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Investigation of Antigenotoxic Potential of Wheatgrass (Triticumaestivum) Powder on Cyclophosphamide Induced Genotoxicity and Oxidative Stress in Mice

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Abstract

The present study was aimed to investigate the effect of Wheatgrass (Triticumaestivum Linn.) powder against genotoxicity and oxidative stress induced by Cyclophosphamide (CP) in mice. Two doses of Wheatgrass (WG) powder were selected for the study (WG-250 and WG-500mg/kg). Animals received 14days pretreatment (oral) of WG, followed by induction of genotoxicity by CP (40mg/kg) 24 hours before sacrifice. Mice bone marrow chromosomal aberration assay and micronucleus assay were employed for the study. Activities of hepatic antioxidant enzymes were also investigated. Results were statistically analyzed by one way ANOVA followed by Tukey's test ($P<0.05$). Results showed that CP produced a significant increase in average percentage of aberrant metaphases and Chromosomal Aberrations (CAs) excluding gap, and Micronuclei (MN) formation in polychromatic erythrocytes, produced cytotoxicity in mouse bone marrow cells. CP also markedly inhibited the activities of Superoxide Dismutase (SOD), and reduced glutathione (GSH) and increased malondialdehyde (MDA) content. Pretreatments with WG in both doses, significantly inhibited the frequencies of aberrant metaphases, CA's, MN formation, and cytotoxicity in mouse bone marrow cells induced by CP. WG also significantly antagonized the reduction of CP-induced SOD, GSH activities and inhibited increased MDA content in the liver. Our studies revealed that WG has protective effect against genotoxicity and oxidative stress induced by CP.

Keywords: Wheatgrass; Cyclophosphamide; Chromosomal aberration; Micronucleus; Antioxidant enzymes

Introduction

Natural products derived from diets are known to exhibit a variety of biological effects including antioxidant, anticarcinogenic, antimutagenic and anti aging activities [1]. In recent years in few European countries, USA and India, wheatgrass is being consumed in the form of ready-made juice, powder or tablets as a health food supplement [2]. Wheatgrass (Triticumaestivum Linn. -Gramineae) is a commonly used traditional herbal medicine and well known for its therapeutic and nutritional values. Wheatgrass (WG) is rich with chlorophyll content that accounts for 70% of its total chemical constituents [3]. WG is a good source of mineral nutrients like calcium, magnesium, iron, vitamin A, C and tocopherols with high vitamin E potency, bio-flavonoids, 17 amino acid (with 8 essential amino acids) and enzymes [4].

Scientists and clinicians have evaluated the efficacy of WG in the treatment of rheumatoid arthritis [5]. WG was found to reduce severity of rectal bleeding in patients with ulcerative colitis [6], and to reduce the frequency of blood transfusions in patients with thalassemia major [7]. A number of scientific researches on wheatgrass establishes it's anticancer potential based on its chlorophyll content, suggesting that WG reduces the risk of breast cancer and provide beneficial effect in liver, colon, stomach and gastrointestinal cancer cases [8-10]. WG stimulates metabolism, improves digestion and its abundance of alkaline minerals helps reduce over acidity in the blood and restores alkalinity [11]. Accordingly, WG is not only removes toxins from the liver and blood [12], but also its detoxifying action removes deposits of heavy metals, drugs, several carcinogens from the body and helps restore healthy cells [11,13]. A study, concluded that WG possesses the ability to control blood glucose in diabetes and has potential to prevent diabetic-associated complications [14]. WG also decreases blood cholesterol by blocking lipid absorption [12]. Peryt et al. (1992), reported that WG extract provides protection against benzo(a)pyrene induced mutation in rats. In addition to this WG sprouts extract was found to be anti-mutagenic in the Ames test [15], capable of inhibiting oxidative DNA damage and responsible for metabolic deactivation of carcinogens [16,17].

The antioxidant activity of WG, at various levels of protection, has been studied in detail. WG contains several antioxidant vitamins such as A, B, C and E, antioxidant content chlorophyll, and antioxidant enzymes such as SOD, cytochrome oxidase and other enzymes [12,18,19]. Since WG presents a wide array of biological activities, especially antioxidant property, in present study, we investigated the antigenotoxic potential of WG powder in mouse models of experimentally Cyclophosphamide (CP)-induced genotoxicity. Moreover, we also evaluated the effect of WG powder on antioxidant defense systems.

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Methods

Drugs and chemicals

Wheatgrass powder was purchased from Patanjali Ayurved Ltd., India. Cyclophosphamide, Colchicine, Bovine albumin Fraction V, Giemsa stain, and May-Grunwald's stain (all purchased from Sigma Aldrich, Germany) were used for the study. All other chemicals (analytical grade) were purchased from the commercial sources.

Experimental design

Swiss albino mice (8–12 weeks old) of either sex were procured from the institutional animal house of the College of Pharmacy, Jazan University, Jazan KSA. Mice were acclimatized for 7 days under standard husbandry conditions (i.e., room temperature maintained at 25±5°C, relative humidity of around 45–55%, and a 12-hour light-dark photoperiod), with water and libitum. For animal experimentation, approval from the local institutional animal ethical committee was taken before the experiments.

Determination of acute drug toxicity of WG

Acute toxicity of WG was determined according to Prieur et al. (1973) and Ghosh (1984) [20,21]. Animals were allowed to fast by withdrawing food for 18 hours. Fasted animals were divided into several groups of 10 each. Each group of animals was given various doses of 100, 200, 500, 750, 1000, and 1500 mg/kg body weight (b.w.) of freshly prepared WG powder in distilled water, orally. Mortality of animals was observed up to 14 days post drug treatment.

Group distribution

Healthy mice [approximately 25g body weight each] were selected and divided randomly into six groups (n = 5) for each study. The group distribution for each study was as follows: Group 1, negative control (distilled water); Group 2, positive control (CP 40mg/kg); Group 3 animals treated with, WG (250mg/kg); Group 4 animals treated with, WG (500mg/kg); Group 5 animals treated with, WG (250mg/kg+CP) and Group 6 animals treated with WG (500mg/kg+CP). Prior to each dosing, 500mg of WG powder was suspended in 10ml distilled water and kept for 6h and shaken well before oral administration, for 14 days (the dose was prepared as per instructions for use of WG powder given for human use). Genotoxicity was induced by administering CP (40mg/kg, intraperitoneally; i.p.) 24 hours before tissue sampling.

Bone marrow CA assay

Colchicine (0.4ml of 0.05%) was administered intraperitoneally to the animals 90 minutes before sacrifice in order to arrest the mitotic process in metaphase. After sacrifice, both femurs were immediately dissected out and bone marrow was extracted in 0.075M of KCl and the cell suspension was incubated for 20min at 37°C. Cells were collected by centrifugation at 1000rpm for 10min and were fixed three times with a solution of methanol/acetic acid (3:1). Chromosome slides were prepared by cell suspension being dropped onto clean chilled slides, which were flame dried, coded and stained in dilute Giemsa solution. The microscopic observations were performed with a magnification of 100X oil immersion. Hundred well spread metaphase were scored per animal (around 500 metaphase per treatment group) at random. The types of aberration were scored and recorded with strict accordance of the method of Tice et al (1987) [22]. All aberrations (chromatid gaps, chromosomal gaps, deletion, chromatid and chromosomal breaks, ring and fragmentation) were considered equal regardless of the number of breakages involved. Percentage of aberrant metaphases and aberrations (excluding gaps) per cell in them were calculated. From the same slides, 1000 cells from each animal were taken into consideration for Mitotic Index (MI) study [23,24].

Bone marrow MN assay

Groups of animals were killed by cervical dislocation. Both femurs were removed and bone marrow was collected in tubes containing 0.2mL of 5% bovine serum albumin and centrifuged at 1000rpm for 5minutes. Smears were prepared and allowed to air dry before fixation and staining with May-Grunwald's/Giemsa solutions. Observations were made within 1week by means of light microscopy at 1000X magnification to assess the presence of micronuclei within polychromatic young erythrocytes (PCEs). Slides were coded and scored blind, and 1000PCEs per animal were examined for the presence of micronuclei. The ratio polychromatic/normochromatic erythrocytes (PCEs/NCEs) were calculated by counting a total of 1000 erythrocytes per animal. Values are expressed as the ratio of PCE/NCE of the total erythrocyte counts to determine a reduction of erythroblast proliferation [25].

Determination of hepatic SOD, GSH activities and MDA content

Firstly, the livers were excised and then perfused with ice cold saline (0.9% sodium chloride). A 10% liver homogenate was made with fresh tissue in 0.1M Tris-HCl buffer at pH of 7.4. The tissue homogenate was used for the estimation of protein content [26], MDA [27], SOD [28] and GSH [29]. The reaction products were determined by spectrophotometry.

Statistical analysis

The average data generated at different end points of the treated groups of mice was calculated and compared with the respective data of negative and positive control groups. For statistical analysis, the one-way ANOVA was first applied, followed by Tukey's test for multiple pair-wise comparisons using GraphPad Prism 6 software. The statistical significance was examined at the P-value of 0.05.

Results

Effect of WG on acute toxicity

Administration of 100–1500 mg/kg of WG to mice did not induce drug-related toxicity in animals, as evident by 100% survival of treated animals. There was no change in behavior, respiration pattern, and neuromuscular coordination. Therefore, it was concluded that WG as such did not induce any toxic manifestations up to a dose 1500mg/kg b.w. The higher dose of WG could not be tested because of problems in drug administration. Hence, 250 and 500mg/kg doses were used for the study.

Bone marrow CA assay

Mice of the negative control group showed 4.82±1.80% aberrant metaphases with 5.89±2.84 aberrations (excluding gaps) per hundred metaphases. The positive control group mice showed 64.69±12.01% aberrant metaphases with 77.93±10.38 aberrations (excluding gaps) per hundred metaphases. The percentages of Aberrant Metaphases (AMs) and CAs in mice of the positive control group were statistically

Table 1: Chromosomal aberration test in mice bone marrow cells pretreated with Wheatgrass powder (2 weeks continuous treatments).

Groups	Dose mg/kg	No. of metaphase analyzed	AM	Avg. percentage of AM	Chromatid		Chromosomal		del	ring	dc	Ex	frag	Total no. of aberrations (excluding gap)	% Avg. aberrations
					gap	break	gap	break							
NC	--	501	24	4.82±1.80	3	7	3	5	4	8	3	-	2	29	5.89±2.84
PC (CP)	40	511	374	64.69±12.01[a]	18	26	29	32	93	76	102	41	27	394	77.93±10.38[a]
WG-250	250	503	19	3.76±2.01[c]	5	5	4	3	7	14	2	3	-	34	7.40±2.85[c]
WG- 500	500	498	18	3.56±1.37[c]	1	7	4	5	-	7	3	1	1	24	4.82±1.27[c]
WG-250+CP	250+40	519	161	31.35±6.79[a,c]	15	18	19	17	33	41	39	7	9	164	35.64±6.24[a,c]
WG-500+CP	500+40	505	102	20.29±3.40[b,c]	9	16	6	20	21	27	19	3	7	113	22.36±4.56[b,c]

Data are expressed as mean±SD (n = 5). Abbreviations: NC: Negative Control, PC: Positive Control, AM: Aberrant Metaphases, del: deletion; dc: dicentric; Ex: Exchange, frag: fragmentation, WG: Wheatgrass, CP: Cyclophosphamide (40mg/kg). *ap*< 0.001; *bp*< 0.01, significant when compared with the control. *cp*< 0.001, significant when compared to positive control group (CP).

Table 2: Mitotic index of bone marrow cells pretreated with Wheatgrass powder.

Groups	Dose mg/kg	No. of cell analyzed	No. of dividing cells	% Mitotic index
NC	--	5000	493	9.86±0.56
PC (CP)	40	5000	146	2.92±1.06[a]
WG-250	250	5000	517	10.34±0.36[b]
WG-500	500	5000	485	9.71±0.86[b]
WG-250+CP	250+40	5000	390	7.8±0.57[a,b]
WG-500+CP	500+40	5000	437	8.74±0.47[b]

Data are expressed as mean±SD (n = 5). WG: Wheatgrass, CP: Cyclophosphamide (40mg/kg). *ap*< 0.001, significant when compared with the control. *bp*< 0.001; significant when compared to positive control group (CP).

Table 3: Percentage of MNPCE in 1000 PCE and the ratio between PCE and NCE in treatment with Wheatgrass powder.

Groups	Dose mg/kg	Individual animal score/1000 PCE	MNPCE	PCE/NCE
NC	--	5,3,8,1,6	4.60±2.70	1.11±0.25
PC (CP)	40	41,32,34,25,29	32.20±5.97[a]	0.53±0.16[a]
WG-250	250	5,8,3,2,4,	4.40±2.30[d]	1.14±0.11[f]
WG-500	500	3,9,3,7,2	4.80±3.03[d]	1.13±0.12[f]
WG-250+CP	250+40	17,19,24,16,20	19.20±3.11[a,d]	0.88±0.15[c,f]
WG-500+CP	500+40	9,17,6,19,12	12.60±5.41[c,d]	0.95±0.21[e]

Data are expressed as mean±SD (n = 5). Abbreviations: MNPCE: Micronucleated Polychromatic Erythrocytes, PCE: Polychromatic Erythrocytes, NCE: Norm chromatic Erythrocytes, WG: Wheatgrass, CP: Cyclophosphamide. *ap*< 0.001; *bp*< 0.01; *cp*< 0.05, significant when compared with the control. *dp*< 0.001; *ep*< 0.01; *fp*< 0.05, significant when compared to positive control group(CP). Five animals per group (representing a total of 5000 PCE) were analyzed for the presence of MNPCE and for the ratio PCE/NCE.

significant (P < 0.001), compared to that of the respective negative control groups of mice (Table 1).

Pretreatment of WG (250 and 500 mg/kg) alone showed comparable results with negative control animals. Average percentage of AMs in WG (250 and 500 mg/kg)-alone animals were 3.76±1.58 and 3.56±1.37 respectively, while aberrations (excluding gaps) per cell were 7.40±2.85 and 4.82±1.27, respectively. WG-250 and 500-induced average percentages of aberrant metaphases in mice were 31.35±6.79 and 20.29±3.40, respectively, after induction of clastogenicity by CP. The average aberrations (excluding gaps) per hundred metaphases in the same mice were 35.64±6.24 and 22.36±4.56, for WG-250 and WG-500 respectively. The percentages of aberrant metaphases and aberrations per hundred metaphases induced by both tested doses of WG in mice were significantly (P < 0.001 to P < 0.01) lower than the positive control group of mice (Table 1).

In mitotic index study, mice of the negative control group showed 9.86±0.56 percentage of MI, whereas their counterparts in the positive control group have shown of 2.92±1.06%, with a significant difference (P < 0.001) from that of the negative control mice. In the group of mice that received WG-250 and WG-500 alone, the average percentages of dividing cells (MI) were 10.34±0.36, 9.71±0.86 respectively and were found to be non-significant compared to negative control animals. Average percentages of dividing cells in the WG-250 and WG-500 mg/kg-treated groups combined with CP were increased to 7.8±0.57 and 8.74±0.47 respectively from that of the positive control mice and were statistically significant (P < 0.001) (Table 2).

Bone marrow MN assay

Average Micro-Nucleated Polychromatic Erythrocytes (MNPCs) per thousand PCEs of the negative control group of mice were 4.60±2.70; whereas the positive control group showed 32.20±5.97, which was significantly (P < 0.001) higher than that of the negative control group. WG-250 and WG-500 mg/kgin combination with CP induced 19.20±3.11 and 12.60±5.41 average MNPCEs respectively. The decrease in MN in both WG-treated groups of mice were found

Table 4: Effects of Wheatgrass powder on hepatic GSH, SOD and MDA in mice.

Groups	Dose mg/kg	GSH n mol / mg protein	SOD (U/mg protein)	MDA n mols / mg protein
NC	--	0.067±0.014	6.82±0.76	2.88±0.56
PC (CP)	40	0.029±0.010[a]	2.90±0.94[a]	7.21±1.09[a]
WG-250	250	0.070±0.009[c]	6.52±1.16[c]	2.89±0.39[b,c]
WG-500	500	0.071±0.012[c]	6.72±1.05[c]	2.47±0.77[c]
WG-250+CP	250+40	0.057±0.071[e]	5.70±0.69[d]	5.59±0.76[e]
WG-500+CP	500+40	0.062±0.015[d]	6.04±1.06[c]	3.68±1.27[c]

Data are expressed as mean±SD (n = 5). Abbreviations: WG: Wheatgrass, CP: Cyclophosphamide. *ap*< 0.001; *bp*< 0.01, significant when compared with the control. *cp*< 0.001; *dp*< 0.01; *ep*< 0.05, significant when compared to positive control group (CP).

to be statistically significant from that of the respective control group (P<0.001); however, both doses of WG (250 and 500 mg/kg) when treated alone did not produce the significant increase in MNPCs (Table 2).

In the current study, erythropoietic cell toxicity was calculated by the PCE/NCE ratio. CP-induced clastogenicity (0.53±0.16) in the positive control animals; whereas, in the animals pretreated with the different doses of WG (250 and 500 mg/kg+CP), significant restoration (0.88±0.15 and0.95±0.21, P<0.05 and P<0.01) in PCE/NCE ratio were observed (Table 3).

Determination of SOD, GSH activities and MDA content

Activities of SOD and GSH were substantially reduced and MDA content was significantly increased in the CP-treated positive control group, compared to the negative control animals (Table 4) (P < 0.001). In the groups given WG in combination with CP, activities of SOD and GSH were significantly increased (P < 0.05 to P < 0.001) and MDA content was significantly reduced (P < 0.05 and P < 0.001) respectively, compared to that in the positive control group. Further, there were no significant changes in activities of SOD, GSH, and MDA content found in mice given WG at 250 and 500 mg/kg alone, compared to negative control.

Discussion

The present study investigated the effect of WG powder against genotoxicity and oxidative stress induced by cyclophosphamide using *in vivo* mice bone marrow Chromosomal Aberration assay (CA) and Micronucleus Assay (MN). Bone marrow chromosomal analysis is based on the ability of a test agent to induce numerical or structural alterations in chromosomes that can be visualized through microscope at 100X [30]. Although, the cellular process and mechanism(s) behind induction of CA is still not understood completely [31,32] but it is assumed that these aberrant chromosomal structures may result of direct DNA breakage and/-or replication on a damaged DNA template and/-or inhibition of DNA synthesis and other mechanisms, like topoisomerase 2 inhibition [33].

CP, the positive control chemical in the present study, is a covalent DNA-binding agent [34] and recommended as a positive control chemical to induce genetic toxicity [35]. In positive control animals, genotoxicity induced by CP resulted in great increase in the number of aberrant metaphase. This high frequency of AM reflected in increased no. of morphological abnormalities in chromosomes (average chromosomal aberrations) in the same animals. This induction of significantly high percentages of aberrant metaphases and CAs, in bone marrow, by CP (40mg/kg b.w. of mice), is in agreement with its earlier reported clastogenicity [34,36]. However, pretreatment of WG in both doses shown reduction in the frequencies of structural CAs and number of aberrant metaphases.

The determination of proliferation rates and/-or Mitotic Indices (MI) in bone marrow cells proved to be a very useful and sensitive indicator of the cytostatic and cytotoxic action of various environmental hazards or therapeutic agents [37,38]. The cytotoxic effect of CP is on account of its ability to inhibit cell division by damaging the DNA of proliferating cancerous cells. However, at the same time it also damages the DNA of the healthy tissues with high cellular turnover such as the bone marrow, the Gastro-Intestinal Tract (GIT) and the germ cells [39]. In present investigation, decrease in MI in the CP-treated positive control group showed that there was a decrease in cellular proliferation in the bone marrow of mice. Whereas, pretreatment with WG in different groups gave a significant improvement in MI. The improvement in mitotic activity of bone marrow cells of animals pre-treated with WG may focus attention on the beneficial effect of WG to overcome one of the most serious problems in cancer chemotherapy, which is the bone marrow suppression. This can be supported by a prospective matched control study done on patients with breast carcinoma on chemotherapy to evaluate the beneficial effect of Wheatgrass Juice (WGJ) reveled that WGJ taken during FAC (5–fluorouracil, doxorubicin and cyclophosphamide) chemotherapy may reduce myelo-toxicity, dose reduction and need for Granulocyte Colony Stimulating Factors (GCSF) support, without diminishing efficacy of chemotherapy [40].

MN is well characterized biomarkers of structural and numerical chromosomal damage; they arise from acentric chromosome fragments or lagging whole chromosome(s) that fail to incorporate in to the daughter nuclei after nuclear division [39]. CP at a dose of 40mg/kg i.p. significantly induced MN formation in the bone marrow cells. Further, the cytotoxicity of CP is evident from the reduction of PCE/NCE ratio in comparison to Negative Control (NC) group. The present *in vivo* studies showed that pre-treatment of WG can protect the DNA damage in dose-dependent manners as evident from MN assay in the bone marrow. Furthermore, the cytotoxicity of CP was also significantly reduced by WG pre-treatment as is evident from restoration in PCE/NCE ratio.

CP is a reactive alkylating agent that can bind to a variety of cellular molecules, but the most important site of binding is DNA. As discussed by several authors, damage of DNA caused by the covalent bonding of alkyl groups to phosphate, hydroxyl, and amino groups of the bases of nucleic acid may result in DNA strand breaks, formation of micronuclei, and ultimately to cell death [41-43]. Several studies

suggest that the important factor for the therapeutic and the toxic effects of CP is the requirement of the metabolic activation by the hepatic microsomal cytochrome P450 mixed functional oxidase system [44-46]. After metabolism, CP generates active metabolites, 4-hydroxy cyclophosphamide (4-OHCP), phosphoramide mustard and acrolein. Out of these acrolein is highly toxic and generates oxidative stress, resulting in DNA damage [44,47-49]. It reacts directly with GSH and forms an adduct glutathionylpropionaldehyde, which induces oxygen radical formation [47,50]. Acrolein and lipid peroxidation product-MDA belongs to carbonyl compounds, which are very reactive and can interact with amino acids of protein causing structural and functional changes in the enzymes [2,51]. Recently, it has been reported that CP affected the distribution of membrane bound phospholipids and impaired the plasma membrane of bone marrow cells in mice [52]. The reduction of SOD activity by CP or its metabolite is either due to the inhibition of enzyme synthesis and/or direct effect of hydrogen peroxide [19,48,49,53].

In the present investigation, treatment with CP leads to oxidative stress as evident from significant increase in MDA level and decrease in GSH and SOD content. However, pre-treatment of WG in different doses significantly restores GSH and SOD levels, as well as decreases the formation of lipid peroxidation byproduct MDA. WG is an excellent source of antioxidant such as SOD, cytochrome oxidase and other enzymes [18,19]. Few clinical trials have reported, WG supplementation to healthy volunteers reduces the lipid per oxidation level in blood [54]. According to Peryt et al. (1992), WG extracts scavenge superoxide anions [15]; have ferric reducing power and inhibit oxidative DNA damage [54]. WG is also rich with Super Oxide Dismutase (SOD) enzyme that scavenges dangerous reactive oxygen species; convert it into hydrogen peroxides anions and kills cancer cells [55]. It was also reported that chlorophyll content of WG inhibits metabolic activation of carcinogens [2,17,56], may be responsible to inhibit the formation of acrolein which reacts directly with GSH [47,50].

Although, the exact mechanism behind this improvement is not well known, yet it can be concluded that chlorophyll content of the wheatgrass may be responsible for inhibition of metabolic activation of the CP. This may inhibit the production of acrolein that leads to potentiation of tissue antioxidant defense system. On other hand the antioxidant properties of WG increases the SOD, GSH levels and reduce the lipid peroxidation that contributes to protection against CP induced genotoxicity.

Conclusion

In conclusion, the present study for the first time provides evidence that WG pre-treatment attenuates the CP-induced oxidative stress and the subsequent DNA damage in the bone marrow cells of mice. The antigenotoxic potential of WG might be due to its antioxidant property. However, further studies using other end points with possible mechanistic evidence are required to elucidate the precise mechanism of protection offered by WG.

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