Clinical Dermatology and Dermatitis

Hesperidin Treatment Abates Radiation-Induced Delay In Healing of Deep Cutaneous Excision Wound of Mice Hemi-Body Exposed to Different Doses of γ-Radiation

Ganesh Chandra Jagetia[1]*
K.V.N. Mallikarjuna Rao[2]

[1]10 Maharana Pratap Colony, Sector-13, Hiran Magri, Udaipur-313002, India
[2]Department of Biochemistry GSL Medical College, Rajmundry-533296, AP, India

Abstract

Potential of hesperidin as a vulnerary agent has been investigated in the irradiated wounds, where Swiss albino mice were given a single administration of 100 mg/kg body weight of hesperidin orally before hemi-body (below rib cage) exposure of animals to 2, 4, 6 or 8 Gy γ-radiation. A full thickness excision circular wound of 15 mm diameter was created on the dorsum of irradiated mice. The wound contraction was assessed by capturing video-images of wounds periodically until the complete healing of wounds. The data regarding mean wound healing time, NO, collagen, hexosamine and DNA syntheses were also collected. Hemi-body irradiation of animals caused a dose-dependent retardation in the wound contraction and prolongation of mean wound healing time, whereas oral administration of hesperidin before irradiation significantly increased the wound contraction, as a result, the mean wound healing time was also reduced. Irradiation of animals to 6 Gy resulted in a significant decline in DNA, collagen, hexosamine and nitric oxide syntheses in the granulation tissue at different post-irradiation days. Hesperidin treatment before irradiation resulted in a significant elevation in collagen, hexosamine, DNA and nitric oxide syntheses in the granulation tissue in comparison to radiation treatment alone. Biochemical observations are supported by histological examination, where hesperidin treatment increased the densities of blood vessels and fibroblasts in the regenerating wounds when compared to non-drug treated irradiated animals. The present study demonstrates that hesperidin acts as a good vulnerary agent and the observed acceleration in wound healing may be due to the enhancement of collagen, hexosamine, DNA and nitric oxide syntheses in the granulation tissue, which are essential components of wound healing.

Keywords

Radiation; Hesperidin; Mice; Wound Contraction; Collagen; Hexosamine; Nitric Oxide; Fibroblast

Introduction

Nuclear weapon testing, nuclear-reactor accidents, the possibility of dirty bomb use by terror groups and an accidental release of radioactive material from sealed radiation sources in hospital or industry result in a large-scale, uncontrolled exposure of humans to ionizing radiations [1-4]. In fact, in the USA alone there have been 36,110 bombing incidents where improvised explosive devices were used to spread terror between 1983-2002. Fortunately, none of these devices contained nuclear material. However, terrorist outfits threatened to explode "dirty bomb" in Moscow in 1995 and Chicago in 2002, which indicates that this kind of threats seems to be real and cannot be overlooked [5]. Inadvertent or intentional irradiation is accompanied by undesirable pernicious side effects and mass casualties and mortality. Skin is the outer most protective covering, which bears the brunt of irradiation as it is the first tissue to come into direct contact during irradiation [6,7].

Exposure of skin to ionizing radiations induces erythema, desquamation, ulceration, telangiectasia, fibrosis and skin carcinomas in the long run [8-10]. The acute radiation exposure in conjunction with combined injuries such as superimposed skin wounds and burn injury act synergistically producing higher mortality than the radiation injury alone would have rendered [9,11]. The normal responses to injury will be negatively altered when ionizing radiations interact with the wounded tissue, leading to a protracted recovery period [12-17]. The wound healing is an intricate process that requires well-coordinated cellular and molecular events, including inflammation, angiogenesis, fibroplasia, wound contraction, epithelialization and matrix remodeling [18-20]. Ordinarily, the response to injury is normal, and wound healing cascade is triggered leading to the healing of wounds without complications. The irradiation disrupts normal responses to injury, producing multiple negative effects on the wound healing processes including diminished vascularity, impairment of proliferative capacity of fibroblasts, and decreased collagen synthesis [4,9,21-23]. Therefore, understanding of biological response during combined injuries is of

Article Information

DOI: 10.31021/cdd.20181104
Article Type: Research Article
Journal Type: Open Access
Volume: 1 **Issue:** 1
Manuscript ID: CDD-1-104
Publisher: Boffin Access Limited

Received Date: 25 May 2018
Accepted Date: 14 June 2018
Published Date: 20 June 2018

*Corresponding author:

Ganesh Chandra Jagetia
Professor
10 Maharana Pratap Colony, Sector-13
Hiran Magri, Udaipur-313002
India
E-mail: gc.jagetia@gmail.com

Citation: Jagetia GC, Rao KVNM. Hesperidin Treatment Abates Radiation-Induced Delay In Healing of Deep Cutaneous Excision Wound of Mice Hemi-Body Exposed to Different Doses of γ-Radiation. Clin dermatol dermatitis. 2018 June;1(1):104

paramount importance in wound management so as to avoid further complications.

Acute radiation exposure is of interest to defense and civil administration, concerned about the aftermath of the use of nuclear weapons. Likewise, it is also of equal concern to radiation safety personnel related to possible accidents involving large doses of radiation and also to radiotherapists performing localized hemi-body irradiation for cancer treatment. Hemi-body irradiation in multiple fractionated doses is frequently used alone or in combination with surgery or other modalities for the treatment of various solid tumors and it produces both acute and late effects on the skin and subcutaneous tissues that have profound effects on the healing of surgical wounds [24,25]. Ionizing radiations triggers several detrimental effects on the wound repair and regenerative responses in the form of suppression of inflammatory reactions, cell and connective tissue proliferation, formation and maturation of granulation tissue, transcription of collagen mRNAs, secretion of collagen neovascularization and matrix metalloproteinase [23,26,27].

Investigations have been directed to neutralize the radiation-induced adverse effects on the wounds repair and regenerative responses and many potential therapies and prophylaxes for irradiated wounds have been tried. However, use of nutraceuticals to positively modulate radiation response of healing wounds did not receive much attention, which indicates the need for continued research in the medical management of radiation casualties including wound healing. Since wound-healing deformities cause great physical and psychological distress to the affected patients and their treatment is extremely expensive, the use of nutraceuticals in the reconstruction of irradiated wounds seems to be a fascinating research area. The ascorbic acid, curcumin and *Nigella sativa* have been found to accelerate the repair and regeneration of excision wounds of mice exposed to different doses of γ-radiation [13-17,22,28]. However, the use of nutraceuticals as vulnerary agents remains largely unexplored and their use may be pragmatic as they are consumed daily, have wide acceptability, better tolerance, non-toxic, more economic, and can be safely manipulated for human use [4,28-30].

Hesperidin also known as hesperitin-7-rhamnoglucoside or hesperitin-7-rutinoside is a secondary metabolite synthesized by several plants of the citrus family. It is a bioflavonoid that abounds in the discarded rinds of the ordinary orange and fruit pulp of Citrus *aurantium L.*, *C. sinensis, C. unshiu* and other species of the Citrus genus [31-35]. It is also found in many plants other than Citrus species, such as in genera *Fabaceae, Betulaceae, Lamiaceae and Papilionaceae* [36-38]. The anti-inflammatory, analgesic, antihypertensive, diuretic, antibacterial, anticancer antioxidant and antiviral properties of hesperidin have been established in various study systems [32,39-44]. Hesperidin is known to induce apoptosis and cell cycle arrest in vitro [45]. It has been known to exert the chemopreventive effect in vivo [46]. Hesperidin has been found to be active against hepatotoxicity, cardiotoxicity, osteoporosis and cognitive impairment [47-50]. Hesperidin has been found to alleviate cholesterol level and trigger bone formation in humans [49,51]. Hesperidin has been reported to reduce bleomycin-induced lung fibrosis in rats [52]. Hesperidin has been found to reduce diastolic blood pressure and endothelium-dependent microvascular reactivity in human volunteers given orange juice or hesperidin for four weeks in a clinical trial ([53]. The human volunteers administered with orange juice or hesperidin alone for 4 weeks led to the alteration in the expression of 3422 and 1819 genes respectively in a French study [54]. Treatment of mice with 5% hesperidin for 13 weeks has been found to be non-toxic [55]. Hesperidin has been reported to be nontoxic in acute toxicity studies and scavenged various free radicals in a concentration-dependent manner. The topical application of hesperidin gel has been found to hasten the healing of irradiated wounds [56]. The various properties attributed to hesperidin stimulated us to investigate the vulnerary potential of hesperidin in mice exposed to different doses of hemi-body γ-radiation and then inflicted with an open full thickness skin excision wound.

Materials and Methods

Chemicals and Drugs

Hesperidin was obtained from Acros Organics Ltd, Geel, Belgium. ρ-dimethylamino-benzaldehyde, diphenylamine, Deoxyribonucleic Acid (DNA), Trichloroacetic Acid (TCA), N-(1-Naphthyl)Ethylenediamine Dihydrochloride (NEDD) and reduced β-Nicotinamide Adenine Dinucleotide Phosphate, (β-NADPH) were procured from Sigma Aldrich Chemical Co. St. Louis, USA, whereas Carboxymethylcellulose (CMC), Hydrochloric Acid (HCl); Sodium Hydroxide (NaOH), Perchloric Acid, Sulphanilamide and Phosphoric Acid were supplied by Merck India, Mumbai.

Animal care and handling

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). 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 had free access to sterile food and water. The food consisted of 50% cracked wheat, 40% Bengal gram, 4% milk powder, 4% yeast powder, 0.75% sesame oil, 0.25% cod liver oil, and 1% salt. 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 Manipal University, Manipal, where the entire study was conducted.

Preparation of Drug and Mode of Administration: The hesperidin (HPD) is sparingly soluble in water. Therefore, the required amount of hesperidin was suspended in 1% CMC immediately before administration. The animals were administered with a single dose of 0.01 ml/g body weight of CMC or HPD orally before irradiation.

Experimental Protocol: The effect of hesperidin was investigated on the alteration in the healing of excision wounds in mice exposed to different doses of hemi-body γ-radiation by dividing the animals into the following groups:

CMC+ irradiation: The animals of this group were orally given 0.01ml/g body weight of CMC before irradiation.

HPD+ irradiation: This group of animals was orally administered with 100 mg/kg body weight of hesperidin before irradiation [57].

Irradiation: One hour after administration of CMC or hesperidin, each animal was placed into a specially designed well-ventilated acrylic restrainer and the lower half of the animal body (below the rib cage) was exposed to 0, 2, 4, 6 or 8 Gy of γ-radiation given 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 hairs of the dorsum (below the rib cage) of each animal were removed using a cordless electrical mouse clipper (Wahl Clipper Corporation, Illinois, USA) before exposure of animals to various doses of γ-radiation. A full-thickness circular excision skin wound was created on the dorsum (below the rib cage) of the animal as described earlier within ten minutes of irradiation [4,57]. In brief, the animals were anesthetized using ketamine and the skin of the entire body was cleaned and decontaminated by wiping the whole body with sterillium (Bode Chemical, Hamburg, 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 full thickness skin flap including *panniculus carnosus* in an aseptic environment using sterile scissors and forceps (Figure 1). Each wounded animal was housed in an individual sterile polypropylene cage until complete healing of the wound.

Experiment 1: Wound contraction

A separate experiment was conducted to study the effect of hesperidin on the contraction of excision wounds of irradiated mice, where the grouping and other conditions were similar to that described above. The animals were inflicted with full thickness

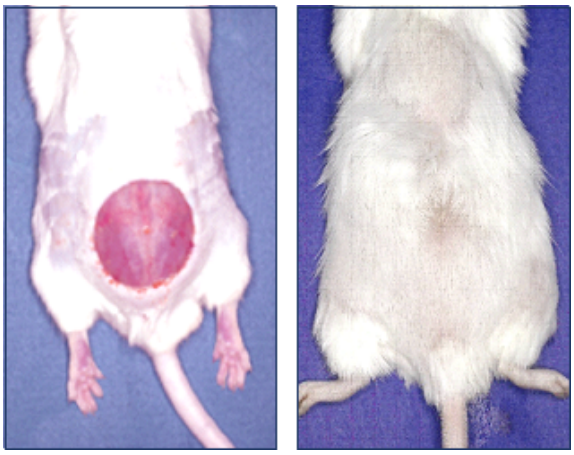

Figure 1: The images of excision wound on the day of creation (left) and the same after complete healing (right)

excision wound after exposure to different doses of γ-radiation. The wound contraction was estimated regularly where the video images of each full-thickness wound were captured using a CCD (charged coupled device) camera connected to a computer. The first image of each wound from different groups was captured one day after wounding, and it was considered as day one. The subsequent images were acquired 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 animals were used to complete this experiment.

Experiment 2: Mean wound healing time

Another 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 left undisturbed and monitored visually until complete healing of wounds (Figure 1) and the day at which each wound healed was recorded. The mean of all the days was 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 utilized to complete this study.

Experiment 3: Biochemical studies

A separate set of experiments was undertaken to study the alteration in the various biochemical profiles of regenerating excision wounds after exposure to 0 or 6 Gy hemi-body γ-radiation and their modulation by hesperidin. Grouping of animals and creation of wounds were essentially similar to that described for wound contraction experiment, except that the hemi-body of the animals was exposed to 0 or 6 Gy of γ-radiation. Wound biopsies were collected on 4, 8 and 12 days post-irradiation and the granulation tissue samples were stored at –70°C until analysis.

Nitric oxide biosynthesis: The stable end products of NO biosynthesis were estimated as both nitrites and nitrates in the regenerating granulation tissue of the wounds [4,22]. Briefly, the preweighed (125 mg) amount of granulation tissue was homogenized in hypotonic saline and centrifuged. Nitrite concentrations were determined with Griess reagent. The supernatant was mixed with Griess reagent (0.1% NEDD, 1% sulphanilamide and 5% phosphoric acid in a 1:1:1 ratio, prepared freshly), incubated at 37°C for 30 min and the absorbance was recorded at 543 nm using a double beam UV-visible spectrophotometer (Shimadzu UV-260, Shimadzu Corp., Tokyo, Japan). 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 tissue homogenate were mixed with the addition of Griess reagent. The mixture was 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 μM/g dry tissue weight. Six animals were used in each group at each assay time and a total of 72 animals were used for each estimation.

DNA synthesis: The DNA contents in the regenerating granulation tissue/s were measured as described earlier [4,58]. Briefly, 125 mg 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. The supernatant was collected used for the determination of DNA contents. The DNA was hydrolyzed with 60% perchloric acid at 80°C for 20 min followed by the addition of Burton's diphenylamine reagent and left overnight at room temperature. 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, which has been expressed as mg/g dry tissue weight. Six animals were used in each group at each assay time and a total of 72 animals were used for this estimation.

Collagen synthesis: The formation of collagen in the regenerating wounds was determined as hydroxyproline contents [4,59]. The 125 mg of granulation tissue was hydrolyzed in 6N HCl at 130°C for 3 h, neutralized (pH 7) with 2.5N 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-dimethylaminobenzaldehyde (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. 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 assay time and a total of 72 animals were used for this estimation.

Hexosamine estimation: Hexosamine contents in the regenerating wound granulation tissues were measured as described earlier with minor modifications [4,60]. In brief, the granulation tissue was weighed, hydrolyzed in 6N HCl for 8 h at 98°C, neutralized to pH 7 with 4N NaOH and diluted further with Milli-Q water. The diluted solution was mixed with acetyl acetone and heated up to 96°C for 40 min and cooled. The 96% ethanol and ρ-dimethylaminobenzaldehyde solution (Ehrlich's reagent) were added in sequence to this mixture. It was mixed thoroughly, kept at room temperature for 1 h and the absorbance was measured at 530 nm using a double beam UV-visible spectrophotometer. The amount 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 assay time and a total of72 animals were used for this estimation.

Experiment 4: Histological studies

A separate experiment was conducted to evaluate the histological alterations during wound healing after exposure to 0 or 6 Gy hemi-body γ-radiation. Grouping of animals and production of wounds were carried out as described for the wound contraction experiment, except that the hemi-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 hematoxylin and eosin. Sections were assessed in a blinded fashion under the 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 hematoxylin and eosin stains were scored for vascular repopulation studies. Three animals were used for each irradiation dose in each group at each post-irradiation time and the total of 36 animals was used for histological examination.

Analysis of Data

Statistical significance between the treatment groups was determined using one-way ANOVA with Tukey's post-hoc test or student's't' test. The Solo 4 statistical package (BMDP Statistical Software Inc., Los Angles, 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 DNA, collagen, hexosamine, nitrate and nitrite and fibroblast and vascular densities in Figures 2-11.

Wound contraction

The wound contraction can be easily estimated by recording the wound area regularly using videography, which is less cumbersome and with a minimum stress to the animals. The capturing of video images of excision wound provides a good measure of wound repair and regeneration. 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 is plotted as a function of days after wounding (Figure 2). Wound repair progressed with time as indicated by a steady contraction of the excision wound with elapse of time in both the CMC+ sham-irradiation and HPD+ sham-irradiation groups (0 Gy). A significant rise in the wound contraction was detectable at 3 ($p < 0.01$), 6 ($p < 0.05$) and 9 ($p < 0.05$) day post-irradiation after hesperidin administration when compared with the CMC+ sham-irradiation group (Figure 2). A clear scab formation was seen in CMC+ sham-irradiation group whereas no such scab formation was observed for HPD+ sham-irradiation group.

Hemi-body exposure of mice to different doses of γ-rays led to a dose dependent retardation in wound contraction (Figure 2, 3). Exposure of animals to 2 or 4 Gy, delayed wound contraction (Figure 2) 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 at day 3 and 15 post-irradiation. Exposure of animals to 6 and 8 Gy significantly delayed wound contraction at all the post-irradiation days in comparison with CMC+ sham-irradiation group (Figure 2). Hesperidin treatment prior to exposure to 2, 4 or 6 Gy irradiation in HPD+ irradiation group resulted in a significant rise in the contraction of wounds at 3 ($p < 0.01$), 6 ($p < 0.001$), 9 ($p < 0.001$), and 12 ($p < 0.05$) days post-irradiation, when compared to CMC+ irradiation group (Figure 3) 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 at all post-irradiation days ($p < 0.001$), except 3 days post-irradiation (Figure2).

Mean wound healing time

Regular monitoring of wounded mice showed complete closure of wounds by 18.7 ± 0.65 days post-irradiation in CMC+ sham-irradiation group, whereas treatment of mice with hesperidin resulted in a significant reduction in the mean wound healing time (16.2 ± 0.39 days post-irradiation) in the HPD+ sham-irradiation group (Figure 4). The hemi-body exposure of mice to different doses of γ-radiation resulted in a dose dependent delay in the complete closure of wounds as a result; the mean wound healing time was also prolonged in CMC+ irradiation group when compared with the CMC+ sham-irradiation group. A mean wound healing time of 20.0 ± 0.28, 21.0 ± 0.29, 22.7 ± 0.47 and 24.4 ± 0.37 days was observed for 2, 4, 6 and 8 Gy, respectively, in CMC+ irradiation group (Figure. 4). Treatment of mice with 100 mg/kg of hesperidin before irradiation

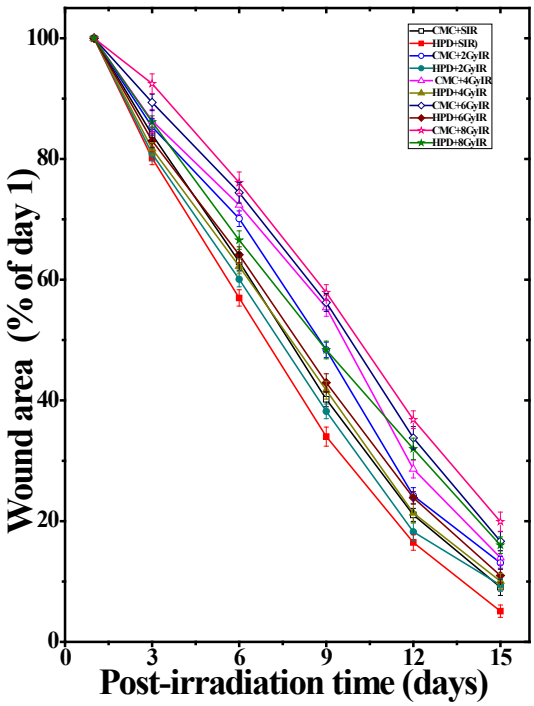

Figure 2: Effect of 100 mg/kg body weight hesperidin treatment on the wound contraction in the full thickness excision wounds of mice exposed to various doses of hemi-body γ-radiation at different post-irradiation days

CMC, carboxymethylcellulose; HPD, hesperidin; SIR, sham-irradiation; IR, irradiation.

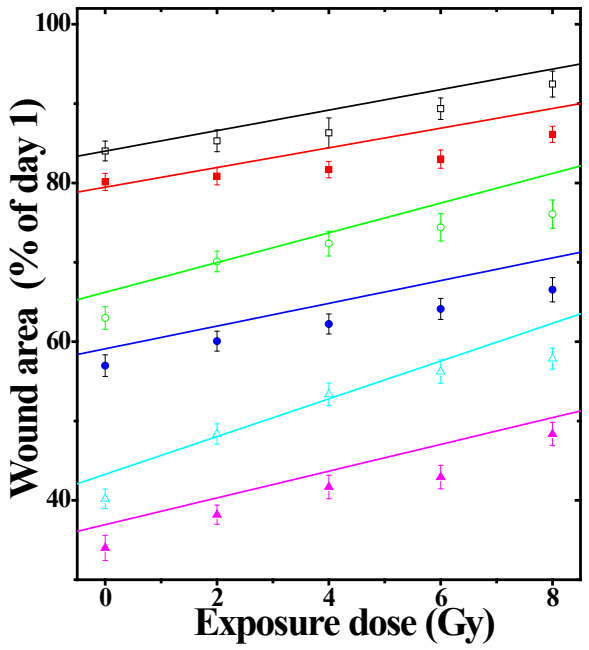

Figure 3: Effect of 100 mg/kg body weight hesperidin treatment on the progression of wound closure (wound contraction) at different post-irradiation days in the full thickness excision wounds of mice exposed to various doses of hemi-body γ-radiation. Open squares: CMC+IR 3 day; Closed squares: HPD+IR 3 day; Open circles: CMC+IR 6 day; Closed circles: HPD+IR 6 day; Open triangles: CMC+IR 9 day and Closed triangles: HPD+IR 9 day

CMC, carboxymethylcellulose; HPD, hesperidin; SIR, sham-irradiation; IR, irradiation.

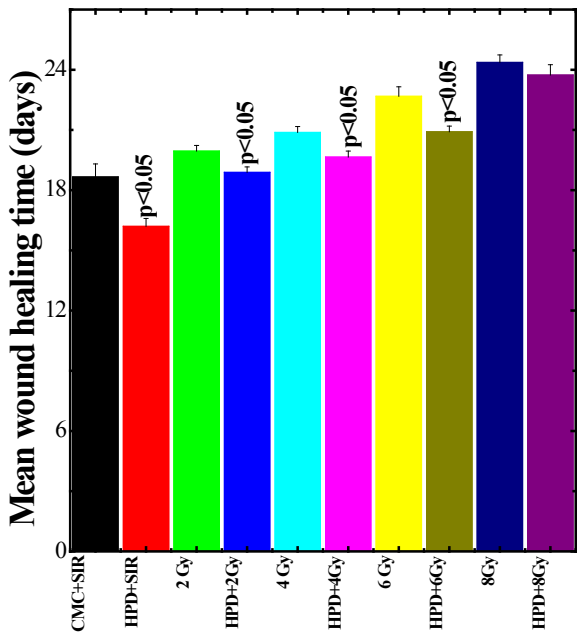

Figure 4: Effect of 100 mg/kg body weight hesperidin treatment on the mean wound healing time in mice exposed to various dose of hemi-body γ-radiation

CMC: Carboxymethylcellulose; HPD: Hesperidin and SIR: Sham-irradiation.

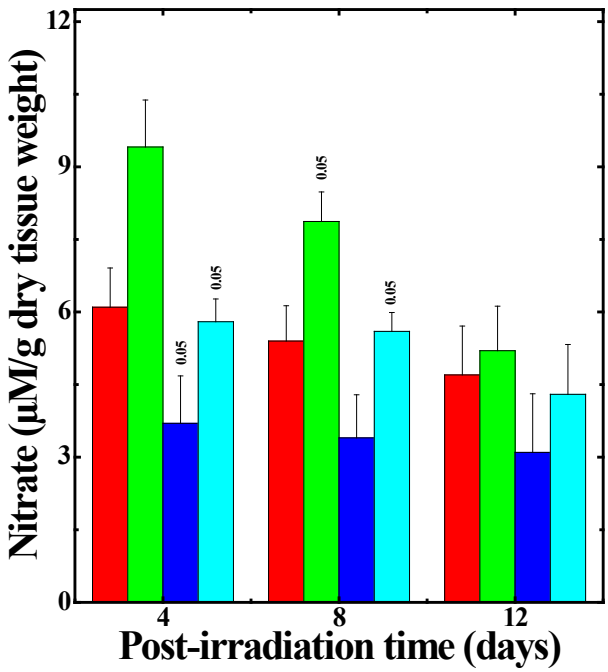

Figure 5: Effect of 100 mg/kg body weight hesperidin treatment on the nitrate synthesis in the regenerating tissue of the full thickness excision wounds of mice exposed to 6Gy hemi-body γ-radiation at different post-irradiation days. Red bars: CMC+SIR; Green bars: HPD+SIR; Blue bars: CMC+IR and Cyan bars: HPD+IR

CMC: Carboxymethylcellulose; HPD: Hesperidin; SIR: Sham-irradiation andIR: Irradiation.

to different doses of γ-radiation accelerated the healing of irradiated excision wounds, as a result there was a decline in mean wound healing time (Figure 4). The mean wound healing time of 19.0 ± 0.27, 19.5 ± 0.3, 20.9 ± 0.28 and 23.7± 0.51 days was observed for 2, 4, 6 and 8 Gy, respectively, in HPD+ irradiation group (Figure 4). This reduction in wound healing time was statistically significant ($p < 0.05$) for 2, 4 and 6 Gy irradiation in HPD+ irradiation group except for 8 Gy, where it was statistically non-significant in HPD+ irradiation group (Figure 4).

Biochemical studies

Nitric oxide biosynthesis: End products of NO synthesis, nitrite and nitrate elevated as early as 4 days post-irradiation in the granulation tissue and the levels of both nitrite and nitrate continued to decline up to 12 days post-irradiation in sham-irradiation group (Figure 5-6). Irradiation of animals to 6 Gy hemi-body γ-radiation resulted in a drastic decline in both nitrite and nitrate contents in the granulation tissues at all post-irradiation times. Administration of 100 mg/kg hesperidin before 6 Gy irradiation resulted in a significant elevation in both nitrite and nitrate contents at all post-irradiation days except 12 days post-irradiation, where this rise was statistically non-significant (Figure 5-6).

DNA synthesis: The DNA synthesis is a good measure of cell proliferation and increase in DNA contents of treated wounds indicates hyperplasia of cells (Figure 7). Exposure of animals to 6 Gy resulted in a drastic decline in the DNA contents in the regenerating granulation tissue of irradiated wounds at day four, which increased abruptly on day 8 and alleviated thereafter on day 12 post-irradiation. Despite this decline at 12 days the DNA synthesis was higher than that of day 4 post-irradiation (Figure 7). Oral administration of HPD before 6 Gy irradiation significantly elevated the DNA synthesis on day 4 by 1.5 times of CMC+ irradiation group and continued to increase up to day 8 where a maximum synthesis of DNA was recorded (Figure 7). Thereafter, a reduction in DNA synthesis was observed on day 12 post irradiation. However, it was still higher than that of day 4 post-irradiation (Figure 7).

Collagen synthesis: The amount of hydroxyproline is an index

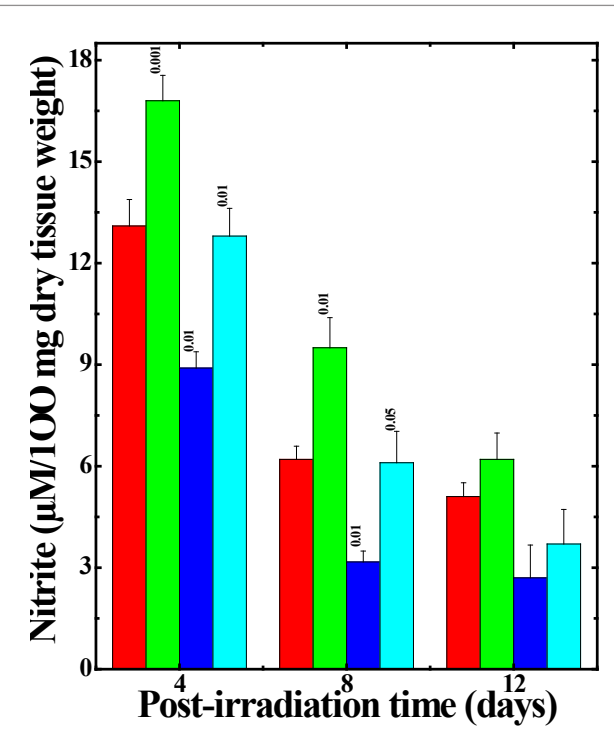

Figure 6: Effect of 100 mg/kg body weight hesperidin treatment on the nitrite synthesis in the regenerating tissue of the full thickness excision wounds of mice exposed to 6Gy hemi-body γ-radiation at different post-irradiation days. Red bars: CMC+SIR; Green bars: HPD+SIR; Blue bars: CMC+IR and Cyan bars: HPD+IR

CMC: Carboxymethylcellulose; HPD: Hesperidin; SIR: Sham-irradiation and IR: Irradiation.

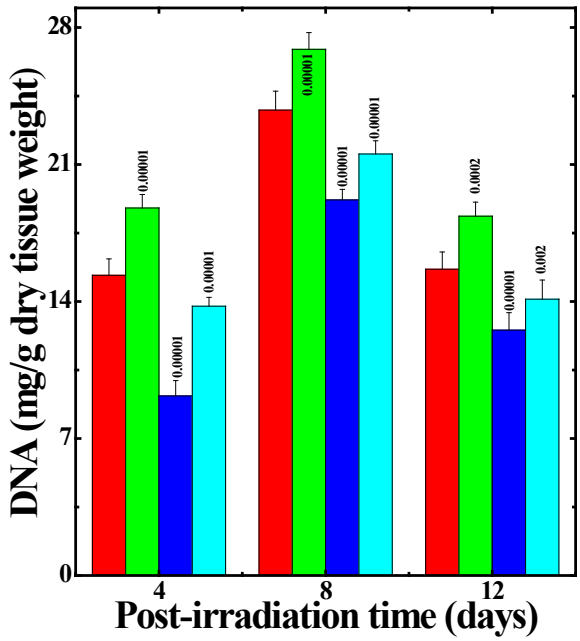

Figure7: Effect of 100 mg/kg body weight hesperidin treatment on the DNA synthesis in the regenerating tissue of the full thickness excision wounds of mice exposed to 6Gy hemi-body γ-radiation at different post-irradiation days. Red bars: CMC+SIR; Green bars: HPD+SIR; Blue bars: CMC+IR and Cyan bars: HPD+IR

CMC: Carboxymethylcellulose; HPD: Hesperidin; SIR: Sham-irradiation and IR: Irradiation.

of collagen content and is also a measure of neo-collagen synthesis. The synthesis of neocollagen increased with time up to day 8 post-irradiation as indicated by the highest hydroxyproline contents in CMC or HPD+ sham-irradiation groups and declined thereafter on day 12 post-irradiation in both the groups (Figure 8). Despite this decline the collagen contents were higher than day 4 (Figure 8). The hesperidin treatment alone significantly increased the synthesis of collagen in the regenerating wound (Figure 8). Irradiation of animals to 6 Gy resulted in a drastic drop in the collagen synthesis at all post-irradiation assay times, which was statistically significant when compared to the CMC+ Sham-irradiation group (Figure 8). Despite this decline in collagen synthesis, a maximum synthesis of collagen was observed on day 8 after wounding in the CMC+ irradiation group; thereafter the formation of new-collagen declined at 12 days post-irradiation but it was significantly higher than 4 days post-irradiation (Figure 8). 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 at all post-irradiation assay times when compared to the concurrent CMC+ irradiation group (Figure 8). Pretreatment of mice with hesperidin could not restore the level of collagen to normal even by 12 days post-irradiation, which was significantly lower than CMC+ Sham-irradiation group ($p<0.001$).

Hexosamine estimation: Hesperidin treatment alone increased hexosamine, the ground substratum for collagen synthesis on day 4, which continued to rise up to day 8 post-irradiation and declined thereafter when compared to CMC+ sham-irradiation control (Figure 9). Irradiation of animals to 6 Gy significantly reduced 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 9). 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, except day 12 when compared to concurrent CMC+ irradiation group (Figure 9).

Histological studies: Histological examination of wound biopsies at various post-irradiation times revealed that hesperidin treatment alone did not alter the histological picture, except that there was an increase in the fibroblast and vasculature densities compared to the non-drug-treated sham-irradiation controls. The density of fibroblasts declined drastically in CMC+ irradiation group when compared with CMC+ sham-irradiation group (Figure 10). A few, large and stellate cells or "radiation fibroblasts" were seen after irradiation at 8 days 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 10-11). However, the histological picture similar to the sham irradiated control was not restored.

Discussion

The detrimental consequences of ionizing radiation on wound healing is multifaceted as ionizing radiation produce direct cytotoxic effects on various cellular/molecular components that play a crucial role in the repair and regeneration of wounds and also indirectly through free radical mediated damage on regenerating wounds. Therefore, a need is felt for a multifunction drug which conforms to all criteria of an optimal radioprotector including effectiveness, non-toxicity, easy availability, wider biological functions and tolerance. The use of natural antioxidants that can specifically target reactive oxygen species and overcome the direct negative effects of ionizing radiations could be an important therapeutic paradigm to improve healing of irradiated wounds. Hesperidin, a citrus flavanone is one such natural product that has been reported to be a good antioxidant and has recently shown its potential as a radioprotective agent [61,62]. Therefore, the present study had evaluated its potential as a vulnerary agent on wound healing in mice hemi-body exposed to different doses of γ-radiation.

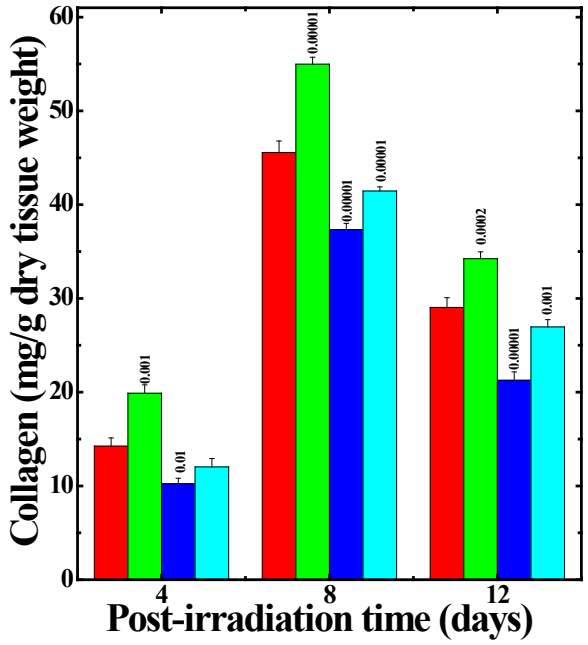

Figure 8: Effect of 100 mg/kg body weight hesperidin treatment on the collagen synthesis in the regenerating tissue of the full thickness excision wounds of mice exposed to 6Gy hemi-body γ-radiation at different post-irradiation days. Red bars: CMC+SIR; Green bars: HPD+SIR; Blue bars: CMC+IR and Cyan bars: HPD+IR

CMC: Carboxymethylcellulose; HPD: Hesperidin; SIR: Sham-irradiation and IR: Irradiation.

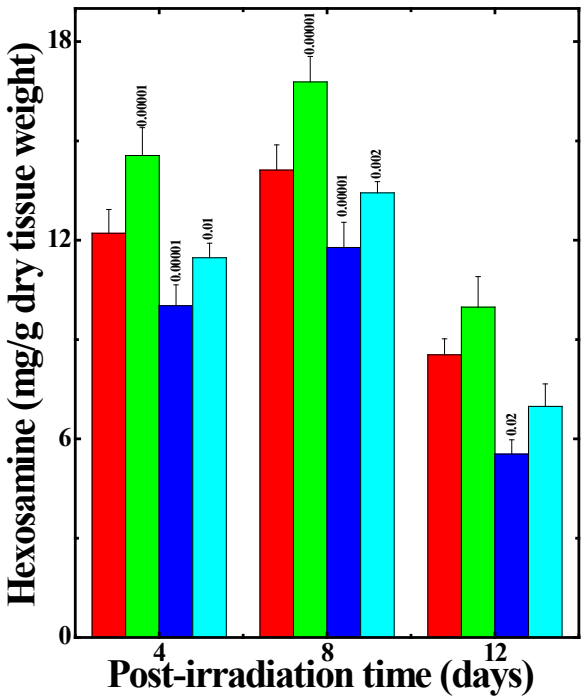

Figure 9: Effect of 100 mg/kg body weight hesperidin treatment on the hexosamine synthesis in the regenerating tissue of the full thickness excision wounds of mice exposed to 6Gy hemi-body γ-radiation at different post-irradiation days. Red bars: CMC+SIR; Green bars: HPD+SIR; Blue bars: CMC+IR and Cyan bars: HPD+IR

CMC: Carboxymethylcellulose; HPD: Hesperidin; SIR: Sham-irradiation and IR: Irradiation.

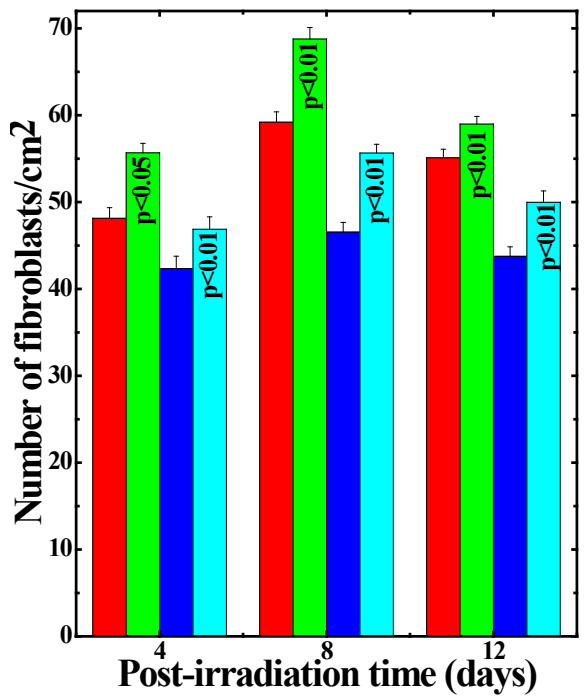

Figure10: Effect of 100 mg/kg body weight hesperidin treatment on the fibroblast density in the regenerating tissue of the full thickness excision wounds of mice exposed to 6Gy hemi-body γ-radiation at different post-irradiation days. Red bars: CMC+SIR; Green bars: HPD+SIR; Blue bars: CMC+IR and Cyan bars: HPD+IR

CMC: Carboxymethylcellulose; HPD: Hesperidin; SIR: Sham-irradiation and IR: Irradiation.

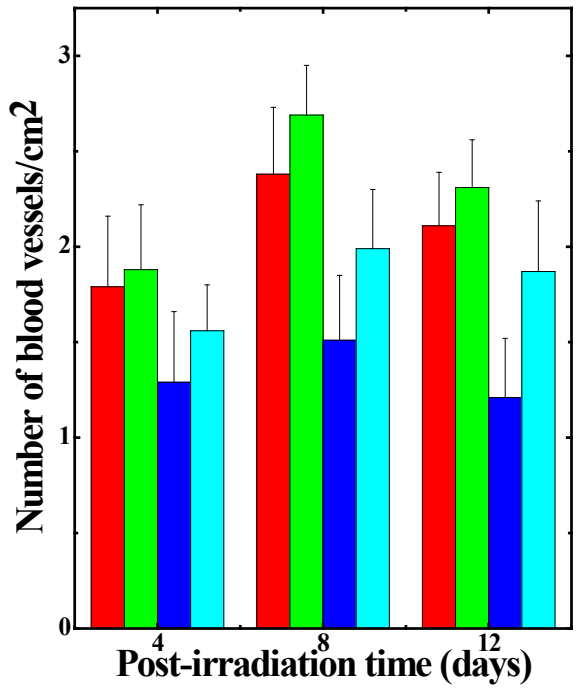

Figure 11: Effect of 100 mg/kg body weight hesperidin treatment on the blood vessel density in the regenerating tissue of the full thickness excision wounds of mice exposed to 6Gy hemi-body γ-radiation at different post-irradiation days. Red bars: CMC+SIR; Green bars: HPD+SIR; Blue bars: CMC+IR and Cyan bars: HPD+IR

CMC: Carboxymethylcellulose; HPD: Hesperidin; SIR: Sham-irradiation and IR: Irradiation.

The cutaneous wound triggers myriad of events to restore its function and clear the foreign material from the wound bed [63]. Wound repair is a meticulous coordination of inflammation, re-epithelization and matrix remodeling [19,64]. Wound repair and regeneration require interplay of numerous molecular, cellular, hormonal, matrix, and enzymatic activities in a well-orchestrated manner [19]. The cutaneous wound healing comprises two important but distinct events, the fibroplasia and epithelialization. The complete closure of a wound is incumbent upon the proliferation and migration of fibroblasts into granulation tissue, followed by a sequential deposition of specific matrix components, and finally contraction and remodeling [19,65]. It is essential that during the repair of nascent wound, the cells of each layer must replicate and migrate to repopulate the wound for its effective closure. The transition of basal keratinocytes allows such repopulation and the invasion of provisional matrix by fibroblasts is the first step in regenerating the future dermal layer. The high levels of numerous growth factors present during repair, including epidermal growth factor (EGF) receptor ligands, seem to be responsible for these mitogenic and motogenic responses [66,67]. 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 [68,69]. The regular assessment of the contraction indicates the progression of wound healing of excision wounds [4,12-17,57]. The dose-dependent delay in wound contraction after hemi-body exposure of mice to different doses of γ-radiation may be due to the inhibition of mitogenic response after irradiation. Ionizing radiations are known to inhibit cell division [70]. A delay in wound contraction after exposure to γ-radiation has been observed earlier, indicating that irradiation alter the local conditions of wound, which is non-conducive to wound repair [4,12-17,57,71,72]. Treatment of mice with hesperidin before exposure to different doses of γ-irradiation resulted in an enhancement of wound healing as was evident by greater degree of

wound contraction and reduction in the mean wound healing time. The hesperidin has been reported to retard the radiation-induced delay in a dose-dependent manner and 100 mg/kg hesperidin had the optimum effect [57]. Similarly, topical application of hesperidin also helped to overcome the radiation induced delay in the repair and regeneration of irradiated wounds [56]. The other nutrient factors like ascorbic acid and curcumin have been reported to accelerate healing of excision wounds after hemi-body irradiation to different doses of γ-radiation [13,22;28]. Likewise, vitamin A supplementation has also been reported to ameliorate the acute radiation-induced delay in wound healing and normalize the breaking strength of wounds after preoperative irradiation [73,74].

Inflammation is one of the most important and first events of wound healing as it initiates a cascade of responses that are absolutely necessary for repair of wounds after injury. The inflammation lasts for several days and its onset is triggered by blood clotting and platelet degranulation in the wound bed [75]. Within few hours of wound creation, the neutrophils transmigrate to the endothelium of the blood vessels, which have been activated due to secretion of proinflammatory cytokines including interleukin factor β, tumor necrosis factor α, and interferon-γ at the wound site. The monocytes at the wound site transform into macrophages and secrete large quantities of nitric oxide (NO), that is finally oxidized to hydroxyl radicals that kill microbes [76]. The NO plays a pivotal role during wound repair and regeneration. Most available evidence suggests 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 [12-14;76,77]. The increased production of NO helps triggers the secretion of vascular endothelial growth factor (VEGF) and angiogenesis that protect wounds from ischemia-reperfusion injury [76]. NO is difficult to measure directly hence the stable end products nitrate and nitrite are estimated as a measure of NO production [77].

The reduced NO production has been indicated to suppress wound repair [76,77]. Exposure of mice to 6 Gy resulted in the suppression of NO synthesis as indicated by the reduced nitrate and nitrite levels. Irradiation has been reported to reduce the level of nitrite and nitrate in healing wounds earlier [4,12-15,22]. The decrease in NO expression has been correlated with radiation-induced impairment in wound healing [4,12-15,22]. The release of NO is an early event and lasts for 1-5 days [76]. Levels of nitrite and nitrate, the stable end products of nitric oxide synthesis, are elevated early and transiently in fluid obtained from sponges implanted in subcutaneous wounds [78]. Administration of hesperidin before irradiation elevated nitrite and nitrate levels in granulation tissue of mice as early as 4 days post-irradiation and declined thereafter. The hesperidin treatment has been reported to increase NO that consistently declined with assay time in the irradiated wound created after whole-body irradiation [4]. A similar effect on nitrite and nitrate levels has been reported with ascorbic acid and curcumin in the irradiated wounds [12-15].

The DNA is the principle components of the cells and an increase in the DNA synthesis during the healing of wounds is a hallmark of cell proliferation [79]. The estimation of DNA contents, therefore, provides important information regarding cell division in the regenerating wounds. The hemi-body irradiation of mice significantly depleted DNA synthesis indicating reduced cell proliferation in the regenerating wounds, that may be responsible for retardation in the wound healing in the CMC+ irradiation group. Irradiation has been reported to reduce the DNA synthesis in the regenerating wounds earlier [4,12-15,22]. Hesperidin treatment significantly raised the DNA contents at all post-irradiation days in HPD+ irradiation group pointing towards increased cell replication and accelerated repair of irradiated wounds. A resembling effect has been observed earlier in the granulation tissues of excision wounds of mice receiving hesperidin before exposure to whole body γ-radiation [4]. The ascorbic acid or curcumin treatment before irradiation has been reported to accelerate the DNA synthesis in the full thickness regenerating wounds earlier [12-15,22].

Extracellular matrix (ECM) proteins and polysaccharides form an important part of the dermis of the skin and provide mechanical strength to the skin [67]. Collagen, a triple helix protein is one of the important ECM components and plays a pivotal role in the healing of wounds. It is a principal fibrillar component of the connective tissue, which provides a structural framework, strength, and milieu to the regenerating tissue [80]. It helps in the migration of cells, contraction of a wound, platelet aggregation, stimulation of various growth factors, cell surface receptors, other ECM molecules and proteins during repair and regeneration of wound [81]. During normal wound healing, a balance between the synthesis and degradation of collagen is maintained. The excess collagen formation results in keloid formation and hypertrophic scar formation, whereas insufficient collagen synthesis may lead to wound dehiscence and chronic non-healing wounds/ulcers [80-82]. Collagen is mainly produced by fibroblasts and assists the wound in gaining tensile strength during wound repair [80,81]. The reduction in hydroxyproline content in the granulation tissue after 6 Gy irradiation indicates depletion in the collagen synthesis. An identical effect has been observed earlier, where irradiation of mice with different doses of γ-radiation resulted in a dose-dependent reduction in the collagen synthesis [12-15,22]. Irradiation to increasing doses of γ- rays have been reported to cause a progressive destruction of the native collagen fibrils [83]. Hesperidin administration before hemi-body irradiation elevated the collagen synthesis at all post-irradiation days when compared to 6 Gy irradiation alone. Earlier, hesperidin has been found to increase the synthesis of collagen in the regenerating wound of mice after whole-body irradiation [4]. Similarly, ascorbic acid, curcumin and *Nigella sativa* were found to inhibit the radiation-induced decline in the collagen synthesis in the regenerating wounds earlier [12-15,17].

The hexosamine serves as a substrate for collagen synthesis and it increases during early stages of wound repair and regeneration and declines thereafter. A similar effect has been observed in the present study, were the regenerating excision wound showed a time-dependent rise in the hexosamine synthesis up to 8 days post-irradiation and declined thereafter, which is in sync with collagen synthesis. Irradiation of mice has been reported to reduce the hexosamine contents in the regenerating excision wounds earlier [12-15]. Administration of hesperidin before irradiation elevated hexosamine contents significantly on day 4 and 8 post-irradiation in comparison with the CMC+ irradiation group. Likewise, hesperidin has been reported to increase the hexosamine contents in the excision wounds of whole body exposed mice [4]. Earlier, ascorbic acid, curcumin and *Nigella sativa* have been reported to elevate the hexosamine contents during reparation of irradiated wounds [12-15,17].

The biochemical observations are supported by histological examination, where irradiation caused a decline in the proliferation of fibroblasts, which are responsible for collagen synthesis in the regenerating wounds. 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 was observed [4,12-15,84]. A direct negative impact of total-body and hemi-body irradiation on cellular influx, vascularization and collagen deposition in mice has been reported earlier [4,12-15,17,85]. 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 [21]. Administration of mice with hesperidin before hemi-body irradiation increased vascularity and fibroblast density and reduced hyalinization. The hesperidin has been reported to increase fibroblast division and vascular density in the whole body irradiated mice earlier [4]. Likewise, ascorbic acid and curcumin escalated the collagen deposition, blood vessel and fibroblast density and reduced hyalinization in the regenerating wounds of mice [12-14].

The exact mechanism of action of hesperidin in augmenting the wound contraction and reducing the MHT in hemi-body irradiated wound is not known. The hesperidin may have used several pathways

to exert its vulnerary action. Ionizing radiations and wounding overproduce reactive oxygen species causing, oxidative stress that results, in the detrimental cytotoxic effects and as a result delay healing of irradiated wounds [86]. Hesperidin treatment may have reduced these reactive oxygen species and thus helped in faster regeneration of the irradiated wounds. The hesperidin has been reported to inhibit the generation of $^{\bullet}OH$, $O_2^{\bullet-}$, DPPH, $NO^{\bullet}$ and $ABTS^{\bullet+}$ *in vitro* [56]. Ionizing radiations have been reported to reduce, a repertoire of antioxidant enzymes including glutathione peroxidase, superoxide dismutase and glutathione that would have adversely affected the healing of hemi-body irradiated wound. Presence of hesperidin before irradiation would have inhibited this radiation induced decline and effected the early repair of irradiated wounds. The glutathione peroxidase, superoxide dismutase and glutathione have been found to be increased in the hesperidin treated regenerating wounds receiving hemi-body irradiation [87]. 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 [21,88]. Histological studies have shown that hesperidin increased the proliferation of fibroblasts and vascular density, which may have led to higher wound contraction and early wound repair. The acceleration in the synthesis of DNA, collagen, hexosamine and NO by hesperidin in the present study may have contributed in accelerated repair and regeneration of the hemi-body irradiated wound. Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors and they are involved in cell proliferation during wound repair. The inhibition of these proteins by irradiation leads to delayed wound healing [89]. The activation of PPARγ by hesperidin may have contributed in the early repair of irradiated wound in the present study. Hesperidin (100 mg/kg) has been reported to activate PPARγ earlier [90]. The wounding and irradiation elicit proinflammatory response by transcriptional activation of NF-κB, COX-II and LOX [9,91-93]. Presence of hesperidin before irradiation might have inhibited the transcriptional activation of NF-κB, COX-II and LOX restoring normal regenerative capacity of irradiated wounds in the present study. Earlier studies have shown that hesperidin inhibits the transcriptional activation of NF-κB, and COX-II [94,95]. Matrix metalloproteinase (MMPs) activation is necessary to debride the wound bed however, their higher expression after irradiation leads to a delay in the healing of wounds [96]. Hesperidin treatment has been reported to suppress MMPs activation that may have also aided in the acceleration of healing of irradiated wounds in the present study [97]. The Nuclear related factor 2 (Nrf2) has been found to be upregulated after excision wounds [98] and irradiation may have inhibited the Nrf2 activation causing retardation in wound healing. The hesperidin treatment may have upregulated the transcriptional activation of Nrf2 accelerating the regeneration and repair of irradiated wound. Hesperidin has been reported to increase the activation of Nrf2 [99].

Conclusions

The present study demonstrates that single administration of hesperidin before different doses of hemi-body γ-radiation accelerates wound healing in mice, as is evident from an improved wound contraction and reduced wound healing time. The early regeneration and repair of wounds by hesperidin may be due to scavenging of radiation-induced free radicals, increased antioxidant status, rise in the synthesis of collagen, hexosamine, DNA, and nitric oxide. Hesperidin may have also blocked the radiation-induced transcriptional activation of NF-κB, a key molecule involved in oxidative stress, inflammation and several other processes involved in the regeneration of wounds. Hesperidin may have also increased the expression of PPAR and thus resulted in an early repopulation of wound bed and repair of irradiated excision wounds. Hesperidin may have upregulated the Nrf2 activity increasing the antioxidant status in the regenerating wounds and accelerating early repair of the excision wounds. Histological examination has demonstrated a rise in proliferation of fibroblasts and increase in vasculature in the wound bed in hesperidin treated animals. The data obtained in this study suggests that hesperidin could be a useful paradigm in the clinical management of normal as well as irradiated wounds.

Acknowledgments

We thank Dr. MS. Vidyasagar, Prof. and Head, and Dr. JGR. Soloman, Department of Radiotherapy and Oncology, Kasturba Medical College, Manipal, India, for providing the necessary irradiation facilities and help in dosimetry. Our thanks are due to Prof. A. Chincholkar, Head of the Department, and Mrs. Geetha J. Nayak, Department of Mechanical Engineering, Manipal Institute of Technology, Manipal, India, for their expert help in calculating the wound area. The financial assistance provided by the Indian Council of Medical Research, Government of India, New Delhi, to carry out the above study is gratefully acknowledged.

Conflict of Interest statement

The authors do not have any Conflict of Interest to declare.

References

1. Evangeliou N, Hamburger T, Talerko N, Zibtsev S, Bondar Y, et al. Reconstructing the Chernobyl Nuclear Power Plant (CNPP) accident 30 years after. A unique database of air concentration and deposition measurements over Europe. Environ pollut. 2016;216:408-418.
2. Ishikawa T. Radiation doses and associated risk from the Fukushima nuclear accident: a review of recent publications. Asia Pacific J Pub Hlth. 2017;29(2S):18S-28S.
3. Richardson L. Radiation exposure and diagnostic imaging. J Am Assoc Nurse Pract. 2010;22(4):178-85.
4. Jagetia GC, Rao KVNM. Hesperidin, a citrus bioflavonoid potentiates repair and regeneration of deep dermal excision wounds of mice whole body exposed to different doses of ^{60}co γ-radiation. Clin Dermatol J. 2018;3(2):000147.
5. Christensen DM, Parillo SJ, Glassman ES, Sugarman SL. Management of ionizing radiation injuries and illnesses, part 2: nontherapeutic radiologic/nuclear incidents. J Am Osteopath Assoc. 2014 May;114(5):383-389.
6. Chao N.J. Accidental or intentional exposure to ionizing radiation: Biodosimetry and treatment options. Exp Hematol. 2007;35(4):24-27.
7. Zhou Y, Ni H, Balint K, Sanzari JK, Dentchev T, et al. Ionizing radiation selectively reduces skin regulatory T cells and alters immune function. PloS One. 2014;9(6):e100800.
8. Collen EB, Mayer MN. Acute effects of radiation treatment: skin reactions. Can Vet J. 2006 Sep;47(9):931-935.
9. Ryan JL. Ionizing radiation: the good, the bad, and the ugly. J Invest Dermatol. 2012 Mar;132(3 Pt 2):985-993.
10. Singh M, Alavi A, Wong R, Akita S. Radiodermatitis: a review of our current understanding. Am J Clin Dermatol. 2016;17(3):277-292.
11. Kumar P, Jagetia GC. A review of triage and management of burn victims following a nuclear disaster. Burns. 1994 Oct;20:397-402.
12. Jagetia GC, Rajanikant GK, Rao KVNM. Modulation of radiation-induced delay in the wound healing by ascorbic acid in mice exposed to different doses of hemi-body γ- radiation. Wounds 2003;15:324-338.
13. Jagetia GC, Rajanikant GK. Curcumin treatment enhances the repair and regeneration of wounds in mice hemi-body exposed to γ-radiation. Plast Reconstr Surg. 2005;115(2):515-528.
14. Jagetia GC, Rajanikant GK, Rao KVNM. Ascorbic acid increases healing of excision wounds of mice whole body exposed to different doses of γ-radiation Burns. 2007;33(4):484-494.
15. Jagetia GC, Rajanikant GK. Role of curcumin, a naturally occurring phenolic compound of turmeric in accelerating the repair of excision wounds in mice whole-body exposed to various doses of γ-radiation. J Surg Res. 2004;120(1):127-138.
16. Jagetia GC, Rajanikant GK. Topical application of curcumin augments healing of deep dermal excision wound of mice

exposed to whole-body γ-radiation. J Nurs Healthcare. 2017:2(1).

17. Jagetia GC, Ravikiran PB. 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. 2015;1:1-7.
18. Singer AJ, Clark RA. Mechanism of disease. Cutaneous wound healing. N Engl J Med. 1999;341(10):738-746.
19. Martin P, Nunan R. Cellular and molecular mechanisms of repair in acute and chronic wound healing. Br J Dermatol. 2015;173(2):370-378.
20. Pang C, Ibrahim A, Bulstrode NW, Ferretti P. An overview of the therapeutic potential of regenerative medicine in cutaneous wound healing. Int Wound J. 2017;14(3):450-459.
21. Rudolph R, Vande Berg J, Schneider JA, Fischer JC, Poolman WL. Slowed growth of cultured fibroblasts from human radiation wounds. Plast Reconstr Surg. 1988 Oct;82(4):669-677.
22. Jagetia GC, Rajanikant GK. Acceleration of wound repair by curcumin in the excision wound of mice exposed to different doses of fractionated γ radiation. Int Wound J. 2012 Feb;9(1):76-92.
23. Jacobson LK, Johnson MB, Dedhia RD, Niknam-Bienia S, Wong AK. Impaired wound healing after radiation therapy: A systematic review of pathogenesis and treatment. JPRAS Open. 2017 Sep;13:92-105.
24. Larson DL, Lindberg RD, Lane E, Goepfert H. Major complications of radiotherapy in cancer of the oral cavity and oropharynx. A 10-year retrospective study. Am J Surg. 1983 Oct;146(4):531-536.
25. Shore RE. Overview of radiation-induced skin cancer in humans. Int J Radiat Biol. 1990;57:809-827.
26. Gu Q, Wang D, Cui C, Gao Y, Xia G, et al. Effects of radiation on wound healing. J Environ Pathol Toxicol Oncol. 1998;17(2):117-123.
27. Di Maggio FM, Minafra L, Forte GI, Cammarata FP, Lio D, et al. Portrait of inflammatory response to ionizing radiation treatment. J Inflamm. 2015;12(1):14.
28. Jagetia GC, Rajanikant GK, Rao SK. Evaluation of the effect of ascorbic acid treatment in the artificially wounded mouse exposed to different doses of fractionated γ- radiation. Radiat Res. 2003;159:371-380.
29. Jagetia GC, Venkatesha VA. Effect of mangiferin on radiation-induced micronucleus formation in cultured human peripheral blood lymphocytes. Environ Mol Mutagen. 2005;46(1):12-21.
30. Jagetia GC. Radioprotective potential of plants and herbs against the effects of ionizing radiation. J Clin Biochem Nutr. 2007 Mar;40(2):74-81.
31. Kanes K, Tisserat B, Berhow M, Vandercook C. Phenolic composition of various tissues of Rutaceae species. Phytochemistry. 1993;32(4):967-974.
32. Emim JADS, Oliveria AB, Lapa AJ. Pharmacological evaluation of the anti-inflammatory activity of a citrus bioflavonoid, hesperidin and the isoflavanoids duartin and claussequinone, in rats and mice. J Pharm Pharmacol. 1994 Feb;46(2):118-122.
33. Nogata Y, Sakamoto K, Shiratsuchi H, Ishii T, YANO M, Ohta H. Flavonoid composition of fruit tissues of citrus species. Biosci Biotechnol Biochem. 2006 Jan;70(1):178-192.
34. Horowitz RM, Gentili B. Flavonoids of Citrus –VI: The structure of neohesperidose. Tetrahedron. 1963;773-782.
35. Kawaguchi K, Mizuno T, Aida K, Uchino K. Hesperidin as an inhibitor of lipases from porcine pancreas and Pseudomonas. Biosci Biotech Biochem. 1997;61:102-104.
36. Bhalla NP, Dakwake RN. Chemo taxonomy of Indigofera Linn. J Indian Bot Soc 1978;57(2):180-185.
37. Pawlowska L. Flavonoids in the leaves of polish species of the genus Betula L. L The flavonoids of B. pendula Roth. and B. obscura Kot. leaves. Acta Soc Bot Pol. 1980;493:281-296.
38. Kokkalou E, Kapetanidis I. Flavonoids of the arial parts of *Acinos suaveolens*. Pharm Acta Helv. 1988;636:170-173.
39. Galati EM, Monforte MT, Kirijavainen S, Forestieri AM, Trovoto A, et al. Biological effects of hesperidin, a citrus flavonoid (note 1): anti-inflammatory and analgesic activity. Framco. 1994;40:709-712.
40. Bae EA, Han MJ, Lee M, Kim DH. *In vitro* anti-Helicobacter activity of some flavonoids and their metabolites. Planta Med. 1999 Jun;65(5):442-3.
41. Ohtsuki K, Abe A, Mitsuzumi H, Kondo M, Uemura K, et al. Glucosyl hesperidin improves serum cholesterol composition and inhibits hypertrophy in vasculature. J Nutr Sci Vitaminol. 2003;49:447-450.
42. Miyagi Y, Om AS, Chee KM, Bennink MR. Inhibition of azoxymethane-induced colon cancer by orange juice. Nutr Cancer. 2000;36(2):224-229.
43. Wilmsen PK, Spada DS, Salvador M. Antioxidant activity of the flavonoid hesperidin in chemical and biological systems. J Agr Food Chem. 2005;53(12):4757-4761.
44. Vabeiryureilai M, Lalrinzuali K, Jagetia GC. Determination of anti-inflammatory and analgesic activities of a citrus bioflavanoid, hesperidin in mice. Immunochem Immunopathol. 2015 Oct;1:107.
45. Wang Y, Yu H, Zhang J, Gao J, Ge X, et al. Hesperidin inhibits HeLa cell proliferation through apoptosis mediated by endoplasmic reticulum stress pathways and cell cycle arrest. BMC Cancer. 2015 Oct;15(1):682.
46. Tanaka T, Tanaka T, Tanaka M, Kuno T. Cancer chemoprevention by citrus pulp and juices containing high amounts of β-cryptoxanthin and hesperidin. BioMed Res Int. 2012.
47. Chen SY, Chyau CC, Chu CC, Chen YH, Chen TH, et al. Hepatoprotection using sweet orange peel and its bioactive compound, hesperidin, for CCl4-induced liver injury in vivo. J Funct Foods 2013;5(4):1591-1600.
48. Agrawal YO, Sharma PK, Shrivastava B, Ojha S, Upadhya HM, et al. Hesperidin produces cardioprotective activity via PPAR-γ pathway in ischemic heart disease model in diabetic rats. PLoS One. 2014 Nov;9(11):e111212.
49. Uehara M. Prevention of osteoporosis by foods and dietary supplements. Hesperidin and bone metabolism. Clin Calcium. 2006 Oct;16(10):1669-1676.
50. Antunes MS, Jesse CR, Ruff JR, de Oliveira Espinosa D, Gomes NS, et al. 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. 2016 Oct 15;789:411-420.
51. Kurowska EM, Spence JD, Jordan J, Wetmore S, Freeman DJ, et al. HDL-cholesterol-raising effect of orange juice in subjects with hypercholesterolemia. Am J Clini Nutr. 2000;72(5):1095-100.
52. Görmeli CA, Saraç K, Çiftçi O, Timurkaan N, Malkoç S. The effects of hesperidin on idiopathic pulmonary fibrosis evaluated by histopathologial-biochemical and micro-computed tomography examinations in a bleomycin-rat model. Biomed Res. 2016;27(3):737-742.
53. Morand C, Dubray C, Milenkovic D, Lioger D, Martin JF, et al. Hesperidin contributes to the vascular protective effects of orange juice: a randomized crossover study in healthy volunteers. Am J Clin Nutr. 2010;93(1):73-80.
54. Milenkovic D, Deval C, Dubray C, Mazur A, Morand C. Hesperidin displays relevant role in the nutrigenomic effect of orange juice on blood leukocytes in human volunteers: a randomized controlled cross-over study. PLoS One. 2011;6(11):e26669.
55. Kawabe M, Tamano S, Shibata MA, Hirose M, Fukushima S, et al.

Subchronic toxicity study of methyl hesperidin in mice. Toxicol Lett. 1993;69:37-44.

56. Jagetia GC, Rao KVNM. 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 OA. 2017;4(3):1-8.
57. Jagetia GC, Rao KVNM. Acceleration in the repair and regenerative responses by different doses of hesperidin in the deep full thickness cutaneous wound of mice whole body exposed to 6 Gy of γ -radiation. Nurs Hlth Care Int J. 2018;2(3):000149.
58. Richards GM. Modifications of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. Anal Biochem. 1974;57(2):369-376.
59. Woessner Jr. JF. The determination of hydroxyproline in tissues and protein samples containing small proportions of this amino acid. Arch Biochem Biophys. 1961;93(2):440-447.
60. Patricia G, Johansen RD, Marshall, Neuberger A. Carbohydrates in protein. 2. The hexose, hexosamine, acetyl and amide-nitrogen content of hen's-egg albumin. Biochem J. 1960;77(2):239-247.
61. Garg A, Garg S, Zaneveld JD, Singla AK. Chemistry and pharmacology of the citrus bioflavonoid Hesperidin. Phytother Res. 2001 Dec;15(8):655-669.
62. Hosseinimehr SJ, Mahmoudzadeh A, Ahmadi A, Mohamadifar S, Akhlaghpoor S. Radioprotective effects of hesperidin against genotoxicity induced by γ-irradiation in human lymphocytes. Mutagenesis. 2009;24(3):233-235.
63. Han G, Ceilley R. Chronic wound healing: A review of current management and treatments. Adv Ther. 2017;34(3):599-610.
64. Janis JE, Harrison B. Wound Healing: Part I. Basic Science. Plast Reconstr Surg. 2016;138(3 Suppl):9S-17S.
65. Tracy LE, Minasian RA, Caterson EJ. Extracellular matrix and dermal fibroblast function in the healing wound. Adv Wound Care. 2016;5(3):119-136.
66. Behm B, Babilas P, Landthaler M, Schreml S. Cytokines, chemokines and growth factors in wound healing. J Eur Acad Dermatol Venereol. 2012;26(7):812-820.
67. Evans ND, Oreffo RO, Healy E, Thurner PJ, Man YH. Epithelial mechanobiology, skin wound healing, and the stem cell niche. J Mech Behav Biomed Mater. 2013 Dec;28:397-409.
68. Peacock EE. Contraction. In: Wound Repair, 3d Edition, Peacock EE, Philadelphia, WB Saunders 1984; 39-55.
69. Tejero-Trujeque R. How do fibroblasts interact with the extracellular matrix in wound contraction? J Wound Care. 2001 Jun;10(6):237-242.
70. Marc S. Rudoltz, Kenneth R. Blank, Gary Kao, Ruth J. Muschel, W. Gillies McKenna, Ionizing radiation and the cell cycle: A review. Radiat Oncol Invest. 1996;4:147-158.
71. Grillo HC, Potsaid MS. Studies on wound healing-IV, retardation of contraction by local X-irradiation and observations relating to the origin of fibroblast in repair. Ann Surg. 1961 Nov;154(5):741–750.
72. Stromberg LR, McLaughlin MM, Donati RM. Combined surgical and radiation injury-3. The effect of pre-irradiation radioprotective drug treatment. Proc Soc Exp Biol Med. 1968;129:140-143.
73. Levenson SM, Gruber CA, Rettura G, Gruber DK, Demetriou AA, Seifter A. Supplemental vitamin A prevents the acute radiation-induced defect in wound healing. Ann Surg. 1984 Oct;200(4):494-512.
74. Taren DL, Chvapil M, Weber CW. Increasing the breaking strength of wounds exposed to preoperative irradiation using vitamin E supplementation. Int J Vitam Nutr Res. 1987;57(2):133-7.
75. Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol. 2007;127(3):514-525.
76. Krausz A, Friedman AJ. Nitric oxide as a surgical adjuvant. Future Sci OA. 2015;1(1).
77. Witte MB, Barbul A. Role of Nitric oxide in wound repair. Am J Surg. 2002;183:406-412.
78. Albina JE, Mills CD, Henry WL Jr, Caldwell MD. Temporal expression of different pathways of L-arginine metabolism in healing wounds. J Immunol. 1990 May;144(10):3877-80.
79. Holm-Pedersen P, Fenstad AM, Folke LE. DNA, RNA and protein synthesis in healing wounds in young and old mice. Mech Ageing Dev. 1974;3:173-185.
80. Fitzgerald RH, Steinberg JS. Collagen in wound healing: are we onto something new or just repeating the past? Foot Ankle Online J. 2009;2(3).
81. Rangaraj A, Harding K, Leaper D. Role of collagen in wound management. Wounds UK. 2011;7(2):54-63.
82. Lazarus GS, Cooper DM, Knighton DR, Margolis DJ, Percoraro RE, et al. Definitions and guidelines for assessment of wounds and evaluation of healing. Wound Rep Reg. 1994;2:165-170.
83. Grant RA, Cox RW, Kent CM. The effects of gamma irradiation on the structure and reactivity of native and cross-linked collagen fibrils. J Anat. 1973 May;115(Pt 1):29-43.
84. Doyle JW, Li Y, Salloum A. The effects of radiation on neo-vascularization in a rat model. Plast Reconstr Surg. 1996 Jul;98(1):129-35.
85. Vegesna V, Withers HR, Holly FE, McBride WH. The effect of local and systemic irradiation on impairment of wound healing in mice. Rad Res. 1993;135:431-433.
86. Trabucchi E, Pallotta S, Morini M, Corsi F, Franceschini R, et al. Low molecular weight hyaluronic acid prevents free radical damage to granulation tissue during wound healing. Int J Tissue React. 2002;24(2):65-71.
87. Jagetia GC, Mallikarjuna Rao KV. Hesperidin, A Citrus Bioflavonoid Reduces the Oxidative Stress in the Skin of Mouse Exposed to Partial Body γ-Radiation. Transcriptomics. 2015;3(111):2.
88. Bumann J, Santo-Holtje L, Loffler H, Bamberg M, Rodemann HP. Radiation-induced alterations of the proliferation dynamics of human skin fibroblasts after repeated irradiation in the subtherapeutic dose range. Strahlenther Onkol. 1995;171:35-41.
89. Linard C, Souidi M. PPARs in irradiation-induced gastrointestinal toxicity. PPAR Res. 2010.
90. Kumar HB, Kumar DB, Diwan PV. Hesperidin, a citrus flavonoid, protects against l-methionine-induced hyperhomocysteinemia by abrogation of oxidative stress, endothelial dysfunction and neurotoxicity in Wistar rats. Pharm Biol. 2017 Dec;55(1):146-155.
91. Zhou D, Brown SA, Yu T, Chen G, Barve S, et al. A high dose of ionizing radiation induces tissue-specific activation of nuclear factor-κB in vivo. Radiat Res. 1999 Jun;151(6):703-709.
92. Rödel F, Hantschel M, Hildebrandt G, Schultze-Mosgau S, Rödel C, et al. Dose-dependent biphasic induction and transcriptional activity of nuclear factor kappa B (NF-kB) in EA.hy.926 endothelial cells after low-dose X-irradiation. Int J Radiat Biol. 2004 Feb;80(2):115-123.
93. Gaddipati JP, Sundar SV, Calemine J, Seth P, Sidhu GS, et al. Differential regulation of cytokines and transcription factors in liver by curcumin following hemorrhage/resuscitation. Shock. 2003;19(2):150-156.
94. Hirata A, Murakami Y, Shoji M, Kadoma Y, Fujisawa S. Kinetics of radical-scavenging activity of hesperetin and hesperidin and their inhibitory activity on COX-2 expression. Anticancer Res. 2005 Sep-Oct;25(5):3367-3374.
95. Ghorbani A, Nazari M, Jeddi-Tehrani M, Zand H. 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. 2012 Feb;51(1):39-46.
96. Gu Q, Wang D, Gao Y, Zhou J, Peng R, et al. Expression of MMP1 in surgical and radiation-impaired wound healing and its

effects on the healing process. J Environ Pathol Toxicol Oncol. 2002;21(1):71-78.

97. Lee HJ, Im AR, Kim SM, Kang HS, Lee JD, et al. The flavonoid hesperidin exerts anti-photoaging effect by downregulating matrix metalloproteinase (MMP)-9 expression via mitogen activated protein kinase (MAPK)-dependent signaling pathways. BMC Complement Altern Med. 2018 Jan 30;18(1):39.
98. Beyer TA, Auf dem Keller U, Braun S, Schäfer M, Werner S. Roles and mechanisms of action of the Nrf2 transcription factor in skin morphogenesis, wound repair and skin cancer. Cell Death Differ. 2007 Jul;14(7):1250-4.
99. Elavarasan J, Velusamy P, Ganesan T, Ramakrishnan SK, Rajasekaran D, et al. Hesperidin-mediated expression of Nrf2 and upregulation of antioxidant status in senescent rat heart. J Pharm Pharmacol. 2012 Oct;64(10):1472-1482.