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Myeloprotective effect of Triticum aestivum Linn. grass against antineoplastic agents induced bone marrow toxicity in mice

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Abstract

Myelotoxicity remains the most important cause of life threatening complications in patients undergoing antineoplastic chemotherapy cycles. Strategies to circumvent or lessen myelotoxicity may improve clinical outcome and quality of life in these patients. The aim of the present study is to investigate myeloprotective effect of *Triticum aestivum* Linn. (wheat) grass against chemotherapeutic agents induced bone marrow toxicity. Swiss albino mice were pretreated with wheatgrass juice at a dose of 20 ml/kg biweekly (b.w.) for 30 days. One hour after the last dose administration of WGJ, animals were injected with a single i.p. dose of cyclophosphamide (50 mg/kg b.w.) and doxorubicin (50 mg/kg b.w.). The reference drug amifostine (350 mg/kg b.w.) was administered 45 min prior to the cyclophosphamide and doxorubicin injection. At 24 h post chemotherapeutics challenge, animals were euthanized after blood sample collection and bone marrow was aspirated from both femurs. Hematologic parameters in blood samples were measured. Chromosomal abnormalities such as chromatid break, chromosomal ring, chromatid gap, chromatid exchange, chromosome break, and number of micronucleated polymorphonuclear erythrocytes formed and polychromatic erythrocytes/normochromatic erythrocytes ratio were recorded in the bone marrow smear. Results of the present study show that pretreatment with wheatgrass juice significantly protected against cyclophosphamide and doxorubicin induced hematologic abnormalities and chromosomal damage in bone marrow stem cells due to its vast array of active principles. By virtue of its anticlastogenic and cytoprotective effects, wheatgrass juice might be considered as a promising candidate for adjuvant therapy without compromising efficacy of chemotherapeutic agents.

Keywords: Chemotherapeutic agent, Chromosomal aberration, genotoxicity, micronucleus, myelotoxicity, wheatgrass.

1. Introduction

Hematological toxicity is one of the main adverse effects of anticancer chemotherapies and often a cause of treatment termination (1). Therefore, myelosupression is regarded as a major factor in treating the cancer with chemotherapeutic agents, as these cytotoxic agents cause hypocellularity of the bone marrow by injuring hematopoietic progenitor stem cells. This leads to decrease in red blood cells, white blood cells (neutropenia or granulocytopenia), and platelets (thrombocytopenia), thereby increasing the risk of anaemia, infection, and uncontrolled bleeding, respectively, which proves to be life threatening to the patients. Hence, myelosuppression continues to be a major dose-limiting toxicity for most chemotherapy regimens (1-4). According to a survey conducted on 1175 patients who completed at least four chemotherapy courses at 64 Italian centres, the association between events during chemotherapy and myelotoxicity indices was assessed by logistic regression. Myelotoxicity was observed in 53.9 %

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patients, anemia (<10 g/dl) in 22.4 %, mild anemia (Hb<12 g/dl) in 58.5 %, and neutropenia in 45.1 %. Dose reductions were observed in 16.9 %, dose delays in 28.7 %, and discontinuations in 13.4 % with no significant difference between age groups. In this study, myelotoxicity accounted for 20 % of treatment withdrawals with no differences between age groups. This study showed a significant association between chemotherapy dose delays, dose reductions and myelotoxicity (5).

Very few options are available to overcome chemotherapy-induced myelotoxicity. Even if available, they are either costly, tedious to comply, or may cause adverse effects. Current guidelines recommend usage of myeloid growth factors prior to the first cycle of chemotherapy for patients with more than 20 % risk of febrile neutropenia (6). Meta-analysis from randomized trials shows that granulocyte colony-stimulating factor (G-CSF) prophylaxis in patients receiving more intensive chemotherapy is associated with a better survival. However, G-CSF also increases the risk of secondary acute myeloid leukaemia (AML) (6). Similarly, erythropoietin may prevent anaemia in high risk patients, but a previous report has raised concerns of serious adverse events such as arterial hypertension, cerebral convulsion/hypertensive encephalopathy, thrombo-embolism, iron deficiency, and influenza-like syndrome on long-term erythropoietin administration (7). Moreover, the cost-effectiveness and safety aspects of current practices including mortality, quality of life, and patient productivity remain doubtful, since much of the available evidences on the use of human recombinant growth factors prophylaxis are partial (8). Thus, the management of cancer is still a major challenge in the patients who are intolerant to chemotherapy induced adverse effects. Considering these views, there is a growing need for natural products that can be used along with chemotherapeutic agents as an effective alternative therapy for preventing myelotoxicity and related life-threatening complications in cancer patients.

Triticum aestivum Linn. (wheat) grass belongs to the family Gramineae. Due to its high chlorophyll content (70% of its chemical constituents), wheatgrass juice is commonly referred to as "green blood" (9) and reported to be a rich source of mineral nutrients (iron, phosphorus, magnesium, manganese, copper, zinc, and selenium), vitamins (A, C, E, and B-complex), antioxidants (beta-carotene), amino acids, and enzymes (10). Medicinal values of *Triticum aestivum* Linn. grass have been recognized in the light of scientific studies conducted to establish it as a valuable and safe therapeutic alternative in the management of chronic diseases. Literature review reveals that *Triticum aestivum* L. grass possesses anti-cancer, anti-ulcer, antioxidant, anti-arthritic, and blood building activity in thalassemia major (10). Therefore, the aim of the present study is systematical investigation of the protective effect of fresh wheatgrass (*Triticum aestivum* L.) juice against chemotherapy induced genotoxicity/clastogenicity in mouse bone marrow stem cells.

2. Material and methods

2.1. Chemicals and reagents

Cyclophosphamide (Cyphos® 200 mg, Intas Pharmaceuticals, Ahmedabad, India), doxorubicin (Adriamycin® 5 mg, Pfizer India, Mumbai, India), amifostine (Amfos® 1000 mg, VHB Life Sciences Limited, Mumbai, India), and colchicine (Yucca enterprise, Mumbai, India) were purchased from commercial sources. Bovine serum albumin, Giemsa ,and May-Grunwald stains were obtained from Hi-Media laboratories, Mumbai, India. All other chemicals/ reagents used were of analytical grade and procured from approved chemical suppliers.

2.2. Animals

Experiments were performed on 10 weeks old Swiss albino mice of either sex weighing 25-30 g. Animals were obtained from Torrent Research Centre, Gandhinagar, India and were housed in clean polypropylene cages under standard environmental conditions (12/12 h light/dark cycles at 22±3 °C and 50±5 % relative humidity). The animals were acclimatized to the laboratory conditions for a week prior to the experiments and fed with standard pellet diets (Keval Sales Corporation, Baroda, India) and water *ad libitum*. Animals were maintained in accordance with Committee for Purpose of Control and Supervision of Experiments on Animals guidelines (CPCSEA), Ministry

of Environment and Forest, India, for the care and use of laboratory animals. The experimental protocol (SJT/69-2012) was reviewed and approved by Institutional Animal Ethics Committee.

2.3. Cultivation of wheatgrass and preparation juice

Wheatgrass was grown in the laboratory according to a reported method (11). Briefly, overnight soaked unpolished wheat grains were spread on the soil surface in a plastic tray, covered with an additional thin layer of soil and watered regularly. The tray was covered with a paper in order to maintain darkness, which helps the sprouting. Once sprouting occurred, the tray was left uncovered and watered every day for 8 days. On the following day, wheatgrass was harvested by cutting the shoots about half inch above the soil surface with a clean pair of scissors. Multiple trays were similarly planted at one day interval to ensure continuous supply of wheatgrass. Twenty grams of fresh wheatgrass was crushed in a mortar and subsequently squeezed through a wet muslin cloth and the filtrate was collected in a clean, sterile container. The residues were resuspended in 3 ml of distilled water twice and squeezed again. The filtrate volume was made up to 20 ml with distilled water. Fresh wheatgrass juice (WGJ) was prepared on each day for administration to mice.

2.4. Experimental design

The animals were divided into following seven groups (n=6).

Groups	Treatment
Group- I	Normal Control (NC) received distilled water, p.o. for 30 days followed by normal saline i.p. on 30th day.
Group-II	Disease Control (DC)-I; received distilled water, p.o. for 30 days followed by cyclophosphamide (CP, 50 mg/kg b.w., i.p.) on 30th day.
Group III	Received WGJ (20 ml/kg b.w., p.o.) for 30 days + CP (50 mg/kg b.w., i.p.) on 30th day.
Group IV	Received distilled water, p.o. for 30 days followed by single dose of amifostine (AMI, 350 mg/kg b.w., i.p.) and CP (50 mg/kg b.w., i.p.) on 30th day.
Group V	Disease control (DC)-II; received distill water, p.o. for 30 days followed by doxorubicin (DXR, 50 mg/kg b.w., i.p.) on 30th day.
Group VI	Received WGJ (20 ml/kg b.w., p.o.) for 30 days + DXR (50 mg/kg b.w., i.p.) on 30th day.
Group VII	Received distilled water, p.o. for 30 days followed by single dose of AMI (350 mg/kg b.w., i.p.) and DXR (50 mg/kg b.w., i.p.) on 30th day.

The WGJ 20 ml/kg b.w. (12) and vehicle were administered by oral gavages for 30 consecutive days. On 30th day, myelotoxicity in mice was induced by single i.p. injection of CP 50 mg/kg b.w. (13) and DXR 50 mg/kg b.w. (14) in respective groups as per the protocol. The standard drug AMI 350 mg/kg b.w. (15) was given 45 min prior to the injection of CP and DXR by i.p. route.

2.5. Estimation of hematologic parameters

After 24 h of chemotherapeutic challenge, blood samples were collected by retro-orbital puncture in ethylenediaminetetraacetic acid (EDTA) containing tubes for the estimation of hematological parameters like RBC (red blood cells), Hb (hemoglobin) Hct (hematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), total WBC (white blood cells), and differential WBC were measured following standard hematological methods (16).

2.6. Chromosome aberration assay

Chromosomal aberration assay was performed according to a previously published method (17). Animals were injected with colchicine (4 mg/kg b.w., i.p.) 1.5 h prior to sacrifice, to arrest the bone marrow cells in the metaphase stage. In the present study, the bone marrow extracted from right femur was used for micronucleus test, while marrow from left femur was subjected to chromosomal aberration assay. Briefly, the animals were euthanized by cervical dislocation under sodium pentobarbital anesthesia and were dissected from the lower abdomen to the upper sternum. Each end of the femur was pruned until bone marrow was exposed as small red dot. The left femur was flushed with 2 ml of 0.56 % KCl (pre-warmed to 37 oC) in a centrifuge tube and subjected to centrifugation at 1000 rpm for 10 min (cooling cen-

trifuge, Remi instruments division, Vasai, India). The supernatant was decanted, and cells pellet was resuspended in 2 ml of 0.65 % KCl (37 °C). Tubes were then incubated at 37 °C for 18 min and again centrifuged at 1000 rpm for 10 min. The supernatant was gently aspirated, without disturbing the cell pellet. The cell pellet was fixed with cold aceto-methanol fixative (absolute methanol: glacial acetic acid, 3:1 (v/v), freshly prepared) and centrifuged at 1000 rpm for 10 min. Fixation and centrifugation was repeated twice with an interval of 30 min. Pellet thus obtained were resuspended in small volume of fixative and stored at 4 °C. Grease free slides were chilled overnight and allowed to collect condensation for 5 sec. Three to four drops of cell suspension from micropipette were dropped evenly on each slide. The fixative was heated on flame to burst open the hypotonically swollen cells in order to fix metaphase spread to the slide. Slides were then allowed to dry at room temperature overnight. On the following day, slides were stained with freshly prepared 5 % Giesma (v/v, stock Giemsa stain/distilled water) for 10 min, washed in distilled water to remove excess stain and air dried. Two to three slides were prepared from each animal. A total of 100 well spread metaphase were scored at 1000x magnification using oil immersion lens for different types of chromosomal aberrations such as chomatid break, chromosomal ring, chromatid gap, chromatid exchange, chromosome break, and chromatid breaks and expressed as % chromosomal aberrations.

2.7. Micronucleus test

Bone marrow harvesting for micronucleus assay was done according to the reported method (18). The contents of right femur were flushed out (penetrated with a 23 G needle) with 2 ml of 5 % Bovine serum albumin (BSA) into pre-marked test tubes and centrifuged at 1000 rpm for 10 min at 4 °C. The supernatant was discarded and pellets were resuspended in one drop of BSA. The resultant cell suspension was used for micronucleus assay. The bone marrow cells were then smeared on coded glass slides to avoid observation bias (two slides per mouse). After 24 h air-drying, bone marrow smear was first stained in 5 % May-Grunwald solution for 15 min and then immersed in 20 % Giemsa solution for 30 min. The slides were then placed in Sorensen's buffer (pH 6.7 and pH 6.8) for 10 sec, rinsed in distilled water, and then dried overnight. Slides were then analyzed under a light microscope (x1000) for the frequency of cells with micronuclei. The frequencies of micronucleated polychromatic erythrocytes (MnPCEs) and micronucleated normochromatic erythrocytes (MnNCEs) in 1000 polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE), respectively, per mouse, were counted. To study cytotoxicity of CP and DXR on bone marrow cells, the ratio of PCE/NCE was calculated by counting 1000 erythrocytes (19, 20).

2.8. Statistics

DData were expressed as mean ± SEM (n=6). Statistical analysis was done by one-way ANOVA followed by Tukey- Kramer multiple comparison test using GraphPad Prism version 6.01 for Windows, GraphPad Software, San Diego, CA, USA. $P<0.05$ was considered statistically significant.

3. Results and discussion

3.1. Effect of the wheat grass juice on hematologic parameters

Table 1 summarizes the effect of WGJ on the hematologic parameters in CP and DXR injected mice.

3.1.1. Effect on RBC count and Hb content

A significant decrease in RBC count and Hb content was observed in the mice injected with single i.p dose of CP and DXR ($P<0.001$ for both) when compared with that of NC group animals. The treatment with WGJ at a dose of 20 ml/kg b.w. for 30 days showed significant elevation in RBC count and Hb content ($P<0.01$ for WGJ + CP group and $P<0.001$ for WGJ + DXR group for both parameters) when compared with the respective DC groups (CP and DXR injected). The group administered with reference drug AMI 350 mg/kg on the 30th day of protocol 45 min prior to CP challenge increased RBCs and Hb content, but statistically not significant. On the other hand, AMI showed significant rise in RBCs ($P<0.01$) and Hb content ($P<0.05$) when compared with DXR injected DC

Table 1. Effect of WGJ pretreatment on CP and DXR induced hematopoietic toxicity in mice.

Groups	Treatment	Hb (g/dl)	RBC ($\times10^6$/μl)	Hct (%)	MCV (fl)	MCH (pg/cell)	MCHC (g/dl)	WBC ($\times10^3$/μl)	Neut ($\times10^3$/μl)	Lym ($\times10^3$/μl)
I	Distilled water	13.4± 0.47	10.10± 0.47	43.13± 1.28	51.69± 1.29	13.65± 0.40	30.65± 0.57	10.64± 0.20	5.28± 0.21	7.03± 0.18
II	Distilled water + CP	9.42± 0.39^b	7.82± 0.22^b	33.62± 1.62^b	32.16± 1.46^b	8.27± 0.23^b	19.68± 1.02^b	8.17± 0.39^b	3.10± 0.58^a	4.58± 0.24^b
III	WGJ (20 ml/ kg b.w.) + CP	12.07± 0.59^{**}	9.76± 0.22^{**}	40.70± 1.12^{**}	40.78± 1.13^{**}	11.74± $0.38^{\#}$	27.49± $1.47^{\#}$	9.97± 0.42^{**}	4.19± 0.43^{ns}	6.12± $0.28^{\#}$
IV	AMI (350 mg/kg b.w.) + CP	11.03± 0.45^{ns}	9.05± 0.18^{ns}	38.96± 1.06^*	38.49± 1.06^*	9.91± 0.30^*	23.40± 0.95^{ns}	9.75± 0.35^*	3.50± 0.65^{ns}	5.54± 0.18^*
V	Distilled water + DXR	10.01± 0.46^b	7.13± 0.36^b	31.74± 0.93^b	31.06± 1.49^b	9.02± 0.17^b	27.78± 1.07^{ns}	7.62± 0.33^b	3.30± 0.36^a	4.44± 0.20^b
VI	WGJ (20 ml/ kg b.w.) + DXR	13.14± $0.45^{\$}$	10.31± $0.43^{\$}$	38.67± $0.92^{‡}$	40.65± $1.46^{\$}$	11.65± $0.41^{\$}$	29.41± 1.59^{ns}	9.65± $0.29^{‡}$	4.25± 0.26^{ns}	5.81± $0.17^{\$}$
VII	AMI (350 mg/kg b.w.) + DXR	12.07± $0.35^{†}$	9.09± $0.34^{‡}$	36.86± $0.99^{†}$	37.32± $1.48^{†}$	10.54± $0.26^{†}$	25.43± 0.52^{ns}	9.24± $0.28^{†}$	3.40± 0.32^{ns}	5.45± $0.16^{†}$

Values are expressed as mean ± standard error of mean (n=6). CP, cyclophosphamide; DXR, doxorubicin; WGJ, wheatgrass juice; AMI, amifostine; Hb, hemoglobin; RBC, red blood cells; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin hemoglobin; WBC, white blood cells; Neut, neutrophils; Lym, lymphocytes. Statistical significance was calculated by one-way ANOVA followed by Tukey-Kramer post hoc test. $^aP<0.05$, $^bP<0.001$ when compared with normal control (Group I); $^*P<0.05$, $^{**}P<0.01$, $^{\#}P<0.001$ when compared with CP injected disease control (Group II); $^{†}P<0.05$, $^{‡}P<0.01$, $^{\$}P<0.001$ when compared with DXR injected disease control (Group V). ^{ns}Not significant when compared with respective disease control groups.

group animals.

3.1.2. Effect on Hct values

Oral administration of WGJ for the 30 days significantly raised Hct values in CP and DXR injected DC groups when compared to that of NC animals ($P<0.01$ for both WGJ + CP and WGJ + DXR groups). Reference drug AMI too proved to be efficient in bringing Hct values to the normal level in CP and DXR injected mice ($P<0.05$ for both groups) when compared to that of respective disease control groups.

3.1.3. Effect on other hematologic parameters

MCV, MCH, and MCHC values were also decreased after the injection of CP ($P<0.001$ for all parameters) and DXR ($P < 0.001$ for MCV, MCH) when compared with NC animals. Treatment with WGJ prior to CP and DXR challenge on the 30th day showed a significant improvement in these parameters (in WGJ + CP, $P<0.01$ for MCV, $P<0.001$ for MCH and MCHC, in WGJ + DXR, $P<0.001$ for MCV and MCH) when compared with respective CP and DXR injected DC groups.

3.1.4. Effect on total WBCs, neutrophil, and lymphocyte counts

In the present investigation, single i.p. injection of CP and DXR in DC group animals significantly lowered total leucocytes ($P<0.001$ for CP and DXR injected DC groups), neutrophil ($P<0.05$ for CP and DXR disease groups), and lymphocyte ($P<0.001$ for CP and DXR disease groups) count when compared to NC group. Intervention with WGJ at the dose of 20 ml/kg b.w. for 30 days significantly prevented a decline in the immune cells count in CP ($P<0.01$ for total WBCs, $P<0.001$ for lymphocytes) and DXR injected mice ($P<0.01$ for total WBCs, $P<0.001$ for lymphocytes in both groups). In case of neutrophil count, a statistically non-significant increase was observed in these treatment groups. Besides, the standard drug AMI was also effective in replenishing leucocytes and lymphocytes in blood ($P<0.05$ for both AMI + CP and AMI + DXR groups) when compared with respective CP and DXR injected DC groups. It was noted that the efficacy of WGJ was higher than the reference drug AMI in normalizing most hemato-

Table 2. Effect of WGJ pretreatment on CP and DXR induced chromosomal aberrations in mouse bone marrow cells.

Groups	Treatment	Chromosomal Aberrations (%)					Total Ab. (%)	% Protection
		Ctb	Csr	Ctg	Cte	Csb		
I	Distilled water	0.28 ± 0.18	0.16 ± 0.13	0.31 ± 0.26	1.57 ± 0.20	0.12 ± 0.09	2.44 ± 0.86	–
II	Distilled water + CP	7.42 ± 0.75^{b}	1.57 ± 0.29^{b}	2.42 ± 0.36^{b}	4.14 ± 0.34^{b}	2.71 ± 0.35^{b}	18.26 ± 2.09^{b}	–
III	WGJ (20 ml/kg b.w.) + CP	$2.42 \pm 0.36^{\#}$	$0.28 \pm 0.18^{**}$	$0.71 \pm 0.28^{**}$	$2.00 \pm 0.21^{**}$	$1.14 \pm 0.14^{*}$	$6.55 \pm 1.17^{\#}$	64.13
IV	AMI (350 mg/kg b.w.) + CP	$4.28 \pm 0.62^{**}$	$0.42 \pm 0.20^{**}$	$1.00 \pm 0.30^{*}$	$2.42 \pm 0.29^{*}$	1.59 ± 0.36^{ns}	$9.71 \pm 1.77^{*}$	53.18
V	Distilled water + DXR	6.57 ± 0.84^{b}	1.12 ± 0.29^{a}	2.28 ± 0.35^{b}	4.00 ± 0.53^{b}	2.54 ± 0.42^{b}	16.51 ± 2.43^{b}	–
VI	WGJ (20 ml/kg b.w.) + DXR	2.14 ± 0.37	$0.14 \pm 0.14^{\dagger}$	$0.71 \pm 0.18^{\dagger}$	$1.85 \pm 0.34^{\ddagger}$	$0.76 \pm 0.28^{\ddagger}$	$5.60 \pm 1.31^{\$}$	66.08
VII	AMI (350 mg/kg b.w.) + DXR	$3.07 \pm 0.28^{\ddagger}$	0.56 ± 0.20^{ns}	$0.85 \pm 0.34^{\dagger}$	$2.48 \pm 0.31^{\dagger}$	1.43 ± 0.40^{ns}	$8.39 \pm 1.53^{\dagger}$	49.81

Values are expressed as mean ± standard error of mean (n=6). CP, cyclophosphamide; DXR, doxorubicin; WGJ, wheatgrass juice; AMI, amifostine; Ctb, chromatid break; Csr, chromosomal ring formation; Ctg, chromatid gap; Cte, sister chromatid exchange; Csb, chromosome break. Statistical significance was calculated by one-way ANOVA followed by Tukey-Kramer post hoc test. $^{a}P< 0.05$, $^{b}P< 0.001$ when compared with normal control (Group I); $^{*}P< 0.05$, $^{**}P< 0.01$, $^{\#}P< 0.001$ when compared with CP injected disease control (Group II); $^{\dagger}P< 0.05$, $^{\ddagger}P< 0.01$, $^{\$}P < 0.001$ when compared with DXR injected disease control (Group V). ^{ns}Not significant when compared with respective disease control groups.

logic parameters.

3.2. Effect of WGJ on chromosomal aberrations

Table 2 summarizes the effect of WGJ on CP and DXR induced chromosomal aberrations in mice.

3.2.1. Effect on chromatid break

IIn the present study, i.p. injection of chemotherapeutic agents- CP and DXR in DC groups significantly increased chromatid break in bone marrow cells (P<0.001 for both DC groups) when compared with NC animals. The animal groups receiving WGJ at the therapeutic dose of 20 ml/kg b.w. showed a significant reduction in the incidences of chromatid break (P<0.001 for WGJ + CP and WGJ + DXR) when compared with respective CP and DXR injected DC groups. The group receiving AMI (350 mg/kg b.w.) before i.p. injection of CP and DXR also showed protection against chromatid break (P<0.01 for CP and P<0.01 for DXR).

3.2.2. Effect on chromosomal ring formation

A significant increase in chromosomal ring formation was observed in CP and DXR challenged animals in respective DC groups when compared with NC group animals (P<0.001 for CP disease control, P<0.05 for DXR disease control) while animal groups treated with WGJ and AMI significantly decreased chromosomal ring formation (P<0.01 for WGJ + CP and AMI + CP, P<0.05 for WGJ + DXR) when compared with that of respective DC groups.

3.2.3. Effect on chromatid gap

Single i.p. injection of chemotherapeutic agents caused chromatid gap when compared with NC group animals (P<0.001 for both DC groups). Treatment with WGJ and standard drug AMI showed significant reduction in the gap formation in chromatids (P<0.01 for WGJ + CP, P<0.05 for WGJ + DXR, AMI + CP and AMI + DXR).

3.2.4. Effect on sister chromatid exchange

A high frequency of chromatid exchange was observed in CP and DXR injected DC mice (P<0.001 for DC groups) when compared with that of NC group animals. Pretreatment with WGJ at dose of 20 ml/kg b.w. for 30 days exhibited significant reduction in chromatid exchanges (P<0.01

for WGJ + CP and WGJ + DXR) when compared with respective DC groups. The reference drug AMI showed reasonable protection against chromatid exchanges ($P<0.05$ for AMI + CP and AMI + DXR groups).

3.2.5. Effect on chromosome break

A significant number of chromosome breaks were produced in CP and DXR injected DC groups when compared with that of NC group ($P<0.001$ for both DC groups). WGJ pretreated animals showed a decrease in the chromosome break with $P<0.05$ for WGJ + CP, $P<0.01$ WGJ + DXR groups when compared with respective DC groups. The efficacy of WGJ was higher than the reference drug in protecting bone marrow cells against chromosomal aberrations.

3.3. Effect of WGJ on the MN formation and PCE/NCE ratio in bone marrow

Clastogenic effect produced by chemotherapeutic agents not only produced structural damage to the chromosomes, but also caused micronucleus formation in the bone marrow cells. Figure 1 and 2 represent the effect of WGJ on the occurrence of MnPCEs and MnNCEs 24 h post single i.p. injection of CP and DXR in mice. The animals exposed to CP and DRX, respectively, showed significant increase in the occurrence of MnPCEs and MnNCEs ($P<0.001$ for both DC groups) when compared with NC animals. Furthermore, CP and DXR induced cytotoxicity was also detected based on terms of decreased PCE/NCE ratio in the bone marrow of these animals ($P<0.001$ for CP and $P<0.01$ for DXR injected mice) as depicted in Figure 3. Animals pretreated with WGJ and AMI significantly reduced frequency of MnPCEs and MnNCEs occurrences ($P<0.001$ for all treatment groups) as well as increase in PCE/NCE ratio in CP and DXR injected animals when compared with respective DC groups.

4. Discussion

The therapeutic effectiveness of radiochemotherapy is often limited due to multiple organ toxicity. One such vulnerable target is hemopoeitic progenitor bone marrow stem cells (21). Myelosuppression is the most anticipated adverse effect of antineoplastic chemotherapeutic agents such as CP and DXR may be manifested as various blood dyscrasias including neutropenia, agranulocytosis, pancytopenia, aplastic anemia, and thrombocytopenia (3, 5, 22, 23). Myelotoxicity could potentially be life threatening because of infection and bleeding complications of neutropenia and thrombocytopenia, respectively (21). However, there is a considerable variability in the severity of bone marrow suppression that they may induce.

CP, an alkylating agent is commonly indicated for the management of chronic and acute leukaemias, lymphomas, multiple myeloma, in preparation for bone marrow transplantation (24). Being inactive prodrug, CP releases active phosphoramide mustard upon enzymatic and chemical activation within the cells, which subsequently causes interstrand and intrastrand DNA crosslinks responsible for the cytotoxic properties of CP (25). On the other hand, DXR is an anthracycline antibiotic commonly used for treating breast and oesophageal carcinomas, Kaposi's sarcoma, osteo-

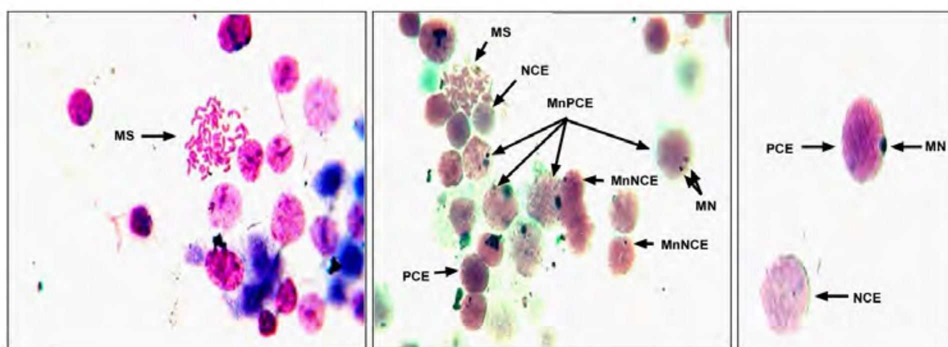

Figure 1. Representative cytology of bone marrow cells (x1000). MS, metaphase spread; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; MN, micronucleous; MnPCE, micronucleated polychromatic erythrocytes; MnNCE, micronucleated normochromic erythrocytes

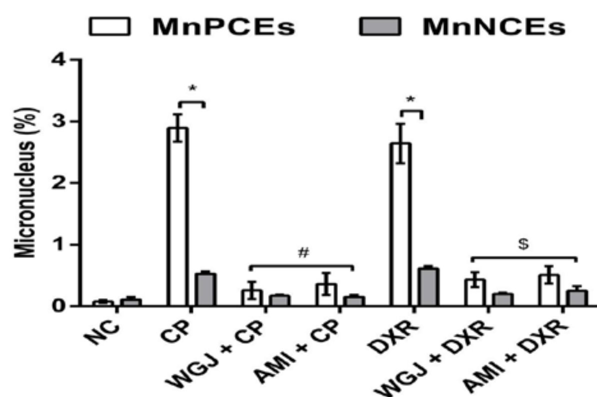

Figure 2. Frequency of micronucleated PCEs and NCEs in WGJ pretreated mice. Values are expressed as mean ± standard error of mean (n=6). NC, normal control; CP, cyclophosphamide (50 mg/kg b.w.); DXR, doxorubicin (50 mg/kg b.w.); WGJ, wheatgrass juice (20 ml/kg b.w.); AMI, amifostine (350 mg/kg b.w.); MnPCEs, micronucleated polychromatic erythrocytes; MnNCEs, micronucleated normochromatic erythrocytes. Statistical significance was calculated by one-way ANOVA followed by Tukey-Kramer post hoc test. **P*<0.001 when compared with normal control; #*P*<0.001 when compared with CP injected disease control (Group II); $*P*<0.001 when compared with DXR injected disease control (Group V).

sarcoma, soft-tissue sarcomas, and Hodgkin's and non- Hodgkin's lymphomas (26). DXR is reported to act through multiple mechanisms including intercalation into DNA, DNA cross-linking, topoisomerase II inhibition, and reactive oxygen species induced DNA damage (25).

As these agents cannot differentiate rapidly dividing normal cells from the cancer cells, they end up damaging bone marrow stromal cells, resulting in deficient hematopoiesis (27). Therefore, development of safe and effective cytoprotective agents is being pursued by many researchers worldwide. The US Food and Drug Association (FDA) approved some of these agents including amifostine, dexrazoxane, and mesna (28).

AMI, an organic thiophosphate was developed to provide selective protection to the normal tissues against chemotherapy and radiation induced toxicities. AMI is dephosphorylated to free thiol group containing active metabolite by capillary membrane bound alkaline phosphatase in normal tissues. The ability of thiol metabolite to selectively accumulate in higher concentration in normal tissues tends to detoxify the reactive metabolites of cisplatin, cyclophosphamide, bleomycin, doxorubicin, and related chemotherapeutic

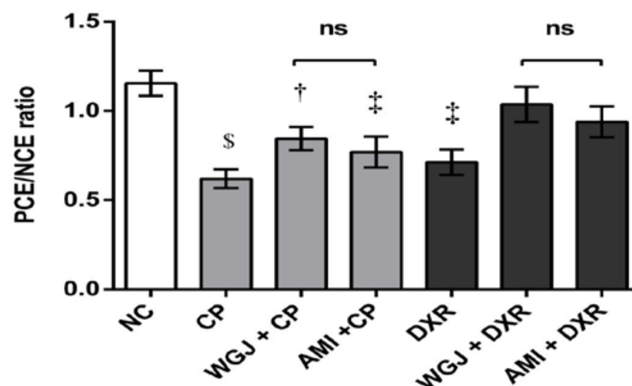

Figure 3. Effect of WGJ on cytotoxicity in mouse bone marrow cells (PCE/NCE ratio). Calculation of PCE/NCE ratio was done based on examination of 1000 erythrocytes in bone marrow smear. Values are expressed as mean ± standard error of mean (n=6). NC, normal control; CP, cyclophosphamide (50 mg/kg b.w.); DXR, doxorubicin (50 mg/kg b.w.); WGJ, wheatgrass juice (20 ml/kg b.w.); AMI, amifostine (350 mg/kg b.w.); Statistical significance was calculated by one-way ANOVA followed by Tukey-Kramer post hoc test. †*P*<0.05, ‡*P*<0.01, $*P*<0.001 when compared with normal control (Group I); ns=not significant when compared with respective disease control.

agents (29). However, reports suggest that despite its high effectiveness in reducing radiochemotherapy induced cytotoxicity, AMI was discontinued due to severe hypotension, vomiting, and allergic reactions (generalized exanthema, fever, and shivering) in patients irradiated for head and neck cancer and receiving concurrent chemotherapy with cisplatin/5-fluorouracil (30). Moreover, another study has suspected of AMI induced Stevens-Johnson syndrome and toxic epidermal necrolysis during head and neck radiotherapy (31). These findings suggest that there is a greater need for safer and effective cytoprotective agents that not only reduce severity of chemotherapy induced bone marrow clastogenicity, but also enhance the overall efficacy of these therapies.

In the present investigation, single i.p injection of CP and DXR in animals significantly decreased hematologic parameters viz., RBC, Hb content, Hct, MCV, MCH, MCHC, WBC, neutrophil, and lymphocyte counts in disease control animals, which is in accordance with the previously reported studies (32,33) . Treatment with WGJ significantly improved hematological status in both animal groups injected with CP and DXR. One of the most remarkable qualities of *T. aestivum* is its high chlorophyll content (70 %), and thus it is recognised as 'green blood'. The compounds chlorophyll and hemoglobin have a striking similarity in having a tetrapyrrole ring structure, with only one difference between two, which is the nature of the central metal atom - magnesium (Mg) in chlorophyll and iron (Fe) in hemoglobin (34). Some researchers have reported that wheatgrass increases HbF level and reduces the frequency of blood transfusion in beta-thalassemia patients. Administration of wheatgrass extract causes 3-5 folds increase in HbF production and improves the quality of life in these patients (9,35). Furthermore, WGJ is enriched with essential vitamins including vitamin B12 and minerals such as iron and amino acids, which may further speed up hemoglobin synthesis (10). Hence, the unique nutritinal profile of WGJ along with myeloprotective property may be attributed to its restorative effect on RBCs, WBCs, Hb content, Hct, MCV, MCH, MCHC, and neutrophil count in CP and DXR challenged animals.

In genetic toxicology, the study of DNA damage at the chromosome level is an essential part, since chromosomal mutation is an important event in carcinogenesis (36). Micronucleus assay has emerged as a preferred method to assess the induction of chromosomal aberrations for hazard identification and risk assessment (37). Micronuclei are formed in dividing cells either spontaneously or as a result of chemical or radiation treatment due to aneugenic damage (whole chromosome unable to migrate with the rest of the chromosomes to the spindle poles during cell division) or clastogenic damage (chromosome breakage resulting from DNA double-strand breakage or from inhibition of DNA synthesis) (38). Individuals exposed to chemotherapy demonstrate higher number of MnPCEs in bone marrow. Furthermore, PCE/NCE ratio is also analysed to assess the cytotoxic effects of chemotherapeutic agents. A study suggests that cytotoxic agents alter the turnover of bone marrow cells and decrease the PCE/NCE ratio. In other words, the number of immature erythrocytes (PCEs) reduces as compared to the number of mature erythrocytes (NCEs), reflecting bone marrow toxicity and suppression of cell proliferation (39). Therefore, in vivo mi¬cronucleus test and chromosomal aberration assay are currently recommended by the International Conference on Harmonization to investigate the genotoxic potential of cytotoxic agents for human use (40).

IIn the present study, single i.p. injection of CP and DXR in animals significantly increaseed the frequency of MNPCEs, MNNCEs, and reduced PCE/NCE ratio in bone marrow. Moreover, both cytotoxic agents induced different types of chromosomal abnormalities such as chromatid break, centric fusion, ring formation, chromotid exchange, chromasomal gap, etc. in these animals. Similar results have been observed in previously reported studies (13,14,17,41,42). Treatment with WGJ afforded better protection against genotoxic/clastogenic effects of CP and DXR and significantly reduced chromosomal aberrasions, prevented occurrence of MnPCEs and MnNCEs, and improved PCE/NCE ratio in mouse bone marrow cells. These observations are suggestive of the protective role of WGJ in bone marrow protection against chemotherapeutic agents . Moreover, WGJ administered groups showed higher efficacy than

AMI treated animals. The differences in the efficacy between WGJ and AMI treated groups could be due to different treatment durations (30 days consequetive adminstration of WGJ vs single i.p injection of AMI).

As mentioned earlier, WGJ contains many antioxidant compounds such as vitamins C and E, beta-carotene, bioflavonoids, tannins, phenolic compunds, thiol-containing amino acids, and superoxide dismutase enzyme (SOD) (9, 10). Protection exerted by flavonoids against DNA damage could principally be due to their free radical scavenging ability in normal cells without affecting efficacy of chemotherapeutic agents on tumours (43). Ample of evidences indicate that diosmin, hesperidin, quercetin prevents hematotoxicity by protecting bone marrow cells from deleterious effects of chemotherapeutic agents (44). Furthermore, other phytochemicals such as tea-derived polyphenols and saponins from Chinese ginseng have shown adaptogenic effect, thereby reducing hematopoietic complications induced by systemic chemotherapy (45, 46).

It is therefore presumed that anticlastogenic and cytoprotective activities of WGJ could at least partially be due to the presence of various polyphenolic antioxidants including flavonoids. The vast array of phytoprinciples of WGJ may have contributed to the myeloprotective effect by scavenging reactive cytotoxic metabolites of CP/DXR and preventing DNA breaks in bone marrow pluripotent stem cells. In addition, a reported study reveals that free thiol containing compunds provide an alternative target to DNA and RNA for reactive moities of alkylating or platimum containing chemotherapeutic agents (29). The WGJ reportedly contains thiol containing amino acids, which might have a role in neutralizing reactive metabolites. The overall efficacy of WGJ extract could be a result of complex integrations (such as synergism, potentiation) among its phytochemicals.

5. Conclusion

The findings of the present study reveal that WGJ affords protection against clastogenic and genotoxic effects of CP and DXR in bone marrow stem cells and may eventually prevent hematologic toxicity related life-threating complications. Therefore, pretreatment with WGJ might be considered as a promising adjuvant therapy without compromising therapeutic efficacy of chemotherapeutic agents.

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Conflict of Interest

None declared.

6. References

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