Therapeutic Effects of Olive Leaf Extract or Bone Marrow Mesenchymal Stem Cells against Lung Damage Induced In Male Albino Rats Exposed To Gamma Radiation

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ABSTRACT

Aim of the work- This study aimed to investigate the histological and histochemical changes in the lung tissue of male albino rats post exposure to gamma radiation and the possible therapeutic effect of both olive leaf extract and bone marrow mesenchymal stem cells.

Material and methods- The current study was carried out on 40 adult male albino rats (Sprague dawely strain); they were divided equally into 5 groups (C group: control rats; O group : rats treated with olive leaves extract( 15 mg /kg body weight/daily) ; R group: rats exposed to a single dose of gamma-radiation(3 Gy); RO group: rats of this group treated with olive extract 15 mg /kg body weight/daily one week before and one week after irradiation; RS group: rats of this group irradiated with 3Gy then treated with bone marrow mesenchymal stem cells (BMSCs) 3x10⁸ cells/ml suspension through caudal vein about 5h post radiation exposure. Histopathological and histochemical changes were studied.

Results- Rats exposed to gamma radiation showed numerous histological and histochemical changes, these changes were ameliorated by using either olive leaf extract or bone marrow mesenchymal stem cells. BMSCs showed more obvious therapeutic effect than olive leaf extract.

Conclusion- The present work showed that olive leaf extract or bone marrow mesenchymal stem cells (BMSCs) have lung tissue radiotherapeutic effects against whole body gamma radiation in male albino rats.

Key words: Gamma radiation - Albino rats - Lung - bone marrow mesenchymal stem cells (BMSCs) – Histology- Histochemistry.

INTRODUCTION

Lung is one of the most radiosensitive organs of the body. Radiation damage to the lung can be described at all levels of organization from the cellular up to the organ level. Ionizing radiation is that type which contains sufficient energy to displace an orbital electron around the nucleus. The most important consequence of this displaced electron on living tissue is the potential damage it can inflict on DNA, which may occur directly or indirectly. [1] Direct damage occurs when the displaced electron breaks a DNA strand. Indirect damage occurs when the electron reacts with a water molecule, creating a powerful hydroxyl radical which then damages the cell’s DNA. Ionizing radiation absorption causes immediate biochemical, subcellular and cellular damage, while its morphological expression and organ dysfunction are often considerably delayed. [2]

Accidental exposure and the therapeutic application of gamma radiation are the main triggers for the production of reactive oxygen species (ROS) in cells. [3] Superoxide anions, hydrogen peroxide and hydroxyl radicals are the principal types of ROS that react with macromolecules, resulting in cell dysfunction and tissue damage. [4] The major targets for ROS include proteins, lipids and nucleic acids, generating DNA strand breakage, DNA- protein cross linking and lipid peroxide production. [5,6] These toxic products affect the balance of antioxidant systems such as glutathione and enzymatic antioxidant defense systems. [7,8]

Mesenchymal stem cells are a population of adult stem cells and they are promising sources for therapeutic applications. These cells can be isolated from the bone marrow and can be easily separated from the hematopoietic stem cells (HSCs) due to their plastic adherence. [9] Adult bone marrow MSCs can differentiate into several types of mesenchymal cells including osteocytes, chondrocytes and...
adipocytes; also they can differentiate into non-mesenchymal cells, such as neural cells, under appropriate experimental conditions.\(^{[10]}\)

The olive tree (\textit{Olea europaea}) produces oleuropein abundantly in its leaves as well as in the olive fruit itself. Oleuropein is the unique molecule that provides olive oil with its multitude of health and life-extending benefits, it is the polyphenol that can help lower bad cholesterol and blood pressure, prevent cancer, protect against oxidative damage and help guard against cognitive decline.\(^{[11]}\) Oleuropein is responsible for most of olive oil’s antioxidant, anti-inflammatory, and disease-fighting characteristics.\(^{[12,13]}\) Oleuropein completely regressed and inhibited different types of mice tumors within 9 to 12 days of its administration.\(^{[14]}\) In addition oleuropein has an antioxidant activity as it is potently and dose-dependently inhibits copper sulphate-induced oxidation of low-density lipoproteins LDL-cholesterol.\(^{[15]}\) Oleuropein has both the ability to scavenge nitric oxide and to cause an increase in the inducible nitric oxide synthases expression in the cell.\(^{[16]}\) Oleuropein has also been reported to have hypoglycemic and antioxidant effects.\(^{[17]}\)

This study aimed to investigate the histochemical and histological changes in the lung tissue of male albino rats which exposed to gamma radiation and the possible therapeutic role of both olive leaf extract and bone marrow mesenchymal stem cells.

**MATERIAL AND METHODS**

**Experimental animals:**

A total of 40 male Swiss albino rats (\textit{Sprague dawely strain}) (weighting 130 ± 5 gm) were obtained from the Egyptian Organization for Biological Products and Vaccines. They were kept for about 15 days, before the onset of the experiment under observation to acclimatize the laboratory conditions. Rats were housed collectively in plastic cages, maintained under standard conditions of light, ventilation, temperature and humidity and allowed free access of standard pellet diet and tap water. All animal procedures were carried out in accordance with the Ethics Committee of the National Research Centre conformed to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health.

**Gamma –irradiation procedure:**

Irradiation process was performed using Gamma Cell-40 achieved by Egypt’s National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt. The gamma cell–40 is a caesium-137 irradiation unit manufactured by Atomic Energy of Canada Limited. The unit provides means for uniform Gamma-irradiation of small animals or biological samples while providing complete protection for operating personnel. The dose rate was 0.54 Gy/min at the time of the experiment. The radiation dose level was 3 Gy single dose.

**Olive leaves (\textit{Olea europaea}) Extraction:**

Olive (\textit{olea europaea}) leaves were weighed and ground to a fine powder in an electric mixer. The powdered plant material was extracted twice (24h each time) in 70% ethanol by soxhlet apparatus,\(^{[18]}\) the extract supplied to groups of animals as a single dose (15 mg/kg b.w.) via intragastric gavages daily for 2 weeks.\(^{[19]}\)

**Mesenchymal stem cells Transplantation:**

Bone marrow transplantation rat donors and recipients were chosen of the same inbred strain, sisters to sisters. The donors were sacrificed and femur bones were dissected out, cleaned and both ends were chipped by bone nibbling forceps. The marrow was blown of the femur into saline solution under sterilized conditions surrounded by ice cubes and mixed by drawing and expelling it several times from the syringe without needle in order to avoid mechanical damage to the cells.

Concentration of mesenchymal stem cells transplantation was 3×10^6 cells/ml suspension they were transplanted into the irradiated rats through caudal vein according to the method of Abdel Aziz et al.\(^{[20]}\) Ten animals received 100 μL cell suspension.

**Experimental design:**

The experimental animals were randomly divided into 5 groups (n=10) as following.

**Group 1**: control rats: normal healthy rats left without any treatment (C).

**Group 2**: rats treated with olive leaves extract (O),for 2 weeks (15 mg /kg body weight/daily).
Group 3: the irradiated group: rats exposed to a single dose of gamma-radiation, 3 Gy (R).

Group 4: olive irradiated rats (RO): rats of this group treated with olive extract 15 mg /kg body weight/daily one week before and one week after irradiation.

Group 5: stem cell-irradiated animals (RS): rats of this group irradiated with 3Gy then treated with transplanted (MSCS) 3x10⁶ cells/ml suspension through caudal vein about 5h post radiation exposure.

The experimental rats were sacrificed at 7 days post irradiation.

Histological and histochemical techniques

Lungs were immediately excised and fixed in 10% neutral formalin. Paraffin sections (5μm in thickness) were prepared for processing the histological and histochemical studies. For general histology, sections were stained with Harris’ hematoxylin and eosin. Collagen fibres were stained by using Mallory's trichrome stain. Polysaccharides were detected by using periodic acid Schiff’s (PAS) reagent. Total proteins were detected by using the mercury bromophenol blue method and DNA materials were detected by using Feulgen's method.

RESULTS

Histopathological observations of the lung

Control group (C): stained sections of the rat lungs showed normal alveoli with their thin inter alveolar septae lined with simple squamous epithelium, clear alveolar sacs. Bronchioles appear with their pseudostratified ciliated columnar epithelial cells. Peri-bronchiolar blood vessels and capillaries are normal in appearance with few numbers of macrophages in the lung interstitium.

Mallory’s trichrome stained sections of the lung tissue of the control group showed normal distribution of the collagen fibres which are supporting walls of the bronchioles, blood vessels and alveolar septae.

Olive leaves extract treated group (O): there were no detectable histological or histochemical changes in lung tissue of this group.

Irradiated group (R): the lungs of 3Gy gamma irradiated rats showed many histopathological changes represented by highly thickened and congested alveolar septae, narrow alveolar sacs, highly thickened arterial and venous walls which appeared congested with haemolysed blood cells inside them. Bronchial walls were highly thickened and some of them showed debris of degenerated cells inside their lumens with granulomatous areas in the lung tissues. Highly increased collagen fibres were demonstrated in the highly thickened and corrugated arterial and bronchial walls, in the interstitium between them and also in the granulomatous areas.

Olive irradiated group (RO): lungs of rats exposed to gamma radiation and treated with olive extract showed some signs of improvement in the lung architecture, where somewhat normal appearance of alveolar sacs, alveolar septae and bronchioles were detected, but congested arteries had thickened walls with haemolysed blood cells inside them.

Moderate increase in collagen bundles deposition was also detected in walls of the bronchioles and alveolar septae.

Stem cells irradiated group (RS): lungs of rats exposed to gamma radiation and treated with stem cells showed well developed architecture of the lung tissues.

Histochemical observations of the lung

Polysaccharides

Normal distribution of PAS +ve materials (magenta color) is seen in the lung tissue of the control rat (Fig. 23) indicated by moderate staining affinity of the bronchial walls and alveolar septae. Increased staining affinity of PAS +ve materials was detected in the thickened walls of bronchioles, walls of arteries and alveolar septae of R group with moderately stained granuloma areas (Figs. 24 & 25). An increase in PAS +ve materials was observed in the walls of bronchioles, alveolar septae and blood vessels of RO group (Figs. 26, 27 & 28).

However, somewhat normal appearance of PAS +ve materials was demonstrated in lung tissues of RS group with the appearance of deeply stained PAS +ve materials in the thickened arterial walls (Figs. 29 & 30).

Total protein

Lung tissue of rats of the control group shows...
normal distribution of the total protein content represented by deeply to moderately stained cells of the bronchial walls, alveolar septae and walls of the blood vessels (Fig. 31). Highly increased staining affinity of total protein was noticed in the thickened and corrugated walls of the pulmonary bronchioles, blood vessels and alveolar septae of R group (Figs. 32, 33 & 34). However, moderate increase in staining affinity of total protein was detected in the bronchial walls and also in some alveolar septae of RO group (Fig. 35). To some extent, normal distribution of the total protein was realized all over the lung tissues of RS group as illustrated in Fig. 36.

DNA
Lung tissue of the control rats showed normal distribution of DNA in the form of magenta color granules (Fig. 37). A noticeable increase in DNA content was detected in the nuclei of the highly thickened walls of arteries, bronchioles, alveolar septae and some nuclei of degenerated cells in the bronchial lumens of R group (Figs. 38 & 39). Lung tissue nuclei of RO group revealed more or less normal appearance of Feulgen stained materials (Fig. 40). In addition normal appearance of DNA content was also detected in the lung tissue nuclei of RS group as demonstrated in Figs. 41 & 42.

DISCUSSION
Radiation induced pulmonary fibrosis is a disease with high morbidity and mortality rates due to progressive breakdown of pulmonary architecture, which results in respiratory failure. Fibrotic lesion formation in irradiated lungs can generally be classified into three stages, a latent phase (less than one week post-irradiation), a pneumonitic phase (2 -16 weeks post-irradiation) and a fibrotic phase (more than 24 weeks post-irradiation). Gamma radiation induces pulmonary fibrosis by direct activation of an epithelial mesenchymal transition in type II alveolar epithelial cells. Transforming growth factor-betalpha1 (TGF-b1) plays an important role both in regulating the transformation of fibroblasts into myofibroblasts and in inducing type II alveolar epithelial cells to undergo an epithelial mesenchymal transition into myofibroblasts, which are contractile cells that cause alveolar collapse through the production of fibres and extracellular matrix. Mesenchymal stem cells (MSCs) are population of multipotent cells which are capable of differentiating into multiple lineages and promoting tissue regeneration. In irradiated tissue, the MSCs differentiated into functional lung cells, including endothelial and epithelial cells. In addition, MSCs attenuate inflammatory responses by increasing the number of regulatory T cells and/or enhancing the levels of anti-inflammatory cytokines, such as Interleukin-10 (IL-10). Moreover, bone marrow derived MSCs can attenuate the inflammatory response in irradiated lungs by up regulating of IL-10 expression in the latent phase and down regulating tumor necrosis factor-alpha (TNF-α), Interferon Gamma (IFN-γ), Interleukin-1β (IL-1β) and Interleukin-6 (IL-6) expression. Mesenchymal stem cells have the potential to limit pulmonary fibrosis and to prevent the irradiated type II alveolar epithelial cells from undergoing epithelial-mesenchymal transition after exposure to ionizing irradiation. Radiation-induced pulmonary damage could affect normal lung tissue and may lead to both early phase pneumonitis and late phase fibrosis months to years post exposure. Lung tissue damage post-irradiation was recorded before. Results obtained in the present work revealed that exposing rats to 3Gy gamma radiation induced many histopathological changes represented by narrow alveolar sacs with highly thickened and congested alveolar septae, highly thickened arterial and venous walls which appeared congested with haemolysed blood cells. Bronchial walls appeared highly thickened, some of pulmonary bronchioles showed debris of degenerated cells with granulomatous areas in the lung interstitium. Such findings come in agreement with those reported earlier following ionizing and non ionizing radiations. Lung neutrophils are the first responding cells injured by irradiation. It was reported that lung histopathological changes following gamma radiation exposure may be due to oxidative stress and subsequent overproduction of reactive oxygen species (ROS) which was postulated as one of the most important mechanisms of radiation toxicity.
realized in the present experiment and this may be due to increased pulmonary arterial pulse pressure. \cite{40} Also, the appearance of degenerated alveolar septae and debris of degenerated cells in the lung bronchioles may be due to highly affected DNA in the nuclei of their cells. \cite{41} Results of the current experiment showed highly increased collagen bundles in the highly thickened and corrugated walls of arteries, around walls of the bronchioles and in the interstitium between them, highly increased collagen fibres deposition was also noted within the granulomatous areas of the lung tissue of irradiated group (R). Increased collagen fibres post exposure to gamma irradiation in the current work comes in coincidence with those reported before. \cite{41,42} Such increase in collagen fibres deposition might lead to rapid healing process where the secretion of collagen subtype within the injury site increases to replace necrotic tissue during the proliferative phase of the wound healing mechanism. \cite{43} Administration of olive extract to irradiated rats in the present experiment showed improvement in the architecture of the lung tissues where somewhat normal appearance of alveolar sacs and bronchioles were detected, but blood vessels appeared congested and contain haemolysed blood cells with thickened arterial walls. In addition, moderately increased collagen fibres deposition was detected in walls of the bronchioles and alveolar septae.

Lung architecture improvement in the present study could be attributed to presence of oleuropein which is the most prominent phenolic compound in the olive leaves extract that has anti-inflammatory and antioxidant properties. \cite{44} Where it reduces infiltration of cells, as well as helped wounds heal more quickly, such phenolic compound has been demonstrated to stimulate fibroblast formation for synthesizing new collagen fibres and influence collagen metabolism and support wound healing. \cite{45} The decrease of infiltrated cells in the wound site could increase the wound healing activity. \cite{46} Mesenchymal stem cell injection of rats showed somewhat normal architecture of the lung tissues of rats of RS group with more or less normal distribution of collagen fibres. Attenuation of abnormal histological appearance of lung tissue following BMSCs injection was reported by \textit{et al.} \cite{47}; the authors attributed such recovery to their radical scavenging activities, which prevent the accumulation of hydroxyproline in the lung tissues.

Results of the current study revealed increased PAS +ve materials in the thickened walls of bronchioles, arteries and alveolar septae with moderately stained granuloma areas in lungs of the irradiated group (R) as compared to the control group. Increase of the lung polysaccharides staining affinity following 2 Gy gamma radiation exposure was reported before. \cite{40} The increase in the PAS +ve materials after gamma radiation exposure could be due to increased thickness of the lung components. Moreover, such increase in the staining affinity may be due to the increase in the RBCs post-exposure to gamma irradiation, increased staining affinity of the granulomatous areas indicating their high polysaccharides content. Increased PAS +ve materials in the thickened walls of bronchioles, alveolar septae and blood vessels were also demonstrated in RO group of the present work whereas restoration of the normal appearance of PAS +ve materials in the lung tissues of rats of RS group was observed following BMSCs administration. Such restoration may also be due to the increase in the activities of antioxidant enzymes including superoxide dismutase (SOD) and the decreases in malondialdehyde (MDA) levels in lung tissues. \cite{47} The current results showed highly increased staining affinity of DNA and total protein in the thickened and corrugated walls of the bronchioles, blood vessels and also increased staining affinity of alveolar septae in the lung of the irradiated R group as compared to the control group. Increased total protein content of lung tissue post exposure to gamma radiation was recorded before by \textit{Mansour}. \cite{48}; the author declared that the increase in staining affinity of total protein of lung tissues following gamma radiation exposure may be attributed to increased RBCs in the blood vessels and also to increased collagen fibres deposition. Increased staining affinity of both DNA and total protein may be due to the generation of ROS and consequent oxidative stress. \cite{49} Gamma radiation can induce DNA damage by the reactive oxygen species (ROS) directly or indirectly. If normal cells failed to repair such damage, it can lead to the
cell cycle inhibiting even premature senescence and cell apoptosis. Free radicals could also react with DNA bases, impairing their structure and leading to mutations. The most common type of damage is DNA oxidation. An improvement of both DNA and total protein contents were noted in the present results following olive extract application and such improvement may due to the action of olive leaf extract on the lung tissue by DNA repairing system and enhancing protein synthesis. Also oleuropein acts as a free radicals scavenger, since DNA materials are the main target of them. In addition, this improvement may also be due to the antioxidant activity of olive leaf extract where oleuropein stimulates endothelium formation as well as synthesis of mRNA and protein.

Bone marrow mesenchymal stem cells treatment nearly restored both DNA and total protein content in the lung tissue. Somewhat normal total protein content was reported earlier in the fetal lung tissue maternally treated with the bone marrow cells post-irradiation. Lung tissue restoration following gamma radiation exposure could be due to the therapeutic effect of BMSCs. As the stem cells can be transplanted to replace nonfunctional or lost stem cells in tissues to enhance tissue healing and restore their original function.

CONCLUSION
According to the results obtained in the current study administration of oil leaf extract or bone marrow mesenchymal stem cells (BMSCs) provides good therapeutic effect against gamma radiation induced histological and histochemical alterations in lungs of male albino rats. A better ameliorative effect was obvious in BMSCs treatment.

REFERENCES:
15. Visioli F, Poli A and Galli C (2002): Antioxidant and other biological activities of
37. Mansoub NH and Sarvestani A H (2011): Effects


Figures 1-12: photomicrographs of lung tissue of the control and treated groups (H&E X100&200)

**Fig.1** - Lung tissue of a control rat showing normal alveolar sacs (as), inter alveolar septae (S) lined with simple squamous epithelium, bronchioles (br) with their pseudostratified ciliated columnar epithelial cells, peribronchiolar blood vessels (bv) and capillaries with few number of macrophages in the lung interstitium (X 100).

**Figs.2-6** - Lung tissues of R group showing highly thickened and congested alveolar septae (S), narrow alveolar sacs (as), highly thickened walls of arteries (A) and veins (V), some of them are congested with haemolysed blood cells (→) and highly thickened walls of bronchioles (br), some of them contain debris of degenerated cells (d) with granulomatous areas (G) in the lung tissues (X 200)
Figs. 7-10- Lung tissues of RO showing improvement in the architecture of the lung tissues, but blood vessels are still congested and contain haemolysed blood cells with thickened arterial walls (arrow). Somewhat normal appearance of alveolar sacs and bronchioles are detected (X 200).

Figs.11, 12- Lung tissues of RS showing well developed architecture of the lung tissues (X 200).
Figures 13-22- Photomicrographs of lung tissue showing distribution of the collagen fibres in the lung tissues of the control and treated groups (Mallory’s trichrome stain X 200)

Fig.13- Lung tissue of a control rat showing: thin collagen fibres supporting walls of bronchioles, blood vessels and alveolar septae (X 100).

Figs.14-18- Lung tissues of R group showing highly increased collagen bundles in the highly thickened and corrugated walls of arteries, bronchioles, in the interstitium between them and in the granulomatous areas (G). Notice: brightly stained red blood cells inside the highly congested blood vessels and fatty cells (F) inside the lung tissue(X 200).
Figs. 19, 20- Lung tissue of RO group showing moderately increased collagen fibres in walls of the bronchioles and alveolar septae (X 200).

Figs. 21, 22- Lung tissues of RS group showing somewhat normal distribution of collagen fibres in the lung tissues of rats of this group (X 200).
Figures 23-30- Photomicrographs of lung tissue showing distribution of PAS +ve materials in the lung tissues of the control and treated groups (PAS X 100, 200)

Fig. 23 - Lung tissue of a control rat showing moderately stained PAS +ve materials in walls of the bronchioles and alveolar sacs (X 200).

Figs. 24, 25- Lung tissues of R group showing increased PAS +ve materials in the thickened walls of bronchioles, arteries and alveolar septae. Notice: moderately stained granuloma areas (X 200).

Figs. 26-28- Lung tissues of RO group showing increased PAS +ve materials in walls of the bronchioles, alveolar septae and blood vessels (X 100, 200).

Figs. 29, 30- Lung tissues of RS group showing somewhat normal appearance of PAS +ve materials in the lung tissues of rats of this group, but thickened arterial walls are still detected with deeply stained PAS +ve materials (X 100).
Figures 31-36-Photomicrographs of lung tissue showing total protein distribution in the lung tissue of the control and treated groups (Mercury bromophenol blue X 100, 200)

Fig.31- Lung tissue of a control rat showing moderately stained total protein in walls of the bronchioles, alveolar septae and blood vessels (X 200).

Figs.32-34- Lung tissues of R group showing highly increased total protein in the thickened and corrugated walls of the bronchioles, blood vessels, granuloma areas and with increased staining affinity in alveolar septae. Notice: dark stained hemosiderin granules inside the blood vessels (X 100).

Fig.35- Lung tissues of RO group showing: a moderate increase in the total protein in the bronchial walls and some alveolar septae (X 200).

Fig.36- Lung tissues of RS group showing: somewhat normal distribution of total protein in the lung tissues of this group (X 200).
Figures 37-42-Photomicrographs of lung tissue showing distribution of DNA materials in the lung tissue of the control and treated groups (Feulgen reaction X 100, 200, 400)

Fig. 37- **Lung tissue of a control rat** showing: normal distribution of the DNA materials in nuclei of the epithelial cells of walls of the bronchioles and thin alveolar septae of the lung tissue (X 200).

Figs. 38, 39- **Lung tissues of R group** showing: increased DNA materials in the highly thickened walls of arteries, bronchioles and alveolar septae. Notice: debris of nuclei of degenerated cells is detected in the lumens of the bronchioles (X 200, 100, 400).

Fig. 40- **Lung tissues of RO group** showing somewhat normal appearance of DNA materials in walls of the bronchioles and pulmonary blood vessels in the lung tissues of this group. Notice: erosion of pseudostratified ciliated columnar epithelial cells of the bronchiole with debris of their nuclei in its lumen (X 100).

Fig. 41, 42- **Lung tissues of RS group** showing normal appearance of DNA materials in the bronchioles and thin alveolar septa in lung tissues of this group. (X 200 & 400).