Brown Rice Extract Ameliorates Oxidative Status in Human Placental Cell Line (JEG-3) Induced by Cadmium Chloride

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Abstract

Cadmium (Cd) can induce oxidative stress by producing reactive oxygen species (ROS) and affect the antioxidant defense systems, leading to cellular damage. Supplementation with antioxidants may protect against Cd-induced oxidative stress. This study aimed to investigate the effect of germinated brown rice (GBR), brown rice (BR), and white rice (WR) extracts against Cd-induced toxicity in human placenta (JEG-3) cells. The cytotoxicity of Cd and rice extracts was measured by MTT assay, and pretreatments with GBR, BR, and WR before Cd exposure were examined. Intracellular ROS levels and the expression of SOD2 and GPx4 were determined by DCFH-DA assay and western blot, respectively. An antioxidant N-acetylcysteine (NAC) was used as a positive control. The antioxidant capacity and total phenolic content of rice extracts were investigated by DPPH and Folin-Ciocalteu methods, respectively. The GBR extract exhibited the highest antioxidant activity and total phenolic content, followed by BR and WR, respectively. Cd decreased cell viability in a dose-dependent manner, whereas incubation with 0.1 - 0.5 mg/mL showed no toxicity in all rice extracts. Moreover, Cd treatments significantly ($p < 0.05$) increased ROS levels. Conversely, treatments with GBR and BR extracts decreased ROS. Similar results were observed for combinatorial treatments of GBR and BR with Cd, except for WR treatment. Additionally, significant increased SOD2 and decreased GPx4 expression levels were evident in the GBR group and in both GBR and BR pre-treated groups, respectively, compared to Cd exposure alone. These results suggest that brown rice pre-treatments may ameliorate oxidative status induced by Cd in cultured human placental cells.

Keywords: Cadmium, JEG-3, Brown rice, Oxidative status, Antioxidant

Introduction

Cadmium (Cd), a toxic metal ion, has been known as a high risk for health problems, through either acute or long-term exposure. Cd can be found in all areas, including air, water, soil, tobacco, and food [1], inducing toxicity in various organs, such as the liver, kidneys, lungs, and gastrointestinal tract, and the reproductive, respiratory, and skeletal systems [2-5]. Cd can induce abnormalities in the fetus when administered during gestation. In pregnant rats, feeding with Cd caused organ malformations of the fetus [6]. In humans, during pregnancy, cadmium exposure via the environment and/or cigarette smoking leads to fetal growth restriction [7] and human infertility [8] and induces spontaneous abortion [9].

It is known that Cd induces oxidative stress by producing reactive oxygen species (ROS) [10,11], such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals ($HO^-$), and affects the antioxidant defense systems, leading to cellular damage by enhancing lipid peroxidation, protein oxidation, and oxidized DNA [12].
Within the cells, antioxidants play a vital role to protect cells against the adverse effects of free radicals. These antioxidants include enzymatic antioxidants [superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)] and non-enzymatic antioxidants [glutathione, uric acid, and bilirubin]. In addition, dietary antioxidants can also contribute to cellular protection against ROS, such as vitamin E, vitamin C, carotenoids, and flavonoids [13].

A previous study [14] demonstrated that vitamin E supplements in rats reduced lipid peroxidation and hemolysis of red blood cells due to oxidative damage induced by cadmium chloride (CdCl₂). Another recent study [15] in pregnant rats revealed that vitamin E and wheat germ oil supplements can eliminate the toxic effects of Cd via increasing antioxidant enzyme activities in both mothers and fetuses, when compared treatment with Cd only. This suggests that antioxidants can exert a protective role against Cd-induced oxidative stress.

Rice (Oryza sativa L.) is a staple food worldwide, especially in Asian countries. Polished rice or white rice (WR) seeds are widely consumed but, at present, brown rice (BR) is popular because of its high nutrition [16]. The outer layer parts of BR grains or rice bran contain beneficial nutritives, but most of these parts are removed during the milling process. One of the major bioactive components in BR is γ-oryzanol, a mixture of ferulate esters and plant sterols [17], and these components are intensely increased in germinated brown rice (GBR) during the germination process [18]. γ-Oryzanol has important biological properties, including cholesterol-lowering [19], anti-inflammatory [20], anticancer [21], antidiabetic [22], and antioxidant activities [23].

The aim of this study was to investigate a possible protective effect of brown rice, germinated brown rice, and white extracts on the antioxidant defense system in human placenta JEG-3 cells treated with Cd. NAC was used as an antioxidant standard. The selected biochemical parameters were intracellular ROS and expressions of antioxidant proteins, SOD2 and GPx4.

Materials and methods

Cell culture

JEG-3 cells (HTB-36; ATCC, Rockville, USA) were cultured in Eagle’s minimal essential medium (MEM) (Corning, USA), containing 10 % fetal bovine serum (Gibco, USA) and antibiotics (Gibco, USA) at 37 °C in a humidified atmosphere of 5 % CO₂ and 95 % air. The study was approved by Naresuan University Institutional Review Board (IRB. No.297/58).

Rice extracts preparation

Thai brown rice (Khao Dawk Mali 105; KDML105) was obtained from Lopburi Rice Research Center, Thailand. The germination method was modified from Patil and Khan [24]. Briefly, 100 g of BR was soaked in 200 mL of sterile water at room temperature for 6 h and germinated for 24 h. The extraction process was modified from a previous study [25]. Firstly, the WR, BR, and GBR were ground into powder and extracted twice in 70 % (v/v) methanol solution. Then, the mixture was shaken for 20 h at 37 °C, filtered, and evaporated by rotary evaporation (BUCHI, Switzerland). The crude rice extracts were weighed and dissolved in dimethyl sulfoxide (DMSO) (final concentration 0.1 %) as the stock solution.

Determination of cell viability

Cells were grown at a density of 2×10³ cells/well in a 96-well plate and treated with various concentrations of CdCl₂ and rice extracts (WR, BR, and GBR) for 24 and 48 h, respectively. Culture supernatants were then removed and replaced by 180 μL of fresh free-serum medium. Following this, 20 μL of MTT solution (5 mg/mL) was added into each well. After 2 h, the unreacted reagent was removed, the insoluble formazan crystals were dissolved in DMSO (100 μL/well), and absorbance at 595 nm was measured using a microplate reader (Biotek, USA). The cell viability was determined as percentage over control.
Assessment of intracellular ROS levels

To study the effects of Cd and 3 rice (WR, BR, and GBR) samples on ROS levels, the 2′, 7′-dichlorofluoroscein diacetate (DCFH-DA) was used. Briefly, cells were divided into 3 treatment groups. In the first 2 groups, cells were treated with Cd and rice samples for 24 h and 48 h, respectively. For the third group, cells were pre-treated with WR, BR and GBR extracts for 48 h, followed by 24 h Cd exposure. For a positive control group, cells were pre-treated with N-acetylcysteine (NAC) for 48 h. After treatments, cells were then washed with 0.1 mL of PBS per well. Following this, 100 μM DCFH-DA was added and the cells were incubated for 30 min at 37 °C. The excess DCFH-DA was then discarded, and cells were washed with PBS. In the presence of ROS, DCFH was oxidized to highly fluorescent dichlorofluorescein (DCF), which was immediately measured using a microplate reader (Biotek, USA) at excitation and emission wavelengths of 485 and 530 nm, respectively. The intracellular DCF fluorescence can be used as an index to quantify the overall ROS within treated cells compared with the control.

Measurement of protein expression

To determine the effects of Cd and rice extracts (WR, BR, GBR) on the expression of protein associated with antioxidant defense system (SOD2 and GPx4), after treatments, cells were lysed in RIPA buffer (Sigma-Aldrich, USA) including protease inhibitor cocktail (AMRESCO, USA & Canada). Total protein concentrations were measured by BCA protein assay kit (Thermo Scientific, USA). 60 μg of total protein was separated by SDS-PAGE and transferred onto 0.45 μm PVDF membrane (Millipore, USA). The membranes were blocked with 5 % non-fat dried milk (BIO BASIC, Canada) in TBST [Tris-buffered saline containing 0.1 % v/v Tween 20 (AMRESCO, USA & Canada)] for 1 h; the membranes were then incubated overnight at 4 °C with specific primary antibodies: GPx4 (Boster Biological Technology, CA, USA), and SOD2 and β-actin (Cell Signaling Technology, USA). After that, horseradish peroxidase conjugated secondary antibodies (Millipore, USA) were incubated for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (Millipore, USA) and a chemiluminescence imager instrument (Image Quant LAS 500, GE Healthcare Life Sciences, Sweden). For quantification, the densities of each band were determined by Image J analysis software (National Institutes of Health (NIH), USA).

Determination of antioxidant activity and total phenolic content

To measure the antioxidant activity of WR, BR, and GBR extracts, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and total phenolic content were selected. Briefly, in a 96-well plate, 10 μl of all rice samples were added to 140 μl methanol solution of DPPH radical solution (0.12 mM final concentration). The mixture was mixed well and left in the dark at room temperature for 30 min. Thereafter, the absorbance for the sample (A\textsubscript{sample}) was measured using a microplate reader at 515 nm, compared to methanol blank. A negative control (A\textsubscript{control}) was 140 μl of DPPH radical solution in the respective extraction solvent. The percent of DPPH scavenging activity of the sample was calculated by the equation:

\[
\frac{(A\textsubscript{control} - A\textsubscript{sample})}{A\textsubscript{control}} \times 100
\]

The free radical scavenging capacity of WR, BR, and GBR extracts was expressed as an equivalent of that of Trolox. The results were calculated and expressed as millimoles curves of Trolox equivalent antioxidant capacity (TEAC) per g of rice extracts using the standard of Trolox. The linearity range of the standard curve was 0.005 to 0.025 mM (r = 0.99)

The total phenolic content was examined by a Folin-Ciocalteu assay using gallic acid as the standard. The mixture of the reaction included sample solution (10 μl) and distilled water (215 μl), and 25 μl of Folin-Ciocalteu solution (25 μl) was mixed and incubated for 30 min at room temperature. The absorbance was then measured at 765 nm, using distilled water as a blank. The total phenolic content of the sample was calculated as the gallic acid equivalents (mg of GAE/g sample) by the standard curve of gallic acid. Linearity of the curve was 0.2 to 1 mg/mL (r = 0.99).
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Statistics
Data were expressed as the means ± S.D. The comparison between groups was performed by ANOVA analysis and Tukey’s HSD multiple comparisons. Differences of \( p < 0.05 \) were considered to be statistically significant.

Results and discussion
In the present study, Cd induced high toxicity in JEG-3 cells, even at very low concentrations. As shown in Figure 1A, viability of JEG-3 cells was dramatically decreased and 50% of cell survival was shown at 0.9 µM Cd exposure (IC50 = 0.9 µM). Meanwhile, cytotoxicity in all rice samples was not seen at concentrations ≤ 0.5 mg/mL (Figure 1B). The cytotoxic effect of Cd was presented by morphology changes (Figure 1C), including cell shape becoming round, dying cells beginning to detach, and the disappearance of nuclear structure [26].

![Figure 1](image)

Figure 1 Viability of JEG-3 cells incubated for 24 h and 48 h with different concentrations of Cd (A) and rice extracts (WR, BR, GBR) (B), respectively. Cell morphology changes were shown after 24 h Cd exposure (C). Solid arrowhead in (C) indicates round-shape and disappearance of nuclear structure. Results are the mean ± SD of the absorbance expressed as the percentage related to control.

Previous studies revealed that Cd, per se, is unable to generate ROS, but can do so through depletion of thiol status, such as glutathione and protein-bound sulphhydryl groups [27] and disturb the antioxidant defense system [28]. As a consequence, oxidative stress induced by Cd causes cell damage and cell death [10,11,14,29,30]. The toxic effect of Cd to placenta cells has been reported in human [31-33], animal [34], and cultured cell [24,35] models. These studies were concerned with the considerable results regarding placental structure and function for pregnancy complications, such as fetal growth restriction or development. Taken together, treatment or supplement with antioxidants may play a role to protect the cells from Cd toxicity. In the present study, pre-treatment with rice extract at a concentration of 0.5 mg/mL was chosen to investigate its effect on ROS levels induced by Cd in cultured JEG-3 placenta cells.

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As shown in Table 1, ROS levels significantly increased (p < 0.05) in JEG-3 cells exposed to various concentrations of Cd in a dose-dependent manner, whereas those pre-treated with the rice extracts decreased their levels in JEG-3 cells after exposure to Cd. The GBR (90.36 % ± 1.56) showed the most efficiency to reduce ROS levels at a concentration of 0.5 mg/mL, followed by BR (91.59 % ± 3.72) and WR (96.63 % ± 3.39) extracts, compared to untreated cells. As a positive control, an antioxidant NAC decreased ROS levels by 26 % (89.08 % ± 4.27).

For pre-treatments with rice extracts, it was indicated that pre-treatment with 0.5 mg/mL of GBR and BR (Table 1) for 48 h before exposure to 0.05 - 0.3 µM Cd was more efficient in decreasing ROS levels than WR when compared with the low concentration of Cd (0.05 µM) treated group. Nevertheless, the protective effect of rice pre-treatments were not presented at high concentrations of Cd (≥ 0.1 µM) exposure. However, positive control NAC pre-treatments effectively decreased ROS levels in all Cd-treated groups. This revealed that pre-treatment with antioxidants can influence antioxidative status in JEG-3 cells induced by Cd, as reflected by lowering ROS.

Brown rice has more nutritional components than white rice, which mainly come from the outer layer of rice grains or rice bran and are lost during polishing [36]. These components include γ-aminobutyric acid (GABA), dietary fiber, phytic acid, tocotrienols, and gamma-oryzanol, and these components may be enhanced in germinated brown rice during the germination process [36,37]. Their biological activities are useful for health effects such as lowering blood pressure, preventing heart disease, and preventing colon cancer [18]. Recent studies reported that rice bran extract could be an effective antioxidant due to its action on free radical-scavenging, reducing capacity, metal ion-chelation, and inhibition of lipid peroxidation [38,39]. Our recent study demonstrated that the levels of GABA and gamma-oryzanol were higher in GBR than BR [40]. In this study, antioxidant activity and total phenolic content in all rice samples were examined. The results demonstrated that GBR had the greatest radical scavenging activity and phenolic contents, followed by BR and WR, respectively. These were in agreement with a previous study [37] which reported that WR had significantly lower levels of phenolics than BR and GBR.

It has been shown that two of the major phenolics found in WR, BR, and GBR were ferulic acid and p-coumaric acid, with them being most abundant in GBR [37]. Ferulic acid, which is ester linked to sterol as part of γ-oryzanol and bound to insoluble fiber forming the cell walls, exhibits antioxidant activity in response to free radicals [41]. In addition, its scavenging activity is found to be similar to that of superoxide dismutase [41-43]. GABA is also a bioactive compound found in whole rice grains and is
highly produced during the grain germination process [24]. Its effect on antioxidative status has been reported [44]. GABA can improve antioxidation status in pigs by increasing the activities of serum GPx, SOD, catalase (CAT), and decreasing malondialdehyde (MDA), a product of lipid peroxidation, levels during heat stress [45].

In the present study, the antioxidant activities of WR, BR, and GBR extracts were determined by DPPH assay. The activity was expressed as TEAC/g rice extracts and NAC was used as a standard antioxidant. As shown in Figure 2, the scavenging DPPH activity by standard Trolox and antioxidant NAC increased with increasing concentrations (Figure 2). From the results, Trolox exhibited the highest scavenging activity, followed by NAC and rice extracts. For rice samples, the DPPH activity was significantly influenced ($p < 0.05$) by type of rice. GBR extract revealed the highest DPPH activity, followed by BR and WR extracts. The DPPH activity of 3 different rice extracts ranged from 2.99 to 78.69 %, whereas Trolox and NAC were from 15.11 to 93.33 % and 13.55 to 85.29 %, respectively. The IC50 values of WR, BR, and GBR extracts against DPPH activity were 10.31, 7.40, and 5.31 mg/mL, respectively, and that of Trolox and NAC were 3.43 and 5.88 mg/mL, respectively (Table 1). For rice samples, the lowest IC50 value was obtained in GBR, indicating that GBR exhibited potent antioxidant capacity compared to that of BR and WR, whereas for standard antioxidant, Trolox had more antioxidant capacity than NAC. While expressed as TEAC, the DPPH value of 0.5 mg/mL of WR, BR, and GBR extracts was 541.33, 865.13, and 1261.66 mmoles TEAC/g of rice extract, respectively. Further, it was found that GBR exhibited the highest amount of total phenolic content (1102.11 mg GAE/g), followed by BR (794.85 mg GAE/g) and WR (470.46 mg GAE/g), respectively (Table 2).

Figure 2 DPPH radical scavenging activity of rice extracts (WR, BR, and GBR), Trolox, and NAC.
Table 2 Total phenolic content of white rice (WR), brown rice (BR), and germinated brown rice (GBR) extracts by Folin-Ciocalteu assay.

<table>
<thead>
<tr>
<th>Type of rice</th>
<th>Total phenolic content (mg GAE/g of rice extract)</th>
<th>DPPH free radical activity IC50 values (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WR</td>
<td>470.46 ± 22.87</td>
<td>10.31</td>
</tr>
<tr>
<td>BR</td>
<td>794.85 ± 102.35</td>
<td>7.40</td>
</tr>
<tr>
<td>GBR</td>
<td>1102.11 ± 43.02</td>
<td>5.31</td>
</tr>
<tr>
<td>Trolox</td>
<td>NA</td>
<td>3.43</td>
</tr>
<tr>
<td>NAC</td>
<td>NA</td>
<td>5.89</td>
</tr>
</tbody>
</table>

NA = Not available

In addition, in this study, antioxidant protein (SOD2 and GPx4) expression was measured to investigate whether GBR, BR, or WR extract have a protective effect on antioxidant defense system in JEG-3 cells induced by Cd. The results were expressed as relative expressions of SOD2 (Figure 3) and GPx4 (Figure 4) proteins to β-actin.

For rice treatment groups, it was indicated that 48 h treatment with GBR significantly up-regulated \((p < 0.05)\) both SOD2 (Figure 3) and GPx4 (Figure 4) expression much higher than BR and WR treated groups, respectively, compared with the control. Meanwhile, for 24 h incubation with 0.3 \(\mu\)M Cd, SOD2 and GPx4 expression were significantly increased \((p < 0.05)\).

Moreover, the results indicated that after 48 h pre-treatment with 0.5 mg/mL GBR, BR, and WR followed by 0.05 \(\mu\)M Cd treated group, GPx4 protein expression was significantly \((p < 0.05)\) decreased compared with the non-pre-treated group (Figure 4), whereas SOD2 expression was up-regulated only in the GBR pre-treated group (Figure 3).

In addition, pre-treatment with BR and WR had no effect on SOD2 expression compared with the Cd-treated group. However, BR showed higher efficiency to scavenge intracellular ROS in JEG-3 cells exposed to Cd than WR (Table 1). This suggests that pre-treatment with GBR may reveal the protective defenses and are proposed to act as the first line to scavenge free radicals induced by Cd. However, at high concentrations of Cd exposure, GBR extract had slight influence on the antioxidant defense system, signifying that Cd is a potent heavy metal ion harmful to the cells. To our knowledge, this study is the first \textit{in vitro} report of the protective effects of GBR and BR on human placental cell lines. However, the role of these rice extracts involved in defense mechanisms during Cd stress still needs to be clarified further, such as the measurement of antioxidant enzyme activities.

In addition, pre-treatment with 10 mM NAC down-regulated GPx4 expression in cells exposed to 0.3 \(\mu\)M Cd compared with Cd treated group (Figure 4). However, expression of SOD2 was unchanged in all NAC pre-treatments (Figure 3). In agreement with rice pre-treatments, these results suggest that supplementation of antioxidants may protect JEG-3 cells from Cd toxicity.
Figure 3 SOD2 protein expression of JEG-3 cells treated with (A) rice extracts (GBR, BR, WR), (B) Cd, and (C) 48 h pre-treated with different concentrations of WR, BR, GBR extracts and NAC before 24 h Cd exposure. Results are the mean ± SD. * = p < 0.05 compared to control; # = p < 0.05 compared to Cd treated group.

Figure 4 GPx4 protein expression of JEG-3 cells treated with (A) rice extracts, (B) Cd, and (C) 48 h pre-treated with different concentrations of GBR, BR, WR extracts and NAC before 24 h Cd exposure. Results are the mean ± SD. * = p < 0.05 compared to control; # = p < 0.05 compared to Cd treated group.
However, at higher concentrations of Cd exposure (0.1 - 0.3 µM), the expression level of SOD2 in all rice pre-treatments was also increased, but not significantly compared to Cd-treated groups. This suggested that a concentration of 0.5 mg/mL of rice extracts may not able to lessen Cd toxicity. Nevertheless, interestingly, GPx4 protein expression was significantly decreased ($p < 0.05$) in all rice pre-treatments in cells exposed to 0.05 µM Cd (Figure 4). This may be due to their role and the level of antioxidant actions in relation to SOD2 [46].

In addition, the present study also demonstrated the association between Cd and oxidative stress in JEG-3 cells. From Pearson correlation analysis, the negative correlation ($r = -0.727, p < 0.01$) demonstrated that high ROS levels in JEG-3 cells induced by Cd (0 - 0.5 µM) dramatically decreased cell viability (Figure 5A). In addition, the antioxidant defense response in oxidative stress induced by Cd was shown by up-regulating the expression of antioxidant proteins SOD2 and GPx4, as indicated in Figure 5B. The results (Figure 5B) showed the positive correlation between ROS levels and expression of SOD2 ($r = 0.672, p < 0.0.5$) and GPx4 proteins ($r = 0.865, p < 0.01$).

Moreover, it also demonstrated that GPx4 (Figure 6A) and SOD2 (Figure 6B) expression were negatively correlated with ROS levels in GBR (SOD2, $r = -0.745, p < 0.01$; GPx4, $r = -0.814, p < 0.01$) and BR (SOD2, $r = -0.817, p < 0.01$; GPx4, $r = -0.651, p < 0.05$) pre-treatment groups after exposure to low concentration Cd (0.05 µM). These results indicated that GBR and BR extracts may affect the cellular defense mechanism to reduce Cd toxicity. However, such a correlation was not seen in WR pre-treatments.

Taken together, these results demonstrated that Cd treatment perturb cellular oxidative status and that pre-treatments with GBR and BR that have antioxidant activity may help to protect cells from Cd-induced oxidative stress through the reduction of ROS and regulation of antioxidant SOD2 and GPx4 protein expression.
Figure 6 Pearson correlation analysis between (A) GPx4, (B) SOD2 protein expression and ROS levels in JEG-3 cells pre-treated with GBR and BR (0.5 mg/mL) before Cd treatments (0.05 - 0.3 µM).

Conclusions

The present study demonstrated that cultured JEG-3 cells, a model for the human placental trophoblast, exposed to Cd increased intracellular ROS levels and induced antioxidant defense response through up-regulation of the expression of antioxidant enzymes SOD2 and GPx4. Nevertheless, cells supplemented with BR and GBR extracts protected cells from Cd toxicity, as reflected by decreasing ROS. By improving oxidative status, BR and GBR affected the antioxidant defense systems within the cells by acting on the basis of line of antioxidant defense through up-regulating SOD2 and down-regulating GPx4 production.

This study presented preliminary results suggesting that pregnant women should avoid exposure to a state of oxidative stress induced by the toxic metal ion Cd and that supplementation by antioxidants may help to protect cells and will not affect pregnancy outcome [3,9,46,47]. Moreover, further studies may need to verify other important mechanisms involved in the beneficial effects of BR and GBR against Cd toxic effects.

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References


