Hesperidin, a Citrus Bioflavonoid Potentiates Repair and Regeneration of Deep Dermal Excision Wounds of Mice Whole Body Exposed to Different Doses of 60Co γ-Radiation

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Abstract

Irradiation adversely affects the repair and regeneration of wounds causing negative impact on the clinical outcome. Therefore, it is necessary to screen newer paradigms that may help to mitigate the deleterious effects of ionizing radiations. Hesperidin (hesperitin-7-rhamnoglucoside or hesperitin-7-rutinoside), a predominant bioflavonoid found in citrus fruits has been evaluated for its wound healing activity in mice whole-body exposed to 2,4, 6 or 8 Gy of γ-radiation. A full-thickness skin wound was created on the dorsum of mice after exposure to various doses of γ-radiation and the wound repair and regeneration was assessed by capturing video images of the excision wounds periodically as a measure of wound contraction as well as by assessing the mean wound healing time. In addition, the biochemical profiles including collagen, hexosamine, DNA, and nitric oxide syntheses were estimated in the granulation tissues at various days post-irradiation after exposure to 0 or 6 Gy. In a separate experiment histological information on fibroblast and blood vessel densities were collected in the regenerating granulation tissue at various post-irradiation days after 0 or 6 Gy irradiation. The whole-body exposure of mice to different doses of γ-radiation resulted in a dose dependent delay in the wound contraction and prolongation of wound healing time, where the highest delay was observed after 8 Gy irradiation. Administration of hesperidin orally before irradiation significantly reduced the radiation-induced delay in the wound contraction and mean wound healing time. The collagen, hexosamine, DNA and nitric oxide syntheses were significantly reduced after exposure to 6 Gy, whereas pretreatment with hesperidin significantly enhanced synthesis of collagen, hexosamine, DNA, and nitric oxide. Histological examination revealed an increased rise in fibroblast and vascular densities after treatment with hesperidin in comparison with 6 Gy irradiation.

The study demonstrates that hesperidin treatment accelerated the healing of irradiated wounds by increasing the collagen, hexosamine, DNA, and nitric oxide syntheses and increasing the densities of fibroblasts and blood vessels in the

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regenerating wounds. The stimulation of a cascade of repair and regeneration processes by hesperidin could be a substantial therapeutic strategy in irradiated wounds.

Keywords: Mice; Hesperidin; Irradiation; Wound; Collagen

Introduction

Ionizing radiation has emerged as an important paradigm in medicine for diagnostic and therapeutic purposes. With the increase in terrorist activities worldwide, the possibilities for deliberate misuse of radioactive materials cannot be underrated [1]. The other possibility of inadvertent human exposure includes nuclear accidents, industrial uses of ionizing radiations, air travel, radon and radiation emanating from cosmos. The acute radiation exposure resulting from any of these sources could cause combined injuries in the form of superimposed skin wounds and/or burn injury, which may lead to serious clinical problems not encountered by most military and civilian physicians. Combined injuries act synergistically, leading to higher morbidity and/or mortality than the radiation injury alone would have produced [2-6]. Interaction of ionizing radiation with wounded tissue disrupts normal responses to injury leading to a protracted recovery period. Irradiation also produces multiple negative effects on the wound healing processes that include inhibition of inflammatory reactions, connective tissue proliferation, formation and maturation of granulation tissue, transcription of collagen mRNAs, secretion of collagen, neovascularization and a host of other processes involved in wound repair and regeneration [7-11].

There have been several attempts to alleviate the radiation-induced delay in wound healing and accelerate the repair and regeneration of irradiated excision wounds. Supplemental vitamin A has been reported to reduce the acute radiation-induced defect in wound healing by aggrandizing the early inflammatory reactions in the wound and increasing the number of monocytes and macrophages at the wound site [12]. Certain radioprotective compounds like mercaptoethylamine, serotonin and WR-2721 have also been found to be useful in combined injuries [13]. Several growth factors and antimicrobial agents have been explored in animal models as potential options to improve wound healing in radiation damaged skin [14-16]. Some of the studies have shown that autologous non-irradiated fibroblasts, bacterial cellulose impregnated membranes, and poviargol accelerate the repair and regeneration of irradiated wounds [17,18]. Phenytoin sodium, vitamin A, C and curcumin have been reported to inhibit radiation-induced defects in wound healing, [3,5,12,19-23]. *Nigella sativa* extract has been reported to enhance the healing of irradiated deep dermal wound [11]. Ascorbic acid and curcumin have been found to accelerate the repair and regeneration of excision wounds of mice exposed to different doses of γ-radiation [20-23].

Despite a plethora of basic research in potential therapies and prophylaxis of irradiated wounds, use of nutraceuticals for wound repair and regeneration of irradiated wounds did not receive a greater attention. This indicates that use of nutraceuticals in wound healing need to be explored for their ability to enhance wound repair and regeneration of irradiated wounds. Since wound-healing deformities cause great physical and psychological stress to affected patients and is extremely expensive to treat, the use of nutraceuticals in the reconstruction of irradiated wounds seems to be an attractive proposition, because, they are non-toxic, consumed daily, have wide acceptability, better tolerance, more economic and can be safely manipulated for human use [24-26].

Hesperidin (hesperitin-7-rhamnoglucoside or hesperitin-7-rutinoside), a predominant bioflavonoid, has been reported to be anti-inflammatory, analgesic, antihypertensive, diuretic antibacterial and antiviral in various study systems [27-30]. Hesperidin helps in plant defense. The orange juice containing pulp is richer in the hesperidin than the juice without pulp. Sweet oranges (*Citrus sinensis*) and tangelos contain larger amount of hesperidin [31]. Hesperidin has been reported to act as an antioxidant, anti-inflammatory, and free radical scavenging agent. It has antiulcer activity and it also suppresses selected cytochrome p450 enzymes [28,32,33]. Hesperidin regulates the capillary permeability and increases their strength [34]. It assists vitamin C in keeping collagen, the intercellular "*cement*" in healthy condition; and is essential for the proper absorption and use of vitamin C. It prevents vitamin C from being destroyed in the body by oxidation; beneficial

in hypertension; helps reduce hemorrhages and in conditions of ruptured capillaries and connective tissues and protects against infections [35]. Hesperidin has been reported to be anti-allergenic, anti-hypotensive, anti-inflammatory, anti-cancerous, anti-microbial, and improves cognitive depression [36-38]. Hesperidin has been found to reduce cholesterol levels in humans and retard bone loss [39,40]. The sub chronic administration of 5% hesperidin for 13 weeks has been found to be non-toxic in mice [41]. The micronized flavonoid fraction, of 90% diosmin and 10% hesperidin has been reported to offer protection against reactive oxygen radicals both *in vivo* and *in vitro* and it has been found to be effective in the healing of clean and infected wounds, both orally and topically [42-44]. Hesperidin has been found to be non-toxic in animals and humans [33,45,46]. The pleotropic properties of hesperidin stimulated us to obtain an insight into the effect of hesperidin in mice exposed to different doses of whole-body γ-radiation and inflicted with deep dermal excision wound as an additional trauma.

Materials and Methods

The animal care and handling were done according to the guidelines of the World Health Organization, Geneva and the INSA (Indian National Science Academy, New Delhi). Generally, eight to ten-week old Swiss albino mice of either sex weighing 30 to 36 g were procured from an inbred colony maintained under the controlled conditions of temperature (23±2°C), humidity (50±5%) and light (12 h of light and dark, respectively). The animals were allowed free access to sterile food (50% cracked wheat, 40% Bengal gram, 4% milk powder, 4% yeast powder, 0.75% sesame oil, 0.25% cod liver oil, and 1% salt) and water. Four animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. The study was approved by the institutional animal ethical committee of the Kasturba Medical College, Manipal, India, where the study was performed.

Preparation of Drug and Mode of Administration

Hesperidin (HPD) was procured from Acros Organics, Geel, Belgium. The required amount of (HPD) was suspended in 1% carboxymethylcellulose (CMC) and the animals were administered with 0.01 ml/g b. wt. of CMC or HPD orally.

Experimental Protocol

The animals were divided into two groups as follows: -
CMC+irradiation: The animals of this group received 0.01ml/g body weight of CMC before irradiation.
HPD+irradiation: The animals of this group were administered with 100 mg/kg body weight of hesperidin before irradiation.

Irradiation

One hour after the administration of CMC or hesperidin, each animal was transferred into a specially designed well-ventilated acrylic restrainer and whole body of the animals was exposed to 0, 2, 4, 6 or 8 Gy of γ-rays delivered at a dose rate of 1.35 Gy/min from a ^{60}Co Teletherapy source (Theratron, Atomic Energy Agency, Ontario, Canada).

Production of Full-Thickness Skin Wound

The fur of the dorsum (below the rib cage) of each animal was cleared with the help of a cordless electric mouse clipper (Wahl Clipper Corporation, Illinois, USA) before exposure to radiation and a full-thickness skin wound was produced on the dorsum (below the rib cage) of the animal as described earlier [33] within ten minutes of irradiation. Briefly, the animals were anesthetized and the skin of the entire body was cleaned and decontaminated by wiping the whole body with sterillium (Bode Chemical, Germany) disinfectant solution. The cleared dorsal surface of the skin was marked with a sterile circular (15-mm-diameter) stainless steel stencil. A full-thickness wound was created by excising the skin flap including *panniculus carnosus* in an aseptic environment using sterile scissors and forceps. Each wounded animal was housed in a separate sterile polypropylene cage until complete healing of the wound.

Wound contraction: The wound contraction was monitored by capturing the video images of each full-thickness wound with a CCD (charged coupled device) camera connected to a computer [21-24]. The first image of each wound from different groups was obtained one day after wounding, and it was considered as day one. The subsequent images were captured on 3, 6, 9, 12 and 15 days post-irradiation. The wound area was calculated using Auto CAD R14 (Autodesk Inc., San Rafael, CA) software. Eight animals were used for each irradiation dose in each group and a total of eighty animal were used for this experiment.

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Mean wound healing time: A separate experiment was carried out to estimate the mean wound healing time where grouping and other conditions remained exactly similar to that described above except that all the wounded animals in each group were monitored until complete healing of wounds and the day at which each wound healed was recorded. The mean of all the days were considered as mean wound healing time (MHT) and expressed in days. Eight animals were used for each irradiation dose in each group and a total of eighty animals were used for this experiment.

Biochemical Studies

A separate set of experiments was undertaken to study the alteration in the various biochemical profiles of regenerating excision wound after exposure to 0 or 6 Gy whole-body γ-radiation and their modulation by hesperidin. Grouping of animals and production of wounds were essentially similar to that described in experimental protocol section, except that the whole-body of the animals was exposed to 0 or 6 Gy γ-radiation. Wound biopsies were collected on 4, 8 and 12 days post-irradiation and the tissue samples were stored at –70°C until analysis. The estimation for collagen, hexosamine, DNA and nitrite syntheses were carried out as follows: -

Collagen: As an indication of total collagen content, hydroxyproline concentration was determined as described earlier [47]. The weighed granulation tissue was hydrolyzed in 6 N HCl for 3 h at 130°C, neutralized (pH 7) with 2.5 N NaOH and diluted with Milli-Q water (18 Ω). The diluted solution was mixed with chloramine-T reagent and incubated for 20 min at room temperature. This was followed by the addition of freshly prepared ρ-dimethylamino-benzaldehyde (Ehrlich's reagent) solution and incubation for 15 min at 60°C. The absorbance of each sample was recorded at 550 nm using a double beam UV-visible spectrophotometer (Shimadzu UV-260, Shimadzu Corp., Tokyo, Japan). The amount of hydroxyproline was determined by comparing the absorbance of samples with a standard curve. Total collagen from hydroxyproline analysis was determined by multiplying with a factor of 6.94. The collagen contents in granulation tissue have been expressed as mg/g dry tissue weight. Six animals were used in each group at each interval.

Hexosamine: Hexosamine contents of granulation tissues were estimated as described earlier with minor modifications [22]. Briefly the preweighed amount of granulation tissue was hydrolyzed in 6N HCl for 8 h at 98°C, neutralized to pH 7 with 4 N NaOH and further diluted with Milli-Q water. It was mixed with acetyl acetone and heated up to 96°C for 40 min. The resultant mixture was cooled, and 96% ethanol and ρ-dimethylamino-benzaldehyde solution (Ehrlich's reagent) were added, then thoroughly mixed, and kept at room temperature for 1 h. The absorbance was measured at 530 nm using a double beam UV-visible spectrophotometer. The quantity of hexosamine was determined by comparing with a standard curve. Hexosamine contents have been expressed as mg/g dry tissue weight. Six animals were used in each group at each interval.

DNA synthesis: The DNA contents in the granulation tissue/s were measured by the method of Richards [48]. A known amount of dry granulation tissue was homogenized in 5% TCA and centrifuged. The pellets were washed with 10% TCA, resuspended in 5% TCA, incubated at 90°C for 15 min and centrifuged again. The resultant supernatant was collected for the determination of DNA content. The DNA was hydrolyzed with 60% perchloric acid at 80°C for 20 min and Burton's diphenylamine reagent was added and left for incubation at room temperature overnight. Next day, 95% ethanol was added and absorbance was read at 600 nm using a double beam UV-visible spectrophotometer. The amount of DNA was determined by comparing with a standard curve and has been expressed as mg/g dry tissue weight. Six animals were used in each group at each interval.

Nitric oxide: The stable end products of NO biosynthesis were measured by estimating both nitrite and nitrate levels in the granulation tissue of wounds. Briefly, the preweighed amount of granulation tissue was homogenized in hypotonic saline and centrifuged. Nitrite concentrations were determined with Griess reagent. The supernatant was mixed with freshly prepared Griess reagent (0.1% NEDD, 1% sulphanilamide and 5% phosphoric acid in a 1:1:1 ratio), incubated at 37°C for 30 min and the absorbance was recorded at 543 nm using a double beam UV-visible spectrophotometer. Sodium nitrite was used as a standard. Nitrite levels were expressed in terms of μM/100 mg dry tissue weight. Nitrate concentrations were quantified using nitrate reductase assay. Briefly, 0.275 mg/ml of β-NADPH in imidazole buffer (pH 6.8), 0.41 U/ml nitrate reductase and test solutions were mixed. Griess reagent was added to this mixture, incubated at 37°C for 30 min and the absorbance was measured at 543 nm. Sodium nitrate was used as standard. Nitrate levels were expressed in terms of mM/g dry tissue weight. Six animals were used in each group at each interval, and a total of 72 animals were used for this estimation.

Histological Studies

A separate experiment was conducted to evaluate the histological alterations during wound healing after exposure to 0 or 6 Gy whole-body γ-radiation. Grouping of animals and production of wounds were carried out as described in experiment 1, except that the whole-body of the animals was exposed to 0 or 6 Gy γ-radiation. The cross sectional full thickness skin biopsies from each group were collected at 4, 8 and 12 days post-irradiation. The samples were fixed in 10% buffered formalin, passed through different grades of alcohol in order to ensure complete dehydration and were embedded in paraffin wax. Medial samples were sectioned (5 μm) perpendicular to the surface, starting from the center of the wound and stained with haematoxylin and eosin. Sections were assessed in a blinded fashion under light microscope using a planimeter for fibroblast proliferation, and neovascularization. Two areas in each section were counted for neo-vascularization and fibroblast proliferation. The elongated or spindle shaped cells with purple nuclei and pink cytoplasm were identified as fibroblasts and scored. Blood vessels that are conspicuous with haematoxylin and eosin stains were scored for vascular repopulation studies. Three animals were used for each irradiation dose in each group and a total of thirty-six animal was used for this experiment.

Analysis of Data

Statistical significance between the treatment groups was determined using one-way ANOVA and student's 't' test. The Solo 4 Statistical Package (BMDP Statistical Software Inc., Los Angeles, CA, USA) was used for data analysis. All data are expressed as mean ± SEM (Standard error of mean).

Results

The results are expressed as wound contraction, mean wound healing time, contents of collagen, hexosamine, DNA, nitrate and nitrite and fibroblast and vascular densities in Figures 1-7.

Wound Contraction

Periodic computation of wound contraction by measuring the wound area can provide a good measure of wound healing. Area of each wound at a specific time has been expressed as the percentage of its original size on day one. The mean corresponding area of wound for each group was plotted as a function of days after wounding. Wound contraction progressed with time and a steady contraction of the excision wound was discerned in both the CMC+Sham-irradiation and HPD+Sham-irradiation groups (0 Gy). Hesperidin treatment resulted in a significant enhancement in wound contraction at 3 ($p < 0.005$), 6 ($p < 0.05$) and 9 ($p < 0.05$) day post-irradiation compared to CMC+Sham-irradiation group (Figure 1). Furthermore, CMC+Sham-irradiation group showed formation of scab whereas no scab formation was observed for HPD+Sham-irradiation group.

Whole-body exposure of mice resulted in a dose dependent delay in wound contraction (Figure1). Exposure of animals to 2 or 4 Gy, delayed wound contraction (Figure 1) accompanied by an early formation of scab in wounds when compared to the CMC+Sham-irradiation group. However, the difference in wound contraction between CMC+Irradiation and CMC+Sham-irradiation group was statistically non-significant. Exposure of animals to 6 or 8 Gy significantly delayed wound contraction at all the post-irradiation times studied (Figure 1). A significant retardation in wound contraction was observed after exposure to 6 or 8 Gy when compared to CMC+Sham-irradiation group (Figure 1).

Hesperidin treatment prior exposure to 2, 4 or 6 Gy irradiation in HPD+Irradiation group resulted in a significant rise in the contraction of wounds at 3 ($p < 0.005$), 6 ($p < 0.05$) and 9 ($p < 0.05$) day post irradiation, when compared to CMC+Irradiation group (Figure 2) and the scab formation was very thin, especially at 2 and 4 Gy irradiation. Hesperidin treatment prior to 8 Gy irradiation resulted in a significant contraction of the wound only at day 3 ($p < 0.05$) post-irradiation (Figure 2).
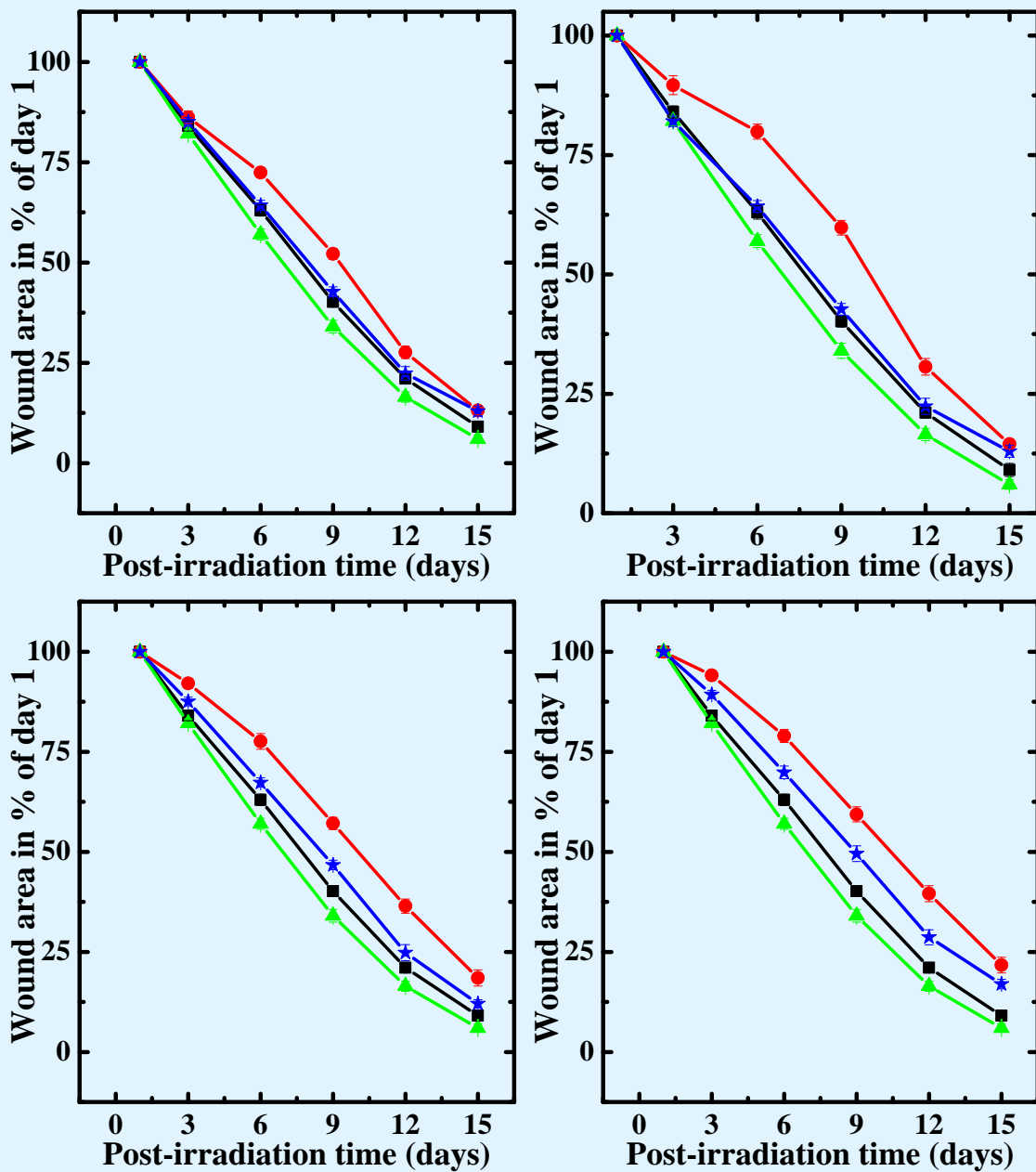
Figure 1: Effect of hesperidin treatment on the contraction of wound with time in mice exposed to various doses of whole-body γ-radiation. Squares: CMC+Sham-irradiation; Circles: CMC+Irradiation; Triangles: HPD+Sham-irradiation and Stars: HPD+ Irradiation. Upper left: 2 Gy; Upper right: 4 Gy; Lower left: 6 Gy and Lower right: 8 Gy.
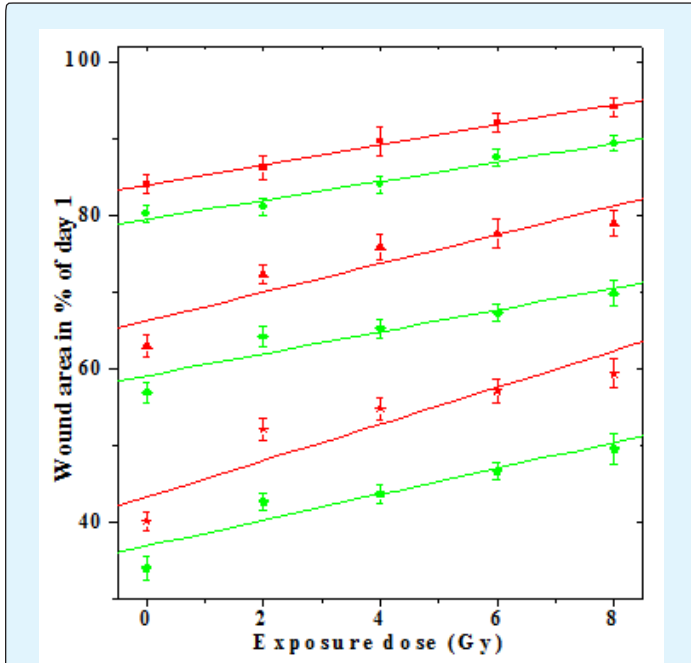
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Figure 2: Effect of hesperidin treatment on the contraction of wound with time in mice exposed to various doses of whole-body γ–radiation. Squares: CMC+Irradiation 3 day; Circles: HPD+Irradiation 3 day; Triangles: CMC+Irradiation 6 day; Diamonds: HPD+ Irradiation 6 day; Stars: CMC+Irradiation 9 day and Pentagons: HPD+ Irradiation 9 day post wounding.

Mean Wound Healing Time

The complete closure of wounds was observed on 18.7 ± 0.61 days post-irradiation in CMC+Sham-irradiation group, whereas oral administration of mice with hesperidin resulted in a significant decline in MHT (16.2 ± 0.36 day post-irradiation) in the HPD+Sham-irradiation group (Figure 3). The whole-body exposure of mice to different doses of γ-radiation delayed the complete closure of wounds in a dose dependent manner as a result the mean wound healing time was also increased in CMC+ Irradiation group when compared with the CMC+Sham-irradiation group (Figure 3). A mean wound healing time of 20.5 ± 0.29, 21.8 ± 0.29, 23.3 ± 0.49 and 25.5 ± 0.33 days was observed for 2, 4, 6 and 8 Gy, respectively in CMC+Irradiation group (Figure 3). The oral administration of 100 mg/kg of hesperidin to mice before irradiation to various doses of γ-radiation accelerated the healing of irradiated excision wounds, as a result there was a reduction in the mean wound healing time and a mean wound healing time of 19 ± 0.28, 20.6 ± 0.3, 21.5 ± 0.26 and 24.6 ± 0.47 days was observed for 2, 4, 6 and 8 Gy, respectively in

HPD+Irradiation group (Figure 3). This improvement in wound healing time was statistically significant ($p < 0.05$) for 2, 4 and 6 Gy, respectively in HPD+Irradiation group. However, this enhancement in wound healing in HPD+Irradiation group was non-significant for 8 Gy irradiation (Figure 3).
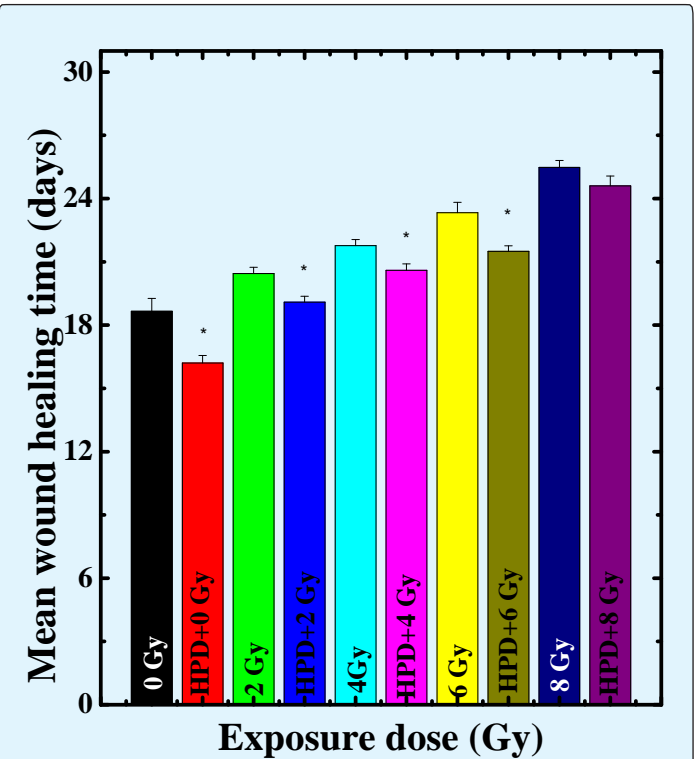

Figure 3: Effect of hesperidin treatment on the progression of mean wound healing time in mice exposed to various doses of whole-body γ-radiation.
*$p < 0.01$ when carboxy methylcellulose groups are compared to hesperidin groups.
CMC: Carboxy methylcellulose; HPD: Hesperidin; SIR: Sham-irradiation; IR: Irradiation.

Biochemical Studies

The amount of hydroxyproline is an index of collagen content and is also a measure of neo-collagen synthesis. A maximum synthesis of collagen was observed at day 8 post-irradiation indicated by the highest hydroxyproline contents in CMC or HPD+Sham irradiation groups; thereafter, synthesis of collagen remained almost unaltered in both the groups. Irradiation of animals to 6 Gy resulted in a drastic drop in the collagen synthesis at all post-irradiation times, which was statistically

significant when compared to the CMC+Sham irradiation group (Figure 4). Despite this decline in collagen synthesis, a maximum synthesis of collagen was observed on day 8 after wounding in the CMC+Irradiation group; thereafter a nadir in the formation of new-collagen was reached at 12 days post-irradiation (Figure 4). The pattern of collagen synthesis was similar in HPD+Irradiation group, except that the treatment of mice with 100 mg/kg hesperidin before 6 Gy irradiation resulted in a significant elevation in collagen synthesis when compared to the concurrent CMC+Irradiation group (Figure 4). Pretreatment with hesperidin could not restore the level of collagen to normal even by day 12 post-irradiation.
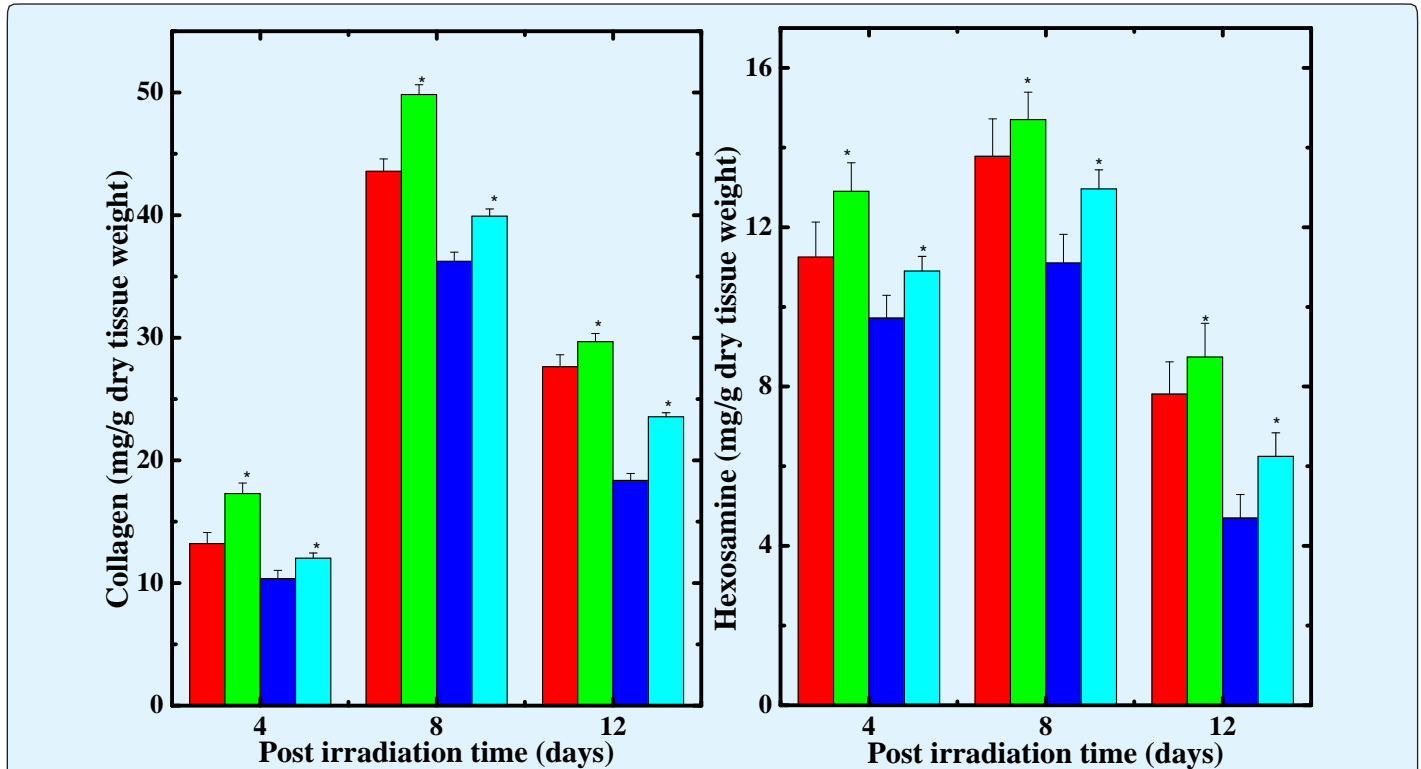

Figure 4: Effect of hesperidin treatment on the biosynthesis of collagen (Left) and hexosamine (Right) in the excision wound of mice whole-body exposed to 6 Gy γ-radiation. Red bars: Carboxy-methylcellulose +Sham-irradiation; Green bars: Hesperidin+Sham-irradiation; Blue bars: Carboxymethylcellulose + Irradiation and Cyan bars: Hesperidin+Irradiation. *p < 0.05 when carboxy methylcellulose groups are compared to hesperidin groups.

Hesperidin treatment alone increased hexosamine, the ground substratum for collagen synthesis on day 4 post irradiation, which continued to rise up to day 8 post-irradiation and declined thereafter when compared to non-irradiated control (0 Gy). Irradiation of animals to 6 Gy caused a significant decline in the hexosamine contents at all post-irradiation days in CMC+Irradiation group. Despite this reduction, the hexosamine contents were maximum on day 8 post-irradiation in this group (Figure 4). The pattern of hexosamine synthesis was similar in the HPD+Irradiation group, except that the hexosamine synthesis was significantly higher at all post-irradiation days when compared to concurrent CMC+Irradiation group (Figure 4).

The increase in DNA contents of treated wounds indicates hyperplasia of cells. Exposure of animals to 6 Gy resulted in a drastic reduction in the DNA contents in the granulation tissue at day four, that showed a sudden surge on day 8 and declined thereafter on day 12 post-irradiation, however it was higher than that of day 4 post-irradiation (Figure 5). Oral administration of HPD before 6 Gy irradiation significantly increased the DNA synthesis on day 4 by 1.5 times of CMC+Irradiation group and continues to increase up to day 4 where a maximum

synthesis of DNA was observed. Thereafter the synthesis of DNA declined however it was higher than that of day 4 post-irradiation (Figure 5).
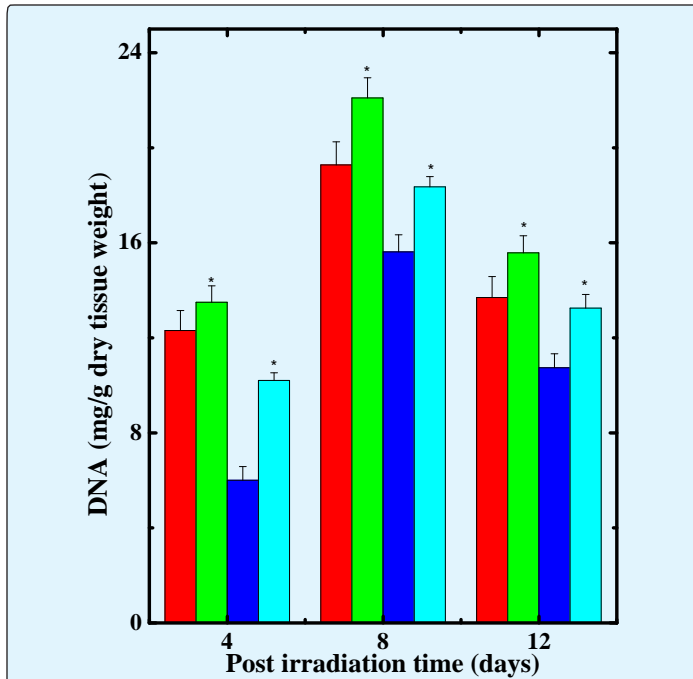

Figure 5: Effect of hesperidin treatment on the biosynthesis of DNA in the excision wound of mice whole-body exposed to 6 Gy γ-radiation. Red bars: Carboxymethylcellulose+Sham-irradiation; Green bars: Hesperidin+Sham-irradiation; Blue bars: Carboxy methylcellulose + Irradiation and Cyan bars: Hesperidin +Irradiation.
*p<0.05 when carboxy methylcellulose groups are compared to hesperidin groups.

End products of NO synthesis, nitrite and nitrate were elevated as early as 4 day post-irradiation in the granulation tissue and the levels of both nitrite and nitrate continued to decline up to 12 day post-irradiation in non-irradiated animals (Figure 6). Irradiation of animals to 6 Gy whole-body γ-radiation resulted in a drastic decline in both the nitrite and nitrate contents in the granulation tissues at all post-irradiation times. However, hesperidin pretreatment resulted in a significant elevation in both nitrite and nitrate contents at all post-irradiation days (Figure 6).
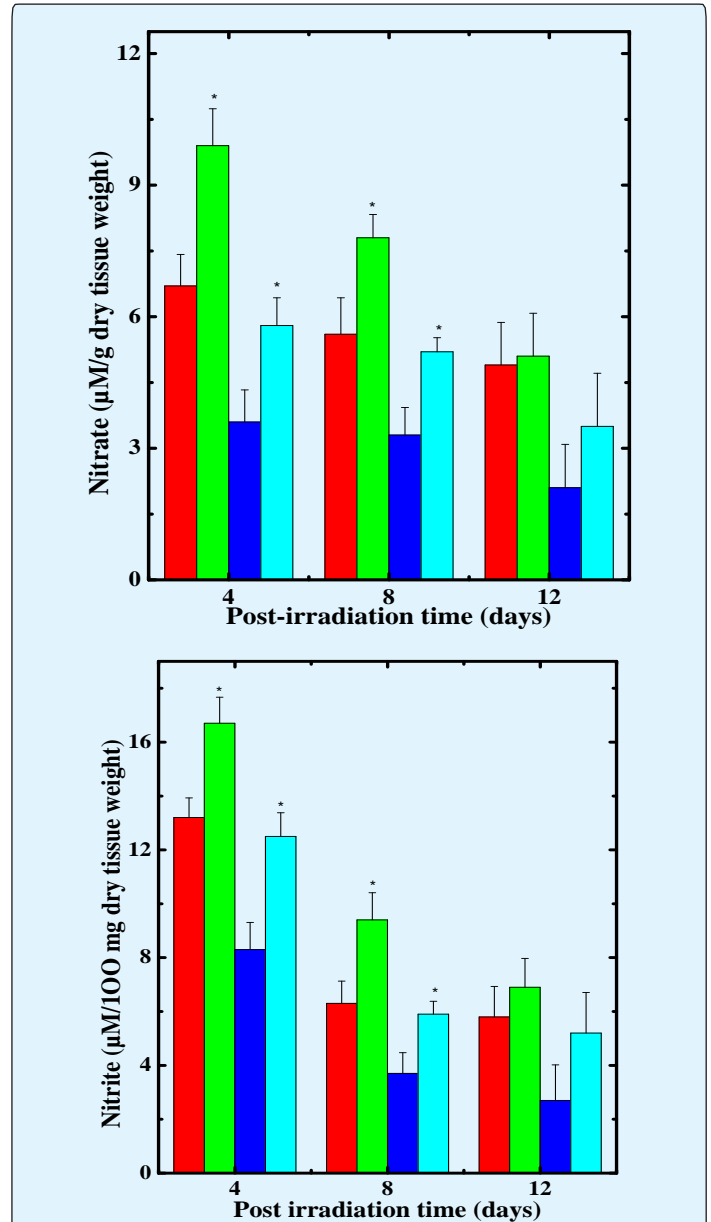

Figure 6: Effect of hesperidin treatment on the biosynthesis of nitrate (Upper) and nitrite (Lower)in the excision wound of mice whole-body exposed to 6 Gy γ-radiation.
Red bars: Carboxy-methylcellulose+Sham-irradiation;
Green bars: Hesperidin+Sham-irradiation;
Blue bars: Carboxymethylcellulose + Irradiation and
Cyan bars: Hesperidin+Irradiation.
*p < 0.05 when carboxy methylcellulose groups are compared to hesperidin groups.

Histological Studies

Histological evaluation of wound biopsies at various post-irradiation times revealed that hesperidin treatment alone did not alter the histology picture except that there was an increase in the fibroblast and vasculature densities compared to the non-drug-treated Sham-irradiation controls (Figure 7). Exposure of mice to 6 Gy caused degeneration of collagen bundles and depletion in fibroblasts and vasculature densities (Figure 7). A few isolated "fragments" of collagen surrounded by unstained spaces were seen at this radiation dose. The density of fibroblasts declined drastically in CMC+Irradiation group when compared with CMC+Sham-irradiation group (Figure 7). Few, large and stellate cells or "radiation fibroblasts" were seen after irradiation at 8 day post-irradiation. A slight variation in epidermal thickness was also evident. A similar trend was observed for vascularization, where blood vessels were larger and more irregular in shape in the irradiated group than in the CMC+Irradiation group. Pretreatment with hesperidin protected the mice against radiation-induced damage to fibroblasts and vasculature as revealed by an increase in the density of fibroblasts and vasculature (Figure 7). However, the histology could not be restored similar to that of sham-irradiated control.
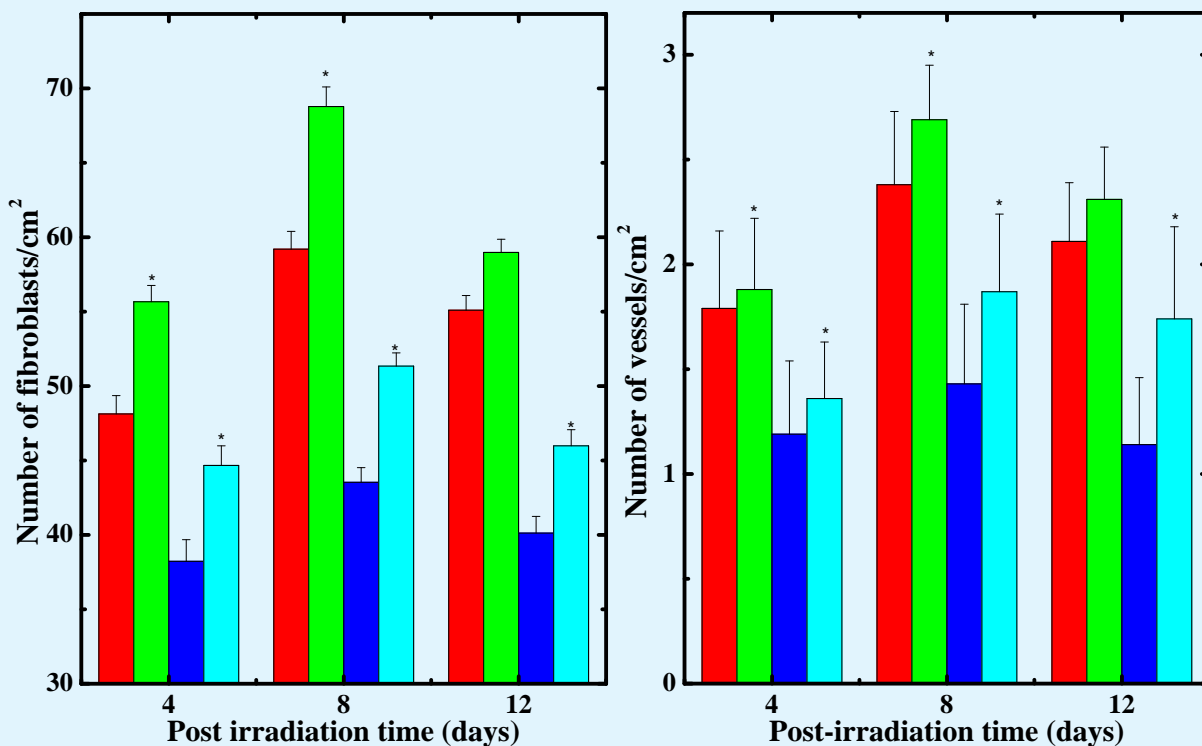

Figure 7: Effect of hesperidin treatment on the fibroblast (Left) and vascular density (Right) in the regenerating excision wound of mice whole-body exposed to 6 Gy γ-radiation. Red bars: Carboxy-methylcellulose +Sham-irradiation; Green bars: Hesperidin+Sham-irradiation; Blue bars: Carboxymethylcellulose + Irradiation and Cyan bars: Hesperidin+Irradiation.
*$p < 0.01$ when carboxy methylcellulose groups are compared to hesperidin groups.

Discussion

Increasing use of radioactive materials in industry, medicine, science, military and in localized areas of high radiation within nuclear facilities has significantly increased the potential of large-scale, uncontrolled exposure to radiation, especially during accidents Chernobyl and Fukushima type. With the spurt in terrorist activities and evolution of new terror outfits frequently, the potential for deliberate misuse of radioactive material

can also not be ruled out. Irradiation of tissue, whether for therapeutic purpose or incidental or accidental raises problems or concern for surgeons in terms of the malignant potential of the irradiated bed and wound healing issues, especially during combined injuries in such situations [49,50]. Irradiation produces both acute and late effects on skin and subcutaneous tissues that have profound implications on surgical wound healing [50-52]. This indicates that strategies are needed that can accelerate the repair and regeneration of the wounded tissue and also counter the effect of ionizing radiation. Therefore, the present study was undertaken to study the application of hesperidin a bioflavonoid in the repair and regeneration of deep dermal excision wound in mice whole body exposed to different doses of γ-radiation.

Periodical and regular monitoring of wound contraction provides the precise information about progress of repair and regeneration of excision wounds. The video imaging to assess wound contraction has been used by us as it produces less trauma to the wounded animal and the chances of detachment of the regenerating wound from wound bed are minimal [5,11,20-23,33]. The exposure of animals to different doses of γ-radiation led to a significant delay in the wound contraction in a dose dependent manner and maximum retardation in wound repair and regeneration was observed for 8 Gy exposure. Similarly, whole-body irradiation has been reported to retard wound healing in mice exposed to different doses of γ-radiation earlier [11,20-22,24]. Wound contraction can be defined as the centripetal movement of the edges of a full thickness wound in order to facilitate closure of the defect [53,54]. A delay in wound contraction after exposure to γ-radiation has been observed in earlier studies. These studies have indicated that irradiation alter the local conditions in the wound milieu that act against the wound repair and regeneration. [13,20-22,55,56]. The similar factors must be responsible for retardation in wound healing after irradiation in the present study. Oral administration of mice with 100 mg/kg body weight of hesperidin before exposure to different doses of γ-irradiation accelerated the wound healing as was evident by greater degree of wound contraction and reduction in mean wound healing time. The topical application of hesperidin has been recently reported to enhance healing of irradiated wound in mice [33]. Likewise, ascorbic acid, curcumin and extract of *Nigella sativa* have been reported to accelerate the healing of excision wounds after whole body irradiation to different doses of γ-radiation [11,20-23]. Vitamin A supplementation has also been reported to ameliorate the acute radiation-induced delay in wound healing [12]. In another study vitamin E treatment has

been reported to normalize the breaking strength of wounds that received preoperative irradiation [57].

The wound repair and regeneration is a concerted event of well-organized physiological processes that are triggered immediately after infliction of the wound and ionizing radiation induces multiple negative effects on all these processes including, collagen, and DNA syntheses, fibroblast proliferation, and endothelial cells and other cytokine factors [5,11,21,22,56,58-61]. Collagen is an important protein which is abundant in the connective tissues and it plays a pivotal role in the healing of wounds [62]. It provides a structural framework, strength and milieu to the regenerating tissues. Collagen is produced by fibroblasts and assists the wound in gaining tensile strength during wound repair [62-63]. Irradiation of animals significantly reduced the collagen synthesis, which is evident from the estimation of hydroxyproline content in the granulation tissue. Irradiation with increasing doses of gamma rays, have been reported to cause a progressive destruction of the native collagen fibrils [64]. An identical effect has been observed earlier, where irradiation of mice after 6 Gy resulted in the decline in the collagen synthesis [11,21,22]. The hesperidin administration inhibited the radiation-induced decrease in collagen synthesis at all post-irradiation days. Ascorbic acid, curcumin and *Nigella sativa* extract have been found to inhibit the radiation-induced decline in the collagen synthesis in the regenerating wounds earlier [11,21,22].

Ground substratum, for collagen synthesis, the hexosamine increases during early stages of wound repair and regeneration and declines thereafter [65]. Irradiation of mice has been reported to reduce the hexosamine contents in the regenerating excision wounds earlier [21,22]. Similarly, hexosamine was found to decline during the regeneration of wounds at different post-irradiation days in the present study. Administration of hesperidin before irradiation elevated the hexosamine contents significantly on day 4 and 8 post-irradiation in comparison with the CMC+Irradiation group. Earlier ascorbic acid and curcumin have been found to elevate the hexosamine contents during reparation of irradiated wounds [21,22,24]. Similarly, hexosamine has been found to increase in wounds of diabetic rats receiving topical application and oral administration of *Aloe vera* [65].

The rise in DNA contents during the healing of wounds serves as an index of cell proliferation [66]. Therefore, estimation of DNA contents could provide crucial information on cell proliferation during wound repair.

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The whole-body irradiation significantly reduced DNA contents in the CMC+Irradiation group indicating reduced cell proliferation in the regenerating wounds, whereas hesperidin treatment significantly increased the DNA contents at day 4 and 8 post-irradiation in HPD+Irradiation group pointing towards increased cell division and repair of irradiated wounds. A resembling effect has been observed earlier after treatment with ascorbic acid, curcumin and *Nigella sativa* extract in the irradiated mice after infliction of excision wounds [11,21,22]. The oral administration and topical application of *Aloe vera* has also been reported to increase DNA contents significantly in the wounds of diabetic rats [65].

Inflammatory responses are central to the repair of wounds after injury and some of the bioregulatory molecule like nitric oxide plays a crucial role during repair and regeneration as it allows the migrations of neutrophils and macrophages in the wound bed for the subsequent wound healing events to take place [61,67,68]. Most available evidences suggest that adequate rate of NO production is essential and promotes processes central to wound healing such as angiogenesis, fibroblast synthetic function, epithelial cell proliferation, collagen formation and wound contraction in various distinct ways [5,20-22,61,69]. The decrease in NO expression has been correlated with radiation-induced impairment in wound healing [70]. Irradiation has been reported to reduce the level of nitrite and nitrate levels in healing wounds and ascorbic acid and curcumin increased their levels during the healing of irradiated wounds [5,20-22,24]. A similar effect has been observed in this study, where irradiation has reduced the formation of NO, whereas pretreatment of mice with hesperidin elevated nitrite and nitrate levels in irradiated mice.

The fibroblasts play a major role in the repair and regeneration of wounds as they are indispensable in breaking of fibrin clot, production of extracellular matrix, synthesis of neocollagen and the myofibroblasts that are important for wound contraction [71-73]. These cells are proliferating cells and ionizing radiation adversely affect them. A similar observation has been made in the present study, where the density of fibroblast declined in the regenerating irradiated wound. The formation of new blood vessels is essential part of wound healing and this process occurs due to neovascularization and angiogenesis in the granulation tissue of the wound and the irradiation hampers neovascularization and retard wound healing [74,75]. This reduction in fibroblast proliferation and vascularization is in agreement with

earlier reports, where a similar decrease in fibroblast proliferation, retardation of collagen maturation, and overall delay in wound repair is observed [5,21,22]. The Cell culture studies on fibroblasts exposed to ionizing radiation have also demonstrated that irradiated fibroblasts have a significantly prolonged generation time when compared to normal fibroblasts [76]. Pretreatment of mice with hesperidin increased fibroblast proliferation and vascular density in the HPD+Irradiation group. An identical effect has been observed earlier with ascorbic acid and curcumin, which have been reported to augment blood vessel formation and fibroblast proliferation in the regenerating wounds of mice [5,21,22,24].

The response of normal tissues to radiation can be viewed as consisting two partially interacting components. The first is a process that resembles the healing of traumatic wounds, perturbed by the radiation treatment. The second is a set of specific injuries that affect virtually all cellular and extracellular components within irradiated volume and that may be responsible for the progression of injury over a period of time. The irradiated wound differs in interesting ways from acute traumatic, thermal or chemical wounds, in which structural tissue damage occurs instantaneously, or nearly so. Injuries that are benign by themselves may become lethal when combined even with relatively small doses of radiation. There are several possible explanations for alterations in wound healing after irradiation. Severe damage to vital tissues, especially those with a high rate of cell division such as the hematopoietic system after exposure to ionizing radiation has been reported [12,77]. This loss of significant numbers of bone marrow cells can lead to an Immunocompromised state in which the individuals become highly susceptible to bacterial infections leading to complications in the healing of wounds. Shielding of bone marrow during acute whole-body X-irradiation has been reported to lower mortality and increase the closure of open dorsal skin wounds of rats [13]. These studies suggest a requirement of radiation-sensitive, bone marrow-derived cells in tissue repair. A delay in fixation of the wound edge to underlying tissue due to a lack of fibroblast proliferation and a decrease in fibroblast synthetic function in the granulation bed could be another possibility. The contraction of open excised wounds has been found to be a function of contractile fibroblasts, known as myofibroblasts [72]. Irradiation is thought to impair wound healing in skin through its cytotoxic effect on fibroblasts. This impairment may be due to the delay in the progression of cells through the cell cycle induced by radiation [76]. A similar effect cannot be ruled out in the

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present study since whole-body of the animal was irradiated. Furthermore, ionizing radiation are known to produce cytotoxic effects by generating oxygen-derived free radicals and the overproduction of free radical results in oxidative stress, producing negative effect on wound healing [78].

The exact mechanism of acceleration in healing of irradiated wound after hesperidin treatment is not known. However, there may be several putative mechanisms acting in concert with each other to reduce the irradiation-induced delay in the repair and regeneration of irradiated wounds treated with hesperidin. The presence of HPD may reduce the radiation and wound injury-induced free radicals resulting in the early closure of wounds. Hesperidin has been reported to neutralize free radicals [33]. The increase synthesis of DNA, collagen, hexosamine and NO after HPD treatment may have contributed in various ways to effect early repair of irradiated wounds. HPD may also have acted as an antioxidant and anti-inflammatory agent [37,79]. Wounding and irradiation have been reported to induce transcriptional activation of NF-κB, COX-II and LOX causing adverse impact on the healing of irradiated wounds [6,80,81]. Administration of hesperidin before irradiation may have suppressed the NF-κB, COX-II and LOX activation and restored normal regenerative capacity of irradiated wounds in the present study. Hesperidin has been reported to inhibit the activation of NF-κB, and COX-II in earlier studies [6,82,83]. Exposure to ionizing radiation alleviates various antioxidants in the irradiated wounds and thus derail and retard normal wound healing [11,79,84]. Hesperidin treatment may have increased various antioxidants and thus may have accelerated healing of irradiated wounds. Our earlier study has reported that hesperidin administration before irradiation raised the antioxidant status in the regenerating skin wounds of irradiated mice [79]. The transcriptional activation of Nrf2 pathways by hesperidin may have increased the antioxidants and improved healing of irradiated wounds [85]. Matrix metalloproteinase are important during healing of wounds and their higher expression after irradiation led to delay in the healing of wounds [61,86]. Hesperidin has been reported to reduce their expression that would have also augmented the healing of irradiated wounds in the present study [87].

Conclusions

The present study demonstrated that single administration of hesperidin before whole-body exposure to different doses of γ-radiation accelerates wound healing in mice, as is evident from an improved contraction of wound and reduced mean wound healing time. This effect of hesperidin may be due to its ability to increase the synthesis of collagen, hexosamine, DNA, and nitric acid, which may have contributed in acceleration of wound repair and regeneration in the present study. The stimulation of proliferation of fibroblasts and vasculature by hesperidin in the wound granulation tissue may have also contributed in the early wound healing. The hesperidin may have reduced the expression of matrix metalloproteinase, NF-κB, and COX-II accompanied by the augmented activation of Nrf2 would have played a major role in the enhanced wound healing. This study suggests that hesperidin could be a useful paradigm in the clinical management of normal as well as irradiated wounds.

References

1. Jagetia GC, Baliga MS (2003) Evaluation of the radioprotective effect of the leaf extract of *Syzygium cumini* (Jamun) in mice exposed to a lethal dose of γ-irradiation. Mol Nutri Food Res 47(3): 181-185.

2. Kumar P, Jagetia GC (1994) A review of triage and management of burns victims following a nuclear disaster. Burns 20(5): 397-402.

3. Jagetia GC, Rajanikant GK (2004) Role of curcumin, a naturally occurring phenolic compound of turmeric in accelerating the repair of excision wound, in mice whole-body exposed to various doses of γ-radiation. J Surg Res 120(1): 127-138.

4. Jarrett DG, Sedlak RG, Dickerson WE, Reeves GI (2007) Medical treatment of radiation injuries-Current US status. Radiat Measurem 42(6-7): 1063-1074.

5. Jagetia GC, Rajanikant GK (2012) Acceleration of wound repair by curcumin in the excision wound of mice exposed to different doses of fractionated γ radiation. Int Wound J 9(1): 76-92.

6. Ryan JL (2012) Ionizing radiation: the good, the bad, and the ugly. J Invest Dermatol 132(3): 985-993.

7. Bernstein EF, Salomon GD, Harisiadis L, Talbot T, Harrington F, et al. (1993) Collagen Gene Expression and Wound Strength in Normal and Radiation-Impaired Wounds. J Dermatol Surg Oncol 19(6): 564-570.

8. Lee S, Thiele C (2010) Factors associated with free flap complications after head and neck reconstruction and the molecular basis of fibrotic tissue rearrangement in preirradiated soft tissue. J Oral Maxillofac Surg 68(9): 2169-2178.

9. Guo SA, DiPietro LA (2010) Factors affecting wound healing. J Dent Res 89(3): 219-229.

10. Maryna S, Shkumat PP, Yuri K, Leonov I, Iryna N, et al. (2013) Prolonged inflammatory cytokine expression during the late phase of wound healing in the diabetic K14/mIGF1 transgenic mice. J Med Biol Sci 6(1): 1-12.

11. Jagetia GC, Ravikiran PB (2015) Acceleration of wound repair and regeneration by *Nigella sativa* in the deep dermal excision wound of mice whole body exposed to different doses of γ-radiation. Am Res J Med Surg 1(3): 1-17.

12. Levenson SM, Gruber CA, Rettura GI, Gruber DK, Demetriou AA, et al. (1984) Supplemental vitamin A prevents the acute radiation-induced defect in wound healing. Ann Surg 200(4): 494-512.

13. Stromberg LR, Woodward KT, Mahin DT, Donati RM (1967) Altered wound healing in X-irradiated rats: The effect of bone marrow shielding. Experientia 23(12): 1064-1065.

14. Cromack DT, Porras-Reyes B, Purdy JA, Pierce GF, Mustoe TA (1993) Acceleration of tissue repair by transforming growth factor β1: identification of in vivo mechanism of action with radiotherapy-induced specific healing deficits. Surgery 113(1): 36-42.

15. Donati RM (1971) Combined Surgical and Radiation Injury: IV. Effect of antimicrobials on the wound healing pattern of the x-irradiated rat. Arch Surg 102(2): 132-135.

16. Vegesna V, McBride WH, Taylor JM, Withers HR (1995) The Effect of Interleukin-1β or Transforming Growth Factor-β on Radiation-Impaired Murine Skin Wound Healing. J Surg Res 59(6): 699-704.

17. Ferguson PC, Boynton EL, Wunder JS, Hill RP, O'Sullivan B, et al. (1999) Intradermal injection of autologous dermal fibroblasts improves wound healing in irradiated skin. J Surg Res 85(2): 331-338.

18. Legeza VI, Galenko-Yaroshevskii VP, Zinov'ev EV, Paramonov BA, Kreichman GS, et al. (2004) Effects of new wound dressings on healing of thermal burns of the skin in acute radiation disease. Bull Exp Biol Med 138(9): 311-315.

19. Song S, Cheng T (1997) The effect of systemic and local irradiation on wound macrophages and the repair promoting action of phenytoin sodium. Zhonghua Yi Xue Za Zhi 77(1): 54-57.

20. Jagetia GC, Rajanikant GK, Rao SK (2003) Evaluation of the effect of ascorbic acid treatment on wound healing in mice exposed to different doses of fractionated gamma radiation. Radiat Res 159(3): 371-373.

21. Jagetia GC, Rajanikant GK, Rao KVNM (2007) Ascorbic acid increases healing of excision wounds of mice whole body exposed to different doses of γ-radiation. Burns 33(4): 484-494.

22. Jagetia GC, Rajanikant GK (2005) Curcumin treatment enhances the repair and regeneration of wounds in mice exposed to hemibody γ-irradiation. Plastic Reconstruct Surg 115(2): 515-528.

23. Jagetia GC, Rajanikant GK (2017) Topical application of curcumin augments healing of deep dermal excision wound of mice exposed to whole-body gamma radiation. J Nursing Healthcare 2(1): 1-7.

24. Jagetia GC, Rajanikant GK, Baliga MS, Rao KV, Kumar P (2004) Augmentation of wound healing by ascorbic acid treatment in mice exposed to γ-radiation. Int J Radiat Biol 80(5): 347-354.

25. Jagetia GC, Venkatesha VA (2005) Effect of mangiferin on radiation-induced micronucleus formation in cultured human peripheral blood lymphocytes. Environ Mol Mutagen 46(1): 12-21.

26. Jagetia GC (2007) Radioprotective potential of plants and herbs against the effects of ionizing radiation. J Clin Biochem Nutri 40(2): 74-81.

27. Galati EM, Monforte MT, Kirjavainen S, Forestieri AM, Trovato A, et al. (1994) Biological effects of hesperidin, a citrus flavonoid. (Note I): antiinflammatory and analgesic activity. Farmaco 40(11): 709-712.

28. Emim JA, Oliveira AB, Lapa AJ (1994) Pharmacological evaluation of the anti-inflammatory activity of a citrus bioflavonoid, hesperidin, and the isoflavonoids, duartin and claussequinone, in rats and mice. J Pharm Pharmacol 46(2): 118-212.

Jagetia GC and Rao KVNM. Hesperidin, a Citrus Bioflavonoid Potentiates Repair and Regeneration of Deep Dermal Excision Wounds of Mice Whole Body Exposed to Different Doses of 60Co γ-Radiation. Clin Dermatol J 2018, 3(2): 000147.

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29. Bae EA, Han MJ, Lee M, KIM DH (2000) In vitro inhibitory effect of some flavonoids on rotavirus infectivity. Biol Pharmaceut Bull 23(9): 1122-1124.

30. Ohtsuki K, Abe A, Mitsuzumi H, Kondo M, Uemura K, et al. (2003) Glucosyl hesperidin improves serum cholesterol composition and inhibits hypertrophy in vasculature. J Nutr Sci Vitaminol (Tokyo) 49(6): 447-450.

31. Nogata Y, Sakamoto K, Shiratsuchi H, Ishii T, Yano M, et al. (2006) Flavonoid composition of fruit tissues of citrus species. Biosci Biotechnol Biochem 70(1): 178-192.

32. Crespo ME, Galvez J, Cruz T, Ocete MA, Zarzuelo A (1999) Anti-inflammatory activity of diosmin and hesperidin in rat colitis induced by TNBS. Planta Medica 65(07): 651-653.

33. Jagetia GC, Rao KVNM (2017) Topical application of hesperidin, a citrus bioflavanone accelerates healing of full thickness dermal excision wounds in mice exposed to 6 Gy of whole body γ-Radiation. Clin Res Dermatol Open Access 4(3): 1-8.

34. So FV, Guthrie N, Chambers AF, Moussa M, Carroll KK (1996) Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. Nutr Cancer 26(2): 167-181.

35. Ameer B, Weintraub RA, Johnson JV, Yost RA, Rouseff RL (1996) Flavanone absorption after naringin, hesperidin, and citrus administration. Clin Pharmacol Ther 60(1): 34-40.

36. Miyagi Y, Om AS, Chee KM, Bennink MR (2000) Inhibition of azoxymethane-induced colon cancer by orange juice. Nutr Cancer 36(2): 224-229.

37. Vabeiryureilai M, Lalrinzuali K, Jagetia GC (2015) Determination of anti-inflammatory and analgesic activities of a citrus bioflavanoid, hesperidin in mice. Immunochem Immunopathol 1: 107.

38. Antunes MS, Jesse CR, Ruff JR, de Oliveira Espinosa D, Gomes NS, et al. (2016) Hesperidin reverses cognitive and depressive disturbances induced by olfactory bulbectomy in mice by modulating hippocampal neurotrophins and cytokine levels and acetylcholinesterase activity. Eur J Pharmacol 789: 411-420.

39. Kurowska EM, Spence JD, Jordan J, Wetmore S, Freeman DJ, et al. (2000) HDL-cholesterol-raising effect of orange juice in subjects with hypercholesterolemia. Am J Clin Nutr 72(5): 1095-1100.

40. Chiba H, Uehara M, Wu J, Wang X, Masuyama R, et al. (2003) Hesperidin, a citrus flavonoid, inhibits bone loss and decrease serum and hepatic lipids in ovariectomized mice. J Nutr 133(6): 1892-1897.

41. Mayumi K, Seiko T, Masa-Aki S, Masao H, Shoji F, et al. (1993) Subchronic toxicity study of methyl hesperidin in mice. Toxicol Lett 69(1): 37-44.

42. Lonchampt M, Guardiola B, Sicot N, Bertrand M, Perdrix L, et al. (1989) Protective effect of a purified flavonoid fraction against reactive oxygen radicals. In vivo and in vitro study. Arzneimitforschung 39(8): 882-885.

43. Cypriani B, Limasset B, Carrie ML, Le Doucen C, Roussie M, et al. (1993) Antioxidant activity of micronized diosmin on oxygen species from stimulated human neutrophils. Biochem Pharmacol 45(7): 1531-1535.

44. Hasanoglu A, Ara C, Ozen S, Kali K, Senol M, et al. (2001) Efficacy of micronized flavonoid fraction in healing of clean and infected wounds. Int J Angiol 10(1): 41-44.

45. Kawabe M, Tamano S, Shibata MA, Hirose M, Fukushima S, et al. (1993) Subchronic toxicity study of methyl hesperidin in mice. Toxicol Lett 69(1): 37-44.

46. Kawaguchi K, Mizuno T, Aida K, Uchino K (1997) Hesperidin as an inhibitor of lipases from porcine pancreas and Pseudomonas. Biosci Biotech Biochem 61(1): 102-104.

47. Woessner JF (1962) Catabolism of collagen and non-collagen protein in the rat uterus during post-partum involution. Biochem J 83(2): 304-314.

48. Richards GM (1974) Modifications of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. Anal Biochem 57(2): 369-376.

49. Ledney GD, Elliott TB (2010) Combined injury: factors with potential to impact radiation dose assessments. Health Phys 98(2): 145-152.

50. DiCarlo AL, Maher C, Hick JL, Handling D, Dainiak N, et al. (2011) Radiation injury after a nuclear detonation: medical consequences and the need for scarce resources allocation. Disast Med Pub Health Prepared 5(1): S32-44.

51. Tibbs MK (1997) Wound healing followed by radiation therapy: A review. Radiother Oncol 42(2): 99-106.

52. Gu Q, Wang D, Cui C, Gao Y, Xia G, et al. (1998) Effects of radiation on wound healing. J Environ Pathol Toxicol Oncol 17: 117-123.

53. Peacock EE (1984) Contraction. In: Peacock EE (Ed), Wound Repair. 3rd (Edn.), Philadelphia, WB Saunders, pp: 39-55.

54. Tejero-Trujeque R (2001) How do fibroblasts interact with the extracellular matrix in wound contraction? J Wound Care 10(6): 237-242.

55. Grillo HC, Potsaid MS (1961) Studies on wound healing-IV, retardation of contraction by local X-irradiation and observations relating to the origin of fibroblast in repair. Ann Surg 154(5): 741-750.

56. Kumar P, Jagetia GC (1995) Modulation of wound healing in Swiss albino mice by different doses of gamma radiation. Burns 21(3): 163-165.

57. Taren DL, Chvapil M, Weber CW (1987) Increasing the breaking strength of wounds exposed to preoperative irradiation using vitamin E supplementation. Int J Nutr Res 57(2): 133-137.

58. Gieringer M, Gosepath J, Naim R (2011) Radiotherapy and wound healing: principles, management and prospects. Oncol Rep 26(2): 299-307.

59. Jacobson LK, Johnson MB, Dedhia RD, Niknam-Bienia S, Wong AK (2017) Impaired wound healing after radiation therapy: A systematic review of pathogenesis and treatment. JPRAS Open 13: 92-105.

60. Pang C, Ibrahim A, Bulstrode NW, Ferretti P (2017) An overview of the therapeutic potential of regenerative medicine in cutaneous wound healing. Int wound J 14(3): 450-459.

61. Wang PH, Huang BS, Horng HC, Yeh CC, Chen YJ (2018) Wound healing. J Chin Med Assoc 81(2): 94-101.

62. Holchstein AO, Bhatia A (2014) Collagen: Its role in wound healing. Wound Manage 4(1): 104-109.

63. Rangaraj A, Harding K, Leaper D (2011) Role of collagen in wound management. Wounds uk 7(2): 54-63.

64. Grant RA, Cox RW, Kent CM (1973) The effects of gamma irradiation on the structure and reactivity of native and cross-linked collagen fibres. J Anat 115(1): 29-43.

65. Chithra P, Sajithlal GB, Chandrakasan G (1998) Influence of Aloe vera on collagen characteristics in healing dermal wounds in rats. Mol Cell Biochem 181(1-2): 71-76.

66. Holm Pedersen P, Fenstad AM, Folke LE (1974) DNA, RNA and protein synthesis in healing wounds in young and old mice. Mechanisms of ageing and development 3: 173-185.

67. Eming SA, Krieg T, Davidson JM (2007) Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol 127(3): 514-525.

68. RohlJ, Zaharia A, Rudolph M, Murray RZ (2015) The role of inflammation in cutaneous repair. Wound Practice & Research: J Australian Wound Managem Assoc 23(1): 8-15.

69. Witte MB, Barbul A (2002) Role of nitric oxide in wound repair. Am J Surg 183(4): 406-412.

70. Schäffer MR, Tantry U, Gross SS, Wasserkrug HL, Barbul A (1996) Nitric oxide regulates wound healing. J Surg Res 63(1): 237-240.

71. Bainbridge P (2013) Wound healing and the role of fibroblasts. J Wound Care 22(8): 407-408.

72. Darby IA, Laverdet B, Bonté F, Desmoulière A (2014) Fibroblasts and myofibroblasts in wound healing.Clin Cosmet Investigat Dermatol 7: 301-311.

73. Kendall RT, Feghali Bostwick CA (2014) Fibroblasts in fibrosis: novel roles and mediators. Front Pharmacol 5: 123.

74. Velazquez OC (2007) Angiogenesis and vasculogenesis: Inducing the growth of new blood vessels and wound healing by stimulation of bone marrow–derived progenitor cell mobilization and homing. J Vas Surg 45(Suppl A): A39-47.

75. Chong DC, Yu Z, Brighton HE, Bear JE, Bautch VL (2017) Tortuous Microvessels Contribute to Wound Healing via Sprouting Angiogenesis. Arterioscl Thromb Vascul Biol 37(10): 1903-1912.

76. Rudolph R, Vande Berg J, Schneider JA, Fischer JC, Poolman WL (1988) Slowed growth of cultured fibroblasts from human radiation wounds. Plastic Reconstruct Surg 82(4): 669-677.

77. Zelman D, Song IC, Porteous DD, Bromberg BE (1969) The effect of total body irradiation on wound healing and the hematopoietic system in mice. Bull NY Acad Med 45(3): 293-300.

78. Trabucchi E, Pallotta S, Morini M, Corsi F, Franceschini R, et al. (2002) Low molecular weight hyaluronic acid prevents free radical damage to granulation tissue during wound healing. Int J Tissue React 24(2): 65-71.

79. Jagetia GC, Rao KVNM. Hesperidin (2015) A citrus bioflavonoid reduces the oxidative stress in the skin of mouse exposed to partial body γ-radiation. Transcriptomics 3(2): 1-6.

80. Zhou D, Brown SA, Yu T, Chen G, Barve S, et al. (1999) A high dose of ionizing radiation induces tissue-specific activation of nuclear factor-kappaB in vivo. Radiat Res. 151(6): 703-709.

81. Gaddipati JP, Sundar SV, Calemine J, Seth P, Sidhu GS, et al. (2003) Differential regulation of cytokines and transcription factors in liver by curcumin following hemorrhage/resuscitation. Shock 19(2): 150-156.

82. Hirata A, Murakami Y, Shoji M, Kadoma Y, Fujisawa S (2005) Kinetics of radical-scavenging activity of hesperetin and hesperidin and their inhibitory activity on COX-2 expression. Anticancer Res 25(5): 3367-3374.

83. Ghorbani A, Nazari M, Jeddi Tehrani M, Zand H (2012) The citrus flavonoid hesperidin induces p53 and inhibits NF-κB activation in order to trigger apoptosis in NALM-6 cells: involvement of PPARγ-dependent mechanism. Eur J Nutr 51(1): 39-46.

84. Jagetia GC, Rajanikant GK (2015) Curcumin Stimulates the Antioxidant Mechanisms in Mouse Skin Exposed to Fractionated γ-Irradiation. Antioxidants (Basel) 4(1): 25-41.

85. Elavarasan J, Velusamy P, Ganesan T, Ramakrishnan SK, Rajasekaran D, et al. (2012) Hesperidin-mediated expression of Nrf2 and upregulation of antioxidant status in senescent rat heart. J Pharm Pharmacol 64(10): 1472-1482.

86. Gu Q, Wang D, Gao Y, Zhou J, Peng R, et al. (2002) Expression of MMP1 in surgical and radiation-impaired wound healing and its effects on the healing process. J Environm Pathol Toxicol Oncol 21(1): 71-78.

87. Lee HJ, Im AR, Kim SM, Kang HS, Lee JD, et al. (2018) The flavonoid hesperidin exerts anti-photoaging effect by downregulating matrix metalloproteinase (MMP)-9 expression via mitogen activated protein kinase (MAPK)-dependent signaling pathways. BMC Compl Altern Med 18(1): 39.