Bioaccessible fraction of parboiled germinated brown rice exhibits higher anti-inflammatory activity than that of brown rice

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<th>Journal:</th>
<th><em>Food &amp; Function</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>FO-ART-12-2014-001194.R1</td>
</tr>
<tr>
<td>Article Type:</td>
<td>Paper</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>04-Mar-2015</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Tuntipopipat, Siriporn; Institute of Nutrition, Muangnoi, Chawanphat; Institute of Nutrition, Thiyajai, Parunya; Institute of Nutrition, Srichamnong, Warangkana; Institute of Nutrition, Charoenkiatkul, Somsri; Institute of Nutrition, Praengam, Kemika; Institute of Nutrition,</td>
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Bioaccessible fraction of parboiled germinated brown rice exhibits higher anti-inflammatory activity than that of brown rice

Siriporn Tuntipopipat, Chawanphat Muangnoi, Parunya Thiyajai, Warangkana Srichamnong, Somsri Charoenkiatkul, Kemika Praengam*

Parboiled germinated brown rice (PGBR) has been suggested as a functional food because it is relatively rich in a number of nutrients and health promoting compounds. Here we compared the bioaccessibility of several of the bioactive compounds in cooked PGBR and brown rice (BR) by simulating oral, gastric and small intestinal digestion. The uptake and retention of bioactive compounds from the bioaccessible fraction also was determined using Caco-2 human intestinal cells. The anti-inflammatory activity of the bioaccessible fraction from digested BR and PGBR was then assessed with Caco-2 cells that were activated with H$_2$O$_2$+IL-1β. PGBR had higher content of GABA, γ-oryzanol, γ-tocotrienol, ferulic acid and p-coumaric acid than BR. The amounts of these compounds transferred to the aqueous fraction during digestion and the quantities accumulated by Caco-2 cells were proportional to those in cooked PGBR and BR. The anti-inflammatory activity of the bioaccessible fraction from digested BR and PGBR was then assessed for Caco-2 cells that were activated with H$_2$O$_2$+IL-1β. Pre-treatment of the cells with the bioaccessible fractions from PGBR and BR suppressed secretion of IL-8 and MCP-1 and the ROS content in activated cells. Inhibitory activities were attenuated to a greater extent after cells had been pre-exposed to the bioaccessible fraction from digested PGBR compared to BR. These results suggest that digested PGBR contains and delivers greater amounts of compounds with anti-inflammatory activity to absorptive epithelial cells than digested BR.

1. Introduction

Brown rice which is also referred to as unmilled rice consists of bran layers, embryo and endosperm. It contains greater amounts of bioactive compounds such as gamma aminobutyric acid (GABA), γ-oryzanol, vitamin E, phenolic acids than white rice. However, it is not consumed as a regular staple compared to white rice due to its hard texture, dark appearance and lengthy cooking time. Germination is a strategy to enhance the amounts of nutrients and bioactive compounds in order to improve the nutritional quality of cereals including brown rice, barley, wheat and oat, as well as enhancing texture and digestibility. Increased amounts of GABA, γ-oryzanol, vitamin E and several phenolic acids have been reported in germinated brown rice. Germinated brown rice also has been shown to be anti-hyperlipidemia in rat, anti-hypertensive in spontaneously hypertensive rats, anti-tumorigenic for small airway epithelial cell lines and in rats with azoxymethane-
induced colon cancer rats,\textsuperscript{27} and anti-diabetic in healthy human subjects\textsuperscript{18} and in free-living patients with impaired fasting glucose or type 2 diabetes.\textsuperscript{19} These findings suggest that germinated brown rice should be considered a healthier choice for staple diets and for the development of functional foods.

Intestinal epithelial cells participate in the metabolism and absorption of dietary compounds and provide a physical and biochemical barriers against microorganisms, antigens and xenobiotics. These cells also secrete inflammatory cytokines in response to pathogenic bacteria, chemical insults and pro-inflammatory cytokines.\textsuperscript{20,21} Excessive production of such inflammatory mediators disturbs gut homeostasis that can induce the onset of intestinal disorders such as inflammatory bowel diseases (IBD).\textsuperscript{22-24} IL-8 or CXCL8, an α-chemokine, is highly expressed in the intestinal mucosa in IBD\textsuperscript{25} and induces persistent infiltration of neutrophils into inflamed areas.\textsuperscript{26} Monocyte chemoattractant protein-1(MCP-1) or CCL2 is another chemokine that recruits monocytes, memory T cells and dendritic cells to inflamed tissues.\textsuperscript{27,28} Elevated expression of MCP-1 occurs in the mucosa of IBD patients\textsuperscript{27,29} and contributes to the pathogenesis of various immunodeficiency and inflammatory diseases.\textsuperscript{30} Increased amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and other markers of oxidative injury, as well as decreased quantities of antioxidants, have been reported in the intestinal mucosa in IBD patients compared to control subjects.\textsuperscript{31} The extent to which antioxidant levels and markers of oxidative stress are altered has been associated with the severity of intestinal inflammation in IBD patients.\textsuperscript{31} Inhibition of the activity of cytokines represents a therapeutic strategy for IBD.\textsuperscript{32,33} However, this strategy is expensive, associated with undesirable side effects and often ineffective.\textsuperscript{34} Consequently, there is increased interest in the identification of dietary compounds that are relatively safe and affordable for the treatment of inflammatory bowel disorders. Various investigators have demonstrated that differentiated cultures of Caco-2 human intestinal cells were used to evaluate the anti-inflammatory effects of dietary compounds in the inflamed human intestinal epithelium. Cultures are pre-treated with compounds of interest before insulting the cells with oxidants and/or pro-inflammatory cytokines to assess possible suppression of the cellular response to such insult.\textsuperscript{35-37}

The first objective of the study was to compare the bioaccessibility and intestinal cellular uptake of GABA, γ-oryzanol, vitamin E and phenolic acids in digested BR and PGBR using the coupled \textit{in vitro} digestion method and Caco-2 human intestinal cell model. The second objective was to compare the effect of pre-treatment of Caco-2 cells with the bioaccessible fraction of digested BR and PGBR on the inflammatory response of Caco-2 cells activated with hydrogen peroxide and interleukin 1-beta (H\textsubscript{2}O\textsubscript{2}+IL-1β). This cell model is well-established for investigating the potential effects of food substances on the activities of small intestinal epithelial cells, including the modulation of intestinal inflammation.\textsuperscript{38,39}

\textbf{2. Materials and Methods}

\textbf{2.1. Chemicals and reagents}
Dulbecco’s modified Eagle’s medium (DMEM), α-amylase, pepsin, porcine bile extract, porcine pancreatin, porcine lipase and protease from bovine pancreas were purchased from Sigma Chemical Co. (St. Louis, MO, USA). L-glutamine, non-essential amino acids penicillin-streptomycin and fungizone were obtained from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Thermo Fisher Scientific, MA, USA). All reagents were either analytical grade or high performance liquid chromatography (HPLC) grade. Human IL-8 and MCP-1 capture and biotin-labeled detection antibodies, and human IL-1β were purchased from Peprotech Inc. (Rocky Hill, NJ, USA).

2.2. Preparation of cooked rice

Thai variety Khao Dawk Mali 105 (KDML 105) brown rice (BR) and parboiled germinated brown rice (PGBR) were provided by RCK Agri Marketing Company, Thailand. The parboiled germination process has been described elsewhere.\(^{60}\) Briefly, rice (80 kg) was soaked in 160 L water for 18 h at 30\(^\circ\)C and water was changed every 4 h until the moisture content of paddy was 30%. After removal of the surface water, this steeped paddy was germinated in presence of flowing air for 42-48 h at 30\(^\circ\)C and before steaming (parboiling) for 30 min under vacuum. Parboiled rice was then dried at 70–75 \(^\circ\)C for 2 h under vacuum and dried in an oven at 40\(^\circ\)C to a moisture content of approximately 13% prior to de-husking. BR and PGBR were cooked using an electronic rice cooker (Sharp KS-19ET size 1.8 L) at ratios of 1:2.2 and 1:2.3 (w/v) of rice and water, respectively. BR and PGBR were cooked for 30 and 33 min, respectively, and then cooled for 15 min at 25 \(^\circ\)C prior to lyophilization. The dried samples were ground with a Cyclotec unit (FOSS, Sweden). Rice powder was stored in aluminum foil in vacuo at -20 \(^\circ\)C.

2.3. In vitro digestion and bioaccessibility

Simulated oral, gastric and small intestinal phases of digestion were conducted according to Chitchumroonchokchai et al. (2004)\(^{41}\) and Ferruzzi et al. (2006).\(^{42}\) Digestion reactions contained 0.7 g freeze dried rice sample with 3% (v:wt) soybean oil. After completion of simulated small intestinal phase of digestion, chyme was centrifuged (Becton Dickinson Dynac Centrifuge, Sparks, MD, U.S.A.) at 10,000 x g for 1 h at room temperature to isolate the aqueous fraction. Control digestion without rice was also conducted to assess the possible cytotoxic effects of compounds in the aqueous fraction. The supernatant after centrifugation was filtered (0.22 \(\mu\)m pores; polytetrafluoroethylene (PTFE) membrane; Millipore Corp., Cork, Ireland) to obtain the fraction with mixed micelles. Filtrate (20 mL) was transferred to a polypropylene tube, the headspace was blanketed with nitrogen gas, and sealed tubes were stored at -80\(^\circ\)C until HPLC analysis. Remaining filtered aqueous fractions was used to assess uptake and anti-inflammatory activity of select compounds from digested rice using differentiated monolayers of Caco-2 human intestinal cells. Bioaccessibility is defined as the amount of the bioactive compound in the cooked rice that partitioned in the filtered aqueous fraction during simulated digestion to become available for uptake and possibly transport across small intestinal absorptive epithelial cells.

2.4 Uptake and retention of bioactive compounds by Caco-2 Cells
Caco-2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were seeded and maintained in complete medium as previously described. Differentiated cultures of Caco-2 cells were used between passages 24-35 and experiments were performed 11-14 days after monolayers became confluent. Cell uptake of indicated bioactive compounds from the diluted aqueous fraction generated during digestion of BR and PGBR was determined for replicate cultures. Monolayers were washed with basal DMEM before addition of 2 mL of diluted aqueous fraction containing (1.5 mL of DMEM and 0.5 mL aqueous fraction) and incubated for 4h at 37 °C in a humidified atmosphere of 95% air/5% CO₂ (v/v). To examine intracellular retention of bioactive compounds, spent medium was removed by aspiration and monolayers were first washed once with phosphate-buffered solution (PBS) containing 2 g/L bovine albumin before adding fresh medium without the bioaccessible fraction of digested rice and incubated for an additional 16 h. Cells were collected and analyzed by HPLC. Cell protein content was measured by bicinchoninic acid (BCA) method.

2.5 Extraction and analyses

The protocol for GABA analysis was modified as described elsewhere. Briefly, uptake of active compounds by Caco-2 cells was examined by incubation of thawed cells at 37 °C with protease from bovine pancreas (10 mg/mL in PBS) for 30 min. Then, 1.5 mL of 1% sodium dodecyl sulfate (SDS)-ethanol was added and mixed for 1 min prior to centrifugation at 6,000 g at 4°C for 10 min. An equal volume of 9-fluorenylmethyl chloroformate (FMOC-Cl) was added to the supernatant and incubated for 15 min. Aliquots (2 mL) were filtered and applied to a Vertical UPSIC18 column (4.6 x 250 mm; 5 µm; Vertiscp™, Bangkok, Thailand) with derivatives detected with excitation at 270 nm and emission at 315 nm. Quantities were determined by comparison of AUC with a standard curve of pure GABA derivatized with FMOC-Cl. Vitamin E and gamma-oryzanol content were analyzed by the method of Chen & Bergman. After thawing, homogenized BR and PBGR and filtered aqueous fraction of chyme were extracted with 2 volumes of hexane: acetone (2:1:1), mixed for 10 min and centrifuged at 5,000 g for 10 min. The supernatant was evaporated to dryness and the film re-solubilized in absolute methanol prior to analysis by HPLC. Thawed cells were extracted with hexane: acetone: ethanol as above. Vitamin E and γ-oryzanol were separated using a C18 90A column (3.9 x 150 mm; inner diameter 5 mm; Water Resolve™). The γ-oryzanol and vitamin E were separated by gradient elution program and quantified by comparing peak areas with calibration curves. Vitamin E was detected by fluorescence with excitation at 298 nm and emission at 328 nm. γ-oryzanol was detected with a photodiode array detector at 325 nm. Phenolic acids were analyzed according to Tian et al. (2004). Thawed and homogenized BR and PBBR and aqueous fraction of chyme were hydrolyzed with 1N NaOH for 3 h and acidified to pH 1.0 with HCl before extracting twice with an equal volume of ethyl acetate for 10 min before centrifugation at 5,000 g for 10 min. The pooled supernatant was dried under a stream of N₂ gas and re-solubilized in 50% methanol prior to analysis by HPLC. To determine the presence of phase II conjugates of phenolic acids, cell pellets were incubated with 425 units of β-glucuronidase and 25 units of sulfatase from Helix pomatia in 0.1 M sodium acetate buffer, pH 5.0, at 37 °C for 16 h before extracting as above. The phenolic contents were separated by Zorbax Eclipase XDB-C18 column (4.6 x 150 mm;
inner diameter 5 mm; Agilent Technologies) at 30 °C using previously described gradient elution programs. Compounds were identified by comparison of retention time and spectra and quantified by comparison of AUC with pure standards of gallic, 4-hydroxybenzoic, chlorogenic, vanillic, caffeic, syringic, p-coumaric, ferulic, sinapic and trans-cinnamic acids at 325 nm.

2.6 Anti-inflammatory activity of bioaccessible fraction with Caco-2 cells

As above, washed monolayers of differentiated cultures of Caco-2 cells were incubated with 2 mL of diluted aqueous fraction generated during simulated digestion of rice for 4h. Spent medium was removed and the monolayer was washed with fresh DMEM. \( H_2O_2 \) (1 mmol/L final concentration) was added to medium for 30 min, monolayers were washed with basal medium and activated by addition of fresh medium containing human IL-1β (10 ng/mL final concentration). Cultures were incubated for an additional 20 h before collecting medium for measuring IL-8 and MCP-1 by ELISA as previously described. Concentrations of IL-8 and MCP-1 were calculated by comparing absorbance with the curve generated using standards.

2.7 Intracellular reactive oxygen species (ROS)

Control and treated monolayers were washed with warm PBS prior to addition with 5 \( \mu \)M dichlorofluorescine diacetate (DCF-DA). After incubating at 37 °C for 30 min, monolayers were washed with PBS and lysed with 0.5% Triton X-100 in cold PBS. Lysate was centrifuged at 14,000 g for 5 min at 4 °C. Fluorescent intensity of supernatant was determined using excitation wavelength of 485 nm and emission wavelength at 530 nm with a microplate reader (BioTek® Instruments, Vermont, USA).

2.8 Cytotoxicity test.

The non-toxic concentration of bioaccessible fraction with BR and PGBR on for Caco-2 cells activated with \( H_2O_2 + \) IL-1β was determined in a preliminary study. Diluted (1:4) filtered aqueous fractions were incubated with fully differentiated monolayers of Caco-2 cells for 4 h prior to activation with 1 mM \( H_2O_2 \) for 30 min and washed with basal medium before activation with 10 ng/mL of human IL-1β for an additional 20 h. Viability of treated cells was assessed by microscopic observation of the monolayer and by the sulforhodamine B (SRB) assay. Caco-2 cells were washed with phosphate buffered saline (PBS) before initiating the SRB assay and the absorbance was monitored at 500 nm. Activated (\( H_2O_2 + \) IL-1β) Caco-2 cells similarly incubated with diluted aqueous fraction generated within rice (control digestion) was arbitrarily assigned the value of 100%. Acceptable viability of treated cells was set at > 90%.

2.9 Data analyses

SPSS version 16 was used for statistical analyses. All parameters were conducted in triplicate and each experiment was independently performed at least twice. The descriptive statistics including mean and SD were calculated for percent
bioaccessibility and cellular uptake of bioactive compounds, IL-8, MCP-1 and ROS. Means were analyzed by one-way
ANOVA when appropriate following with Tukey’s multiple comparisons or t-test. Differences were considered significant at
\( p < 0.05 \).

3. Results

3.1 Content of GABA, γ-oryzanol, γ-tocotrienol and phenolic acids in BR and PGBR

GABA, γ-oryzanol, γ-tocotrienol and phenolic acids were present in both BR and PGBR. The amounts of GABA, γ-oryzanol,
γ-tocotrienol, ferulic acid, p-coumaric acid in PGBR were 76%, 10%, 12%, 15% and 84% greater (\( p < 0.01 \)) in PGBR than
in BR (Table 1).

3.2 Bioaccessibility of GABA, gamma-oryzanol, gamma-tocotrienol and phenolic acids

The relative efficiency with which GABA (approx. 60%), γ-oryzanol (approx. 50%) and γ-tocotrienol (approx. 38%) in BR
and PGBR were transferred to the aqueous fraction of chyme was not significantly affected by differences in the two food
matrices (Table 2). However, because the actual amounts of these three compounds in PGBR were greater than in BR (Table
1), the quantity of each compound in the bioaccessible fraction was significantly higher in the aqueous fraction of digested
PGBR. The efficiency of transfer and the quantities of ferulic acid and p-coumaric acid from the cooked matrix to the
bioaccessible fractions of digested BR and PGBR (10% – 15%) were markedly less than those for GABA, γ-oryzanol and γ-
tocotrienol. To confirm that the bioactive compounds in the filtered aqueous fraction of digested rice were bioaccessible,
apical uptake and cellular retention of GABA, γ-oryzanol, γ-tocotrienol, ferulic acid and p-coumaric acid were assessed
using monolayers of differentiated Caco-2 cells. Relative extent of apparent uptake from medium during 4h incubation
differed for the test compounds with γ-oryzanol, γ-tocotrienol > GABA > ferulic acid and p-coumaric acid and independent
of type of rice (Table 3). The cell content of all five compounds and the aqueous fraction generated by simulated digestion
of PGBR were greater than for BR after exposure to diluted aqueous fraction from digested PGBR and BR. There was no
evidence of cytotoxicity as evidenced by changes in gross morphology of cells, reduction of SRB and protein content per
well when cells were exposed to medium containing aqueous fractions from control digestion (no rice) or aqueous fractions
generated from digested BR and PGBR for 4 h. Treatment of cell pellet with β-glucuronidase and sulfatase revealed that
both ferulic and p-coumaric acids were conjugated by phase II enzymes. Cells retained 70-90% of such active compounds
after overnight incubation in medium without rice, except for ferulic acid from PGBR for which only 45% was retained in
cells suggesting considerable metabolism, efflux and/or degradation of this compound (Table 4). Treatment of cells and
spent medium at 16 h with β-glucuronidase and sulfatase revealed that phase II conjugates of ferulic and p-coumaric acids
accumulated in cells and that both free and conjugated forms effluxed from cells exposure into medium after addition of
fresh medium. The amounts of all five compounds from digested PGBR retained in the cells were significantly greater than in cells incubated in medium with aqueous fraction from digested BR (Table 4).

3.3. Pre-incubation of Caco-2 cells with the bioaccessible fraction of BR and PGBR suppresses secretion of IL-8 and MCP-1 in response to H$_2$O$_2$+IL-1β.

Following incubation of cultures of Caco-2 cells with the diluted bioaccessible fraction from digested rice for 4h, spent medium was removed and washed monolayers were first exposed to H$_2$O$_2$ for 30 min followed by IL-1β 10 ng/mL. After overnight incubation, medium was collected to quantify IL-8, a pro-inflammatory chemokine. Control cells incubated with the oxidant + IL-1β secreted 63-fold more IL-8 into medium than control cultures (Fig. 1). Pre-incubation of Caco-2 cells with the bioaccessible fraction containing compounds from digested BR and PGBR resulted in 20% and 30%, respectively, declines in secretion of IL-8 in response to H$_2$O$_2$+IL-1β (Fig. 1A). The suppressive effect of pre-treatment of cells with diluted aqueous fraction from digested PGBR was significantly greater than that of BR. Similarly, Caco-2 cells activated with H$_2$O$_2$+IL-1β secreted 35-fold more MCP-1 than control cultures. Pretreatment of the monolayer with the bioaccessible fraction from digested BR and PGBR inhibited MCP-1 secretion by 25% and 35%, respectively (Fig. 1B). The inhibitory activity of the diluted aqueous fraction from digested PGBR was significantly greater than that from digested BR.

Collectively, these data suggest that the anti-inflammatory activity of the bioaccessible fraction generated by digesting PGBR was more potent than that of digested BR due to the increased concentrations of the bioactive compounds of interest.

3.4. Bioaccessible fraction of BR and PGBR decreases H$_2$O$_2$+IL-1β induced intracellular accumulation of ROS.

Incubation of Caco-2 cells with H$_2$O$_2$+IL-1β significantly elevated intracellular ROS compared to that in the control culture (Fig.2). Pre-incubation of cultures with the bioaccessible fraction of digested BR and PGBR before exposure to H$_2$O$_2$+IL-1β decreased intracellular ROS by 20% and 35%, respectively, compared with the H$_2$O$_2$+IL-1β activated cells (Fig. 2). The suppressive effect of pre-treatment with digested PGBR was significantly greater than that of BR. These results suggest that exposure of Caco-2 cells to the bioaccessible fraction from digested PGBR provided cells with greater scavenging capacity than that of digested BR.

4. Discussion

Rice is a staple food consumed by more than one half of the global population. Although brown rice is more nutritious than that of polished or white rice, its poor texture, low digestibility and difficulty of cooking brown rice result in less preference of the consumer for brown compared to white rice. Germination is a strategy for improving cereal quality. Several studies have demonstrated anti-inflammatory and antioxidant activities of GABA, 50,51 γ-oryzanol, 52-54 γ-tocotrienol, 55,56 and ferulic acid 57,58 and their concentrations are elevated in germinated brown rice. Thus, the present study measured GABA, γ-
tocotrienol, γ-oryzanol, ferulic acid and p-coumaric acid by HPLC and confirmed that the quantities of such compounds in cooked parboiled germinated brown rice (PGBR) was significantly greater than that in cooked brown rice (BR). Also, the amounts of these bioactive compounds that partitioned in the bioaccessible fraction of chyme generated during *in vitro* digestion of cooked PGBR and were subsequently accumulated and retained by Caco-2 intestinal cells was proportionally greater than that for digested BR. Finally, the intracellular quantities of GABA, γ-oryzanol, γ-tocotrienol, phenolic acids and likely other unknown compounds after digesting both cooked rices were correlated with the extent of suppression of IL-8 and MCP-1 secretion by the Caco-2 cells, as well as ROS production, in response to activation by H2O2 + IL-1β.

The cooked PGBR contained significantly greater GABA than cooked BR. Previous studies also have reported increased GABA content after soaking and germination of brown rice.8,59,60 However, it is difficult to compare the absolute amount of GABA content with other previous studies because they analyzed GABA content from the uncooked rice grain and the condition for germination of brown rice differed among studies. Cooked PGBR had 10% γ-oryzanol higher than that of cooked BR which confirms a previous report.8 As also previously reported, γ-tocotrienol was the predominate form of vitamin E in BR and PGBR and cooked PGBR contained 12% greater quantity of this compound than that in cooked BR.61-65 We detected ferulic acid and p-coumaric acids in both rices with cooked PGBR containing a greater amount than cooked BR which also agrees with previous reports.12,66-68

γ-amino butyric acid (GABA) is a major inhibitory neurotransmitter in the adult human brain,69 but it is excitatory in the developing brain.70,71 Beside the central nervous, GABA is also found in several organs including pancreas, pituitary, testes, gastrointestinal tract, ovaries, placenta, uterus and adrenals.72 In addition, GABA was reported to modulate immune response by inhibiting pro-inflammatory CD4+ T cell responses, modulating the cytotoxicity of CD8+ T cells *in vitro* and inhibiting cell autoimmunity and inflammatory responses in a mouse model of type-I diabetes.73 Oral GABA treatment also down-regulated inflammatory responses in a mouse model of rheumatoid arthritis.74 The transfer of GABA and γ-oryzanol in BR and PGBR to the aqueous fraction during digestion was relatively efficient (~ 60% for GABA and ~50% for γ-oryzanol). These results differ from those of Mandak and Nyström75 who reported negligible bioaccessibility of γ-oryzanol from rice.75 γ-oryzanol is a mixture of ferulic acid esters of sterol and triterpene alcohols in rice bran oil and presumably requires co-consumption of oil for transfer to mixed micelles during digestion like other dietary fat soluble compounds.76,77 Thus, the observation of Mandak and Nyström of poor bioaccessibility of γ-oryzanol in rice was likely resulted from the absence of exogenous oil during digestion. The bioaccessible fraction of PGBR also contained a higher amount of γ-tocotrienol than that of BR and 37-38% of γ-tocotrienol was transferred to the micelle fraction. The present results agree with a recent report that 42% of α-tocopherol in salad puree containing 3% soybean oil was bioaccesssible.78 In the present study, intracellular content of ferulic acid and p-coumaric acid was less than 1% that in the medium. However, treatment of medium and cells with β-glucuronidase/sulfatase indicated extensive conjugation and efflux as reported.79 Dihydroferulic acid has been
reported to be the most abundant conjugate followed by dihydroferulic acid-4-O-sulfate, ferulic acid-4-O-sulfate and a trace amount of ferulic acid-4-O-glucuronide.\(^{80}\)

Caco-2 intestinal cells have been used as a model to investigate the potential anti-inflammatory activities of compounds in foods via modulation of pro-inflammatory cytokine and chemokine production.\(^{39,81}\) The fully differentiated human intestinal epithelial Caco-2 cell increases secretion of numerous chemokines and cytokines in response to exposure to pro-inflammatory and chemical insults.\(^{39,81}\) Both IL-8 and MCP-1 are potent chemokines that induce migration of leukocytes to sites of inflammation.\(^{82,83}\) Attenuation of the secretion of such chemokines represents a promising therapeutic strategy for gut inflammatory disorders.\(^{84}\) To our knowledge, this is the first study to assess anti-inflammatory and antioxidant activity of digested BR and PGBR using this cell model. GABA has been reported in LPS-induced RAW264.7 cell line by to suppress LPS-induced iNOS, IL-1β, and TNF-α mRNA expression in RAW264.7 cells.\(^{51}\) The same study also found that topical daily application of 0.1-10 mmol GABA for 10 days on the excisional open dorsal wounds of Sprague-Dawley rats accelerated healing in a dose dependent manner by suppressing inflammation and stimulating re-epithelialization to a greater extent than treatment with epidermal growth factor. Also, dietary administration of gamma-oryzanol or ferulic acid were found to markedly inhibit the severity of dextran sodium sulfate (DSS)-induce colitis.\(^{53}\) Similarly, dietary intake of coumaric acid suppressed DSS-induced oxidative DNA damage \((p < 0.01)\), over-expression of COX-2 and restored superoxide dismutase gene expression in colitis rats.\(^{85}\) Our observation that pre-treatment of Caco-2 cells with aqueous fraction from digested PGBR and BR decreased ROS after exposure to \(\text{H}_2\text{O}_2 + \text{IL-1}\beta\) aligns with several in vitro and in vivo observations demonstrating the anti-oxidant activity of these foods. Germinated brown rice extracts increased hydroxyl radical scavenging activities in \(\text{H}_2\text{O}_2\)-treated HepG2 cells.\(^{86}\) Consumption of cooked brown rice and germinated brown rice improved glycaemia and kidney hydroxyl radical scavenging activities, and prevented the deterioration of total antioxidant status in diabetic rats.\(^{87}\) We also have found that dietary PGBR was more efficacious than an equivalent amount of BR for attenuating CCL\(_4\)-induced liver fibrosis in rats (manuscript in preparation). Collectively, our results suggest that bioaccessible GABA, \(\gamma\)-oryzanol, \(\gamma\)-tocotrienol, ferulic acid and \(\beta\)-coumaric acid from BR and PGBR contribute to the suppressive response of the combined oxidative and pro-inflammatory stress. However, other unidentified compounds in the bioaccessible fractions from the digested cooked rice may have contributed to the anti-inflammatory and antioxidant activities observed in the Caco-2 cell model. Additional pre-clinical and clinical studies are needed to further confirm the efficacy of BR and PGBR for gut and systemic inflammatory disorders.

### Acknowledgments

This work was supported by Agricultural Research Development Agency (ARDA). The authors would like to thank Professor Mark Failla at The Ohio State University for his helpful commentary during manuscript preparation.
Notes and references

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Tables and figures

Table 1 Content of GABA, γ-oryzanol, γ-tocotrienol and phenolic acids (µg/g dry weight) in cooked BR and PGBR

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<th>Compounds</th>
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<th>PGBR</th>
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<td>GABA</td>
<td>162 ± 2.0</td>
<td>286 ± 11.9b</td>
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<tr>
<td>γ-oryzanol</td>
<td>687 ± 43.5</td>
<td>756 ± 26.5a</td>
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<tr>
<td>γ-tocotrienol</td>
<td>117 ± 6.9</td>
<td>130 ± 6.1a</td>
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<tr>
<td>Ferulic acid</td>
<td>233 ± 15.9</td>
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<tr>
<td>p-coumaric acid</td>
<td>56 ± 1.5</td>
<td>104 ± 9.9b</td>
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Data are the mean ± SD (n=9) of three independent experiments. Statistical analysis of each bioactive compound between BR and PGBR was analyzed by unpaired t-test. Superscript indicated bioactive content in BR and PGBR differs significantly; a, \(p < 0.01\); b, \(p < 0.001\).

Table 2 Percent bioaccessibility and content of GABA, γ-oryzanol, γ-tocotrienol and phenolic acids of BR and PGBR

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<th>% Bioaccessibility</th>
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<tr>
<td></td>
<td>BR</td>
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<tr>
<td>GABA</td>
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<td>γ-oryzanol</td>
<td>50 ± 1.3</td>
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<td>γ-tocotrienol</td>
<td>37 ± 1.8</td>
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<tr>
<td>ferulic acid</td>
<td>11 ± 0.9</td>
<td>13 ± 0.7(^*)</td>
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<td>p-coumaric acid</td>
<td>10 ± 0.4</td>
<td>15 ± 1.8(^*)</td>
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</tbody>
</table>

Data are the mean ± SD (n=9) of three independent experiments. Means of the relative and actual amounts of each compound partitioning in the filtered aqueous fraction after digestion of BR and PGBR were analyzed for significant differences by unpaired t-test. Asterisk (*) as superscript indicates that the % bioaccessibility of each compound in BR and PGBR differs significantly \((p < 0.01)\). Superscripts (a) and (b) indicate significant differences in the quantity of each active compound in filtered aqueous fraction generated during simulated digestion of BR and PGBR; a \((p < 0.01)\); b \((p < 0.001)\).
Table 3  Apparent uptake and amounts of GABA, γ-oryzanol, γ-tocotrienol and phenolic acids in Caco-2 cells incubated with diluted aqueous fraction from digested BR and PGBR.

<table>
<thead>
<tr>
<th>Bioactive compound</th>
<th>% apparent cell uptake</th>
<th>Amount (pmol/mg cellular protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BR</td>
<td>PGBR</td>
</tr>
<tr>
<td>GABA</td>
<td>5.1 ± 0.5</td>
<td>6.0 ± 0.5*</td>
</tr>
<tr>
<td>γ-oryzanol</td>
<td>10.8 ± 0.4</td>
<td>11.4 ± 0.6</td>
</tr>
<tr>
<td>γ-tocotrienol</td>
<td>9.5 ± 0.8</td>
<td>10.9 ± 0.6*</td>
</tr>
<tr>
<td>ferulic acid</td>
<td>0.11± 0.01</td>
<td>0.16 ± 0.01*</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>ND#</td>
<td>0.14 ± 0.01*</td>
</tr>
</tbody>
</table>

Data are the mean ± SD (n=6) of two independent experiments. Statistical analysis of each bioactive compound between BR and PGBR was analyzed by unpaired t-test. # ND, below level of detection. Asterisk (*) as superscript indicates that the apparent percentage of cellular uptake of each compound in the diluted aqueous fraction of digested BR and PGBR differs significantly (p < 0.01). Superscript (a) indicates a significant difference in the amount of the active compounds in cells exposed to aqueous fraction of digested BR and PGBR; p < 0.001.
Table 4 Percent cell retention and amount of GABA, γ-oryzanol, γ-tocotrienol and phenolic acids by Caco-2 cells after 16 h exposure to aqueous fraction of BR and PGBR.

<table>
<thead>
<tr>
<th>Bioactive compound</th>
<th>% Cell retention</th>
<th>Amount (pmol/mg cellular protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BR</td>
<td>PGBR</td>
</tr>
<tr>
<td>GABA</td>
<td>89.8 ± 2.0</td>
<td>90.4 ± 0.5</td>
</tr>
<tr>
<td>γ-oryzanol</td>
<td>70.6 ± 2.2</td>
<td>70.7 ± 5.3</td>
</tr>
<tr>
<td>γ-tocotrienol</td>
<td>86.2 ± 3.5</td>
<td>86.4 ± 3.0</td>
</tr>
<tr>
<td>ferulic acid</td>
<td>87.6 ± 6.4</td>
<td>45.4 ± 4.01</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>ND</td>
<td>86.3 ± 2.85</td>
</tr>
</tbody>
</table>

Data are the mean ± SD (n=6) of two independent experiments. Statistical analysis for each bioactive compound between BR and PGBR was analyzed by unpaired t-test. # ND, below level of detection. Means of the relative and actual amounts of each compound retained in cells 16 h after 4 h pre-incubation with diluted aqueous fraction from digested BR and PGBR. Asterisk (*) as superscript indicates that the relative extent of retention of each compound differs significantly (p<0.001). The presence of a letter as superscript indicates that the mean amount of the compound retained in the cell after pre-treatment with aqueous fraction generated during digestion of BR and PGBR differ significantly; a, p<0.01; b, p<0.001.
Fig. 1. Pre-treatment of Caco-2 cells with the bioaccessible fraction of digested BR and PGBR suppresses the secretion of IL-8 and MCP-1 in response to exposure to H$_2$O$_2$+IL-1β. Differentiated cultures of Caco-2 cells were incubated for 4 h either with control medium (bars 1 and 2) or with the bioaccessible fraction from digested BR (bar 3) or PGBR (bar 4). Medium was removed after 4 h before addition of fresh medium without (control; bar 1) or with H$_2$O$_2$ + IL-1β as described in Methods. Medium was collected after 20 h to quantify IL-8 (A) and MCP-1 (B). Data represent mean ± SD for 6 replicate cultures. Different letters above the error bars indicate that the mean quantities for the indicated treatments differ significantly (p<0.05).
Fig. 2 Intracellular ROS produced by Caco-2 cells exposed to H$_2$O$_2$+IL-1β is decreased by pre-treatment with the bioaccessible fraction of BR and PGBR. Caco-2 cells were treated as described in legend for Figure 1. Medium with DCF-DA was added to the washed monolayers to measure intracellular ROS as described in Methods. Data represents mean ± SD for 6 replicates. Different letters above the error bars indicated that mean ROS differ significantly ($p<0.05$).
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