

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

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Abstract: The effect of kalonji (*Nigella sativa*) extract (NSE) was studied on dermal excision wound of mice whole body exposed to different doses of γ -radiation. The optimum dose was selected by administering mice with different doses of NSE for five consecutive days before whole-body exposure to 6 Gy γ -radiation and creating a full thickness deep dermal wound of 15 mm diameter on the dorsum of mice after irradiation. The estimation of wound contraction and mean wound healing time showed that 150 mg/kg NSE was the optimum dose as it caused an early closure of the wound and reduced the mean wound healing time to a maximum extent than the other NSE doses. Irradiation of mice to 0, 2, 4, 6 and 8 Gy resulted in a dose dependent delay in the wound contraction and mean wound healing time, whereas administration of mice with 150 mg/kg b. wt. NSE before exposure to 0, 2, 4, 6 and 8 Gy γ -radiation, reduced the radiation induced delay in the wound contraction and mean healing time, significantly especially for 6 and 8 Gy irradiation. The estimation of total collagen and hexosamine contents at 3, 5, 10 and 15 days post-wounding in the granulation tissues of regenerating wounds, revealed that irradiation of mice to 6 Gy γ -radiation resulted in a significant decline in the collagen, and hexosamine contents at all post-irradiation times, whereas pre-treatment of mice with 150 mg/kg b. wt. NSE resulted in a significant elevation in the collagen and hexosamine synthesis when compared to the concurrent irradiation alone group. The irradiation caused a reduction in glutathione concentration in the granulation tissue at 6, 12, 48 and 72 h post-treatment, whereas glutathione peroxidase and superoxide dismutase activities increased at 6 h post-irradiation. NSE treatment arrested the decline in GSH concentration, and, further elevated glutathione peroxidase and superoxide dismutase activity. The irradiation resulted in a dose dependent elevation in lipid peroxidation and pretreatment of mice with NSE significantly reduced radiation induced lipid peroxidation.

The NSE administration reduced the radiation induced delay in wound contraction and mean wound healing time, which may be due to enhanced synthesis of collagen and hexosamine and increased anti-oxidants and alleviation in the lipid peroxidation.

Keywords: Mice, Radiation, *Nigella sativa*, Collagen, antioxidants, and lipid peroxidation.

I. INTRODUCTION

Since the discovery of ionizing radiations, ionizing radiations have emerged as one of the most powerful tools in the diagnosis of diseases, and treatment of cancer or other similar conditions preoperatively or postoperatively. Many a times irradiation is the only modality for the treatment of cancer (Ryan 2012; Liauw *et al.*, 2013). Apart from therapy, humans may experience combined injuries during nuclear accidents, atomic bomb explosion and release of radioactive material. Irradiation along with other injuries acts synergistically, resulting in much greater morbidity and/or mortality than the radiation injury alone (Kumar and Jagetia 1994, Jarrett 1999, Jagetia and Rajanikant 2004, 2012; Hall and Giaccia 2006, Cox and Ang, 2010, Ryan, 2012). In normal course, the wounds heal naturally as the body works to heal itself. However, the healing process may be delayed or complicated in conditions of radiation exposure (for treatment or otherwise), age, body size, chronic diseases, low nutritional status, vascular insufficiencies, and immunosuppression that may require specialized treatment and care (Lee and Thiele 2010, Guo and DiePietro, 2010, Maryna *et al.*, 2013).

Irradiation inhibits inflammatory reactions, connective tissue proliferation, formation and maturation of granulation tissue, transcription of collagen mRNAs, secretion of collagen and neovascularization (Jagetia *et al.*, 2003; Jagetia

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and Rajanikant, 2004, 2012; McQuestion, 2011; Liauw *et al.*, 2013). The irradiation initiates acute degenerative changes in the basement membrane, edema of vascular walls and thrombosis with the progressive loss of vessels and decreased neovascularization (Doyle 1996; Mack and Maytin 2010; Lee and Thiele 2010). Interaction of ionizing radiation with wounded tissue disrupts normal responses to injury leading to a protracted recovery period (Power *et al.*, 1967; Jagetia *et al.*, 2003; Jagetia and Rajanikant 2004, 2012; Ryan 2012).

Numerous attempts have been made to reduce the radiation-induced complications in healing of irradiated wounds. Bacterial cellulose impregnated membranes, poviargol, and autologous non-irradiated fibroblasts have been shown to accelerate the healing of irradiated wounds earlier (Ferguson 1999; Legeza *et al.*, 2004). Vascular endothelial growth factor (VEGF-A) and stromal cell-derived growth factor stromal cell-derived growth factor 1 α (SDF-1 α) have been also shown to accelerate wound healing in animal models (Hunt *et al.*, 1984; Beaudry *et al.*, 2010), whereas platelet derived growth factor (PDGF) has been successfully used to clinically treat wounds (Ma *et al.*, 2015). A major shortcoming of these approaches is that these treatments only supply individual factors in short boluses, whereas wound healing is a complex interplay of multitude of factors that requires pleotropic agent to tackle wound healing effectively. Phenytoin sodium, vitamin A, C and curcumin have been reported to inhibit radiation-induced defects in wound healing (Levenson *et al.*, 1984; Song and Cheng 1997; Jagetia *et al.*, 2003, 2007; Jagetia and Rajanikant 2004, 2005, 2012).

Natural plant products have been used by humans since the advent of human history for various purposes including healthcare. Having coevolved with life, these natural products are billions of years old. Tens of thousands of them are produced as secondary metabolites by the higher plants as a natural defense against disease and infection (Dixon 2001). Medicines derived from plants have played a pivotal role in the human healthcare since the ancient times and continue to do so in the modern era. Therefore, use of herbal products in the regeneration and restoration of irradiated wounds is an attractive proposition, because, they have wide acceptability, better tolerance, do not have side effects and can be safely manipulated for human use (Jagetia and Venkatesha 2005). The kalonji, seeds of *Nigella sativa* Linn. (family: Ranunculaceae), commonly known as black seed or black cumin, are used in folk (herbal) medicine all over the world for the treatment and prevention of a number of diseases including asthma, diarrhoea and dyslipidaemia. It is also an antioxidant, antiparasitic, antimicrobial, antiulcer, anti-inflammatory, and analgesic in nature (Ali and Blunden, 2003). Seeds of *N. sativa* contain 36–38% fixed oil, proteins, alkaloids, saponins and 0.4–2.5% essential oils. The anti-oxidant property of essential oils obtained from seeds of *N. sativa* has been investigated in cell-free systems (Burits and Bucar 2000, Ramadan *et al.*, 2003, Abdel-Wahhab and Aly 2005, Kanter *et al.*, 2006). It has been reported to modify the oxidative damage in irradiated rats after intra-peritoneal injection of *N. sativa* oil (Cemek *et al.*, 2006). Ethanol extract of *N. sativa* showed significant free radical scavenging activity and protection against DNA damage in cell free systems. In addition, *ex vivo* treatment of mouse splenic lymphocytes with an ethanol extract of *N. sativa* showed significant prevention of the formation of lipid-peroxides and intracellular reactive oxygen species (Rastogi *et al.*, 2010). The effect of *N. sativa* on the irradiated wound has not been evaluated. Therefore, the present study has been undertaken to obtain an insight into the wound healing and regenerative potential of kalonji in Swiss albino mice whole body exposed to different doses of γ -radiation and then inflicted with deep dermal excision wound.

II. MATERIALS AND METHODS

2.1. Chemicals

Chloramine-T, ρ -dimethylaminobenzaldehyde, hydroxyproline, acetylacetone, hexosamine, glutathione, 2-thiobarbituric acid (TBA), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), phenazine methosulphate, ethylene diamine terta acetic acid (EDTA), diethylenetriaminopentaacetic acid (DETAPAC) nitroblue tetrazolium (NBT), butylated hydroxytoluene (BHT), sodium azide and tetraethoxypropane were purchased from Sigma Aldrich Chemicals Co., Bangalore, India. The other routine chemicals were procured from Sd Fine Chemcals, Mumbai, India.

2.2. Animal Care and Handling

The animal care and handling were done according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Usually, six to eight weeks old Swiss albino mice weighing 22 to 24 g of either sex were selected from an inbred colony maintained under the controlled conditions of temperature ($23 \pm 2^\circ\text{C}$), humidity ($50 \pm 5\%$) and 12 h of light and dark cycle, respectively. The animals had free access to the sterile food and water, five 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 Manipal University, Manipal, India.

2.3. Preparation of Extract

The dried seeds of kalonji, *Nigella sativa* (family: Ranunculaceae) were commercially procured and the details are given elsewhere (Jagetia and Ravikiran, 2014).

2.4. Mode of Administration

NSE was weighed, dissolved in double distilled water (DDW) and 0.01 ml/g b. wt. of NSE or DDW was administered orally.

2.5. Experimental Design

The wound healing and regenerative potential of NSE was studied by undertaking various experiments described below.

DDW+irradiation: This group of animals received 0.01 ml/g body weight of DDW prior to 6 Gy γ - radiation.

NSE+irradiation: The animals of this group were administered orally NSE once daily for 5 consecutive days before 6 Gy γ -radiation.

2.6. Irradiation

One hour after the last administration of DDW or NSE, each animal was placed into a specially designed well-ventilated acrylic restrainer and the whole body of the prostrate animals was exposed to 0 or 6 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).

2.7. Production of Full-Thickness Skin Wound

The fur of the dorsum (below the rib cage) of each animal was removed with a cordless electric mouse clipper (Wahl Clipper Corporation, Illinois, USA) before exposure to different doses of γ -radiation and a full-thickness skin wound was created on the dorsum of each animal within 1 h of irradiation as described earlier (Jagetia *et al.*, 2003, 2007). Briefly, the animals were anesthetized using diethyl ether and the skin of the entire body was cleaned and decontaminated by wiping the whole animal body with sterillum disinfectant solution (Bode Chemie, Germany). 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 *penniculus carnosus* in an aseptic environment using sterile scissors and forceps. The whole procedure was carried out in an aseptic environment. Each wounded animal was housed in an individual sterile polypropylene cage.

The wound healing and regenerative potential of NSE was determined as described below:-

2.8. Selection of Optimum Dose

An experiment was performed to select the optimum wound healing dose of NSE in the irradiated wounds, where the grouping and other conditions were essential similar as described in the experimental design section, except that the animals of NSE+irradiation group were orally administered with 25, 50, 100, 150, 250 or 500 mg/kg b. wt. of NSE before 6 Gy of whole body γ -radiation and monitored regularly for wound contraction until complete contraction of the wound/s.

2.9. Wound Contraction

Wound contraction was monitored by capturing the video images of each full-thickness wound with a charged coupled device (CCD) camera connected to a computer (Jagetia *et al.*, 2003, 2004, and 2007; Jagetia and Rajanikant, 2012). The first image of each wound from different groups was obtained one day after wounding, and that day was considered as day one. The subsequent images were captured on 2, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27 days post wounding. The wound area was calculated using Auto CAD R14 (Autodesk Inc., San Rafael, CA) software. Six animals were used in each group at each exposure dose and a total of eighty four animals were used for the whole experiment.

2.10. Mean Wound Healing Time

A separate experiment was performed to determine the mean wound healing time in mice receiving NSE or not before exposure to 0 or 6 Gy γ -radiation, where grouping and other conditions were essentially similar to that described above, except that all the animals in each group were monitored regularly until complete healing of

wounds and the day by which each wound healed completely was recorded. Mean of all healed wounds was calculated and expressed as mean wound healing time in days. Five animals were used in each group at each exposure dose and a total of seventy animals were used for the whole experiment.

2.11. Effect of NSE on Irradiated Wound

Two individual experiments were conducted to evaluate the wound healing potential of 150 mg/kg b. wt. of NSE in mice exposed to different doses of γ -radiation, where the grouping and other conditions remained essentially similar to that described above, except that the animals were exposed to 0, 2, 4, 6 and 8 Gy of γ -radiation before creation of the wound. Ten animals were used for each group and a total of 100 animals were used for each experiment and a total of 200 animals were used for both the experiments.

The wound contraction and mean wound healing time were determined for both the experiments as described above for optimum dose selection experiments.

2.12. Biochemical Analyses

To understand the alteration in various biochemical parameters by 150 mg/kg b. wt. of NSE in irradiated wound, separate experiment was carried out, where the grouping and other conditions were essentially similar to that described in experimental section, except that the animals were exposed to 0 or 6 Gy γ -radiation after treatment with 150 mg/kg b. wt. NSE for collagen and hexosamine estimations.

2.13. Preparation of Animals

The hairs were depilated from the lower half of the dorsal surface of the animals before irradiation and a full thickness wound was created as described previously (Jagetia *et al.*, 2003, 2007). The granulation tissue from regenerating wounds was collected at 3, 5, 10 and 15 days after the exposure. The skin was freed from *panniculus carnosus* and flash frozen in the liquid nitrogen. The skin was weighed and homogenized in the phosphate buffered saline. A total of five animals were used in each group at each interval and a total of 40 animals were used for the whole experiment. The following estimations were carried out:-

2.14. Estimation of Collagen

As an indication of total collagen content, hydroxyproline concentration was determined as described by Woessner, (1961). The details of procedure are given elsewhere (Jagetia and Rajanikant, 2012).

2.15. Estimation of Hexosamine

For estimation of hexosamine, the weighed granulation tissues were hydrolyzed in 6N HCl for 8 h at 98°C, neutralized to pH 7 with 4 N NaOH and diluted with Milli-Q water. Hexosamine contents of granulation tissues were estimated by the method of Elson and Morgan (1933) with minor modifications.

2.16. Estimation of Antioxidant Status

A separate experiment was conducted to evaluate the intracellular antioxidant status in irradiated wounds treated with 150 mg/kg b. wt. of NSE, where animal grouping and other conditions were essentially similar to that described in experimental design section, except that the animals were whole body exposed to 2, 4, 6, or 8 Gy γ -radiation. The skin biopsies from the healing wounds were collected on 6, 12, 24, 48 and 72 h post treatment and the proteins, glutathione (GSH), glutathione peroxidase (GSHPx), superoxide Dismutase (SOD) and lipid peroxidation (LOO) were estimated using standard protocols (Jagetia and Rajanikant, 2015)

III. ANALYSIS OF DATA

Statistical significance between the treatments was determined using one way ANOVA and Bonferroni's post-hoc test was applied for multiple comparisons wherever necessary. The Student's 't' test was used for biochemical estimations. The Solo 4 Statistical Package (BMDP Statistical Software Inc., Los Angeles, CA, USA) was used for data analysis, and the data are expressed as mean \pm SEM (Standard error of mean).

IV. RESULTS

The results of wound contraction, mean wound healing time and different biochemical analyses are expressed as mean \pm standard error of the mean in Figures 1-10.

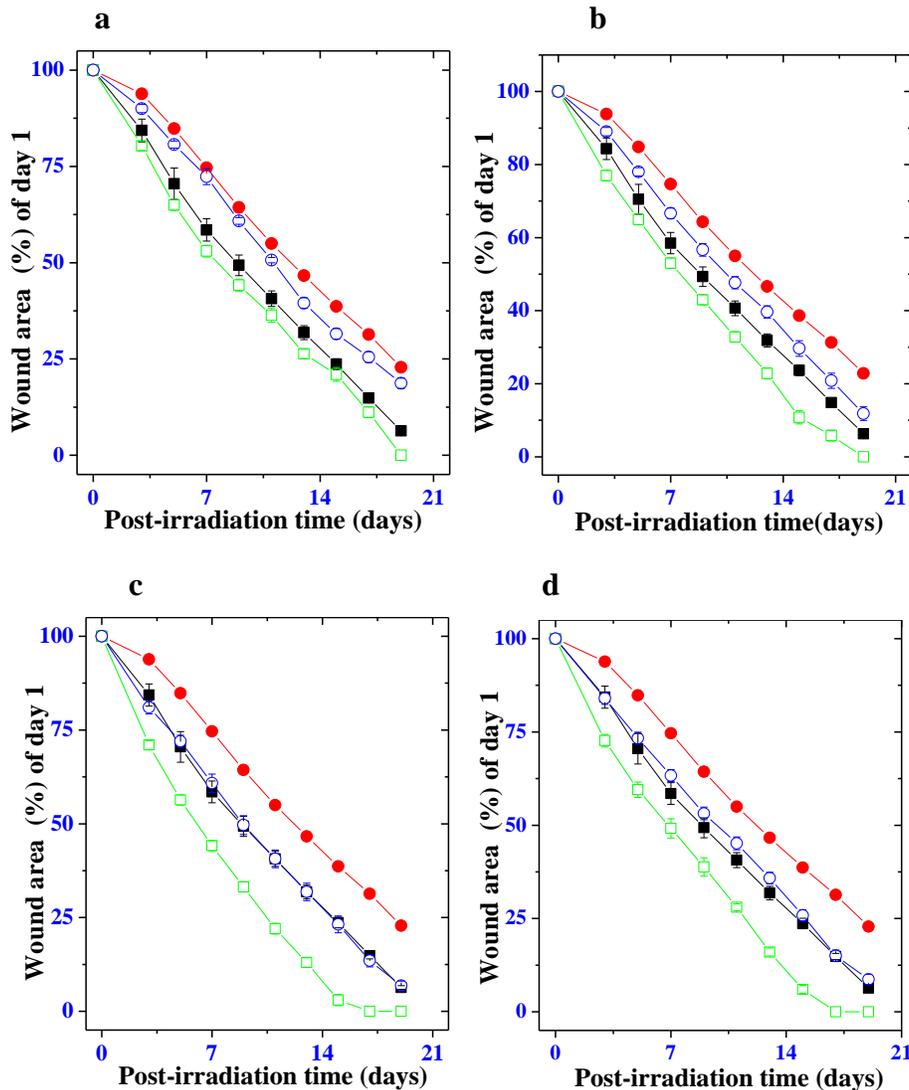
4.1. Optimum Dose Selection

The results of the experiments conducted to select the optimum dose of NSE for wound contraction are described below.

4.2. Wound Contraction

Progression of the healing of excision wounds can be determined by the periodic computation of wound area which is a measure of contraction. The area of each wound at a specific time has been expressed as the percentage of its original size on day 1. The mean corresponding area of wound for each group was plotted as a function of days after wounding.

Treatment of mice with DDW or various doses of NSE alone resulted in a steady contraction of excision wounds with the elapse of time after wounding. The administration of 25, 50, 100, 150, 200 or 500 mg/kg NSE enhanced the wound contraction significantly when compared to untreated sham-irradiated controls depending on the NSE dose. NSE treatment showed a progressive reduction in the scab formation with increasing dose, and the scab formation was almost absent for 150 mg/kg NSE and thereafter. In contrast the non-drug treated animals (NSE+sham-irradiation) showed a thick scab formation. The irradiation of animals to 6 Gy retarded the wound contraction causing delayed wound healing in this group when compared to sham-irradiated group (0 Gy). The administration of different doses of NSE resulted in a progressive acceleration in the wound contraction as evidenced by a continuous reduction in the open wound area with time and an early closure of wound when compared with DDW+irradiation group. The wound contraction accelerated with an increase in NSE dose up to 150 mg/kg, where a significant enhancement of wound contraction was observed at day 3 ($p < 0.001$), 6 ($p < 0.01$) and 9 ($p < 0.05$) post-irradiation when compared to DDW+irradiation (Figure 1). Therefore, this dose of NSE was considered as an optimum dose and further experiments were performed using this dose.



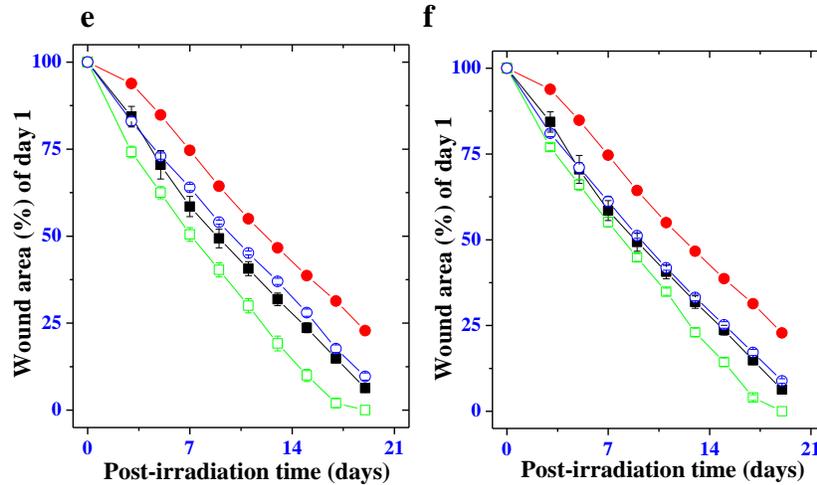


Fig 1: Alteration in the contraction of skin excision wound of mice treated with different doses *Nigella sativa* extract (NSE) before 6 Gy of whole-body γ -radiation. Closed Squares: DDW+Sham-irradiation; Open Squares: NSE+Sham-irradiation; Closed circles: DDW+irradiation and Open circles: NSE+irradiation. a. 25 mg/kg NSE Gy, b. 50 mg/kg NSE, c. 100 mg/kg NSE d. 150 mg/kg NSE; e. 250 mg/kg NSE and f. 500 mg/kg NSE. Error bars indicate standard error of mean. (n = 8 animals in each group).

4.3. Mean Wound Healing Time

The complete closure of wounds was observed by 19.17 days post-irradiation in DDW+sham irradiation group, whereas treatment of mice with 25, 50, 100, 250 or 500 mg/kg NSE did not alter the mean wound healing time significantly when compared with the DDW+sham-irradiation group. However, a significant reduction in the mean wound healing time was observed (17 days post-irradiation) for 150 mg/kg NSE when compared with the other doses of NSE. A further increase in NSE dose did not alter the mean healing time significantly.

Whole-body exposure of mice to 6 Gy γ -radiation resulted in a significant delay in the complete closure of wounds as a result the mean wound healing time was also increased by 5 days (24.33 days post-irradiation) in DDW+irradiation group when compared with the DDW+sham-irradiation group (19.17 days post-irradiation). Treatment of animals with different doses of NSE before 6 Gy irradiation reduced the mean wound healing time in a dose dependent manner until 150 mg/kg NSE where a shortest wound healing time of 21.67 days was recorded when compared with concurrent DDW+irradiation group. A further increase in the NSE dose did not change the mean healing time significantly that remained almost unaltered up to a dose of 500 mg/kg, the highest NSE dose evaluated (Figure 2).

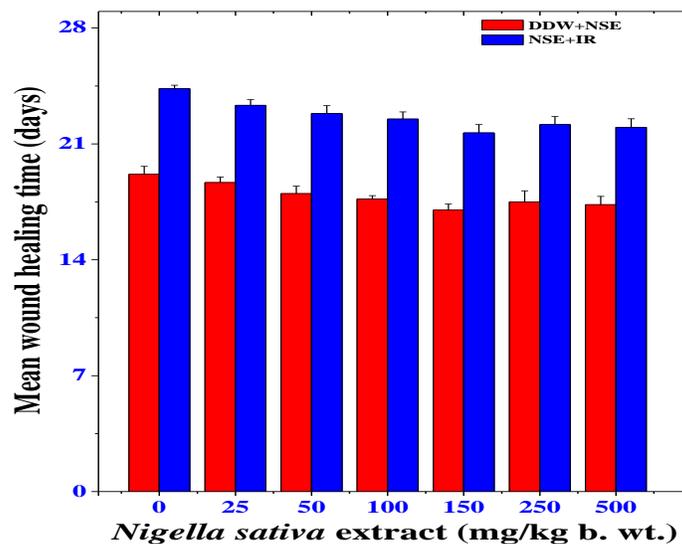


Fig 2: Effect of different doses of *Nigella sativa* extract (NSE) on the contraction of excision wound in the skin of mice exposed to 6 Gy of whole-body γ -radiation.

4.4. Effect Of NSE on Irradiated Wound Contraction

Irradiation resulted in a dose dependent scab formation and it was thicker at higher doses, especially 6 and 8 Gy irradiation. Treatment of mice with 150 mg/kg b. wt. NSE reduced the thickness of scab formation, which was very thin for 2 and 4 Gy irradiation. Whole-body exposure of mice resulted in a irradiation dose dependent delay in the wound contraction (Figure 3), and the longest delay in wound contraction was recorded for 8 Gy. However, the difference in wound contraction between DDW+irradiation and DDW+sham-irradiation group was statistically non-significant. A further increase in exposure dose to 6 or 8 Gy resulted in a significant delay in the wound contraction at all the post-irradiation times studied when compared to DDW+sham-irradiation group (Figure 3). Treatment of mice with 150 mg/kg NSE before exposure to 2, 4, 6 or 8 Gy irradiation resulted in a significant rise in the contraction of wounds at 3, 6 and 9 days post-irradiation, when compared with the DDW+irradiation group. However, NSE treatment prior to 8 Gy irradiation resulted in a significant contraction of the wound only at day 6 post-irradiation (Figure 3). The wound contraction profile of NSE+6 Gy irradiation was almost similar to that of DDW+sham-irradiation group as the both curves are almost superimposed to each other (Figure 3).

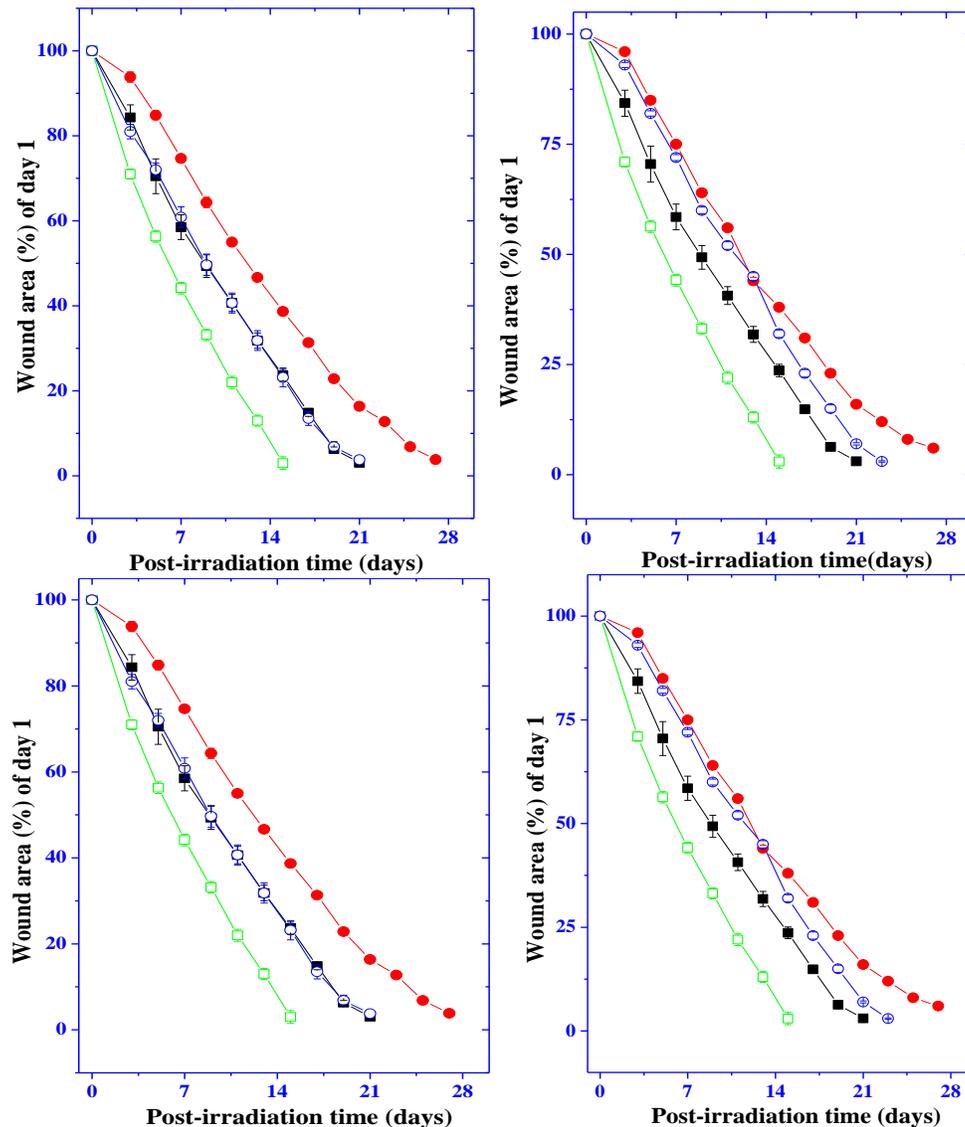


Fig3. Effect of *Nigella sativa* extract (NSE) treatment on the contraction of excision wound in the skin of mice exposed to different doses of whole-body γ -radiation. Closed Squares: DDW+Sham-irradiation; Open Squares: NSE+Sham-irradiation; Closed circles: DDW+irradiation and Open circles: NSE+irradiation. A. 2 Gy, b. 4 Gy, c. 6 Gy and d. 8 Gy. Error bars indicate standard error of mean. (n = 8 animals in each group).

4.5. Mean Wound Healing Time

The DDW+sham-irradiation group showed a complete closure of wounds by 19.17 day post-irradiation, whereas oral administration of mice with 150 mg/kg NSE resulted in a significant decline in the mean wound healing time, where the excision wound closed by 17.0 days post-irradiation. 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 also increased in DDW+irradiation group when compared with the DDW+sham-irradiation group. This delay in the mean wound healing time was 2 (21.2 ± 0.75), 3 (22.4 ± 0.42), 4 (23.1 ± 0.50) and 7 (26.1 ± 0.53) days, for 2, 4, 6 and 8 Gy irradiation, respectively, in DDW+irradiation group (Figure 4). The oral administration of 150 mg/kg of NSE to mice before irradiation to various doses of γ -radiation accelerated the healing of irradiated excision wounds leading to a reduction in the mean wound healing time. The mean wound healing time of 20.72 ± 0.45 , 21.4 ± 0.62 , 22.3 ± 0.15 and 25.7 ± 0.25 was observed for 2, 4, 6 and 8 Gy, respectively in NSE+irradiation group (Figure 3.4). This improvement in wound healing time was statistically significant ($p < 0.05$) for 2, 4 and 6 Gy, respectively in NSE+irradiation group, except for 8 Gy irradiation, where this enhancement in wound healing time was statistically non-significant.

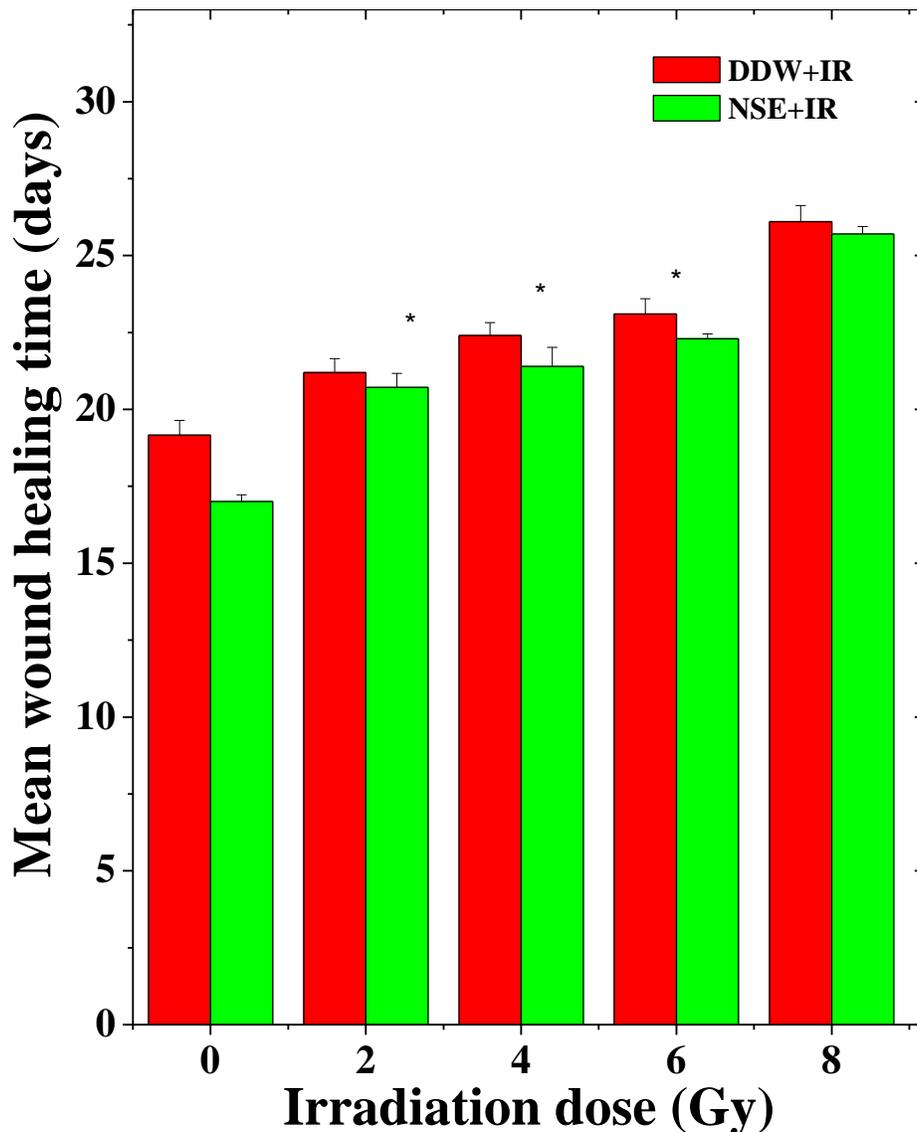


Fig4. Effect of optimal dose of NSE (150mg/kg) on the mean wound healing time of wounded mice exposed to 2, 4, 6 and 8Gy whole-body γ -radiation. All results are shown as mean \pm SEM. The significance * = $p < 0.05$, when compared with respective DDW+irradiation group.

4.6. Estimation of Collagen

The amount of hydroxyproline is an index of collagen content and is also a measure of neo-collagen synthesis. The synthesis of collagen increased in a time dependent manner up to day 10 post-irradiation, where the highest hydroxyproline contents were estimated in sham-irradiation groups; thereafter, the synthesis of collagen declined on day 15 post-irradiation. The pattern of collagen synthesis after 6 Gy irradiation was similar to that of sham irradiation groups, except that the amount of collagen synthesis was lower than the sham-irradiation groups at all the post-irradiation time points evaluated. The NSE administration before 6 Gy exposure resulted in an elevation in the collagen synthesis indicated by the increased hydroxyproline contents at all post-treatment times. However, this increase in collagen synthesis was statistically significant at 5 and 10 days post-irradiation only (Figure 5).

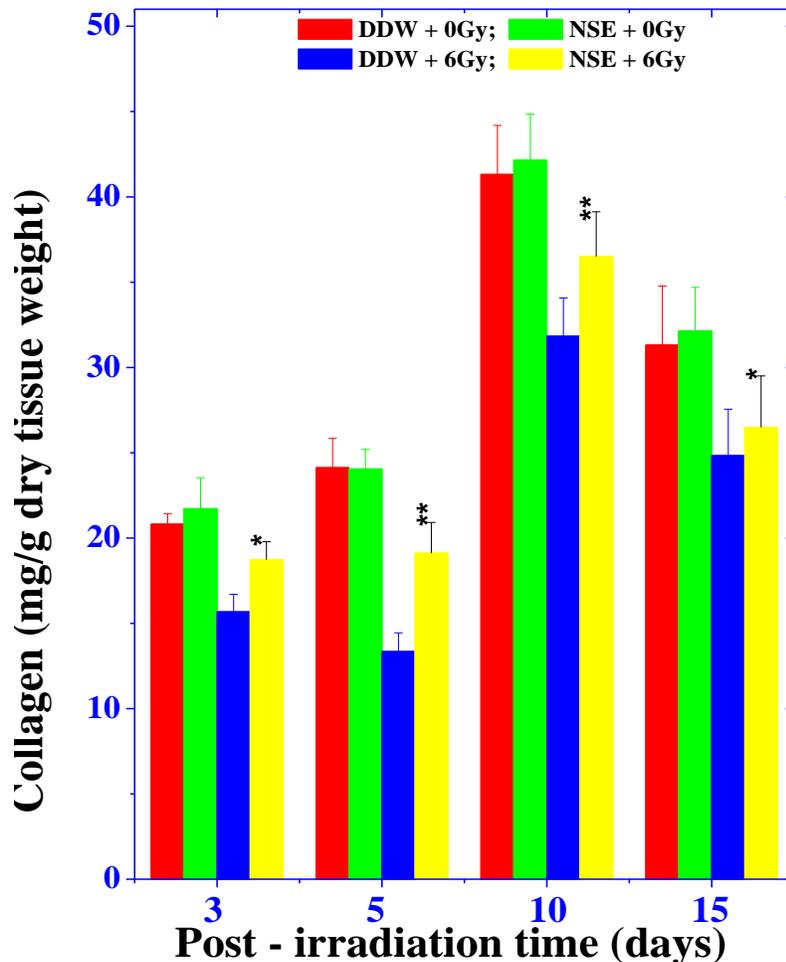


Fig5. Effect of NSE treatment on biosynthesis of collagen in the wound exposed to 6 Gy γ -radiation. All results are shown as mean \pm SEM. The significance * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and No symbol = Non significant, when compared with respective DDW+irradiation group.

4.7. Estimation of Hexosamine

NSE treatment alone increased hexosamine, the ground substratum for collagen synthesis on day 3 post-irradiation when compared to non-irradiated control (0 Gy), and continued to rise up to day 10 post-irradiation and declined thereafter (Figure 6). Irradiation of animals to 6 Gy caused a significant decline in the hexosamine contents at all post-irradiation days in DDW+irradiation group in comparison with the sham-irradiation group (Figure 6). Despite this reduction, the hexosamine contents showed a time dependent increase up to day 10 post-irradiation and a decline thereafter. The pattern of hexosamine synthesis was similar in the NSE+irradiation group, except that the hexosamine synthesis was significantly higher at all post-irradiation days when compared to concurrent irradiation group (Figure 6).

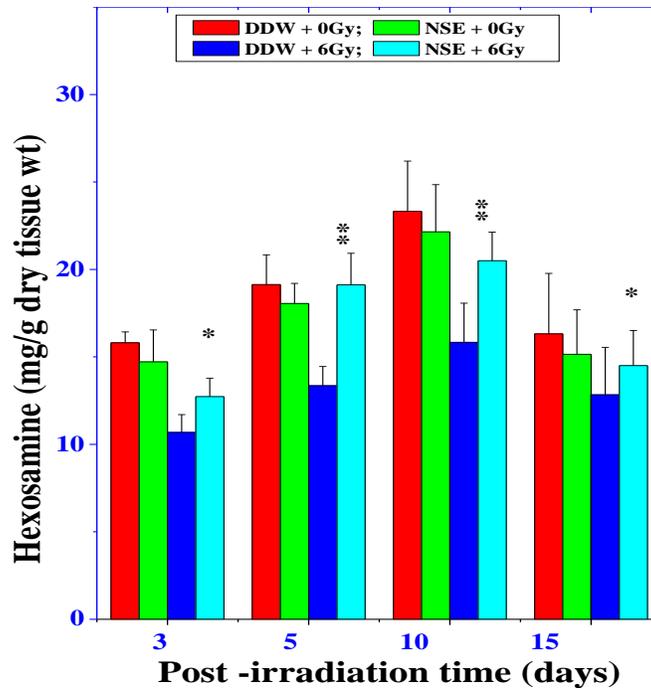


Fig6. Effect of NSE treatment on the biosynthesis of hexosamine in the wound exposed to 6 Gy γ -radiation. All results are shown as mean \pm SEM. The significance * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and No symbol = Non significant, when compared with respective DDW+irradiation group.

4.8. Glutathione (GSH)

Irradiation of animals reduced the GSH contents in a dose dependent manner and a highest decline was observed for 8 Gy irradiation. The GSH concentration showed a maximum reduction at 6 h post-irradiation, where it was lowest than all other post-irradiation times. Thereafter the GSH concentration increased with time up to 72 h post-irradiation. Despite this elevation, the GSH concentration was lower than the DDW+ sham-irradiation group (Figure 7). The pattern of GSH decline and elevation in NSE+irradiation group was similar to that of DDW+irradiation group except that the administration of NSE significantly elevated the GSH concentration at all the post-irradiation times, when compared to the DDW+irradiation group (Figure 7).

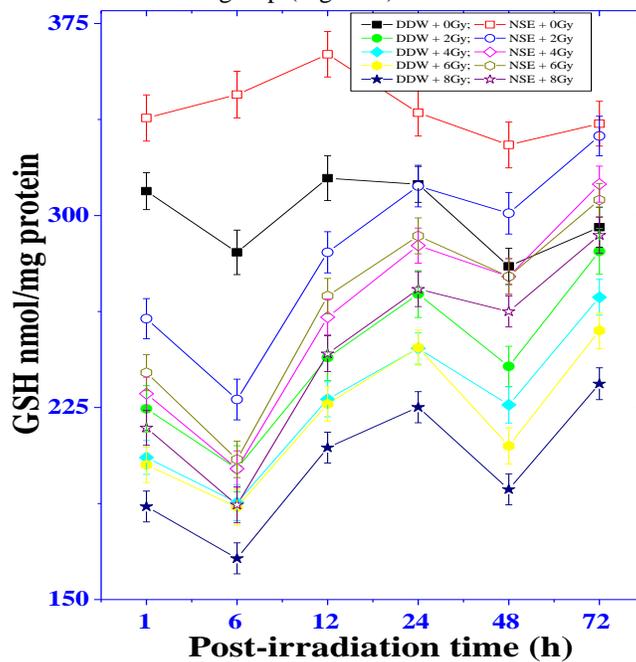


Fig7. Effect of NSE on the Glutathione activity in the mice skin exposed to different doses of whole-body γ -radiation.

4.9. Glutathione Peroxidase (Gshpx)

The GSHpx declined after irradiation and a maximum decline was observed for 2 Gy, whereas increase in exposure dose resulted in an elevation in the GSHpx activity, especially for 6 and 8 Gy which was greater than 0 h at 6 Gy post-irradiation (Figure 8). With increase in assessment time this pattern was not followed and at 72 h post-irradiation, all exposure doses showed a reduced activity of GSHpx (Figure 8). NSE pretreatment significantly elevated the GSHpx activity for all exposure doses and at all post-irradiation times, when compared to the DDW+irradiation group (Figure 8).

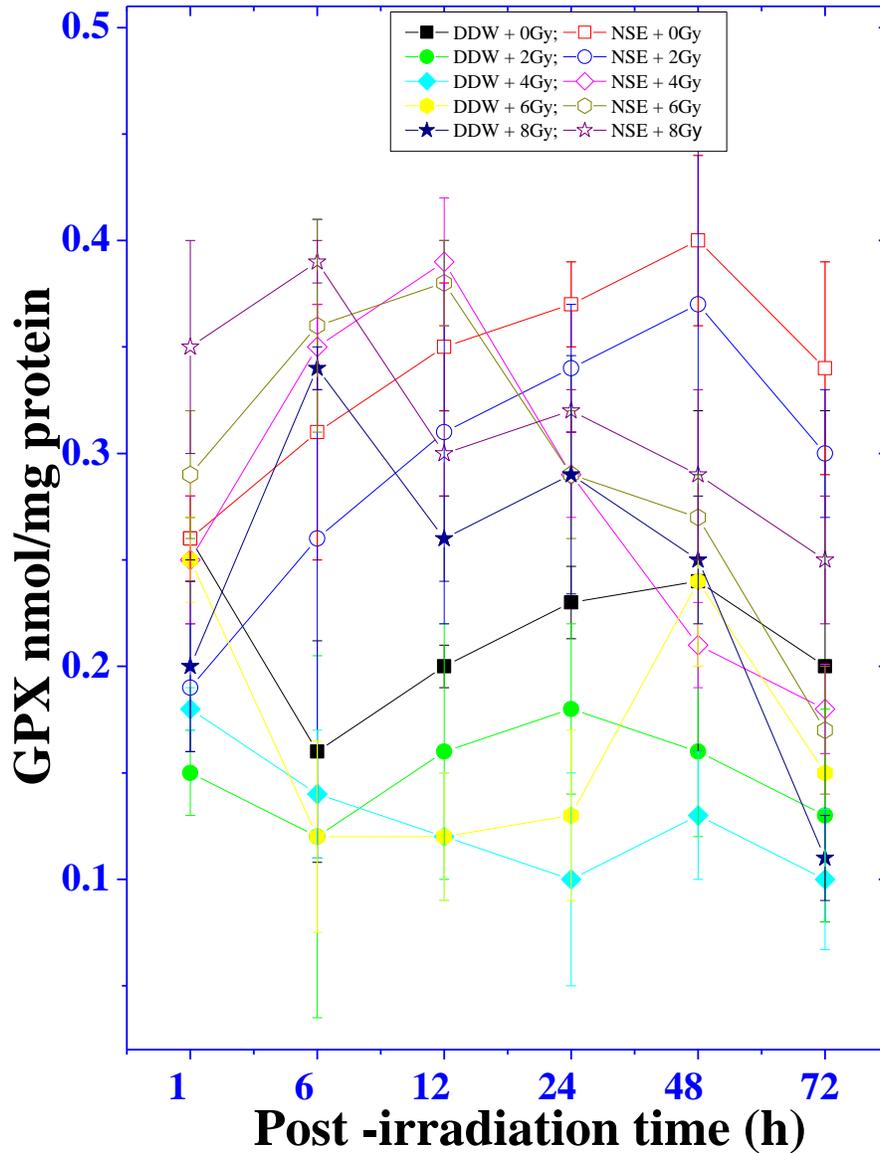


Fig8. Effect of NSE on the Glutathione peroxidase activity in the mice skin exposed to different doses of whole-body γ -radiation.

4.10. Superoxide Dismutase (Sod)

Infliction of excision wound itself increased the SOD activity in the sham- irradiation groups. Irradiation elevated the SOD activity significantly and a maximum increase was observed for 2 Gy at 6 h post-irradiation. This increase in SOD activity showed a radiation dose dependent decline and almost normal levels were restored by 72 h post-irradiation (Figure9). The pattern of SOD activity in NSE+irradiation group was almost similar to that of DDW+irradiation group, except that the activity was significantly higher in the former than the latter (Figure 9).

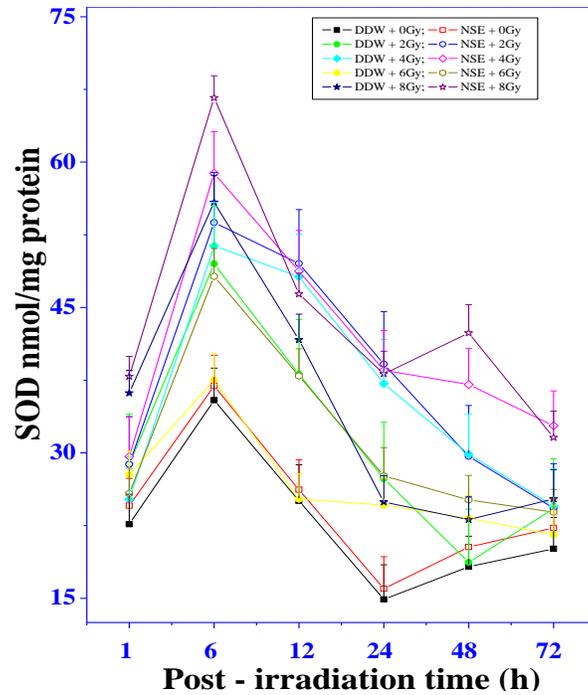


Fig9. Effect of NSE on the super oxide dismutase activity in the mice skin exposed to different doses of whole-body γ -radiation.

4.11. Lipid Peroxidation (Loo)

Irradiation caused a dose dependent elevation in the lipid peroxidation in the wounds at all post-irradiation times. LOO also increased with the assessment time and the highest LOO was observed at 72 h post-irradiation in both the groups (Figure 10). Treatment of animals with NSE significantly reduced the LOO in the wounds of NSE+irradiation group when compared with DDW+irradiation group at all the post-irradiation times (Figure 10).

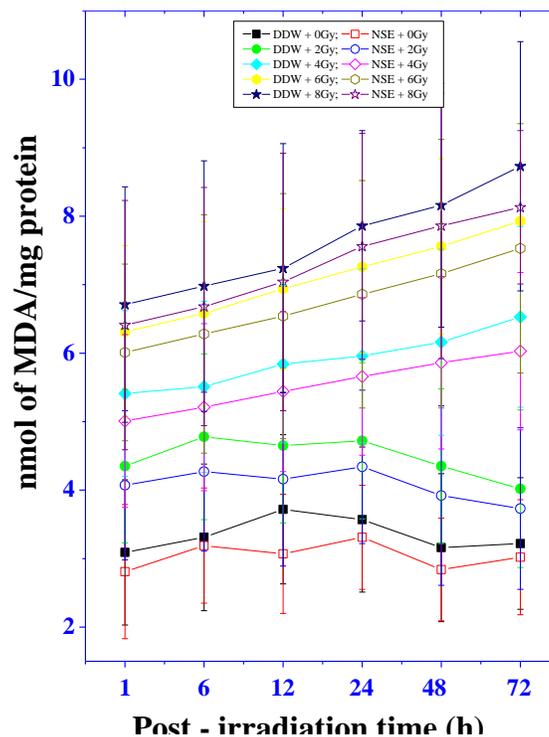


Fig10: Effect of NSE on the lipid peroxidation in the mice skin exposed to different doses of whole-body γ -radiation.

V. DISCUSSION

Natural products have been the major source of medicine for human healthcare and several modern drugs have been isolated from the plant products as they have been found to be nontoxic and have been in constant use since the inception of human civilization. Kalonji, the seeds of *Nigella Sativa* Linn. (Ranunculaceae) has been used as a spice and food preservative as well as a curative remedy for several disorders in the Ayurvedic system of medicine in India. However, its healing activity in irradiated wounds remains unexplored, therefore, the present study was undertaken to elucidate the effect of NSE on healing of irradiated excision wounds in mice.

Wound healing is a concerted effort of a sequence of various physiological processes including inflammation, metabolism, regeneration and remodeling leading to complete wound closure (Haubner *et al.*, 2012). 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 (Peacock, 1984; Tejero-Trujeque, 2001). The progression of wound healing can be determined by the periodic assessment of contraction of excision wounds using various methods. In the present study, the wound contraction was measured by capturing the video images of excision wound periodically (Jagetia and Rajanikant, 2012). The exposure of mice to 6 Gy retarded the healing of wound and this delay in wound contraction is in good agreement with earlier reports, where a similar effect has been reported (Grillo and Potsaid 1961, Stromberg *et al.*, 1968, Kumar and Jagetia, 1995; Jagetia *et al.*, 2004, 2007, Jagetia and Rajanikant, 2012). Treatment of mice with different doses of NSE prior to whole body 6 Gy irradiation resulted in a dose-related acceleration in wound healing up to 150 mg/kg, as was evident by an early closure of wound in the NSE+irradiation group. The studies regarding the amelioration of wound healing by NSE treatment after whole body irradiation are lacking. The other nutrient factors including ascorbic acid and curcumin accelerated early repair of irradiated wounds in a dose related manner and a maximum effect was observed up to a certain dose (Jagetia and Rajanikant, 2003; Jagetia *et al.*, 2004). Similarly, vitamin A supplementation improved the acute radiation-induced delay in wound healing (Levenson *et al.*, 1984). Likewise, phenytoin sodium has been also reported to accelerate healing of irradiated wounds earlier (Song and Cheng 1997).

Wound healing involves a cascade of well-orchestrated biochemical and cellular events leading to the growth and regeneration of wounded tissue in a specific manner and ionizing radiation have been reported to produce multiple negative effects on wound healing including diminished vascularity, impairment of the proliferate capacity of fibroblasts and hematopoietic cells and decreased collagen synthesis (Rudolph *et al.*, 1988, Doyle *et al.*, 1996, Kumar and Jagetia 1995; Jagetia *et al.*, 2004,2007; Jagetia and Rajanikant, 2012). The retardation in wound healing after irradiation may be due to the multiple negative effects elicited by irradiation on various important physiochemical events leading to delayed healing of wounds.

The inflammatory response is generated immediately after wounding by neutrophils and macrophages that collect in the wound bed after injury. This inflammation is caused by releasing H₂O₂ and other ROS species. (Woo *et al.*, 2012). The excess generation of ROS by irradiation in addition to wounding, may be one of the causes of delayed wound healing in the irradiated wounds in the present study. The continued inflammation in the form of persistence increase in ROS has been reported in chronic non healing wounds (Trabucchi *et al.*, 2002; Williams and McBride, 2011; Ryan, 2012). Pretreatment of mice with NSE may have neutralized radiation-induced free radicals by increasing the antioxidant enzymes and subsequently reduced the radiation-induced delay in irradiated wounds. The NSE has been reported to scavenge various free radicals in vitro (Jagetia and Ravikiran, 2015)

Ionizing radiation induces severe damage to vital tissues, especially those with a high rate of cell division, such as hematopoietic tissue (Levenson *et al.*, 1984; Ryan, 2012) that play an essential role in healing of wounds. The loss of significant number of bone marrow cells can lead to an immunocompromised state that could make the animals more susceptible to bacterial infections, leading to complications in the healing of wounds. In fact 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 (Stromberg *et al.*, 1967). These studies suggest that radiation-sensitive bone marrow-derived cells play an important role in tissue repair and regeneration. Other possibility includes a delay in fixation of the wound edge to the underlying tissue after irradiation, which may be due to lack of fibroblast proliferation and a decrease in fibroblast synthetic function in the granulation bed. Recently, the role of bone marrow derived mesenchymal stem cells has been confirmed in irradiated mice; where these cells have been reported to reduce wound healing time (Liauw *et al.*, 2013).

The contraction of open excised wounds has been found to be a function of contractile fibroblasts, known as myofibroblasts (Gabbiani *et al.*, 1972; Darby *et al.*, 2014). Irradiation is thought to impair wound healing in skin through its cytotoxic effect on fibroblasts, which may be due to the delay in the progression of cells through the cell

cycle after irradiation (Rudolph *et al.*, 1988). Irradiation may also induce adverse effects on the fibroblasts through bone marrow depression, since some fibroblasts of the normal subcutaneous connective tissue, participating in wound healing have been shown to originate from the bone marrow (Lange *et al.*, 1979; Ryan 2012). The fibroblasts play an important role during wound healing by secreting various collagens, fibronectins, heparin sulphate proteoglycans, tenascin and connective tissue growth factors that are essential for healing of wounds (Kendall and Feghali-Bostwick, 2014; Darby *et al.*, 2014). Therefore, destruction of fibroblasts by irradiation may have been responsible for delayed healing of irradiated wounds. Furthermore, fibroblasts have been reported to secrete various angiogenic factors like VEGF, transforming growth factor- β (TGF- β) and platelet derived growth factor (PDGF) that aid in the formation of new blood vessels during tissue repair (Haubner *et al.*, 2012).

The proteins are one of the most important nutrient factors affecting wound healing. A deficiency of proteins can impair capillary formation, fibroblast proliferation, proteoglycan synthesis, collagen synthesis, and wound remodeling. A deficiency of protein also affects the immune system, with resultant decreased leukocyte phagocytosis and increased susceptibility to infection (Gogia, 1995). Collagen is the major protein component of connective tissue and is composed primarily of glycine, proline, and hydroxyproline. Collagen synthesis requires hydroxylation of lysine and proline, and co-factors such as ferrous iron and vitamin C. Impaired wound healing results from deficiencies in any of these co-factors (Campos *et al.*, 2008). Also, reduction in collagen and DNA syntheses may be another reason for delayed healing after irradiation. Irradiation has been reported to produce negative effects on collagen and DNA syntheses (Reily 1994, Jagetia *et al.*, 2003, 2005, 2007, Jagetia and Rajanikant, 2005, 2012). In this study, the NSE administration resulted in a significant elevation in the collagen and hexosamine synthesis when compared to the concurrent DDW+irradiation group, which may be one of the reasons of early wound closure in NSE treated group.

Cells are equipped with antioxidant defence systems to detoxify the endogenous and exogenous oxidative challenges during aerobic metabolism or when they encounter stress inducing agents (Mates *et al.*, 1999). The increase in GSHpx and SOD after irradiation is a short-term measure to counter the oxidative stress induced by wounding coupled with irradiation. Alterations in the activity of these enzymes would modify the cellular redox state and the net effect of radiation. Lipid hydroperoxides are a well-known marker of oxidative stress formed from unsaturated phospholipids, glycolipids and cholesterol, through peroxidative reactions under oxidative stress (Girotti, 1998; Jagetia *et al.*, 2003; Rastogi *et al.*, 2010). The NSE pretreatment has increased GSH concentration, GSHpx, catalase and SOD activities in the present study, which may have contributed to reduced inflammation and early wound repair. The ascorbic acid and curcumin treatment have been reported to elevate GSH, and GSHpx and superoxide dismutase in irradiated wounds (Jagetia *et al.*, 2003; Jagetia and Rajanikant, 2015) The induction of lipid peroxide after irradiation is well known phenomenon and it has increased in a dose and time dependent manner. A similar effect has been observed earlier in irradiated wounds treated with curcumin (Jagetia and Rajanikant, 2015). The active constituents of *Nigella sativa*, thymoquinone could act as a free radical and superoxide radical scavenger, as well as preserve the activity of various antioxidant enzymes such as catalase, glutathione peroxidase and glutathione-S-transferase (Houghton *et al.*, 1995). NSE treatment has reduced the formation of lipid peroxides and the decline in the lipid peroxide may have accelerated the wound repair in NSE+irradiation group.

Thus acceleration in wound healing after NSE treatment may not be due to a single mechanism, but it could be due to interplay of multiple mechanisms during the healing of irradiated wounds. The presence of NSE may have scavenged the radiation induced free radicals thereby neutralizing their negative effect and accelerating the repair and regeneration of irradiated wounds. NSE may have curtailed the inflammatory response elicited by wounding and irradiation by increasing the antioxidant status and reducing the lipid peroxidation as observed in the present study. The Nrf2 is involved in transcriptional regulation of antioxidant genes and irradiation has been reported to suppress Nrf2 pathway genes (Canales-Aguirre, 2012; Khan *et al.*, 2015). The thymoquinone the main phytochemical of NSE has been recently reported to upregulate the transcription of Nrf2 gene (Kundu *et al.*, 2014). NSE might have alleviated the radiation-induced inhibition in Nrf2 leading to increased antioxidant status and reduced the delay in healing of irradiated wounds in the present study. This may have accelerated wound repair. Although molecular pathways employed by NSE during wound healing have not been investigated in the present study, the presence of NSE before irradiation may have blocked the down modulation of cell cycle regulatory protein mRNAs retaining the capacity of fibroblast and endothelial cell division that are essential for wound repair. Moreover, wounding and irradiation have been reported to activate the transcription of NF- κ B, COX- II and LOX mRNAs, and NSE may have inhibited their transcriptional activation leading to an early repair and regeneration of wounds in this group. The thymoquinone one of the active principles of NSE has been reported to block the transcriptional activation of NF- κ B, COX- II and LOX-5 (Haughton *et al.*, 1995; Ryan 2012). NSE may have aided in the transcription of

mRNAs related to collagen, DNA, NO syntheses therefore it would have helped in matrix remodeling and early repair of irradiated wounds. NSE treatment may have also struck a balance between regeneration and apoptosis that are essential for wound repair and thus accelerated the repair and regeneration of wounds.

The present study demonstrates that NSE retarded the radiation induced delay in healing of irradiated wounds in a dose dependent manner and the greatest augmentation in wound healing was observed for 150 mg/kg. This healing effect may be due to scavenging of radiation-induced free radicals, reduced inflammatory activity, increased antioxidant status due to increased Nrf2 transcription and upregulation other mRNAs needed for wound healing. The active principle of NSE, the thymoquinone may have suspended the transcription of NF- κ B, COX- II and LOX5 and accelerated wound repair and regeneration of irradiated wound in the present study. The NSE would have added in early deposition of collagen and other proteins required during wound healing.

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