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PhD THESIS

CONTRIBUTIONS RELATED TO THE EFFECTS OF ULTRAVIOLET RADIATION EXERTED ON HEALTHY AND TUMORAL ORAL CELLS

– A B S T R A C T –

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**T i m i ș o a r a
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TABLE OF CONTENTS

ABSTRACT

Oral tissues are exposed to ultraviolet radiation during various procedures such as dental treatments. Thus, the investigation of the changes that occur after exposure to different types of ultraviolet radiation is of interest, the mechanisms involved being still incompletely understood.

One of the major advantages of ultraviolet radiation (UV irradiation techniques) is related to its simplicity and efficiency in terms of combating microbes, respectively countering infectious diseases to a certain extent. Ultraviolet light irradiation techniques at different wavelengths could inactivate microbes by exercising various mechanisms of action. Type C radiation and a certain part of type B radiation have the property of directly affecting microbes, causing damage by interrupting the DNA replication process, in a very short period of time. At the same time, it notes an increased efficiency regarding the inactivation of most types of microbes, even at low doses. In the case of irradiation with type B ultraviolet radiation, an innate immune response could be triggered, thus inducing the expression of certain AMPs, a process by which certain types of microbes are eradicated. Type A ultraviolet radiation and part of type B ultraviolet radiation, in association with a photosensitizer, can lead to the generation of reactive oxygen species, thus causing oxidative damage to pathogenic microbes. By comparison with the previously exposed mechanisms, the process of DNA damage following the action of ultraviolet radiation shows an increased performance of microbial inactivation on a varied range of microbes.

The doctoral thesis is structured according to the methodological norms in four main parts: (i) the general part, (ii) the original research part, (iii) personal contributions and conclusions and (iv) the references.

The general part comprises two chapters that describe the current notions related to: (a) oral cavity and ultraviolet radiations and (b) preclinical studies related to the study of oral diseases – methodology and relations with effects of ultraviolet radiations on oral cells. The original research part, comprises different experimental studies, namely: (a) analysis of cell viability in the presence of ultraviolet radiation, (b) analysis of cell morphology and confluence in the presence of ultraviolet radiation, (c) evaluation of changes at the level of nuclei and actin filaments in the presence of ultraviolet radiations, (d) evaluation of

apoptotic processes and (e) an introduction to the effect of ultraviolet radiation: from the promotion of malignant skin processes to the use in dentistry. The final part of the thesis integrates the conclusions and personal contributions, and the current cited bibliographic references that were studied for background documentation, the selection of the research methods applied and for the interpretation of the obtained results.

The experimental part of the thesis was conducted using standard internationally validated methods. The study of malignant diseases has traditionally been based on different preclinical in vitro models that were the starting point in evaluating the effect of certain therapies, in facilitating diagnosis, being also recognized as highly performing screening methods. Currently, despite the spectacular evolution within molecular biology that has helped uncover the impressive relevance of the cellular and molecular nature of the tumor microenvironment, these preclinical in vitro models are still widely used for various purposes (e.g., drug discovery, progression analysis disease, the development of personalized therapies).

The first chapter from the experimental part - *Analysis of cell viability in the presence of ultraviolet radiation* - sought to examine the impact of ultraviolet B radiation on pharyngeal and tongue carcinoma cells and, in parallel, the effect on healthy gingival fibroblast cells. Accordingly, the viability of tumor and healthy cells was assessed immediately after UVB exposure and the effect on nuclei and cytoskeleton structure was analyzed. Furthermore, despite the existing evidence regarding the harmful effects of UV radiation on normal skin cells and their role in malignant skin pathologies, data comparing the response of healthy and cancerous skin cells in the individual and the combination of UVA-based treatments and UVB remains limited, limiting current knowledge regarding the influence of UVR on skin health and disease. Thus, in order to provide a deeper understanding of the harmful effects of UVR and at the skin level and how normal and pathological cells behave after irradiation, the analysis of the phototoxic potential of three UVR treatments on immortalized human keratinocytes and skin tumor cells. As a result of cell viability assessment, UVB exposure was found to decrease the viability of both pharyngeal and tongue carcinoma cells, as well as healthy gingival fibroblasts, where the strongest cytotoxic effect was observed.

The most significant decrease in cell viability was observed when cells were exposed to UVB at a dose of 5 J/cm^2. In contrast, gingival fibroblasts were not as strongly affected, with a viability value of approximately 81%. Another important hypothesis underlying the cell viability assessment study was that healthy skin cells respond differently to UVA, UVB and UVA/UVB irradiation compared to skin cancer cells, thus aiming to evaluate the in vitro phototoxic effects of of these UV treatments on two skin-derived cell lines - immortalized human keratinocytes and human malignant melanoma cells. These 2D in vitro models were chosen based on their characteristics. HaCaT cells are spontaneously immortalized keratinocytes of human origin that preserve the morphology and functional activity of isolated keratinocytes and were used in this study considering that keratinocytes represent the main defense mechanism of the skin against UVR damage. The main experimental findings of this study suggest that at 24 h post-irradiation UVA had no cytotoxic effect on both type cells in terms of cell viability, UVB exerted significant toxicity, which is similar in both cells, the most cytotoxic effect was observed when the cells were exposed to both UVA and UVB. To assess whether UVA, UVB, and UVA/UVB treatments affect the viability of cells 24 h after irradiation, an MTT assay was performed. The results showed that UVA had no effect on the viability of cells which remained around 100%, while UVB and UVA/UVB treatments significantly reduced their viability. The different response of cells to direct exposure to UVR could be explained by the differential characteristics of UVA and UVB radiation: the former exerts its toxicity by generating ROS that could have been counteracted by the cells' innate defense mechanisms, while UVB is more genotoxic by directly affecting cellular DNA.

Conclusions. It can be concluded that type B ultraviolet radiation (5 J/cm^2) has a cytotoxic effect on both pharyngeal carcinoma and tongue squamous carcinoma tumor cells, as well as on gingival fibroblasts, which results in a series of changes including and a significant reduction in cell viability, especially in healthy cells. Type A ultraviolet radiation, at the dose used in the present studies, does not induce cytotoxic effects in either human keratinocytes or tumor cells. However, irradiation with type B ultraviolet radiation produces cytotoxic effects in both types of cells, even at a low dose.

The second chapter from the experimental part - *Analysis of cell morphology and confluence in the presence of ultraviolet radiation* – was necessary to support cell viability in the case of healthy cells and reduce cell viability in the case of pathological cells, and the analysis of these aspects in real time is very important. The structure, morphology and exerted properties were followed in cells exposed to ultraviolet radiation. After observing the cytotoxic effects of UVB on cells, the next step was to determine the effect of UVB on morphology. Upon exposure to 2.5 J/cm^2 of UVB, morphological structural changes occur, especially in tumor cells. Regarding the impact of UVB on tumor cells, exposure to 5 J/cm^2 UVB was observed to lead to a marked decrease in cell number as well as changes in shape, with cells becoming rounded and detaching from the plate. It was also indicated that healthy gingival fibroblast cells underwent important morphological changes following UVB exposure, including rounding, plaque detachment, shrinkage, and a reduction in cell number. Consistent with the viability results, the morphology and confluence of keratinocytes and melanoma cells were not affected by UVA exposure. However, 24 h after UVB irradiation, several signs of cytotoxicity were observed in both cell lines, such as cell rounding, shrinkage, and loss of confluence. Interestingly, UVA/UVB had a differential phototoxic effect in the irradiated cell lines. Although cell confluence was not significantly reduced, keratinocytes showed signs of necrosis, while in melanoma cells a significant reduction in confluence accompanied by enucleation was observed. Apoptosis remains the most widely studied type of cell death, however, other mechanisms could also be involved in the cytotoxic effect exerted by chemical or physical agents. In this study, two different types of cell death induced by UVA/UVB irradiation were observed in human skin cells 24 h after irradiation: necrosis in keratinocytes and enucleation in malignant melanoma cells. However, to our knowledge, this is the first study to report enucleation as the underlying mechanism of UVR-induced phototoxicity in skin tumor cells.

Conclusions. Regarding the impact of ultraviolet B radiation on oral tumor cells, it was noted that exposure to a higher dose produced a marked decrease in the number of cells, as well as changes in shape, with the cells becoming rounded and detached from the plaque. Healthy gingival fibroblast cells were also observed to undergo important morphological changes following UVB exposure,

including rounding, plaque detachment, shrinkage, and a reduction in cell number. According to the results obtained from the viability analysis, the morphology and confluence of healthy (keratinocytes) and tumor (melanoma) model cells were not affected by exposure to type A ultraviolet radiation. However, after exposure to type B ultraviolet radiation, observed several signs of cytotoxicity in both cell lines, such as cell rounding, shrinkage and loss of confluence. Interestingly, the combination of the types of radiation had a differential phototoxic effect in the irradiated cell lines. Although cell confluence was not significantly reduced, keratinocytes showed signs of necrosis, while a significant reduction in confluence accompanied by enucleation was observed in tumor cells.

The third chapter was *Evaluation of changes at the level of nuclei and actin filaments in the presence of ultraviolet radiation*. Apoptosis is characterized by cell shrinkage, which is one of the most well-known features of the process and another consequence of the decrease in cell size is the loss of contact with neighboring cells, as well as detachment from the extracellular matrix. After detachment, focal adhesions undergo reorganization, resulting in morphological changes such as rounded cell shapes. Next, membrane blebbing occurs, which requires reorganization of the actin cytoskeleton as well as activation of myosin chains by phosphorylation. Finally, strong cell condensation leads to the formation of apoptotic bodies. To study the process of apoptosis, fluorescence microscopy is a popular technique. Thus, DAPI (4,6-diamidino-2-phenylindole-dihydrochloride) binds to cell nuclei with a strong affinity. Visually, apoptotic nuclei may appear to be smaller than normal nuclei, and condensed chromatin will appear brightly fluorescent. Upon exposure to UVB, all three types of cell lines (pharyngeal carcinoma, tongue carcinoma and fibroblasts) showed a reduction in nuclear area as well as a slight reduction in circumference. To distinguish between apoptotic and necrotic cell death, it is important to observe these changes at the nuclear level. Consequently, in the case of apoptosis the nucleus undergoes early morphological changes, while in the case of necrosis the nucleus remains relatively unaltered, while the membranes and organelles show early signs of alteration. To further assess the impact of UVRs on the appearance of cellular components, immunofluorescence staining highlighting cell nuclei as

well as cytoskeletal F-actin was performed 24 hours after irradiation. UVA/UVB produced massive condensation of chromatin and F-actin in keratinocytes, whereas punctate disintegration of F-actin and nuclear condensation were observed in melanoma cells. These observations confirm UVB and UVA/UVB phototoxicity in these cell lines. However, a difference could be observed between the effects of these treatments on cellular components, as the integrity of keratinocytes and melanoma cells is significantly affected only by UVA/UVB irradiation.

Conclusions. Following the nuclear staining, a series of changes were observed. A higher dose (5 J/cm^2) induced a strong condensation on the nuclei of the oral tumor cells and on the actin filaments, which were condensed in the form of a ring around the periphery. A similar pattern of changes was observed in gingival fibroblasts, nuclei and actin filaments showing areas of condensation. The changing morphological features were used to identify the type of cell death that was responsible for the decrease in cell viability. Therefore, during apoptosis, cells have undergone stereotypical changes that occur mainly in the nucleus and cytoplasm. In the case of UVB irradiation both in keratinocytes and in tumor cells, the destabilization and aggregation of F-actin in stress fibers accompanied by nuclear condensation was observed. The combination of the two types of radiation produces a massive condensation of chromatin and f-actin in keratinocytes, while in tumor cells the disintegration of F-actin in punctate spots and nuclear condensation were observed. These observations confirm UVB and UVA/UVB phototoxicity in these cell lines.

Chapter four was *Evaluation of apoptotic processes*. An investigation of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) gene expression was performed to more precisely determine the type of cell death induced by UVB exposure. A dose of 2.5 J/cm^2 increased the expression of the pro-apoptotic marker Bax, especially in tumor cells, while the expression of the Bcl-2 gene was relatively decreased compared to the control, but not significantly different. In addition, UVB (5 J/cm^2) decreases Bcl-2 gene expression, while the pro-apoptotic gene, Bax, increases expression in all cell types exposed to UVB. Based on these results, UVB exposure may induce cytotoxicity by stimulating apoptosis. To elucidate the possible mechanisms behind the phototoxic events observed following UVB

irradiation of keratinocytes and melanoma cells, an RT-qPCR analysis was performed. In accordance with the results obtained so far, 24 hours after irradiation, UVB exerted a similar effect in both irradiated cell lines – a significant increase in Bax mRNA expression accompanied by a decrease in Bcl-2 mRNA expression – illustrating its activity pro-apoptotic also supported by previous results. In addition to these changes, another feature of the apoptosis process is the activation of a family of intracellular cysteine endopeptidases known as caspases. Caspases 8 and 9 play the role of gate initiators, while caspases 3 and 7 play the role of an executor. In the activation of caspases, Bax is known to play an important role. To detect the possible mechanism of UVA/UVB-induced cell death in keratinocytes and melanoma cells, the Annexin V RealTime-Glo™ apoptosis and necrosis assay was applied. The variations observed in both cells were not specific to an apoptotic phenotype. In keratinocytes irradiated with UVA/UVB, an initial increase in luminescence signal was observed from 3 h to 6 h, followed by a decrease at 24 h. The fluorescence signal in these cells increased over time. These results complement the observations made during cell morphological assessment where necrotic-like features were detected in UVA/UVB irradiated keratinocytes. In melanoma cells, a concomitant increase in luminescence and fluorescence signals was detected, indicating an alternative mechanism of cell death to apoptosis or necrosis. These findings indicate that UVA/UVB exposure differentially affects normal and tumoral cells due to distinct mechanisms involved. Although these results comparatively illustrated how healthy and malignant cells respond to the phototoxic effects of UVR 24 h after irradiation, the small number of cell lines used in the experiments and the short time interval after irradiation at which UVR-induced toxicity was assessed could constitute potential limitations of the study. A deeper insight into the skin toxicity exerted by UVR could be obtained by increasing the number of cell lines evaluated, by diversifying the skin cell types used in the study, and by extending the post-irradiation incubation time (over 24 hours).

Conclusions. It can be concluded that UVB (5 J/cm^2) has a cytotoxic effect both on tumor cells (tongue and pharyngeal carcinoma) and on gingival fibroblasts, which results in a series of changes including increased expression pro-apoptotic gene (Bax) followed by decreased anti-apoptotic gene expression (Bcl-2), indicating that UVB induces apoptosis in both tumor and healthy cells.

Apoptotic changes characteristic of both intrinsic and extrinsic pathways are induced by UVB exposure, as corroborated by the expression pattern of caspases that play a critical role in these processes. However, further studies are needed, both to understand the biological mechanisms underlying UVB effects and to determine whether these changes persist over the long term.

The last chapter from the original part - *An introduction to the effect of ultraviolet radiation: from the promotion of malignant skin processes to the use in dentistry* – was purported to highlight the effects produced by ultraviolet radiation, through the lens of its use for diagnostic and therapeutic purposes in the field of dental medicine, but without neglecting the negative effects produced on the skin, the first defence barrier of the human body against harmful factors in environment. At the same time, the connection between the deterioration of oral quality due to exposure to environmental ultraviolet radiation and the state of knowledge in the field regarding the effectiveness of the use of ultraviolet radiation in the newest techniques in dentistry is analysed. Regarding the processes that take place in the skin following exposure to ultraviolet radiation, the mechanisms involved are multiple, some of them understood, others incompletely elucidated. What is known for certain is that exposure to ultraviolet radiation stimulates the natural production of vitamin D. Therefore, the beneficial effects exerted are closely related to this process, bearing in mind that vitamin D has multiple roles in the body, among which it regulates calcium metabolism, increases immunity, stimulates cell proliferation, maintains blood pressure within normal parameters, etc. As for the beneficial effects on the oral cavity, they are related to the beneficial action on calcium metabolism and the induction of cathelicidin (antimicrobial peptide) capable of fighting the bacteria responsible to produce dental caries. Despite these known data, it is still not known exactly whether the additional administration of vitamin D has a significant contribution in fighting dental caries or in reducing the risk of their occurrence.

Conclusions. Studies reveal a link between natural ultraviolet (UV) radiation and the occurrence of malignant processes. Oral cells are significantly more sensitive to ultraviolet radiation than skin cells. At the same time, the use of ultraviolet radiation in the dental field, both for diagnostic and therapeutic purposes, is free of significant adverse effects. The antimicrobial activity in the

dental sphere of ultraviolet radiation has been shown to be pronounced, especially when used in combination with classic disinfection treatments. However, there is a need to deepen the mechanisms involved in the damage of oral cells by the two types of ultraviolet radiation of clinical interest, UV type B and UV type A, considering their origin (natural/synthetic), exposure time and dose used.