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Protection against Radiation-induced Genotoxic Damages in Cultured Human Fibroblast Cells by Treatment with Pallidium-lipoic Acid Complex

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ABSTRACT

The present study was aimed to evaluate the radioprotective efficacy of Pall-lipoic acid complex against X-ray radiation-induced cellular damage in cultured MRC5 cells. Pall-lipoic acid (5%) was pre-incubated with the cultured cells for 1h prior to irradiation. A dose dependent increase in the total micronucleus frequency was observed in the X-ray irradiated (0.5, 1, 1.5 and 2 Gy) groups. The γH2AX, can be visualized as discrete foci with the use of specific antibodies with fluorescent tags and directly counted in the fluorescence microscope at different time points (0.5 h, 1h, 6h and 24 h) after exposure to X-ray dose of 0.5 Gy. The obtained results indicated that pall-lipoic acid showed optimum protection by effectively decreasing the MN frequencies and γH2AX foci. In addition, a significant difference in surviving fraction between non-treated cells and 5 % pall-lipoic acid at 2 Gy was observed. This is due to the powerful antioxidant effect found in Pallidium-lipoic complexes which can be partly attributed to its lipoic acid fraction.

INTRODUCTION

Humans are constantly exposed to ionizing radiations both from natural sources such as cosmic rays (during space travel), radioisotopes found in the earth crust and also from a wide variety of artificial sources. Radiation can be electromagnetic (X-rays, gamma rays) or particles (electrons, photons, neutrons, alpha particles and heavy ions). Although ionizing radiation has been found to produce deleterious effects on the biological systems; this property of ionizing radiation has been ingeniously exploited to an advantage in the treatment of various neoplastic disorders in humans [(1)]. Presently, more than one-half of all

cancer patients are treated with radiation therapy.

Ionizing radiation causes damage to living tissues through a series of molecular events, such as photoelectric, Compton and Auger effects, depending on the radiation energy. Because human tissues contain 80% water, the major radiation damage is due to the aqueous free radicals, generated by the action of radiation on water. The major free radicals resulting from aqueous radiolysis are OH, H, eaq–, HO_2, H_3O^+, *etc*. These free radicals react with cellular macromolecules, such as DNA, RNA, proteins, membrane, etc, and cause cell dysfunction and mortality. These reactions take place in tumor as well as normal cells when exposed to radiation [(2)].

The most potent form of DNA damage which can arise from ionizing radiation exposure is DSBs[(3)], which can lead to chromosomal aberrations and loss of genetic material[(4)]. Damaged DNA efficiently activates a complex signaling network known as DNA damage response which temporarily shuts down DNA replication process, thereby facilitating the repair of the damaged site[(5)] by a highly coordinated process involving different DNA repair enzymes. Normal DNA repair is protective against genotoxic effects of carcinogens, while reduced DNA repair capacity results in increased mutation rates and genomic instability which may lead to the development of cancer [(6)].

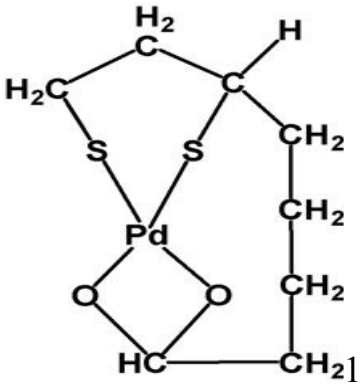
Increasing interest to look for protocols, elements and compounds may play a role in radioprotection by decreasing or delaying the radiation injury has accompanied with the development in instruments and techniques to benefit from peaceful uses of atomic energy in all life fields, especially, in the radiotherapy and the individual protection in internal and external contamination in radiation incidents. Thus, since more than three decades, several laboratories and research centers in the world have developed and tested numerous drugs and chemical compounds having radioprotective properties. Early developments of such agents focused on thiol synthetic compounds, such as amifostine. This compound reduced mortality; however, there were difficulties in administering aminothiols that led to adverse effects. Hence, the search for new radioprotectors that are less toxic than the currently available compounds is crucially needed to develop better strategies for protecting exposed populations from radiation injury [(7)].

Therefore it is necessary to design another suitable agent in order to reduce the radiation side effects. Hence the aim of our present study was to investigate the radioprotective effect of Pallidium-lipoic acid against X-rays

radiation-induced cellular in cultured human epithelial cells.

The Pallidium-lipoic acid was originally designed as a non-toxic chemotherapeutic agent, in a prescription version called DNA Reductase, and consists of a palladium bonded to both end-groups of a lipoic acid (LA), the two sulfurs of the thiolane ring and the carboxyl of the pentanoic chain in a 1:1 ratio, Scheme 1[8,9]. This arrangement is unique in that it allows the molecule to be both water and lipid soluble. The presence of the free radical scavenger, LA, and the addition of an alternative energy source, palladium, led to consider that LAPd can be used in the treatment of various cancers [10, 11, 12, 13].

The LA, ligand in the LAPd complex, is a well established antioxidant that may act as a buffer in cancer therapy[14] where the drugs used are known promoters of oxidative stress, or in therapy of diseases associated with oxidative stress, either directly as a free radical scavenger or indirectly due to its synergistic action with other antioxidants [15, 16].



Scheme 1. Proposed structure of LAPd.

MATERIALS AND METHODS

Pallidium-lipoic acid formulation was obtained as a gift from Garnett McKeen Laboratory, Inc., USA and all other chemicals used were of reagent grade.

Cell preparation

Human embryonic lung fibroblast cell lines (MRC5) derived from normal lung tissue of a 14-week-old male fetus by J. P. Jacobs in September 1966 (J. P. Jacobs et al., 1970). Cells were maintained in MEM supplemented with 10% FBS, 100 IU/ml penicillin G and 100 ug/ml streptomycin, 1% L-glutamine, in an incubator under a humidified atmosphere of 5% CO_2 and 95%

air at 37°C. Unless otherwise noted, cells were passaged and removed at 70-80% confluency.

Immunofluorescence

Cells with different treatments were plated on cover slips and put in 35mm dishes at a density of 1×10^5 cells/dish. 16 h later, the cells were irradiated with X-rays, fixed for 20 min in 4% paraformaldehyde, permeabilized for 20 min in methanol at -20°C, blocked for 1 h with 5% skim milk, and stained with mouse anti-γH2AX antibody (Upstate Biotechnology, Lake Placid, NY, USA) for 2 h. The bound antibody was visualized using Alexa Fluor® 488 anti-mouse (Molecular Probes, Eugene, OR, USA), and cell nuclei were counterstained with DAPI solution (PharMingen). Slides were observed under a fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). At least 100 cells were scored for each sample, and the average number of foci per cell was calculated.

Micronuclei

Cells were plated on cover slips and put in 35mm dishes at a density of 1×10^5 cells/dish. 3 μg/mL of cytochalasin B (Cyto-B, Sigma) was added into each well right after cells were exposed to X-rays (0, 0.2, 0.5, 1, 1.5, and 2Gy). 48 h later, cells were washed once with PBS and then fixed with methanol-glacial acetic acid (9:1, V/V). Stained with 3 μg/mL Acridine Orange (Sigma), at least 500 of binucleated cells for each sample were counted.

ROS

The generation of ROS was detected by dichlorofluorescein diaacetate assay (DCF-DA). Cells were incubated for 1h at 37 ◦C with 5% Pall-lipoic acid. Cells were then incubated with DCF-DA (10μM) at 37 ◦C for 15 min and irradiated at 2 Gy. The change in fluorescence intensity of the resultant 2_,7_-dichlorofluorescin was measured in fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Colony formation assay

Overall cell survival was assessed by routine colony formation assay. Identical number of MRC5 cells were exposed to X-rays (0, 1, 2, 4, 6, 8 Gy), and then cultured in 60-mm dishes containing 5 mL MEM media supplemented with 10% FBS for 13 days. Cells were then fixed with 70% ethanol for 5 min and then stained with 1% crystal violet for 20 min. Colonies containing more

than 50 cells were counted as survivors. At least 3 parallel dishes were scored in 3 or 4 repetitions for each treatment.

Irradiation and with drug

The irradiation of the cells was carried out using 100 KVp X-rays (Faxitron RX-650, Faxitron Bioptics, Lincolnshire, IL, USA).

RESULTS AND DISCUSSION

Ionizing radiation interacting with water in cells can produce reactive free radicals, such as hydroxyl radicals, hydrogen radicals and the toxic substance, hydrogen peroxide, all of which can damage critical macromolecules. The elimination of the free radical species from the cell environment can inhibit the side effects induced by irradiation. The presence of sulfhydryl groups or other molecules capable of scavenging the radiolysis radicals arising from irradiation of water molecules can confer protection to radiation. Due to very short life of the radicals, such protective agents need to be present in the cell environment before the production of free radicals in order to neutralize their destructive properties [(17)].

A cytological consequence of the induction of the chromosomal aberrations is the formation of micronuclei that are observed in the interphase cells. The cytokinesis-block micronucleus assay has been widely used to assess the in vitro radiation-induced chromosomal damage, and satisfactory dose relationships have been reported[(18)]. In this study, the alteration in the level of the micronucleus (MN) frequencies in the MRC5 cells is shown in Fig.1. X-ray irradiated MRC5 cells showed a significant elevation in the levels of MN frequencies whereas pre-incubation of Pallidium-lipoic acid (5%) with MRC5 cells prior to X-ray radiation significantly decreased the levels of MN frequencies. A dose dependent increase in the total MN frequency was observed in the X-ray irradiated (0.5, 1, 1.5 and 2 Gy) groups. When compared to their corresponding radiation groups, pre-treatment of Pallidium-lipoic acid (5%) prior to different doses showed a significant decrease in the MN.

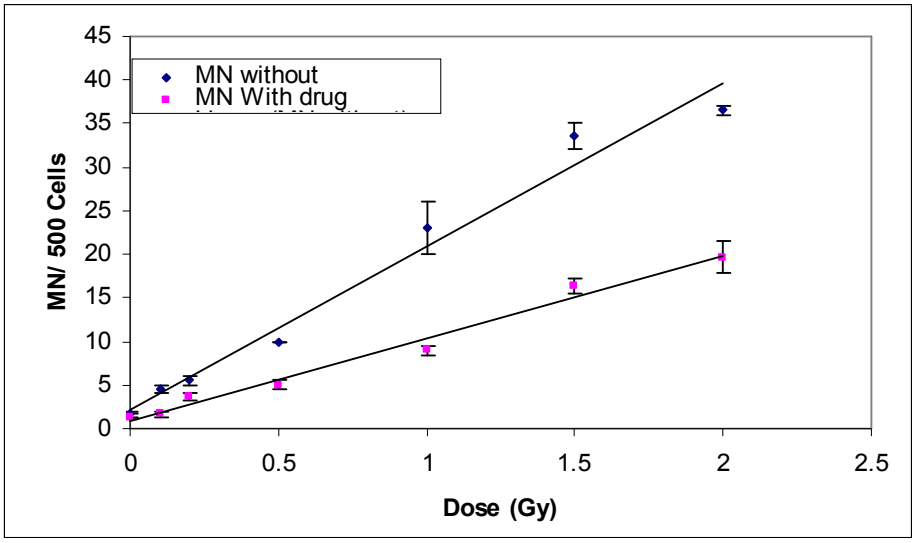


Fig. (1): Micronuclei (MN) of MRC5 cells after irradiation with X-rays. The cells incubated with Palladium-lipoic acid 5% before irradiation.

An immediate result of IR exposure is the DNA double strand breaks (DSB) that can be detected with an antibody to γH2AX, a phosphorylated form of the histone H2A variant H2AX which forms rapidly at the sites of DNA DSBs[19]. *In vitro* studies show that γH2AX formation peaks 15-30 min after IR proportionally to the dose, and then declines over several hours [20]. γH2AX is required for the concentration and stabilization of DNA repair proteins and plays a role in both non-homologous end-joining (NHEJ) and homologous recombination (HR) repair pathways[21]. The ratio of DNA DSBs to visible γH2AX foci is close to 1:1, which forms the basis of a sensitive quantitative method for detection of DNA DSBs in mammalian cells [22]. To study the repair kinetics, induction and loss of γH2AX foci was investigated in the MRC5 cells at different time points (0.5 h, 1h, 6h and 24 h) after exposure to X-ray dose of 0.5 Gy. Fig. 2 shows that the radiation-induced foci increased until 1h after irradiation, reaching a maximum number of 12 foci/cell. Then, the number of foci decreased gradually. The pre-treatments with Pallidium-lipoic acid 5% reduced the number of foci significantly.

The obtained results are in agreement with the results of Lakshmy Ramachandran *et al* 2010[23] who studied the protective effect of Poly-MVA using the comet assay. POLY-MVA protected the cellular DNA of spleenocytes and bone marrow cells of irradiated animals against radiation-induced damages when administered prior to a lethal dose of 8 Gy whole-body gamma-radiations.

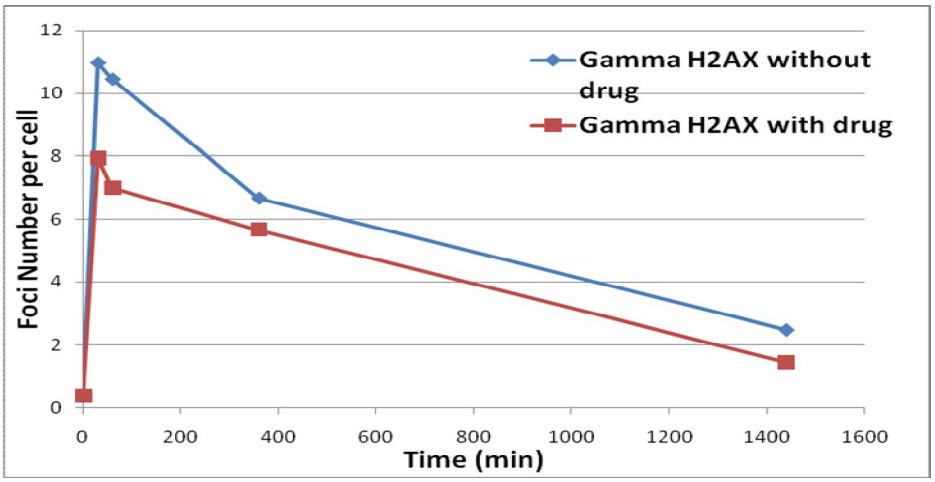


Fig. (2): γH2AX foci after irradiation of the MRC5 cells by 0.5 Gy X-rays. The cells incubated with Pallidium-lipoic acid 5% for 1h before irradiation.

Radioprotecive effect of Pallidium-lipoic acid was observed in MRC5 cells in colony formation, the obtained data showed the enhancement of cell survival by Pall.lipoic acid of fibroblast cells irradiated with x-rays, which reflected in the DNA repair process, (Fig. 3). Significant difference in surviving fraction was observed in 2 Gy irradiated condition between non-treated cells and 5 % Pallidium-lipoic acid.

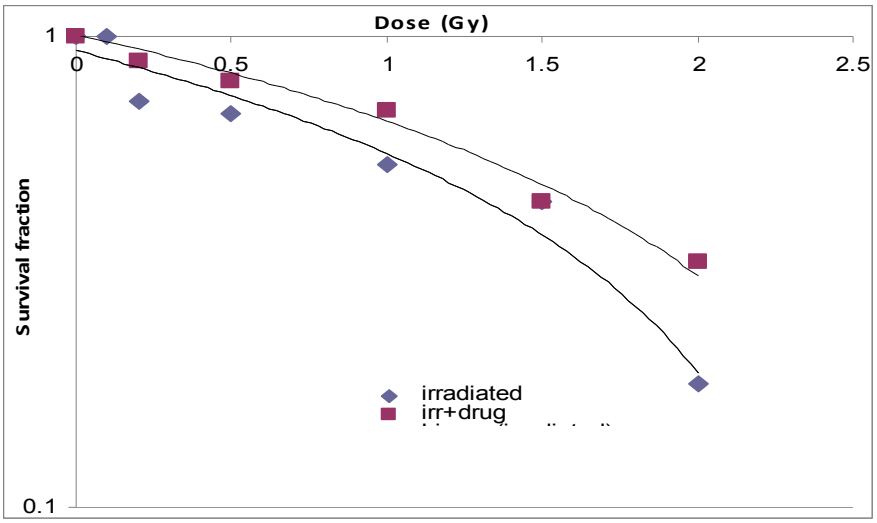


Fig. (3): Effects of 5% Pallidium-lipoic acid on surviving fractions after X-ray irradiation.

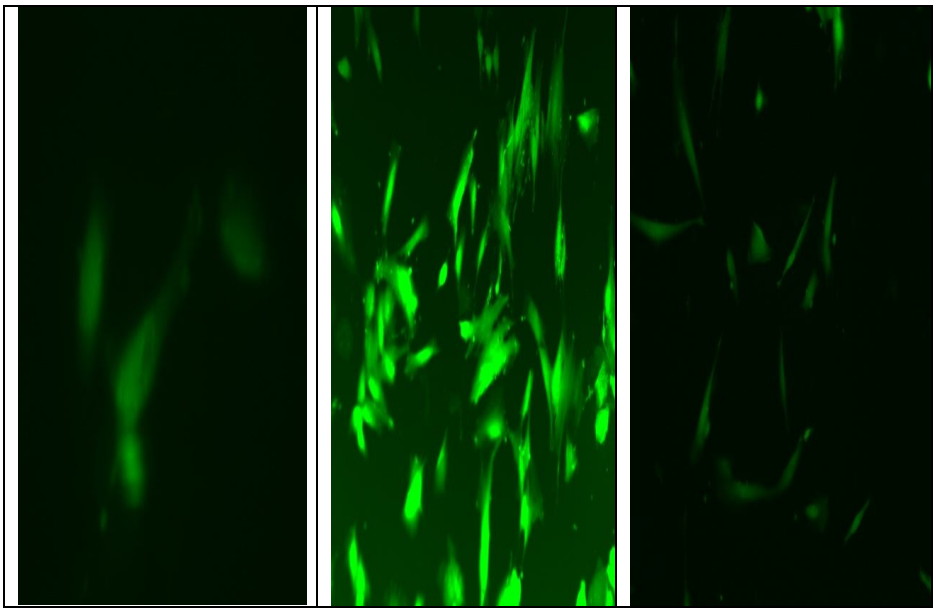


Fig (4) :DCF-DA fluorescence image for control, irradiated with 2Gy and irradiated with Pall-lipoic acid.

The ROS was detected using DCF-DA (Fig.4), the images showed that the brighter fluorescence associated with high ROS production due to the exposure of the cells to 2Gy gamma radiation. Whereas the incubation of the cells with Pall-lipoic acid resulted in lower level of ROS due to the antioxidant efficiency of Pall-lipoic acid.

Palladium-lipoic acid is considered as a sort of "liquid transistor" consisting of a metal and an organic compound (a metallo-organic compound). This liquid transistor (combination of the metal palladium and the organic molecule alpha-lipoic acid) would act as an enzyme and affect the electron transfer to DNA. In other words, Poly-MVA works in cancer cells by transferring excess electrons from membrane fatty acids to DNA via the mitochondria. Therefore, it can both quench radicals as well as provide energy to the mitochondria [(24)].

The unusually powerful antioxidant effect found in palladium lipoic complexes can be partly attributed to its lipoic acid fraction. Since this naturally occurring acid is soluble in both fat and water, palladium-lipoic complexes are able to pass across cell membranes and work intracellularly. Antonawich and Valane (2007)[(25)] reported that the Oxygen Radical Absorbance Capacity

(ORAC) analysis which provides a measure of the scavenging capacity of antioxidants against the peroxyl radical, which is one of the most common ROS found in the body. The ORAC are 5.65, 1.6, 1.4, 1.12 and 1.0 for Pall-lipoic acid formulation, Vitamin A, α-lipoic acid, Vitamin C، and Vitamin E, respectively. Palladium α-lipoic acid complex is considered a unique electronic and redox properties appear to be the key to its physiological effectiveness (26,27,28).

In summary, our results indicate that exposure to different doses of X-rays results in increased in MN frequencies, DSBs which visualized by γH2AX and cell killing in cultured human fibroblast. The pre-incubation of the cells with Pallidium-lipoic acid 5% before irradiation caused protective effect action of the cells. These findings suggest that supplementation of the radiotherapeutic patients with Pallidium-lipoic acid could offer some protection against the expected adverse effects for normal cells during treatment.

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