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Attenuated Antioxidant property of UV-B Irradiated Piperine in Ischemia-Reperfusion Injury in SD rat

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ABSTRACT

PIP is well known antioxidant that showed neuroprotection against cerebral ischemia (2h) /reperfusion (24h) (I/R) injury. However, it can degrade very easily in presence of UV-R/sunlight. Here, comparative study of non-irradiated and UV-B (0.6mW/cm^2) irradiated PIP was performed against cerebral I/R injury. First, the dose dependence evaluation of neuroscore was investigated after the non-irradiated PIP intravenously (2.5, 5, 10 mg/kg b w, i.v.) treatment. The neuroprotective outcome of UV-B irradiated and non-irradiated PIP (5 mg/kg b w, i.v.) against cerebral I/R in SD rats was investigated through evaluation of neuroscore and quantification of infarct volume by TTC staining. The result of photodegradation of PIP under UV-B irradiation revealed the formation of photoproducts. There were no changes in Cortical and mitochondrial ROS level and antioxidant defence enzyme (CuZn-SOD, Mn-SOD, and catalase) and non-enzyme (LPO, protein carbonyl and reduced glutathione) activities in UV-B irradiated compare vehicle group. Thus, our results demonstrated that PIP could lose its parent structure in presence of UV-B and attenuates its neuroprotective efficacy. Thus, UV-B exposure must be avoided during dietary intake of PIP to obtain best neuroprotective response.

1) INTRODUCTION

I/R injury produces large amount of reactive oxygen species (ROS) in peri-infarct cortical region [1]. ROS induced the inflammatory and oxidative injury in infracted brain tissue [2]. Small concentration of ROS can produces changes in cellular redox state that may affect the activity of enzyme and interaction of different protein-protein and DNA-protein with transcription factors. Under the normal condition, a base line natural defence system was provided by antioxidant enzyme such as glutathione peroxidase (GPx), superoxide dismutase (Mn-SOD, CuZn-SOD) and catalase (CAT). 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 imbalance between ROS production and available antioxidant defence capacity, antioxidant (enzymatic and/or non-enzymatic) cannot potentially nullify the increased ROS level in neuronal cells. The increased ROS level could be nullified by the several endogenous antioxidants such as alpha-tocopherol and ascorbic acid [3]. The stored cellular ROS cause oxidative damage to lipid membrane, proteins and DNA of neuronal cell, which leads to brain dysfunction and cell death. Furthermore, increased ROS level also impaired the functionality of endogenous antioxidant defence enzyme activities in neuronal cell via oxidative damaged [4]. Previous studies supported that antioxidant defence decreases in I/R injury for several folds [5, 6]. Antioxidants control the level of ROS in normal physiological state. Several studies reported to nullify the level of ROS by using different antioxidant molecule against cerebral I/R injury animal model.

Over the last few decades due to ozone depletion the frequency of UV-B radiation at the earth's surface has been increased. Excessive exposure of UV-B can be detrimental to human beings by inducing premature skin aging, inflammation, and cancer and cell death [7]. Previous studies reported that phytochemicals such as PIP has tendency to absorb ultraviolet light causing loss of its molecular structure and formation of multiple ionic photoproducts [8].

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Piperine (PIP) is widely used as spice in cooking and has important medicinal fundamental inhibitory effects on *p*-glycoprotein and many other drug metabolizing enzyme systems [9,10]. PIP exerts as bioavailability enhancer and important effect for biotransformation including chemoprevention, detoxification, and enhancement of the absorption and bioavailability of herbal and conventional drugs [11, 12]. The neuroprotective role of PIP was examined via activation of anti-apoptotic pathway in various brain injury models [13]. Several phytochemicals, having benzenoid ring structure susceptible for absorbance of UV-Visible light, may be responsible for alteration of molecular structure and loss of neuroprotective efficacy. Furthermore, PIP can be photosensitive and degrade very easily in presence of sunlight. Therefore it is essential to investigate the neuroprotective role of UV-B exposed PIP whether it enhances or attenuates the efficacy. Several studies documented the neuroprotective role of phytochemicals via activation of antioxidant defence enzyme system in different neurological animal model [14, 15, 16, 17]. Therefore, it is important to investigate the comparative neuroprotective effect of UV-B (0.6mW/cm^2 for 1h) irradiated PIP against ischemic stroke rat model.

In present study, the comparative neuroprotective effect of UV-B irradiated and non-irradiated PIP was investigated against experimental cerebral stroke rat model. Simultaneously, The antioxidant enzymatic and non enzymatic level in both UV-B irradiated and non-irradiated with alpha-tocopherol was also measured. We evaluated neuroscore and estimated cortical and mitochondrial ROS level with intravenously treatment of UV-B irradiated and non-irradiated PIP (5mg/kg, i.v.) and alpha-tocopherol (20mg/kg, i.v.).

2) MATERIALS AND METHODS

2.1 Chemicals and Reagents

PIP (Cat no. P49007**)**, Nembutal, alpha-tocopherol and 2, 3, 5-triphenyl tetrazolium chloride (TTC) were all procured from Sigma Company. Monofilament purchased from Ethicon Company. Both dye 2´,7´dichlorofluorescein diacetate (Cat no. D6883) and dihydrorhodamine 123 (Cat no. D1054) was procured from Sigma. 2,4-dinitrophenylhydrazine (Cat no. D199303) and thiobarbituric acid reactive substances (Cat no. T5500) was procured from sigma.

2.2 Radiation Source and Dosimetry

The UV-irradiation system comprised an array of 1.2 m long UV-B emitting tubes manufactured by Vilber Lourmat (France). The intensity of emitted light was measured by a microprocessor-controlled RMX-3W radiometer (Vilber Lourmat) equipped with calibrated UV-B detecting probe. The spectral emission of UV-B sources ranged from 290 to 320 nm with a peak at 312 nm, respectively. 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 sunlight at Lucknow (26^{o}45'N latitude and 80^{o}50'E longitude at 146 m above the mean sea level).

2.3 Radiation Exposure

UV-B exposure was carried out in a temperature controlled (25^{0}C ± 2^{0}C) radiation chamber. The samples were put at a minimum distance of 22.0 cm from the source. Glass petri dishes (60 x 15 mm) were used for photochemical reactions.

2.4 UV-visible Analysis of PIP

PIP was dissolved in 70% ethanol (20mg in 1ml) followed by dilution in saline (0.9% NaCl) to concentration 10mg/ml. PIP (10mg/ml) was exposed under UV-B in 25 ml volume for 60 min. Photodegradation spectrum of the drug was recorded between 200 to 700 nm.

2.5 Experimental animal groups

Fourty eight SD male rats weighing 250–300 g were obtained from the National Laboratory Animal Centre, CSIR-Central Drug Research Institute (CDRI), Lucknow used for experiment. The experimental animals for the study were approved by Institutional Animal Ethical Committee (IAEC) and all animal experiments were carried out in accordance with the institutional guidelines on the care and use of experimental animals. The experimental rats were divided into four groups, vehicle, non-irradiated, UV-B irradiated PIP (5 mg/kg, i.v.) and combinatorial treatment of both UV-B irradiated PIP and alpha-tocopherol (20mg/kg, i.v). The rats were divided into four groups of 12 rats each. UV-B irradiated, non-irradiated and alpha-tocopherol was injected i.v. before ischemia and after 30min of reperfusion. It is possible that PIP may pass through BBB [18] more easily than under normal condition. Half of all rats were killed 24 h after middle cerebral artery occlusion (MCAO) surgery and others are monitored for 7days to see neurological deficit score.

2.6 MCAO Surgery Procedures

Rats were anesthetized with nembutal (2ml/kg, i.p.) before MCAO procedure. The skin incision was made to create a 1.5-cm opening in central midline of neck region. Common carotid artery was traced followed by the external and internal common carotid artery (ECCA and ICCA) [10]. ECCA was ligated with thread to block the insertion of Ethicon monofilament (4/0 in Number, Ethicon). After this, a small pick was created by 2ml sterile syringe needle at proximal end of CCA, to insert the monofilament**.** The filament inserted a distance of 20 to 22 mm from the CCA bifurcation, resulted transient blockage of blood supply for 2h and reperfusion was allowed by withdrawal of the filament. The incision site was then closed with suture and the animal was allowed to recover from anaesthesia. Irradiated and non-irradiated PIP was administered intravenously (i.v.) route 30 min before ischemia and after the reperfusion injury.

2.7 Evaluation of neurological deficit

Neurological deficit score was tested by three to four blind observer, who were unaware of the identity of the groups after 2h ischemia/ 1, 2, 3 and 7 day reperfusion and scored as follows: 1, no observable neurological deficit (normal); 2, failure to extend left forepaw on lifting the whole body by tail (mild); 3, circling to the contra lateral side (moderate); 4, leaning to the contra-lateral side at rest or no spontaneous motor activity (severe) [20]. Each animal score was estimated approximately for 2 min, and repeated another 5 times for confirming consistency. Score of 1 represent normal neurological status, and score of 4 represent severe behavioural deficit.

2.8 Measurement of infarct volume

Each group of animals were anaesthetised and decapitated after 2h ischemia /24 h reperfusion. The brain was removed and placed in -20°C for freezing. The brain slicing was done immediately after half an hour of freezing. Four slices of 2mm thickness of sections were made from posterior to olfactory

bulb. All slices were incubated for 30 minutes in a 2% solution of TTC dissolved in physiological saline at 37°C and then fixed in 4% formalin. The stained slices were photographed by digital camera (Sony cyber- shot, Model No. DSC-W210) and subsequently measured for the infarct area of each brain slice including ischemic region by image analyser software.

2.9 Cortical mitochondria preparation

Rats were sacrificed and mechanically dissociated tissue [21] was used for brain live cells preparation [22]. Three rats were used in each group treated with non-irradiated, UV-B irradiated, UV-B irradiated PIP with and without alpha-tocopherol were euthanized, and brain were rapidly removed. Estimation of ROS in live 100 mg brain cortical tissue was based on several methods [23, 24] with some modifications. Cytoplasmic and mitochondrial ROS level was measured by using the fluorescent probes 2´,7´-dichlorofluorescein diacetate (DCFDA) and dihydrorhodamine 123 (DHR123). Rat brain cells were incubated for 25 min in the presence of 15µM DCFDA or for 10 min in the presence of 10µM DHR123 at 37°C in a shaking water bath [25]. After washing twice with HBSS, the formation of the fluorescent product DCF was monitored by a BMG fluorescence spectrometer with excitation wavelength of 488 nm and emission wavelength of 510 nm. DCF is trapped mainly in the cytoplasm and is oxidized by several ROS, most notably hydrogen peroxide [26]. 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 500nm and 536nm, respectively. Production of ROS was expressed as fluorescence unit/mg protein.

2.10 Oxidative Stress

To evaluate the extent of oxidative stress after 2 h ischemia followed by UV-B irradiated PIP with alpha-tocopherol, estimation of lipid peroxidation, protein carbonyl, and reduced glutathione levels and biochemical estimation of activity of superoxide dismutase, catalase, and glutathione peroxidase was carried out in cortical brain.

2.10.1 ***Non-enzymatic assay of Lipid Peroxidation, Protein Carbonyl, and Reduced Glutathione Levels in cortical region of rat brain***

For the measurement of malondialdehyde (MDA), levels of thiobarbituric acid reactive substances (TBARS) were estimated following the method of Ohkawa et al [27]. Briefly, cortical tissue brain homogenate in 0.1 M phosphate buffered saline (10% w/v) was incubated with 8.1% sodium dodecyl sulfate (SDS, w/v) for 10 min at room temperature followed by the addition of 20% acetic acid. Thiobarbituric acid (TBA, 0.8%, w/v) was added in the reaction mixture after vortexing mixture of the tube. The tubes were kept in a boiling water bath for 1 h and the intensity of pink color as chromogen formed during the reaction was read at 532 nm. The amount of TBARS was calculated using a molar extinction coefficient of 1.56×10^5 m/cm.

Protein carbonyl amount in peri-infarct cortical region was measured following the method of Levine et al [28] using 2,4-dinitrophenylhydrazine (DNPH) as a substrate. The difference in absorbance between the DNPH-treated and the HCl-treated samples was determined spectrophotometrically at 375 nm and the amount of carbonyl contents was calculated using a molar extinction coefficient of 22.0 mM^{-1} cm^{-1} for aliphatic hydrazones.

Reduced glutathione level was measured in rim of cortical brain tissue by method of Hasan and Haider [29]. The brain homogenate (10%) was added with an equal volume of trichloroacetic acid (TCA, 10%) for deproteinization and allowed to incubate at 4ºC for 1 h. The cortical brain homogenate was centrifuged at 3,000xg for 15 min. The supernatant (0.25 ml) was added to 2.5 ml of Tris buffer (0.4 mM, pH 8.9) containing EDTA (0.02 M) followed by the addition of DTNB (0.01 M). The volume was made up to 3 ml by addition of 0.25 ml of distilled water and absorbance of yellow color read on a spectrophotometer at 412 nm. The results are expressed as µg GSH/g cortical brain tissue.

2.10.2 Estimation of antioxidant enzymes (Mn-SOD, CuZn-SOD and CAT) in cortical region

Rat brain mitochondria were isolated from mitochondrial brain fraction following the procedure as described by Stahl et al [21]. Dissected brain regions were homogenized (10% w/v) in ice-cold buffer containing Tris–HCl (10 mM, pH 7.4), sucrose (320 Mm), EDTA (5 mM), and BSA (0.1%). Brain homogenate was centrifuged at 1,000 xg for 15 min at 4ºC, pellets discarded and the supernatant was further centrifuged at 14,000 xg for 15 min at 4ºC. The post-mitochondrial fraction was separated and preserved for estimation of CuZn-SOD. Crude mitochondrial pellets were separated and washed with buffer and resuspended in Tris–HCl buffer (10 mM, pH 7.4) containing sucrose (0.44 M) and used for the estimation of Mn-SOD activity. Mn-SOD activity was measured in the mitochondrial fraction using NADH as a substrate by using the method of Kakkar et al [30]. Activity of catalase in cortical region of brain was measured spectrophotometrically in post mitochondrial fraction as substrate following the method of Aebi [31]. The activity is expressed in µmol/mg protein.

2.11 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 $p<0.01$.

3) RESULTS

3.1 Absorption spectra of PIP

Absorption spectrum of PIP under-vis-spectrophotometer (5mg/ml) in saline (Fig .1). It is characterized by one intense absorption peak at visible range. Photodegradation study was performed under UV-B ($0.6mW/cm^2$) and Sunlight for 1h. Result showed 1 h irradiated PIP, absorption maxima shifted towards UV range Further confirmation was done through LC-MS/MS analysis (Not shown here).

3.2 Effect of UV-B exposed PIP on neurological deficit score

Petullo et al [20] method was used to evaluate the neurological deficit scores in different groups after 1, 2, 3 and 7 day reperfusion times [32]. Neuroscore successively decreases from 1 to 7 day significantly ($p< 0.05$) in non-irradiated PIP compare to vehicle (Fig.2b). Furthermore, the value of neuroscore was significantly increases in UV-B irradiated PIP. On combinatorial treatment of UV-B irradiated PIP (5 mg/kg, i.v.) and alpha-tocopherol (20mg/kg, i.v.), the value of neuroscore was increased (Fig.3b).

3.3 Effect of UV-B exposed PIP on percentage Infarct volume

The TTC staining was used as a marker to determine the dead (white colour) and live tissue (red colour) and to estimate infarct volume. The percentage infarct volume was significantly reduced in non-irradiated PIP (16.67±1.38, p<.05) as reported in earlier studies (Fig.3a). However, no significant reduction was observed in UV-B (27.78±1.78, p<.05) irradiated PIP treated groups than vehicle (Fig.3a). And another group of animal treated with combination of UV-B irradiated PIP and alpho-tocopherol, percentage infarct volume was significantly (p< 0.05) decreased (6.78±.78, p<0.05).

3.4 Effect of UV-B irradiated PIP on Mitochondrial and cortical tissue ROS

ROS production was estimated in different treatment group with non-irradiated, UV-B irradiated PIP with and without alpha-tocopherol. The estimated ROS level was decreases (23.49%, p<.05 for cortical tissue; 15.46%, p<.05 for mitochondrial) significantly in non-irradiated PIP treated group (Fig.4a and b). Further, UV-B irradiated PIP treated group showed increased in the ROS level (17.46%, p<.05 for cortical tissue; 2.17%, p<.05 for mitochondrial ROS) (Fig.4a and b). However, the combinatorial treatment of both UV-B irradiated PIP and alpha-tocopherol the ROS (30.05%, p<.05 for cortical tissue; 17.37%, p<.05 for mitochondrial ROS) level decreases significantly (Fig.3a and b).

3.5 Oxidative stress

UV-B irradiated PIP effect on antioxidant (Mn-SOD, CuZn-SOD and CAT) enzyme and non-enzymatic antioxidant

The activity of superoxide dismutase and CAT was significantly increases (22.24%, p<.05 for Mn-SOD, 23.55%; p<0.05 for CuZn-SOD; 34.98%, p<0.05 for CAT) in non-irradiated PIP treated group compare to vehicle (Fig. 5). Further, activity of both SOD and CAT was found low (11.54%, p<0.05) for CuZn-SOD; 19.77%, P<.05 for Mn-SOD; 28.98%, p<.05 for CAT) in UV-irradiated PIP compare compare to non-irradiated. On combinatorial treatment of UV-B irradiated with alpha-tocopherol the level of Mn-SOD and CuZn-SOD was significantly increases (27.62% for CuZn-SOD; 22.16% for Mn-SOD) suggesting antioxidant property of alpha-tocopherol on oxidative stress developed after the PIP photoproducts treatment.

Effect of UV-B irradiated PIP on Lipid Peroxidation, Protein Carbonyl, and Reduced Glutathione Levels in cortical Brain tissue of Rats

TBRAS level was decrease in non-irradiated PIP (15.48%, p<0.05) compare to vehicle. The increase in TBRAS level was more marked in rats treated with UV-B irradiated PIP (8.2%, p<.05) at 3mg/kg i.v dose suggested that photodegraded PIP could be generate oxidative stress in brain. Further, TBRAS level was significantly decreases (24.48%, p<.05) after the combinatorial treatment of UV-B irradiated PIP with alpha-tocopherol and exhibited its antioxidant property.

The estimation of protein carbonyl level in cortical brain tissue suggested that its level significantly decrease (16.5%, p<.05) in non-irradiated PIP treated group. However, its level was increases in UV-B irradiated PIP (5.64%, p<.05). But, the combinatorial treatment of UV-B irradiated PIP with alpha-tocopherol showed that protein carbonyl level significantly deceases (19.08%, p<.05) in cortical brain tissue. The decrease in level of protein carbonyls in UV-B irradiated tissue was correlated with more oxidative stress generated after treatment of photodegraded PIP. But another group of animals showed that decrease in level of protein carbonyls after combinatorial treatment of UV-B irradiated PIP with alpha-tocopherol (Fig.6).

The significant increase (16.7%, p<.05) in the level of reduced glutathione was observed in cortical tissue of rat treated with non-irradiated PIP compare to control (Fig. 6). However, there is no significant change (8.24%, ns) was observed with UV-B irradiated PIP compare to vehicle control animals. However, rats were treated with both UV-B irradiated PIP and alpha-tocopherol showed significant increased (12.64%, p<.05) level of reduced glutathione (Fig. 6).

4) DISCUSSION

PIP, main alkaloid (piper nigrum and Piper longum. Lnn.) showed anti-inflammatory and antianalgesic, anti-ischemic, cytoprotection, antioxidant and bioavailability enhancer [33, 34, 35, 36, 37]. PIP having multiple effects such as inhibitory effect of P-glycoprotein (p-g) and drug metabolizing enzyme [12]. Recent study reported that pregnane X receptor (PXR) has been activated by PIP through activation of PXR (hPXR)-mediated CYP3A4 and MDR1 expression in human hepatocytes, intestine cells, and a mouse mode [38]. Preclinical studies reported that 10mg/kg PIP showed neuroprotective role in cerebral ischemia rat model [13]. Intravenously injected PIP was increased 97% bioavailability compare to other doses [39]. It is also reported that PIP enhances the bioavailability from 20 to 200%. The whole blood volume of a human needed 20 min to exposed with sunlight which flow beneath the dermis of skin [40]. Thus, the intravenously injected PIP into the blood may become the substrate for photochemical degradation. Patients are normally unconscious about penetration of UV-R/Sunlight through clouds, window glass and thin clothing and may produce photosensitization and degradation of photosensitive compounds [41]. UV radiation at 254 nm was used to induce cis−trans geometric isomerization as a function of light intensities and time of exposure. So the light of above information it could be possible to affect the neuroprotective effect [43, 44].The present study was done with UV-B irradiated PIP (5mg/kg i.v.) to investigate its neuroprotective effect in cerebral ischemia in SD rat.

Neuroscore was evaluated for different doses of non-irradiated PIP which suggested that 5mg/kg was significantly effective dose for neuroprotection. All treatments were given 30 min before ischemia and also 30 min after reperfusion suggested that leakage BBB facilitated the bioavailability of PIP [18]. It was also suggested that PIP could enhance the bioavailability of different flavanoids as like Quercetin and curcumin [11, 12]. TTC staining demonstrated that significant reduction of % infarct volume in non-irradiated PIP treated groups (Fig. 3a).The maximum degradation of PIP in sunlight was due to cumulative effects of UV-A, UV-B other visible light radiation (data not shown). However, the UV-B irradiated PIP was used to observe the effect of neuroprotective efficacy of PIP. Furthermore, % infarct volume was significantly increases after the treatment of UV-B irradiated PIP. However, the combination of both PIP and alpha-tocopherol (20mg/kg, i.v.) was found to significantly decrease the infarct

volume due to its antioxidant property [42]. The decrease in % infarct volume was due to the anti-ischemic property of alpha-tocopherol modulated by antioxidant defence mechanism. The increase in % of infarct volume was related with photodegraded product of PIP could be due to in free radical generation. Next reductions of neurological parameter could be associated with photodegradation of PIP under UV-R/sunlight. This was also confirmed by both ROS estimation in cortical tissue. I/R injury generate the ample amount of free radicals in brain that results in neuronal apoptotic cell death. Previously, It was known that PIP scavenges the increased ROS level and alleviate oxidative damaged treated with PIP and quercetin [11, 12]. However, previous report also suggested that these antioxidant enzymes couple with other antioxidant such as ascorbic acid and alpha -tocopherol to reduce the level of ROS [13]. Non-irradiated PIP treatment normalizes the level of antioxidant enzyme (Mn-SOD, CuZn-SOD, GPx and catalase) activities and non-enzymatic

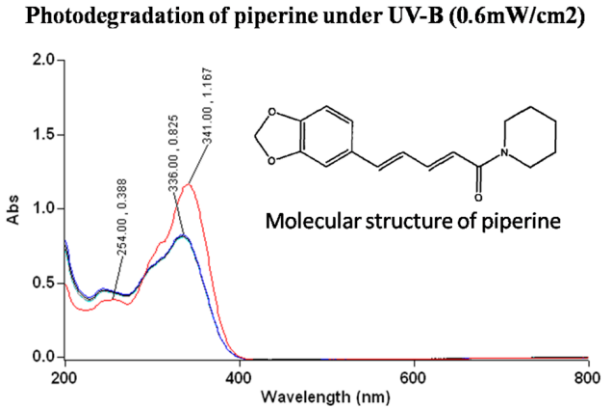


Figure 1: (A) Photodegradation spectra of PIP (3mg/ml) by UV-Vis-Spectrophotometer.

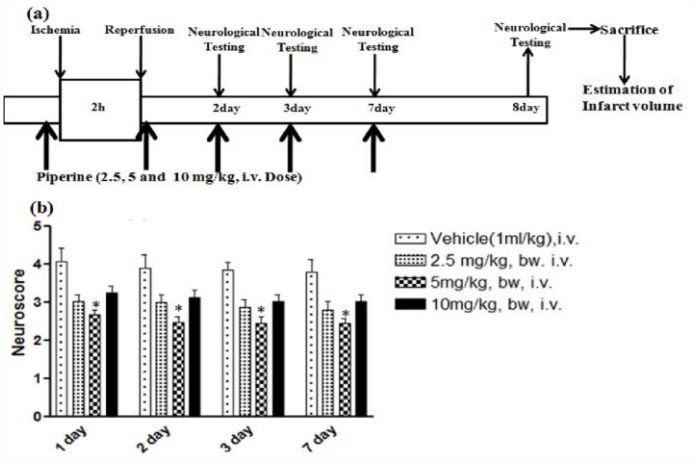


Figure 2: Treatment schedule and total neuroscore for 7 days. (A) Treatment regime of PIP with different doses (2.5, 5 and 10 mg/kg, i.v.) after transient I/R injury for 7 days. (B) Total neuroscore evaluated from animal subjected to ischemia surgeries by the different doses of PIP treatment. Values are the mean±SEM, * P<.05 vs. 2.5 and 10 mg/kg, intravenously (n=4).

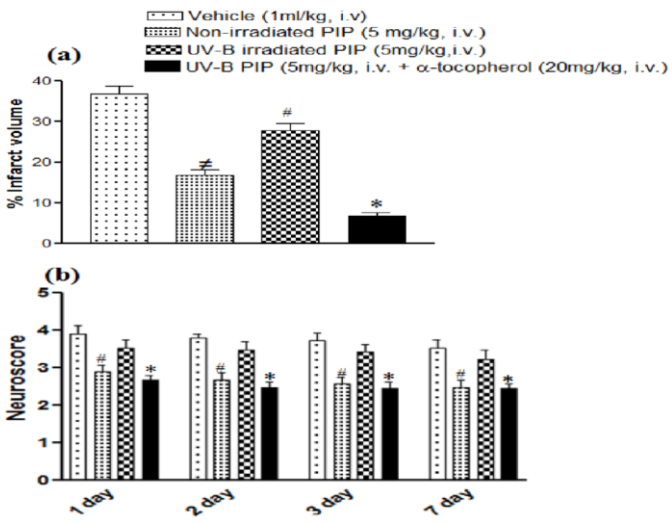


Figure 3: Estimation of percentage infarct volume and neurological parameters (Total neuroscore, % infarct volume) were examined in non-irradiated, UV-B irradiated PIP (5mg/kg, i.v.) and UV-B irradiated PIP with alpha-tocopherol (20mg/kg, i.v.) treated groups. (A) TTC staining of four coronal brain sections and quantitative analysis of infarct of UV-B and non-irradiated PIP treated TTC stained brain sections. (B) Evaluation of total neuroscore of non-irradiated, UV-B irradiated PIP and UV-B irradiated PIP with alpha-tocopherol in Post-ischemic treated groups for 1, 2, 3 and 7 day reperfusion. Values are the mean±SEM, $^{\neq}$P<.05 vs. Vehicle treated group, $^{\#}$ P<.05 vs. Non-irradiated PIP treated group,* P<.05 vs. UV-B irradiated PIP, (n=6).

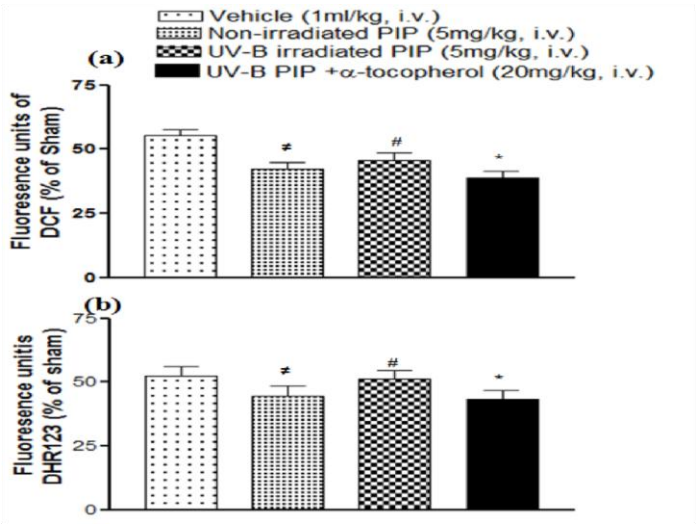


Figure. 4: Non-irradiated PIP scavenges the ROS formation in **(A)** Mitochondrial fraction sample and in **(B)** in cortical tissue of 2h ischemia and 24h reperfusion injury in SD rat. The rats were subjected to 2h MCAO and 24h reperfusion injury. Non-irradiated PIP treatment reduces the ROS production in 5mg/kg, i.v. dose. Alpha-tocopherol (20mg/kg, i.v) was given with UV-B irradiated to estimate the level of ROS in both cytosolic and mitochondrial fraction. Data are expressed as mean±SD P<0.05 vs. Vehicle treated, $^{\#}$P<0.05 vs. non-irradiated PIP, *P<0.05 vs. UV-B irradiated PIP, n=4 for each group. (n=4).

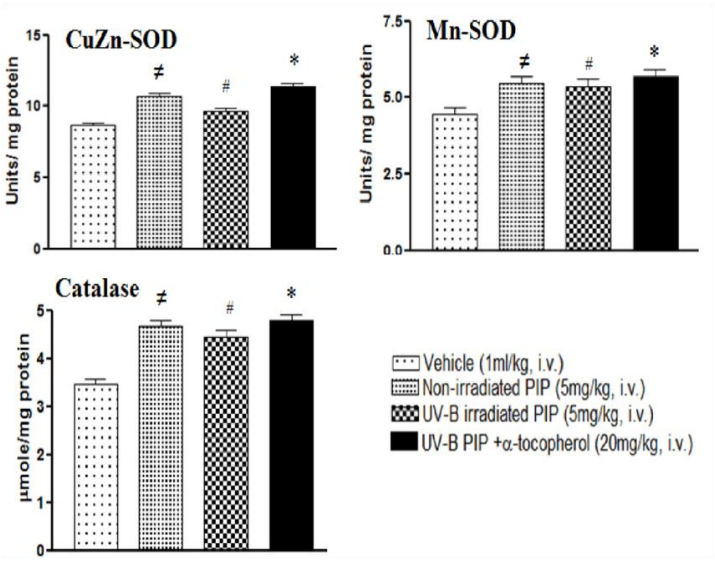


Figure. 5: Effect UV-B irradiated CUR on the endogeneous antioxidant enzyme activity of Mn-SOD, Cu/Zn-SOD and CAT in I/R injury SD rats. After 24 h reperfusion injury the level of different antioxidant Mn-SOD, CuZn-SOD, CAT level decreased significantly in I/R injury group treated with vehicle. Non-irradiated PIP increases the level of antioxidant compare to UV-B-irradiated 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. UV-B irradiated PIP, n=4 for each group.

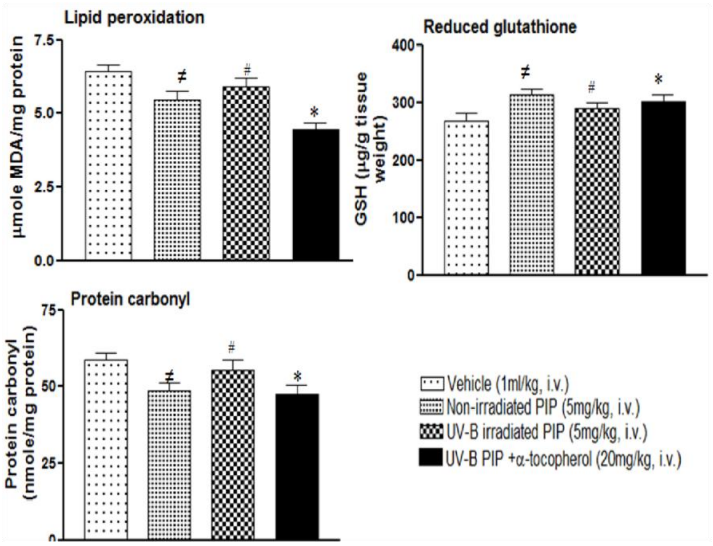


Figure. 6 Effect of lipid peroxidation**,** protein carbonyl and reduced glutathione in cortical brain subjected 2h ischemia and 24h reperfusion with treatment of UV-B irradiated and non-irradiated treatment. Ischemic rats with 30min reperfusion treated with UV-B irradiated and non-irradiated PIP (5mg/kg, i.v.) with alpha-tocopherol (20mg/kg, i.v). Data are expressed as mean±SD. ≠P<0.05 vs. Vehicle treated, #P<0.05 vs. non-irradiated PIP, *P<0.05 vs. UV-B irradiated PIP, n=4 for each group. (n=4).

(GSH, protein carbonyl and Lipid peroxidation) from decreased in vehicle treated group (Fig. 5 and 6). But the interesting results obtained that UV-B irradiated PIP was unable to change these enzymatic and non-enzymatic antioxidant (Fig. 5 and 6). Further, co-treatment of both UV-B irradiated PIP and alpha-tocopherol significantly increase the antioxidant enzyme level. This might be due alpha-tocopherol performed ROS scavenging, generated after the cumulative effect of both ischemic incident and photoproducts toxicity. This was possibly may be due to formation of multiple and ionic photoproducts. These photoproducts could be toxic to neuronal cells and can nullify the effect of antioxidant and anti-inflammatory property of PIP.

5) CONCLUSION

This is the first report on neuroprotective effect of UV-B irradiated PIP against experimental cerebral stroke. Non-irradiated PIP showed neuroprotection via reducing infarct volume and neuroscore. However the UV-B irradiated attenuated the neuroprotective efficacy via downregulating the antioxidant defence enzyme and increase the ROS level. Present study illustrated if patients are taking PIP and simultaneously exposed with sunlight its neuroprotective efficacy reduced and that will prolong the treatment process. Thus we suggested that after PIP therapy patients should not be exposed with sunlight.

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