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Research Article

The Effect of Irradiation on the Skin upon Breast Cancer Radiotherapy Studied by FTIR Spectroscopy

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Abstract

Breast cancer affects the female population worldwide. Radiotherapy (RT) is part of the therapeutic modality in the management of breast cancer, after radical mastectomy or conserving surgery. The FTIR spectroscopic "marker bands" will lead us to approach the mechanism of skin damage due to the interaction of ionizing radiation and skin, on a molecular level at the very early stages. FT-IR spectroscopy, breast digital pictures, and ImageJ software were used in the study. Healthy breast skin was irradiated *ex-vivo* with a 4 Gy dose of a γ-^{60}Co course Gammachamber 4000A. The FT-IR spectra showed that the low-dose irradiation induces skin dehydration, collagen secondary structure changes and advanced glycation end products (AGEs) as a result of free radicals as mediated products. The infrared

"marker bands" at about 1743, 1160, and 870 cm^{-1} are characteristic, indicating the development of inflammation, glycations, and peroxidations respectively, due to ionizing radiation-induced oxidative stress. ImageJ analysis provided the sharp surface of the skin after RT irradiation in contrast to the smooth surface of the non-irradiated healthy skin. The most important damages, induced by radiotherapy, were connective tissue lesions, glycosylation, and phosphorylation processes in the skin. The reactive oxygen species (ROS) free radicals prefer to abstract H atoms from lipids, sugar rings of glycoproteins, and base ribose of DNA. The produced intermediate free radicals, as a result of ROS reactions, led to the formation of AGEs and peroxides.

Keywords
Spectroscopy; Fourier Transform Infrared (FTIR); radiotherapy; skin irradiation and aging; ImageJ

1. Introduction

Breast cancer is the most common cancer in the female population. Radiotherapy is a highly effective method in the multifactorial therapeutic approach and plays a key role in the postoperative setting, such as breast-conserving surgery or radical mastectomy, with or without chemotherapy [1]. The side effects induced in the local adjuvant treatment after surgery have been well known since the discovery by the physicist Emil Grubbé in Chicago in 1895. He described the first symptoms of the harmful results of X-rays on the skin, which were erythema, edema, hyperemia, and hyperesthesia, whereas a few days later, the skin presented bleb [2]. It has been observed that dermatitis occurred in nearly 90% of breast cancer patients receiving radiation therapy [1, 3, 4]. It is well documented that the collisions of ionizing radiations with the skin cause electronic excitation and ionization of biological components of the cells. However, in biological systems due to the high concentration of water (>70%), the major process taking place is the production of reactive oxygen species (ROS), such as $HO^{\cdot}$, $HO_2^{\cdot}$, $O_2^{-\cdot}$ and the reductant species hydrated electrons (e_{aq}^-) and hydrogen atoms, which by the indirect interactions with biological molecules, provoking changes of the redox potential and increasing the radiation-induced damage [2, 5]. Skin models have been used to study the effects induced by ionizing radiations *in vitro* [6]. It has been shown that irradiation affects epidermal homeostasis by compromising the regulatory mechanism that balances proliferation and cell loss [7]. Such as, a study on cultured human breast skin demonstrated that epidermal homeostasis perturbation begins 24 h after a single dose of γ-rays (2 Gy) [8].

The pathophysiology of radiation-induced dermatitis is based on an inflammatory response process, activating several mediators (NF-κB, iNOS, COX-2) leading to the secretion of pro-inflammatory cytokines (IL-1, IL-2, IL-6, TNF-α, IFN-γ) and factors that regulate the immune response in the irradiated microenvironment [9-11]. Macrophages and monocytes play an important role in acute inflammation by secreting pro-inflammatory cytokines release [12], due mostly to huge cell death in rapidly dividing cells, followed, by the enhanced movement of leukocytes from the blood into the irradiated tissues, as a response to radiation. Under this condition, just a few hours after

exposure, there was an increased cellular population of macrophages, neutrophils, lymphocytes and DCs locally [13, 14].

Many studies have demonstrated that fibrosis is a complex phenomenon and is the most important injury of RT [15-18] exposure. Fibrosis is related mostly to the reactions between biological molecules and the produced ROS upon radiolysis of skin water molecules, which account for about 60-70% of the total damage while their accumulation increases the damaging effects [19, 20]. ROS accumulation, by increasing the irradiation dose during RT, affects the redox potential of cells and leads to a series of membrane peroxidations and other chain reactions that finally causes non-reversed damage [21, 22]. For higher RT-dose administration**,** DNA *in vitro* and *in vivo* studies have shown that phosphorylation and methylations are some of the early irradiation effects among intermediate products [2, 23-27].

Although the clinical effects of ionizing radiation on the skin are known, however, the mechanism at a molecular level is not yet clear. Fourier Transform Infrared (FT-IR) spectroscopy is an appropriate method to study the changes induced by irradiation on skin tissues. FT-IR spectroscopy is a non-invasive physicochemical method, that is reproducible, extremely rapid, and does not require any special treatment or labeling of the samples as in histopathology [28-35]. The method is based on the interactions of infrared radiation taking place within the matter and the obtained spectrum contains all the vibrations of the molecules present in a sample, which may be simple as a single cell or more complicated, such as a tissue and body liquid providing information on all components simultaneously [28-35] of complex shapes of groups, such as NH, NHCO, COOH, CH_3, CH_2, PO_2-, etc. of biomolecules in cells and tissues simplify the analysis. This makes FT-IR spectroscopy an extremely valuable tool for lipid, protein, DNA, and membrane structural determination studies in the pre-diagnosis and diagnosis of diseases [32-35]. In this research paper, to simulate the effects of ionizing radiation on the skin during RT, the skin was irradiated *ex-vivo* with γ-^{60}Co rays. FT-IR spectroscopy was used to provide the changes at a molecular level induced by ionizing radiation.

ImageJ analysis was applied to study the macromolecular changes of the architecture morphology of the skin together with the infrared study.

2. Materials & Methods

The samples used in this experiment were normal breast skin samples obtained from five healthy women, who underwent breast aesthetic plastic surgery. The skin samples were fixed in 10% formalin and not embedded in paraffin. Then they were washed with distilled water and dried under a vacuum at room temperature for further investigation. The samples were irradiated *ex vivo* in the solid state with a dose of 4 Gy (Grey unit) using a Gammachamber 4000 A γ-^{60}Co course. The radiation dose was calculated by using Fricke dosimetry, taking G(Fe^{3+}) = 15.6 [36].

The FT-IR spectra were recorded in absorbance mode at room temperature with a Thermo-Scientific Nicolet 6700 spectrometer, equipped with an Attenuated Total Reflection (ATR) accessory in the infrared region of 4000-800 cm^{-1}. The sample was placed on the ATR crystal plate of the instrument, without any other handling, as described elsewhere [37, 38]. There is the possibility to record the spectra in different positions of the same sample. Each infrared spectrum at the specific site on the specimen was the average of 120 co-added spectra with a resolution of 4 cm^{-1}. The OMNIC 7a workstation software (included in the instrument) was used for data analysis.

The open-access platform of ImageJ-Fiji software [39] was used to analyze the digital breast photographs collected from 15 women, who underwent radiotherapy for breast cancer, aiming to study and reveal the skin alterations related to the processes that occur during radiotherapy.

The samples were obtained according to the principles of the Helsinki declaration and the Greek law of ethics for *ex vivo* clinical research.

3. Results

To understand the effect of ionizing radiation on the skin, human healthy skin was irradiated *ex-vivo* with a single dose of 4 Gy. The dose is lower than the one used in breast cancer radiotherapy treatment schedules since there are not much data concerning the very early effects of radiotherapy at a molecular level. The representative FT-IR spectra of non-irradiated (A) and *ex-vivo* irradiated (B) skin with γ-rays are given in Figure 1. A comparison of the two spectra showed considerable frequency, intensity, and shape changes of bands in all spectral regions between 4000-800 cm^{-1}. Particularly, in the regions 3700-3000 cm^{-1}, 3000-2850 cm^{-1} and 1800-800 cm^{-1}.

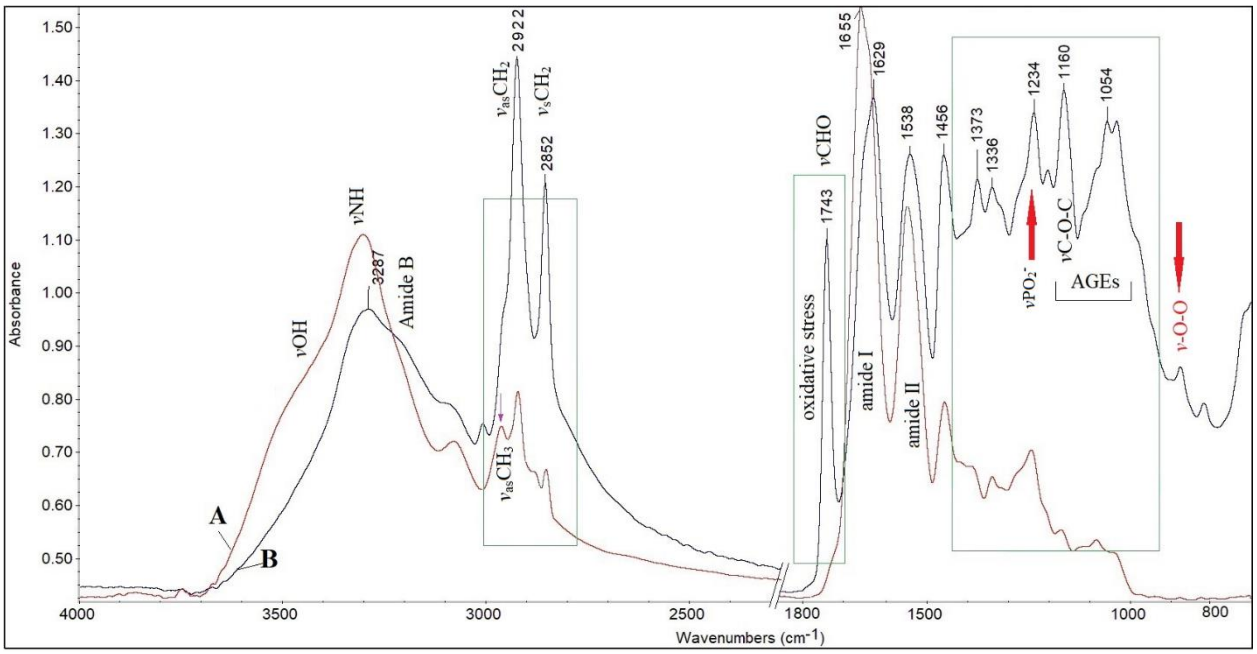

Figure 1 Representative FT-IR spectra of A) non-irradiated skin and B) *ex-vivo* irradiated skin with a single dose of 4 Gy in the region 4000-800 cm^{-1}.

The spectrum of irradiated skin is characterized by the decrease of the absorption spectral bands in the region 4000-3000 cm^{-1}. In this special region located the stretching vibration bands of *ν*OH groups resulting from the water molecules and glycoproteins of the skin. The intensity reduction of this characteristic band is related mostly to skin dehydration [30, 31]. In this case, collagen exists in the α-helix conformation and the stretching vibration of *ν*NH groups normally occurs within the range of 3300-3000 cm^{-1}, which is characteristic of collagen α-helix conformation [30-34, 37, 40]. The intensity reduction of this band at 3300 cm^{-1} and the peak shifts from about 3287 cm^{-1} to lower wavenumbers, followed by the new broadband near 3200 cm^{-1}, indicates that the conformational structure of collagen modified from Amide A to Amide B configuration. This is the result of the

energy change of the protein backbone hydrogen bond between the hydrophilic and hydrophobic donors which stabilizes the collagen strands together [36-38, 40].

In the next important spectral region between 3000-2850 cm^{-1} are shown the stretching vibration bands of asymmetric and symmetric vibrations of methyl ($\nu_{as,s}CH_3$) and methylene ($\nu_{as,s}CH_2$) groups mainly from membrane lipids and phospholipids [28-35]. After skin irradiation, the asymmetric and symmetric vibrations of methyl ($\nu_{as,s}CH_3$) almost disappeared and the corresponding intensities of methylene stretching vibration bands ($\nu_{as,s}CH_2$) increased in intensity, concerning the increasing lipophilicity of the membrane environment [26-35, 40, 41].

The highly intense new band at 1743 cm^{-1} is attributed to the stretching vibration of the aldehyde *v*CHO group formed during skin irradiation, due to lipid and protein peroxidations [22, 42, 43]. The bands at 1655 cm^{-1} and 1545 cm^{-1} are assigned to amide I and amide II, respectively, and have been proposed to be related to the α-helix conformational structure of the native proteins [28-35, 42, 43]. These bands shifted to lower frequencies 1629 cm^{-1} and 1538 cm^{-1}, respectively, upon irradiation, which is the β-sheet configuration formation [41-43]. These shifts in combination with the observed enriched lipophilic environment, as it resulted from the increasing intensity of the stretching absorption bands of $\nu_{as,s}CH_2$ groups, lead to the suggestion that this environment supports the aggregate and amyloid protein formation [44].

In the fingerprint region between 1400 and 800 cm^{-1}, many spectral intensity and shape changes were observed. The intensity increase of the band at about 1234 cm^{-1} (Figure 1B) after irradiation revealed phosphorylation, a phenomenon connected strongly with ROS activity, inducing DNA damage by γ-radiolysis [2, 21, 23, 28, 42]. The phosphate release is the result of $HO^{\cdot}$ radical attacks with C(3') and C(5') hydrogens, leading to strand fragmentation [2, 45] and the production of new free radicals and phosphate anions. Another important observation was the intensity increase of the band at 1160 cm^{-1}, which is assigned to the *v*C-O-C vibrational mode, where the oxygen atom is linked to two carbon atoms of the sugar moiety of glycosaminoglycans together with the exocyclic C-O-C intermolecular group [21]. This band confirmed the glycosylation and the production of advanced glycation end products (AGEs) and the shift of this band to higher wavenumbers is related to the progression of the lipophilic environment upon irradiation [28-30, 42, 43]. Glycosaminoglycans are important single-strand polysaccharides of connective skin tissue that produced free radicals **1** by reacting with ROS [2]. Free radicals 1 are unstable and usually cause the formation of **2** and **3** radicals, as follows in Figure 2:

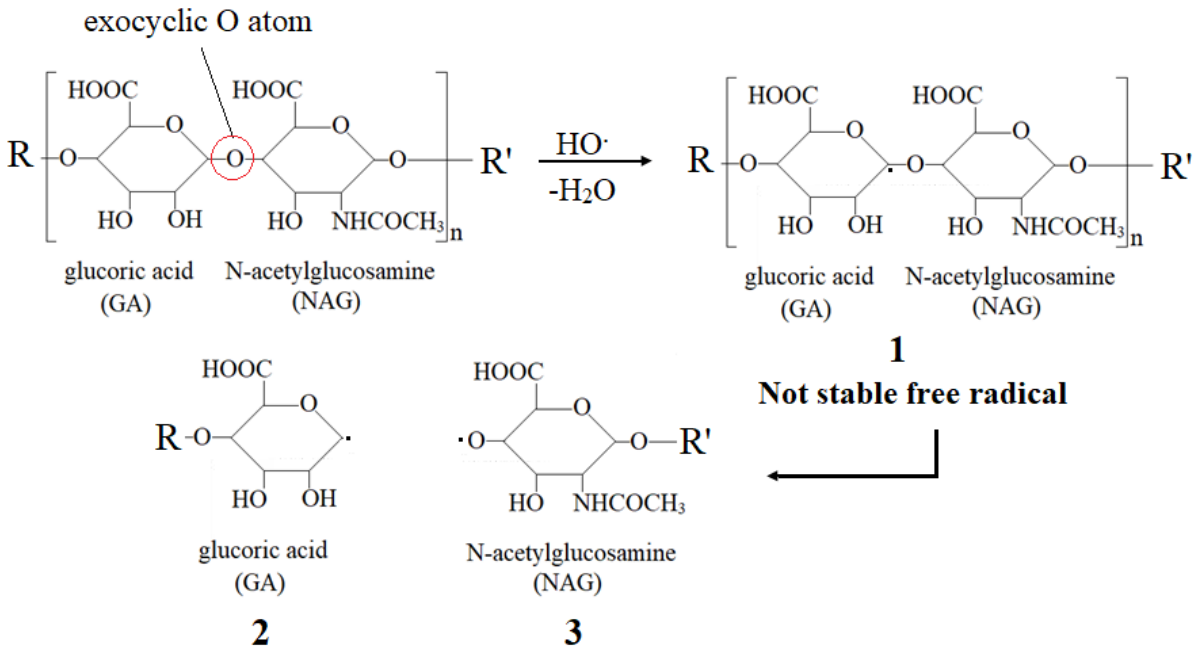

Figure 2 The reactions of the disaccharide, the monomer of glycosaminoglycans with hydroxyl free radicals, and the formation steps of new sugar radicals 1, 2, and 3. The exocyclic oxygen atoms are located between the two sugar rings. R and R' correspond to different chain lengths [21].

The free radicals 2 and 3 are also unstable and further interact with lipids, DNA-bases, and protein intermediate fragments produced from radiolysis, finally leading to stable AEGs production that aggravates the effect of RT on skin. Notably, the appearance of the band at about 870 cm^{-1} is assigned to the stretching vibration of *v*-O-O peroxide groups confirming the production of peroxides [46]. Molecular oxygen, a double free radical, reacts in a diffusion control reaction with intermediated free radicals to produce a series of peroxides that stabilize the damaging effect of ionizing radiation and cellular oxygen depletion.

Scientists tried to find an easy and less invasive technique to study the changes induced on the skin upon RT in the first days. Figure 3 shows the digital photographs of a patient's breast after surgery and RT. The right breast corresponds to the irradiated breast (1) and the left breast to the healthy one (2). The 1' and 2' correspond to the square labeled regions 1 and 2 of ImageJ analysis.

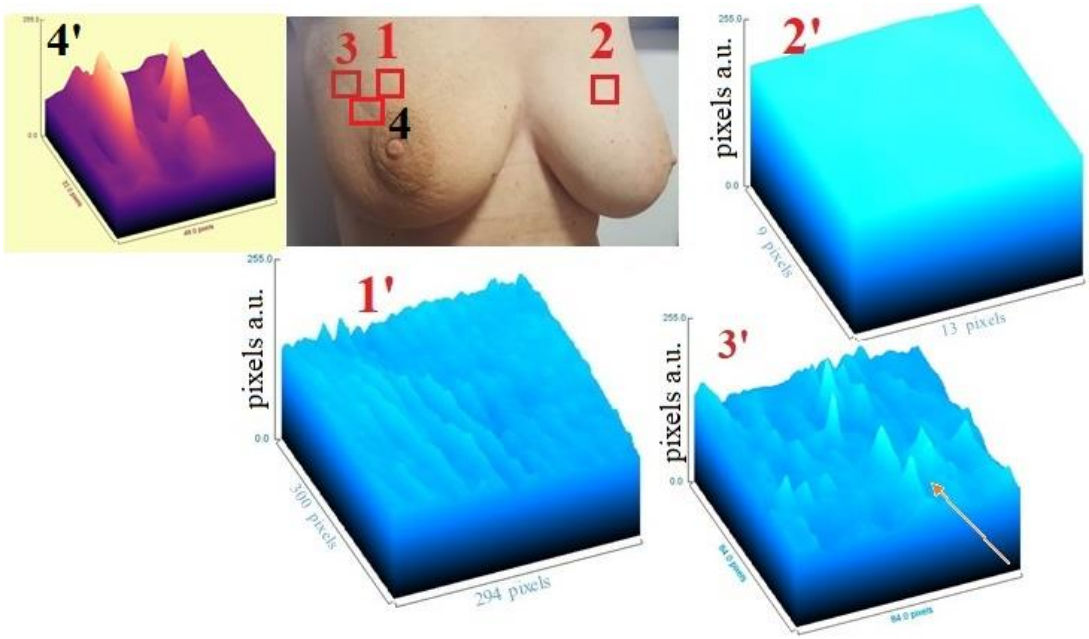

Figure 3 ImageJ analysis of photographs received of patient's breasts skin. 1 is the irradiated right breast, 2 is the healthy left breast, 3 and 4 are on the scar due to surgery. 1', 2', 3' and 4' are the corresponding squares. The irradiated epidermis appears sharp and the non-irradiated epidermis is smooth.

A comparison of the 1' and 2' pictures (Figure 3) showed that the irradiation affected the architecture of the epidermis, which appeared sharp with a pattern channel, while the healthy one appeared homogeneous with a smooth superficial surface. The irradiated skin morphology leads to the suggestion that the glycosidic bond that stabilizes the connective tissue is more susceptible to RT, which is in agreement with the observed FT-IR spectra (the band confirmed the glycosylation and the production of AGEs). These data are consistent with those derived from irradiated cartilage, another important glycosaminoglycan-rich tissue, which gave similar damage on FT-IR spectra and ImageJ picture [22]. An important finding was the analysis of the scar, which showed the appearance of sharp formations like "hills". These "hills" indicated the increase of tissue conductivity of the scar upon RT, concerning the deposit of calcium (Ca^{2+}) cations [22, 42].

4. Discussion

Since the use of ionizing radiation for cancer treatment, scientists have been interested in understanding the mechanism of skin damage, since the skin is the first and most important part of the body that interacts with rays in external beam radiotherapy (EBRT). The effects of ionizing radiation are direct when the rays interact with the molecules or indirect when the rays pass through the species produced by the radiolysis of water molecules, as shown in Equation 1 [2, 21].

$$H_2O \rightsquigarrow HO^{\cdot}, H, e_{aq}^{-}, H^{+}, H_2O_2 \quad (1)$$

The produced hydroxyl free radicals ($HO^{\cdot}$) are strongly oxidative species and interact with organic molecules and metal ions changing the redox potential of the cell membranes. Radiolysis of water leads to skin dehydration, not only because of the transepidermal water loss but also, as suggested

by the decreased intensity in the stretching absorption bands of *v*OH groups, due to H_2O consumption through the radiolysis process. The FT-IR spectra revealed that hydrogen bonds of proteins are affected by destabilizing the peptide bonds and strands. This became clear from the changes in the absorption bands in the regions 3300-3000 cm^{-1} and 1700-1500 cm^{-1}, which characterize the protein secondary structure and amyloid formation. The hydrated electrons (e_{aq}^-) interact only with the protonated amino groups of protein amino acids, leading to the elimination of NH_2 groups by releasing ammonia (NH_3) [2, 46-49]. These results are in agreement with the reduction of the intensity band at 3300 cm^{-1} observed in the irradiated skin spectrum (Figure 1B). These intermediate protein free radicals lead to the cleavage of the polypeptide chain supporting the cross-linking process of protein aggregates.

The FT-IR spectral "marker band" at 1743 cm^{-1}, confirms the inflammation of tissues as a result of lipids and proteins peroxidation and that one of the most important products is malonaldehyde, which has been observed in many diseases [21, 30, 42, 43]. The inflammation has been observed clinically in almost all patients and it is related to dose rates, while the accumulation of free radicals seems to provoke endogenous defense systems [9-13]. In the oxygenated cells, the molecular oxygen reacts with the DNA, lipid, protein, sugar, and other free radicals to yield peroxides (R_1O-OR_2). The peroxidation of skin was confirmed by the absorption band at about 870 cm^{-1} which is assigned to the stretching vibration of the *v*O-O peroxide bond [46]. Furthermore, the hydrated electrons reacted with molecular oxygen by electron transfer reaction to produce superoxide anions, as shown in Equation 2.

$$e_{aq}^- + O_2 \longrightarrow O_2^{-\cdot} \quad (2)$$

The superoxide anions are long-lived free radicals that can move long distances by changing the redox potential of the cells undergoing other enzymatic or non-enzymatic pathways [21]. The metal center of enzymes and metalloproteins are site-specific to superoxide anions attack and lead to the inactivation of their biological role by reducing their redox potential.

Skin exposure to RT promoted oxidative stress and the excess of ROS caused depletion of endogenous defense, dysregulating the Ca^{2+}-ATPase energy signaling pathways, leading to elevated ATP depletion and extensive Ca^{2+} efflux through channels [50]. Depletion of ATP is followed by increases in monophosphate (PO_4^{3-}) anions which release triggering heterotopic calcification [51]. Dehydration and aggregate formation influence the hydrophilic and lipophilic endogenous molecules, such as vitamins C and E, respectively, inhibiting their role in the intervention of skin recovery [52].

The effects of γ-^{60}Co irradiation on skin damage are summarized in Figure 4. The FT-IR spectra provide the important "marker bands", which provide information about the early effects of RT. The dehydration and deamination combined with the increased lipophilic environment increase irritation and skin aging.

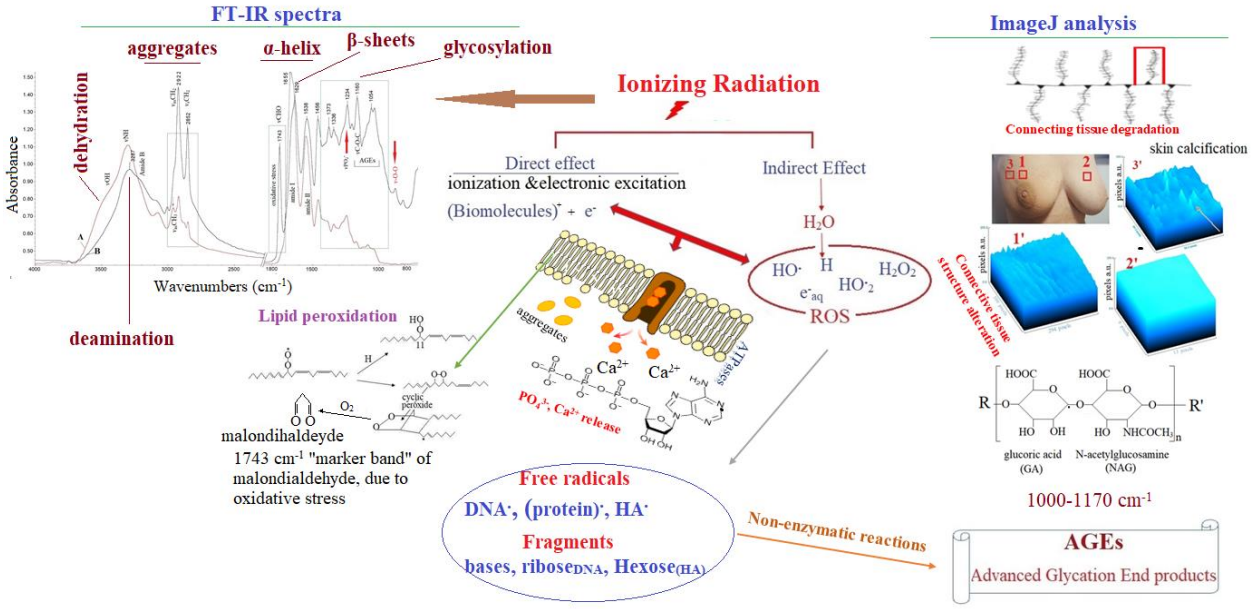

Figure 4 Interactions of ionizing radiation on the skin upon radiotherapy and the micro-pictures reveal the multifunctional problem at hand.

5. Conclusions

FT-IR spectroscopy emphasizes the effects of ionizing radiation on the skin. Our findings, such as skin dehydration, protein deamination, peroxidations, inflammation, lesions of connective tissues, glycosylation, and phosphorylation are processes describing the early effects of ionizing radiation on the skin during a radiotherapy course. The ROS free radicals prefer to abstract H atoms from lipids, sugar rings of glycoproteins, and DNA base-ribose. The "marker band" at about 1743 cm^{-1}, characteristic of lipid and protein peroxidation upon oxidative stress induced by skin ionizing radiation, is assigned to the aldehyde group CHO stretching vibration.

ImageJ analysis indicated an accumulation of calcium ions in the irradiated skin and the protein and lipids peroxidation affected ATPase and induced the calcium ion release [42]. The mediated free radicals through various pathways led to fibril formation and the AGEs enhanced skin aging. A combination of FT-IR spectroscopy and ImageJ analysis data could lead to the development of a mathematical skin simulation model for non-invasive monitoring of the progression of radiation effects on the skin upon radiotherapy. Our findings could contribute to the development of new anti-inflammatory and radio-protective pharmaceuticals to prevent radiotherapy-induced skin pathogenesis.

Author Contributions

Athina Markouizou, MD, PhD, Radiotherapist, part of her PhD, Evrydiki Michali, MD, Hemaologists she did contribution of immune system, Panayiwta Kolovou, MD, PhD, Radiodignost, her PhD was reffered to bone ionizing radiation interactions, Christina Mamareli, MD, specializing in dermatology, Ioannis Mamarelis, MD,PhD, oxidative stress, Professors Jane Anastassopoulou and Theophile Theophanides, supervisors, disined the paper, specialists on Bio-infrared spectroscopy.

Competing Interests

The authors have declared that no competing interests exist.

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