

RESEARCH COMMUNICATION

Red Strain *Oryza Sativa*-Unpolished Thai Rice Prevents Oxidative Stress and Colorectal Aberrant Crypt Foci Formation in Rats

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Abstract

Oxidative stress has been proposed to be involved in colorectal cancer development. Many dark pigments of plants have potent oxidative stress preventive properties. In this study, unpolished Thai rice was assessed for antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) methods. Red strain unpolished Thai rice was also administered to rats exposed to azoxymethane (AOM) for induction of aberrant crypt foci (ACF). Serum malondialdehyde (MDA) and ferric reducing antioxidant power (FRAP) were investigated for cellular oxidative stress and serum antioxidants, respectively. Red pigment unpolished Thai rice demonstrated high antioxidant activity and was found to significantly and dose dependently decrease the total density and crypt multiplicity of ACF. Consumption of Thai rice further resulted in high serum antioxidant activity and low MDA cellular oxidative stress. Interestingly, the density of ACF was strongly related to MDA at $r = 0.964$, while it was inversely related with FRAP antioxidants ($r = -0.915$, $p < 0.001$). The results of this study suggest that the consumption of red strain of unpolished Thai rice may exert potentially beneficial effects on colorectal cancer through decrease in the level of oxidative stress.

Keywords: Aberrant crypt foci - colorectal cancer - oxidative stress - unpolished Thai rice - Thailand

Asian Pacific J Cancer Prev, 13, 1929-1933

Introduction

The bioequilibrium of antioxidant and prooxidant has a crucial role in cellular function with free radical defense (Valko et al., 2006). Oxidative stress is defined by the excess of free radicals and antioxidant depletion. It was implicated on biomolecules such as protein, lipid, and nucleic acid (Sies, 1997; Finkel & Holbrook, 2000). Cellular oxidative stress eventually caused tissue injury and progressed to chronic diseases including cancer (Halliwell, 1987; Valko et al., 2006).

Colorectal cancer is described as the malignant neoplasm which arising from an inner lining of colonic epithelium (Rajamanickam & Agarwal, 2008). The early stage of colorectal cancer is generally related to the aberrant crypt foci (ACF), the clusters of colonic epithelial cells with an enlarged and thicker layer of mucosal epithelium than the surrounding normal crypts (Cappell, 2007). The consumption of diet rich in high fat and low phytochemicals is associated with the risk of colorectal cancer (Cappell, 2007; Kim & Milner, 2007). Phytophenolic substances were claimed to prevent

clinically oxidative disease (Ames et al., 1993; Tian et al., 2004). Several epidemiological studies have been reported that a high intake of natural phytochemicals was reduced risk of colon cancer (Liu, 2003; Williams & Hord, 2005; Vainio & Weiderpass, 2006; Nishino et al., 2007). Chemopreventive agents and their ingredient of phytochemical plants have been reported to interfere with various molecular pathways that involved with colorectal cancer initiation and progression (Gustin & Brenner, 2004).

The red color strain of unpolished Thai rice was the high source of phytophenolics and their potent with antioxidant activities. Thai rice had a crucial role in oxidative stress prevention. The consumption of the red pigment of unpolished Thai rice in rats has been associated with low levels of oxidative stress marker (Suwannalert et al., 2010). Thus, the red strain of unpolished Thai rice supplementation may benefits to prevent colorectal cancer. In this study, total antioxidant of unpolished Thai rice was obtained for radical scavenging. Oxidative stress markers and the aberrant crypt foci (ACF) were also investigated in rats consumed unpolished Thai rice for obtaining the

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Materials and Methods

Unpolished Thai rice

The color strains of unpolished Thai rice were obtained from the CT-Chang Thong Co., Ltd., Thailand. The rice samples were defined into three groups according to their pigments: red, black and yellowish-white colors. All samples were grounded by an electric blender and extracted with 95% ethanol by 1:2 (w/v) and shaken at 150 rpm for 24 h. The mixed extract was centrifuged at 3000 rpm for 15 min and filtered by polytetrafluoroethylene (PTFE) filter nylon 0.45 µm before use.

Assessment of total antioxidant activity by DPPH method

Total antioxidant activity was obtained by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Rattanachitthawat et al., 2010). The extract samples 20 µl were mixed with 167 µM of DPPH in 180 µl ethanol. The mixture was immediately measured by using a Multimode Detector (Beckman, DTX 880, Australia) at 540 nm. Vitamin C equivalent antioxidant capacity was used as a reference for DPPH radical scavenging activity of sample test.

Assessment of total antioxidant activity by ABTS method

Total antioxidant activity of the rice extracts was investigated by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method (Suwannalert & Rattanachitthawat, 2011). The working ABTS solution was freshly prepared by equal volumes of 7 mM ABTS and 2.4 mM potassium persulfate and allowed to react for 12 h at room temperature in dark condition. The mixed reaction was diluted with 95% ethanol to obtain an absorbance of 0.95 ± 0.01 units at 734 nm. The extracted sample 70 µl were reacted with 630 µl of the mixed solution. After 30 min, the absorbance was measured at 734 nm spectrophotometrically (Spectro UV-Vis Scanning 2650, U.S.A). Trolox equivalent antioxidant capacity was used as a standard.

Animal experiment

Male Sprague-Dawley rats, aged 4 weeks were purchased from National Laboratory Animal Centre, Mahidol University, Nakhon Pathom, Thailand. The experimental protocol is shown in Figure 1. After 1 month of acclimation, Thirty six animals were randomly divided into 4 groups; normal control (CN), positive control (CA), low dose (LD) and high dose (HD) groups. Rats in CN and CA groups were fed with commercial diet. Rats in LD and HD groups were fed with the mixed of 20% and 70% red pigment of unpolished Thai rice with commercial diet, respectively. Four weeks after treated, the rats in groups of CA, LD and HD were subjected to once a week for 2 times of subcutaneous injections of azoxymethane (AOM) at a dose of 15 mg/kg each. At 33 weeks after the second AOM induced, all animals were sacrificed; their large intestine and serum were collected.

Identification of colorectal aberrant crypt foci (ACF)

ACF analysis was performed according to Bird (1987)

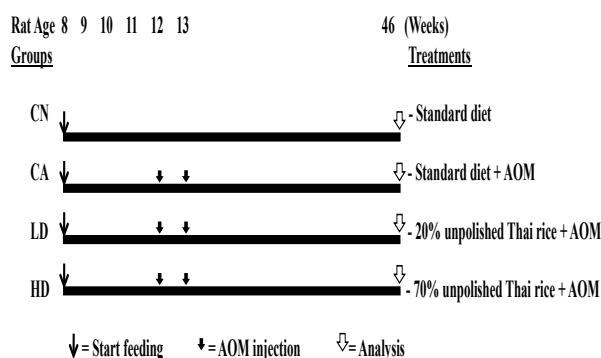


Figure 1. Experimental Protocol of Animals Treatment

Groups. Animals were divided into 4 groups and fed standard diet or diet supplemented with different doses of red strain of unpolished Thai rice; CN = Negative control group, CA = Positive control group, LD = Low dose group, HD = High dose group.

with slightly modified. The large intestine from caecum to anus was longitudinally opened and fixed in 10% buffered formalin for at least 24 hours before ACF determination. The formalin-fixed colonic tissues were cut into ascending and descending segments and stained in 0.1% methylene blue solution for 5 min and counted ACF under a light microscope. ACF were identified with the following morphological characterization as the crypts with enlarged and elevated epithelial lining and their lumen often had oval or slit-like lumens with an increased pericryptal space from the surrounding normal crypts (Bird, 1995; Cheng & Lai, 2003; Cappell, 2007). The variables used to assess the aberrant crypt were their total density (number of ACF per cm²) and number of crypts in each focus, which were categorized as ACF containing 1, 2, 3 and ≥4 aberrant crypts (ACs).

Measurement of serum oxidative stress by malondialdehyde (MDA) assays

Serum MDA were obtained for cellular oxidative stress. The concentration of MDA based on the reaction of thiobarbituric acid (TBA) was determined according to the modification of previous studied (Suwannalert et al., 2010). Serum 100 µl and 50 µl of 7.2% butylated hydroxytoluene (BHT) were mixed with 1.5 ml of 25 nmol/L TBA, 1.5 ml HCl, 550 µl distilled water and 200 µl of 8.1% SDS. The reaction mixture was incubated at 90°C for 20 min and rapidly cooled for 10 min, then 0.5 ml of distilled water and 3 ml of n-butanol in pyridine were added to the reaction mixture. The mixtures were mixed and centrifuged at 3000 g for 15 min. The MDA product was measured by using a Multimode Detector (Beckman, DTX 880, Australia) at 520 nm excitation and 550 nm emission.

Measurement of serum antioxidant by FRAP assays

Ferric reducing antioxidant power (FRAP) in serum were obtained for serum antioxidant. The method was investigated the reduction of a ferric 2,4,6-tripyridyl-striazine complex (Fe³⁺-TPTZ) to ferrous (Fe²⁺-TPTZ) at the low pH (Suwannalert et al., 2010). Working FRAP reagent was freshly prepared by mixing a solution of 2.5 ml of 20 mM ferric chloride hexahydrate (FeCl₃.6H₂O)

Table 1. Total Antioxidant Activities in Color Strains of Unpolished Thai Rice

Unpolished Thai rice (pigment strain)	Antioxidant capacity (Mean±SD)	
	DPPH	ABTS
	(mg VitC/g sample)	(mg Trolox/g sample)
Red	9.382±0.103 ^{a,b*}	12.231±0.201 ^{a,b*}
Black	6.171±0.024 ^{c*}	5.531±0.137 ^{c*}
Yellowish-white	1.973±0.045	2.502±0.018

* ^ap-value of red and black strains, ^bp-value of red and yellowish-white strains, ^cp-value of black and yellowish-white strains, ^{*}statistically significant at p≤0.001

and 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ solution) and 25 ml of acetate buffer pH 3.6. Serum sample was mixed with FRAP reagent in 96 well plates of a multimode detector model of Beckman, DTX 880 and then incubated in dark conditions at room temperature for 15 min. The absorbance of ferrous complex was determined by a multimode detector at 595 nm and compared to that of standard ferrous sulfate heptahydrate (FeSO₄·7H₂O). The FRAP activity was expressed as μM Fe²⁺/g sample equivalent.

Ethics

Ethical approval for the study was obtained from the Ethics Committee of the Faculty of Veterinary Medicine, Chiang Mai University (ACUC-R3/2554) that the animals used in the study conformed to international and national guideline for ethical conduct on the care and use of animals.

Statistical analyses

All results were presented as Mean ± SD. The difference among groups and data correlation were obtained by one-way ANOVA and Pearson correlation, respectively. Statistical significance was considered at p < 0.05.

Results

Antioxidant activity of unpolished Thai rice

In this study the total antioxidant activity of unpolished Thai rice was determined by DPPH and ABTS methods. The results were given in Table 1. The red strain of unpolished Thai rice showed highest antioxidant activity. Additionally, the black color strain was significantly higher than that of yellowish-white strain.

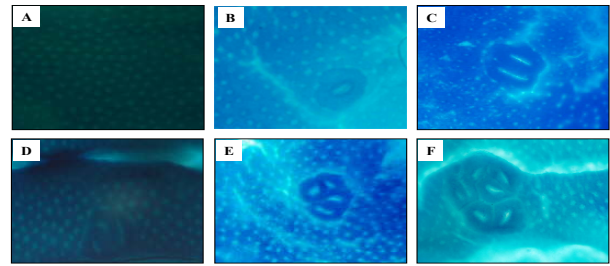
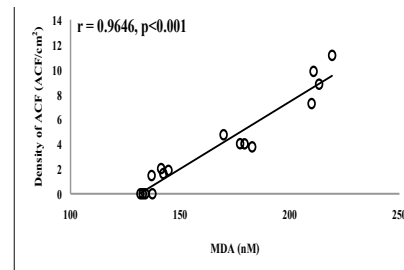
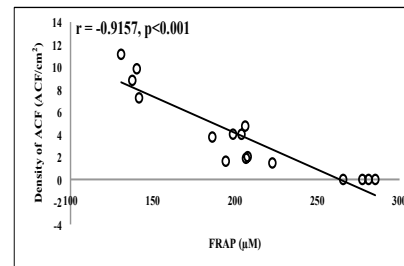


Figure 2. Topographic View of Methylene Blue Stained Mucosa of Whole Mount Colons Exhibiting the Presence of Normal Crypts and ACF. (A) Normal colonic mucosa from a control animal. (B to F) ACFs in the colonic mucosa of AOM-treated animal were categorized as a focus consisting of one (B), two (C), three (D), four (E) and more than four crypts (F). Magnification: × 40.



3a. Correlation between total density of ACF and MDA



3b. Correlation between total density of ACF and FRAP

Figure 3. Correlation Between Total Density of ACF and MDA Oxidative Stress and FRAP Antioxidant Markers.

Aberrant crypt foci (ACF) determination

The average body weights of rats in all 4 groups were not significant (data not show). The morphological change of ACF were identified and shown in Figure 2. In present study, treatment of rats with AOM induced ACF only in the descending colon. Table 2 is shown the total density of ACF (ACF/cm²) and the number of aberrant crypts (ACs)/foci which containing 1, 2, 3, and ≥4 ACs. No ACF was detected in control group that fed with commercial diet alone. The total density of ACF in HD (1.748 ± 0.251 ACF/cm²) and LD (4.139 ± 0.421 ACF/cm²) groups

Table 2. Total Antioxidant Activities in Color Strains of Unpolished Thai Rice

Groups	Descending colon (Mean±SD)				Serum (Mean±SD)		
	Density of ACF (ACF/cm ²)	Aberrant crypts (ACs)/Foci				MDA (nM)	FRAP (μM)
		1 AC	2 Acs	3 Acs	≥ 4 ACs		
CN	0.00±0.00 ^{***}	0.00±0.00 ^{***}	0.00±0.00 ^{***}	0.00±0.00 ^{***}	0.00±0.00 ^{***}	134.06±2.89 ^{***}	277.39±10.31 ^{***}
CA	9.26±1.64	0.69±0.44	1.10±0.32	1.21±0.56	3.49±0.63	213.36±5.17	137.55±5.94
LD	4.14±0.42 ^{b***}	0.29±0.29	0.98±0.39	0.50±0.25 ^{b**}	2.80±0.97	177.32±6.75 ^{b**}	198.76±10.95 ^{b**}
HD	1.75±0.25 ^{c***, d**}	0.27±0.09 ^{c*}	0.75±0.18	0.39±0.22 ^{c**}	2.98±1.03	141.32±3.94 ^{c***, d**}	207.90±14.21 ^{c**}

CN = Negative control group, CA = Positive control group, LD = Low dose group, HD = High dose group, ^ap-value of CN and CA, ^bp-value of LD and CA, ^cp-value of HD and CA, ^dp-value of HD and LD, ^{*}statistically significant at p≤0.05, ^{**}statistically significant at p≤0.01, ^{***}statistically significant at p≤0.001

were significantly and dose dependently decreased when compared with the positive control (9.258 ± 1.639 ACF/cm²) at $p \leq 0.001$. Additionally, total density of ACF in HD group also presented significantly lower than LD group at $p \leq 0.01$. The numbers of 1, 2, 3, and ≥ 4 ACF/foci have the tended to decrease in rats consumed unpolished Thai rice.

Serum oxidative stress and antioxidant in rats

The serum MDA levels of rats in the CN, CA, LD and HD groups were 134.064 ± 2.888 , 213.355 ± 5.165 , 177.324 ± 6.747 and 141.318 ± 3.940 nM, respectively (Table 2). The rats that consumed unpolished Thai rice tended to have low levels of MDA in serum. The serum MDA in HD group was significantly lower than those of the LD group ($p = 0.003$) and the CA group ($p \leq 0.001$). In addition, the group of LD showed lower MDA level than that CA group at $p = 0.002$.

The FRAP level, serum antioxidant was lowest in CA group. The treated with unpolished Thai rice showed high levels of FRAP depend on dose manner (Table 2). FRAP level in HD group was 207.900 ± 14.212 μ M and was significantly higher than CA group (137.547 ± 5.943 μ M) at $p = 0.006$. Although the rats that consumed unpolished Thai rice tended to have high level of FRAP in serum, no statistic significance was found between the HD and LD groups ($p = 0.430$).

The correlations between the density of ACF and serum MDA and FRAP levels were computed. The linear correlation coefficients (r) were illustrated in Figure 3. Serum MDA level was strongly correlated with ACF density at $r = 0.964$ ($p \leq 0.001$). While, serum antioxidant of FRAP showed inversely associated with the density of ACF ($r = -0.915$, $p \leq 0.001$).

Discussion

High fat diet, low fiber, chronic inflammation and also infection can initiate cellular oxidative stress and progress to colorectal cancer (Bartsch & Nair, 2006; Morgillo et al, 2007; Umar, 2009). The previous studies have been reported the phytochemical agents can reduce the risk of cancer developed in both early and late stages (Liu, 2004; Lila, 2007; Nishino et al., 2007). Unpolished Thai rice, a high source of phenolic compounds, play a crucial role in antioxidant activities (Rattanachitthawat et al., 2010). In this study, the red and black strains of unpolished Thai rice showed high antioxidant activity than that yellowish-white strain. The dark pigments including red and black color strains of phytochemicals had a potent to scavenge free radicals (Rattanachitthawat et al., 2010; Suwannalert & Rattanachitthawat, 2011). The red pigment of unpolished Thai rice, high antioxidant activity might be a beneficial source for colorectal cancer prevention.

Aberrant crypt foci (ACF) have provided as biomarkers in the screening studies of various carcinogens and colorectal cancer prevention (Bird, 1995; Raju, 2008). The phytochemicals of natural products have been widely used to test as sources of bioactive colorectal prevention by using AOM-induced ACF in rat model (Velmurugan et al., 2008; Xiao et al., 2008). The present study was

proposed to evaluate the phytopreventive effect of AOM induced ACF in rats that consumed the red strain of unpolished Thai rice. The results showed that dietary administration with red pigment of rice significantly inhibited ACF formation. Interestingly, unpolished Thai rice supplementation was dose dependently to prevent the development in crypt multiplicity of small ACF (1, 2, and 3 ACF/foci) than a large ACF (≥ 4 ACF/foci). The preneoplastic lesions of large ACF have a higher tendency to progress to malignancy of colon cancer (Bird & Good, 2000). Our result indicated that red pigment of unpolished Thai rice can prevent the ACF formation especially in the early stage. In addition, AOM-induced rats that consumed red strain of unpolished Thai rice tended to decrease the MDA-cellular oxidative stress and increase FRAP-serum antioxidant. These finding suggest that the consumption of red strain of unpolished Thai, potent antioxidant may beneficial effects on carcinogenesis inhibition through oxidative stress induced.

In conclusion, the rats consuming red pigment strain of unpolished Thai rice possessed a low level of oxidative stress marker and high levels of serum antioxidant. It also inhibited the ACF formation. Thus, dietary red color strain of rice supplementation may have a beneficial role in the prevention of colorectal cancer through oxidative stress defense mechanisms.

Acknowledgements

This research project is supported by Mahidol University and Faculty of Science, Mahidol University. Thank also go to the Department of Pathobiology, Faculty of Science, Mahidol University and Faculty of Pharmacy, Chiang Mai University for providing laboratory space and facilities for this work.

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