

## Effect of a topical treatment in organotypic culture of human breast skin after exposure to gamma-rays

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The early radiation of epidermal reactions can lead to healing of the lesion or radiation necrosis. There is no general agreement for either the prevention and/or treatment of radiation skin response, also as little is known about the immediate phases of this phenomenon. We investigated the early effects exerted by Healing and Wound Emulsion (HWE) on human skin response after ionizing radiation. Epidermal morphology, Heat Shock Protein (HSP) 70, and Transforming Growth Factor- $\beta$ 1 (TGF- $\beta$ 1) gene expression were investigated in organotypic human skin cultures undergoing a double dose of gamma-rays (2 Gy). HSP70 gene expression tended to be induced in the HWE group 6 hours after cream administration and was significantly up-regulated after 48 hours, when epidermal morphological alterations were evident. TGF- $\beta$ 1 seems not affected in cream treated samples. HWE may stimulate skin to mount an early defensive response against damage induced by gamma rays.

**Key words:** radiodermatitis, epidermis, ionizing radiations, TGF- $\beta$ 1, HSP70.

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Radiotherapy is employed in approximately 50 to 60 percent of patients with cancer both to control the tumour and for the management of tumour-related symptoms (Porock *et al.*, 2002).

Radiotherapy induces reactions in many of the treated patients; one of the most common side effects is an acute skin response, referred to as radiodermatitis, which affects up to 95% of patients (De Conno *et al.*, 1991; King *et al.*, 1985). Acute radiation reactions typically appear 10-14 days after radiotherapy, continue to increase in severity until the completion of treatment (Porock *et al.*, 1999), and may persist for up to 4 weeks after the end of treatment. These early effects are characterized by epilation and erythema, followed by dry and moist desquamation of the epidermis that can lead to either healing of the lesion or radiation necrosis (Porock *et al.*, 1999; Sivan *et al.*, 2002). These side effects induce a condition ranging from mild irritation to severe pain, and may require a temporary or permanent withdrawal of the therapy (Williams *et al.*, 1996). During the next few months or years, late effects appear, characterized by fibrosis which involves the connective compartment of the skin.

In order to minimize the effects of acute skin reactions, a number of management strategies have been suggested. At present, there is no general agreement for the prevention and/or treatment of radiation skin response (Barkham 1993; Lavery 1995; Schmuth *et al.*, 2002; for review see Bolderston *et al.*, 2006); most managements rely on topical skin treatments. However, these treatments vary among cancer institutions and are not based upon principles but rather upon historical practice and personal preference. Currently, management is focused on the attempt to prevent the skin deterioration to higher grades of dermatitis, to relieve the pain and itch, and on addressing any infections. This is done by advising

patients to keep the skin clean and moist throughout their radiotherapy treatment and afterwards.

The assessment of the early skin response to gamma-rays is a difficult endeavour mainly for ethical reasons, and, therefore, experimental models mimicking the clinical setting are needed in defining this issue. Recently, we have characterized in our laboratory an experimental model of human organotypic skin cultures, which offers the advantage of the tridimensional arrangement and of epithelial-mesenchymal interactions (Bedoni *et al.*, 2007).

Using this model, we found a marked inhibition of epidermal proliferation after a single standard clinical dose of gamma-rays (2 Gy) within the first 24 hours (Donetti *et al.*, 2005), indicating that skin homeostasis is rapidly altered by ionizing rays.

The aim of this study was to investigate the main morphological features of the epithelial compartment and to perform molecular analysis of specific target genes in organotypic human skin cultures undergoing a double standard clinical dose of gamma-rays (2 Gy) after the application of an innovative compound, Healing and Wound Emulsion (HWE, provided by Sinclair Pharma Group). As target genes, we analyzed the expression of transforming growth factor beta 1 (TGF- $\beta$ 1) and of the heat shock protein (HSP) 70. TGF- $\beta$ 1 is a multi-functional cytokine involved in the maturation of epidermis, which promotes the initiation and differentiation of keratinocytes by blocking their proliferation (Barkham 1993). HSP70 is expressed in the skin and induced under stressful conditions (Daugaard *et al.*, 2007), thus representing a useful stress marker after ionizing radiation.

The main components of the cream HWE are hyaluronic acid, vitis vinifera, shea butter, telmesteine, glycyrrhetic acid, and bisabolol in a hydrolipidic base.

## Materials and Methods

### Organotypic skin cultures

Human breast skin samples were obtained from cosmetic surgery of 30–40 year old healthy women (n=4) after informed consent, accordingly with the Helsinki declaration. Immediately after excision, skin was transported in cold culture medium and cut into 0.5–1 cm<sup>2</sup> fragments using a scalpel. Skin biopsies were cultured in a Transwell system (Costar, Corning Inc, NY, USA) at 37°C and 5% CO<sub>2</sub> overnight to reduce the acute effects of explantation,

as previously described (Donetti *et al.*, 2005; Bedoni *et al.*, 2007). All skin fragments were cultured in DMEM culture medium containing 10% foetal bovine serum (FBS) (Invitrogen, Milan, Italy) supplemented with hydrocortisone, amphotericine B, and glutamine (Sigma Aldrich, Milan, Italy). Skin biopsies were placed epidermal side up at the air liquid interface. Medium was changed every day and the stratum corneum remained constantly exposed to the air. After the overnight incubation, samples were divided in irradiated and non irradiated groups. Each donor was represented in triplicate in all experimental groups at all considered time points.

### Gamma-irradiation protocol

All irradiated samples were twice exposed to a 2 Gy dose emitted by <sup>137</sup>Cs-irradiator, with the second irradiation administered 24 hours after the first one, when irradiated samples were further divided in two experimental groups: IRR and HWE groups. The IRR group was only irradiated, whilst HWE group was irradiated and then HWE cream was applied on the epidermal surface for one hour after each irradiation. At the end of the HWE application, samples were thoroughly washed with phosphate buffer saline (PBS) 0.1 M, pH 7.4 and were maintained in Transwell systems either for 6 or 48 hours after the second irradiation.

Non irradiated skin biopsies (CT) were kept at room temperature during gamma-irradiation and incubated for 6 or 48 hours after the second irradiation under the same conditions, representing controls.

CT, IRR, and HWE skin fragments harvested at the different time points were processed either for the morphological analysis or immediately frozen in liquid nitrogen and stored at –80°C until use.

### Morphological analysis

For each donor, 2 replicates/treatments were fixed in 4% formalin in 0.1M PBS (pH 7.4) for 5 hours at room temperature, routinely dehydrated, paraffin embedded, and 5  $\mu$ m serial sections were obtained. Haematoxylin-eosin stained sections were analyzed by two independent investigators, using a light microscopy Nikon Eclipse E600 equipped with a Nikon digital camera DXM1200 (Nikon, Tokyo, Japan).

### PCR analysis

For each donor, 1 frozen replicate/treatment was

homogenized with an Ultraturrax and total RNA was extracted by a modification of the guanidine-isothiocyanate method (Tri-Reagent, Sigma, Milan, Italy). Total RNA was obtained for each single skin fragment obtained from each donor. Total RNA (1  $\mu\text{g}$ ) was DNase digested, and first-strand DNA was carried out following the manufacturer's protocol (Promega Italia, Milan, Italy). PCR amplification was performed with primers specific for TGF- $\beta$ 1 and HSP70. 28s was used as an internal control.

Primer sequences were the following: TGF- $\beta$ 1 forward CAG AAA TAC AGC AAC AAT TCC TGG, reverse TTG CAG TGT GTT ATC CCT GCT GTC (186 bp); HSP70 forward CGACCTGAACAA-GAGCATCA, reverse AAGATCTGCGTCTGCTTG-GT (213 bp); 28s forward TTA CCA AAA GTG GCC CAC TA, reverse GAA AGA TGG TGA ACT ATG CC (345 bp). PCR products were resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide and quantified by densitometric analysis. TGF- $\beta$ 1 and HSP70 mRNA levels were normalized on 28s gene expression.

### Statistical analysis

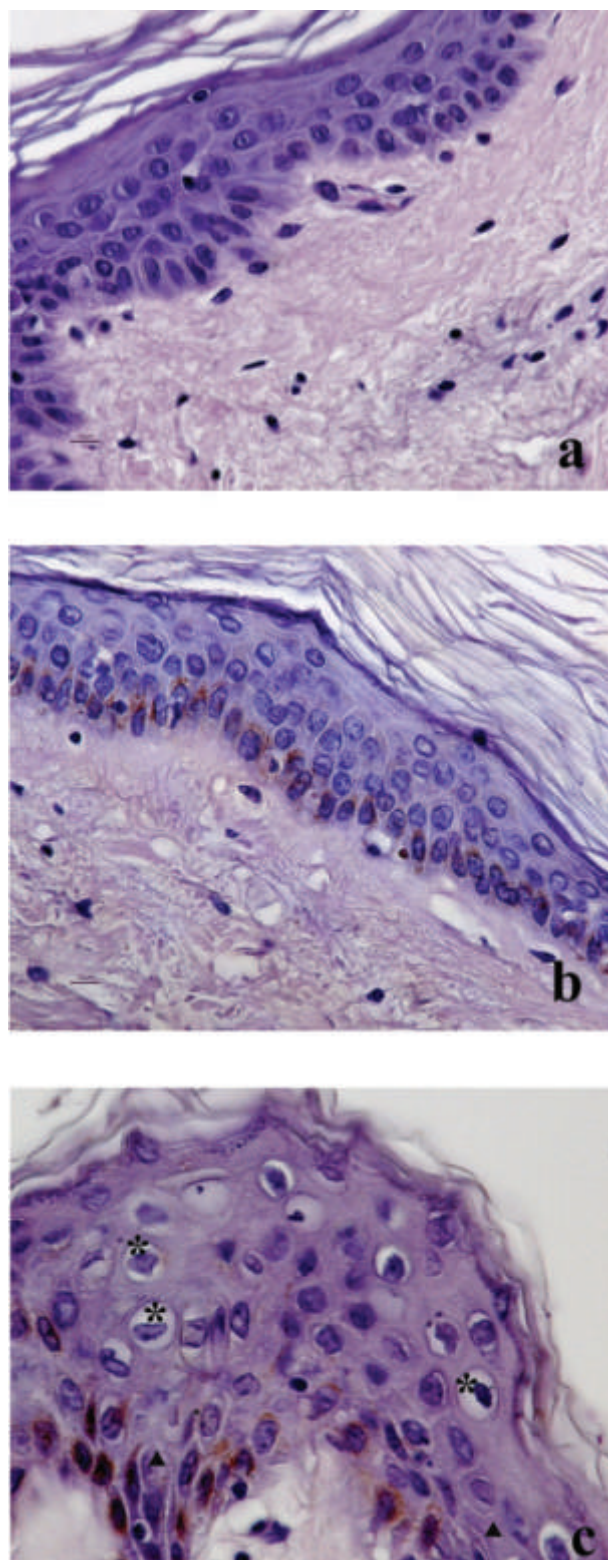
Experimental groups were compared by one-way ANOVA, followed by the Student-Neumann-Keuls post test. Results are expressed as mean  $\pm$  SEM. A  $p$  value  $<0.05$  was considered significant.

## Results

### Morphological analysis

A quantitative assessment of morphological changes between IRR and HWE groups at different time points is reported in Table 1. Among the different experimental groups, no evident differences were detected in the overall distribution of the basal brownish pigmentation due to melanin.

**6 hours.** Light microscopic analysis of the skin samples of the IRR group harvested 6 hours after the double gamma-irradiation revealed that epidermal architecture was not yet affected (Figure 1a) compared to the CT group (Figure 1b). In both experimental groups, a profound basal monolayer composed of regular cylindrical keratinocytes, multilayered spinous/granular layer, and an outer stratum corneum were well preserved. Compared to the IRR group, the HWE samples showed a slight enlargement of intercellular spaces (arrowheads in Figure 1c). Scattered keratinocytes with high condensed chromatin and pale cytoplasm were present



**Figure 1.** Photomicrographs of haematoxylin/eosin stained paraffin sections (5  $\mu\text{m}$  thick) of human breast skin harvested 6 hours after a double dose of 2 Gy of gamma-rays. (a) Irradiated epidermis; (b) non irradiated epidermis; (c) irradiated epidermis with HWE. In (c), asterisks indicate keratinocytes with nuclear condensation and pyknosis, and arrowheads show enlargement of intercellular spaces. Original magnification 40X, bar 10  $\mu\text{m}$ .



in the basal/suprabasal epidermal layers (see asterisks in Figure 1c).

**48 hours.** When comparing the epithelial morphological features of the IRR and CT groups, a slight increase of basal/suprabasal pyknosis were observed in the former samples (compare Figure 2a and 2b). In all irradiated samples harvested 48 hours after the double gamma-irradiation, a moderate widening of intercellular spaces occurred throughout all the epithelium. At this time point, in HWE group, epidermis was present, but nuclear pyknosis massively occurred in the suprabasal layers (Figure 2c).

**Molecular biology analysis**

*TGF-β1 gene expression:* the effect of HWE on TGF-β1 gene expression after gamma-radiations is shown in Figure 3a. TGF-β1 mRNA levels, although with some interindividual differences, were similarly expressed in all the experimental groups at the time points investigated.

*HSP70 gene expression:* 6 hours after irradiation, the administration of HWE appeared to induce HSP70 gene expression (Figure 3b), while 48 hours after radiations, the treatment with HWE induced a significant level of HSP70 mRNA levels ( $p < 0.05$  for HWE vs CT and IRR) (Figure 3c).

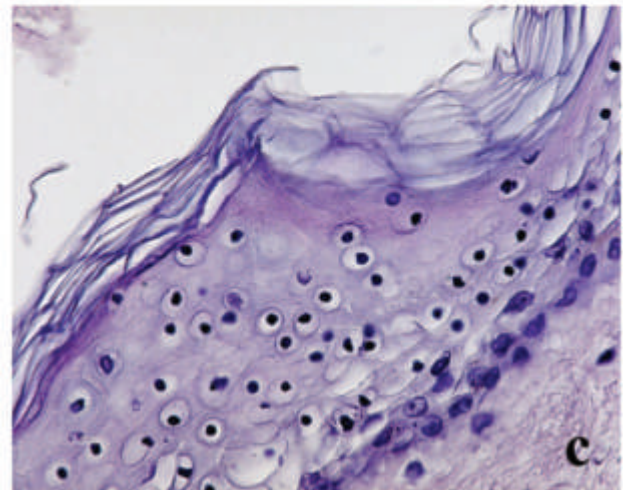
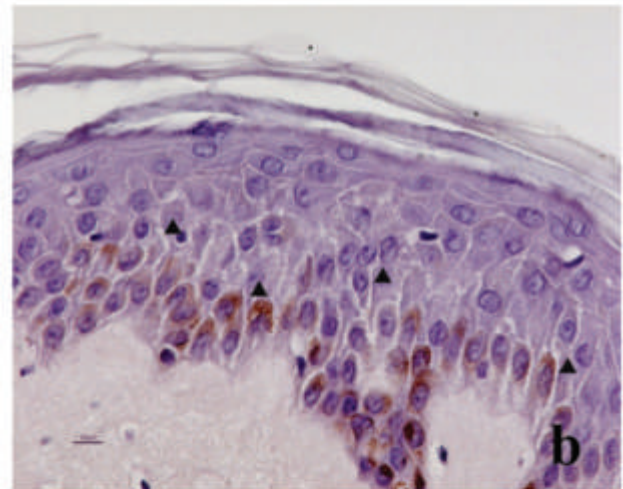
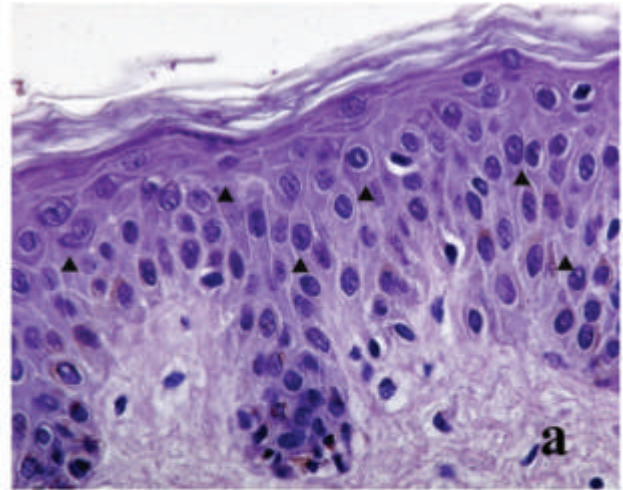
**Discussion**

The knowledge of the early acute epidermal responses after ionizing radiation induced by radiotherapy might elucidate the mechanisms leading to late damage (Archambeau *et al.*, 1995) and may contribute to the prevention and/or treatment of the radiation skin response. The early response induced by environmental damaging stimuli can be

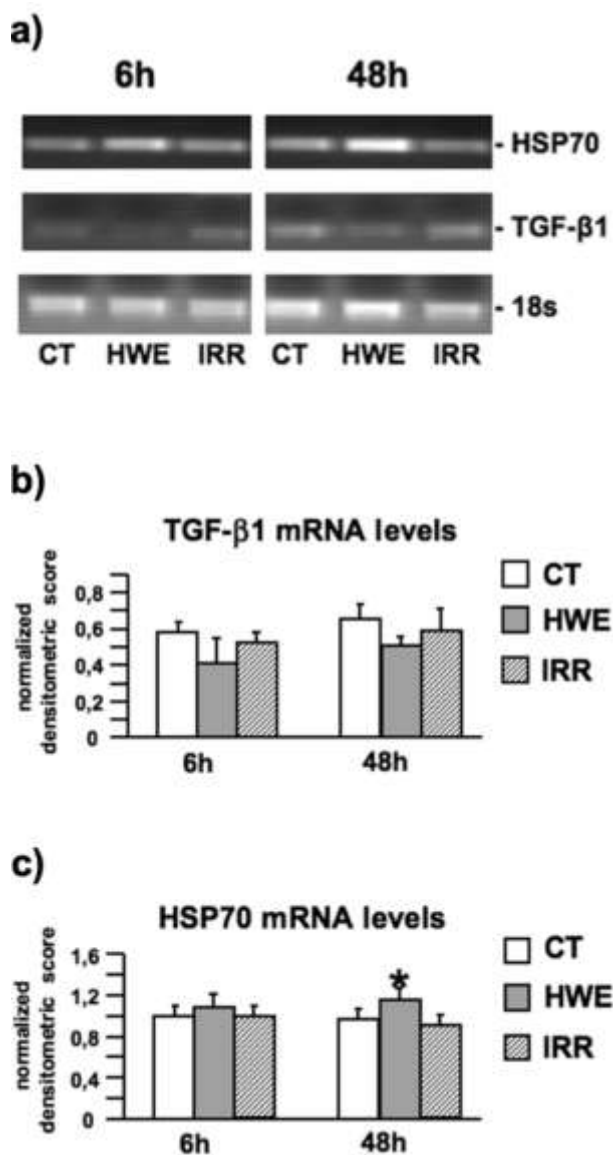
**Table 1. Morphological evaluation of epidermal features in irradiated samples.**

Time	Parameter	IRR group	HWE group
6 hours	Basal pyknosis	±	++
	Suprabasal pyknosis	±	++
	Widening of intercellular spaces	±	++
48 hours	Basal pyknosis	±	++
	Suprabasal pyknosis	±	++
	Widening of intercellular spaces	++	+++

IRR group: irradiated skin; HWE group: irradiated and HWE treated skin after each irradiation (see Methods section for details). Histological assessment was scored as follows: ±: scattered; ++: moderate; +++: severe.



**Figure 2. Photomicrographs of haematoxylin/eosin stained paraffin sections (5 μm thick) of human breast skin harvested 48 hours after a double dose of 2 Gy of gamma-rays. (a) Irradiated epidermis; (b) non irradiated epidermis; (c) irradiated epidermis with HWE. In (a) and (b) arrowheads show enlargement of intercellular spaces. Original magnification 40X, bar 10 μm.**



**Figure 3.** a) Representative RT-PCR for TGF- $\beta$ 1 and HSP70. Bar graphs showing TGF- $\beta$ 1 (b) and HSP70 gene expression (c) in human breast skin harvested 6 and 48 hours after a double dose of 2 Gy of gamma-rays. TGF- $\beta$ 1 and HSP70 mRNA levels are expressed as normalized densitometric units in relation to 28s gene expression. Data are expressed as normalized densitometric score. \* $p < 0.005$  vs CT, HWE and IRR groups.

investigated in skin organotypic cultures which have been already characterized (Bedoni *et al.*, 2007) and could be exploited as a good tool to investigate the whole dermal/epidermal events (Moll *et al.*, 1999; Bäckvall *et al.*, 2002; Donetti *et al.*, 2005). The maintenance of the three dimensional arrangement is pivotal since the cross-talking between the epidermal and dermal compartments of the human skin plays a key role in physiological and pathological conditions.

To our knowledge, no experimental evidence is available about i) the morphological epithelial features and molecular mechanism(s) induced in human skin and ii) the effect of a topical treatment immediately after exposure to ionizing rays. The present study represents the first effort to elucidate these issues.

The evaluation of HSP70 gene expression gave insights into the effect of HWE in the early phases of skin stress response. Ionizing radiation induces a skin stress response, similarly to elevated temperatures, alcohol, heavy metals and oxidants. All organisms respond to such stimuli with the transcription of Heat Shock Proteins (HSPs), among which HSP70 is the most abundant and investigated member (Lindquist 1986, Maytin 1995). The transient induction of HSPs, in fact, produces a state of increased resistance to further stress and the heat shock response is generally thought to represent an adaptive mechanism to counteract adverse environmental conditions. Like in any other organ system, HSPs and, in particular HSP70, are expressed in the skin: under stressful conditions they are induced in epidermal and dermal cells (Lindquist 1986; Kwon *et al.*, 2002).

As early as 6 hours after the double exposure to gamma-rays, cream application slightly increased HSP70 mRNA levels. This observation suggests that HWE rapidly stimulates the skin to produce a response for counteracting a noxious stimulus, as gamma rays. Interestingly, at longer time points (48h), a significant increase of HSP70 gene expression was accompanied by evident epidermal morphological alterations. Partially, the morphological changes induced by HWE application can also be due to an occlusive effect exerted by the cream. However, we wonder that a possible explanation for this finding is that the application of HWE on irradiated epidermis immediately after the exposure to ionizing rays can help in preserving and/or restoring the epidermal resistance to further stress. Actually, mice deficient for inducible HSP70 proteins display an increased sensitivity to radiation (Hunt *et al.*, 2004). In our experimental conditions, HSP70 up-regulation can be involved in the early and intense skin stress response mechanisms triggered by ionizing radiation, possibly by preventing protein misfolding and denaturation (Barral *et al.*, 2004). Therefore, HSP70 up-regulation is consistent with a higher ability to respond to damage, and high levels of HSP70 mRNA in the skin may be

correlated to a protective condition and can thus provide a subsequent defensive response (Matsumoto *et al.*, 1995; Calini *et al.*, 2003).

TGF- $\beta$  is a multi-functional cytokine that controls cell growth and differentiation. In mammals, three isoforms were isolated, among which TGF- $\beta$ 1 is the best characterized (Roberts *et al.*, 1993; Martin *et al.*, 1997). TGF- $\beta$ 1 activity varies according to the cell type and to the tissue context and, in keratinocytes, it is involved in their initiation/differentiation by blocking their proliferation (Fuchs *et al.*, 1994; Doi *et al.*, 2003). Our data show almost unchanged TGF- $\beta$ 1 gene expression in the early phases of radiation skin response in all the experimental groups and is consistent with previous data (Donetti *et al.*, 2005) suggesting that TGF- $\beta$ 1 may play a role in later phases of the radiation skin response. Sivan and coll. studied, from a histopathological point of view, the scarring epidermis of patients from 7 months to 27 years after gamma-rays exposure (Sivan *et al.*, 2002). In this study, suprabasal activation of keratinocytes, epidermal hyperproliferation, abnormal keratinocyte terminal differentiation, and TGF- $\beta$ 1 overexpression were described.

HWE administration appeared to modulate HSP70 and TGF- $\beta$  expression in irradiated skin. Among HWE components, hyaluronic acid (HA) is a major constituent of the extracellular matrix of the skin. HA displays remarkable rheological, viscoelastic and hygroscopic properties which are relevant for dermal tissue function. Topical applications of HA improved wound healing, particularly in acute radioepithelitis (Weindl *et al.*, 2004), and its hygroscopic properties restore the normal free water balance of the derma, lost during radiotherapy. Liguori and coll. demonstrated in a randomized, double-blind, placebo-controlled study that the prophylactic use of a cream containing 0.2% HA during RT postpones the early signs of skin reactions, thus improving the compliance and quality of life in patients (Liguori *et al.*, 1997). This component can thus play a central role in the induction of a protective condition in irradiated skin in our experimental model.

*Vitis vinifera* extracts are known for their antibacterial, antiviral, anticarcinogenic and anti-inflammatory actions. Moreover, the presence of epigallocatechin gallate in *vitis vinifera* extract exerts anti-degenerative effects (Cooper *et al.*, 2005; F'guyer *et al.*, 2003).

Telmesteine (3-ethoxy-carbonyl-4-carboxyl-1-thia-3-aza-cyclopentane) is an anti-oxidant. Its association with *vitis vinifera* has demonstrated significant activity against the enzymes responsible for the degeneration of the derma. Glycyrrhetic acid is a triterpene glycoside that protects from oxidant-mediated toxicity and displays antiinflammatory and antiviral properties. Both glycyrrhetic acid (Armanini *et al.*, 2002) and bisabolol (chamomile component) (O'Hara *et al.*, 1998) can reduce the inflammation caused by radiotherapy.

Altogether, our results suggest that a topical treatment with HWE in irradiated skin can elicit a very early skin response inducing a protective condition aimed at counteracting damage triggered by ionizing radiation in the late phases of RT.

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