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M. KARBARZ[1], J. MYTYCH[2], P. SOLEK[2], K. STAWARCZYK[1], A. TABECKA-LONCZYNSKA[2], M. KOZIOROWSKI[2], L. LUCZAJ[1]

CEREAL GRASS JUICE IN WOUND HEALING: HORMESIS AND CELL-SURVIVAL IN NORMAL FIBROBLASTS, IN CONTRAST TO TOXIC EVENTS IN CANCER CELLS

[1]Department of Botany, University of Rzeszow, Rzeszow, Poland;
[2]Department of Animal Physiology and Reproduction, University of Rzeszow, Kolbuszowa, Poland

Natural products and traditional medicines are of great importance. Recent studies have demonstrated, that cereal grass juice improves wound healing, however the cellular and molecular mechanisms underlying these processes have not been fully characterized. Also, the full phytochemical characteristics of freshly squeezed juices obtained from cereal grasses is still missing. Thus, in this study a multi-dimensional analysis of juice parameters like refraction value, pH, chlorophyll and flavonoids content as well as antioxidant properties was performed. The results demonstrate that the effect induced by freshly squeezed cereal juices is strictly cell type-dependent. In this study, it is shown for the first time, that in normal fibroblasts (BJ cells) low dose cereal grass juices exhibit strong adaptive response through hormetic mechanism mediated by NF-κB/HO-1 and insulin/IGF-1 anti-oxidant pathways. As consequence, the process of wound healing is significantly upregulated. In cancer cells (ES-2 cells), despite anti-oxidant defense mechanism activation, levels of ROS and RNS are elevated. This leads to enhanced O-GlcNAcylation, DNA damage and cell cycle arrest, and as a result impaired wound healing. This study provides insights into the underlying mechanisms through which cereal grass juices activate hormetic adaptation response in normal fibroblasts, and induce cytotoxic and genotoxic events in cancer cells.

Key words: *cereal grass juices, fibroblasts, cancer fibroblasts, hormesis, wound healing, phytotherapy, nuclear factor-kappa B, oxidative stress, nitrosative stress*

INTRODUCTION

Over the last few decades, cereal grass juices have been recognized as a most successful source of potential preventive and therapeutic drug leads. They have been shown to act as co-suppressors of many oxidative stress-related health disorders including inflammation, obesity, heart and neurological diseases and diabetes, as well as cancer. This has been associated with their remarkable antioxidant abilities, resulting from significant quantities of anti-oxidant enzymes, as well as non-enzymatic anti-oxidants (1). At the cellular level, cereal grass juices have been reported to act directly on cells by various mechanisms. In cancer cells, winter wheat and barley were demonstrated to induce p53-dependent G0/G1 cell cycle arrest (2), mitochondrial pathway-mediated (3) and tumor necrosis factor α (TNF-α)-dependent extrinsic (4) apoptosis. In terms of anti-inflammatory properties, barley was shown to modulate TNF-α release and to repress lipopolysaccharide (LPS)-induced nuclear factor-kappa B (NF-κB) activation (5). Similar observation have been made for winter wheat (6). Interestingly, recent *in vivo* studies have linked cereal grass juices with one more pro-health activity: improved normal wound healing and skin repair. Although phytochemicals and naturally derived substances have already been demonstrated as wound healing acceleration agents, these reports point this ability in cereal grass juices for the first time. Winter wheat juice has been shown to accelerate wound healing process and skin recovery when topically administered (7), whereas barley-supplemented diet was reported to inhibit atopic dermatitis-like lesions (8). These beneficial effects were primarily attributed to the anti-inflammatory and anti-oxidant properties of cereal grass juices, nonetheless, the precise cellular and molecular mechanisms underlying these processes were not fully characterized. According to the literature and previous results, this mechanism seems to be associated with a phenomenon called hormesis.

Hormesis is defined as a process in which exposure to a low dose of a chemical agent or environmental factor, which is damaging at higher doses, induces a beneficial effect on the cell or organism. Some of the main hormetic agents include exercise, ethanol, heat, irradiation, pharmacological agents, antioxidants and dietary components (9). At the cellular level, low dose-induced mild stress disturbs homeostasis and in response, the cell strives to normalize the situation by up-regulating its defence and repair mechanisms. As a consequence, an adaptive response typically involving several kinases, deacetylases and transcription factors followed by a synthesis of cytoprotective and restorative proteins, is initiated, resulting in the improved maintenance, repair and function of cells (10).

Therefore, taking into account previous observations and literature data, the hypothesis is that cereal grass juice-mediated

improvement in the normal wound healing process results from mild stress-induced hormesis. Hence, the present preliminary *in vitro* study was aimed at analysis of the potential hormetic effects of fresh cereal grass juices (winter wheat, khorasan wheat, barley and oat) on human fibroblasts, cells critical in supporting the wound healing process. As cereal grass juices seem to exert pleiotropic effects depending on cellular status, the effects in two fibroblast cell lines, one normal (BJ cell line) and one cancerous (ES-2 cell line) were compared. Additionally, in the light of the lack of literature data on comprehensive study on fresh cereal grass juices phytochemical parameters a multi-dimensional analysis of juice parameters like refraction value, pH, chlorophyll and flavonoids content as well as antioxidant properties was performed.

MATERIAL AND METHODS

Chemical reagents

The reagents were purchased from Sigma (Saint Louis, Missouri, USA), unless stated otherwise.

Cereal grass cultivation and juice preparation

Thirty grams of organic winter wheat (*Triticum aestivum* L.), khorasan wheat (*Triticum turanicum* Jakubz), barley (*Hordeum vulgare* L.), and oat (*Avena sativa* L.) seeds were rinsed with distilled water and soaked for 12 hours. Seeds were sown into trays with commercially available universal soil. On the third day, young seedlings were uncovered and cultivated under controlled conditions: temperature 20°C, ND (night/day) 12/12 h, and photosynthetically active radiation 150 µmol m^{-2} s^{-1}. After 4 days, seedlings were cut approximately 2 cm above soil and squeezed with a hand juicer Lexen Healthy Juicer 3g. The juice extracts were centrifuged for 5 min at 8500 × g and the obtained supernatants were immediately used for analyses.

Chlorophyll fluorescence

Just before cutting, chlorophyll fluorescence was measured using with a Plant Efficiency Analyser, Handy PEA (Hansatech Instrument, King's Lynn, UK) on the penultimate leaf segment. The leaf segments were clipped in the middle using leaf dark clips (Hansatech Instrument) for 30 min at room temperature. The maximum quantum efficiency of PSII photochemistry (Fv/Fm) and PI (overall performance index of PSII photochemistry) was measured using a PPFD of 3000 µmol m^{-2} s^{-1} as saturating flash for the duration of 1 second. Relative chlorophyll content (CI - Chl index, Greenness index,) was measured noninvasively by at LEAF CHL PLUS (FT Green LLC, Wilmington, Delaware, USA), that uses optical density difference at two wavelengths (640 and 940 nm) can be an indicator of the plant's condition. The atLEAF values were measured 5 times for central part of each leaf. Results were converted to chlorophyll concentration meter SPAD units according to the Zhu *et al.* equations (11). The total chlorophyll content was also calculated by converting the at LEAF CHL values into SPAD and considering the relationship among chlorophyll content and SPAD units.

pH and refraction value

The pH of the juice samples was measured in triplicate (Elmetron CPR-411 apparatus, Zabrze, Poland) in accordance with AOAC protocols (1995) (12). The pH meter was calibrated using standard buffer solutions of pH 4.0 and 7.0. All measurements were performed in a 50 ml cup, at temperature 25°C. The refraction value (°Brix) was determined at 21°Cusing portable digital refractometer 96801 (Hanna Instruments) (0-320 °Bx). The refractive index was measured by placing a drop of the sample on the refractometer prism.

Chlorophyll content

The concentrations of pigments (mg/L): chlorophyll a (Chl a), chlorophyll b (Chl b) were determined according to Kamble *et al.* method (13). To 1 ml of fresh juice, 6 ml of 80% acetone (Chempur) was added. The material thus obtained was centrifuged at 4°C, 2000 rpm for 10 min. Subsequently, the 2 ml supernatant was transferred into 2.5 ml cuvettes (Varian) and assayed. Absorbance was determined by spectrophotometer (UV-Vis Cary 300, Varian), using a wavelength of 645 for chlorophyll b, 663 for chlorophyll a. The concentration of chlorophyll a, b and total chlorophyll were evaluated using the following equations (14):

Total Chlorophyll: 20.2(A645) + 8.02(A663)
Chlorophyll a: 12.7(A663) – 2.69(A645)
Chlorophyll b: 22.9(A645) – 4.68(A663)

Chlorophyll degradation was evaluated by adding one drop of 96% HCl (Honeywell) to the sample. Acetone (80%) was used as a blank. The chlorophyll content measurement was repeated three times for each sample.

Total phenolic content

Ten mL of sample juice extracts was pipetted into a 96-well plate, followed by 100 µL of 0.2 M FCR. After 3 min, 90 µL of saturated Na_2CO_3 solution was added to each well. The samples were incubated for 1 hour at room temperature, after which the optical density was measured at 620 nm on Multi-detector microplate reader - VICTOR™ X4 (PerkinElmer, Waltham, Massachusetts, USA). TPC was calculated using a gallic acid standard curve, with concentrations ranging from 50 to 500 µg/mL and was reported as milligrams of gallic acid equivalents per 100 mL of juice. Three analytical replications were measured for each biological replicate.

Antioxidant properties

The antioxidant capacity of juices was quantified by the 2.20-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS) radical assay. The ABTS solution was prepared according to Ku *et al.* (15). The ABTS solution was generated by the treatment of 7 mM ABTS with 2.45 mM potassium persulfate. The mixture was then allowed to stand for 12 – 16 h for full color development (dark blue-green). The solution was diluted with PBS until the absorbance (measured at 620 nm) reached 1.0 ± 0.02 for use in the assay reaction. 10 mL of the aqueous sample extract was treated with 190 uL of 7 mM ABTS. The samples were incubated for 6 min at 21°C and then optical density was read at 620 nm on a microplate reader. The antioxidant capacity was calculated as millimolar 6-hydroxy- 2,5,7,8-tetramethylchroman-2-carbonsaure (Trolox) equivalents per 100 mL of juice, based upon a Trolox standard curve with concentrations ranging from 0.5 to 5.5 mM. All tests were performed in triplicate.

Cell culture and cereal grass juice treatment

Normal human fibroblasts - BJ cell line (ATCC, Manassas, Wirginia, USA; CRL-2522) and carcinoma human fibroblasts - ES-2 cell line (ATCC, Manassas, Wirginia, USA; CRL-1978), were cultured in a humidified atmosphere in the presence of 5% CO_2 at 37°C in high-glucose DMEM, supplemented with 10% FBS and antibiotic mix solution (100 U/ml penicillin, 0.1 mg/ml streptomycin, 29.2 mg/ml L-glutamine), until they reached

confluence. For experiments, cells were seeded at a constant density of 3×10^3 cells/cm^2 and after 24 h treated with cereal grass juices (diluted in complete DMEM).

MTT assay

Juices cytotoxicity was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assay. BJ or ES-2 cells were seeded into 96-well plates at a density of 1×10^3 cells/well, grown for 24 hours and then the medium was discarded and replaced with fresh medium containing juices (0.25, 0.5, 1 and 2%) or plain DMEM (control). After 48 hours incubation, MTT assay was performed accordingly to (16). Briefly, cells were supplemented with MTT solution (working concentration 500 µg/ml) and the cells were incubated for another 4 h at 37°C. Then, the medium was removed and crystals were dissolved in DMSO (Sigma, Poland) (5 min, 300 rpm, room temperature). Absorbance was measured at 595 and 620 nm (measurement and reference wavelengths, respectively) using Victor microplate reader. Metabolic activity was calculated as A595 – A620 and metabolic activity at standard conditions (control) is considered as 100%. Further studies, based on MTT results, were performed with the use of 1% fresh cereal grass juices.

Oxidative and nitrosative stress parameter measurement

Superoxide, free thiol levels and nitric oxide were measured using fluorogenic probes: dihydroethidium, Thiol Tracker Violet (Thermo Scientific, Waltham, Massachusetts, USA) and DAF-2 diacetate (Cayman Chemical, Ann Arbor, Michigan, USA), respectively, as described in the manufacturer's protocols and previously published paper (16). Briefly, cells were washed twice with Hank's Balanced Salt Solution (HBSS) and suspended in HBSS, supplemented with 5 µM of fluorescent probes. After 15 min incubation in the dark at room temperature, digital images were captured and quantitative analysis was conducted using InCell Analyzer 2000 software of minimum 1000 cells, and presented as relative fluorescence units (RFU).

Western blotting

The Western blot protocol was used as previously described (17). In general, after trypsinization, cells were homogenized in RIPA buffer, and 20 µg of proteins were separated by 10% SDS-PAGE and electro-blotted to nitrocellulose membranes. Next, membranes were blocked in 1% BSA, then incubated with the primary antibody and further with a secondary HRP-conjugated antibody. Protein bands were visualized using the ECL substrate and Fusion Fx7 system. The relative protein expression levels were normalized to the levels of β-actin using GelQuantNET software.

The primary antibodies used were: anti-HO-1; 1:1000 (#MA1-112), anti-HO-2; 1:1000 (#PA5-19156), anti-NF-κB p65; 1:1000 (#14-6731-81), anti-O-GlcNAc; 1:2000 (#MA1-072), anti-β-actin; 1:10 000 (#PA1-16889) (Thermo Scientific, Waltham, Massachusetts, USA) and anti-IGF-1Rβ; 1:500 (#sc-9038) (Santa Cruz, Santa Cruz, California, USA). Secondary antibodies: HRP-conjugated were: anti-mouse; 1:40,000 (#A9044), anti-rabbit; 1:40 000 (#A0545) (Sigma, Saint Louis, Missouri, USA), and anti-goat; 1:5000 (#sc-2768) (Santa Cruz, Santa Cruz, California, USA).

Micronuclei detection and cell cycle distribution

Micronuclei detection and cell cycle distribution were controlled accordingly to previously published paper (18). Briefly, cells were washed twice with PBS and suspended in DMEM w/o FBS supplemented with 1 µg/ml Hoechst 33342. After 20 min incubation in the dark at 37°C, the staining solution was removed, cells were covered with PBS and micrographs were captured with InCell Analyzer 2000. Quantitative analysis was conducted with ImageJ and presented as % of cells in each of the G0/G1, S and G2/M phases (cell cycle distribution) or as % of micronuclei positive cells (micronuclei detection).

Wound healing scratch assay

The extent of cell migration was described by employing a so-called wound healing scratch assay as previously described (10). Briefly, 1×10^5 cells were seeded in each well of a 12-well plate, after 24 h a scratch was made using a 10 µl tip, and the medium was replaced with medium supplemented with 1% cereal grass juices. The changes were monitored with Zeiss Axiovert 40 CFL inverted microscope and computer image analysis system Zeiss Axiovert 40 CFL, immediately after the scratch, then after 10, 24 and 48 hours. Quantitative analysis was conducted with ImageJ software and results were presented as % of wound closure.

Statistical analysis

The results represent the mean ± SD from at least three independent experiments. Statistical analysis of the results was performed using GraphPad Prism ver. 6.0. Differences between control and test samples were assessed with one-way analysis of variance followed by Tukey's (phytochemical parameters) or Dunnett's (studies on cell lines) comparison post-tests. A P-value of < 0.05 was considered as statistically significant between groups: ***$P < 0.001$, ** $P < 0.01$, * $P < 0.05$, no indication - no statistical significance.

RESULTS

Plant condition and phytochemical parameters

The results obtained by non-invasive methods demonstrating the condition of plants (Fv/Fv, PI, CI, SPAD, Total Chl) intended for the juice production are presented in *Table 1*.

In the analyzed juices, the pH was determined to be in the range of 6.20 to 7.30 (*Fig. 1A*). In the case of this research, the highest Brix index values of the fresh juice extract were noted for khorasan wheat samples and the smallest for oat juices (*Fig. 1B*). The highest chlorophyll content was found in khorasan wheat and winter wheat and the lowest in oat juices (*Table 1*). The results obtained for khorasan wheat and winter wheat do not differ (*Fig. 1C-1D*). The obtained results indicate differences in the chlorophyll 'a' to chlorophyll 'b' ratio, in tested plant juices. The highest is in oats, the lowest in barley (*Fig. 1E*). There were no differences in the chlorophyll degradation between the values for plants (*Fig. 1F*). The largest amount of polyphenols was determined in the juices from khorasan wheat, while the least polyphenols contained fresh barley juice (*Fig. 1G*). In analyzed fresh cereal juices, the antioxidant activity was at a level ranging from 2.15 in wheat juice to 2.4 mM Trolox/mL in barley juice (*Fig. 1H*).

Fresh cereal grass juices-mediated biphasic dose-response in normal cells and dose-dependent cytotoxicity in cancer cells

In this study, using a MTT assay, the effect of fresh cereal juices on two fibroblast human cell lines: normal and cancerous (BJ and ES-2, respectively) was evaluated. Firstly, as shown on *Fig. 2A*, all fresh cereal grass juices analyzed in this study

Table 1. Physiological parameters of plants.

Species	F_V/F_m	PI	CI	SPAD	Total chlorophyll mg/cm^2
Triticum turanicum J. (khorasan wheat)	0.82 ± 0.008	1.702 ± 0.150	38.933 ± 6.029^{cd}	28.467 ± 5.953^{de}	0.02 ± 0.007^{bd}
Avena sativa L. (oat)	0.835 ± 0.01^{a}	1.895 ± 0.107	25.583 ± 1.358^{c}	15.233 ± 1.358^{ad}	0.008 ± 0.001^{b}
Hordeum vulgare L. (barley)	0.828 ± 0.01 ^{a}	1.658 ± 0.385	32.75 ± 3.909^{b}	22.333 ± 3.880^{c}	0.016 ± 0.004
Triticum aestivum L. (winter wheat)	0.815 ± 0.021	2.173 ± 0.207^{a}	35.817 ± 3.607^{a}	25.4 ± 3.579 ^{ab}	0.019 ± 0.004^{a}

Values are means (n = 3) ± SD. Different letters in the same column mean significant differences at P ≤ 0.05. Letters are assigned (*e.g.*, a, b, and c) to highlight significant differences. Those means that are not significantly different are assigned a common letter. In other words, two treatments without a common letter are statistically significant at the chosen level of significance.
Abbreviations: Chl, chlorophyll; CI, Chl index - Greenness index; PI, overall performance index of PSII photochemistry; SPAD, chlorophyll concentration meter.

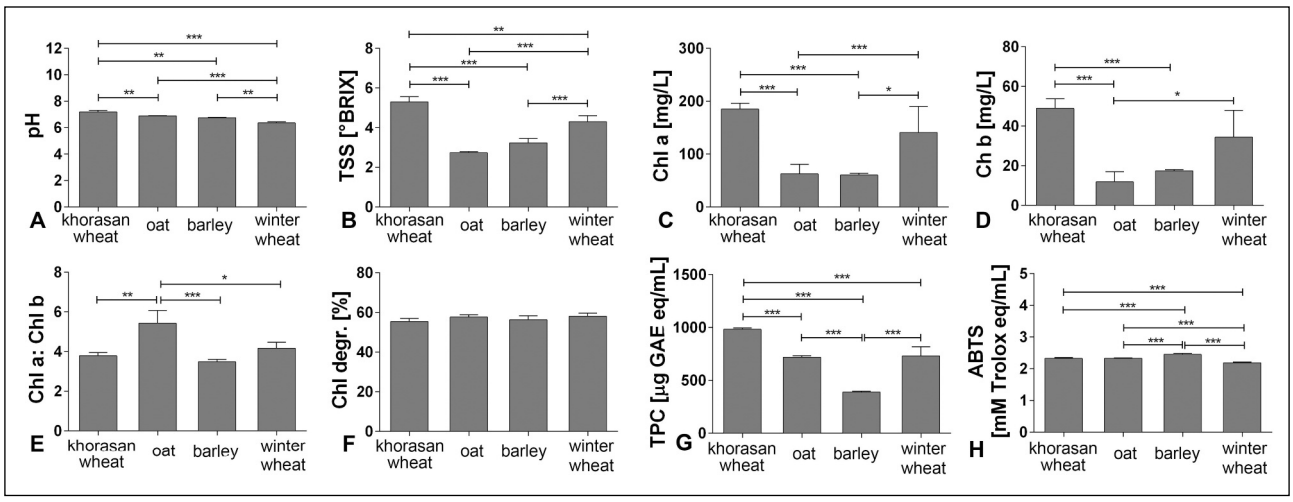

Fig. 1. Cereal grass juices parameters: (A) pH values of juices; (B) Brix values of juices; (C) Chlorophyll a content; (D) Chlorophyll b content; (E) Chlorophyll a:b ratio; (F) Chlorophyll degradation values of juices; (G) Total phenolic content in juices; (H) Antioxidant capacity (ABTS assay) of juices.

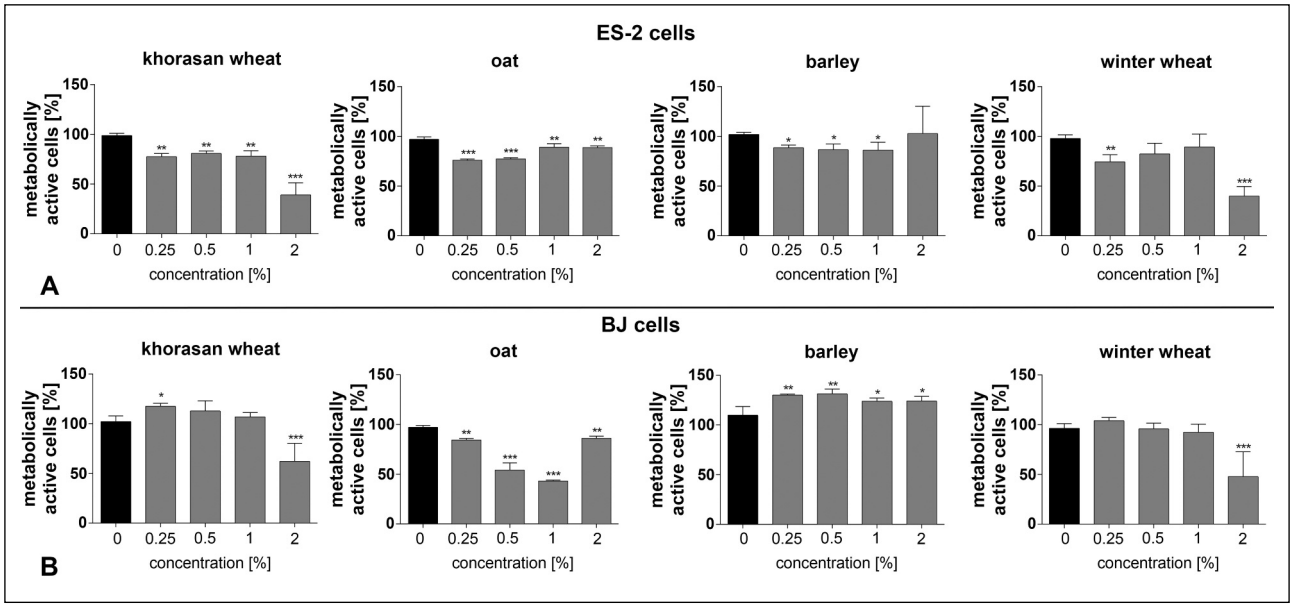

Fig. 2. Effects of a 48 h exposure of BJ and ES-2 cells to cereal grass juices in terms of MTT activity. BJ or ES-2 cells were seeded at the density of 3000 cells/cm^2 and treated for 48 h with wide range of fresh cereal grass juices (0.25 – 2%). Then, MTT assay was performed. Bars indicate SD, n = 3, ***P < 0.001, **P < 0.01, *P < 0.05, no asterisk indication - no statistical significance (one-way ANOVA and Dunett's a posteriori test).

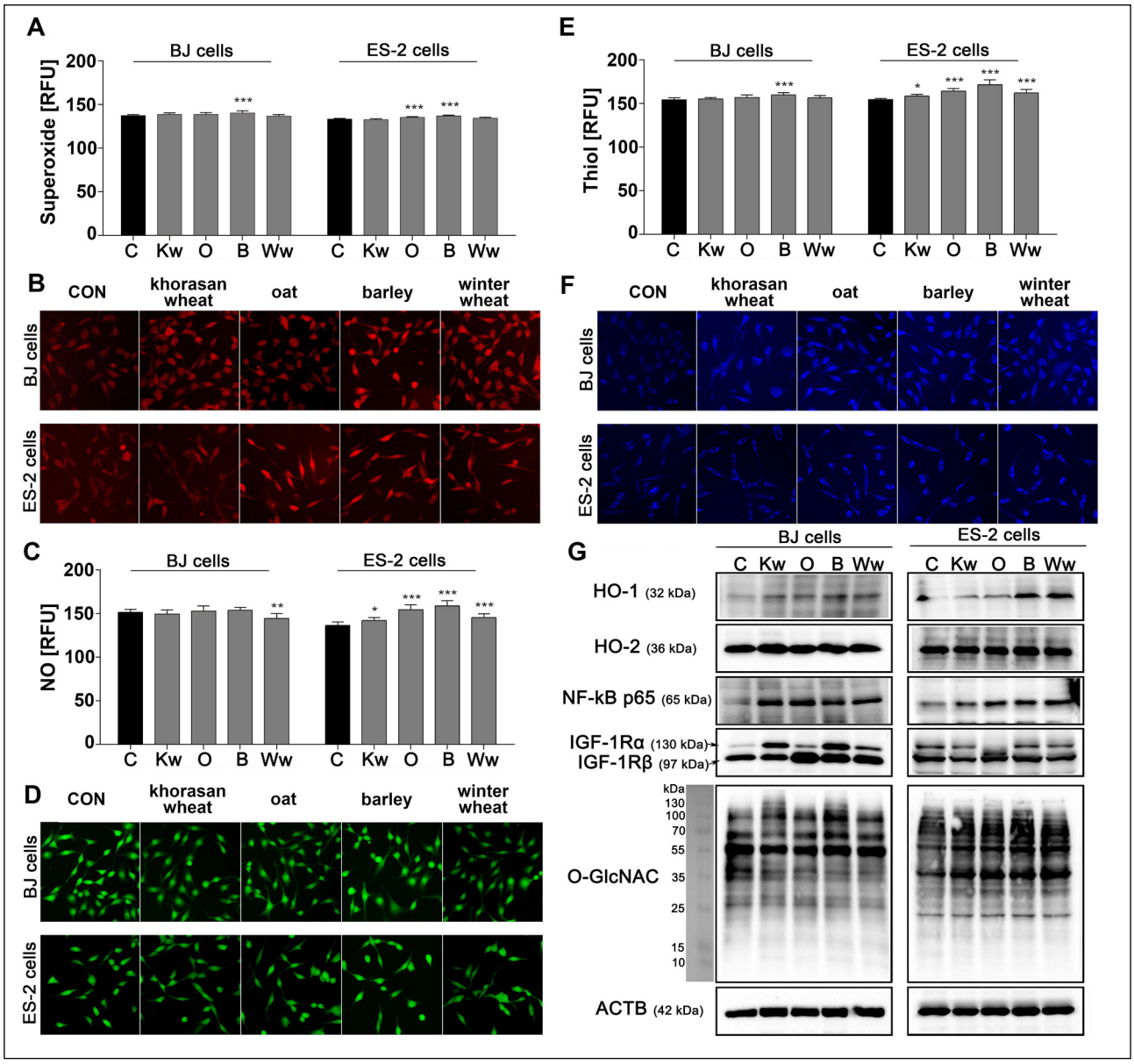

Fig. 3. Cereal grass juice-mediated changes in ROS, RNS and Thiols production in normal and cancer fibroblasts and activation of antioxidant pathways and oxidative protein damage. BJ or ES-2 cells were seeded at the density of 3×10^3 cells/cm^2 and treated for 48 h with 1% cereal grass juices. Then, cells were stained with (A) 5 μM dihydroethidium to control ROS levels; (B) representative images: red fluorescence - dihydroethidium (ROS); (C) 5 μM DAF-2 diacetate to control RNS levels; (D) representative images: green fluorescence - DAF-2 diacetate (RNS); or (E) 5 μM Thiol Tracker to control levels of reduced GSH (thiols); (F) representative images: blue fluorescence - Thiol Tracker (GSH, Thiols). Digital images were taken with InCell Analyzer 2000, quantitative analysis was performed with InCell Analyzer 2000 analysis software, and statistical with GraphPad Prism. Bars indicate SD, n = 6, ***P < 0.001, **P < 0.01, *P < 0.05, no indication - no statistical significance (one-way ANOVA and Dunett's a posteriori test). (G) The expression of proteins involved in cellular mechanisms against oxidative stress was evaluated with Western blot method. Representative blots are presented. Cropped blots are grouped and delineated with clear dividing lines. The full-length images are available upon request. C, control; Kw, Khorasan wheat; O, Oat; B, Barley; Ww, Winter wheat.

significantly reduced the metabolic activity of cancer ES-2 cells (*Fig. 2A*). Secondly, three out of four analyzed cereal juices affect normal human fibroblast in completely different manner to cancer cells. As it can be seen on *Fig. 2B*, winter wheat, khorasan wheat and barley when added to BJ cell line at low concentrations enhance metabolic activity. Further, initial increase in MTT activity is followed by its decrease when higher doses of grass juices are applied. In contrast, oat-treated BJ cells did not show the same tendency. All doses applied (0.25 – 2%) led to statistically significant decrease in metabolic activity of BJ cells, thus hormesis effect has not been confirmed in this case (*Fig. 2B*).

Cereal grass juices induce oxidative and nitrosative stress in cancer but not normal cells

Then, the redox balance of normal and cancer fibroblasts treated for 48 hours with 1% cereal grass juices was controlled

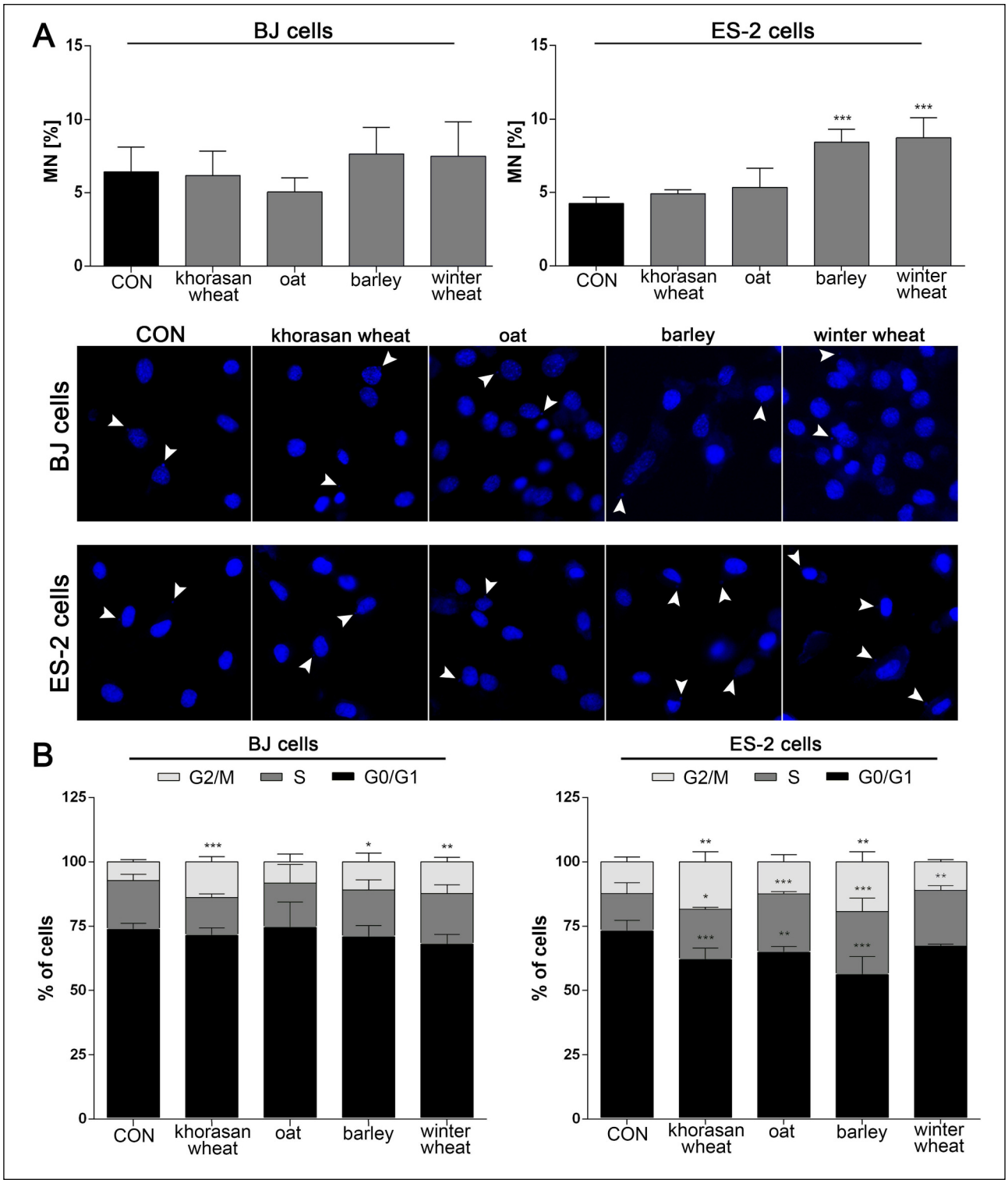

Fig. 4. Cereal grass juice-induced genotoxicity in cancer fibroblasts: (A) micronuclei formation and (B) cell cycle profile alterations. BJ or ES-2 cells were seeded at a density of 3×10^3 cells/cm^2 and treated for 48 h with 1% cereal grass juices. After that, cells were stained with 1 µg/ml Hoechst 33342 and digital images were taken with InCell Analyzer 2000. Quantitative analysis was performed with ImageJ, and statistical with GraphPad Prism. Bars indicate SD, n = 3, ***P < 0.001, **P < 0.01, *P < 0.05, no indication - no statistical significance (one-way ANOVA and Dunett's a posteriori test). Representative images are presented, magnification of the objective lens × 20, blue fluorescence - Hoechst 33342.

(*Fig. 3*). Superoxide level reflecting the reactive oxygen species pool were not affected in normal fibroblasts, except cells treated with barley (P < 0.001). However, in cancer cells a slight but statistically significant up-regulation in superoxide generation was observed after treatment with oat and barley juices (P < 0.001) (*Fig. 3A* and *3B*). At the same time, treatment with 1%

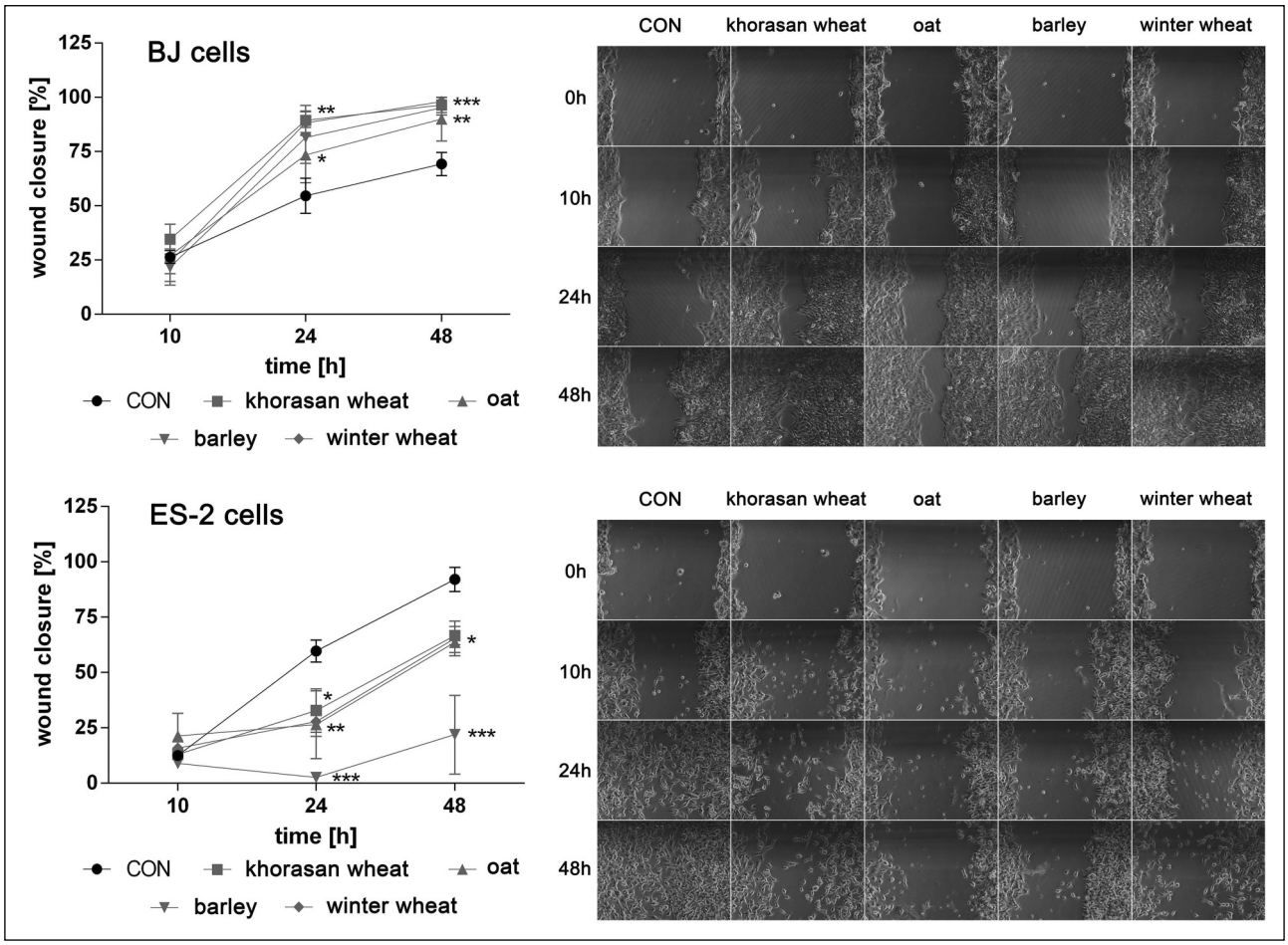

Fig. 5. Wound healing ability in normal and cancer cells affected by 1% cereal grass juice treatment. 1×10^5 BJ or ES-2 cells were seeded into each well of 12-well plate, 24h later a scratch was made and cereal grass juices were added. Wound closure was controlled at 0, 10, 24 and 48 hours. Quantitative analysis was performed with ImageJ, while statistical with GraphPad Prism. Bars indicate SD, n = 3, ***P < 0.001, **P < 0.01, *P < 0.05, no indication - no statistical significance (one-way ANOVA and Dunett's a posteriori test). Representative images are presented, magnification of the objective lens × 10.

winter wheat led to downregulation of nitric oxide production in BJ cells (P < 0.01). In contrast to normal cells, in cancer fibroblasts treatment with all cereal grass juices resulted in intracellular nitric oxide pool upregulation. As one can observe in *Fig. 3C* and *3D*, all differences turned out to be statistically significant. Moreover, an augmentation in the levels of thiol pools, but only in cancer cells was confirmed. In cancer fibroblasts, the level of thiols reflecting reduced glutathione (GSH) content was increased after treatment with all cereal grass juices used (*Fig. 3E* and *3F*).

As cereal grass juice-mediated increase in reduced glutathione levels may be part of an adaptive response involving the activation of mechanisms modulating oxidative stress response, the expression level of proteins involved in such pathways was controlled (*Fig. 3G*). Firstly, cereal grass juices promoted heme oxygenase 1 (HO-1) up-regulation in both normal and cancer cells. While in BJ cells the observed elevation was approximately 1.35-fold, in ES-2 cells the observed effect was significantly higher. The smallest up-regulation of HO-1 in ES-2 cells was noted after khorasan wheat treatment - a 1.66-fold increase, while the highest was a 5.16-fold increase after treatment with barley. Simultaneously, cereal grass juices did not promote any changes in heme oxygenase 2 (HO-2) synthesis, which is believed to be constitutively expressed, independently of any conditions. This was observed in both cell lines tested. Further, as an adaptive response, the activation of NF-κB p65 transcription factor was observed. Similarly to HO-1, the effect was more pronounced in cancer cells when compared to normal cells, but just slightly. In detail, NF-κB p65 pools in BJ cells were enhanced approximately 2.15-fold, while in ES-2 cells it was a 2.33-fold increase. In ES-2 cells, the highest augmentation was reported for barley (a 2.71-fold increase), and the lowest for khorasan wheat (a 1.94-fold increase) when compared to non-treated cells. Also, cereal grass juice treatment resulted in employment of insulin growth factor 1 (IGF-1) pathway, but only in normal cells. All tested cereal grass juices activated IGF-IR precursor synthesis (IGF-1Rα), however the level of matured IGF-1R (IGF-1Rβ) was elevated in BJ cells only after treatment with oat, barley and winter wheat. Moreover, although antioxidant systems were activated, cereal grass juice treatment resulted in increased protein O-GlcNAcylation. In cancer cells, O-GlcNAc modification of proteins with high molecular weight (> 60 kDa) was enhanced approximately 1.39-fold when compared to non-treated cells. Interestingly, the most pronounced effect was observed in the case of barley treatment (a 1.44-fold increase). The level of O-GlcNAc in normal fibroblasts remained unaffected (*Fig. 3G*).

Cereal grass juice-mediated genotoxicity in cancer cells

Persistent oxidative and nitrosative stress may lead to DNA damage. Additionally, recent studies point directly to the crucial role of O-GlcNAcylation in maintaining genomic stability. Therefore, it was controlled whether cereal grass juices treatment may result in genotoxic events in normal and cancer fibroblasts. Indeed, an increased micronuclei formation, but only in cancer cells, as a result of barley (a 1.99-fold increase) and winter wheat (a 2.05-fold increase) treatment was confirmed. Simultaneously, no cereal grass juice stimulated micronuclei formation in BJ cells (*Fig. 4A*).

Most cells exhibit transient or permanent cell cycle delays as a response to DNA damage. In this study, cereal grass juices altered the cell cycle distribution in cancer cells by inducing S and/or G2/M phase cell cycle arrest with a concomitant drop in the pool of cells in the G0/G1 phase. As expected, the most pronounced effect was observed in the case of barley treatment. When compared to control cells, the population of barley-treated cells in the G0/G1 phase decreased by 16.80% ($P < 0.001$), while the number of cells in S and G2/M phases increased by 9.72% ($P < 0.001$) and 7.08% ($P < 0.001$), respectively. Similarly, due to khorasan wheat treatment, the number of ES-2 cells in G0/G1 phase dropped, while the population of cells in S and G2/M phases rose. In oat- and winter wheat-treated cells, a decrease in the population of cells in G0/G1 phase was accompanied by S phase arrest ($P < 0.01$). On the other hand, in BJ normal cells, the same tendency was not observed, and the pool of cells in G0/G1 phase remained unaffected (*Fig. 4B*).

Cereal grass juices promote the wound healing process in normal cells, and inhibit it in cancer cells

There is much evidence to support the critical role of reactive oxygen species in modulating wound healing and infection control at the wound site. Thus, the effect of cereal grass juices on normal and cancer fibroblasts' wound healing ability was evaluated in the next step of this study. The analysis performed revealed a cereal grass juice-mediated promoting effect in normal fibroblasts, and an inhibiting effect in cancer fibroblasts on the *in vitro* wound healing process. In the case of normal cells, a promoting effect was reported for all cereal grass juices tested. Although after 10 h, a slight increase in percentage wound closure could be already seen, this difference became significant after 24 hours, and finally at the last time point analyzed (48 h), the observed % of wound closure was 25.77% ($P < 0.001$), 24.23% ($P < 0.001$), 22.93% ($P < 0.001$) and 17.72% ($P < 0.01$) higher in khorasan wheat-, barley-, winter wheat- and oat-treated cells, respectively, than in control cells. On the other hand, in cancer cells, all cereal grass juices exhibited inhibitory effects on the wound healing process (*Fig. 5*).

DISCUSSION

In this study, it is reported for the first time that fresh cereal grass juices affect both, normal and cancerous human fibroblasts, but in different ways. In normal fibroblasts, oxidative stress response-mediated adaptation results in improved wound healing. In cancer cells, on the other hand, despite activated anti-oxidant defense mechanisms, levels of reactive oxygen and nitrogen species are up-regulated and lead to elevated O-GlcNAcylation, DNA damage, cell cycle arrest and wound healing inhibition.

Studies and these results suggest that cereal grasses have high antioxidant activity due to different phenolic acid compounds and flavonoid presence in the samples (19). From the results we obtained, the main substances responsible for the antioxidant potential value cannot be clearly determine. In juices the content of chlorophyll was directly proportional to the TPCs specified in it. However, these results do not translate directly into the value of the antioxidant potential. At the cellular level, the oxidation process and redox imbalance may be inhibited through a variety of mechanisms, however naturally derived substances mostly induce anti-oxidative defensive responses by producing hydrogen peroxide upon free radical and ROS neutralization (20). In this study, it was confirmed that NF-κB transcription factor acts as a cereal grass juice-mediated anti-oxidative response regulator in normal fibroblasts. Further, the expression of HO-1 catalyzing heme degradation, finally resulting in the formation of bilirubin, a potent anti-oxidant, was up-regulated by NF-κB. Typically, the expression of NF-κB target genes attenuate ROS to promote cellular survival through the inhibition of c-Jun N-terminal protein kinase (JNK) (21). The NF-κB/HO-1 pathway observed in this study has also been proposed by others (22), however Nrf2/HO-1 and upstream signaling pathways were mainly shown to be engaged (23). Further, in cereal grass juice-treated normal fibroblasts the insulin/IGF-1 pathway activation, which was demonstrated to enhance both NF-κB signaling (24) and Nrf2/HO-1 expression (25) is confirmed. As a result of anti-oxidant adaptation, the final production of reactive oxygen and nitrogen species was unaffected in normal cells. Moreover, cereal grass juices exerted beneficial effects in terms of enhanced cellular proliferation and wound healing. This observation confirms the activation of hormetic adaptation response through stimulation of anti-oxidant defenses, except oat-treated cells. Moreover, results by Olas *et al*. suggest that phenolic fraction of *Hippophae rhamnoides* fruit reveals anti-adhesive properties of this plant preparation on blood platelet activation (26). To date, the hormetic effect on wound healing has been shown in only a few studies. Firstly, Tian *et al*. demonstrated 1,25-dihydroxyvitamin D3-dependent acceleration in wound treatment (27). Rattan *et al*. reported the hormetic effect of glyoxal and heat shock on the wound-healing capacity of skin fibroblasts, and on the angiogenic ability of endothelial cells (28). Similarly, a curcumin-mediated effect on wound healing also seems to be hormesis-related and dependent on the Nrf2/OH-1 stress response pathway (29).

It is worth pointing out that increased proliferation may lead to pathophysiological events, especially in cancer. However, in this study, cereal grass juices inhibited proliferation and thus the wound healing process in cancer fibroblasts, in contrast to normal fibroblasts. In detail, cereal grass juice treatment resulted in NF-κB/OH-1 pathway activation followed by a compensatory increase in GSH pools, however the levels of ROS and RNS in cancer cells were elevated, implying that defense mechanisms were not strong enough to contend with oxidative stress. In such a situation, the cell typically induces the formation/opening of the mitochondrial permeability transition pore, as an efficient way to decrease ROS production, by decreasing the mitochondrial membrane potential. On one hand, this increases the overall oxidative state in the cell (30), as was observed in this study. Elevated protein O-GlcNAcylation was also confirmed, which could be initiated directly by phytochemicals from cereal grass juices (31) or indirectly by ROS/RNS (32). It was shown that the transcription factor skinhead-1 (SKN-1), the ortholog of human Nrf2, is regulated by O-GlcNAcylation, and thus modulates lifespan and oxidative stress resistance (33). Additionally, since O-GlcNAcylation was shown to modify and activate c-Rel, a sub-unit of NF-κB (34), it is highly probable that the activation of NF-κB and downstream HO-1 observed in this study results not only from oxidative stress response mechanisms but also from enhanced O-GlcNAcylation. Further, homeostatic disruption of

the redox balance resulted in micronuclei formation. The observed genotoxicity could be also associated with an elevated level of O-GlcNAcylation, since loss of O-GlcNAcase, an enzyme catalyzing the hydrolytic cleavage of O-GlcNAc from post-transitionally modified proteins leads to mitotic defects, including cytokinesis failure and binucleation, increased lagging chromosomes and micronuclei formation (35). In turn, DNA damage elicits the prompt activation of DNA damage response (DDR), which arrests the cell cycle in order to avoid entering the next phase of cell cycle with damaged DNA. Here, both S and G2/M phases of cell cycle arrest in cancer fibroblasts due to the cereal grass juice treatment was confirmed. Cell cycle arrest was certainly mediated by upregulated O-GlcNAcylation. As Miura *et al*. demonstrated, this post-transitional modification activates ataxia-telangiectasia mutated (ATM) kinase, an important element of early response to DNA damage, and thus initiates cell cycle arrest (36). Interestingly, another traditional plant *Tribulus terrestris* has been shown to be capable of attenuating the oxidative DNA damage (37). Moreover, increased O-GlcNAcylation results in growth defects linked to delays in G2/M progression, altered mitotic phosphorylation and cyclin expression (38). O-GlcNAcylation was also found to promote apoptosis through attenuating the phosphorylation of protein kinase B (AKT) and the Bcl-2 associated death promoter (39, 40). This, together with the fact that G2/M phase arrest leads the cell to follow the apoptosis pathway, confirms apoptotic cell death as the reason for cell migration and proliferation inhibition in cancer fibroblasts treated with cereal grass juices. On the other hand, normal cells do not undergo apoptosis suggesting anti-apoptotic effects of cereal grass juices similar to phenolic fraction of another plant *Tropaeolum majus* L. extract (41).

In summary, it is demonstrated for the first time, that in normal human fibroblasts, relatively low doses of cereal grass juice (except oat) exhibit strong adaptive activity through activation of hormetic mechanisms involving up-regulated NF-κB/HO-1 and insulin/IGF-1 anti-oxidant pathways. In the case of oat-treated cells, proliferation of normal fibroblasts is inhibited and accompanied by induced cellular movement. Thus, this study provides new insight into the mechanisms for cereal grass juice-mediated improvement in wound healing. Additionally, a new evidence that the same low dose of cereal grass juices induces cytotoxic and genotoxic events leading to a significant reduction in cancer fibroblasts' viability, and impaired wound healing is provided.

Abbreviations: ABTS, 2.20-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid); BJ cells, normal human fibroblasts; Chl, chlorophyll; CI, Chl index - Greenness index; ES-2 cells, cancer human fibroblasts; FBS, fetal bovine serum; FCR, Folin-Ciocalteu reagent; GSH, reduced glutathione; HO-1, heme oxygenase 1; HO-2, heme oxygenase 2; IGF-1, insulin growth factor 1; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappaB; NO, nitric oxide; O-GlcNAc, O-linked N-acetylglucosamine; PI, overall performance index of PSII photochemistry; PPFD, photosynthetic photon flux density; RFU, relative fluorescence units; RNS, reactive nitrogen species; ROS, reactive oxygen species; SPAD, chlorophyll concentration meter; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; TPC, total phenolic content.

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Authors M. Karbarz and J. Mytych share first authorship.

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Author's address: Dr. Jennifer Mytych, Department of Animal Physiology and Reproduction, University of Rzeszow, Werynia 502, 36-100 Kolbuszowa, Poland.
E-mail: jennifermytych@gmail.com