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Original Research Article

Red sticky rice (*Oryza sativa* L.) bran extract attenuates cellular oxidative stress in human hepatocellular carcinoma cell line via Nrf-2 and HO-1 pathway

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Abstract

Purpose: *To investigate the molecular mechanism underlying the antioxidant effect of red sticky rice bran extract (RRBE; a pigmented strain of Oryza sativa L.) in human hepatocellular carcinoma cell line.*
Methods: *Human hepatocellular carcinoma HuH-7 cells were treated with the ethanol extract of RRBE in the presence or absence of EX-537 (Sirtuin 1 inhibitor), dexamethasone (NF-κB inhibitor), brusatol (Nrf-2 inhibitor), or HO-1 inh (HO-1 inhibitor) before exposure to hydrogen peroxide-induced oxidative stress. Intracellular ROS and glutathione levels were assessed using CellROX™ green reagent and GSH-Glo™ glutathione assay, respectively. Levels of nuclear factor erythroid 2-related factor 2 (Nrf-2) and expression of heme oxygenase-1 (HO-1) were determined using immunofluorescent assay and real-time polymerase chain reaction (RT-PCR), respectively.*
Results: *None of the tested inhibitors affected the ability of RRBE to reduce the level of ROS. Treatment with RRBE significantly decreased intracellular ROS and glutathione levels in HuH-7 cells undergoing oxidative stress (in the presence of BST and HO-1 inh; $p < 0.05$). Furthermore, Nrf-2 expression and HO-1 gene were significantly enhanced and upregulated respectively in HuH-7 cells pre-treated with RRBE ($p < 0.05$). Conversely, these effects were attenuated in the presence of brusatol.*
Conclusion: *Antioxidant property of RRBE is mediated through the activation of Nrf-2 and HO-1 pathways. These insights pave way for the development of functional foods or supplementary medicines aimed at preventing and treating NCDs in the future.*

Keywords: *Red sticky rice, Oxidative stress, Hepatocellular carcinoma, Nrf-2, HO-1*

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INTRODUCTION

Rising levels of environmental pollution, coupled with contemporary lifestyle changes, have a significant impact on human health. These factors are emerging as a primary cause of chronic non-communicable diseases (NCDs), which are the leading causes of mortality worldwide [1]. The fundamental pathology of NCDs involves an imbalance between free

radicals and antioxidants, leading to oxidative stress at a cellular level, which is a pivotal mechanism driving cellular dysfunction and organ damage [2]. In recent times, scientists have explored the antioxidant properties of various natural products, highlighting their potential as therapeutics [3]. Recent study has shown that antioxidant effect involves reducing reactive oxygen species (ROS) levels while concurrently enhancing an essential antioxidant, glutathione [4]. This study investigated the ethanolic extract of bran from red sticky rice (RRBE; a pigmented variant of *Oryza sativa* L.) in mitigating oxidative stress in liver cancer cells (HuH-7). This study aims to further investigate the molecular mechanisms underlying the antioxidant properties of RRBE by employing specific inhibitors that target intracellular signaling pathways.

EXPERIMENTAL

Extraction of RRBE

Red sticky rice bran was milled from red sticky rice collected from Ban Dok Bua in the Ban Tun sub-district of Muang Phayao district, Phayao, Thailand. The sample (1 kg) was extracted thrice in 6 L of 50 % ethanol solution at room temperature. The extract was filtered through filter paper (Whatman No.1) using a laboratory vacuum filtration system. After rotary evaporation, the samples were freeze-dried to obtain an extract powder, which was stored at −80 °C. The powder was dissolved in dimethylsulphoxide (DMSO) and sterilized by filtration through a 0.22 µm membrane. The stock solution was kept in a -20 °C freezer, and protected from light until further use [5].

Cell culture

Human hepatocellular carcinoma HuH-7 cells (JCRB0403, Japanese Collection of Research Biosources Cell Bank) were a gift from Assoc. Prof. Thawornchai Limjindaporn, Department of Molecular Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. The HuH-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco; Thermo Fisher Scientific, USA) supplemented with 10 %v/v heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, USA), 1 % v/v penicillin (100 U/mL), and streptomycin (100 mg/mL; Gibco; Thermo Fisher Scientific, USA). The cells were maintained in a humidified incubator at 37 °C with 5 % CO_2, subpassage following standard trypsinization procedures and counted using a trypan blue exclusion assay.

Treatment of cells with RRBE and inhibitors

The HuH-7 cells were seeded in a 96-well plate for 24 h. Subsequently, the medium was discarded, and the cells were treated with 1 µg/mL RRBE in the presence or absence of 10 µM of EX-537 (MedChemExpress, USA), dexamethasone (DEX; MedChemExpress, USA), brusatol (BST; MedChemExpress, USA), or HO-1 inhibitor (HO-1 inh; MedChemExpress, USA). Thereafter, 2 % v/v DMSO served as the control for the experiment. After 24 h treatment, the cells were incubated with 10 µM hydrogen peroxide (H_2O_2; Siribuncha Corporation, Thailand) for 5 h, and intracellular ROS and glutathione levels were measured.

Evaluation of parameters/indices

Intracellular ROS level

Reactive oxygen species (ROS) levels were measured using CellROX™ Green Reagent (Thermo Fisher Scientific, USA) according to manufacturer's instructions. CellROX Green (2 µL of 5 mM) was added to the cells and incubated at 37 °C for 30 min. Subsequently, the fluorescent signal was measured using a multimode microplate reader (Biotek, USA) at $485_{Ex}/520_{Em}$ nm. Intracellular ROS levels were compared to the control cells, which were set as 100 % of baseline level.

Intracellular glutathione level

Glutathione levels were measured using GSH-Glo™ Glutathione Assay (Promega, USA) according to manufacturer's instructions. GSH-Glo™ reagent (50 µL) composed of lysis buffer, glutathione S-transferase, and Luciferin-NT, was added to the cells and incubated at 25 °C for 30 min. Thereafter, 50 µL of luciferin detection reagent was added, and the mixture was incubated in the dark for another 15 min. The luminescent signal was measured from the reaction using multimode microplate reader (Biotek, USA). Intracellular glutathione levels were compared to control cells, which were assigned as 100 % of baseline level.

Determination of Nrf-2 protein level

The HuH-7 cells were cultured on a glass coverslip for 24 h. Thereafter, the cells were treated with RRBE, H_2O_2, RRBE and H_2O_2, and RRBE, BST, and H_2O_2. After treatment, Nrf-2 protein expression was investigated using an immunofluorescence assay. The cells were fixed and permeabilized using 4 % paraformaldehyde and 0.1 % Triton-X, respectively. Thereafter, the

cells were incubated with a 1:500 dilution of rabbit anti-human Nrf-2 (Abclonal, USA) at 37 °C for 60 min. After washing, the cells were incubated with 1:1000 donkey anti-rabbit IgG-Alexa488 (Jackson ImmunoResearch, USA) in the presence of nucleus staining dye, Hoechst 33342 (Thermo Fisher Scientific, USA). The coverslip was mounted onto a glass slide, and the fluorescence image was captured using a Nikon ECLIPSE Ni fluorescent microscope with NIS imaging software (Nikon, Japan).

Determination of HO-1 mRNA expression

The HuH-7 cells were seeded in a 6-well plate and treated with RRBE, H_2O_2, RRBE and H_2O_2, and RRBE, BST, and H_2O_2. Thereafter, total RNA was isolated using the Quick-RNA Microprep Kit (Zymo Research, USA). Total RNA concentration and purity were measured using Nanodrop spectrophotometry (Thermo Fisher Scientific, USA). Equal amounts of 100 ng of total RNA from each condition were reverse transcribed to cDNA using the SuperScript™ First-Strand Synthesis System (Thermo Fisher Scientific, USA). The cDNA was then mixed with LightCycler® 480 SYBR Green I Master (Roche Applied Science, Germany), which contained primers specific to genes in Table 1.

Table 1: Primer sequence of gene used

Primer	Sequence (5'-3')
HO-1 forward	TTTGAGGAGTTGCAGGAGC
HO-1 reverse	AGGACCCATCGGAGAAGC
GAPDH forward	CGACCACTTTGTCAAGCTCA
GAPDH reverse	AGGGGTCTACATCGCAACTG

Real-time PCR was carried out on LightCycler® 480 System (Roche Applied Science, Germany) with the temperature profile of pre-incubation at 95 °C for 10 min, followed by 45 amplification cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s. The *HO-1* mRNA was calculated as relative gene expression compared to control cells, using the $2^{-\Delta\Delta Ct}$ formula. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) served as a housekeeping gene.

Statistical analysis

All determinations were done in triplicate. Data were expressed as mean ± standard deviation (SD) and compared using one-way ANOVA. Bar graphs were created using GraphPad Prism 5 software (GraphPad Software, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Red sticky rice bran extract (RRBE) treatment increased intracellular glutathione levels in HuH-7 cells

Treatment with RRBE significantly decreased level of intracellular ROS, but none of the tested inhibitors affected the potential of RRBE to reduce the level of ROS in HuH-7 cells undergoing oxidative stress ($p < 0.05$; Figure 1 A). Also, treatment with RRBE in the presence of BST and HO-1 inh significantly decreased intracellular levels of glutathione in HuH-7 cells undergoing oxidative stress ($p < 0.05$; Figure 1 B).

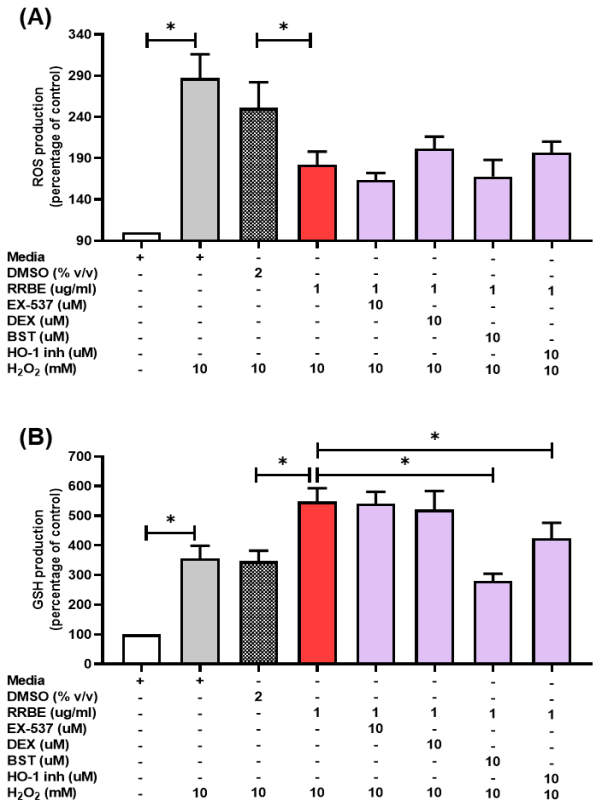

Figure 1: Intracellular levels of (A) ROS and (B) glutathione were assessed in HuH-7 cells following treatment with RRBE in the presence or absence of EX-537, DEX, BST, and HO-1 prior to inducing oxidative stress by H_2O_2 treatment ($p < 0.05$)

Red sticky rice bran extract (RRBE) treatment increased Nrf-2 protein in HuH-7 cells undergoing oxidative stress

Fluorescent images of Nrf-2 protein expression determined using immunofluorescent assay are shown in Figure 2. Based on the fluorescent intensity (green represents Nrf-2 protein and blue represents the nucleus), Nrf-2 protein increased in HuH-7 cells treated with H_2O_2, suggesting an

antioxidant response against oxidative stress (Figure 2 B). Also, Nrf-2 protein expression was strongly enhanced when the cells were pre-treated with RRBE (Figure 2 C), and this up-regulation was diminished when the pre-treatment was done in the presence of BST (Figure 2 D).

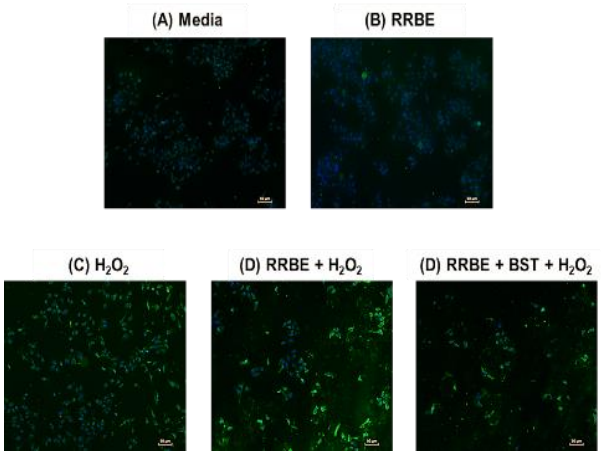

Figure 2: Nrf-2 protein expression was assessed in (A) HuH-7 cells without treatment, (B) HuH-7 cells with RRBE treatment, (C) HuH-7 cells with H_2O_2-induced oxidative stress, (D) HuH-7 cells with RRBE pre-treatment before H_2O_2-induced oxidative stress, and (E) HuH-7 cells with RRBE and BST pre-treatment before H_2O_2-induced oxidative stress. Fluorescent images were captured using a fluorescence microscope (NIKON) at 400× magnification (scale bar = 50 µm); green represents Nrf-2 protein, and blue represents the nucleus

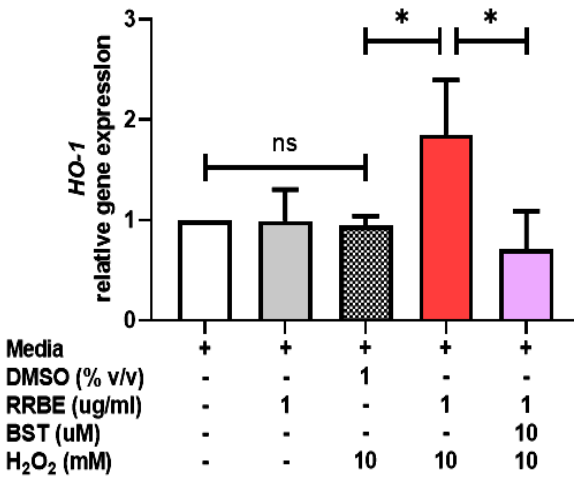

Figure 3: *HO-1* gene expression was assessed in HuH-7 cells following pre-treatment with RRBE in the presence or absence of BST, before inducing oxidative stress with H_2O_2 treatment ($p \geq 0.05$; *$p < 0.05$)

Red sticky rice bran extract (RRBE) treatment upregulated HO-1 gene in HuH-7 cells undergoing oxidative stress

The HO-1 gene expression was determined using a real-time RT-PCR assay. The results showed that HO-1 gene was expressed 0.95 ± 0.09-fold in HuH-7 cells with H_2O_2-induced oxidative stress. However, this expression was not significant, indicating that HO-1 gene was not altered during oxidative stress (Figure 3). Furthermore, HO-1 gene was significantly up-regulated in HuH-7 cells pre-treated with RRBE. This up-regulation significantly decreased when the cells were pre-treated with BST (Figure 3).

DISCUSSION

Comparative study of antioxidant activity in various pigmented rice varieties has identified red rice as the most potent in exerting antioxidant effects. The antioxidant effect has been attributed to the various constituents (phenolic compounds, flavonoids, and proanthocyanidins) of RRBE [5]. These compounds play a pivotal role in mitigating the generation of ROS, thereby enhancing cellular antioxidant defense [6]. In a previous study, RRBE exerted anti-inflammatory effects in RAW 264.7 macrophage cells by downregulating expression of several cytokine genes, such as *IFN-α*, *IFN-γ*, *Mx1*, *CXCL10*, *IRF3*, *IRF5*, *IRF7,* and *NO* through the inhibition of sting protein phosphorylation [5].

However, RRBE stimulates the expression of *INSR*, *IRS1*, *IRS2*, and *GLUT4* genes, resulting in improved insulin resistance and reduced blood sugar in rats fed a high-fat diet [7]. Also, RRBE lowers expression of cytokine genes associated with inflammation, such as *MCP-1*, *TNF-α,* and *iNOS,* through the NF-κB p65 pathway [8]. Thus, RRBE exerts its pharmacological effects by modulating specific gene expression through various intracellular signaling pathways.

Previous studies revealed that extracts from different colored rice varieties affect various intracellular signaling pathways, such as Sirt1, NF-κB, Nrf-2, and HO-1 pathways [9-12]. In this study, specific inhibitors for these pathways were used to investigate the pharmacological mechanism of RRBE. The results indicated that inhibition of Sirt1, NF-κB, Nrf-2, and HO-1 pathways did not reduce intracellular ROS levels. This suggests that RRBE may inhibit ROS generation through other uninvestigated pathways, such as iNOS, NAPDH, NOX, and MAPK pathways [13]. Inhibiting the Nrf-2 and HO-1 pathways affected glutathione levels in the cells (Figure 1 B), suggesting that RRBE

promotes glutathione production through the Nrf-2 and HO-1 pathways. This finding is consistent with previous studies showing that γ-oryzanol, a key component in rice, interferes with interaction between Nrf-2 and Keap1 proteins, enabling Nrf-2 to act as a primary transcription factor for antioxidant defense [10].

Downstream genes of Nrf-2 include various genes related to the glutathione system such as *GSR*, *GCLM*, *GCLC*, *GPX2*, and *SLC7A11* [14]. Further studies are necessary to determine whether RRBE activates Nrf-2 to induce the expression of these genes, leading to increased glutathione production. Also, HO-1, which is regulated by Nrf-2 signaling, functions as an antioxidant by inhibiting ROS through the suppression of iNOS and facilitating the production of ferritin from iron [15]. Previous reports have demonstrated that both brown rice [16] and black rice [17] promote the expression of the *HO-1* gene. Further reports suggest that HO-1 increases glutathione production by increasing the expression of the *GCLC* gene [18].

CONCLUSION

Red sticky rice bran extract (RRBE) significantly decreases intracellular ROS and glutathione levels, enhances Nrf-2 expression, and upregulates HO-1 gene in HuH-7 cells. This elucidated molecular mechanism of RRBE will facilitate the development of functional foods or supplementary medicines aimed at preventing and treating NCDs.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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