Fig. 4 b). While, rats treated with L-car then irradiated showed a moderate positive expression of Bcl-2 (immunopositivity indicated by brown color) indicating a reduced myocardial damage comparable to irradiated group (Fig. 4 d). In the same concern, myocardial tissue of rats that treated with L-car and Vit E then irradiated showed a strong positive expression of Bcl-2 (immunopositivity indicated by brown color) indicating the marked reduction in myocardial damage (Fig. 4 f).

**Bcl-2 determination by immunohistochemical staining of lung sections**

The effect of L-car and Vit E on pulmonary tissue following gamma irradiation inducing a pulmonary damage was examined by immunohistochemical analysis of Bcl-2 expression in the lung tissue (Fig. 5). Lung tissue of the normal control group (a) showed a strong positive expression of Bcl-2 (a negative immunohistochemical reaction) indicating normal biological processes (Fig. 5 a). In contrast, the tissue of the irradiated group showed a weak positive expression of Bcl-2 (immunopositivity indicated by brown color) indicating abnormal nuclear and pulmonary ultra-structural damage (Fig. 5 b). While, rats treated with L-car then irradiated showed a moderate positive expression of Bcl-2 (immunopositivity indicated by brown color) indicating reduced pulmonary damage comparable to the irradiated group (Fig. 5 d). In the same trend, the lung tissue of the rats treated with L-car and Vit E then irradiated showed a strong positive expression of Bcl-2 (immunopositivity indicated by brown color) indicating the marked reduction in pulmonary damage (Fig. 5 f).

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**Fig. 3. Effect of vitamin E (Vit E) and/or L-carnitine (L-car), gamma irradiation (IRR) and their combination on caspase-3 and Bax activity (Data are presented as mean±S.E., n= 6, a and b indicate a significant change from control, gamma irradiation respectively at P≤0.05 using ANOVA followed by Tukey-Kramer as a post ANOVA test).**
Fig. 4. Effect of vitamin E (Vit E) and/or L-carnitine (L-car), gamma irradiation (IRR) and their combination on Bcl-2 by immunohistochemical staining in heart of rat: (a) Control group showed a strong positive expression of Bcl-2, (b) IRR group showed weak positive expression of Bcl-2, (c) L-car group showed a strong positive expression of Bcl-2, (d) L-car+IRR group showed a moderate positive expression of Bcl-2, (e) L-car+VitE group showed a strong positive expression of Bcl-2 and (f) L-car+VitE+IRR group showed a strong positive expression of Bcl-2 (immunopositivity indicated by brown colour) (arrow) (X 400).

Fig. 5. Effect of vitamin E (Vit E) and/or L-carnitine (L-car), gamma irradiation (IRR) and their combination on Bcl-2 by immunohistochemical staining in lung of rat: (a) Control group showed a strong positive expression of Bcl-2, (b) IRR group showed a weak positive expression of Bcl-2, (c) L-car group showed a strong positive expression of Bcl-2, (d) L-car+IRR group showed a moderate positive expression of Bcl-2, (e) L-car+VitE group showed a strong positive expression of Bcl-2 and (f) L-car+VitE+IRR group showed a strong positive expression of Bcl-2 (immunopositivity indicated by brown color) (arrow) (X 400).

Discussion

In the present study; whole body γ-irradiation (5Gy) of rats induced significant increases in serum activities of CK-MB and LDH and in the levels of serum cholesterol, triglycerides and LDL-C associated with a significant decline in the HDL-C level compared to the control group. This increase indicates an injury or damage to cardiac cells by gamma-irradiation which may be due to the inhibition of nucleic acid and protein synthesis (Lee et al., 2007). Mansour (2013 a) attributed the increase of CK-MB and LDH to the excessive production of free radicals and lipid peroxides that might have caused a leakage of cytosolic enzymes and to the membrane cell damage. Previous studies suggested that ROS play an important role in cellular damage caused by ionizing radiation. These ROS induces changes in membrane fluidity, and loss of plasma membrane integrity and causes DNA and protein damage (Partyka et al., 2017). The alterations of the lipid profiles could be attributed to the fact that the hydroxyl radicals (OH•) greatly enhance the NADH-dependent microsomal lipid peroxidation and thus initiates a lipid radical chain reaction causing an oxidative damage to cell membranes and increase the rate of cholesterol biosynthesis in the liver and other tissues, as an early reaction necessary for restoration of biomembranes (Hadi et al., 2012). In addition, radiation modifies LDL-C and HDL-C metabolism indirectly via the action of various inflammatory products and might decrease the lipoprotein lipase activity in adipose tissues, leading to a reduction in the uptake of lipids (Mansour et al., 2014).

Administration of Vit E and/or L-car induced no significant change in other serum lipid profiles and activities of LDH and CK-MB compared to the control group. The administration of Vit E and/or L-car prior to IRR resulted in a significant recovery in cholesterol, triglyceride and LDL-C levels, LDH and CK-MB activities and increased HDL-C levels compared to the irradiated groups. These alterations could be valuable in preventing irradiation complications as well as in improving lipid metabolism. The effect of L-car could be due to its ROS-scavenging activity and antioxidant properties (Mansour, 2013 b). It was also reported that the antioxidant properties of L-car may be related to the transport of fatty acids into mitochondria for β-oxidation and thus to the decrease of lipid usage and protection of the cell membrane against toxic ROS and other free radicals (Tousson et al., 2014 b). Moreover, L- car protects cell membranes against ROS damage and decreases the availability of lipids for peroxidation by reducing the formation of hydrogen peroxide (Augustyniak & Skrzydlewska, 2010; Zhang et al., 2016 and Sahebkar et al., 2016).

Previous studies have suggested beneficial effects of L-carnitine supplementation on the inflammatory parameters (DiNicolantonio et al., 2013), on the prevention of Cardio-vascular disease (CVD) (Shang et al., 2014) and on serum lipid profile in hemodialysis patients (Huang et al., 2013).

On the other hand, vitamin E allows free radicals to abstract a hydrogen atom from the antioxidant molecule rather than from poly unsaturated fatty acids, thus breaking the chain of free radicals reaction (Hadi et al., 2012). Vitamin E induces a cellular defense mechanism that prevents peroxidative processes by sequestering free radicals, which detoxifies peroxides and protects cells from subsequent deleterious effects (Yildiz et al., 2011).

The Bcl-2 family of proteins is central regulators of apoptosis because they integrate diverse survival and death signals that are generated outside and inside the cell (Cory & Adams, 2002). Bcl-2 family is subdivided into two classes, anti-apoptotic members such as Bcl-2 and Bcl-xL, which protect cells from apoptosis, and pro-apoptotic members such as Bax and Bak and the BH3- only death proteins, which trigger or sensitize cells for apoptosis (Puthalakath & Strasser, 2002). The main action of Bcl-2 family members is to control mitochondrial membrane permeability. Loss of mitochondrial membrane potential, DNA fragmentation, caspase activation, and increase in membrane permeability are the most significant changes related to apoptosis (Partyka et al., 2017).

In the irradiated group, the expression of Bax and caspase-3 activity increased significantly, whereas Bcl-2 expression decreased markedly. Consistent with these findings, it has been reported that cell apoptosis induced by radiation is regulated by a complex balance between pro-apoptotic factors, including caspase-3, c-caspase-3, Bax and anti-apoptotic factors, including the Bcl-2 family (Stahnke et al., 2004).
It has been shown that induction of apoptosis in T cells was associated with imbalance activation of caspase and Bcl-2 (Wu et al., 2014). Additionally, during the apoptotic process, Bcl-2 prevents the opening of the mitochondrial membrane pores, whereas Bax induces their opening. In many cases, oxidative stress induces caspase activation through cytochrome c and apoptosis inducible factor (AIF) - release from the mitochondrial intermembrane space into the cytosol (Zhang et al., 2014). In the present study, the authors observed that L-car and/or Vit E reduced radiation-induced caspase-3 and Bax activation and enhanced Bcl-2 expression.

L-Carnitine has been shown to reduce apoptosis in different cells and organelles (Surai, 2015 a and Fattah et al., 2017). A previous study showed that L-car could prevent apoptosis of skeletal muscle cells and plays a role in the treatment of congestive heart failure-associated myopathy (Vescovo et al., 2002). Protective mechanisms of L-carnitine include the inhibition of mitochondrial membrane permeability, the decrease of oxidative stress, inhibition of ROS production, prevention of free radical formation in the electron transfer chain, and the prevention of pro-apoptotic protein expression (Chao et al., 2011; Coşkun et al., 2013; Surai, 2015 b and Tousson et al., 2014 b).

On the other hand, previous study reported that, L-car exerts protective effects on endoplasmic reticulum stress-mediated myocardial injury after cardiopulmonary resuscitation in rats by decreasing the level of caspase-12 (Zhang et al., 2015).

Vit E not only protects intact tissues by decreasing apoptosis induced by injurious conditions, but also increases apoptosis with a direct selective action on cancer cells (Osakada et al., 2003 and Kang et al., 2004). It has been reported that Vit E protects DNA from free radicals’ attack either by scavenging lipid peroxy radicals, thereby terminating lipid peroxidation chain reaction that creates DNA damaging products or by inactivating ROS and reduced apoptosis (Badgujar et al., 2017).

In accordance with previous studies (KLuciński et al., 2005 and Valente et al., 2015), gamma irradiation significantly increased C-reactive protein (CRP) level. CRP, so called positive protein of the acute phase, dramatically increases in serum as a marker of tissue damage, inflammation and has been increasingly associated with prognosis in a variety of solid tumors (Hall et al., 2013). It should be noted that ionizing radiation induces the production of interleukin 1b (IL-1b) proinflammatory cytokine, tumor necrosis factor a (TNF-a) and interleukin 6 (IL-6), thereby contributing to the development of inflammation and possibly increasing CRP concentration (KLuciński et al., 2005 and Valente et al., 2015).

Administration of L-car and/or Vit E induced a significant decrease in CRP compared to the irradiated group, which might be due to their antioxidant and anti-inflammatory activities.

In the present study, gamma irradiation induced a significant increase in potassium level. Irradiation can lead to efflux of potassium ion into extracellular space, leading to transfusion-associated hyperkalemia (Kuttath et al., 2015). Irradiation is known to damage RBC membranes, resulting in the release of potassium, haemoglobin (Hb) and lactate dehydrogenase (LDH) from the RBCs (Winter et al., 2015). The side effects of elevated potassium in the serum can be mild to severe, mild to moderate abnormalities include muscle weakness and occasionally respiratory muscle involvement. However, the dreaded side effects of hyperkalemia are its effect on cardiac rhythm. A mild elevation of potassium is reflected in electrocardiogram changes while more severe changes observed are ventricular fibrillation and asystole, which may lead to cardiac arrest and death (Kuttath et al., 2015).

Administration of L-car and/or Vit E induced a significant decrease in potassium level compared to the irradiated group, which might be due to their antioxidant activities.

**Conclusion**

In this study, the data have shown that Vit E and/or L-car modulates gamma irradiation-induced apoptosis by modulation of antiapoptotic and proapoptotic proteins and consequently reduced oxidative damage.

**Authors’ Contribution**

Heba H. Mansour and Shereen Galal conceived and designed the research. Heba H. Mansour,
Shereen El Kiki and Shereen Galal performed the experiments. Heba H. Mansour and Shereen Galal analyzed the data. Heba H. Mansour, Shereen El Kiki and Shereen Galal wrote the manuscript.

**Declaration of Interest**

The authors report no conflicts of interest. The authors are the sole responsible for the content of the paper.

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