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CONCISE COMMUNICATION

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Molecular effects of photon irradiation and subsequent aftercare treatment with dexpanthenol-containing ointment or liquid in 3D models of human skin and non-keratinized oral mucosa

Sebastian Huth[1] | Yvonne Marquardt[1] | Laura Huth[1] | Laurenz Schmitt[1] | Kirsten Prescher[2] | Philipp Winterhalder[3,4] | Timm Steiner[3,4] | Frank Hölzle[3,4] | Michael Eble[2] | Jens Malte Baron[1,3]

[1]Department of Dermatology and Allergology, Medical Faculty, RWTH Aachen University, Aachen, Germany

[2]Department of Radiation Oncology, Medical Faculty, RWTH Aachen University, Aachen, Germany

[3]Interdisciplinary Center for Laser Medicine, Medical Faculty, RWTH Aachen University, Aachen, Germany

[4]Department of Oral and Maxillofacial Surgery, Medical Faculty, RWTH Aachen University, Aachen, Germany

Correspondence
Sebastian Huth, Department of Dermatology and Allergology, Medical Faculty, RWTH Aachen University, Pauwelsstrasse 30, 52074 Aachen, Germany.
Email: shuth@ukaachen.de

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Abstract

This study aimed to investigate the molecular effects of radiation and subsequent aftercare treatment with dexpanthenol-containing ointment and liquid on established full-thickness 3D skin models depicting acute radiodermatitis and mucositis. To mimic radiomucositis and radiodermatitis, non-keratinized mucous membrane and normal human skin models were irradiated with 5 Gray. Afterwards, models were treated topically every second day with dexpanthenol-containing ointment or liquid in comparison with placebo and untreated controls. On day 7 after irradiation, histological examination showed impairments in irradiated models. In contrast, models treated with dexpanthenol-containing ointment or liquid showed a completely restored epidermal part. While gene expression profiling revealed an induction of genes related to a pro-inflammatory milieu, oxidative stress and an impaired epidermal differentiation after irradiation of the models, aftercare treatment with dexpanthenol-containing ointment or liquid revealed anti-oxidative and anti-inflammatory effects and had a positive effect on epidermal differentiation and structures important for physical and antimicrobial barrier function. Our findings confirm the potential of our established models as in vitro tools for the replacement of pharmacological in vivo studies regarding radiation-induced skin injuries and give indications of the positive effects of dexpanthenol-containing externals after radiation treatments as part of supportive tumor treatment.

KEYWORDS
3D skin model, dexpanthenol-containing externals, radiomucositis, radiodermatitis, supportive tumor treatment

Michael Eble and Jens Malte Baron are senior authors contributed equally.

1 | BACKGROUND

Radiodermatitis is a significant side effect of ionizing radiation on the skin, which occurs mainly during radiotherapy in the treatment of cancer and affects the patient's quality of life during and after treatment.[1,2] Radiation therapy induces damage to cellular macromolecules, especially DNA breaks, which occur in all cell types within the epidermis and dermis.[3] As a result, DNA damage interferes proliferation and differentiation of basal keratinocytes leading to impairments of the physical skin barrier.[4] Depending on the duration of the skin reaction, two forms of radiodermatitis are distinguished: an acute reaction developing a few hours or weeks after radiation and a chronic form that develops months or years after radiation.[5] Early effects of the acute form result from damage to the mitotic ability of the basal keratinocytes in the epidermis, which leads to disturbances in the self-renewing property of the skin.[6] The most common skin reactions include erythema, edema, epilation, desquamation, dryness, burning, itching and hyperpigmentation.[2,3] A common side effect of systemic chemotherapy and of radiation therapy of the head and neck region is oral mucositis.[7] Mucositis is a complex condition involving all cells and tissues of the mucosa.[8] Symptoms vary in severity from oral soreness and erythema to life-threatening oral ulcers that prevent oral intake.[9]

A large number of topical agents and wound dressings are used for the treatment of radiation-induced skin injuries without being consistently effective.[10] Many small studies have investigated the use of topical therapies on the patient outcome without including the molecular level of treatment effects. Considering that tissue samples and biopsies are required for in-depth studies at the molecular level, clinical trials with chemotherapy patients are limited due to ethical restrictions and risks associated with invasive procedures. It has already been shown that dexpanthenol, which is widely used in topical dermatological treatments, stabilizes the barrier function of the skin,[11] stimulates the regeneration of the skin [12] and facilitates wound healing.[13] In contrast to a study by Lokkevik et al. which showed no beneficial effects of dexpanthenol during radiotherapy,[14] a later clinical trial by Schmuth et al. exhibited that the use of a dexpanthenol-containing emollient ameliorates radiodermatitis without determining the underlying molecular mechanisms.[15] In another study comparing different skin care conceptions in patients undergoing radiotherapy, the authors came to the conclusion that the application of basic creme products containing dexpanthenol is justified.[16]

The aim of the present in vitro study was to examine the molecular effects of a dexpanthenol-containing ointment and liquid on established three-dimensional (3D) full-thickness skin models depicting acute radiodermatitis and mucositis.

2 | QUESTIONS ADDRESSED

We herein address the molecular effects of radiation and subsequent aftercare treatment with dexpanthenol-containing ointment and liquid on in vitro models mimicking acute radiodermatitis and mucositis.

3 | EXPERIMENTAL DESIGN

The methods for establishing radiodermatitis and mucositis-like 3D skin models and all analyses carried out are described in detail in Data S1.

4 | RESULTS

To mimic radiodermatitis-like skin conditions, we established 3D skin models with NHEK and NHDF cells and irradiated these models with 5 Gy (Figure 1). Histological examination on day 7 after irradiation showed significant impairments in irradiated skin models, especially in the epidermal part with an ablated stratum corneum. Since irradiation-induced DNA damage interferes with the proliferation and differentiation of basal keratinocytes,[4] we additionally performed immunofluorescence analyses with antibodies directed against proliferation marker Ki67 and differentiation marker laminin 5, an epithelial-derived basement membrane component. Compared to non-irradiated controls, the expression of Ki67 and laminin 5 was almost lost in the models on day 7 after irradiation. In contrast, models treated with a dexpanthenol-containing ointment (dco) showed a completely restored epidermal part with an intact stratum corneum on day 7 after irradiation. Consistently, dexpanthenol-containing ointment-treated models showed a re-expression of Ki67 and laminin 5 (quantification of Ki67-positive cells is shown in Figure 1B). To prove that these effects were dexpanthenol-dependent, we chose an additional approach where we treated the models with a placebo ointment that featured the identical formulation and galenics, but without dexpanthenol. In opposite to dexpanthenol-containing ointment-treated models, we observed impairments in the epidermal part of placebo-treated models that were almost similar to ointment-untreated models.

Addressing the molecular effects of radiotherapy and the subsequent treatment with the dexpanthenol-containing ointment, we performed a transcriptomic gene expression profiling (Figure 3A,B). In radiated skin models, microarray analysis revealed an upregulation of genes that are associated with the immune response and wound healing, such as chemokines and cytokines (e.g. CXCL8, CCL20, IL6) as well as growth factors (e.g. FGF2, FGF5) (Figure 3A). In addition, we detected an upregulation of matrix metalloproteinases (MMP3, MMP1), playing a major role in the remodelling of the extracellular matrix (ECM). On the other side, expression profiling revealed a downregulation of genes associated with epidermal differentiation and skin barrier function (e.g. LOR, FLG, DSC1, KRT10, COL14A1). Consistently, gene ontology (GO) analysis showed an impact of radiotherapy on biological processes such as “response to wounding”, “response to mechanical stimulus”, “cellular response to hydrogen peroxide”

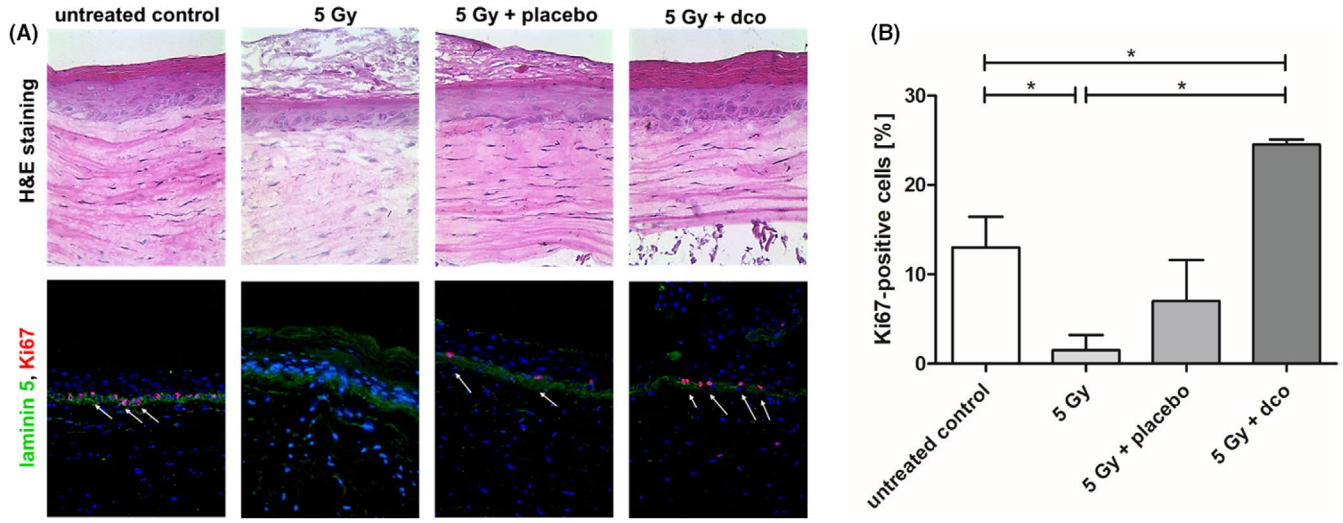

FIGURE 1 Histological and immunofluorescence analysis of 3D skin models mimicking radiodermatitis. A, Upper row shows exemplarily HE stainings of untreated controls, models that were irradiated with 5 Gy, and models that were additionally treated either with placebo or a dexpanthenol-containing ointment (dco) on day 7 after irradiation. Lower row shows immunofluorescence examination of laminin 5 and Ki67 in the respective models. Representative images of three independent experiments performed in duplicates are shown. B, Quantitative Ki67 immunohistomorphometry, Ki67-positive cells were counted in clearly defined reference areas per image. $^*p < 0.05$

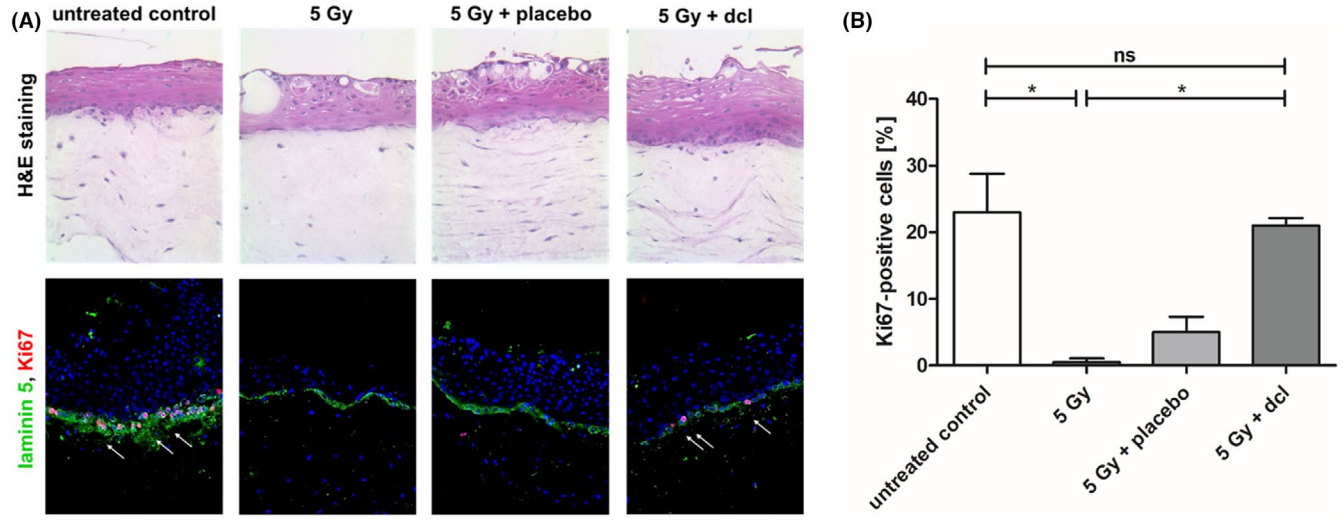

FIGURE 2 Histological and immunofluorescence analysis of 3D skin models mimicking mucositis. A, Representative HE stainings of untreated controls, models that were irradiated with 5 Gy, and models that were additionally treated either with placebo or a dexpanthenol-containing liquid (dcl) on day 7 after irradiation are shown in the upper row. Lower row shows immunofluorescence examination of laminin 5 and Ki67 in the respective models. B, Quantitative Ki67 immunohistomorphometry, Ki67-positive cells were counted in clearly defined reference areas per image. $^*p < 0.05$, ns, not significant

and "collagen metabolic process" (Figure S1a). Investigating the molecular effects of the dexpanthenol-containing ointment treatment in irradiated skin models, we observed anti-inflammatory responses by a downregulation of pro-inflammatory cytokines and chemokines (e.g. CXCL-1, IL6) (Figure 3B). A positive impact of the dexpanthenol-containing ointment treatment on epidermal differentiation and remodelling was accentuated by a downregulation of MMPs (MMP-1, -3, -7) and an upregulation of differentiation markers (e.g. LCE6A, LCE3B) and a stimulator of DNA synthesis (KRT7). Emphasizing these data, GO analysis showed an impact of the dexpanthenol-containing ointment treatment on categories like "positive regulation of cell communication" and "cellular response to DNA damage stimulus" (Figure S1b).

Radiation therapy in the head and neck region often provokes oral mucositis as a side effect.[7] To study dexpanthenol-induced effects on this skin condition, we established 3D skin models of oral mucositis with NHOK and NHOF cells and irradiated these models with 5 Gy (Figure 2). As previously described, the epithelium of such a mucosa model features the distinctive layers of oral mucosa — the lamina propria as a fibrous connective tissue, a basement membrane and a stratified squamous epithelium.[17] Consistent to the radiodermatitis model, we detected prominent impairments

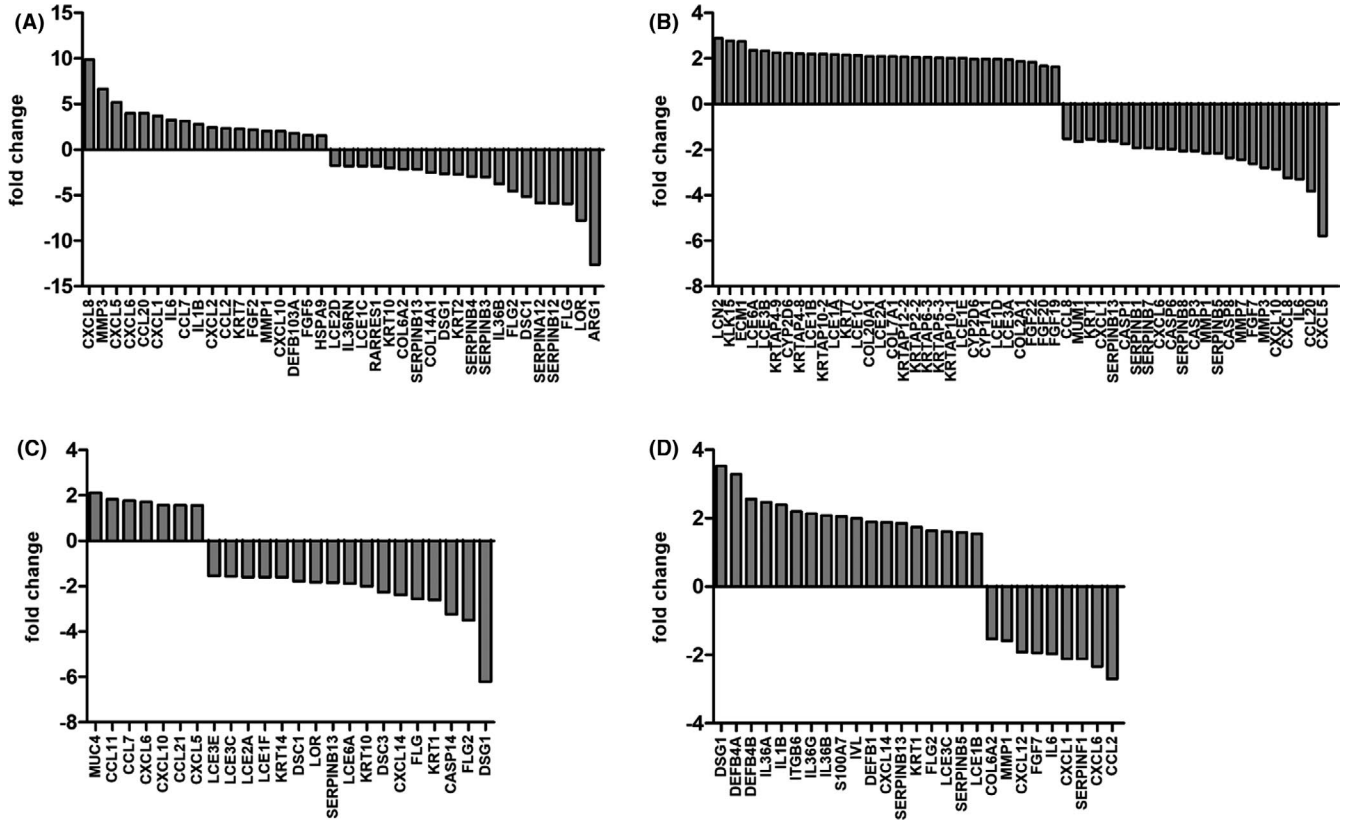

FIGURE 3 Gene expression profiling of radiodermatitis and mucositis models. A, Microarray analysis in radiodermatitis models 7 days after irradiation with 5 Gy in comparison with untreated controls. B, Microarray analysis in radiodermatitis models 7 days after irradiation with 5 Gy and additional treatment with a dexpanthenol-containing ointment in comparison with irradiated models. C, Microarray analysis in mucositis models 7 days after irradiation with 5 Gy in comparison with untreated controls. D, Microarray analysis in mucositis models 7 days after irradiation with 5 Gy and additional treatment with a dexpanthenol-containing liquid in comparison with irradiated models

with blistering in the epithelial part of the models at day 7 after irradiation. Performing immunofluorescence analyses, the expression of Ki67 and laminin 5 was reduced in these models. In contrast, models treated with a dexpanthenol-containing liquid (dcl) showed a completely restored epidermal part with an intact stratum corneum on day 7 after irradiation. In agreement, dexpanthenol-containing liquid-treated models showed a re-expression of Ki67 and laminin 5 (quantification of Ki67-positive cells is shown in Figure 2B). In contrast, the epithelial part of placebo-treated models exhibited nearly no improvements.

In mucositis models, gene expression profiling indicated a stress response by upregulation of chemokines (e.g. CCL11, CXCL6) and downregulation of differentiation and skin barrier markers (e.g. KRT10, FLG) (Figure 3C). In GO analysis, over-represented gene annotations confirmed an impact of radiation on biological processes like "response to stress", "chemotaxis", "epidermis morphogenesis" and "establishment of skin barrier" (Figure S2a). Similar to our observations with the dexpanthenol-containing ointment, treatment of radiated mucosa models with the dexpanthenol-containing liquid showed beneficial effects on the molecular level by upregulating epidermal differentiation markers (e.g. IVL, KRT1) and skin barrier function-related genes (e.g. DEFβ4A, DEFβ4B, S100A7A) (Figure 3D). Accordingly, we detected anti-inflammatory effects of dexpanthenol-containing liquid treatment by downregulation of chemokines and cytokines (e.g. CXCL1, IL-6). GO analysis supported these findings by showing a regulation of dexpanthenol-containing liquid treatment on biological processes like "positive regulation of epithelial cell proliferation", "tissue remodelling" and "regulation of extracellular matrix disassembly" (Figure S2b).

It is well known that radiation therapy leads to the release of reactive oxygen species (ROS).[18] Thus, we wanted to determine the effects of calcium pantothenate, an analog of dexpanthenol, on the release of ROS in our in vitro radiodermatitis model. While radiation with 5 Gy exhibited an increase in ROS production, the addition of calcium pantothenate significantly reduced the release of ROS (Figure S3).

5 | CONCLUSIONS

In order to find promising treatment options for side effects of radiotherapy, the pathophysiology of radiodermatitis and mucositis needs to be better understood, which in turn requires the establishment of suitable in vitro models for both conditions. Previous studies that attempted to mimic clinical conditions used animal models.[19–22] It is worth mentioning that the skin of laboratory animals differs in a

variety of biological properties from human skin, making it difficult to translate findings of animal experiments to the clinic.[23]

In our present study, we tested the effects of a dexpanthenol-containing ointment in a skin model mimicking acute radiodermatitis as well as the effects of a dexpanthenol-containing liquid in a skin model depicting acute oral mucositis. Former studies proved that organotypic skin models are a reliable and standardized in vitro tool to investigate the effects of various topical therapies on skin morphology, physiology and gene expression.[17,24,25]

Acute radiodermatitis develops 6–12 days after irradiation,[26] which is why we investigated the morphological and molecular effects in our models at day 7 after irradiation. Histological examination exhibited significant impairments in irradiated radiodermatitis and mucositis mimicking models, especially in the epidermal part with an ablated stratum corneum. Our observations are in line with the results of clinical studies showing that radiation-induced DNA damage interferes proliferation and differentiation of basal keratinocytes leading to impairments of the physical skin barrier.[4] Interestingly, while in clinical routine an inhibition of epidermal cell proliferation is usually observed after 3–4 weeks, we detected this disorder in our models after 7 days. In our study, treatment with a dexpanthenol-containing ointment or liquid led to a reconstitution of these disorders that was not detectable in models that were treated with the placebo, attributing the effects only to dexpanthenol. These data clearly reveal that a dexpanthenol-containing ointment and liquid are able to prevent radiation-induced skin damage. To address the underlying molecular mechanisms, we performed transcriptomic gene expression profiling. Matching to the histological abnormalities, irradiated models showed a deregulated expression of genes that could be attributed to a stress response, a disturbed epidermal differentiation and barrier function and the induction of wound healing. Interestingly, treatment with a dexpanthenol-containing ointment and liquid inverted these effects by upregulating genes associated with epidermal differentiation, skin barrier function and DNA synthesis as well as downregulating genes that are responsible for a pro-inflammatory milieu. These data indicate that dexpanthenol is able to confer anti-inflammatory effects and strengthen the physical and antimicrobial barrier in radiation-damaged skin. Previous in vitro studies with laser wounded 3D skin models have already examined the impact of dexpanthenol and calcium pantothenate on wound healing mechanisms by showing a modulation of genes that accelerate wound closure.[25] Our new study reveals for the first time beneficial molecular effects of dexpanthenol in the prevention and treatment of radiodermatitis and mucositis.

Radiation therapy leads to the release of reactive oxygen species (ROS).[18] We could show that the addition of calcium pantothenate to the cell culture medium of our models significantly reduced the release of ROS, which supports the previous described anti-oxidative effects of dexpanthenol.[27]

In conclusion, we present for the first time an in vitro study with self-developed 3D skin models of radiodermatitis and mucositis, which allow the investigation of the underlying molecular mechanisms of radiotherapy on skin and mucosa and the testing of topical treatments. These models are standardized tools enabling reliable data and avoiding animal experiments and clinical trials. After irradiation of these model systems, topical treatment with dexpanthenol-containing ointment and liquid showed protective effects against radiodermatitis and mucositis by providing anti-oxidative and anti-inflammatory effects and by strengthening the physical and antimicrobial barrier function. Our data suggest that dexpanthenol-containing topical preparations are a promising aftercare treatment option for radiation-induced skin damage as part of supportive tumor treatment.

ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

No conflict of interest.

AUTHORS' CONTRIBUTIONS

SH drafted the manuscript. YM performed the experiments. SH, YM, LH, LS, KP, PW, TS, FH, ME and JMB analysed and interpreted the data. JMB and ME designed the research study. All authors read, critically revised and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The microarray datasets have been submitted to Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) and are available under accession number GSE150738.

ORCID

Sebastian Huth https://orcid.org/0000-0003-4701-3864

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1 Gene ontology (GO) analysis of radiodermatitis models. (a) GO analysis of microarray results of models 7days after irradiation with 5Gy in comparison with untreated controls. (b) GO analysis of microarray results of models 7days after irradiation with 5Gy and additional treatment with a dexpanthenol-containing ointment in comparison with irradiated models

Figure S2 Gene ontology (GO) analysis of mucositis models. (c) GO analysis of microarray results of models 7days after irradiation with 5Gy in comparison with untreated controls. (d) GO analysis of microarray results of models 7days after irradiation with 5Gy and additional treatment with a dexpanthenol-containing liquid in comparison with irradiated models

Figure S3 Measurement of reactive oxygen species (ROS) production. 3D skin models were either treated with H2O2 (positive control), 5Gy, calcium pantothenate or 5Gy and calcium pantothenate. Assessment of ROS release was measured by using a fluorescent probe. Two independent assays were done in triplicate. **p<0.01

Data S1 Material and Methods

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