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Original Paper

Glycyrrhizic Acid Prevents Oxidative Stress Mediated DNA Damage Response through Modulation of Autophagy in Ultraviolet-B-Irradiated Human Primary Dermal Fibroblasts

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Key Words
Primary Human Dermal Fibroblasts • Ultraviolet-B • Photo-damage • Oxidative stress • DNA damage • Autophagy

Abstract
Background/Aims: Excessive exposure to UV radiation negatively affects the human skin, characterized by photo-damage (premature aging & carcinogenesis). UV-B radiation causes about 90% of non-melanoma skin cancers by damaging de-oxy ribonucleic acids (DNA). We have previously reported that UV-B radiation induces skin photodamage through oxidative & Endoplasmic Reticulum (ER) stresses and Glycyrrhizic acid (GA), a natural triterpene, protects skin cells against such stresses. UV-B radiation elicits signalling cascade by activation of proteins involved in sensing, signalling, and repair process of DNA damage. In this study, we explored the effects & mechanisms of Glycyrrhizic acid (GA) against UV-B –induced photo-damage using a well established cellular model. ***Methods:*** We used primary human dermal fibroblasts as a cellular model. The cells were cultured in the presence or absence of GA for 3,6, & 24 h. Effect of UV-B was assessed by examining cell viability, cell morphology, oxidative stress, ER stress, DNA damage & cellular autophagy levels through biochemical assays, microscopy & protein expression studies. ***Results:*** In this study, we have determined the effect of GA on autophagy mediated DNA damage response system as the main mechanism in preventing photodamage due to UV-B -irradiation to primary human dermal fibroblasts (HDFs). GA treatment to UV-B exposed HDFs, significantly inhibited cell death,

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oxidative & ER stress responses, prevented Cyclobutane Pyrimidine dimer (CPD) DNA adduct formation, and DNA fragmentation via modulation of UV-B induced autophagic flux. Present results showed that GA treatment quenched reactive oxygen species (ROS), relieved ER stress response, improved autophagy (6 hr's post-UV-B -irradiation) and prevented UV-B induced DNA damage. ***Conclusion:*** The present study links autophagy induction by GA as the main mechanism in the prevention of DNA damage and provides a mechanistic basis for the photo-protective effect of GA and suggests that GA can be potentially developed as a promising agent against UV-B induced skin photo-damage.

Introduction

Ultraviolet (UV) radiation induces a sequence of morphological and ultrastructural alterations in human skin leading to photo-damage (premature aging, referred to as photo-aging & photo-carcinogenesis). These alterations are primarily due to ultraviolet (UV-B) radiations, which cause damage to DNA, alter antioxidant balance & signal transduction pathways, negatively impacting immunity and the extracellular matrix (ECM). The DNA alterations include UV irradiation induced formation of Cyclobutane Pyrimidine dimers (CPD's) and 6, 4-photoproducts (6, 4-PP's) [1]. UV-B -irradiation induced DNA photo-damage activates DNA damage response mechanism (DDR), cell cycle arrest and apoptotic pathways, allowing the cell to recognize and repair the damaged DNA or to initiate apoptosis depending upon the extent of the damage. UV-B induced cellular stress activates several well-orchestrated responses aimed at either restoring cellular homeostasis or committing to cell death [2]. These processes include the unfolded protein response (UPR) and autophagy, which are part of the global endoplasmic reticulum (ER) stress response (ERS). During normal metabolic activities, reactive oxygen species (ROS) are produced as a result of mitochondrial oxidative phosphorylation. However, levels of ROS are substantially enhanced upon UV-B exposure that alters the molecular structure and causes damage to nucleic acids, proteins, and lipids [3]. Numerous studies have established the deleterious effects of UV-B radiation and oxidative stress on the skin that can inappropriately activate signalling pathways, interferes with genome maintenance and affect apoptosis [4]. UV-B induced oxidative stress has been linked to age-related loss of skin elasticity, defective cellular signalling and photo-damage response. This damage has a serious bearing on the very integrity of the cell and elicits activation of molecular signaling pathways involved in sensing, signaling, and repair of DNA damage [5]. The activation events following DNA damage are well elucidated, whereas, the primary signal which triggers DNA damage response signaling remains incompletely understood. Human skin contains well-organized antioxidant defense systems to deal with enhanced levels of oxidative stress due to environmental or toxic insults. However, prolonged stress can rout ROS defense mechanisms contributing to the development of cutaneous disorders [6]. ROS and RNS (reactive nitrogen species) are induced immediately or secondarily upon UV-B irradiation resulting in subsequent endoplasmic reticulum stress, representing the primary source of cellular damage in human skin [7]. Different situations that induce oxidative stress manifested ER stress have been found to induce autophagy response in cells [8]. It has also been recently recognized that autophagy is required for efficient repair of UV-B induced DNA damage by nucleotide excision repair (NER) [9]. Autophagy deficiency in skin cells has been found to inhibit NER and recruitment of DNA damage sensing protein machinery for efficient repair of DNA damage in skin cells. It has been proposed that the cellular genetic background may determine whether autophagy exerts a pro or anti-apoptotic effect in response to genomic stress [10].

Natural products, including plant-based agents, have been shown to induce autophagy in human HDFs as a protective mechanism against UV-B induced cell death. Glycyrrhizic acid, (hereafter referred to as GA), a plant-derived triterpenoid saponin glycoside has been shown to exhibit potential photo-protective effect in HDFs against UV-B induced photo-damage in our previous reports [7, 11]. In the present study, we have explored the autophagy

inducing potential of GA in primary HDFs as a mechanistic link in the protection offered by GA against UV-B induced photo-damage. The present study demonstrates for the first time the therapeutic signalling mechanisms involved in the protection offered by GA against UV-B induced photo-damage in HDFs via modulation of autophagy.

Materials and Methods

Chemicals

Human primary dermal fibroblast cell line from the juvenile foreskin, (HDF) was obtained from HiMedia India. Primary fibroblast expansion media (HiMedia), fetal bovine serum (FBS), penicillin–streptomycin, trypsin–EDTA, 3-(4, 5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT), GA ($C_{42}H_{62}O_{16}$. NH_3, FW=840.0), phosphatase-protease cocktail, RIPA buffer were purchased from Sigma–Aldrich Chemicals (St. Louis, MO). Antibodies against P62, BECN 1, phospho ATM, phospho ATR, phospho p53, Phospho Chk1, phospho Chk2, Bcl-2, and secondary antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Fura 3 AM Calcium dye from (Thermo Scientific). TUNNEL assay & CPD Elisa kits were procured from Abcam. All other biochemicals used were of high purity biochemistry grade. All antibodies were purchased from Santa Cruz Biotechnology (USA) except Anti β-actin that were purchased from Sigma Aldrich.

Cell culture

HDF cells were maintained in primary fibroblast expansion media from (HiMedia) supplemented with L-Glutamine, glucose (3.5 g/L), Hepes (15 mM), Penicillin (120mg/L), Streptomycin (270mg/L) and Fetal bovine serum (10% v/v) at 37 C in a humidified atmosphere of 5% CO_2.

UV-B treatment/Exposure

Cells were exposed to UV-B using Daavlin UVA/UVB Research Irradiation Unit (Bryan, OH, USA) having digital control. The Lamps were maintained at a fixed distance of 24 cm from the surface of cell culture dishes. Majority of the resulting wavelengths (>90%) were in UV-B range (280–320 nm). UV-B irradiation of 10mJ/cm^2 was used for the majority of experiments as it is the environmental (i.e., physiological dose) [12]. For UV-B exposure, cell mono-layers were first washed with Dulbecco's phosphate buffered saline (DPBS) and then irradiated with UV-B under a thin layer of pre-warm DPBS. After irradiation, cells were again washed twice with DPBS and incubated in fresh medium with or without Glycyrrhizic acid (GA). To assess the protective effects of GA, cells were pre-treated with GA at 25 μM & 50 μM concentrations for 16 h and then subjected to UV-B exposure and post-treated for 3, 6 & 24 h with GA.

Cell viability Analysis

Cell viability analysis was determined by MTT assay as described earlier [7]. Briefly, the cells were seeded and incubated overnight. After treatment with GA or UV-B or both, the cells were further incubated for 24h. Cell viability was evaluated by assaying for the ability of functional mitochondria to catalyze the reduction of MTT to form formazan salt by an enzyme mitochondrial dehydrogenase, and determined by Multiskan Spectrum plate reader (Thermo Electron Corporation) at 570 nm using DMSO as detergent.

Reactive oxygen species measurement using fluorescence microscopy

Dichlorofluorescin diacetate (DCFDA) was used for the measurement of ROS generation, as described previously [12]. Briefly, cell monolayers were pre-treated with GA at different concentration and then exposed to UV-B. After UV-B irradiation, cell monolayers were washed twice with DPBS and incubated with fresh media supplemented with GA for further 3, 6 & 24 h. After treatment, the cells were stained with 5 μM DCFDA for 30 min at 37°C. The cells were then washed twice with DPBS and observed immediately under a fluorescent microscope (Evos FL Color Imaging System from Thermo Scientific). Five random microscopic fields were selected and the intensity of fluorescence was quantified using the Image J software.

Florescent microscopy imaging of intracellular Ca^{2+}

Changes in Ca^{2+} levels were determined by the Ca^{2+} indicator Fura 3 AM (Thermo Scientific) using florescent microscopy imaging. Briefly, after the cells were seeded to sterile cover slips & UV-B treatment was given as required, HDFs were loaded with fluorescent Ca^{2+} indicator dye Fura 3 AM at 5 μM for 45 min before imaging post 6 h UV-B –irradiation. Cells were washed three times with live cell imaging solution for imaging using an Evos FL Color Imaging System (Thermo Scientific). Imaging was done by using excitation at 488 nm, emission at 515-565 nm, and 40X oil lenses.

Detection of DNA Fragmentation by DAPI staining

In DAPI staining, briefly, cells were harvested, centrifuged onto glass slides, fixed with 4 % paraformaldehyde for 10 minutes, washed with PBS & stained with 1 mg/ml 4', 6-diamidino-2-phenylindole hydrochloride (DAPI) for 10 minutes at 37°C. Slides were then washed with PBS, air dried, covered with cover slips & mounted for analysis by florescence microscope (Evos FL Color Imaging System from Thermo Scientific).

Preparation of whole cell lysates for western blotting

After treatment, cells were washed twice with ice-cold DPBS, trypsinized from the dishes and transferred to micro-centrifuge tubes in cell lysis buffer (25 mM HEPES, pH 7.2, 2.5 mM MgCl2, 75 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol (DTT) and 20 mM b-glycerophosphate), supplemented with 10 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mg/ml pepstatin A and 1 mM, phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate. Following 20 min of incubation at 4°C, cell homogenates were centrifuged at 14, 000×g at 4°C for 20 min, and supernatants were collected and used as whole cell lysates. Protein concentration was measured with Bradford Reagent (Sigma Aldrich Chemicals Private Limited, St. Louis, MO) as per instructions of the manufacturer. The BSA was used as standard. For protein expression by western blotting, about 40–60 μg of protein samples was electrophoresed using 7–15% SDS–PAGE gel and transferred to PVDF membrane (Millipore, Billerica, MA, USA). The PVDF membranes were probed with appropriate primary antibodies followed by a secondary horse radish peroxidase (HRP)-conjugated antibody. The blots were then analyzed using a chemiluminescent HRP substrate (Millipore) and visualized by ChemiDocTM XRS+. Densitometric analysis of the blots was performed using Image LabTM software, version 3.0 (BioRad Hercules, CA, USA).

Immuno-cytochemical detection of P62, BECN1, phosphorylated histone (γH_2AX), activated ATR & DDB2

Cultured cells were seeded on coverslips in chamber slides and incubated in the presence or absence of indicated concentrations of GA and exposed to UV-B -irradiation for either 6 or 24 h. Cells were washed twice with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized in PBS with 0.1% TritonX-100 at room temperature for 10 min. Nonspecific binding sites were blocked by incubating the cells with 1 % BSA. Cells were incubated with p62, Beclin1 & χpH_2AX and DDB2 antibody at a dilution of 1:100 in 0.1% Triton X-100 in PBS for overnight at 4°C, then washed and incubated with Alexa Fluor 488 conjugated anti-rabbit secondary antibody at a dilution of 1:500 in PBS for 2 h at room temperature. Cells were then washed three times with PBS and stained with DAPI 1 μg/ml in PBS. The coverslips were mounted on glass slides, and cells were imaged by a florescent microscope (Evos FL Colour Imaging System from Thermo Scientific).

TUNEL assay

TUNEL assay was performed with an In Situ Direct DNA Fragmentation (TUNEL) Assay Kit (ab66108) from (Abcam) according to the manufacturer's instruction. Briefly as follows: cell smears after fixation, blocking and permeabilization were incubated with TUNEL reaction mixture for 1 h in the dark at 37 °C and counterstained with RNase/PI solution for 20 minutes. Substrate solution was added and cells were imaged by a florescent microscope (Evos FL Colour Imaging System from Thermo Scientific).

ELISA based estimation of Cyclobutane Pyrimidine dimers (CPD's)

UV-B induced CPD adducts were quantified with an in situ OxiSelect™ UV-Induced DNA Damage staining kit (CPD quantification kit from Cell Bio Labs, Inc. San Diego, CA, USA) according to the manufacturer's instruction. Briefly, after corresponding treatments of HDFs, DNA was isolated and incubated with the anti-

CPD antibody for overnight on an orbital shaker at room temperature. Then the cells were washed and incubated with secondary FITC-conjugated antibody for 2 hours. The absorbance was measured at 450 nm using the Multiskan Spectrum plate reader (Thermo Electron Corporation).

Statistical analysis

The data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed by using INSTAT statistical software. Data are represented as Mean• ±S.E. from three independent experiments. Statistical comparisons between two groups was performed by student's *t*-test and among groups by One-way ANOVA. p≤ 0.05, p<0.01, p<0.001 was considered as statistically significant.

Results

GA helps HDFs in cell survival upon UV-B –irradiation

We first investigated the cytotoxic effect of UV-B -irradiation on HDFs at different doses. We found the viability of cells decreased significantly upon increasing the intensity of UV-B. UV-B at 10 mJ/cm^2 shows 16.5% cytotoxicity & at 20 mJ/cm^2 shows 30.7% cytotoxicity as compared to normal cells, (*p<0.05, **p<0.01 as compared to untreated), (Fig. 1A). We further tested the cytotoxic effect of GA at various concentrations on primary HDFs. Upon treatment of cells with GA up to 50 µM concentration, the viability of cells remained unchanged (Fig. 1B). Thus, GA exhibited no significant cytotoxic effects on HDFs upto 50µM. We then tested whether GA treatment to UV-B (10mJ/cm^2) irradiated HDFs can prevent cell death and observed that GA significantly protects cell death in UV-B -irradiated HDFs by about 60% & 90% at 25µM & 50µM concentrations respectively (*p<0.05 as compared

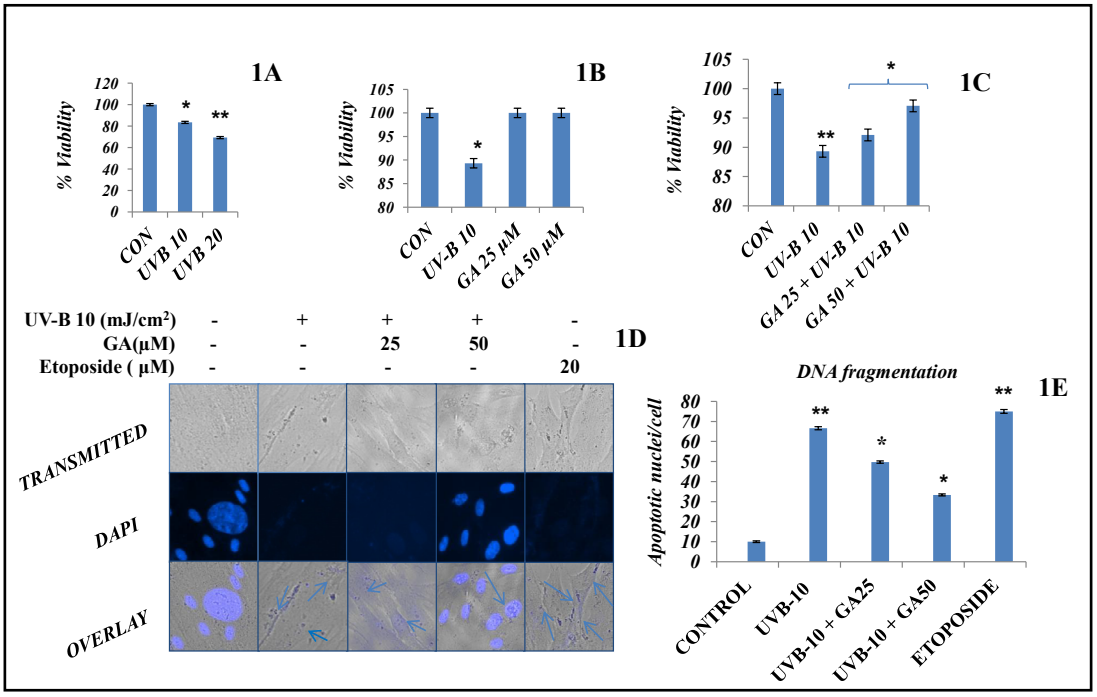

Fig. 1. GA helps HDFs in cell survival upon UV-B –irradiation. UV-B treatment to primary fibroblast cells at 10 mJ/cm^2 shows 16.54% cytotoxicity & at 20 mJ/cm^2 shows 30.72% cytotoxicity (Fig. 1A), (*p<0.05, **p<0.01 as compared to untreated). GA treatment to HDFs does not induce any cytotoxicity upto 50 µM (Fig. 1B). GA pre-treatment to HDFs significantly restores the cell viability in HDFs upto 60% at 25 µM and by 90% at 50 µM, (Fig.1C), (*p<0.05 as compared to UV-B treated). DAPI staining showed higher apoptotic nuclei in UV-B treated HDFs as compared to GA treated cells (Fig. 1D & 1E), (**p<0.01 as compared to UV-B treated, *p<0.05 as compared to GA treated).

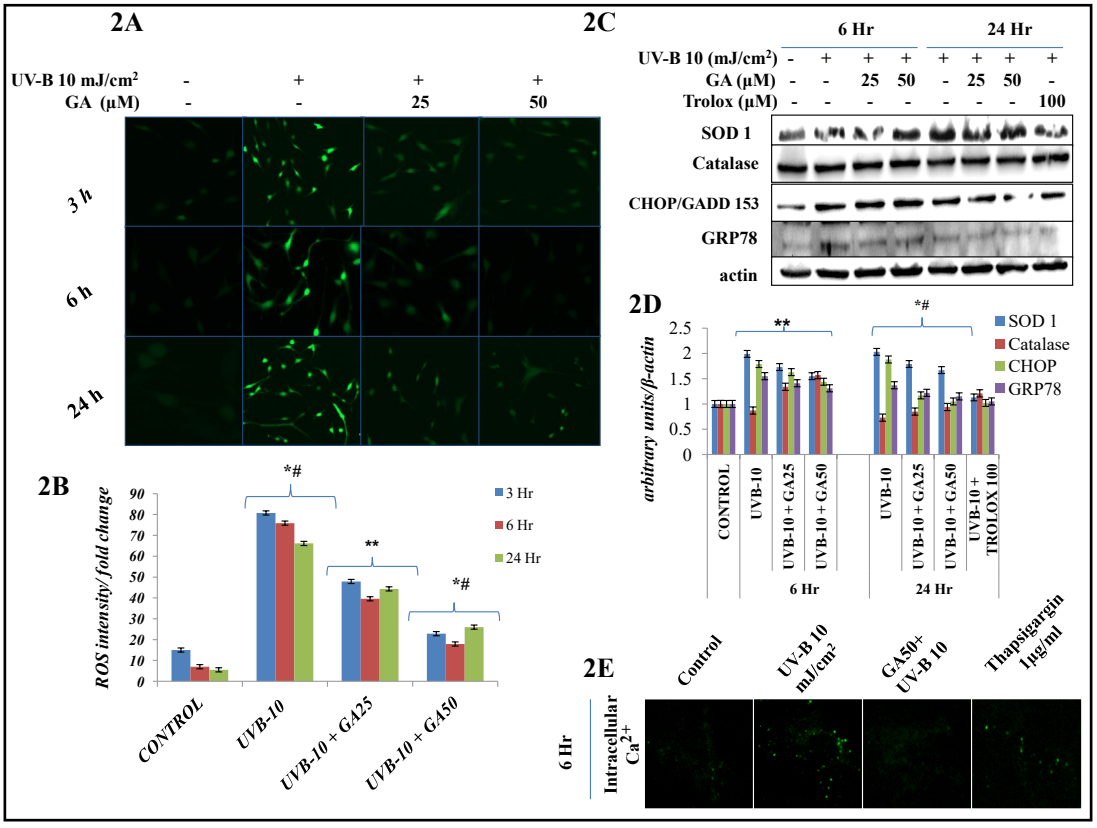

Fig. 2. GA prevents HDFs from UV-B induced Oxidative stress (ROS) mediated ER stress response. UV-B -irradiation to HDFs induces immediate as well as secondary ROS in 3, 6, & 24 h post UV-B -irradiation, which is significantly quenched by GA pre-treatment to HDFs in an intensity dependent manner,(Fig. 2A & 2B), (*#p<0.001 as compared to UV-B treated), depicting its role as strong antioxidant. UV-B –irradiation up-regulates the anti-oxidant defence protein SOD1 and down-regulates the Catalase post-UV-B –irradiation whereas GA maintains the anti-oxidant balance in HDFs in both 6 & 24 Hr post UV-B -irradiation (Fig. 2C & 2D). UV-B -irradiation to HDFs induces ER stress response in 6 & 24 h post UV-B -irradiation whereas GA pre-treatment significantly relieves HDFs from oxidative stress-mediated manifestation of ER stress, (Fig. 2C, 2D & 2E), (**#p<0.001, **p<0.01 as compared to UV-B treated alone). Thapsigargin (1μg/ml) was used as a positive control for comparative analysis of intracellular Calcium changes.

to UV-B treated), (Fig. 1C). We also found that pretreatment with GA, protects HDFs from UV-B induced DNA fragmentation as is clear in DAPI staining (Fig. 1D & 1E), (**p<0.01 as compared to UV-B treated).

GA prevents HDFs from UV-B induced Oxidative stress (ROS) & ER stress responses

ROS generation is the hallmark in the photo-damage study due to UV-B -irradiation. The anti-oxidant potential of any compound can be assessed by its ability to quench the ROS. We measured the anti-oxidant potential of GA by DCFDA dye in HDFs at 3, 6, & 24 h post-irradiation of UV-B. UV-B irradiation to HDFs at 3,6 & 24 h caused 6, 8 & 12 fold increase in ROS generation respectively (*#p<0.001 as compared to control), (Fig. 2A & 2B). GA treatment decreased ROS levels significantly to approximately 2.5 & 3.75 folds at 25 & 50 μM concentration respectively in 3 h, 2 & 3.1 folds at 25 & 50 μM concentration respectively in 6 h & by 1.75 & 1.15 folds at 25 & 50 μM concentration of GA in 24 h post-irradiation of UV-B to HDFs, (*#p<0.001, **p<0.01, as compared to UV-B only). We also found that immediate ROS was more profound at early time intervals and then decreases in 24 h post UV-B -irradiation. This indicates that immediate ROS is responsible for UV-B induced oxidative stress and its significant quenching by GA offers cell protection to HDFs. It was

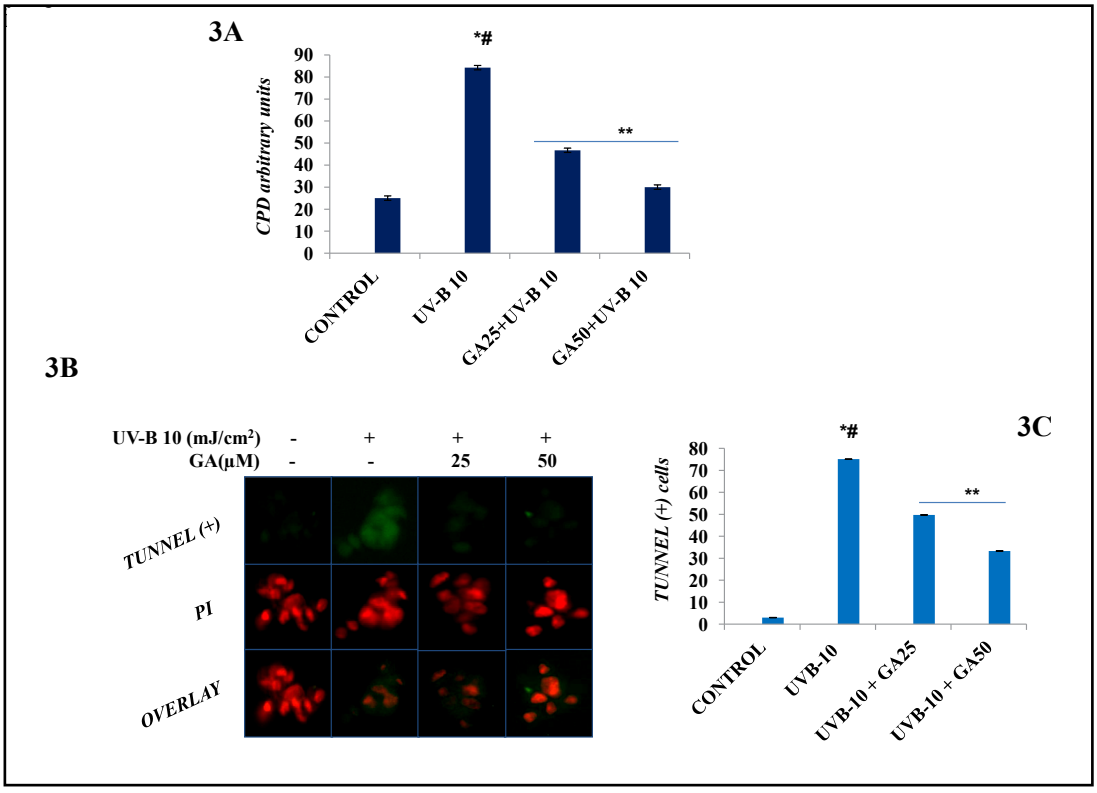

Fig. 3. GA prevents HDFs from UV-B induced Cyclobutane Pyrimidine dimmers (CPD) & apoptosis. UV-B irradiation to HDFs induces significant CPD's whereas GA pre-treatment protects the HDFs from UV-B -irradiation induced DNA adducts in an intensity dependent manner in ELISA quantification, (Fig. 3A), (*#p<0.001 as compared to UV-B treated). GA pre-treatment significantly protects HDFs from UV-B –irradiation induced apoptotic nuclei suggesting its role in protecting HDFs from UV-B induced DNA fragmentation, (Fig. 3B). GA decreases the TUNNEL positive cells in UV-B -irradiated HDFs in an intensity dependent manner by double fold at 50 µM concentration as compared to UV-B alone, (Fig. 3C), (*#p<0.001 as compared to UV-B treated, **p<0.01 as compared to GA treated).

also revealed through western blotting that GA significantly down-regulates oxidative stress marker protein SOD1 but up-regulates Catalase in 6 & 24 h post UV-B –irradiation (Fig. 2C & 2D), (*#p<0.001, **p<0.01, as compared to UV-B only). We also found that UV-B induced ER stress in 6 & 24 h post UV-B -irradiation is significantly relieved by GA pre-treatment as is evident by western blotting analysis of ER stress markers CHOP & GRP78 (Fig. 2C & 2D). We also looked for intracellular Ca^{2+} level changes through florescent microscopy and found that GA treatment significantly maintains the intracellular Ca^{2+} homeostasis in HDFs in 6 h post UV-B –irradiation as compared to UV-B treated alone (Fig. 2E).

GA prevents HDFs from UV-B induced Cyclobutane Pyrimidine dimers (CPD) & apoptosis

Since UV-B light induces the formation of CPDs which represent 70–80% of the total UV-B induced photoproducts. The effect of GA on UV-B -induced CPD formation was determined. ELISA analyses using a CPD-specific antibody revealed that cells exposed to UVB (10mJ/cm^2) had a higher number of CPD's than non-exposed control cells (Fig. 3A), (*#p<0.001 as compared to control). Pre-treatment of GA to the HDFs inhibited UV-B -induced CPD formation significantly as compared to UV-B alone. It was also revealed that GA pre-treatment to UV-B -irradiated HDFs significantly decreased TUNNEL positive cells in the estimation of UV-B -induced apoptosis than in UV-B treated alone (Fig. 3B & 3C), (*#p<0.001, **p<0.01 as compared to untreated), Taken together, these results indicate that GA protects HDFs against UV-B -induced DNA damage.

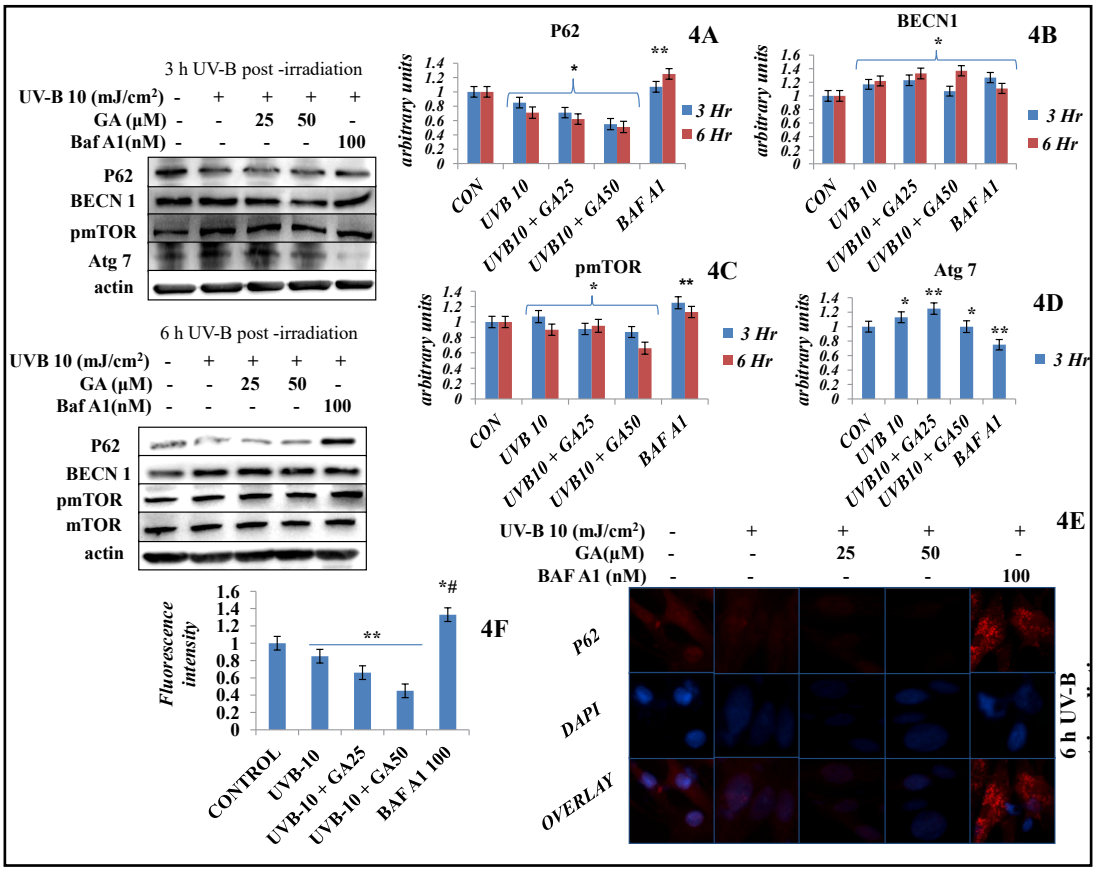

Fig. 4. GA modulates instant autophagic flux upon UV-B -irradiation in HDFs. GA pre-treatment to HDFs modulates UV-B induced autophagic flux at initial time points (3 to 6 h post-irradiation of UV-B), (Fig. 4A to 4D). UV-B induced autophagy in response to oxidative stress-mediated manifestation of ER stress is significantly upregulated in GA pre-treated HDFs as is clear from western blotting analysis of P62 (Fig. 4A), Becn1 (Fig. 4B), mTOR (Fig. 4C) & agt7 (Fig. 4D) in 3 h post-irradiation up to 6 h, (**p<0.01 as compared to UV-B only), (*p<0.05 as compared to GA treated). Immunofluorescence of P62 protein in 6 h post-irradiation also suggests modulation of UV-B induced autophagic flux in GA treated HDFs, (Fig. 4E & 4F), (**p<0.01 as compared to both UV-B & GA treated, (*#p<0.001 as compared to BAF A1 treated).

GA modulates instant autophagic flux after UV-B irradiation in HDFs

Oxidative & ER stress induced upon UV-B -irradiation could induce autophagy in HDFs as a protective response. It prompted us to investigate whether autophagic flux could be detected. We found that GA pre-treatment at 50 µM concentration to HDFs potentiates UV-B induced mild autophagy response contextually to protect HDFs from UV-B -irradiation induced cell death effects as is clear in western blotting analysis of P62, BECN1, pmTOR & atg7 proteins in 3 h but significantly in 6 h post-irradiation of UV-B (Fig. 4A, 4B, 4C & 4D), (*p<0.05, **P<0.01 as compared to untreated). We checked the expression levels of autophagy protein P62 in immunofluorescence in 6 h post-irradiation of UV-B and found that it's expression decreases significantly in GA treated cells as compared to UV-B alone (Fig. 4E & 4F), suggesting induction of flux in GA treated HDFs. From the above results, it is clear that GA pre-treatment modulates the autophagic flux induced upon UV-B -irradiation to HDFs as a protective mechanism against oxidative & ER stress-induced cell death effects.

Autophagy flux vanishes in prolonged post-irradiation of UV-B

Autophagy induction at early time point's upto 6 h post-irradiation protects the HDFs from UV-B induced oxidative & ER stress effect. We found that autophagy flux vanishes on

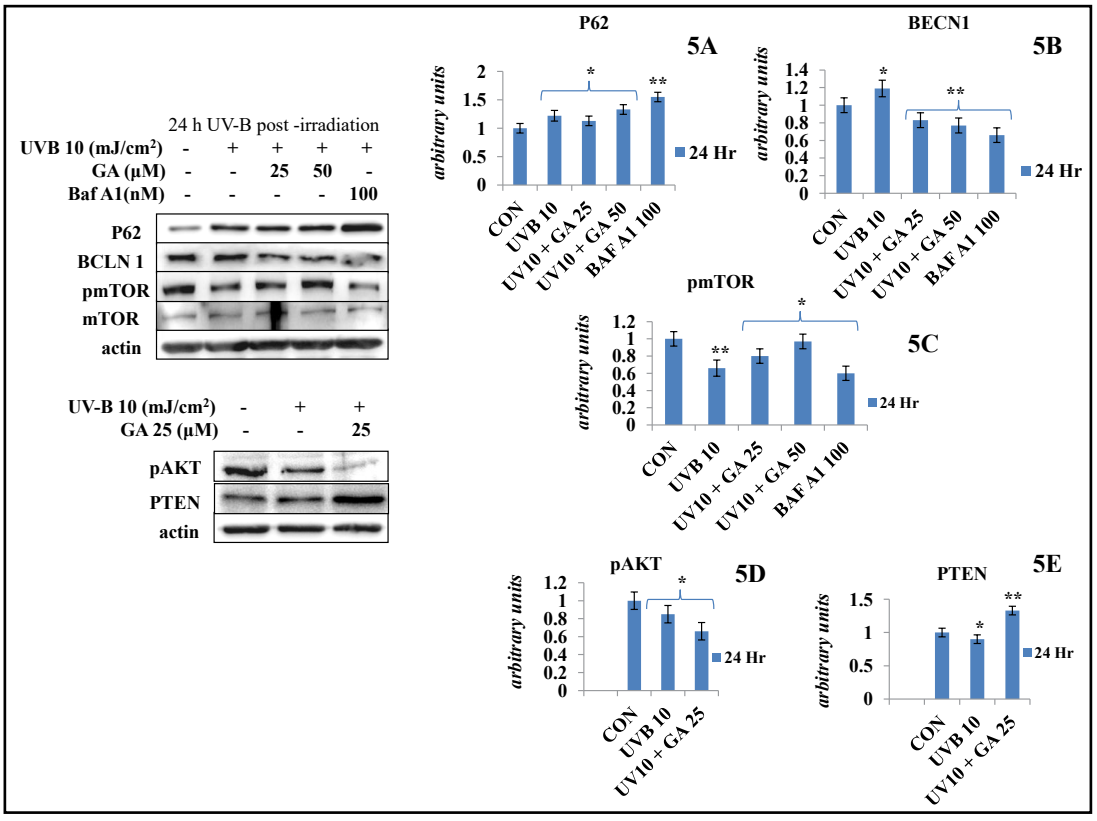

Fig. 5. Autophagy flux vanishes in prolonged post-treatment of UV-B in GA treated HDFs. UV-B irradiation to HDFs impairs autophagic flux in prolonged exposure (24 h). GA pre-treatment blocks the autophagy response in UV-B -irradiated HDFs as is evident from western blotting analysis of P62, BECN1 & mTOR proteins, (Fig. 5A, 5B & 5C respectively), *(*p<0.05, (**p<0.01* as compared to UV-B only & GA treated). UV-B -irradiation to HDFs suppresses the AKT/PTEN axis whereas GA stabilizes it owing to its cell protection effect in HDFs as is evident from western blotting analysis, (Fig. 5D & 5E), *(*p<0.05* as compared to untreated, *(*p<0.05*, ***p<0.01* as compared to GA treated).

increasing the post-irradiation time period as is evident in the western blotting analysis of P62, becn1 & autophagy sensor protein mTOR (Fig. 5A, 5B & 5C), (*p<0.05, **p<0.01 as compared to UV-B treated). We got similar results in immunofluorescence of P62 & Becn1 (Fig. 6A, 6B & 6C), (*p<0.05, as compared to UV-B treated), supporting our western blotting results that autophagy flux dwindles in 24 h post -irradiation to HDFs. GA pre-treatment could not either modulate the flux in UV-B -irradiated HDFs, possibly due to its cyto-protection at early time points (3 & 6 h) upon UV-B irradiation. We used Bafilomycin A1 (100nM) as positive control to rule out any experimental error. AKT protein is important in imparting protection to HDFs against cell death effects of UV-B [13]. GA pre-treatment to HDFs significantly down-regulated AKT (Fig. 5D), (*p<0.05 as compared to UV-B alone), suggesting that GA imparted protection to cells at early time points may be responsible for down-regulation of AKT as there is no requirement for AKT mediated modulation of mTOR to give protection to HDFs upon UV-B irradiation. Earlier findings have revealed that UV-B radiation suppresses PTEN expression in Skin Keratinocytes [13] and the ERK/AKT/PTEN axis may form a positive feedback loop following UV-B -irradiation as a protective mechanism. We found that the expression of PTEN increases significantly in GA pre-treated HDFs than in UV-B alone (Fig. 5E), (*p<0.05, **p<0.01, as compared to untreated), suggesting that PTEN up regulation by GA could have dwindled the expression of AKT as there is early protection offered by GA to HDFs in 6 h post -irradiation of UV-B.

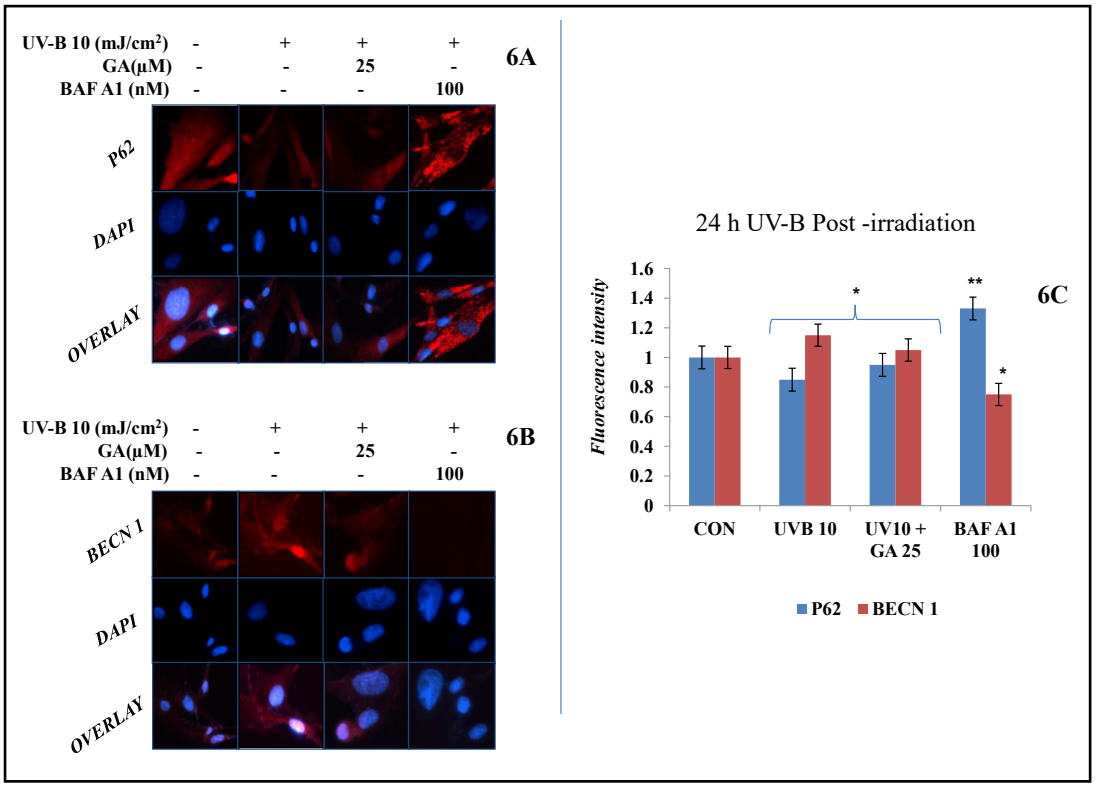

Fig. 6. Immunoflorescence of P62 & BECN1 proteins in 24 h post-irradiation of UV-B in GA treated HDFs. Immunofluorescence of P62, (Fig. 6A & 6C) & BECN1, (Fig. 6B & 6C) showing that autophagic flux vanishes in 24h post-irradiation of UV-B in HDFs, *(*p<0.05* as compared to UV-B alone).

GA protects HDFs from UV-B induced DNA damage

The protective effects of GA in UV-B -irradiated HDFs via autophagy induction and AKT-PTEN pathway modulation prompted us to directly test whether GA could immediately rescue primary HDFs from UV-B induced photo-damage at the molecular level. We found that GA pre-treatment protects HDFs from UV-B -irradiation induced DNA damage in 3 & 6 h post-irradiation of UV-B as is clear in western blotting analysis of key DNA damage marker proteins, ATM, ATR, Chk1, Chk2, DDB2, P53 & χH_2AX (Fig. 7A to 7G) respectively, (*p<0.05, **p<0.01, as compared to untreated). GA mediated protection to HDFs was more prominent in 6 h post-irradiation of UV-B, indicating that increased autophagic flux in 6 h post-irradiation of UV-B than in 3 & 24 h, is directly responsible for imparting protection from DNA damage in HDFs. We got similar results reflected in Immunofluorescence of $p\chi H_2AX$, pATR & DDB2 proteins (Fig. 8A, 8B & 8C respectively). The DNA damage sensor protein $p\chi H_2AX$ was found to be more recruited in UV-B only cells than in GA treated, suggesting that GA pre-treatment sensitizes HDFs & prepares them for UV-B -irradiation induced deleterious effects, suggesting a proto-protective role of GA upon UV-B -irradiation. These results suggest that GA acts as a strong anti-photo-damage molecule by protecting primary HDFs from UV-B induced photodamage.

Chloroquine mediated autophagy inhibition potentiates UV-B induced DNA damage whereas pharmacological activator Rapamycin alleviates UV-B induced DNA damage in HDFs

We confirmed the role of improved autophagy flux by GA upon UV-B –irradiation as the mechanistic link in offering protection to HDFs. We blocked the autophagic flux by chemical mediator Chloroquine (50 μM) in GA treated HDFs and found that on blocking autophagy

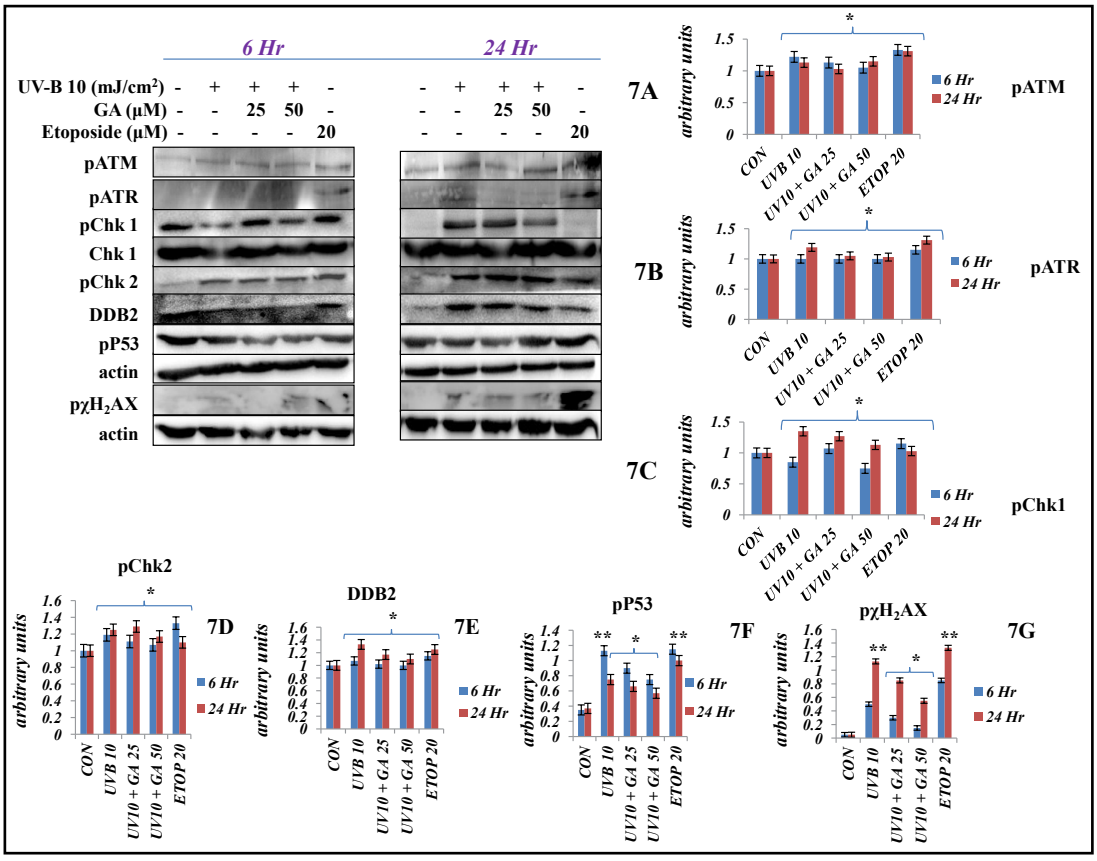

Fig. 7. GA protects HDFs from UV-B induced DNA damage. GA protects the UV-B -irradiated HDFs from DNA damage as is clear from western blotting analysis of key DNA damage response proteins, pATM (Fig. 7A), pATR (Fig. 7B), pChk1 (Fig. 7C), pChk2 (Fig. 7D), DDB2 (7E), pP53 (Fig. 7F) & χH_2AX (Fig. 7G), (*$p<0.05$* as compared to untreated, *(*$p<0.05$, **$p<0.01$* as compared to UV-B only). We found that GA pre-treatment significantly downregulated the expression of DNA damage marker proteins by imparting protection to cells against oxidative stress & ER stress-mediated cell death effect. The protection by GA from UV-B induced DNA damage was more prominent in 6 h post-treatment by about double fold at 50µM concentration.

response, the expression of DNA damage sensor protein χH_2AX significantly increases by double fold in GA pretreated HDFs upon UV-B irradiation than in UV-B alone (Fig. 9A & 9C), (**p<0.01 as compared to UV-B alone). The expression of P53 protein that guards the cell against any deleterious effect also increases double fold in GA pre-treated HDFs (Fig. 9D), (*p<0.05 as compared to UV-B alone), suggesting that autophagy induction is responsible for GA mediated protection to HDFs against UV-B induced DNA damage. We further intervened by using pharmacological activator of autophagy Rapamycin (100 nM) and found that it significantly decreases the expression of pChk2 & pP53 proteins and potentiates the photo-protective effect of GA upon UV-B -irradiation (Fig. 9B, 9E, & 9F), (**p<0.01 as compared to UV-B alone), suggesting that Rapamycin and GA in combination potentiates the autophagic flux & in turn give additional protection to cells from UV-B induced photodamage effects. These results in combination suggest that blockade of autophagy potentiates the UV-B induced DNA damage, whereas activation of autophagy alleviates the HDFs from UV-B induced photodamage.

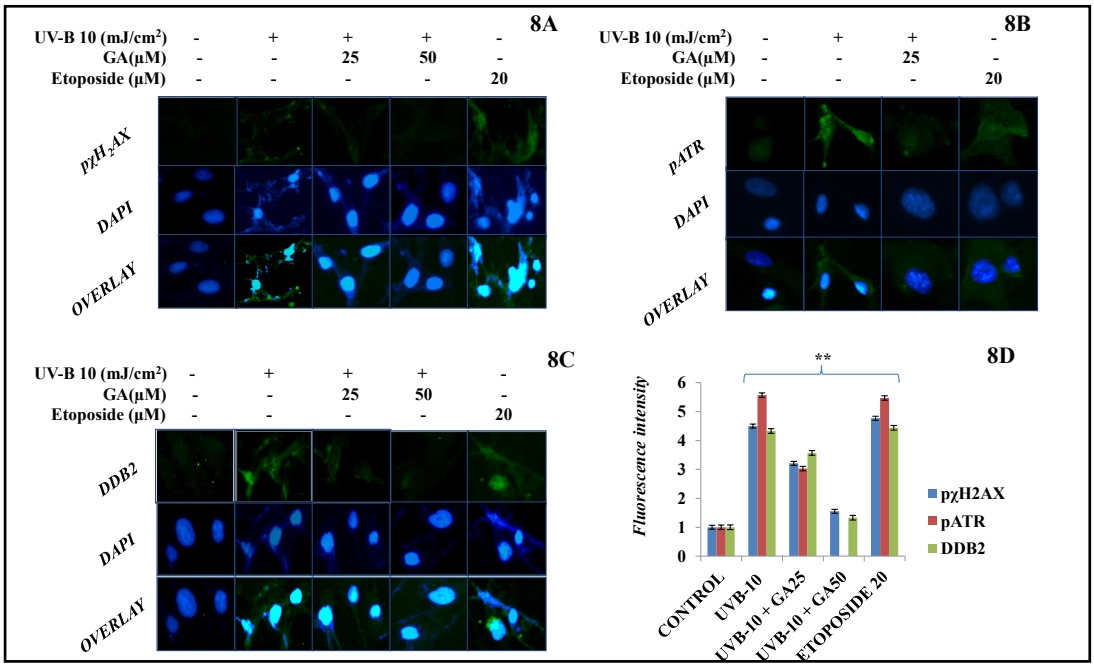

Fig. 8. Immunoflorescence of DNA damage response proteins in 24h post-irradiation of UV-B treated HDFs. Immunofluorescence of χH_2AX (Fig. 8A), pATR (Fig. 8B), & DDB2 (Fig. 8C) showing that GA significantly down-regulated the expression of these marker proteins as compared to UV-B only, indicating that GA protects the HDFs from UV-B -irradiation induced DNA damage.

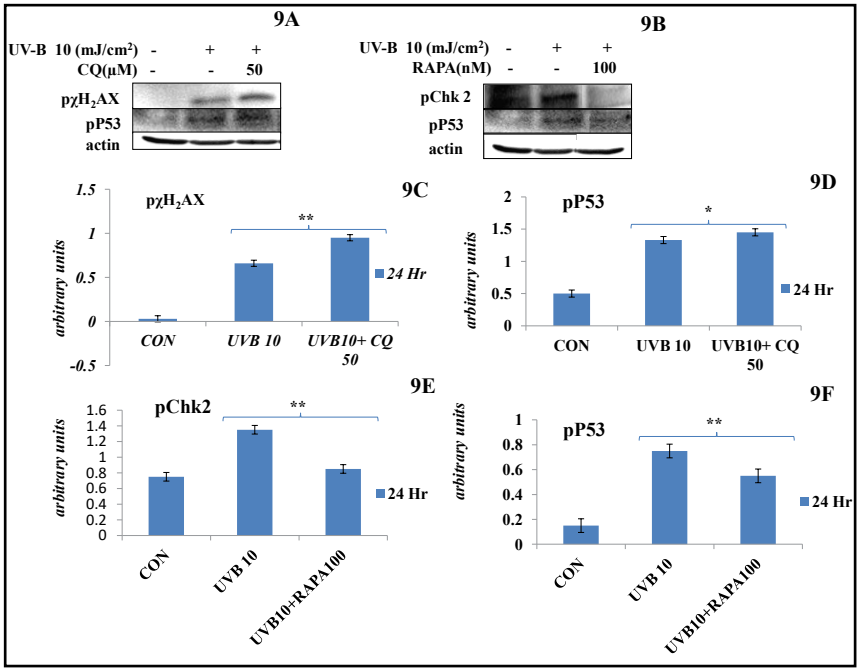

Fig. 9. Chloroquine mediated autophagy inhibition potentiates UV-B induced DNA damage whereas pharmacological activator Rapamycin alleviates UV-B induced DNA damage in primary HDFs. Chloroquine (50 µM) pre-treatment upregulates the expression of DNA damage sensor proteins & potentiates the UV-B induced DNA damage in GA pre-treated HDFs as is evident from western blotting analysis of χH_2AX & pP53 proteins (Fig. 9A, 9C & 9D), *(*p<0.05, **p<0.01* as compared to UV-B only). Rapamycin pre-treatment (100 nM) significantly downregulated the expression of DNA damage sensor protein $p\chi H_2AX$ as well of pP53 protein that acts as guardian of cell (Fig. 9B, 9E & 9F), *(**p<0.01* as compared to UV-B only) suggesting that modulation of UV-B induced autophagy flux is necessary for HDF survival upon UV-B -irradiation as well as for GA to offer protection to HDFs from UV-B induced insult.

Discussion

Various environmental factors such as air pollutants, environmental toxicants, and UV radiation can trigger damages to human skin, leading to premature skin aging & photo-carcinogenesis [14]. Particularly, the role of UV-B in skin cancer has been widely reported because of the increasing incidence of photo damage [15]. Overexposure to UV-B induces severe DNA damage in epidermal cells and cause cytotoxic symptoms [16]. The results of the present study are consistent with these findings. We have found that exposure to UV-B radiation leads to significant cell death of HDFs in an intensity dependent manner (Fig. 1A). In this study, we have investigated the molecular mechanism(s) by which GA, a potent plant-derived anti-oxidant, protects HDFs from UV-B induced cell death. In a therapeutic/ cosmaceutic perspective, it is essential for any agent to have no or negligible cytotoxicity on normal cells. We considered human primary dermal fibroblast cells (HDFs) as a reference model to study the UV-B –induced photo-damage & GA induced autophagy mediated photo-protective effect in HDFs. The predominant reasons underlying photo damage is that UV-B irradiation induces oxidative damage to different organelles, including nucleus [17]. UV-B irradiation causes the accumulation of unfolded proteins in the ER lumen and in response to this disturbance, an adaptive response, unfolded protein response (UPR) gets triggered to restore the ER homeostasis [18]. This adverse impact could damage the vital cellular machinery, including functional cellular molecules as DNA, RNA & proteins [8]. We found that GA protects the HDFs from UV-B mediated induction of oxidative & ER stress responses in a concentration dependent manner in 6 & 24 hr post UV-B -irradiation as was found in western blotting & florescent microscopy analysis (Fig. 2A, 2B, 2C, 2D & 2E). UV-B up regulates the expression of anti-oxidant protein SOD1 whereas down regulates the expression of Catalase. The possible explanation for UV-B mediated upregulation of SOD1 is that UV-B irradiation produces an increase in the expression of copper-zinc superoxide dismutase (Cu/Zn SOD), an enzyme that catalyses the dismutation of superoxide anion radical ($O2^{-}$) to dioxygen (O_2) and hydrogen peroxide (H_2O_2) and downregulates the expression of Catalase, an enzyme that cleaves H_2O_2. This way UV-B induces the expression of SOD1 in HDFs as a defence response against UV-B induced stress [19]. UV-B manifested stress is mostly responsible for DNA damage and leads to the formation of various DNA adducts like CPD's & 6,4 PP's that subsequently destabilize the genomic stability leading to pro-cancerous effects in the cell [20]. However, there are several cellular defense mechanisms that maintain the balance & repair the cellular damage [21]. We found that GA significantly protects the HDFs from UV-B -irradiation induced CPD DNA adducts (Fig. 3A) and also imparts protection from UV-B -irradiation induced DNA fragments by reducing tunnel positive cells in TUNNEL staining of apoptotic nuclei (Fig. 3B & 3C), indicating that GA acts as a strong photo-protectant against UV-B -irradiation induced DNA damage. Our results indicate that GA modulates the UV-B induced autophagy response in HDFs in 3 & 6 h post-UV-B -irradiation by increasing instant autophagic flux (Fig. 4A, 4B, 4C & 4D, 4E & 4F). It has been shown that in case of severe damage, the cell can't repair it beyond a threshold limit & autophagy process is induced by cells under stress to initiate a cascade of cell protection pathways to maintain the genomic integrity of cell [9]. However, if accumulated cellular debris & worn out cellular organelles exceeds a limit, there is a need to annihilate the cell to curb the adverse carcinogenic effect. Hence autophagy acts as a double-edged sword, and its role is context dependent [22]. In the present scenario, our study confirms that autophagy response vanishes (Fig. 5A to 5C, 6A to 6C) as the time period of UV-B irradiation to HDFs is increased (24 h) and there is no sustenance of autophagic flux in UV-B irradiated HDFs treated with GA, indicating that GA as a strong anti-oxidant relieves the cell from instant oxidative stress-mediated induction of ER stress & diverts the cellular signaling mechanisms sustaining autophagy response. It has been earlier reported that UV-B exposure destabilizes AKT/PTEN axis to cope up with the adverse cell death effect [13]. Our findings indicate that GA modulates the AKT/PTEN axis signifying photo-protection against UV-B -irradiation (Fig. 5D & 5E). Our results also indicate that GA protect HDFs against UV-B irradiation induced DNA damage in 6 & 24 h time points

post GA treatment as is clear in western blotting & immunofluorescence experiments (Fig. 7A to 7G, 8A to 8D respectively) and this finely orchestrated protection system perfectly fits the needs of a cell attempting to find a new homeostatic state. Genomic DNA cannot be destroyed or entirely replaced like other cellular molecules. Therefore, its integrity should be maintained and any damage be accurately repaired [23]. Different classes of proteins are implicated in DNA damage response (DDR), among which the protein sensors specifically recognize the damaged lesions of DNA, whereas the mediators and the effectors transduce the signal from the nucleus to the cytosol where several processes are contextually activated in order to better face up to adverse conditions [24]. Cell cycle checkpoints are activated to block proliferation until lesions are repaired. However, if DNA is severely damaged or unrepaired, cells remain quiescent or undergo cell death [25]. Many lines of evidence argue that autophagy can delay apoptotic cell death mechanism upon DNA damage by sustaining the energy demand required to support DNA repair response [26]. Conversely, in cells where DNA is unrepaired, DNA damage -induced autophagy has been reported to contribute to cell death [27]. Our results indicate that GA protects the cells first hand from UV-B -irradiation induced DNA damage, so here autophagy induction may be attributed to play a protective role. It is worth noting that impairment of autophagy results in DNA damage, leading to the assumption that the interplay might be broader and suggesting that many molecular players could exist to bi-univocally link the two processes. In particular, it has been demonstrated that the deficiency of autophagy-related genes lead to DNA damage accumulation and promote tumorigenesis [28]. These previous findings clearly indicate & support our results that autophagy induction by GA in UV-B -irradiated HDFs is a protective one & imparts protection to HDFs against UV-B -induced deleterious cell death effect. Blocking autophagic flux by Chloroquine (50μM) evanesced the GA mediated photo-protection upon UV-B -irradiation to HDFs by up-regulating proteins that signify DNA damage (Fig.9A, 9C & 9D) whereas pharmacological activator Rapamycin (100 nM) augmented the photo-protective role of GA via activating autophagic flux (Fig. 9B, 9E & 9F). Recently, it has been revealed that the interaction between apoptosis and autophagy is attracting more attention [27]. Autophagy, as a cellular digestive process and defined as the second type of programmed cell death, is required for complete cell degradation and researchers observed enhanced autophagy in cells undergoing death, supporting that molecules that induce flux upon UV-B irradiation are protective one [29, 30]. Present results indicate that GA an anti-oxidant molecule potentially modulates cellular response towards UV-B irradiation most notably due to the reduction of oxidative stress-induced DNA damage, modulation of inflammation and regulation of pro-apoptotic and autophagic signaling [7, 11]. Considering the dual effects of GA on ATM-p53 down-regulation and autophagy induction, we suggest that GA has a balancing potential towards stress-induced alterations in mammalian cells. Therefore, it exhibits efficacy in protecting HDFs from UV-B -induced photo-damage and inflammation, possibly by adjusting the adaptation of cells to stresses (Fig. 10).

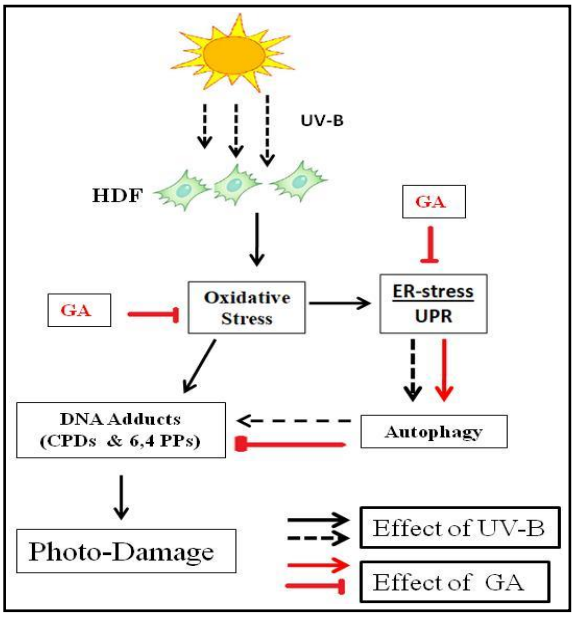

Fig. 10. Molecular mechanism of UV-B induced oxidative stress mediated DNA damage response in HDFs. GA prevents HDFs from oxidative stress mediated DNA damage by improving the cellular autophagy levels.

Conclusion

Above findings provide the first line of evidence that indicate the regulatory and functional role of autophagy to prevent skin photo-damage disorders and support the potential role of GA as promising therapeutic and cosmeceutical molecule in the protection of skin cells from UV-B -irradiation induced cell death effects.

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Disclosure Statement

The authors declare that no conflict of interest exists.

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