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Photo-damage to Keratinocytes by Quinine Photosensitization under mild Ultraviolet-B Exposure

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ABSTRACT

Simultaneous and sufficient exposure to sunlight and drug cause phototoxic reactions in the skin. In the present study, the photosensitizing activity of quinine (Q) was studied. Evidences obtained by reduction of cell viability, mitochondrial damage, DNA damage, ROS generation, lysosome damage and phosphatidyl serine translocation as a marker of apoptosis indicates that exposure of quinine treated keratinocytes to UV-B radiation results in quinine photosensitization and subsequent reactions which alter normal cellular processes. Quinine absorbs strongly in UV-B region (330 nm). It reduced viability of cells significantly as observed through MTT assay. Reduction of cell viability was confirmed through accumulation of Rhodamine 123 dye which showed that quinine and UV-B treated cells accumulate less than control cells confirmed that mitochondrial membrane potential was disturbed. Increased intracellular ROS production was measured through DCF fluorescence. Early apoptosis was confirmed through phosphatidyl serine translocation in the membrane. Increased lysosome damage was observed through acridine orange accumulation in the lysosmes. Single stranded damage was confirmed through comet assay and apoptosis was confirmed through EB/AO staining. These results show that UV-B radiation elicits phototoxic effects in keratinocytes through reactive oxygen species generation.

1) INTRODUCTION

Quinine is used in the treatment of malaria for hundreds of years but, little information is available regarding the interactions of this drug with the cellular components. The importance of quinine has been revitalized and is used in the treatment of severe and complicated malaria. Other than as an antimalarial drug, quinine is also used in rheumatoid arthritis, systemic lupus erythematosus (SLE) and a number of other diseases. Quinine remains bound to alpha amino acid component of the blood plasma. It has long half life and remains in blood stream for longer even if the drug has been discontinued. Quinine has been reported o cause skin irritation [1].

Solar ultraviolet light is subdivided into UV-A (320–400), UV-B (280–320), and UV-C (190–280 nm). UV-C is filtered through stratospheric ozone layer and do not earth's surface. UV-A is more penetrating in skin than UV-B. UV-A has been considered for reactive oxygen mediated alterations in cellular pathways whereas, UV-B reacts directly with various macromolecules including nucleic acids of the cell. Therefore, UV-B irradiation is a major risk factor for induction of skin cancers [2]. The exact mechanism of adverse skin reactions is unknown. Some skin reactions are result of interaction of photo-activated drug with various molecular entities of the cell. UV radiation can induce expression of several proteins involved in oxidative damage to lipids and proteins of cell membrane, and nuclear components.

If UV induced damage is mild, DNA repair enzymes are activated but, if the damage is too severe, apoptosis is induced. If, apoptosis and repair are not induced damages may accumulate in the form of cancer induction [3]. UV radiation-induced damages result in alterations in gene expression, cell-cycle regulation, apoptosis, DNA synthesis and repair.

Photoactivated drugs or their metabolites may act as haptens when bind to proteins and thereby, induce immunological response [4]. Adverse phototoxic effects of photosensitive antimalarial drugs have been recognized as undesirable side effects, and predicting which is gaining importance as new drugs are introduced to the market. Testing of photochemical and photobiological properties can be effective screening method for the prediction of phototoxic/photosensitive potential.

The protection of keratinocytes against UVB damage is controlled through apoptosis with simultaneous growth inhibition, mitochondrial, lysosomal and DNA damage. The

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results of the present study establish apoptosis as the main response in UVB-induced cellular damage which is initiated by generation of reactive oxygen species through indirect mechanism of photosensitization of quinine by UV-B radiation. The purpose of the present study was to confirm whether the drug quinine is phototoxic in human skin cell line under mild UV-B exposure or not. If so, patients under quinine treatment should use sun protection measures.

2) MATERIALS AND METHODS

Chemicals and reagents

Quinine (Q), N,N-dimethyl-p-nitrosoaniline (RNO), superoxide dismutase (SOD), nitro-blue tetrazolium (NBT), fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM F-12 HAM), Hank's balanced salt solution (HBSS), antibiotic and antimycotic solution, trypsin (0.25%), L-histidine, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), carbonate and phosphate buffers, RNase, ethidium bromide (EB), Annexin V, propidium iodide, 2 7-dichlorodihydrofluorescein diacetate (carboxy-H_2 DCFDA), 2'-deoxyguanosine (2'-dGuO), low melting point agarose (LMPA) and normal melting point agarose (NMPA), Rhodamine 123 were procured from Sigma Chemical Co. (St. Louis, MO, USA). MilliQ double-distilled deionized water was used throughout the study. All plastic wares including 96 and 6 well plates, 92 mm Petri plates and 75 and 25 cm^2 (all polystyrene coated) culture flasks were purchased from Nunc (USA).

Cell culture

Human skin keratinocytes (HaCaT) were procured from National Centre for Cell Sciences, Pune, India and sub cultured to maintain in the cell culture facility available at our laboratory. Cell culture medium consisted of DMEM F-12 HAM supplemented with 10% FBS plus 1.5% antibiotic-antimycotic solution. Cells, for all the time were kept in CO_2 incubator (37°C, 5% CO_2 and 95% relative humidity) for proper division and differentiation.

Source and method of radiation exposure

All the UVB exposure was carried out in a temperature controlled radiation chamber (25°C±2°C). The radiation source consisted of UV-R emitting tubes (1.2 m long) manufactured by Vilber Lourmat (France) and a microprocessor-controlled RMX-3W radiometer (Vilber Lourmat) equipped with calibrated UV-R measuring probes to measure the intensity. Cells were sub-cultured and maintained in 25 cm^2 and 75 cm^2 culture flasks. For cell culture experiments 90 mm, 35 mm, 96 and 6 well culture plates were used. During UV exposure, all the samples were kept at minimum distance of 22 cm to avoid thermal effect. The intensity of UVB radiation was measured before each experiment at the level of the cells.

UV-absorption of Q

Solution of Q (25 µM) (20 ml) was exposed to UVB (0.6 mW/cm^2). After irradiation samples were scanned over 200-400 nm wavelength through UV-Vis spectrophotometer (Varian UV-visible- Carry-300).

MTT assay

Cells were seeded in two 96-well plates (2 x 10^4 cells/well), each for dark and UVB then placed in CO_2 incubator for proliferation. After 24 h, the medium was aspirated and cells were washed twice with HBSS. Cells were treated with different concentration of Q (5, 10, 25 µM) and incubated for 30 min at 37°C. Drug treated cells were exposed to UVB (0.6 mW/cm^2) and control samples were kept in dark. Treated cells were analyzed for mitochondrial dehydrogenase activity (MTT assay). A stock of MTT reagent was prepared in HBSS and then diluted with DMEM F-12 HAM medium to a final concentration of 500 µg mL^{-1}. A total of 100 µL MTT reagent was added to each well. The culture plates filled with MTT reagent were incubated at room temperature for a period of 4h. After incubation, the cells were washed with HBSS twice. DMSO was added (100 µL) was added to wells and kept on rocker shaker (NuRS-60, Nulife) for 20 min to dissolve the formazan crystals. The absorbance was read at 570 nm by micro plate reader (Fluostar Omega- BMG Labtech).

Generation of intracellular ROS

Cells were grown in 35 mm Petri plates at 2 x 10^4cells/well. Cells were incubated with non-fluorescent dye carboxy H2-DCFDA (2',7'-dichlorodihydrofluoresceindiacetate; 5µM) for 30 min at 37°C, then treated with Q (10.14 µM), and exposed to UVB (0.6 mW/cm^2). After irradiation, cells were washed twice with HBSS. Fluorescence of DCF in the cells was observed under the fluorescence microscope and photographs were acquired [5].

Phosphatidyl serine translocation

Cells were cultured in 35 mm culture discs. Confluent cells were treated with Q (10.14 µM), and exposed to UVB (0.6 mW/cm^2). HBSS was aspirated and fresh medium was for 2h. After incubation, DMEM was removed, 30µl annexin V (5µl stock was diluted in 1X binding buffer provided) was added and kept for exactly 10 min. Cells were washed with HBSS twice. 10µl of PI was added and incubated for next 15 min. Cells were washed with HBSS twice and observed under fluorescence microscope and photographed for PS translocation [6].

EB-AO staining

Cells were cultured in 35 mm culture discs and treated with Q (10.14 µM) and exposure to UVB (0.6 mW/cm^2). Dark control samples were also prepared parallel to experimental sets. HBSS was replaced by culture medium and cells were incubated for 2 h. After incubation, the cells were washed with HBSS twice. Cells were stained with EB/AO (mixture of EB and AO 100 µg mL^{-1} prepared in HBSS), incubated for 15 min at RT and observed under fluorescence microscope and photographed at 40X magnification [5].

Photodegradation of 2'-dGuanosine

Photo-oxidative degradation of 2'-dGuO by Q under UVB was measured. Carbonate buffer (0.01M, pH 10.0) containing 2'-dGuO and Q (10.14 µM) was irradiated under UVB (0.6 mW/cm^2). Absorbance of samples was monitored spectrophotometrically at 260 nm.

Comet assay

Alkaline SCGE assay was performed to confirm the single stranded DNA damage by Q. Cells were treated with Q (10.14 µM) under UVB (0.6 mW/cm^2), and incubated for 2h in fresh culture medium. Cells were scored for DNA damage by an image analysis system (Komet-5.0, Kinetic Imaging, Liverpool U.K.) connected to fluorescence microscope (DMLB, Leica, Germany) [7].

Lysosomal damage

Cells were treated with Q (10.14 µM) and irradiated under (0.6 mW/cm^2) for 30 min. After treatment, the cells were stained with Acridine Orange (10 µgmL^{-1}), and incubated for

15 min at RT. Cells were observed for lysosomal damage under fluorescence microscope.

Mitochondrial trans-membrane potential

Mitochondria membrane potential was measured by Rhodamine 123 fluorescence. Cells were treated with Q (10.14 µM) and irradiated under (0.6 mW/cm^2) for 30 min. Cells were incubated in fresh culture medium for 2 hr. Rhodamine 123 (5 µM) prepared in HBSS was added to the cells and kept for 10 min at RT. The cells were washed and analyzed under fluorescence microscope for mitochondrial membrane potential [8].

3) RESULTS

Absorbance spectra of Q

Absorbance spectra of Q showed two peaks (λ_{max}) at 279 and 330 nm, respectively, which confirmed that Q absorbs UVB radiation.

Cell viability

Count of cells after drug and UV treatment was calculated as percent cell viability. Significant reduction in viable cells was observed as concentration increased from 5 to 25 µM. Lethal dose (LD_{50}) was calculated from the data obtained, which came to be 10.14 µM. For all the post experiments, value of LD_{50} was used as the main concentration. Chlorpromazine (5 µg/ml) and L-Histidine (50 µg/ml) were used as positive and negative controls, respectively.

Mitochondrial membrane potential

Figure 3 shows mitochondrial membrane damage in the form of decreased red fluorescence of Rhod 123 dye. Q treated cells showed a marked decrease in fluorescence as compared to dark and UVB control samples because damaged mitochondria did not accumulate dye as compared to control which contain intact mitochondria.

Intracellular determination

Intracellular ROS determination has been depicted in figure 4. It is measured in the form of increased green fluorescence of DCF dye. In dark control cells fluorescence was almost non-observable but, Q treated cells have shown a significant increase in green fluorescence. Whereas, in UVB treated cells, fluorescence was less as compared to Q treated cell.

PS translocation

Figure 5 shows early apoptosis marker in the form of translocation of phosphatidyl serine from inner surface to outer surface of the membrane. Dark control cells did not show any fluorescence but, Q and UVB treated cells have shown annexin V (green) and EB (red).

Lysosome damage

Figure 6 shows lysosome membrane damage in the form of accumulation of AO dye. AO gives red fluorescence in control cells but, as lysosomes get damage by Q, AO comes out in the cytosol. In Q treated cells, increased green but, decreased red fluorescence was observed.

DNA damage

Single stranded DNA damage was observed in the form of comet. In dark control sample, no DNA damage was observed whereas, in Q treated sample, significant single stranded damage was observed. UVB treated cells showed only slight damage in DNA. The images were observed as comet in Q treated cells. Concentration dependent damage to 2'-dGuo was observed. Higher concentration (25 µM) showed more damage as compared to lower.

AO/EB staining

Figure 8 shows apoptosis induction measured through AO/EB staining. Dark control cells stain only AO which gives cells green appearance. In UVB exposed cells, green fluorescence with traces of red fluorescence (EB) was observed. Whereas, Q treated cells stain with both, AO and EB which gives cells orange-green appearance. EB binds with DNA when enters in the cell interior due to membrane damage. AO can enter the cell passively which gives green color to control cell.

4) DISCUSSION

In this study, we generated a qualitative data for human skin keratinocytes in response to quinine treatment under normal UV-B exposure to examine phototoxic potential of quinine. Our data revealed that quinine is phototsensitized by UV-B exposure at normal levels and subsequently resulting into ROS generation which alter normal cellular events leading into prevention of cells through apoptosis. We observed that quinine photosensitized with UV-B radiation to reduce the viability of cells after damage to vital organelles, DNA and induction of apoptosis. This effect was most pronounced at higher concentrations than control sets, and has potential implications for understanding the response of keratinocytes to photo-induced DNA damage, and the response of photosensitized quinine to cellular processes. There have been some reports on photosensitized drug induced genototoxicity and alterations in signaling pathways in various cell lines but the effects are mainly result of reactive oxygen generation through Type I and Type II photosensitization mechanisms and their reaction to macromolecules [9]. Our data however, specify that UV-B photosensitized quinine can induce membrane damage including cell membrane; membrane bound organelles and nuclear membrane together with DNA and associated molecules. The exact mechanism of cell survival during drug photosenstitization remains unclear, but since quinine is found to almost every part of the cellular compartments; it is obvious that its photosensitization by UV-B radiation may alter important functions of the cell which may initiate apoptotic pathways. Depending on these observations, we observed that UV-B photosensitization caused cells to undergo death through apoptosis in quinine treated cells rather than dark control and UVB control cells. Treatment of cells with UV-B photosensitized quinine resulted in more than 50% reduction in viability of the cells indicating that higher concentrations may be even more toxic. The reduction in cells of co-treated with quinine and UV-B may therefore, may at least partially explain the dramatically increased apoptotic cell death than control. Interestingly, photosensitized quinine induced single stranded DNA damage in keratinocytes at LD_{50} (10.14 µM). Although, keratinocytes were used to assess the single stranded DNA damage, photosensitized quinine also induced damage in 2'-dGuo which is used as a model for DNA damage *in vitro*. There are reports indicating photosensitized drug induced single stranded DNA damage at higher doses [10]. These findings suggest that quinine induced DNA damage is increased when it is photosensitized with UV-B radiation which may be a possible cause for apoptosis induction.

Signs of both early and late apoptosis were observed following treatment of cells with quinine and UV-B. These events eventually resulted in reduction of viable cells and survival of cells from oxidative stress caused by ROS generation. Early

apoptosis was marked through phosphatidyl serine translocation from inner to outer surface of the cell membrane. Probably, ROS generated intracellularly, attack on energy rich species of the cell membrane which made phosphatidyl serine to translocation. Another important feature observed was chromatin condensation and nuclear blebbing which established that early apoptotic events continued towards complete apoptotic event which ended cells into death. Photo-activated quinine damaged not only to mitochondria but also to lysosomes. Lysosomes are important organelles in controlling drug induced changes in the cytosol [11]. Damage to these vital organelles allowed the drug to remain in the cytosol to react with various macromolecules rather than to expel it to outside through engulfment. All these different events may be dependent or independent of each other which remain to be further elucidated.

5) CONCLUSION

In conclusion, our study expands these findings by demonstrating that UV-B radiation photosensitizes quinine even when it is inside the cellular environment which generated ROS. These reactive oxygen species exert effect by attacking membrane components and DNA which ultimately follow the path for apoptosis for survival and to combat the phototoxic effects of photosensitized quinine. UV-B mediated photosensitization effects are the result of indirect photosensitization of quinine rather than direct mechanism.

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Conflict of interest: The authors declare that there are no conflicts of interest.

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FIGURES

Figure 1: Absorption spectra of quinine scanned from 200-400 nm wavelength range.

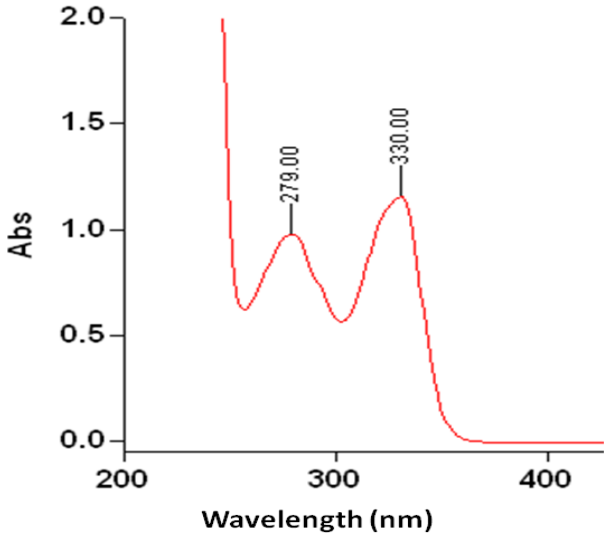


Figure 2: Percent cell viability of cells after quinine and UV-B exposure compared with dark control sets. CPZ and L-His has been used as positive and negative controls, respectively.

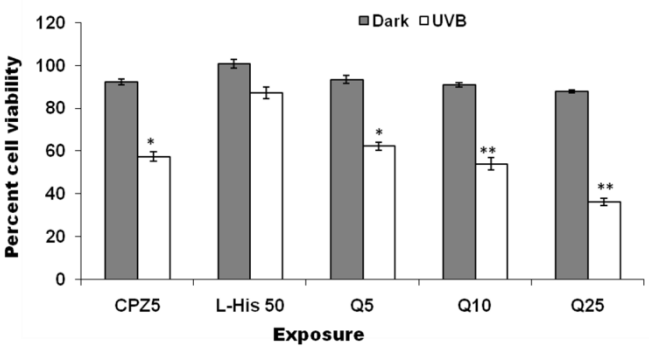


Figure 3: Mitochondrial membrane potential after co-exposure of quinine and UV-B radiation.

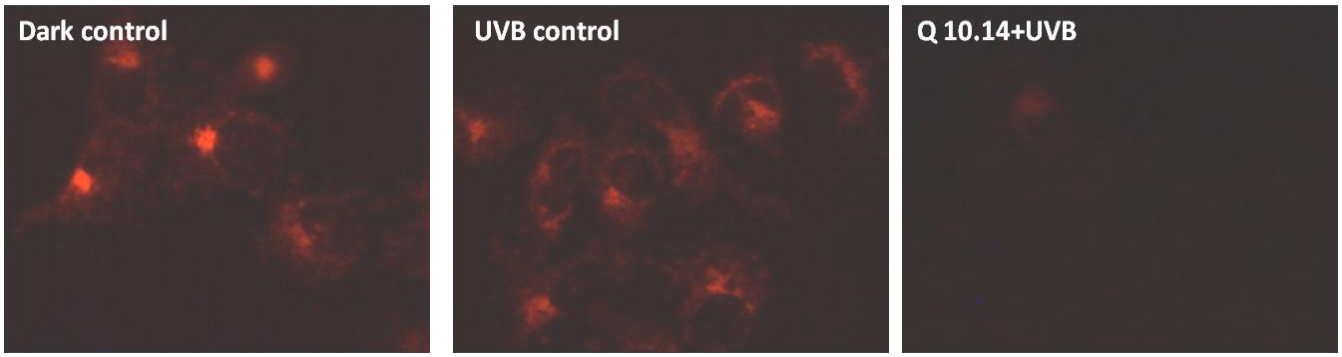


Figure 4: Intracellular ROS generation measured through DCF fluorescence.

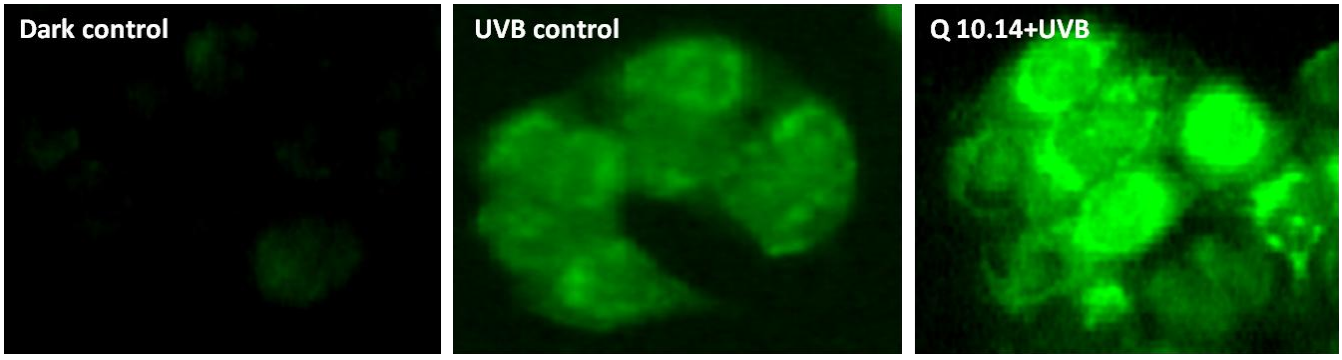


Figure 5: Phosphatidyl serine translocation after quinine and UV-B exposure.

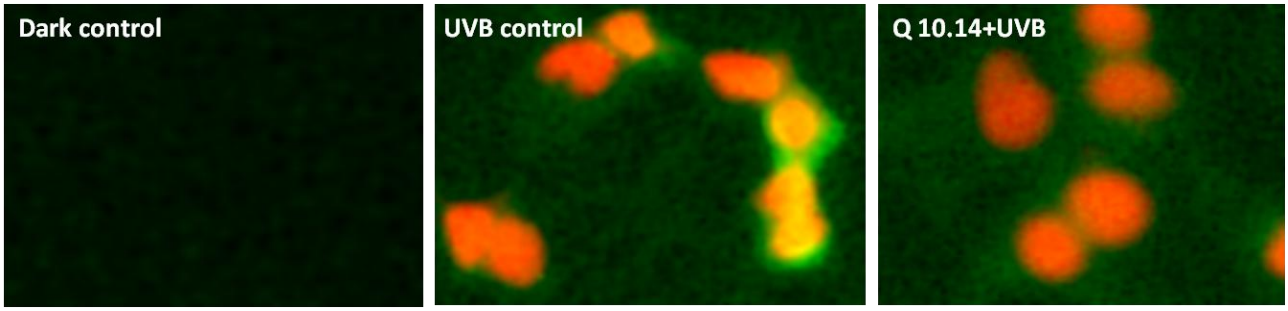


Figure 6: Lysososmal membrane damage measured through accumulation of AO.

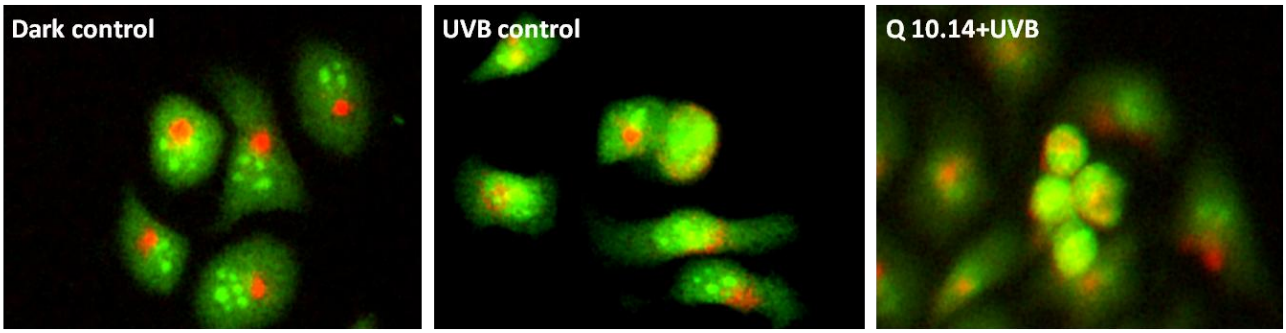


Figure 7: Single stranded DNA damage (comet assay) and 2'd-Guo photodegradation .

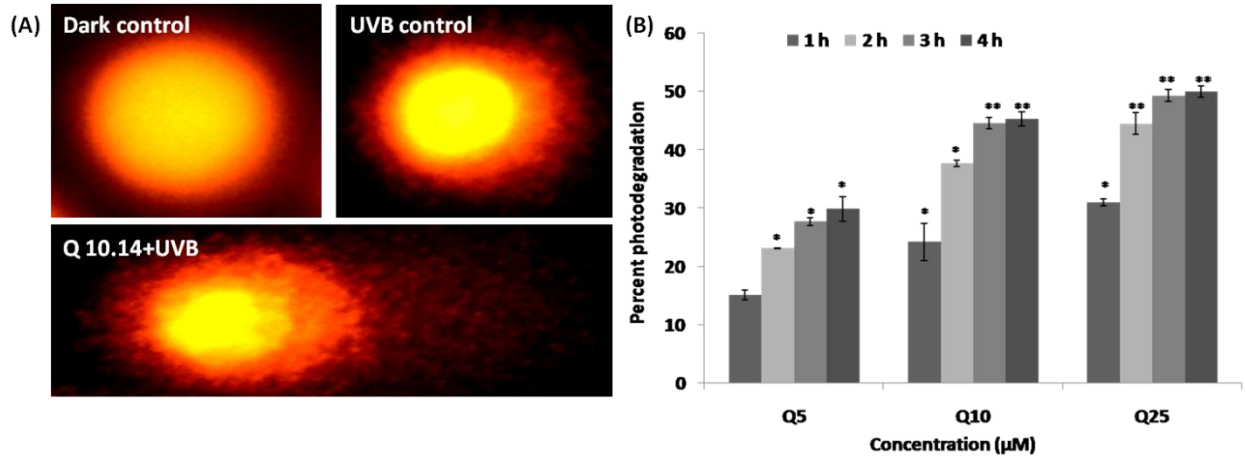


Figure 8: EB/AO staining for the measurement of apoptosis in keratinocytes after quinine and UV-B exposure.

