# Involvement of attenuated antioxidant and Bcl2 signalling property in UV-R/ sunlight irradiated piperine treated ischemia/ reperfusion rat model.

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## ABSTRACT

*Introduction:* Piperine (PIP) is well known multifunctional antioxidant and anti-inflammatory phytochemical that showed neuroprotection. Pre-treatment of UV-R irradiated piperine (UVR-PIP) 10 mg/kg body weight bw, intravenously showed attenuated neuroprotective effects compared to that of PIP (10µM) in PC12, cortical neuronal culture *in-vitro* and a rat model of focal cerebral ischemia *in-vivo*. *Method:* Neurological parameters were evaluated for UVR-PIP and PIP treated against transient focal cerebral ischemia of SD rats through quantification of infarct volume by TTC staining. *In-vitro* results deal with reduction of cell viability on UVR-PIP treatment in PC12 and cortical neuronal cell. The result of photodegradation of PIP under UV-R irradiation revealed the formation of photoproducts. Estimation of Mitochondrial ROS, antioxidant enzyme and non-enzyme activities in UVR-PIP and PIP treated brain tissue. *Results:* Results indicated OGD induced primary cortical neuron cultures and PC12 cells showed that SUN-PIP treatment decrease cell viability and increased LDH secretion compared to that of the PIP pre-treatment. In addition, Bcl-2 expression was decreases after the SUN-PIP treatment. Thus, our results demonstrated that SUN-PIP preconditioning failed to increase levels of Bcl-2 and normalized the endogeneous antioxidant, mediating the neuroprotective effects of PIP. UV-R irradiation attenuated the neuroprotective effects of PIP through modulation of the antioxidant and Bcl-2 mediated pathway. *Conclusion:* Thus, the ability of UV-R to serve as a modulator of this neuroprotective signaling pathway. Piperine loses some of its neuroprotective ability when irradiated by UV radiation and care has to be taken during storage to avoid exposing piperine to the sun.

**Keywords:** Piperine, UV-R/sunlight, Cerebral Ischemia, Neuroprotection.

## INTRODUCTION

Cerebral stroke, a leading cause of death and disability worldwide involves cerebral ischemia-reperfusion injury and impaired blood flow resulting in neuronal cell death.[1,2] Despite the recent efforts for improvement of treatment strategies for cerebral stroke, prognosis of cerebral ischemia patients has remained largely unsatisfactory. This is certified to the lack of effective neuroprotective agents for salvaging neuronal cell death as in most cases only the recombinant tissue plasminogen activator is routinely used for treatment.[2] Therefore, the need for expedited development of effective neuroprotective agents for cerebral stroke is critical.

Understanding of the complex pathophysiology of ischemic stroke is imperative for identifying promising neuroprotective treatments.[2] I/R injury releases huge amount of

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reactive oxygen species (ROS) that induced inflammatory and oxidative injury in peri-infarct cortical region.[3] ROS may alter the antioxidant enzyme activity and interaction of different protein-protein and DNA-protein with transcription factors which may lead to brain dysfunction and cell death.[3] These antioxidant enzymes are basal line natural defence system such as glutathione peroxidase (GPx), superoxide dismutase (Mn-SOD, CuZn-SOD) and catalase (CAT) under the normal condition. SOD converts the hydroxyl redicals to $H_2O_2$ whereas GPx and CAT convert the $H_2O_2$ to water and oxygen. When there is difference between ROS production and available antioxidant capacity, antioxidant (enzymatic and/or non-enzymatic) cannot nullify the augmented ROS level in neuronal cells. In addition, increased ROS level could also be neutralised by the other endogenous antioxidants such as alpha-tocopherol and ascorbic acid.[4,5]

At present rates, it takes a rationally extensive period of time for a lead compound to be developed into a clinically approved drug for cerebral stroke.[2] A possibly faster path to development is to depot dietary agents or neutraceuticals as neuroprotective agents for improvement of cerebral stroke outcome.[6] The insufficiency of natural dietary agents and antioxidants leads to increased cognitive injury in stroke patients.[6,7]

PIP is a dietary spice alkaloid derived from piper (*piper nigrum*) and exhibits multifunctional antioxidant, anti-inflammatory, anti-ischemic and antifibrotic properties.[6-9] In addition to this PIP has tendency to act as bioavailability enhancer, inhibitory effect on p-glycoprotein and drug metabolizing enzyme.[10-16] Further, intravenously administered PIP was found to be 97% bioavailability compare to other route.[14] Previously reported that PIP have neuroprotective ability through reduction of ischemic brain injury in focal cerebral ischemia.[9] The recent interest on the relationship of PIP and stroke is focused for the development of novel neuroprotective agents.[7-9] However, due to its light sensitive nature and cis-trans geometric isomerisation at 254 nm UV radiation, we hypothesized that the exposure predominant UV-R irradiation may alter the functional properties of PIP influencing its neuroprotective effects.[10] Cis-trans geometric isomerisation might be related to less bioavailability that alter neuroprotective ability of PIP.[17] It is also possibility that UVR-PIP photoproduct would be attributed to modulating effect of p-glycoprotein and drug metabolizing enzyme or may be neurotoxic to neuronal cell.

In the present study, we examined the neuroprotective effects of PIP and UVR-PIP in a rat model of cerebral ischemia *in-vivo* and cortical neurons and investigated the molecular mechanisms involved in this process.

## METHODS

### Chemicals and Reagents

Piperine, nembutal , 2, 3, 5-triphenyl tetrazolium chloride (TTC), 2,4-dinitrophenylhydrazine, dihydrorhodamine 123 and thiobarbituric acid reactive substances (TBRAS) were all procured from Sigma Company. Monofilament purchased from Ethicon Company. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) assay from Promega.

### Radiation Source, Dosimetry and Exposure

The UV-irradiation system comprised an arrangement of 1.2 m long UV-R emitting tubes procured by Vilber Lourmat (France). The intensity of emitted light was measured by a microprocessor-controlled RMX-3W radiometer (Vilber Lourmat) equipped with calibrated UV-R detecting probe. The spectral emission of UV-R sources ranged from 240 to 320 nm. The radiation dose was measured in $J/cm^2$. Intensity selected for irradiation was based on dosimeter carried out at our laboratory's roof top between 12.00 noon to 1.00 PM and was parallel to the ambient intensity of UV-A and UV-B reaching in UV-R at Lucknow (26°45'N latitude and 80°50'E longitude at 146 m above the mean sea level). UV-R exposure was carried out in a temperature controlled (25°C ± 2°C) radiation chamber. The PIP solution was put at a minimum distance of 22.0 cm from the source. Glass petri dishes (60 x 15 mm) were used for photochemical reactions.

### Cell Culture

Rat pheochromocytoma PC-12 cells were obtained from the American Type Culture Collection. PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; high glucose formulation) supplemented with 10% horse serum and 10% FBS along with 1% antibiotic and antimycotic (Gibco, NY, USA). Cultures were maintained in 5% CO2 and humidified in an incubator at 37°C. PIP was dissolved in DMSO and stock solutions (10 mM) were further diluted in the culture media prior to the use in experiments. The cells were plated at $1x10^4$ cells per well in 96-well or 5x105cells per well in 6 well microtiter plates for the assays. For the primary cortical neuronal cultures, embryonic day 16–18 pups were obtained from pregnant SD rats anesthetized with tribromoethanol (350 mg/kg, i.p.). Meninges were carefully

removed and isolated cerebral cortices were dissociated with 8.2 U/ml papain (Worthington Biochemical, Lakewood, NJ) for 30 min at 37°C in a shaking water bath. Subsequently, fetal bovine serum and trypsin inhibitor were used to stop digestion. The tissue suspension was then triturated thoroughly using a pasteur pipette. Freshly dissociated cells were seeded at $2\times10^5$ cells/$cm^2$ into 96-well plastic plates coated with L-polyornithine (10 µg/ml) and then incubated in neurobasal medium (Invitrogen, Carlsbad, CA) with 2% B-27 supplement, Glutamax (1:100) (Invitrogen, Calsbad, CA), penicillin, and streptomycin at 37°C with 5% $CO_2$ and 95% air. The medium was changed 24 h after plating, and half of the medium was changed every 3 days. Experiments were conducted after three changes of media. Immunocytochemical analysis of neuronal marker protein gene product 9.5 (PGP 9.5) (Chemicon International, Inc., Temecula, CA) was used to confirm the purity of neuronal cells.

### Measurement of Cell viability and LDH secretion

The cell viability and LDH secretion were quantified in the PC12 cell line and cortical neuron cultures using the Cell Counting Kit-8 (Dojindo, Molecular Technologies, MD, USA) and the LDH quantification kit (Biovision, CA, USA) as per the manufacturer's instructions.

### Oxygen Glucose Deprivation

For oxygen glucose deprivation (OGD) experiments, the media of cultured PC12 cell line or cortical neurons were replaced with pre gassed 1X Hank's balanced salt solution (HBSS, 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, 30 µM glycine, pH 7.4) and placed in a Billups-Rothenberg modular incubator chamber (Del Mar, CA) and flushed with a gas mixture of 5% $CO_2$ and 95% $N_2$ for 10 min. The chamber was then sealed and placed into a humidified $CO_2$ incubator at 37°C. After 60 min in the hypoxic chamber, the OGD treatment was stopped by replacing HBSS with the respective cell culture media. The cells were then placed back to normoxic conditions and incubated for 24 h for the functional assays.

### Focal cerebral ischemia and neurological deficit score evaluation

Focal cerebral ischemia was simulated in a rat model of cerebral stroke through the middle cerebral artery occlusion (MCAO). Adult male Sprague-Dawley (SD) rats (220 ± 20g) were obtained from the National Laboratory Animal Centre, Central Drug Research Institute (CDRI), Lucknow, used for experiment. The experimental animals were approved by Institutional Animal Ethical Committee (IAEC) and all animal experiments were carried out in accordance with the institutional guidelines. Rats were housed in cages in a temperature-controlled (25ºC±1ºC) environment, provided free access to food and purified drinking water ad libitum. The rats were divided into 4 groups of 6 rats each as follows: Group I: Sham operated group; handled as other groups, except MCAO was not done. Group II: Ischemic brain damage induced by MCAO, treated with saline as vehicle. Group III: Ischemic brain damage, treated with PIP (10mg/kg bw, i.v.) 30 min before MCAO. Group IV: Ischemic brain damage, treated with UVR-PIP (10mg/kg bw, i.v.) 30 min before MCAO. The induction of MCAO and evaluation of the neurological deficit score was conducted as per a standardized protocol.[18] At the end of the experimental period the animals were sacrificed through decapitation.

### TUNEL Assay

Rats were subjected to cerebral ischemia injury and divided in to three group vehicle, PIP and UVR-PIP treated group. All animals were sacrificed, dissected and transferred to formalin followed by 10, 20 and 30% sucrose. Mid cortical region was selected for 10 micron cryosection. DNA fragmentation was estimated by TUNEL assay in ipsilateral ischemic penumbra region of all brain sections. At least ten microscopic fields (40 x magnifications) were counted in a double-blinded manner and averaged in each section.

### Cortical mitochondria preparation

Rats were sacrificed and mechanically dissociated tissue was used for brain live cells preparation.[19, 20] Three rats were used in each group treated with PIP, UVR-PIP and alpha-tocopherol were euthanized, and brain were rapidly removed. Estimation of ROS in live 100 mg brain cortical tissue was based on several methods with some modifications.[21, 22] Cytoplasmic and mitochondrial ROS level was measured by using the fluorescent dihydrorhodamine 123. DHR localizes to mitochondria and fluoresces when oxidized by ROS, particularly peroxynitrite, to the positively charged Rhodamine 123 derivate. The fluorescence was measured at excitation and emission wavelengths of 500 nm and 536 nm, respectively. Production of ROS was expressed as fluorescence unit/mg protein.

To evaluate the extent of oxidative stress after 2 h ischemia followed by PIP, estimation of lipid peroxidation and biochemical estimation of activity of superoxide dismutase and catalase was carried out in cortical brain.[23, 24]

### RT-PCR analysis of infarcted cortical brain tissue

Total RNA was extracted using TRIzol (Life Technologies, USA). The concentration of isolated RNA was determined by nano-drop spectrophotometer (ND-1000 Thermo Scientific, USA) at 260 nm. RNAs were stored at −20 ◦C. cDNA was synthesized by high-capacity cDNA Reverse Transcription Kit (Sigma Aldrich). Each RNA sample was assayed in triplicates and cycle threshold (cT) values and normalized by to housekeeping (GAPDH) and expressed as the ratios of Bcl-2 mRNA/GADPH. Bcl-2 Primer sequences used in this study was: 5´-ATACCTGGGCCACAAGTGAG-3´ (Forward Primer) 3´-TGATTTGACCATTTGCCTGA-5´( Reverse Primer).

The thermal parameters were: 94°C for 5 min, followed by 30 cycles of 94°C 40 s, 58°C 40 s, 72°C 40 s and 72°C 10 min old change was calculated using $2^{\Delta\Delta c_T}$ method.[25]

### Statistical analysis

The data obtained from all the parameters were expressed as mean ± SE. Statistical analysis was conducted using prism software for both one-way analysis of variance and Newman Keuls Multiple Comparison Test at the level of $p < 0.05$ and $p<0.01$.

## RESULTS

### UV-R irradiation induces degradation of PIP and decreases its neuroprotective effects in vitro

PIP is known to exert neuroprotective effects and is vulnerable to photodegradation.[8-10] We therefore assessed the effects of UV-R irradiation induced photodegradation of PIP. The absorption spectrum of PIP (5μg/ml) diluted in physiological saline at different intensities (Fig. 1A) is characterized by one intense absorption peak at 267nm in UV-B and UV-A region. The photodegradation study was performed under UV-R (0.6mW/cm$^2$) for 1, 2, 3 and 4h. The result showed 1h irradiation of PIP shifted the absorption maxima towards the UV range (Fig. 1A). Next, we explored whether the photodegradation of PIP influenced its neuroprotective effects. We subjected PC12 cells to oxygen glucose deprivation/reperfusion (OGD/R) and assessed the neuroprotective effects of UVR-PIP and PIP through quantification of the LDH release and cell viability. The results indicated that OGD significantly increased cellular stress indicated by the increased (*$p<0.01$*) LDH secretion (Fig.1D) in control cells. Pre-treatment of the PC12 cells with PIP (10μM) before OGD reduced the OGD-induced LDH release by ~46.2 % while in the UVR-PIP treated group the reduction of LDH secretion was only ~15.9 % (Fig. 1D). Similarly, OGD significantly ($p<0.01$) reduced (~31.8%) the cell viability in the control group. Treatment of PC12 cells with PIP significantly ($p<0.01$) increased cell viability (~67.3%) compared to that of the UVR-PIP treatment (~40.4%) (Fig. 1D). Collectively, these results showed that UVR-PIP irradiation decreased the cytoprotective effect of PIP in PC12 cells.

### UVR-PIP has attenuated neuroprotective effects in a rat model of cerebral ischemia in-vivo

PIP is known to have neuroprotective effects in a rat model of cerebral ischemia.[8,9] As our results indicated attenuated neuroprotective effects of UVR-PIP in vitro, we next tried to assess, the neuroprotective effects of UV-R irradiation in a rat (middle cerebral artery occlusion) model of cerebral ischemia. The results showed that PIP treatment (10 mg/kg body weight, i.v.) showed significant neuroprotective activity compared to that of the UVR-PIP (10 mg/kg body weight, i.v.) as indicated by the decreased neuronal cell death in single coronal brain section and TUNEL staining (Fig. 2D). UVR-PIP treatment also failed to reduce the infract volume (Fig. 2C) and edema volume (Fig. 2C) confirmed by TUNEL staining (Fig. 3). Further, the evaluation of the neurological deficit scores (on days 1, 2, 3 and 7) clearly indicated that PIP treated experimental groups had significantly improved neurological scores compared to that of the of the UVR-PIP treated groups (Fig. 2B). These results collectively suggested that UVR-PIP had attenuated neuroprotective effects in a rat model of cerebral ischemia *in vivo*.

### UVR-PIP fails to restore increased ROS and attenuate antioxidant level in cortical brain tissue

ROS production was estimated in different treatment group with PIP and UVR-PIP. UVR-PIP treated group showed increased in the ROS level (~37%, $p<.05$ for mitochondrial ROS) compare to PIP (Fig.4). The activity of superoxide dismutase and CAT was significantly increases (~44.84%, $p<.05$ for SOD and~34.98%, $p<0.05$ for CAT) in PIP treated group compare to vehicle (Fig.4). Further, activity of both SOD and CAT was found low (~11.54%, $p<0.05$ for SOD; ~28.98%, $p<.05$ for CAT) in UVR-PIP compare compare to PIP. TBRAS level was decrease in PIP treated (~15.48%, $p<0.05$) compare to vehicle. The increase in TBRAS level was more marked in rats treated with UVR-PIP (~8.2%, $p<.05$) at 5mg/kg dose suggested that photodegraded PIP could be generate oxidative stress in brain (Fig. 4). Further, TBRAS level

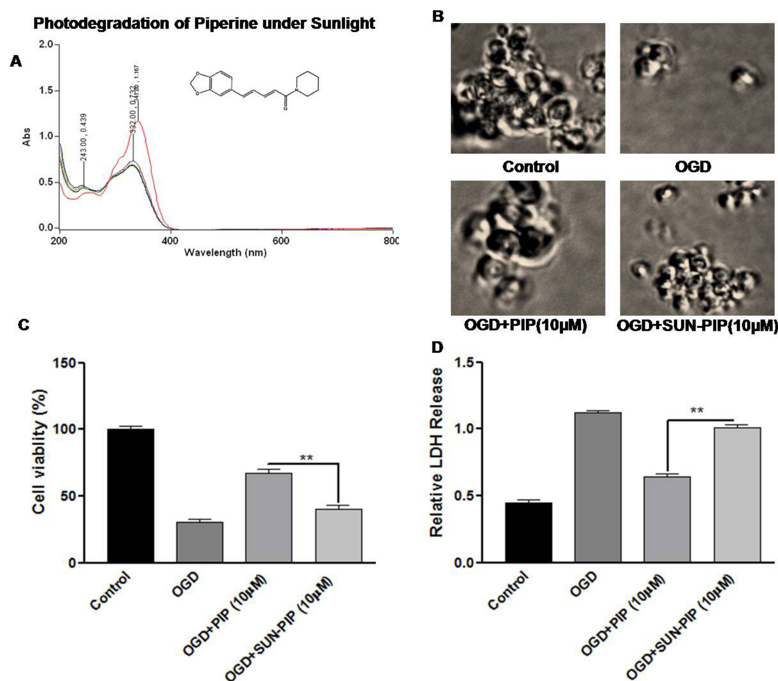


**Figure 1.** Absorbance spectra of PIP under UV-R by UV-vis-Spectrophotometer. (A) Absorbance spectra of PIP (5μg/ml) showed absorbance in UV-R region indicate their photosensitization and alter the neuroprotective efficacy. (B) Morphological picture of PC12 cells. (C) PIP treatment significantly inhibits OGD induced LDH release compared to that of UVR-PIP treatment (D) The average of three independent experiments performed in triplicate. Statistical significance was analyzed by ANOVA. Values are the mean±SEM *p<0.01 vs. PIP treated group.

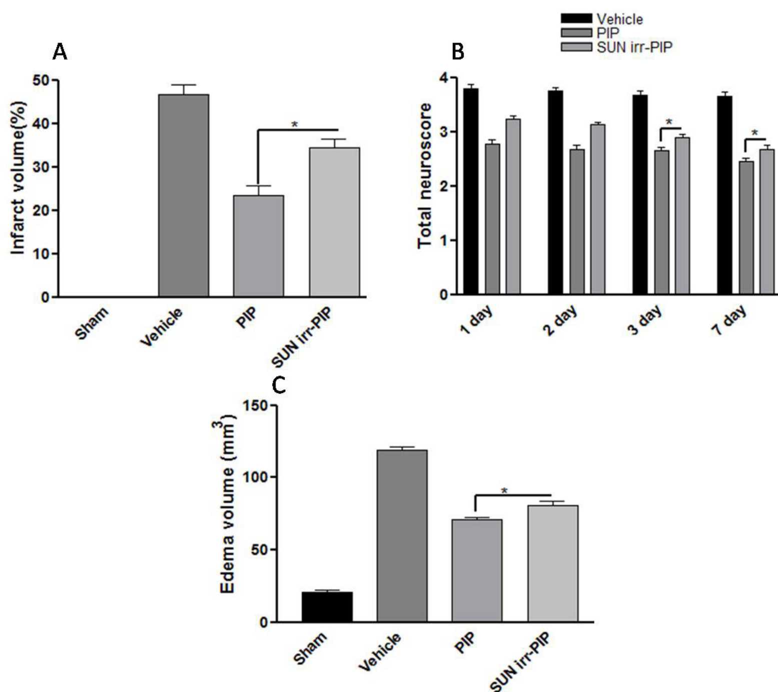


**Figure 2.** UV-R irradiation reduces the neuroprotective effects of PIP. (A) TTC staining of coronal brain section treated with either PIP or UVR-PIP. (B) Total neurological deficit score was evaluated to see neurological impairment after 1 to 3 days successive treatment. (C) Effect of pretreatment with vehicle, PIP (10mg/kg, i.v) and UVR-PIP (10mg/kg) on infarct volume (mm3) and Edema volume (mm3) calculated from image-J analysis softwere. Statistical significance was analyzed by ANOVA. Values are the mean±SEM. *p<0.05 vs. PIP treated group.

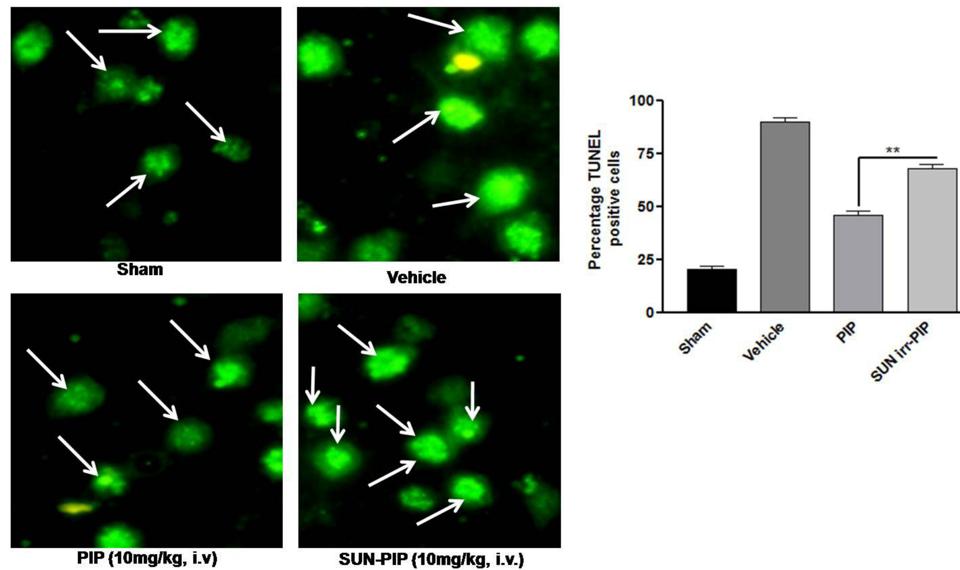


**Figure 3.** Effects of PIP and UVR-PIP on DNA fragmentation (×40) in the cortical region of brain subjected to ischemic injury. The upper row indicates the representative brain images showing TUNEL-positive cells in cortical penumbral region in vehicle (saline), PIP (10mg/kg, bw, i.v.) treated I/R groups, and UVR-PIP (10mg/kg, bw, i.v.) respectively. The fragmented DNA was labelled with green fluorescence in vehicle, PIP and UVR-PIP treated group. Statistical significance was analyzed by ANOVA. Values are the mean±SEM. *p<0.01 vs. PIP treated group.

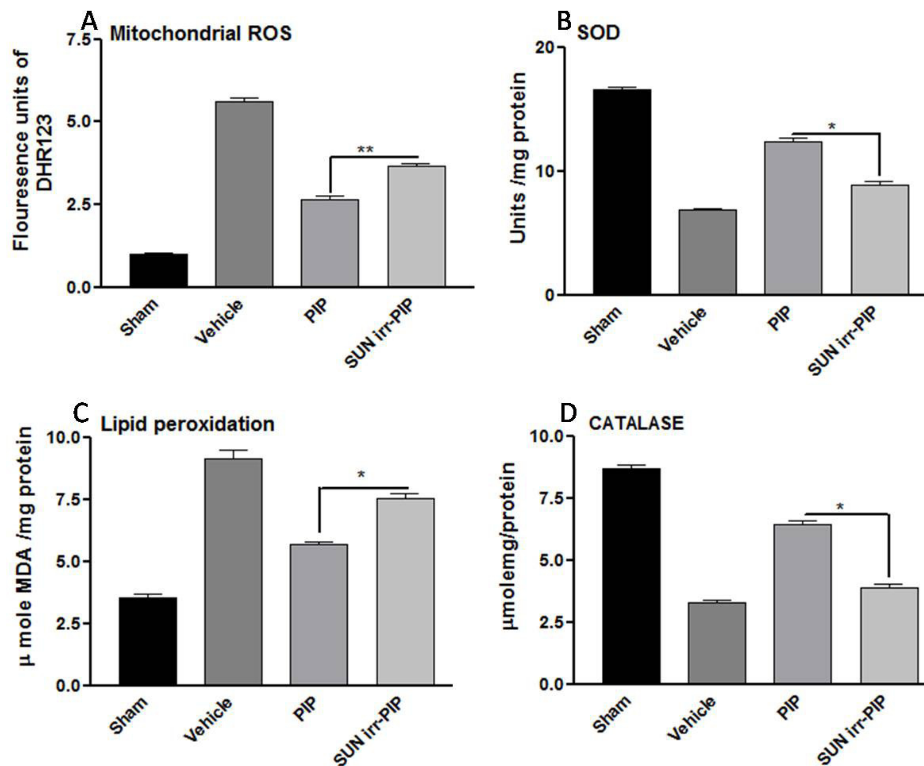


**Figure 4.** Effect UVR-PIP (10mg/kg, bw i.v.) on the ROS, endogeneous antioxidant enzyme activity (SOD, CAT and LPO). After 24 h reperfusion injury the level of different antioxidant decreased significantly in ischemic group treated with vehicle. Non-irradiated PIP increases the level of antioxidant compare to UV-R-PIP. Data have been analyzed by one-way analysis of variance followed by Newman–Keuls multiple comparison test. Values are expressed as mean ± SEM of six animals in each group, $P<0.05$ vs. Vehicle treated, $^{\#}P<0.05$ vs. non-irradiated PIP, $^{*}P<0.05$ vs. PIP treated.

was significantly decreases (~24.48%, p<.05) after the combinatorial treatment of UVR-PIP with alpha-tocopherol and exhibited its antioxidant property.

## DISCUSSION

Bcl-2 gene expression has been shown to regulate cell proliferation, differentiation and death.[26] Recently shown that several natural phytochemicals regulate neuroprotective effects via modulation of Bcl-2 expression. Consistent with this finding, our study showed that PIP treatment in neuronal cells induced significantly decreased OGD induced LDH secretion and increased cell survival compared to UVR-PIP treatment. We further, investigate the synergistic neuroprotective role of curcumin with PIP in cortical neuronal cell culture to show significantly increase cell viability compare to individual PIP and curcumin

. This may represent a critical step in the development of novel neuroprotective therapy based on synergistic combination of natural antioxidant molecules such as curcumin and others with PIP. [7, 8, 14] Previous studies supported that the bioavailability of several phytochemicals increases including curcumin in presence of PIP. [7,8] However, UVR-PIP was devoid of this effect. Thus, our results suggest a novel idea: PIP preconditioned neuronal cells have decreased cell viability while UVR-PIP preconditioned increased in thereby leading to differential effects on Bcl-2 increased expression and neuroprotection. In addition to this, co-administration of PIP and curcumin increased the cell viability against OGD induced neuronal cell death. Obviously, bioavailability enhancing effect of PIP was associated with the increased mRNA expression of Bcl-2 and cell viability (Fig 5). The neuroprotective efficacy of co-administered PIP and curcumin should be further subject of study *in vivo* and *in vitro*. Previously reported, miRNA are regulatory molecule that affects the cell differentiation, apoptosis and neurodegeneration was modulated by various phytochemicals.[27,28] Recent study supported that miR-15b was downregulated by sevoflurane thereby activating Bcl-2 mRNA expression in cerebral ischemia of rat model.[29] So from light of above study it can be hypothesized that piperine and its co-administration with other phytochemicals might be downregulated the miR-15b and activation of the Bcl-2 mRNA level leading neuroprotection.

Interestingly, we found PIP treatment both *in-vivo* and *in-vitro* significantly increased cell viability and subsequent Bcl-2 activation leading to neuroprotection. However, the UVR-PIP treatment fails to restore the decreased the cell viability and Bcl-2 m-RNA expression. From above study it should be noted that UV-R irradiated phytochemicals can be harmful to brain and can be neurotoxic. So it is concluded that the light sensitive phytochemicals can be sensitive to the neurons and avoid for dietary intake. Further studies will be needed to understand whether this signaling pathway is a target of other neuroprotective or neurotoxic agents.

## CONCLUSION

Collectively our studies suggest that (i) UV-R irradiation induces attenuation of the neuroprotective effects of PIP, (ii) Co-administration of both PIP and curcumin reduced cell viability, (iii) through the activation of the Bcl-2 gene expression. Piperine loses some of its neuroprotective ability when irradiated by UV radiation and care has to be taken during storage to avoid exposing piperine to the sun. Importantly, later investigations are required to assess the translational value of the therapeutic activation of the Bcl-2 signaling pathway for neuroprotection.

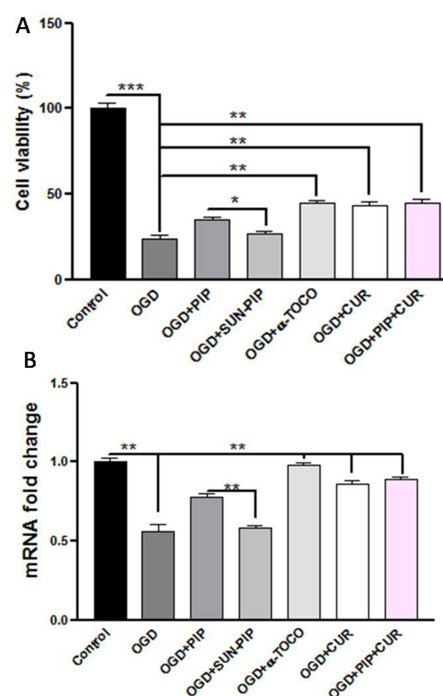


**Figure 5.** UV-R irradiation modulates the Bcl-2 signaling pathway for altering neuroprotective effect of PIP. (A) Cortical neuronal cells were subjected to OGD and treatment with PIP (10µM), UVR-PIP (10µM), alpha-tocopherol (10µM), curcumin (25µM) and PIP (10µM) + curcumin (25µM) treatment. Cell viability of cortical neurons was measured using the CCK-8 kit. (B) Bcl-2 mRNA fold change in different groups. Statistical significance was analyzed by ANOVA. Values are the mean±SEM. *p<0.05, ** p<0.01 *vs*. PIP treated group and **p<0.01 vs. Control.

## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

## ACKNOWLEDGEMENTS

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## Abbreviation

UVR-PIP-UV-R radiation irradiated Piperine
OGD-Oxygen glucose deprivation,
TBRAS-Thiobarbituric acid reactive substances
MCAO-Middle cerebral artery occlusion
I/R-Ischemia/Reperfusion
DMSO-Dimethyl sulfoxide
LDH-Lactate dehydrogenase.

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