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Modulation of UVB-induced Oxidative Stress by Ursolic Acid in Human Blood Lymphocytes*

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Abstract: UV radiation-induced damages may result in pre-cancerous and cancerous lesions and acceleration of skin aging. It involves an imbalance of the endogenous antioxidant system that leads to the increase of free radical levels. Antioxidant pretreatment might inhibit such imbalance. In the present study, the photoprotective effect of ursolic acid (UA; 3β-hydroxy-urs-12-en-28-oic acid), a dietary polyphenolic phytochemical, has been examined in the UVB-(280-320 nm) irradiated human blood lymphocytes. Lymphocytes pretreated with increasing concentrations of ursolic acid (1, 5 and 10 μg mL^{-1}) for 30 min, were irradiated and lipid peroxidation and antioxidant defense were examined. UVB-irradiated lymphocytes exhibited increased levels of lipid peroxidation and disturbances in antioxidant status. Ursolic acid pretreatment resulted in significant reduction in thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LPH) levels. Further, antioxidants like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), vitamin-C (Vit-C) and vitamin-E (Vit-E) were normalised in ursolic acid pretreated plus UVB-treated lymphocytes. The maximum dose of ursolic acid (10 μg mL^{-1}) normalized the UVB induced lipid peroxidation, indicating the photoprotective effect of ursolic acid in human peripheral lymphocytes under *in vitro* condition.

Key words: Ursolic acid, lymphocyte, UVB-radiation, photoprotection, lipid peroxidation, antioxidants, oxidative stress

INTRODUCTION

Phototoxic effect induced by UVB (280-320 nm) radiation involve the generation of Reactive Oxygen Species (ROS) resulting in oxidative damage (Wu *et al*., 2006; Steenvoorden and Beijersbergen Van Henegouewwn, 1997). ROS generated due to UVB irradiation results in DNA damage and lipid peroxidation (Katiyar *et al*., 2007). Further, reactive oxygen species are shown to activate transcription factors such as AP-1 and NF-κB, which may contribute to cell proliferation and/or apoptotic cell death (Ichihashi *et al*., 2003). It has been demonstrated previously that oxidative stress induced by UVB-radiation can lead to alteration in antioxidant enzyme levels, apoptosis and cell death (Cejkava *et al*., 2000; Kimura *et al*., 2000).

Herbal medicine derived from plant extracts is being increasingly utilized to treat a wide variety of clinical disease with relatively little knowledge of their modes of action. Polyphenols are complex group of chemicals that are widely distributed throughout the plant kingdom and thus form an integral part of the human diet (Manach *et al*., 2004). It has been suggested that dietary polyphenol protect against a variety of diseases including cancer and cardiovascular disease and there has been an increased interest in these compounds from both consumers and food manufactures (Geleijnse *et al*., 2002).

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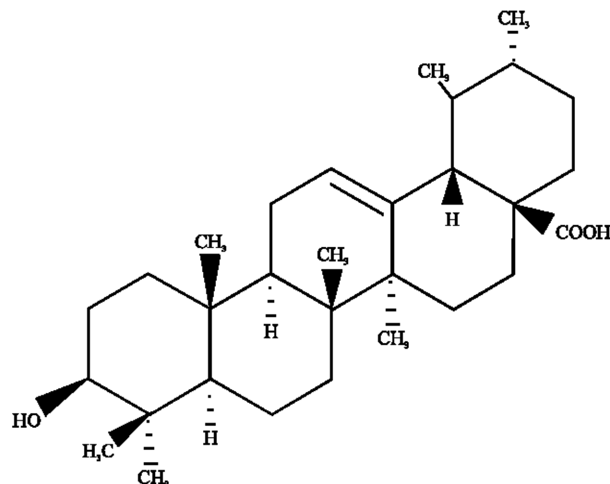

Fig. 1: Urosolic acid (3 β-hydroxy-urs-12-en-28-oic acid)

Ursolic acid (UA; 3 β-hydroxy-urs-12-en-28-oic acid), a pentacyclic triterpenoid, exists widely in natural plants, which is present in many kinds of medicinal plants, (Fig. 1) such as *Eriobotrya japonica*, *Rasmarinus officinalis* and *Glechoma hederaceae* (You *et al*., 2001) in the form of free acid or as aglycones of triterpenoid saponins (Ovesna *et al*., 2006). It exhibits antiinflammatory (Wang *et al*., 2005), anticarcinogenic (Liu, 2005), antiulcer (Hsinshiha *et al*., 2004), antihyperlipidemic (Somova *et al*., 2003) and hepatoprotective (Sarananan *et al*., 2006) activities. Although protective effect of ursolic acid against UVA was evaluated in HaCaT human keratinocytes (Lee *et al*., 2003), no sufficient work has been carried out to study its protective effect against UVB-mediated oxidative stress in human lymphocytes. Lymphocytes have been used to develop non-invasive bioassays to screen human population for toxicant exposure and these cells have been used to determine exposure and susceptibility to the toxicants (Rajendra Prasad *et al*., 2005). Lymphocytes are most studied and contain variety of redox and free radical scavenging systems (Halliwell and Gutteridge, 1998). Hence, studies on lipid peroxidation and antioxidant enzymes in blood lymphocytes could be of immense significance in identifying intracellular oxidative damage in the individuals, who could be at risk to UVB induced oxidative damage. The purpose of the present study was to evaluate the impact of ursolic acid on UVB-mediated oxidative stress in human lymphocytes under *in vitro* condition

MATERIALS AND METHODS

Chemicals

Ursolic acid, heat inactivated fetal calf serum (FCS), thiobarbituric acid (TBA), phenozine methosulphate (PMS) nitroblue tetrazolium (NBT), 5,5-dithiobis 2-nitrobenzoic acid (DTNB) and nicotinamide adenine dinucleotide (NAD) were purchased from (Sigma chemical Co., St. Louis, USA). Other chemicals for blood lymphocyte cultures (RPMI-1640, penicillin, streptomycin, L-glutamine) and reduced glutathione (GSH) were purchased from (Himedia, Mumbai). All other chemicals and solvents were of analytical grade and obtained from (SD Fine Chemical, Mumbai and Fisher. Inorganic and Aromatic Limited, Chennai).

Blood Samples

Blood samples were aseptically collected in heparinized sterile tubes from median cubital vein of non smoking healthy individuals (22-25 years). Lymphocytes were isolated using Ficoll–Histopaque (Sigma, USA) and cultured as described provisionally (Boyum, 1968). Blood was diluted 1:1 with Phosphate Buffered Saline (PBS) and layered onto histopaque/with ratio of blood and PBS; Histopaque maintained at 4:3. The blood was centrifuged at 1340 rpm for 35 min at room temperature. The lymphocyte layer was removed and washed twice in PBS at 1200 rpm for 10 min each and then washed with (RPM1-1640) media.

Study Design

Cultured lymphocytes were divided into six groups; in each group six samples were processed.

Group 1: Normal lymphocytes without any treatment.
Group 2: Normal lymphocytes with 10 μg mL^{-1} of ursolic acid.
Group 3: UVB-irradiated lymphocytes for 30 min.
Group 4: UVB-irradiated lymphocytes pretreated with 1 μg mL^{-1} of ursolic acid.
Group 5: UVB-irradiated lymphocyte pretreated with 5 μg mL^{-1} of ursolic acid.
Group 6: UVB-irradiated lymphocytes pretreated with 10 μg mL^{-1} of ursolic acid.

Treatment of the Cells

Thirty minutes prior to irradiation three test-doses (1, 5 and 10 μg mL^{-1}) of ursolic acid were added to the grouped normal lymphocytes. Preliminary studies were carried out to ensure that whether this concentration had any toxic effect by trypan blue dye exclusion test. Before exposure to UV light, the cell cultures were washed twice with PBS. Non-irradiated lymphocytes showed decrease in viability over the 30 min period of incubation.

Irradiation Procedure

For UVB irradiation cells were irradiated in 35 mm Petri dishes containing 2 mL of PBS and covered with a UV permeable with a UV permeable membrane to prevent contamination. A battery of TL 20W/20 fluorescent tubes (Heber scientific) served as UVB source which had a wave length range set 280-320 nm peaked at 312 nm and an intensity of 2.2 mW cm^{-2} for 9 min. The total UVB-irradiation was 19.8 mJ cm^{-2}, corresponding to an average value of 1.52×0^{-3} mJ $cell^{-1}$. After irradiation the lymphocytes were kept at room temperature for 30 min and then subjected to biochemical assays.

Biochemical Estimation

Lymphocytes were suspended in 130 mM KCl plus 50 mM PBS containing 0.1 mL of 0.1 M dithiothreitol and centrifuged at 20,000 x g for 15 min (4°C). The supernatant was taken for biochemical estimations. In each group six samples (n = 6) were processed. The level of lipid peroxidation was determined by analyzing TBA-reactive substance according to the protocol of Niehaus and Samuelson (1968). The pink coloured chromogen formed by the reaction of 2-TBA with breakdown products of lipid peroxidation was measured. The lipid hydroperoxides (LPH) levels were determined by analyzing BHT-reactive substance according to the protocol of Jiang *et al*. (1992). Superoxide dismutase (SOD) activity was assayed by the method of Kakkar *et al*. (1984), based on the inhibition of the formation of (NADH-PMS-NBT) complex. Catalase (CAT) activity was assayed by the procedure of Sinha (1972) quantifying the hydrogen peroxide after reacting with dichromate in acetic acid. The activity of glutathione peroxidase (GPX) was assayed by the method of Rotruck *et al*. (1973) a known amount of enzyme preparation was allowed to react with hydrogen peroxide (H_2O_2) and GSH for a specified time period. Then the GSH content remaining after the

reaction was measured. The total GSH context was measured by the method of Elliman (1959). This method was based on the development of a yellow colour when 5,5-dithiobis 2-nitrobenzoic acid was added to compounds containing sulphydryl groups. The ascorbic acid was estimated by the methods of Roe and Kuether (1969) the red coloured compound when treated with sulphuric acid and then adding 2,4 - dinitrophenyl hydrazine in the presence of thiourea solution. α-tocopheral was estimated by the method described by Baker *et al*. (1980).

Statistical Analysis

Statistical analysis was performed by one-way (ANOVA) followed by DMRT taking $p<0.05$ to test the significant difference between groups.

RESULTS

In this study, the concentration of TBARS and LPH increased significantly in UVB irradiated lymphocytes (Table 1). Ursolic acid pretreated lymphocytes showed progressively decreased concentrations of TBARS and LPH when compared with UVB-irradiated cells and even 1 μg mL^{-1} of ursolic acid pretreatment significantly decreased the levels of lipid peroxidation indices in UVB-irradiated lymphocytes. UVB-exposure significantly decrease the SOD, CAT activities in this study and pretreatment with ursolic acid results in significant increase in the SOD, CAT activities as ursolic acid concentration increases (Table 2). Present study also shows (Table 3) that UVB-irradiation caused

Table 1: Effect of ursolic acid on the levels of TBARS and LPH in normal, UVB-irradiated and ursolic acid pretreated lymphocytes

Groups	TBARS (nmol mg^{-1} protein)	LPH (nmol mg^{-1} protein)
Normal	1.50 ± 0.26^{a}	4.87 ± 0.60^{ab}
Normal+ursolic acid (10 μg mL^{-1})	1.45 ± 0.2^{a}	4.36 ± 0.44^{a}
UVB-irradiation	5.65 ± 0.32^{d}	9.34 ± 0.51^{d}
UVB+ursolic acid (1 μg mL^{-1})	3.25 ± 0.43^{c}	7.33 ± 0.49^{c}
UVB+ursolic cacid (5 μg mL^{-1})	1.90 ± 0.22^{b}	6.58 ± 0.35^{bc}
UVB+ursolic acid (10 μg mL^{-1})	1.60 ± 0.22^{a}	5.14 ± 0.26^{b}

Values are given as means±SD of six experiments in each group; Values not sharing a common superscript different significantly at $p<0.05$ (DMRT)

Table 2: Effect of ursolic acid on the activities of SOD and CAT in normal, UVB-irradiated and ursolic acid pretreated lymphocytes

Groups	SOD (unit* mg^{-1} protein)	CAT (unit** mg^{-1} protein)
Normal	13.98 ± 1.22^{a}	12.02 ± 0.76^{a}
Normal+ursolic acid (10 μg mL^{-1})	14.44 ± 1.30^{a}	12.24 ± 0.33^{a}
UVB-irradiation	8.78 ± 0.84^{d}	7.06 ± 0.51^{de}
UVB+ursolic acid (1 μg mL^{-1})	10.30 ± 0.81^{c}	9.22 ± 0.60^{d}
UVB+ursolic acid (5 μg mL^{-1})	12.34 ± 0.84^{b}	10.33 ± 0.38^{c}
UVB+ursolic acid (10 μg mL^{-1})	13.45 ± 0.67^{a}	11.82 ± 0.43^{ab}

Values are given as means±SD of six experiments in each group; Values not sharing a common superscript different significantly at $p<0.05$ (DMRT); *: Enzyme concentration required for 50% inhibition of nitroblue tetrazolium reduction in 1 min; **: μmol of hydrogen peroxide consumed per min

Table 3: Effect of ursolic acid on the GPx activities and GSH levels in normal, UVB-irradiated and ursolic acid pretreated lymphocytes

Groups	GPx (unit*** mg^{-1} protein)	GSH (mg dL^{-1})
Normal	12.13 ± 1.06^{ab}	19.49 ± 0.96^{a}
Normal+ursolic acid (10 μg mL^{-1})	12.75 ± 0.89^{a}	19.75 ± 0.56^{a}
UVB-irradiation	7.41 ± 0.88^{d}	12.17 ± 1.08^{d}
UVB+ursolicacid (1 μg mL^{-1})	8.85 ± 0.74^{c}	13.46 ± 0.78^{c}
UVB+ursolic acid (5 μg mL^{-1})	11.44 ± 0.84^{b}	16.26 ± 0.78^{b}
UVB+ursolic acid (10 μg mL^{-1})	12.28 ± 0.81^{ab}	18.97 ± 0.81^{a}

Values are given as means±SD of six experiments in each group; Values not sharing a common superscript different significantly at $p<0.05$ (DMRT); ***: μg of glutathione consumed per min

Table 4: Effect of ursolic acid on the levels of Vit-C and Vit-E in normal, UVB-irradiated and ursolic acid pretreated lymphocytes

Groups	Vit-C (mg dL^{-1})	Vit-D (mg dL^{-1})
Normal	2.46±0.11^{ab}	1.54±0.04^{a}
Normal+ursolic acid (10 µg mL^{-1})	2.98±0.22^{a}	1.65±0.07^{a}
UVB-irradiation	0.81±0.19^{d}	0.36±0.04^{e}
UVB+ursolic acid (1 µg mL^{-1})	1.17±0.21^{c}	0.58±0.02^{cd}
UVB+ursolic acid (5 µg mL^{-1})	1.68±0.21^{b}	0.93±0.07^{b}
UVB+ursolic acid (10 µg mL^{-1})	2.12±0.13^{a}	1.45±0.05^{ab}

Values are given as mean±SD of six experiments in each group. Values not sharing a common superscript different significantly at $p \leq 0.05$ (DMRT)

a significant decrease in the GPx activities and GSH levels when compared with the normal lymphocytes. Ursolic acid pretreatment significantly restored the GPx activities and GSH levels to normal when compared with UVB-exposed groups. UVB-irradiated group decrease vit-C, vit-E levels and pretreatment with ursolic acid result in significantly increases in the vit-C, vit-E levels as ursolic acid concentration increases (Table 4).

DISCUSSION

The studies on development of novel agents with anti-photoaging capabilities particularly from natural resources including various plants have been intensively performed. The UVB radiation is the most described physical attack it causes cellular damage resulting in both pre-cancerous and cancerous lesions and acceleration of aging (Casagrande *et al*., 2006). Probably the genesis of pathologies due to UVB exposure is a consequence of the generation of free radicals. The resulting imbalance between oxidants and antioxidants shifts the redox-sensitive signal transduction pathways and gene expression. These molecular changes may be involved in the pathogenesis of photo damages (Fuchs, 1998).

In this study the levels of lipid peroxidation has been significantly increased in UVB irradiated cells (Table 1). The increase in the levels of TBARS and LPH indicates the activation of lipid peroxidation in UVB-irradiated lymphocytes. Lipid peroxidation induced by UVB-radiation is known to be due to the attack of free radicals on the fatty acid component of membrane lipids. Present results shows that ursolic acid renders protection against UVB-radiation induced oxidative stress. This may be due to its antioxidative property. The antioxidant effects of ursolic acid on lipid peroxidation in liver microsomes, leukemic cells and myocardial cell were already documented (Sarananan *et al*., 2006; Ovesna *et al*., 2006; Senthil *et al*., 2007).

The free radical scavenging and antioxidant property of ursolic acid have been recently proved by Dufour *et al*. (2007). It was thought that this antioxidant property is due to the polyphenolic methyl group present in ursolic acid (Zhang *et al*., 2001). In this study reduced SOD, CAT, GPx activities and GSH levels were observed in UVB-irradiated lymphocytes (Table 2 and 3). Similar results were obtained by Cajkova *et al*. (2000) in corneal epithelium cells and by Isoherranen *et al*. (1997) in He La cells, when these cells were exposed to UVB-irradiation. SOD protects the cells against superoxide radical, which can damage the membrane (Michaelson, 1977). CAT primarily causes decomposition of hydrogen peroxide (H_2O_2) to H_2O at a much faster rate GPx also plays an important role in the removal of lipid hydroperoxides. Therefore a reduction in the activity of these enzymes during UVB-exposure can result in a number of deleterious effects due to the accumulation of superoxide radicals and H_2O_2. Pretreatment with ursolic acid increased the activities of SOD, CAT in UVB-irradiated lymphocytes and thus ursolic acid could exert a beneficial action against pathological alterations caused by the UVB-radiation. Further the increased activity of SOD, CAT, GPx and GSH in UVB-irradiated lymphocytes is mainly because of the antioxidant sparing action of ursolic acid. Since ursolic acid prevents the formation of ROS the syntheses of these enzymes are not affected (Mortin-Aragon *et al*., 2001).

Further present study shows (Table 4) UVB-irradiation caused a significant decrease in the levels of Vit-C and Vit-E in irradiated groups when compared with the normal lymphocytes. The observed decrease in the levels Vit-E and Vit-C may be due to their increased utilization for scavenging hydroxy and/or oxygen derived radicals. Vitamin-C and Vit-E may play a role in preventing lipid peroxidation under experimental and clinical conditions. Lymphocytes with ursolic acid (1, 5 and 10 μg mL^{-1}) prior to irradiation protected Vit-C and Vit-E depletion resulting from the radiation effect. In this study 10 μg mL^{-1} of ursolic acid pretreatment protects Vit-C and Vit-E levels in UVB-irradiated lymphocytes. The results shows that ursolic acid renders protection against UVB-radiation induced oxidative stress. Previously, ursolic acid and other triterpenes have been reported to show photoprotective activity by inhibiting UV-modulated signal transduction pathways in various experimental models (Both *et al*., 2002; Yarosh *et al*., 2000). Studies shows ursolic acid has significantly suppressed the UVA-induced reactive oxygen species production, lipid peroxidation and p53 accumulation in HaCaT human keratinocytes (Lee *et al*., 2003).

CONCLUSION

It is evident from the present study that ursolic acid offers a remarkable protection against UVB-induced oxidative stress. According to our data and those previously reported in the literature, the photoprotective activity in terms of inhibition of lipid peroxidation and sustaining antioxidant status could explain the beneficial action of ursolic acid against pathological alterations caused by the presence of free radicals which occur during UVB exposure.

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