Hesperidin, A Citrus Bioflavonoid Reduces the Oxidative Stress in the Skin of Mouse Exposed to Partial Body $\gamma$-Radiation

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Abstract

Generation of reactive oxygen species (ROS) is one of the important and early events after exposure to ionizing radiations and this ROS production is responsible for the degenerative changes ensuing irradiation. An attempt has been made to modulate the radiation-induced-ROS by hesperidin (hesperitin-7-rhamnoglucoside), a bioflavonoid in the wounded skin of irradiated mouse. The lower half of the animals was shaved and the animals were orally administered or not with 100 mg/kg body weight of hesperidin before exposure to 6 Gy of partial body gamma-radiation. The activities of glutathione peroxidase, superoxide dismutase and glutathione concentration as well as lipid peroxidation were estimated in the skin of mouse at 0, 1.5, 3, 6, 12, 24 and 48 h post-irradiation. Irradiation of mouse to 6 Gy caused a significant depletion in the activities of glutathione peroxidase, superoxide dismutase as well as glutathione concentration. Exposure of mouse to 6 Gy resulted in a significant elevation in lipid peroxidation when compared to the base line levels of lipid peroxidation. Administration of hesperidin before hemi-body exposure to 6 Gy $\gamma$-rays significantly raised the activities of glutathione peroxidase, superoxide dismutase and glutathione concentration, whereas hesperidin pretreatment caused a significant reduction in the radiation induced lipid peroxidation. The present study demonstrates that hesperidin pretreatment reduces the radiation induced oxidative stress in the irradiated wounds of mouse and may be useful paradigm to reduce the radiation-induced oxidative stress before or after surgery.

Keywords: Hesperidin; Mice; Wound; Irradiation; Glutathione; Superoxide dismutase; Lipid peroxidation

Introduction

Although radiation injury has been an infrequent occurrence in our society, an expanding use of radioactive materials in industry, medicine, science, military and localized areas of high radiation within nuclear facilities, has significantly increased the potential of exposure to excessive radiation. Fortunately, there is always the potential for deliberate misuse of radioactive materials in public by terrorists, which could lead to undesirable side effects or even mass casualties depending on the irradiation dose and duration of exposure [1]. Since the skin provides a protective barrier over the body surface, it is the first tissue that will be directly exposed to ionizing radiations during accidental or intentional exposures [2,3]. Most of the cell damage caused by ionizing radiation is mediated by the reactive oxygen species (ROS) generated from radiolysis of water that are responsible for inducing oxidative stress in the skin [4,5]. Some of the general events associated with an early phase of oxidative stress response in the skin are depletion of endogenous intra- and intercellular antioxidants, enhancement of intracellular lipid peroxidation levels [6-8] and the induction of specific signal transduction pathways that can negatively modulate inflammatory, immune suppressive or apoptotic responses in the skin [9,7].

Although skin is equipped with an extensive and most effective network of antioxidant system [10,11], the excessive production of free radicals by various agents including ionizing radiations may escape this surveillance system of skin, and induce substantial damage to different cutaneous constituents. This failure of surveillance system may cause overwhelming of skin defense mechanisms leading to extensive damage to skin depending on irradiation dose. Under normal conditions, the generation of free radicals is counterbalanced by the presence of adequate cutaneous endogenous antioxidants [10,11]. However, when the generation of free radicals exceeds beyond the capacity of the defense systems, these highly reactive radicals may produce substantial damage to the skin, which may be reversible or irreversible depending on the irradiation dose. Safe and effective exogenous supply of antioxidants and protectants that scavenge ROS as well as restore normal redox state in the skin could be required to maintain antioxidant/pro-oxidant balance in vivo and improve the cellular defense system of the skin [12-14].

Hesperidin (hesperitin-7-rhamnoglucoside or hesperitin-7-rutinoside), is a predominant bioflavonoid found in various citrus fruits [15]. It and has been reported to possess significant anti-inflammatory, analgesic, antihypertensive, diuretic, antibacterial, antioxidant, antidepressant and antiviral activities [16-21]. Hesperidin has been reported to inhibit tumor initiation and promotion and reverse the neoplastic transformation of C3H10T1/2 fibroblasts in vitro [22]. Hesperidin has been found to reduce cholesterol levels in humans [23] and retard bone loss [24]. Hesperidin possesses beneficial effects on the abnormal capillary permeability, fragility and protects against various traumas and stresses [25]. Hesperidin has been found to be non-toxic in animals and humans [26,27]. The extracts from citrus plants, which are rich in flavonoids, are being increasingly used in the cosmetics including skin creams [28]. Both the hesperidin and its aglycone hesperitin have been reported to possess a wide range of pharmacological properties. The pleiotropic action, non-toxic nature, presence of hesperidin in citrus fruits and practical/medical importance of the effect of radiation on wound healing stimulated us to investigate the effect of hesperidin on the antioxidant status and lipid peroxidation during the healing of excision skin wounds of mice hemi-body exposed to 6 Gy of gamma radiation.

Materials and Methods

Chemicals

Hesperidin and trichloroacetic acid (TCA) were procured from Acros Organics Ltd, Geel, Belgium, whereas glutathione, 2-thiobarbituric acid (TBA), 5,5-dithiobis(2-nitrobenzoic acid) chemicals

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were removed by an electrical hair clipper (Wahl Clipper Corporation, Illinois, USA) before irradiation. Skin biopsies from each group were collected at 0, 1.5, 3, 6, 12, 24, and 48 h after the exposure.

**Biochemical estimation**

The skin was freed of *panniculus carnosus* and flash frozen in the liquid nitrogen. The skin was weighed and homogenized in the phosphate buffered saline. Six animals were used in each group. The antioxidant status and lipid peroxidation were estimated in the mouse skin as follows:

**Glutathione (GSH)**

GSH concentration was estimated by the method of Moron et al. [29]. Briefly, proteins were precipitated by 25% TCA, centrifuged and the supernatant was collected. The supernatant was mixed with 0.2 M sodium phosphate buffer pH 8.0 and 0.06 mM DTNB and incubated for 10 minutes at room temperature. The absorbance of the sample/s was read against the blank at 412 nm in a double beam UV-visible spectrophotometer (Shimadzu UV-260, Shimadzu Corp, Tokyo, Japan), and the GSH concentration was calculated from the standard curve.

**Superoxide dismutase (SOD)**

For the estimation of SOD the method described by Fried [30] has been followed. Briefly, 900 µl phosphate buffer was mixed with 100 µl each of tissue homogenate (T), nitroblue tetrazolium (NBT), phenazine methosulphate and NADH. The control (C) comprised of buffer and the homogenate without any reagents. The absorbance of the sample/s was recorded against the blank at 560 nm using a double beam UV-visible spectrophotometer and the enzyme activity has been expressed in units (1 U = 50% inhibition of NBT reduction).

**Glutathione peroxidase (GSHPx)**

GSHPx activity was estimated by the method of Szazuka et al. [31]. Briefly, 100 µl skin homogenate was mixed with 200 µl each of EDTA, sodium azide, GSH, H₂O₂ and 400 µl buffer. The reaction mixture was incubated at 37°C for 10 minutes followed by the addition of 10% TCA. After centrifugation, the supernatant was collected and mixed with 3 ml disodium hydrogen phosphate and 1ml DTNB. The absorbance of the sample/s was recorded against the blank at 412 nm using double beam UV-visible spectrophotometer. The enzyme activity has been expressed as µmol GSH/mg protein.

**Lipid peroxidation (LOO)**

LOO was measured by the method of Beusege and Aust [32]. Briefly, the tissue homogenate was mixed with TCA-TBA-HCl solution, butylated hydroxy toluene (BHT, 3.5 mM, 0.1 ml) and diethylenetriaminopentaacetic acid (DETAPAC, 70 µmol, 0.1 ml). The mixture was heated for 15 min in a boiling water bath. After centrifugation the absorbance was recorded at 535 nm using a UV-visible spectrophotometer.

**Estimation of protein**

Protein contents were measured by the method of using bovine serum albumin (BSA) as the standard.

**Analysis of Data**

The statistical significance between various groups was determined using one way ANOVA. Solo 4 statistical package (BMDD Statistical Software, Inc., Los Angeles, CA, USA) was used for data analysis. All the data are expressed as mean ± SEM (standard error of the mean).
Results

The results are expressed as GSH concentration (nM/g tissue), activities of superoxide dismutase (U mg/protein), glutathione peroxidase (mM GSH/mg protein) and lipid peroxidation (MDA nM/mg protein) in Table 1 and Figures 1-4.

Glutathione (GSH)

Infliction of wound resulted in an immediate decline in the GSH concentration up to 3 h that elevated at 6 h post-irradiation in DDW+sham-irradiation group. A second decrement in GSH concentration was observed at 48 h post-irradiation. Treatment of mice with hesperidin before sham-irradiation resulted in a significant (p<0.01) elevation in the GSH contents up to 24 h post-irradiation and reached to baseline levels thereafter (Figure 1). The irradiation of animals to 6 Gy whole-body gamma-radiation caused a drastic reduction in the GSH concentration and a nadir was reached at 3 h post-irradiation with a significant elevation thereafter Hesperidin pretreatment resulted in a significant (p<0.05) elevation in GSH concentration when compared with DDW+irradiation group (Figure 1). However, normal levels could not be restored even by 48 h post-irradiation in both DDW+irradiation and HPD+irradiation groups (Table 1).

Glutathione peroxidase (GSHPx)

The activity of GSHPx fluctuated with time in DDW+sham-irradiation group, where a maximum elevation was observed at 1.5 h post-irradiation. An abrupt decline in GSHPx activity was observed at 3 h that continued up to 6 h post-irradiation. The GSHPx activity increased thereafter up to 24 h and remained unaltered (Figure 2). Hesperidin treatment caused a significant elevation in the GSHPx activity in HPD+sham-irradiation group when compared
to DDW+sham-irradiation group. This increase in GSHPx activity was approximately 2.2 and 3.6 folds at 1.5 and 24 h post-irradiation, respectively. Exposure of animals to 6 Gy resulted in a decline in GSHPx activity at all post-irradiation times (Figure 2). Treatment of mice with hesperidin before irradiation caused a significant elevation in GSHPx activity and this increase was 2.5 and 4 folds at 1.5 and 24 h post-irradiation, respectively, when compared to DDW+irradiation group (Table 2).

**Superoxide dismutase (SOD)**

The activity of SOD showed a sudden rise at 1.5 h post-irradiation after wound creation and a decline thereafter up to 12 h, where a nadir was observed. There has been an abrupt increase in the SOD activity at 24 h that decreased at 48 h post-irradiation in DDW+sham-irradiation group. However, the SOD activity was significantly lower in the DDW+sham-irradiation group when compared to the base line activity. The pattern of elevation in HPD+ sham-irradiation group was similar to that of DDW+ sham-irradiation group except that hesperidin treatment resulted in an elevation in SOD activity (Figure 3). The exposure of mice to 6 Gy whole-body radiation caused a significant alleviation in the SOD activity when compared with the DDW+ sham-irradiation group. Hesperidin treatment before exposure to radiation caused a significant (p<0.05, for 1.5 h post-irradiation) augmentation in the SOD activity in HPD+irradiation group when compared with concurrent DDW+ irradiation group (Figure 3). The pattern of SOD activity in HPD+irradiation group was similar to that of DDW+irradiation group (Figure 3). An increase in SOD activity in all groups was biphasic, first increase was observed at 1.5 h, where a peak SOD activity was observed and a second phase of elevation was observed at 24 h post-irradiation. However, this elevation was lesser than that of 1.5 h post-irradiation (Table 1). Treatment of mice with HPD before 6 Gy irradiation caused a significant elevation in the SOD activity from 1.5 to 24 h post-irradiation (Table 3).

**Lipid peroxidation (LOO)**

Infliction of excision wound caused an elevation in LOO in DDW+sham-irradiation group. Induction of LOO fluctuated with time in both the DDW+sham-irradiation and HPD+sham-irradiation groups, and a highest rise was observed at 3 h post-irradiation. The LOO showed a gradual decline thereafter. Hesperidin treatment caused a significant (p<0.05) decrement in the spontaneous levels of LOO up to 48 h post-irradiation in HPD+sham-irradiation group. Irradiation of animals to 6 Gy γ-rays resulted in a significant rise in LOO and a highest increase in LOO was observed at 3 h post-irradiation, where an approximate 1.5 fold in LOO elevation was observed when compared to DDW+sham-irradiation group (Table 4). The hesperidin pretreatment afforded significant (p<0.05) protection against the radiation-induced rise in the lipid peroxidation. However, the normal levels could not be restored even by 48 h post-irradiation (Figure 4). The pattern of lipid peroxidation was similar in all the groups (Figure 4).

### Discussion

The skin act as barrier between the external and internal environments of the body and it is the first body tissue, which has to bear the brunt of radiation exposure during diagnosis and treatment Ryan [7]. In spite of the presence of an elaborate antioxidant defense network to deal with oxidative stress Shindo et al. [10], exposure of skin to ionizing radiation produces a burst of free radicals that can overwhelm the cutaneous antioxidant system, leading to oxidative damage, which could lead to immunosuppression, premature skin aging and skin cancer [33,34]. Therefore, it seems reasonable to assume if oral supplementation with herbal antioxidants could be a useful paradigm in the prevention or treatment of skin injuries especially those mediated by ionizing irradiations. Hesperidin (hesperitin-7-rhamnoglucoside or hesperitin-7-rutinoside), a predominant citrus bioflavonoid has been investigated for its ability to modulate the radiation-induced oxidative stress in the skin of mice exposed to 6 Gy hemi-body gamma irradiation.

The possibility of direct quantitative comparison of results of the present study with those reported by other investigators may not be prudent due the use of varying experimental protocols and assay conditions (pH, temperature, reagent concentration etc.) and different unit definitions. Despite this the results of our study are qualitatively in conformation with those obtained in other similar type of studies [13,35]. The ionizing radiations induce free radicals and OH radical is one of the predominant radical species produced by radiation and the interaction of OH free radicals with biological molecules gives rise to peroxy radicals leading to the formation of lipid peroxidation in the biological systems [13,14,36]. An abrupt decline in the SOD, and GSHPx activities and glutathione concentration has been observed.

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**Table 2:** Effect of Hesperidin on the Glutathione peroxidase and Superoxide dismutase activity in mice skin exposed to 6 Gy hemi-body gamma irradiation.

<table>
<thead>
<tr>
<th>Post-irradiation time (h)</th>
<th>Glutathione peroxidase activity (µM GSH/mg protein) ± SEM</th>
<th>SOD activity (µmol Mn/mg protein) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DDW</td>
<td>Hesperidin (HPD)</td>
</tr>
<tr>
<td>0</td>
<td>0.31 ± 0.03</td>
<td>0.32 ± 0.11</td>
</tr>
<tr>
<td>1.5</td>
<td>0.37 ± 0.09</td>
<td>0.81 ± 0.10a</td>
</tr>
<tr>
<td>3</td>
<td>0.20 ± 0.12</td>
<td>0.57 ± 0.08a</td>
</tr>
<tr>
<td>6</td>
<td>0.17 ± 0.02</td>
<td>0.50 ± 0.10a</td>
</tr>
<tr>
<td>12</td>
<td>0.19 ± 0.07</td>
<td>0.55 ± 0.09a</td>
</tr>
<tr>
<td>24</td>
<td>0.20 ± 0.11</td>
<td>0.56 ± 0.11a</td>
</tr>
<tr>
<td>48</td>
<td>0.18 ± 0.33</td>
<td>0.34 ± 0.03</td>
</tr>
</tbody>
</table>

*p<0.05 when DDW group compared with HPD group. DDW: Distilled water; HPD: Hesperidin; IR: Irradiation.*

**Table 3:** Effect of Hesperidin on the superoxide dismutase activity in mice skin exposed to 6 Gy hemi-body gamma irradiation.

<table>
<thead>
<tr>
<th>Post-irradiation time (h)</th>
<th>Superoxide dismutase activity (U/mg protein) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DDW</td>
</tr>
<tr>
<td>0</td>
<td>23.01 ± 0.51</td>
</tr>
<tr>
<td>1.5</td>
<td>34.57 ± 0.56</td>
</tr>
<tr>
<td>3</td>
<td>25.46 ± 0.40</td>
</tr>
<tr>
<td>6</td>
<td>24.30 ± 0.58</td>
</tr>
<tr>
<td>12</td>
<td>21.21 ± 0.64</td>
</tr>
<tr>
<td>24</td>
<td>30.82 ± 0.53</td>
</tr>
<tr>
<td>48</td>
<td>29.78 ± 0.99</td>
</tr>
</tbody>
</table>

*p<0.05 when DDW group compared with HPD group. DDW: Distilled Water; HPD: Hesperidin; IR: Irradiation.*
in the mouse skin exposed to hemi-body γ-radiation. Irradiation of mice has been reported to reduce the activities of GSHPx, SOD as well GSH concentration earlier [13,14,36,37]. An identical effect was observed in the erythrocytes of rats exposed to radiation [38]. Increase in lipid peroxidation in the skin after 6 Gy irradiation is in agreement with earlier studies where exposure of mice to gamma-rays has been reported to increase lipid peroxidation in vivo [13,14,36]. Ionizing radiations have been reported to enhance lipid peroxidation in various study systems earlier [13,36,39].

The reports regarding the effect of hesperidin on the radiation-induced changes in skin antioxidants are not available and this is the first report where hesperidin pretreatment has been found to protect against the radiation-induced elevation in lipid peroxidation and the radiation-induced decline in GSHPx and SOD activity and GSH concentration. These observations are in conformation with earlier report where, curcumin has been reported to protect against the radiation-induced alleviation in the GSHPx and SOD activities, GSH concentration and radiation-induced rise in the lipid peroxidation in irradiated skin [14]. However, hesperidin treatment has been reported to protect against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine induced decline in GSH, GSHPx and SOD in mice [40]. Several other nutraceuticals including naringin, ascorbic acid, ginger, and Aegle marmole has been reported to counteract the negative effect of ionizing radiation on GSHPx and SOD activities and GSH concentration and protect against the radiation-induced lipid peroxidation earlier [13,36,37].

Glutathione, a tripeptide is an important biomolecule most abundant non-protein thiol that acts synergistically with other endogenous antioxidants. It also acts as a cofactor with the enzyme glutathione peroxidase to neutralize excess free radicals. GSH is actively involved in the, detoxification of xenobiotics [41,42]. The depletion in skin GSH peroxidase also plays an important role in protecting the cell proteins and membrane against oxidation [43]. Glutathione peroxidase also plays an important role in the transcription of antioxidant related genes [51]. Irradiation has been reported to downregulate the Nrf2 pathway [52]. Therefore upregulation of Nrf2 pathways by hesperidin may have increased the GSHPx, SOD activities and GSH concentration. A recent study showed that hesperidin increased the expression of SOD mRNA and Nrf2 in rat cardiac tissue [53].

The exact mechanism of action of hesperidin in increasing the antioxidant status of irradiated wound is not known. The hesperidin would have employed multiple putative mechanisms to attenuate the radiation-induced oxidative stress in the skin. The most important mechanism of action may be the neutralization of radiation-induced free radicals by hesperidin. The hesperidin has been reported to scavenge free radicals earlier [49]. Ionizing radiation has been reported to elevate several inflammatory cytokines and chemokines in the skin [7] that would have led to the depletion of various antioxidants and increase in lipid peroxidation, whereas hesperidin pretreatment may have arrested the radiation-induced rise in their expression in the present study. Hesperidin has been reported to reduce 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced inflammatory cytokines [50]. The increased antioxidant status may be due to the increase in mRNA of GSH, GSHPx and SOD by hesperidin treatment. The Nrf2 signaling pathway plays a significant role in the transcription of antioxidant related genes [51]. Irradiation has been reported to downregulate the Nrf2 pathway [52]. Therefore upregulation of Nrf2 pathways by hesperidin may have increased the GSHPx concentration, and GSHPx and SOD activities. A recent study showed that hesperidin increased the expression of SOD mRNA and Nrf2 in rat cardiac tissue [53].

In conclusion, the present study demonstrates that hesperidin administration prior to irradiation significantly raised the activities of GSHPx, SOD and elevated GSH contents accompanied by a reduced lipid peroxidation in mice skin exposed to hemi-body gamma radiation. The radiation induced decline in GSHPx, and SOD activities and GSH concentration may be due to down regulation of Nrf2 and subsequently their mRNA and also their consumption in radiation-induced radical scavenging, whereas pretreatment of irradiated mice with hesperidin may have upregulated the Nrf2 transcription and subsequently the mRNAs of GSH, GSHPx and SOD causing their significant elevation in presence study. Our study indicates that hesperidin may be suitable drug to protect the skin against the radiation-induced oxidative stress in clinical conditions.

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