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VITIS VINIFERA SEEDS EXTRACT FOR THE MODULATION OF CYTOSOLIC FACTORS BAX-α AND NF-KB INVOLVED IN UVB-INDUCED OXIDATIVE STRESS AND APOPTOSIS OF HUMAN SKIN CELLS

HANA DECEAN[1,2], EVA FISCHER-FODOR[3], CORINA TATOMIR[3], MARIA PERDE-SCHREPLER[3], LIDIA SOMFELEAN[4], CLAUDIA BURZ[3,5], TUDOR HODOR[5], REMUS ORASAN[5,6], PIROSKA VIRAG[3]

[1]Department of Physiology/Functional Sciences, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania
[2]Emergency Military Hospital, Cluj-Napoca, Romania
[3]Prof. Dr. I. Chiricuta Oncology Institute, Cluj-Napoca, Romania
[4]Babes-Bolyai University, Cluj-Napoca, Romania
[5]Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania
[6]Dermatology Clinical Hospital, Cluj-Napoca, Romania

Abstract

Background and aims. *The depletion of the ozone layer allows overexposure of the skin to UV radiation, which is prolonged due to the increasing life expectancy, together with inappropriate life habits contribute to the increasing incidence of cutaneous malignancies. Plant extracts with antioxidant capacities are frequently employed as a means to protect skin against ultraviolet (UV) radiations, thus preventing skin cancers. In the present study we assessed a red grape seed extract (GSE) potential capacities to reduce ultraviolet B (UVB) radiation-induced reactive oxygen species (ROS) and subsequent apoptosis in a human keratinocytes cell line (HaCaT). We identified molecules and pathways modulated by the GSE through which this may exert its photoprotective effect.*

Methods. *The GSE was standardized according to its polyphenolic content and the most important biologically active compounds, such as epigallocatechin and epicatechin, catechin hydrate, procyanidin B and gallic acid were evidenced by high-performance liquid chromatography. According to the plant extract cytotoxicity on the HaCaT cell line, two concentrations were selected for testing from the non-toxic range: GSE_1 (37.5 μgEqGA/ml) and GSE_2 (75 μgEqGA/ml). The level of ROS was evaluated with CM-H_2DCFDA assay, while apoptosis, Bax-α and NF-kβ p65 proteins with ELISA and confirmed by western-blot.*

Results. *Both concentrations of the extract decreased the level of ROS in UVB-irradiated keratinocytes ($p<0.001$), whereas apoptosis and Bax-α pro-apoptotic protein were only reduced by the higher concentration (GSE_2). The NF-kB p65 protein level registered increasing values in time after UVB exposure of the cells, while the tested plant extract re-established its level when its smaller concentration was used (GSE_1).*

Conclusion. *These results encourage further studies on this extract in order to identify other molecules and pathways through which this extract might exert its beneficial effects and also recommend its use as a potential photoprotective agent.*

Keywords: red grape seed extract, ROS, apoptosis, Bax-α, NF-kB

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Address for correspondence: virag.piroska@yahoo.com

1. Background and aims

Skin represents one of the main defense systems against external pollutants, including solar ultraviolet (UV) radiation [1]. Considering the depletion of the ozone layer, increasing amounts of UV radiation reache to Earth, causing severe health problems, including cutaneous malignancies [2]. UVA (320-400 nm), the largest spectrum of solar UV radiation (90-95%) penetrates deeper into the dermis and induces reactive oxygen species (ROS) generation, damaging proteins, lipids, and DNA [3,4]. UVB (280-320 nm) represent 5% of the total solar spectrum and suppresses immunologic functions and also acts as tumor promoter and co-carcinogen. Its effects are inflammation, sunburn cell formation, hyperpigmentation, induction of oxidative stress, which ultimately lead to the induction of skin cancers [3,5]. UVC (<280 nm), although mutagenic in nature is almost completely absorbed by the ozone layer and does not affect the skin [3].

ROS production holds the central role in the UVA and UVB-triggered deleterious effects. ROS comprise a number of active metabolites including hydroxyl radical, superoxide anion and peroxyl radical and their active precursors (singlet oxygen, hydrogen peroxide and ozone). When the ROS production exceeds the antioxidant defense capacity of the skin, a cascade of events is initiated which finally leads to photocarcinogenesis [6].

According to the current knowledge UV-generated ROS affects mitogen-activated protein kinase (MAPK) signaling cascades [7]. These have been shown to activate nuclear kappa B (NF-kB), as well as c-Jun N-terminal (JNK) [8] and p38 MAPK followed by the activation of transcription factor activation protein 1 (AP-1) and cyclooxigenase-2 (COX-2) [9]. The balance between these processes varies according to the dose of UV, cell type and the involved proteins. Of great importance is apoptosis which plays a key role in the elimination of damaged or abnormal cells and the apoptosis regulatory proteins, such as pro-apoptotic Bax-α protein, which exert its action mostly at mitochondrial level [10].

Several photoprotective agents and strategies were elaborated in order to reduce the detrimental effects of UV radiation and prevent skin disorders, including cancers. Particularly proanthocyanidins, natural compounds found in fruits and vegetables gained great interest, added to diet or by topical applications. Regarding their chemical origin they are oligomers or polymers of polyhydroxy flavan-3-ol units, such as (+) catechin and (-) epicatechin, belonging to the class of phenolics [2]. Grapes (*Vitis vinifera*) cultivated worldwide represent one of the richest sources of phenolics, fruits being consumed as a dietary supplement, while seeds containing the highest concentration of proanthocyanidins (70-95 %) are employed in herbal medicine [9,11]. Recent studies have revealed that grape seed extract due to its antioxidant capacities [12] exerts photoprotective effects and modulates NF-kB/ERK in nude mice and in human keratinocytes [13,14,15], and it has anti-carcinogenic [13], anti-viral and anti-microbial activities [11,16].

In the present study the effects of a red grape seed extract (GSE) were evaluated on UVB-induced oxidative stress and apoptosis in a human keratinocyte cell line (HaCat). The apoptosis regulatory protein (Bax-α) and a transcription factor (NF-kB) were also assessed in order to decipher some of the molecules and pathways through which this extract might exert its beneficial effects. The study represents the continuation of a previous work of the authors in which the effects of the UVB radiation on ROS production, apoptosis induction and apoptosis regulatory proteins were assessed in the human keratinocytes cell line (HaCaT) [17]. Here we demonstrated that an important amount of the UVB-induced deleterious effects could be prevented by the administration of the GSE, which exerted ROS reducing and apoptosis preventive effects, due to the modulation of Bax-α anti-apoptotic protein and re-establishment of the UV-B affected NF-kB level.

2. Methods

2.1. Cell cultures

The spontaneously transformed human keratinocytes cell line (HaCaT) was purchased from the Cell Line Service of the German Cancer Research Centre in Heidelberg (Germany) [18]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 4,500 mg glucose, L-glutamine, NaHCO3 and pyridoxine. Media was supplemented with 10% Fetal Calf Serum and 1% Penicillin/Streptomycin. Cells were seeded in triplicate in 24-well plates at a cell population density of 125 x 10^3/well in 1 ml cell culture media/well. A plate was considered control (non-irradiated) and others were irradiated with UVB.

2.2. UVB irradiation of the cells

In order to irradiate cells, the cell culture medium was removed and cells were washed and overlaid with a thin layer of Phosphate Buffered Saline (PBS) (1 ml/well in the 24-well plates). After irradiation fresh cell culture medium was added to the wells. A Waldmann Medizintechnik UVB 181 broadband device (Waldmann GmbH, Germany) was used for irradiation. This lamp employed for therapeutic purposes, emits a continuous spectrum from 280 to 350 nm, with a peak emission at 320 nm, i.e preponderantly in UVB. The emitted radiation dose was measured with a Variocontrol radiometer (Waldmann GmbH, Germany) before each experiment. Irradiation doses were calculated using the formula:

Dose (mJ/cm^2) = Exposure time (sec) x Intensity (mW/cm^2).

We previously evaluated the cytotoxicity of UVB irradiation on the HaCaT cells [19] and the half maximal inhibitory concentration (IC_{50}) (217 mJ/cm^2) was considered for the selection of the dose we used in this study. Therefore, a dose below the IC_{50} value, within the physiological range

of UVR exposure usually experienced by human skin and approximately equivalent to the minimal erythema dose (MED) [20] was chosen (100 mJ/cm^2).

2.3. Evaluation of ROS production with CM-H_2DCFDA

CM-H_2DCFDA, a chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a general oxidative stress indicator. Inside the cells it is cleaved by esterases and its chloromethyl group reacts with glutathione and other thiols, resulting in a fluorescent adduct. In our study, CM-H_2DCFDA was added to the cells seeded identically to those described in sub-chapter 2.1 in a concentration of 10 μM/wells and samples were incubated 30 min at 37°C and 5% CO_2 atmosphere. The fluorescence was recorded using Biotek Synergy 2, a multi-mode microplate reader (Winooski, USA), at 488 nm.

2.4. Detection of apoptosis with ELISA

Apoptosis was assessed with M30 Apotosense ELISA solid-phase sandwich enzyme immunoassay device (PEVIVA, Bromma, Sweden), which quantitatively measures the apoptosis-associated caspase-cleaved keratin 18 (ccK18) in cell lysates, following the manufacturer protocol. Briefly, cells were seeded and irradiated as described in sub-chapter 2.1 and then incubated for 2, 4 and 8 h. The supernatants were removed and cells lyses were carried out with a non-ionic polioxietilen-based surfactant solution NP40, 10%. ELISA was performed accordingly to the manufacturer's indications (PEVIVA, Bromma, Sweden). Briefly, cell lysates were added to a solid phase capture antibody M5 directed against ccK18. The secondary monoclonal antibody was represented by the horseradish peroxidase conjugated M30, directed against the K18Asp396 neo-epitope. The absorbance was recorded at 450 nm with an ELISA microplate reader (Tecan Sunrise, Grödig/Salzburg, Austria).

2.5. Determination of the intracellular Bax-α level with ELISA

The level of Bax-α from the cell lysates was determined with sandwich-ELISA technique according to the manufacturer indications (R&D Systems, Abingdon, UK). Cells were seeded and irradiated as described in sub-chapter 2.1. After 2, 4 and 8 h from UVB exposure, the cells were lysed with a lyses buffer based on 1 mM EDTA, 0.005% Tween 20, 0.5% Triton X-100, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin, 100 μM PMSF and 3 μg/ml Aprotinin in PBS, ph 7.2-7.4. A mouse anti-human capture antibody and a biotinylated detection antibody (Streptavidin-HRP) specific for Bax-α were used and the fluorescence was recorded at 450 nm with an ELISA microplate reader (Tecan Sunrise, Grödig/Salzburg, Austria).

2.6. Measurement of NF-kB p65 with ELISA

NF-kB p65, the most abundant member of the NF-kB transcription family, was measured with PhosphoTracer NF-kB p65 assay kit (Abcam, UK), according to the manufacturer's indications. This kit detects the endogenous level of this molecule in cellular lysates using an immuno-sandwich technique. Briefly, cells were seeded and irradiated as described in sub-chapters 2.1. and 2.2., respectively. After 2, 4, 8 and 24 h from UVB exposure, the cells were lysed with 100 μl lysis solution containing a combination of detergents, phosphatase inhibitors, salts and buffers, available in the ELISA kit. 50 μl/well cell lysates and 50 μl/well antibody mix (capture antibody + detection antibody) were added to PhosphoTracer assay microplate. Fluorescence was recorded at 530-540 nm with an ELISA microplate reader (Tecan Sunrise, Grödig/Salzburg, Austria).

2.7. Western blot analysis

Cytosolic extracts from the cells were prepared in lyses buffer based on 10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1% Triton X-100, 10 μg/ml Leupeptin, 1 μg/ml Pepstatin and 60 μg/ml Aprotinin (pH 7.4), at 4 °C, with sonication. The protein concentration in each lysate was determined with Coomassie brilliant blue G-250. Loading buffer (containing 125 mM Tris-HCl, 10% Glycerol, 10% SDS, 10% 2- Mercaptoethanol and 0.04% Coomassie brilliant blue G-250) was added and an equal amount of protein (50 μg) was loaded and separated in 10% SDS-PAGE gel and electro-transferred onto nitrocellulose membranes. The blots were blocked with 5% non-fat dry milk and incubated with a monoclonal antibody against RelA/NF-kB p65 (R&D Systems, Abingdon, UK). Thereafter, the blot was exposed to HRP-conjugated secondary antibodies, and the protein bands were detected with DAB (3,3'-diaminobenzidine) reaction. The presence of these molecules was confirmed with pre-stained molecular weight markers.

2.8. The plant extract

In the present study an extract obtained from the seeds of red grapes *Vitis vinifera* (GSE) was tested. The description of the extract preparation was made elsewhere [13,21]. The total polyphenolic content (TPC) was determined with Folin-Ciocalteu method [21]. The most important biologically active compounds were evidenced by high-performance liquid chromatography (HPLC). Thus, for the GSE extract the TPC was standardized as 3 mg GAEq/ml of which 2.02 mg/ml were catechins, 1.073 mg/ml proantocyanidine (35.76% of the TPC) and 3.17 μg/ml antocyanidine [21]. HPLC analysis detected peaks for epigallocatechin and epicatechin, catechin hydrate, procyanidin B and gallic acid (GA) [14]. Antioxidant activity was measured by 2,2-dipheniyl-l-picryl-hydrazyl (DPPH) radical assay (0.072±0.002 mM/mM DPPH) [13] and Trolox-equivalent antioxidant capacity (52.89±0.02 mM Trolox eq) [19].

2.9. Assessment of the plant extract cytotoxicity on the HaCaT cells

The cytotoxicity of the GSE was determined on the HaCaT cells and the calculated IC_{50} value was considered as guide-mark of the tested concentrations. As IC_{50} represents

a concentration which inhibits the survival of 50% of the cells, concentrations lower than this were selected for testing aiming to obtain protective and not cytotoxic effects of the plant extract. Briefly, the cells were seeded in triplicate in 96-well flat-bottom plates, at a cell population density of $15x10^3$ in 200 µl cell culture media/well. After 24 h, variable concentrations of the GSE extract (0.001-522 µgEqGA/ml) were added to the wells and then were incubated for additional 24 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) 1 mg/ml was added to the wells, then were incubated for 1 h and the absorbance was recorded with an ELISA plate reader, at 490 nm wavelength (Tecan Sunrise, Grödig/Salzburg, Austria).

In order to assess the potential protective effects of the GSE extract on the ROS and apoptosis production and on the levels of Bax-α and NF-kB proteins, the cells were treated for 30 min before UVB irradiation with two concentrations selected according to the IC_{50} value of the extract on the tested cell line. N-acetylcysteine (NAC), a well-known antioxidant and selective inhibitor of ROS [22] was used as positive control (5mM). The measurements were done at 100 mJ/cm^2 UVB dose at different time points after irradiation ranging between 1 h – 8 h.

2.10. Statistical analysis

Statistical processing of the experimental data was done using GraphPad Prism software program, version 5.0 (GraphPad, San Diego, CA, USA). Statistical comparisons between groups were made by one-way Anova Test ($p<0.05$ statistical significance threshold) and column statistics was also assessed.

3. Results

3.1. Cytotoxicity results of the GSE extract

The IC_{50} value of the GSE extract on the HaCaT cell line was: 113 µgEqGA/ml. Aiming to obtain protective and not cytotoxic effects of the extract on the irradiated cells we used concentrations below the measured IC_{50} values. Hence, two concentrations of GSE were selected for testing in the present study: GSE_1 (37.5 µgEqGA/ml) and GSE_2 (75 µgEqGA/ml).

3.2. Evaluation of ROS induced by UVB and the effects of the GSE extract

The ROS production after the exposure of keratinocytes to 100 mJ/cm^2 UVB radiation was evaluated earlier at several time points following the exposure (1, 2 and 4 h). Results showed significant ROS productions at 1 and 2 h and slighter differences as compared to control at 4 h [17]. None of the selected concentrations of the grape seed extract showed ROS reducing effects at 1 or 2 h after UVB radiation (data not shown). At 4 h both concentrations considerably reduced the level of ROS: GSE_1 determined 1.15 fold decrease (from 3,249±112.6 to 2,806±26.21 fluorescence units) while GSE_2 caused 1.14 fold decrease (from 3,249±112.6 to 2,847±68.54 fluorescence units) vs irradiated cells ($p<0.001$ for both extracts). NAC, a well-acknowledged selective ROS inhibitor determined a less important reduction in ROS following UVB irradiation, both extracts having superior effects (Figure 1).

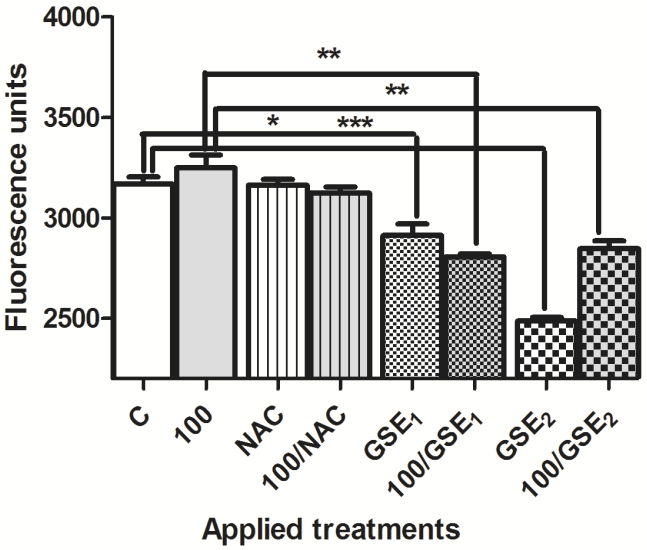

Figure 1. The level of ROS production 4 h after the following treatments of the HaCaT cells: control (C); irradiated with 100 mJ/cm2 UV-B (100); treated with N-acetylcysteine (5 mM) (NAC); treated with N-acetylcysteine (5 mM) followed by irradiation with 100 mJ/cm2 UV-B (100/NAC); treated with GSE1 (37.5 µgEqGA/ml); treated with GSE1 (37.5 µgEqGA/ml) followed by irradiation with 100 mJ/cm2 UV-B (100/GSE1); treated with GSE2 (75 µgEqGA/ml); treated with GSE2 (75 µgEqGA/ml) followed by irradiation with 100 mJ/cm2 UV-B (100/GSE2). Statistical comparisons between groups were made by Unpaired t test, *p<0.05; **p<0.001; ***p<0.0001. (Means ± SEM, n=3).

3.3. Evaluation of apoptosis induced by UVB radiation and the effects of the GSE extract

Because the highest level of the ROS was registered 2 h after UVB exposure [17], apoptosis, quantified as intracellular level of ccK18 was evaluated at this time point and later. Thus, at 2 h this molecule increased from 253±16.43 U/L to 411.8±4.43 U/L, corresponding to 1.62 fold increase ($p<0.001$) as compared to control. At 4 h the increase was from 253±16.43 U/L to 778.4±7.6 U/L ccK18, corresponding to 3.07 fold change ($p<0.0001$) and at 8 h from 253±16.43 U/L to 598.2±10.26 U/L ccK18, corresponding to 2.36 fold change ($p<0.001$) in irradiated cells as compared to control (Figure 2).

Among the tested plant extracts GSE_2 proved its efficiency in apoptosis reduction, causing significant decreases in ccK18 levels for all the tested intervals. Thus at 2 h the level of ccK18 molecule decreased from 411.8±4.43 to 131±5.9 U/L ($p<0.0001$), at 4 h from 778.4±7.6 to 199.1±30.48 U/L ($p<0.001$) and at 8 h from 598.2±10.26 to 89.79±1.49 U/L ($p<0.0001$). The most marked decrease evaluated as fold change in cells treated previously with the plant extract vs. solely irradiated cells was recorded 8 h after irradiation (6.66 fold changes, respectively). GSE_1 showed no effect in apoptosis reduction (data not shown).

3.4. Evaluation of Bax-α protein level after UVB exposure and treatment of the cells with the GSE extract

UVB exposure resulted in statistically significant changes in the Bax-α pro-apoptotic protein level at 4 h after UVB, when this molecule increased from 0.39±0.0007 to 0.84±0.06 pg/ml, representing 2.13 fold increase vs control ($p<0.05$) [17]. Similarly to apoptosis results, the higher concentration of the plant extract (75 µgEqGA/ml) determined statistically significant changes in Bax-α protein level. Thus, GSE_2 reduced this pro-apoptotic protein's level from 0.84±0.06 to 0.3±0.14 pg/ml ($p<0.05$), which corresponds to 2.83 fold decrease (Figure 3).

3.5. Evaluation of NF-kB p65 level after UVB exposure and pre-treatment of the cells with GSE extract

The exposure of the HaCaT cells to 100 mJ/cm^2 UVB triggered a time dependent increase in the NF-kB level of the cells. The highest value was measured at 8 h post-irradiation, when 121,905±4,295 fluorescence units were recorded vs 99,404±10,031 fluorescence units measured in control cells (Figure 4). Among the tested plant extracts, only GSE_1 showed efficiency in the re-establishment of the NF-kB p65 level previously affected by UVB radiation. Statistically significant changes were recorded at 4 h, when NF-kB p65 decreased from 116,941±5,656 to 95,080±3,024 fluorescence units ($p<0.05$). GSE_2 was inefficient in this setting, its effects being added to the damaging effects of the UVB irradiation (data not shown).

3.6. Results of Western blot analyses

The expression of NF-kB p65 protein was also confirmed by Western blot analysis. The results demonstrated the expression of this molecule in control cells, UVB exposed cells and cells pre-treated with the plant extract and afterward irradiated with UVB. Confirmation by Western blot was done for GSE_1 which showed efficiency according to the quantitative ELISA results. (Figure 5).

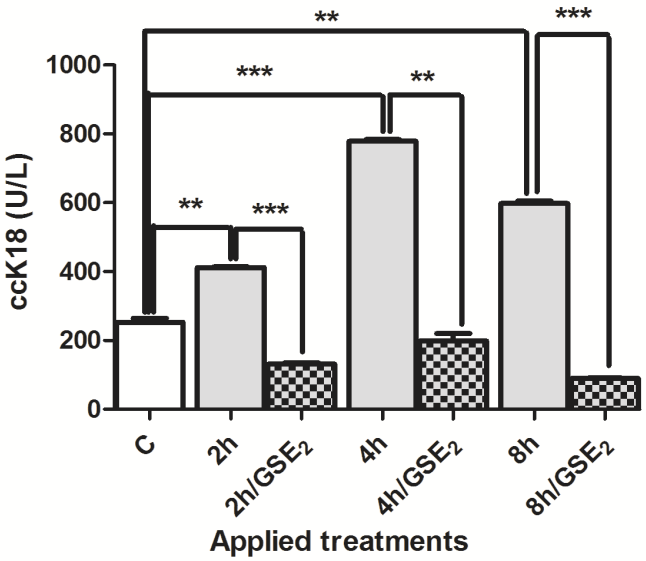

Figure 2. Apoptosis production after the following treatments of the HaCaT cells: control (C); irradiated with 100mJ/cm^2 UV-B at various time points (2h, 4h and 8h); treated with GSE_2 (75 µgEqGA/ml) followed by irradiation with 100 mJ/cm^2 UV-B at 2h, 4h and 8h (2h/GSE_2, 4h/GSE_2, 8h/GSE_2). Statistical comparisons between groups were made by Unpaired *t* test, **$p<0.001$, ***$p<0.0001$. (Means ± SEM, n=2).

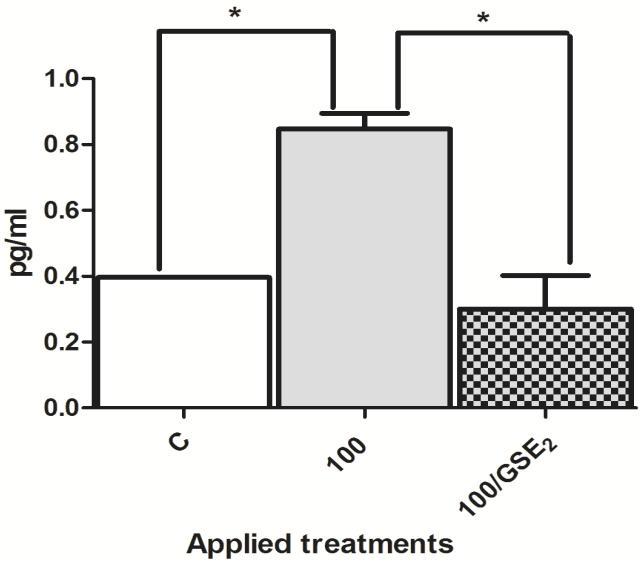

Figure 3. Expression of Bax-α pro-apoptotic protein 4 h after the following treatments of the HaCaT cells: control (C); irradiated with 100 mJ/cm^2 UV-B (100); treated with GSE_2 (75 µgEqGA/ml) followed by irradiation with 100 mJ/cm^2 UV-B (100/GSE_2). Statistical comparisons between groups were made by Unpaired *t* test, *p<0.05. (Means ± SEM, n=2).

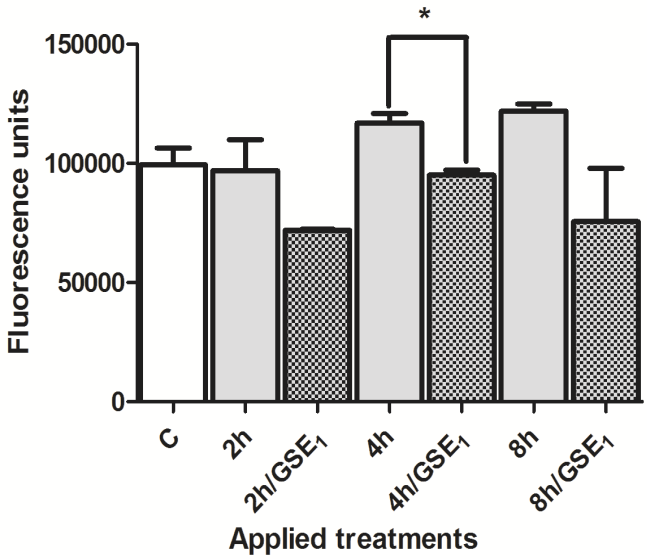

Figure 4. Expression of NF-kB p65 total protein at 2, 4, 8 and 24 h after the irradiation of HaCaT cells with 100 mJ/cm^2 UVB. The applied treatments were: control (C); irradiated with 100 mJ/cm^2 UV-B at various time points (2h, 4h and 8h); treated with GSE_1 (37.5 µgEqGA/ml) followed by irradiation with 100 mJ/cm^2 UV-B at 2h, 4h and 8h (2h/GSE_1, 4h/GSE_1, 8h/GSE_1). Statistical comparisons between groups were made by Unpaired *t* test, *p<0.05. (Means ± SEM, n=2).

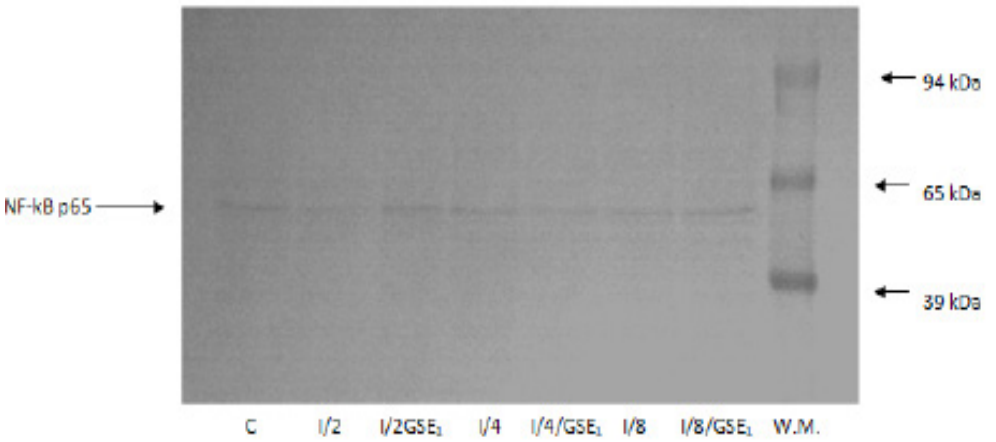

Figure 5. Western blot analysis representing the expression of NF-kB protein level in HaCaT cells subjected to the following treatments: control (C); irradiated with 100 mJ/cm^2 UV-B at various time points (2h, 4h and 8h); treated with GSE_1 (37.5 µgEqGA/ml); weight marker (W.M.) followed by irradiation with 100 mJ/cm^2 UV-B at 2h, 4h and 8h (2h/GSE_1, 4h/GSE_1, 8h/GSE_1).

4. Discussion

Cutaneous malignancies (melanoma and non-melanoma skin cancers) represent a major public health problem, one of the contributing factors appearing to be the overexposure of the skin to UV radiation. The use of efficient chemopreventive and chemotherapeutic agents lately is successfully combined and completed with dietary phytochemicals as a means to protect skin against UV radiations, thus preventing skin cancers.

DNA represents the direct target for UVB, but this type of radiation also causes important ROS mediated damages. In normal conditions lesions are repaired by nucleotide excision repair mechanism [23] or cells are removed entering apoptosis.

UVB influences multiple cellular structures, including protein kinases, transcription factors and receptors, which may contribute synergistically or independently to UV-induced apoptosis [24,25]. Among these structures a central role is played by Bcl/Bax proteins, found in the cytosol or localized in the membrane of cellular organites, exerting pro- or anti-apoptotic effects [26].

Exposure to UV radiation leads also to gene induction through the activation of transcription factors, such as NF-kB, Cox-2, AP-1 and MAPKs. NF-kB is sequestered in the cytosol of the resting cells while it's translocation to the nucleus allows the transcription of some target genes [6]. The whole mechanism of NF-kB activation by UVB is not well established yet, except that the upstream signal is initiated by ROS and that NF-kB activation inhibits UV radiation-induced apoptosis [27,28].

It was previously established that the exposure of HaCaT cells to UVB radiation within the physiological range of exposure and equivalent to the minimal erythema dose (100 mJ/cm^2) results in significant ROS production and consequently apoptosis, one of the contributing molecules appearing to be the Bax-α pro-apoptotic protein [17]. As the present study showed, another element which undergoes changes and most likely contributes to the complexity of the events experienced by human skin exposed to UVB radiation is the NF-kB p65 protein. On the other hand, the treatment of the skin cells, i.e. keratinocytes, with a red grape seeds extract with proved antioxidant capacity prior to UVB exposure considerably decreased ROS levels and consequently apoptosis, as compared to the solely irradiated cells. Bax-α and NF-kB p65 were also regulated by this compound, the effects being materialized in the protection of the cells from UVB-induced apoptosis and restoration of the initial, pre-irradiation level of NF-kB p65 protein.

Numerous compounds were tested throughout the years for their ROS scavenging capacities and photoprotective effects against UVB radiation, such as α-lipoic acid, NAC [29] gingerol [30], luteolin [31], epigallocatechin-3-gallate [27], pomegranate fruit extract [32] etc. As regarding the numerous biologic effects of grape seeds extracts, the following were considered by us and others: spectroscopic characterization and tumor growth inhibition [21,33], evaluation of antioxidant capacities [12], chemopreventive effects and modulation of NF-kB/ERK in nude mice [13,15,34,35,36] and in human keratinocytes [37] and prevention of the UVB radiation induced DNA lesions [14,19].

Studies on SKH-1 mice showed oxidative stress reducing and apoptosis inhibiting effects for red grape seeds extract [15,36]. Similar effects were also demonstrated in HaCaT cells using higher doses of UVB, the extract reducing the UVB-induced lipid peroxidation [14,19]. Mantena and Katyiar [37] observed inhibitory effects of grape seed proanthocyanidins on UVB-induced hydrogen peroxide, lipid peroxidation, protein oxidation in normal human epidermal keratinocytes.

In the present study some features of the GSE mechanisms of action on ROS reduction and apoptosis inhibition were ascertained. Thus, at the highest levels of ROS, both GSE_1 and GSE_2 were inefficient. Their effects became obvious when UVB-induced ROS levels were in decline.

The extracts effects on apoptosis were comparable to those exerted on ROS production, at least from the point of view that the most marked effect was recorded when the ccK18 protein's level wasn't at its maximum level. Regarding the underlying mechanisms and molecules through which this extract might have exerted its effect on apoptosis, the present study demonstrated that one potential molecule could be the Bax-α pro-apoptotic protein. This molecule was down regulated subsequently to the treatment with the tested plant extract.

Other research teams reported comparable results, i.e. preventing apoptosis by grape seed extract through Bax-α protein: in SKH-1 mice [36]; on the contrary, on the preneoplastic mouse epidermal cell line (JB6 C141) grape seed extract induced apoptosis through up-regulation of Bax and down-regulation of Bcl-2/Bcl-x_L proteins [38].

In the present study the total concentration of NF-kB p65 in UVB-irradiated skin cells was evaluated which showed increasing values in time. The NF-kB p65 activating effect of UVB in the skin cells is well documented [39,40], although some authors sustain that only damaging UVB doses could induce this process [41]. On the other hand, NF-kB activation was shown to inhibit UV radiation-induced apoptosis but this anti-apoptotic activity is seemed to be limited to low dose UVC radiation [42]. Hence, the precise role of NF-kB in keratinocytes apoptosis in the context of UVB radiation is still under debate, especially in the view of the fact that NF-kB plays a critical role in the epidermal cells' growth and differentiation, as well [43]. Nonetheless, it is unanimously accepted that the control of NF-kB expression is essential in homeostasis.

In the present study, the tested vegetal extract contributed to the re-establishment of the initial level of total NF-kB demonstrating its regulatory effect on this protein's expression, therefore a potential role in the skin's homeostasis. Similar results were obtained by others in keratinocytes [39] and in SKH-1 hairless mice skin [44] whereas grape seed extract was shown as MAPK, NF-kB and ERK modulator. Other data proved the NF-kB blocking effects of alpha-lipoic acid, NAC and silymarin [41], pomegranate fruit extract [32], luteolin [45], epigallocatechin-3-gallate [27] and a polypeptide from *Chlamys farreri* [46] on HaCaT cells.

5. Conclusion

The tested vegetal extract exerted protective effects on HaCaT cells subjected to UVB radiation (minimal erythema dose) materialized in significant reduction of ROS production and apoptosis induction. According to these findings, the red grape seed extract manifested its beneficial effects through ROS reduction and apoptosis prevention rather than through facilitation of removing UVB-damaged cells through entering apoptosis. Furthermore, these effects occurred, at least to a certain extent, due to the modulation of the Bax-α protein and through the re-establishment of the initial, pre-irradiation level of total NF-kB p65 protein essential for the epidermis homeostasis. These results encourage further studies on this extract in order to identify other molecules and pathways through which this extract might exert its beneficial effects and also recommend its use as a potential photoprotective agent.

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