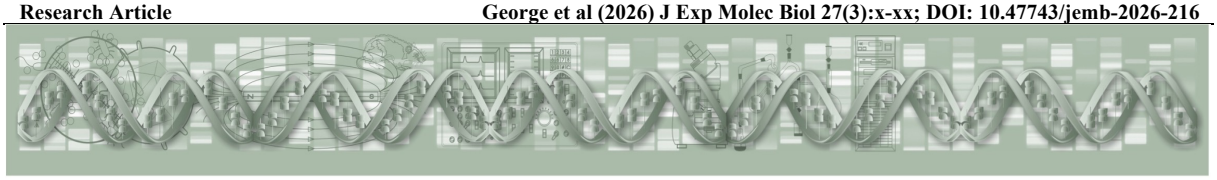

SENOLYTIC EFFECTS OF BETAINE AMELIORATES DNA DAMAGES AND PHOTOAGEING IN FIBROBLAST CELLS- AN INVITRO STUDY

Angel Reena George[1], Anchu Anil Reeja[1], Nithin Vijayakumar[2], Renjini Ambika Padmanabhan[2], Rajesh Ramachandran[2*]

[1]Centre for Professional and Advanced Studies, Kottayam, Kerala
[2]Center for Research on Molecular and Applied Sciences, Thiruvananthapuram, Kerala, India-695006
Corresponding author*
Rajesh Ramachandran- info@crmas.in

Abstract

Ageing is the sequential deterioration of biological functions indispensable for persistence and reproduction. When deterioration reigns above synthesis, the organism ages; UV-induced photoageing is one of the causes of DNA damages leading to premature ageing and there is increased demand for use of phytochemicals as protective agents as senescence inhibitors. Several phytochemicals are being under active research for senolytic effects. In the present study, we focussed to identify the senolytic effects of betaine in UV induced experimental ageing models. L929 cells were subjected to UV treatment to mimic photoageing and the cells were treated with varying concentrations of betaine from 6.25 to 100 µg.mL-1. The impact of betaine on UV exposed cells was determined by reactive oxygen species (ROS) generation using 2'-7'-dichlorodihydrofluorescein diacetate (DCFDA) staining. Comet assay and galactosidase staining was done to analyse the extent of DNA damages and senescence respectively. Betaine-associated protection against UV photoaged L929 cells was evident with concomitant increase in cell viability and significant restoration of cellular morphology. With UV exposure, ROS generation was found to be increased and was reduced in betaine co-treated groups. Betaine significantly reduced UV induced DNA damage as evident from decreased tail length in cotreated groups compared to the increased tail length in UV irradiated groups. X gal staining confirmed the senolytic effects of betaine. The experimental results validate that betaine can curb both oxidative and DNA damages triggered by UV rays proving the potential anti-ageing properties as well as its prospective uses in therapeutic and cosmetic settings.

Keywords: Ultraviolet rays, Photoageing, Betaine, Reactive oxygen species, DNA damage, Comet Assay

Introduction

A combination of unique clinical, histopathological and functional cutaneous changes brought on by prolonged sun exposure is known as photoageing (Berneburg, M et al 2000). The production of reactive oxygen species (ROS) and genetic alterations are the main harmful consequences of UV radiation on skin. As one of the many forms of solar radiation, UVA often causes the skin to become over exposed to ROS, which can change the structure and functions of genes and proteins. Deep wrinkles, skin laxity and hyperpigmentation all of which are

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indicative of photoaged skin are the end result of this process, which also causes the dermis to lose some of its collagen and elastic fibers(Gu, Y et al. 2020).

UV radiation triggers the skin to produce excessive ROS, thereby activating the NF-KB and MAPK signaling pathways (Chen, T et al. 2016). These two pathways aid in the activation of NF-KB and activator protein-1 [AP-1], which in turn controls the transcription of MMPs, with a focus on upregulating MMP1, MMP3 and MMP9 (Fisher, GJ et al. 1996). This mechanism causes an increase in TNFα and MMP expression, which accelerates the ageing process of the skin and degrades the extracellular matrix. The cGas-STING pathway and NF-KB are activated in a similar way by UV radiation, leading to cytoplasmic DNA induction and consequent skin photodamage (Skopelja-Gardner, S et al. 2020). To conclude, the ageing process is accelerated by UV radiation-induced DNA damage, genomic instability, oxidative stress and extracellular matrix degradation (Tang et al. 2024).

In accordance with recent findings, it is reported that natural products and phytochemicals are preferred for longevity and anti-ageing effects over pharmacological agents but need further scientific studies to identify potent senolytic compounds(Huang Y et al. 2016). Scientific research on these compounds has expanded over the past decade. Phytochemicals, found in plants, play a crucial role in growth and development (Süntar, I., & Yakıncı, Ö. F. (2020)), and have been shown due to their antioxidant and anti-inflammatory properties (Zhao, G et al. 2018; Abruzzo, PM 2020). Trimethylglycine or betaine is a stable nontoxic natural chemical found in plants, animals and microorganisms; it is one of the natural products. Although betaine was originally discovered in beets *(Beta vulgaris)* in the 19th century presence of Betaine is already reported in other plants also (Craig, SA. 2004; Cholewa JM et al. 2018). Recent studies suggest that betaine supplementation may improve endurance, reduce fatigue and enhance muscle power (Arumugam MK et al. 2020). It supports liver function, protects against liver damage and has anti-inflammatory and antioxidant properties (Ueland, PM. 2011; Heidari, R et al. 2018). Reports suggest the role of betaine in LKB1-AMPK activation inducing autophagy in human epidermal keratinocyte thereby inhibiting UV mediated senescence (Kim, K et al. 2018). Also betaine protects testicular and ovarian cells from hyperglycemia induced senescence which helps in the management of infertility (Mohammadi, N et al. 2024). Eventhough most of the studies has confirmed the antioxidant and cytoprotective effects of Trimethylglycine, the senolytic effects and anti photoageing affects needs to be studied in details and in this context the present study investigates the cytoprotective and genotoxicity of betaine with emphasis on the ability of betaine to reduce UV-induced DNA damage in fibroblast cells.

MATERIALS AND METHODS

Chemicals

Betaine was sourced from Sigma-Aldrich (Cas No: 107-47-3), while all other chemicals were acquired from Invitrogen, Sigma-Aldrich, and HiMedia.

Cell lines

The murine fibroblast cells, L929, were acquired from National Centre for cell science (NCCS) in Pune, India and it cultured in Dulbecco's modified Eagle's medium (DMEM) from Sigma-Aldrich, USA. The medium is enriched with FBS, penicillin, and streptomycin. They were trypsinized using 0.25% and 0.5mM EDTA, then sub-culturing. The cells were then seeded in a 96-well tissue culture plate and maintained at 37°C. The process involved rinsing with PBS, adding trypsin, collecting cells and seeding cells at $5x10^3$ cells per well followed by incubating plate in a humidified 5% CO_2 incubator.

Induction of in vitro UV toxicity and betaine co-administration

L929 cells were exposed to UV light for 30 minutes as per the protocol of Sunil Kumar et al (Sunilkumar et al. 2024); the media was removed and replaced with complete medium and varying concentration of betaine. The cells were then incubated at 37^0C, 5% CO_2 for 24hours.

SRB assay for cell fixation and staining

SRB assay was done as per protocol mentioned earlier (Nikhitha, JN et al 2021). For cell fixation and staining, 10% TCA was added to a well plate and incubated at 4°C for 1 hour. The plates were then washed, dried, and then 0.057% SRB solution was added and incubated for 30minutes to stain the cells. The SRB was removed and rinsed with 1% acetic acid, followed by addition of 200 µL Tris base solution. The plates were shaken for 5 minutes to solubilize the protein-bound dye, and OD was measured using a microplate reader at 510nm

ANTIOXIDANT ASSAY

Preparation of cell lysate

Cells were treated with Trypsin-EDTA solution, collected, and centrifuged. The supernatant was discarded, and the pellet was suspended in lysis buffer (0.1M Tris, 0.2M EDTA, 2M NaCl and 0.5% Triton)(Padmanabhan, RA et al. 2011). The samples were incubated at 4ºC for 20 minutes, and the resulting cell lysate was used for study.

Estimation of SOD

SOD assay was performed as per the protocol of Mishra and Fridovich (Mishra and Fridovich 1977). Briefly, in 50 µL of cell lysate, 84 µM potassium ferric cyanide, 45 µM methionine, 5.3 mM riboflavin, and, phosphate buffer (pH- 7.8) was added. After that, the tubes were incubated for 10 minutes at 25^0C, and the absorbance was measured at 600 nm using spectrophotometer.

Estimation of catalase activity

Catalase was analysed as per the protocol of Ogata and Takahara (OGATA, M., & TAKAHARA, S. 1962). The enzyme reaction was initiated with 1.2 ml of 0.01Mm phosphate buffer (pH 7.0) and 0.5 ml of cell lysate, followed by 0.1 ml of 0.2 mM H_2O_2. The absorbance decrease was measured at 240 nm and the enzyme blank was run simultaneously with 0.1ml distilled water instead of H_2O_2.

Determination of ROS by DCFDA staining method

L929 cells were seeded onto 96-well plates and incubated for 24 hours at 37°C. The cells were then washed with PBS and treated with 100 µM DCF-DA for 1 hour at 37°C. The resulting fluorescence was imaged on an Olympus CKX41 camera connected to an Optika Pro5 camera, and quantified using a Qubit™ 3 Fluorometer. UV-exposed cells were used as a positive control.

Comet assay

Comet assay was carried out according to the method described earlier(Ramachandran, R., & Saraswathi,M.2017). Cells were cultured in a 6-well plate, treated with a protective concentration of sample, and incubated overnight. They were then trypsinized and washed with fresh media before being used for a comet assay. Microscope slides were precoated with 0.7% normal melting agarose and stored at 4°C. After each step, slides were incubated at 4°C for 10 minutes to allow agarose to set. Slides were then placed in a cold lysing solution containing 12.5 mM NaCl, 10mM Na2 -EDTA, 10 mM tris base pH 10, and 1% SDS. After lysis, slides were placed in an electrophoresis buffer for 20 minutes to allow DNA unwinding. Electrophoresis was conducted in the same buffer by applying an electric current for 20 minutes. Slides were washed in neutralization buffer three times for 5 minutes, dried, and stained with 50 µl Ethidium Bromide. The slides were photographed using an inverted Epifluroscent microscope and scored using Tritek comet scoring software.

X-gal assay

X-gal assay to determine the extent of senescence was done according to protocol of Sunilkumar et al (Sunilkumar et al. 2024). After washing the cells twice in phosphate buffered

saline (PBS), they were fixed for three to five minutes at room temperature using a solution of 2% formaldehyde and 0.2% glutaraldehyde in PBS. After washing the fixed cells in PBS twice, 1 mg/mL X-gal was added to the solution along with 2 mM MgCl2, 150 mM NaCl, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 40 mM citric acid, and sodium phosphate buffer at pH 6.0. The cells were stained for overnight at 37˚C and dried after being washed twice with water. Subsequently, the cells were examined using an Olympus CKX41 inverted phase contrast tissue culture microscope equipped with an Optika Pro5 CCD camera. The microscopic observations were captured as photographs.

RESULTS

Identification of novel senolytic compounds can in turn prevent premature ageing and associated degenerative diseases and our study was focused on assessing the effect of betaine on experimental photoageing induced by UV radiation in fibroblast cells in vitro.

In Vitro Cytoprotective Effect Of Betaine On Cultured L929 Cells Exposed To Uv Using Srb Assay. Using the SRB assay, betaine's protective effects on L929 cells were evaluated after they were subjected to UV radiation. Cell viability was significantly reduced to 48.59% by UV radiation exposure alone. Nevertheless, the viability of the cells significantly increased to 85.87% when they were treated with betaine and UV simultaneously. According to this observation, betaine improved cell viability under UV exposure conditions by 37%, pointing to possible cytoprotective qualities of the substance (figure 1A). Furthermore, an epi-fluorescent microscope (Olympus CKX41) fitted with a Pro5 CCD camera was used to observe morphological alterations brought about by different concentrations of betaine, as shown in the figure 1B. Here after the protective concentration of 100µg/mL of betaine was further used for all the other studies.

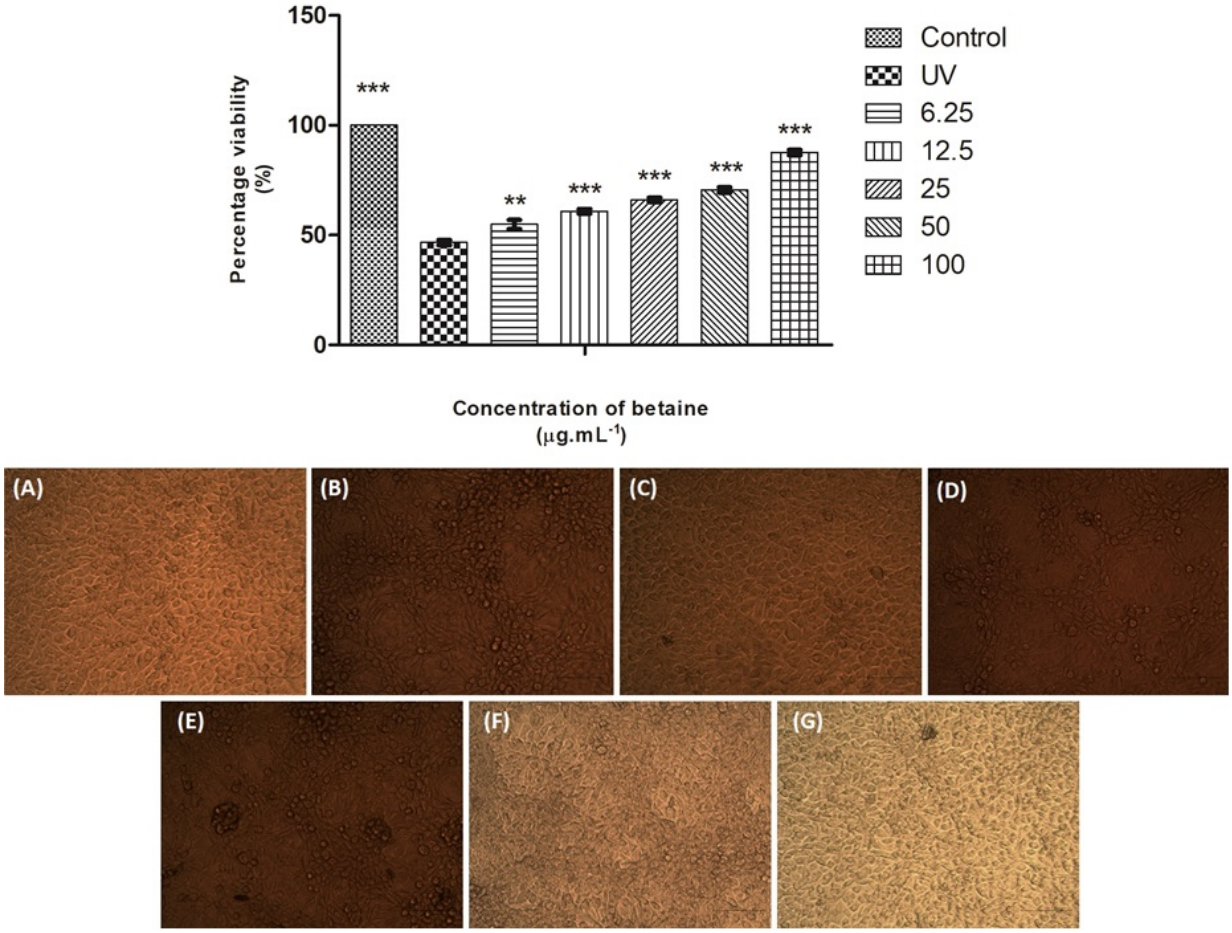

Figure1: ***In vitro*** **cytoprotective effect of betaine on L929 cells exposed with UV**- (1A) Graphical representation depicts the *in vitro* cytoprotective effect of betaine in terms of percentage viability (%) on L929 cells exposed with UV, (1B) - Photomicrographs depicting the effect of betaine co-administration on cultured L929 cells exposed to UV (A) Untreated control cells (B) UV exposed cells and UV exposed cells co-administrated with varied concentration of betaine (C) 6.25 (D) 12.5 (E) 25 (F) 50 and (G) 100 µg/mL

EFFECT OF BETAINE CO-ADMINISTRATION ON ANTIOXIDANT MARKERS

SOD activity

Research has indicated that the consumption of betaine supplements has the potential to enhance the activity of Superoxide Dismutase (SOD), an essential enzyme within the antioxidant defence system and on the contrary UV irradiation is reported to deplete the cellular antioxidant mechanisms. Superoxide radicals are extremely reactive molecules that have the potential to cause oxidative stress and cell damage. SOD aids in their neutralisation as can be seen from the results, L929 cell lines exposed to UV radiation had much lower SOD activity (0.834 Enzyme units) than untreated control cells (1.539 Enzyme units). When betaine was co-administered with UV-exposed cells, however, SOD activity was noticeably higher (1.44 enzyme units), indicating that betaine had increased cellular antioxidant activity (figure 2A).

Estimation of Catalase

L929 cells exposed to UV light both alone and in conjunction with betaine had their catalase activity measured. When compared to untreated control cells, the results show that UV exposure significantly reduced catalase activity. On the other hand, catalase activity was significantly elevated when betaine was added in addition to UV exposure (figure 2B).

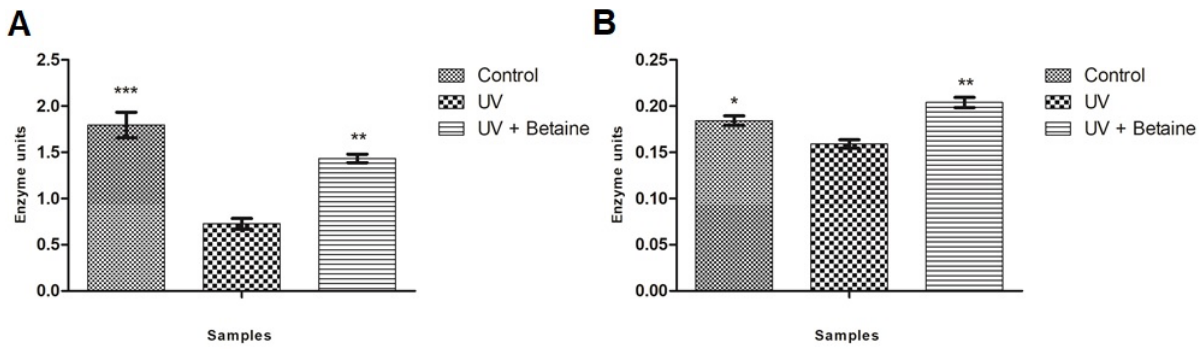

Figure 2 A: Graphical representation depicting the A. SOD level B: Catalase level in terms of enzyme units on the betaine co-administration

DETERMINATION OF ROS GENERATION IN UV EXPOSED L929 CELLS CO-TREATED WITH BETAINE

The effects of betaine on intracellular reactive oxygen species (ROS) levels and the formation of ROS in cells exposed to UV light were investigated through experiments. The amount of ROS was measured and expressed as an intensity of fluorescence (AU). The maximum fluorescence intensity of the UV-exposed cells was 37023.02 AU, suggesting a strong generation of reactive oxygen species. Nevertheless, there was a noticeable drop in fluorescence intensity—which registered at 10187.14 AU—when cells were exposed to both UV light and betaine. This decrease in fluorescence intensity points to a decrease in ROS generation in betaine-treated cells, highlighting betaine's potential as a preventive measure against ROS-induced damage initiated by UV-induced cytotoxicity (Figure 3A & B).

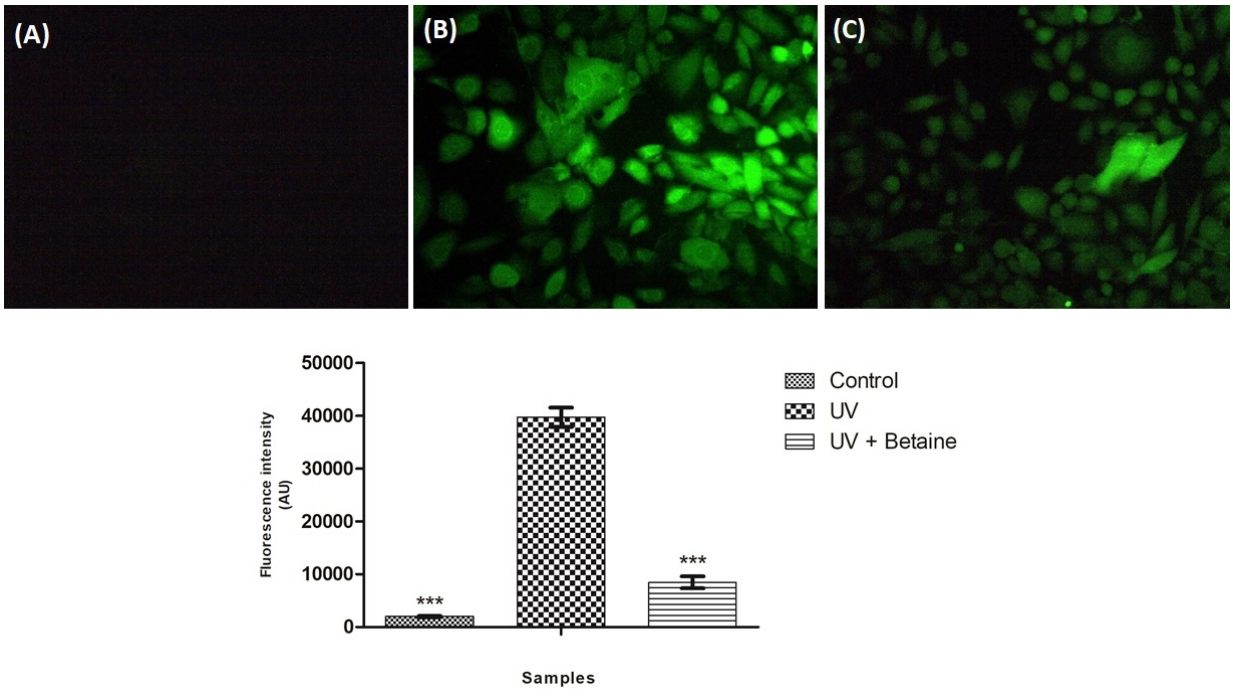

Figure 3 A: Determination of ROS generation by DCFDA staining method; (A) Untreated control cells (B) UV exposed cells (C) UV exposed cells co-treated with betaine B: Graphical representation depicting the generation of ROS on the betaine co-treatment on UV exposed L929 cells

COMET ASSAY

Results showed UV-exposed cells had longer comet tails, indicating more DNA damage which is in accordance with previous findings(Patton WP et al 1999).

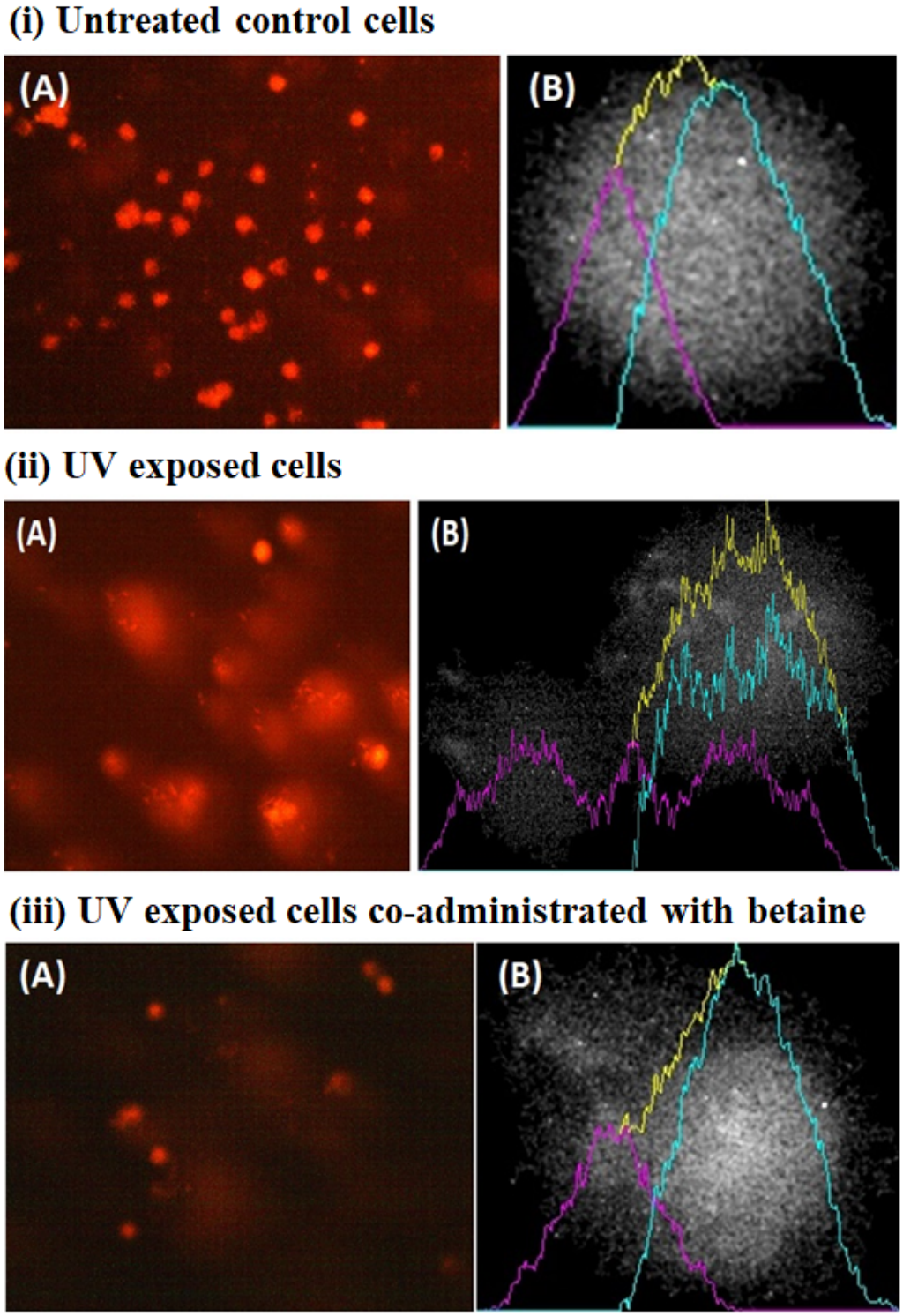

Figure 4: Comet Assay for the determination of genotoxicity; (i) untreated control **(A)** representative fluorescent image **(B)** representative scored image of a selected cell(ii) UV exposed **(A)** representative fluorescent image: **(B)** representative scored image of a selected cell (iii) UV exposed co-administered with betaine **(A)** representative fluorescent image: **(B)** representative scored image of a selected cell

However, when cells were treated with betaine, metrics like comet length (figure 5A), tail length (figure 5B), and olive moment (figure 5C) were decreased. The results suggest that betaine provides a positive protective effect against UV-induced DNA damage (figure 4).

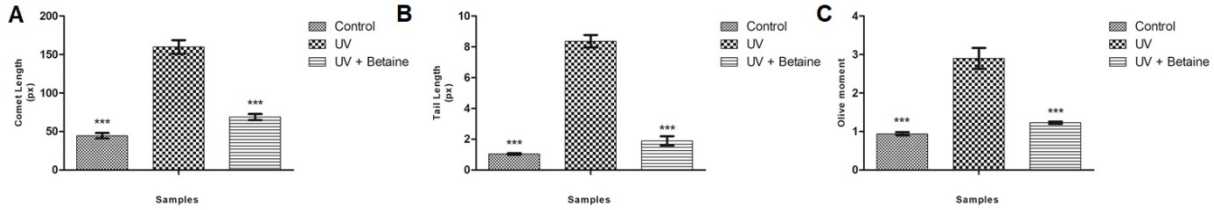

Figure 5 Graphical representation illustrating **A:** comet length **B:** tail length C: Olive moment

X-GAL STAINING

Cellular senescence is indicated by Senescence-Associated β-galactosidase (SA-β-gal), which is usually measured at pH 6. Lysosomal compartments and other low pH conditions are where it exhibits its highest action. Using a chromogenic substrate like as X-gal, researchers assess SA-β-gal activity using in situ staining techniques. Cultured L929 cells exhibit a blue-stained senescent phenotype upon exposure to UV light, suggesting that UV exposure triggers senescence (figure 6). Nevertheless, the number of these blue-stained, senescent cells drops when betaine is also given UV radiation, indicating that betaine may have a preventive impact on UV-induced senescence in L929 cells (Figure 6).

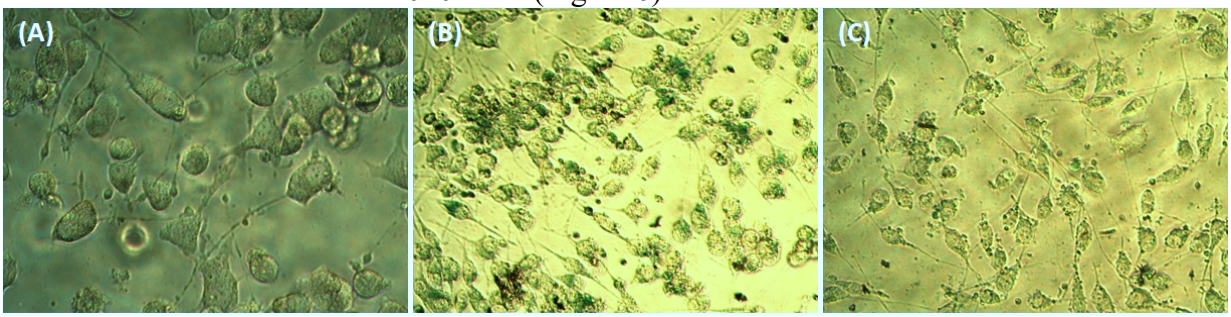

Figure 6: X-gal staining; (A) untreated control cells **(B)** L929 cells exposed to UV radiation **(C)** L929 cells exposed to UV radiation co-administrated with betaine.

DISCUSSION

Our study strives to explore the possible senolytic effects of betaine in lowering UV-induced DNA damage and avoiding cellular degradation in L929 cells, which opens pathways for revolutionary therapeutic treatments in degenerative pathologies.

A naturally occurring substance present in microbes, marine life, and plants is called betaine, often referred to as trimethylglycine. In times of osmotic stress, it is an essential osmolyte that aids cells in preserving fluid equilibrium (Zeisel, SH., & Da Costa, KA 2009). Along with helping to methylate DNA and synthesise neurotransmitters (Lever, M et al. 2005), it also serves as a methyl donor in the methylation cycle. Homocysteine levels are a risk factor for cardiovascular illnesses, and betaine may help lower them (Arumugam MK et al. 2020). Moreover, by promoting cholesterol metabolism and lessening hepatic steatosis, betaine may benefit liver health. Supplementary research on betaine is warranted due to its various physiological roles and possible health advantages but least studied for its potential effects on photoageing and associated changes.

The L929 cell line was chosen for its anti-ageing properties. The cells were exposed to different concentrations of betaine and UV radiation to assess its protective effects. Treatment with 100 µg/ml of betaine significantly improved cell viability by 37%. However, UV radiation without

betaine treatment reduced viability by 48.59%, accompanied by visible cell morphology changes, suggesting potential apoptosis or cell death.

The study found that endogenous antioxidants, including catalase and SOD, play a crucial role in the body's defense against UV radiation's harmful effects on fibroblast cells. The levels of these antioxidants increased significantly in groups co-treated with betaine compared to those exposed solely to UV radiation.

Intraoperative fibroblast pathophysiology is significantly influenced by reactive oxygen species (ROS), which are produced at higher levels when exposed to UV light. Our data unmistakably show that cells exposed to UV light produce more ROS. Nevertheless, as compared to untreated control cells, co-treatment with betaine reduced the production of ROS which can be attributed to the free radical scavenging potential of betaine. Significant cryoprotection and enhanced cell viability resulted from this reduction in ROS production. It was already reported that betaine can attenuate expression of age related NF kappaB activities induced by oxidative stress thereby preventing ageing and inflammatory activities (Go, EK et al. 2005)

Senescence progresses more quickly when DNA damage occurs. We used single cell gel electrophoresis, an alternative name for the comet test, to assess this damage. Utilising Tritek Comet scoring software, DNA damage is evaluated by staining with ethidium bromide and analysis. The comet tail's notable extension following UV radiation exposure, which suggests more DNA damage, is proven by our findings. That comet tail length, however, significantly decreased by 50% after betaine was added, indicating that it had a protective effect against UV-induced DNA damage. Recent reports suggest the oral administration of betaine to reduce UVB-induced skin damage promise its use in cosmetic applications (Im, AR et al. 2016).

When fibroblast cells undergo replicative senescence, there is a rise in detectable beta-galactosidase (beta-gal) activity at pH 6.0, as determined by cytochemical techniques. For assessing cellular senescence in both in vitro and in vivo settings, this increase in activity is a reliable indicator. In order to identify senescence and assess the degree to which betaine attenuates UV-induced senescence, we utilised X-gal staining in recent research. What is evident from the findings is that beta-galactosidase activity is significantly elevated in groups exposed to UV light. Simultaneous betaine administration, on the other hand, successfully lowers X-gal production, indicating a reduction in cellular ageing. Betaine is one of crucial metabolite in natural ageing process and it was reported that the target of rapamycin (mTOR) signaling pathway, forkhead box transcription factor (FoxO) and p38-mitogen-activated protein kinase (MAPK) are the three coregulated metabolic pathways in ageing and the anti-ageing drugs have raised the betaine levels consistently (Lan, W et al. 2024). These results confirm the reduction in cellular senescence observed in the betaine-treated groups and highlight the potential anti-ageing effects of betaine. Recent research into senolytics, agents that selectively eliminate senescent cells, aligns with these findings, emphasizing their role in mitigating ageing and related diseases.

CONCLUSION

The study demonstrates that betaine or Trimethyl glycine which is a plant-derived phytochemical, exhibits significant potential in counteracting UV-induced photo ageing and DNA damages. UV exposure typically reduced cell viability, but treatment with 100 µg/ml of betaine was found to restore cell viability to 85.87% with concomitant improvement in cell morphology. Additionally, betaine enhanced antioxidants like SOD and catalase while reducing cellular ROS generation in contrary to UV alone treated groups. Importantly, the study highlights betaine's ability to slow cellular ageing and preserve DNA integrity through senescence assays. These findings suggest that betaine has promising anti-ageing properties and potential applications in both therapeutic and cosmetic contexts.

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Nil

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