

IN VIVO RADIOPROTECTIVE EFFECTS OF WHEATGRASS (*TRITICUM AESTIVUM*) EXTRACT AGAINST X-IRRADIATION-INDUCED OXIDATIVE STRESS AND APOPTOSIS IN PERIPHERAL BLOOD LYMPHOCYTES IN RATS

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ABSTRACT

Objectives: The present study was undertaken to investigate the possible protective potential of wheatgrass extract against radiation-induced toxicity in peripheral lymphocytes of rats exposed to a fractionated dose of X-rays.

Methods: Effects of the X-irradiation with and without wheatgrass were studied on various parameters in peripheral lymphocytes including antioxidant defense system and apoptosis. Male Sprague-Dawley rats were divided into four different groups: Normal control group, X-ray-irradiated group (21 Gy over a span of 7 days), wheatgrass-treated group (80 mg/100 g bodyweight for 2 weeks), and X-rays-irradiated + wheatgrass-treated group. All the biochemical indices which included lipid peroxidation (LPO), reduced glutathione, reactive oxygen species (ROS), and activities of antioxidant enzymes were investigated in lymphocytes. Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay was carried out to assess the apoptosis in lymphocytes following whole-body X-irradiation.

Results: Whole-body X-irradiation to rats resulted in significant increase in LPO with concomitant depression of antioxidant enzymes activities, namely, superoxide dismutase, catalase, and glutathione peroxidase (GPx) in lymphocytes. Further, the present study witnessed a significant increase in the number of apoptotic lymphocytes in the X-irradiated animals. However, wheatgrass supplementation lowered the LPO levels, restored cellular antioxidant status, and provided significant protection against radiation-induced apoptosis.

Conclusions: Based on these observations, the present study suggests that wheatgrass extract has the potential to be used as an effective radioprotectant against radiation-induced oxidative stress and apoptosis in peripheral lymphocytes of whole-body X-ray-exposed rats.

Keywords: Wheatgrass, Ionizing radiation, Lymphocytes, Radioprotection, X-irradiation.

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INTRODUCTION

Radiotherapy is an important non-surgical modality for the treatment of human malignancies. However, associated side effects limit its applications. Most common side effect is the suppression of hematopoietic system, which is comprised of rapidly proliferating progenitor cells and is the most radiosensitive tissue in the body [1,2]. Ionizing radiation-induced hematopoietic injury causes myelosuppression and dose-dependent depletion of circulating blood cells that result in anemia and increased susceptibility to infections [3-5]. Further, exposure to ionizing radiation has been known to inflict unprecedented long-term effect on cellular pathways, resulting in genomic instability which may later manifest into hereditary diseases or various forms of cancers [6-8]. Exposure to ionizing radiation triggers radiolysis of water in the cells that result in the generation of highly reactive free radicals OH[•], H[•], and H₂O[•] collectively known as reactive oxygen species (ROS) which trigger prooxidant and antioxidant imbalance [9]. ROS is known to react with various critical biomolecules such as DNA, RNA, and protein, thereby setting off disruption of cellular homeostasis [10]. Consequently, free radicals and altered molecules as an outcome of compromised cellular antioxidant defenses necessitate the development of countermeasures to minimize radiation-induced damage.

Many drugs of synthetic nature have been tested both in *in vitro* and *in vivo* models to mitigate injuries caused by ionizing radiations [11,12]. However, synthetic drugs have reported certain side effects

[13]. Therefore, alternative sources, especially natural herbal sources, need to be explored to be used as effective and safe radioprotectors to combat the deleterious effects of ionizing radiations. Wheatgrass is a natural herbal source and is reported to exhibit a variety of effects which are anti-inflammatory, antioxidant, antibacterial, and anti-carcinogenic in nature [14-18]. Wheatgrass is rich in flavonoids, minerals, amino acids, and vitamins [19,20]. Medicinal property of wheatgrass can be attributed to the presence of antioxidants, which include vanillic acid, coumaric acid, caffeic acid, and ferulic acid [21,22]. Wheatgrass is also known as a "green blood" as it contains a high concentration of chlorophyll and thus helps in building red blood cells and rejuvenates hematopoietic system [23,24]. There is no detailed study on radioprotective activity of wheatgrass. Moreover, there is a considerable interest in development of non-toxic radioprotector effective against adverse effects induced by ionizing radiation. Thus, the present study was conducted to explore the role of wheatgrass in mitigating the adverse effects of radiation exposure on the lymphocytes, the most susceptible targets in the blood.

METHODS

Chemicals

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) blue apoptosis detection kit was procured from Trevigen USA. Ficoll-Histopaque-1077, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), bovine serum albumin, nicotinamide adenine dinucleotide phosphate (NADPH), and glutaraldehyde were procured from Sigma-

Aldrich USA. Glutathione (GSH), nitroblue tetrazolium (NBT), and 5,5-dithiobis 2-nitrobenzoic acid (DTNB) were purchased from SRL Chemicals (India). Wheatgrass tablets were procured from a local source, manufactured and supplied by AYUSH, India. All the routine chemicals used were of analytical grade and were procured from the local supplier.

Animals

A total of 32 male Sprague-Dawley rats weighing 100–150 g were obtained from the central animal house of Panjab University Chandigarh and were maintained under standard laboratory conditions. The animals were fed with standard pellet diet (Ashriwad Industries, Punjab, India) and water *ad libitum*. All the experiments on animals were conducted with strict adherence to the ethical guidelines as approved by the Institutional Ethics Committee of Panjab University, Chandigarh (Ref: IAEC/347-370 dated 12/02/2013). The animals were segregated randomly into four groups, and each group consisted of 8 animals. The animals in group I served as Normal control and were fed standard laboratory feed and water *ad libitum*. The animals in group II were subjected to whole-body X-rays exposure to deliver a total dose of 21 Gy by giving a daily exposure of 3 Gy spanned over a period of 7 days [25]. The animals were anaesthetized using mild ether anesthesia so as to immobilize them during X-ray exposure. The group III animals were given wheatgrass extract at a dose of 80 mg/100 g bodyweight everyday orally for 2 weeks [26]. The group IV animals were given a combined treatment of whole-body X-ray exposure as well as wheatgrass with similar doses as were given to group II and group III, respectively. Wheatgrass treatment was started one week prior to the start of first radiation exposure and continued for a total duration of two weeks. All the experimental studies were carried out 24 h after the last X-ray exposure in lymphocytes.

X-ray machine calibration using ferrous sulphate-benzoic acid-xylene orange (FBX) dosimeter

X-ray machine (Allengers Company, India) was calibrated using the FBX dosimeter as described by Gupta *et al.* [27]. Briefly, FBX solution in a plastic Petri dish was exposed to X-rays at a constant current 20 mA and varying voltages from 40 to 110 kV. Thereafter, optical density (OD) was measured after 30 min of exposure at 548 nm wavelength. Measured OD was then compared with OD of FBX samples exposed to known different doses from LINAC in the Department of Radiotherapy, Post Graduate Institute of Medical Education and Research. After calibration, a dose of 3 Gy was delivered to animals by X-ray machine at a constant voltage of 80 kV and a constant current of 20 mA.

Lymphocyte extraction and preparation of lysate

The blood samples were drawn at 24 h post irradiation by puncturing the ocular vein of rats with fine sterilized capillary, and the lymphocytes were isolated from heparinized blood samples according to the method of Hudson and Hay [28]. Briefly, 2 ml of blood was diluted with 2 ml of normal saline and then layered carefully on the 3 ml histopaque 1077. The samples were centrifuged at 1800 rpm for 20 min at 20°C. White milky layer of mononuclear cells (i.e. lymphocytes) was carefully removed, and the cells were washed twice with the saline and then centrifuged at 1200 rpm for 10 min to get the pellets of lymphocytes. The lymphocytes were lysed in a hypotonic solution of distilled water for 45 min at 37°C and then were centrifuged at 10,000 rpm for 2 min at 4°C. The supernatants were taken for the biochemical assays.

Biochemical assays

Lipid peroxidation (LPO)

LPO was assayed using the method of Wills [29]. Briefly, 10% trichloroacetic acid was added to lysate and precipitated proteins were removed by centrifugation. To the supernatants, 2 ml of 0.67% 2-thiobarbituric acid (TBA) was added and the pink color was developed at 100°C. The samples were allowed to cool and OD of pink-colored TBA complex was read at 532 nm. The amount of product formed was calculated on the basis of the molar extinction coefficient of TBA complex (1.56×10^5 M/cm).

Reduced GSH

The levels of reduced GSH were measured according to the method of Ellman [30]. Briefly, 25% TCA was added to the lysates to precipitate proteins and the samples were centrifuged to obtain supernatants. Freshly prepared DTNB was added to the supernatants and the OD of the yellow-colored complex was measured at 412 nm. Molar extinction for GSH is 13600/M/cm.

Catalase (CAT)

CAT estimation was done by the following method as described by Catalase [31]. Briefly, lysates were added to the reaction mixture containing 50 mM phosphate buffer and 12 mM H₂O₂. CAT present in the sample decomposes H₂O₂ and the resulting decrease in OD was measured at 240 nm for 3 min. Decomposition of H₂O₂ was calculated using the molar extinction coefficient (0.0394/mM/cm).

Glutathione peroxidase (GPx)

The activity of GPx was measured by coupled enzyme assay described by Flohé and Günzler [32]. GPx decomposes H₂O₂ by oxidizing GSH into GSSG, and this reaction is coupled with a reduction of GSSG into GSH by GSH reductase by utilizing NADPH. Utilization of NADPH results in a decrease in absorbance of NADPH which was measured at 340 nm.

Superoxide dismutase (SOD)

SOD activity was estimated according to method of Kono [33]. Briefly, NBT is reduced to blue-colored formazon by superoxide ions generated by photo oxidation of hydroxylamine hydrochloride, and the change in absorbance was measured at 560 nm. The addition of SOD in the sample inhibited the reduction of NBT mediated by superoxide ions. The extent of inhibition was taken as a measure of enzyme activity. One unit of enzyme was expressed as inverse of amount of protein (mg) required to inhibit the reduction rate of NBT by 50%.

Protein estimation

The estimation of protein concentration was done by the following method of Lowry *et al.* [34]. Briefly, lysates were diluted in distilled water to make a total volume up to 700 µl. Lowry reagent (prepared by mixing 98 ml of 2% sodium carbonate in 0.1 M NaOH, 1 ml of 1% copper sulphate and 1 ml of 2% sodium potassium tartarate) was added to sample. Sample were then thoroughly mixed and allowed to stand for 10 min at room temperature. 0.25 ml 1N Folin reagent was added followed by immediate mixing and incubation at room temperature in the dark for 30 min. The absorbance of the blue-colored complex was measured at 620 nm using spectrophotometer.

ROS

ROS were estimated by the method of Driver *et al.* [35]. using DCFH-DA. The lymphocytes were incubated with 20 µM DCFH-DA for 30 min at room temperature in the dark. ROS present in the samples activated esterases which cleaved acetate group of non-fluorescent DCFH-DA converting it into fluorescent dichlorofluorescein (DCF). The fluorescence was measured using a spectrofluorimeter at excitation wavelength 488 nm and emission wavelength 521 nm. The fluorescence intensity units of DCF/mg protein were taken as the measure of ROS in different treatment groups.

TUNEL assay

Apoptosis was assessed using Trevigen TUNEL blue apoptosis detection kit. Briefly, lymphocytes were fixed in 4% formaldehyde at room temperature. The cells were then resuspended in 80% ethanol and fixed on polylysine coated slides. For TUNEL assay reaction, the slides were immersed in terminal deoxynucleotidyltransferase (TdT) enzyme labeling buffer following which slides were covered with the TdT labeling reaction mixture containing a brominated nucleotide (BrdU). The reaction was stopped after 30 min by putting slides in the TdT stop buffer. The labeled apoptotic cells were stained with biotinylated anti-

BrdU antibody followed by counterstaining with nuclear fast red stain. The blue apoptotic positive cells were counted for apoptotic analyses.

Statistical analysis

Statistical analysis of the data was carried out using one-way analysis of variance on SPSS followed by multiple *post hoc* analyses (Student-Newman-Keuls). The results were expressed as mean±standard deviation.

RESULTS

The results of different treatment groups were compared with the normal control group. In addition, the results from wheatgrass + X-ray-treated group were compared with radiation-treated group.

Changes in the level of LPO are presented in Table 1. A statistically significant ($p \leq 0.001$) increase in level of MDA (17.10 ± 2.14) was observed in lymphocytes from irradiated rats against the control values (7.85 ± 1.13). However, on wheatgrass extract supplementation to X-ray-exposed rats, a significant ($p \leq 0.001$) decrease in MDA levels (9.67 ± 2.87) was observed and the indicative of its normalization.

The activities of enzymes associated with the antioxidative defense system, namely, CAT, SOD, and GPx as well as the levels of GSH were found to be significantly decreased following exposure to X-rays when compared to the normal control rats (Table 1). In irradiated group GPx activity was 2.41 ± 0.13 , SOD activity was 23.98 ± 2.12 , whereas CAT activity 252.00 ± 50.08 . Interestingly, wheatgrass extract supplementation to X-ray-irradiated rats resulted in a significant increase in the activity of CAT (416.75 ± 45.17), GPx (3.07 ± 0.26), and SOD (36.78 ± 5.27) in lymphocytes when compared to X-irradiated rats. The GSH content (15.18 ± 2.16) was significantly ($p \leq 0.001$) depleted in X-irradiated rats when compared with control rats. Treatment of rats with wheatgrass extract before X-ray exposure resulted in significant increase ($p \leq 0.001$) in GSH level (19.93 ± 2.34) when compared with irradiated group. In addition, depression in inherent antioxidant defense system in lymphocytes was accompanied by an elevated level of ROS (68.34 ± 6.88) (Table 2) in X-ray-irradiated rats when compared to normal control group (13.01 ± 4.28) as indicated by fluorimetric analysis of ROS by DCFH-DA assay. Wheatgrass extract supplementation exhibited a significant protection and lowered the levels of ROS (28.79 ± 2.79) in irradiated rats.

The results of TUNEL assay in peripheral lymphocytes are shown in Fig. 1. An increase in TUNEL-positive cells was clearly noticeable in the

X-irradiated group. However, a significant reduction was observed in a number of apoptotic cells in X-ray-irradiated rats supplemented with wheatgrass when compared to X-irradiated rats, thereby indicating reduced apoptosis.

DISCUSSION

Exposure to ionizing radiations is known to suppress antioxidant defense system leading to the unhindered propagation of the deleterious effects resulting in cytogenetic instability. Wheatgrass extract has a major concentration of active components such as polyphenols, vitamins, essential elements, and chlorophyll [19-23]. In earlier studies, antioxidant and cell signal modulatory property of various components present in wheatgrass has been extensively investigated in various pathological conditions [36]. The present study investigated the protective efficacy of the wheatgrass extract in ameliorating radiation inflicted injury to X-ray-exposed lymphocytes in rats.

Ionizing radiations inflict damage to biological tissue by the way of reactive ROS produced as a consequence of interaction of radiation with water molecule [9]. ROS is known to produce prooxidant and antioxidant imbalance, resulting in cascade of biomolecular changes finally culminating in cellular dysfunction and apoptosis [10]. In addition, ROS is known to attack polyunsaturated fatty acids in Hydroxyl radical reacts with polyunsaturated fatty acid leading to formation of lipid free radicals, which are reduced by nearby fatty acids, thus starting the self-propagating process of LPO. In the present study, a marked increase was observed in MDA levels following whole-body X-ray exposure (Table 1). Various studies have reported that exposure to ionizing radiation results in damage to membrane lipids [37-39]. In addition, the formation of hydrophilic peroxide from lipophilic unsaturated fatty acid changes membrane dynamics and results in loss of structural as well as functional integrity of membrane [40]. Alterations in membrane physicochemical properties affect function of membrane bound enzymes, permeability, and surface receptors leading to alterations in cell proliferation, differentiation, and apoptosis [41,42]. Interestingly, we observed that administration of wheatgrass extract to the X-irradiated rats resulted in the moderation of MDA levels. This could be accredited to active components of wheatgrass such as coumaric acid, caffeic acid, and ferulic acid which work synergistically to effectively scavenge ROS. Furthermore, elemental analysis of wheatgrass has reported a high content of essential elements such as Fe and Zn which are known to enhance cellular repair capacity [43,44]. In an earlier study from our laboratory, Zn has been reported to render

Table 1: Effect of wheatgrass extract on antioxidant status of lymphocytes of normal control and experimental rats

Groups	LPO (10^{-2} nmoles/mg protein)	GSH (nmoles/mg protein)	SOD (U ^a /mg protein)	CAT (U ^b /mg protein)	GPx (U ^c /mg protein)
Normal control	7.85 ± 1.13	23.64 ± 1.24	41.52 ± 9.35	549.50 ± 52.50	3.59 ± 0.33
X-ray irradiated	$17.10 \pm 2.14^{***}$	$15.18 \pm 2.16^{***}$	$23.98 \pm 2.12^{***}$	$252.00 \pm 50.08^{***}$	$2.41 \pm 0.13^{***}$
Wheatgrass	8.82 ± 1.91	21.18 ± 1.17	36.67 ± 6.69	555.33 ± 60.88	3.50 ± 0.16
X-ray irradiated+Wheatgrass	$9.67 \pm 2.87^{###}$	$19.93 \pm 2.34^{###}$	$36.78 \pm 5.27^{###}$	$416.75 \pm 45.17^{**},^{###}$	$3.07 \pm 0.26^{##}$

U^a=50% inhibition of nitroblue tetrazolium reduction in 1 min. U^b=μmoles of hydrogen peroxide consumed per minute. U^c=μmoles of NADPH utilized/min/mg protein. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ when compared to normal control group. # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$ when X-ray irradiated wheatgrass-treated group compared to X-ray-irradiated group. Values are expressed as mean±SD, n=8 animals. LPO: Lipid peroxidation, GSH: Glutathione, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, CAT: Catalase, SD: Standard deviation, NADPH: Nicotinamide adenine dinucleotide phosphate

Table 2: Effect of wheatgrass extract on ROS levels in lymphocytes in the Normal control and experimental rats

Groups	Lymphocytes (DCF fluorescent intensity units/mg protein)
Normal control	13.01 ± 4.28
X-ray irradiated	$68.34 \pm 6.88^{***}$
Wheatgrass	23.35 ± 4.74
X-ray irradiated+Wheatgrass	$28.79 \pm 2.79^{***},^{###}$

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ when compared to normal control group. # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$ when X-ray irradiated wheatgrass-treated group compared to X-ray irradiated group. Values are expressed as mean±SD, n=8 animals. SD: Standard deviation, ROS: Reactive oxygen species, DCF: Dichlorofluorescein

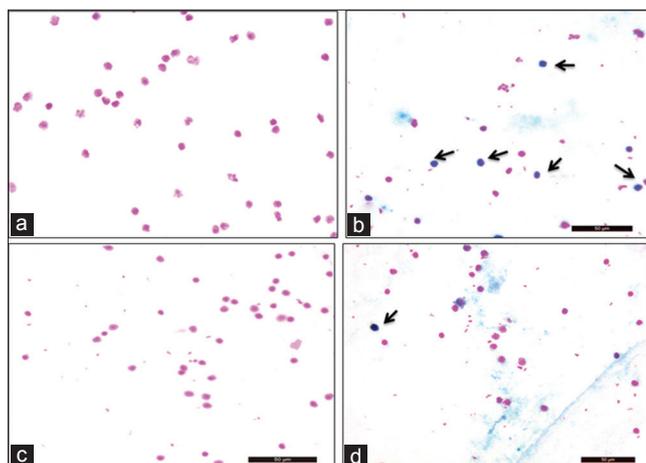


Fig. 1: Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of lymphocytes of (a) normal control, (b) X-ray irradiated, (c) wheatgrass-treated, and (d) X-ray+wheatgrass-treated rats, respectively ($\times 40$). Lymphocytes with 3'OH tagged with brominated nucleotide are TUNEL-positive cells and appear blue, and lymphocytes with intact DNA strand are TUNEL negative and appear pink with nuclear fast red stain

protection against¹³¹I-induced radiation damage to cell membrane [45]. Further, Zn has been observed to stimulate antioxidant defense system to contain free radicals induced damage to biological tissue. Therefore, augmented effect of antioxidants and micronutrients present in the wheatgrass extract reverses cellular membrane damage.

To counteract increased ROS, cells possess comprehensive enzymatic (SOD, CAT, and GPx) and non-enzymatic (GSH) defense system. GSH, a potent endogenous antioxidant is involved in direct reduction of ROS while being oxidized itself [46]. In the present study, we observed radiation-induced oxidative stress that led to increased utilization of GSH and consequently depleted GSH levels (Table 1). Further, depleted GSH levels have been linked with increased LPO which corroborates well with our findings of increased LPO [47]. Antioxidants present in wheatgrass counteract free radicals in X-ray-exposed rats and maintain optimal GSH levels. GSH is known to work in coordination with GPx enzyme which maintains is involved in reduction of GSSG and depleted GSH is known to inhibit GPx activity [48]. Thus, in the present study, we observed dramatic depression in the activity of GPx. The activities of endogenous antioxidant enzymes are closely linked, and thus, decreased GPx activity also results in a significant decline in the activities of SOD and CAT (Table 1). The plausible explanation of decreased activity of SOD and CAT could be due to overwhelming of these enzymes to neutralize the increased amounts of superoxide ions and hydrogen peroxide that are formed by radiolysis of water as a consequence of interaction with X-rays [49]. The present study clearly indicates that wheatgrass supplementation significantly improves the activities of SOD, CAT, and GPx. Another plausible factor for the amelioration of enzymatic activity could be attributed to the presence of micronutrients such as Mn, Fe, and Zn which are associated as cofactors in the activation of enzymes SOD and CAT [50,51].

Severity of radiation damage leads to break in DNA strand, thereby leading to initiation of apoptosis [52]. TUNEL assay is cited as a highly specific biomarker for apoptosis detection by labeling apoptotic cell at genetic level. In our study, we have seen an increase in TUNEL positive cells following X-irradiated which, however, were brought down appreciably on simultaneous wheatgrass supplementation (Fig. 1). Furthermore, the end product of LPO, MDA and 4-hydroxynonenal binds to DNA base pairs and initiates apoptotic signal transduction [53]. Apoptotic cells in irradiated group were brought down appreciably on simultaneous wheatgrass supplementation. Although

the exact mechanism by which wheatgrass prevents apoptosis is not clear, it is understandably due to the antioxidant activities of bioactive components of wheatgrass or DNA repair enhancing property of micronutrients such as Mg and Zn [54,55]. In addition, wheatgrass is a rich source of chlorophyll which is known for inducing DNA repair and has the ability to trap mutagenic substances, thus preventing indirect DNA damage induced by MDA [56].

CONCLUSION

On the basis of the results from the study, we conclude that whole-body X-ray exposure compromises antioxidant defense system and results in ROS-induced apoptosis in lymphocytes. Wheatgrass supplementation offers the non-toxic radioprotective ability to protect lymphocytes from X-irradiation-induced damage. However, further studies are warranted with regard to other definitive bioassays including protein expressions and documentation of specific molecular markers to establish the exact mechanism for wheatgrass-mediated radioprotection.

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AUTHOR CONTRIBUTION

All the persons who have participated in the study have been listed as authors.

- Conception and design of the study : Chandresh Shyam, Devinder K Dhawan, Vijayta D Chadha
- Revision and Drafting of the manuscript : Chandresh Shyam, Devinder K Dhawan, Vijayta D Chadha.

CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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