Full Length Research Paper

Antiproliferation and apoptosis induction of phytic acid in hepatocellular carcinoma (HEPG\textsubscript{2}) cell lines

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Phytic acid is a naturally occurring polyphosphorylated carbohydrate, present ubiquitous in plants and animals. It is not only a natural antioxidant, but may also be the precursor/storage of intracellular inositol phosphates, important for various cellular functions and potential as anticancer compound. A prominent anticancer action of phytic acid has been demonstrated both \textit{in vivo} and \textit{in vitro} in a variety of tumor types, possibly through inhibition of tumor cell growth and differentiation. In this study, the growth inhibitory effect of phytic acid extracted from rice bran on hepatocellular cell lines (HEPG\textsubscript{2}), cell cycle modulation and apoptosis induction were undertaken. Phytic acid prove to induce growth inhibition and differentiation in HepG\textsubscript{2} in a dose and time-dependent manner with IC\textsubscript{50} value of 17.0 \(\mu\)g/mL. Analysis of flow cytometry was performed for the analysis of cell cycle and apoptosis. Treatment of phytic acid against HepG\textsubscript{2} also resulted in cell cycle arrest in HEPG\textsubscript{2} cell at G\textsubscript{2}/M phase cell cycle arrest. Besides, Annexin V-assay and propidium iodide confirmed the apoptosis occurred early and late in the cell line. In conclusion, with the results taken from our findings, phytic acid extracted from rice bran was revealed as a potent candidate for adjuvant chemotherapy and prevention of cancer.

Key words: Phytic acid, rice bran, apoptosis, anticancer, cell cycle.

INTRODUCTION

In cancer prevention, a better strategy is an important ways for controlling cancer other than finding newer and better therapeutics. Cancer prevention can be approached through prophylactic supplements and vitamins. A series of epidemiological studies confirmed by clinical interventions, and animal model studies have pointed that major components of fiber including phytic acid present in wheat bran, grains and legumes may protect against cancer (Matejuk and Shamsuddin, 2010). Besides, phytic acid is also shown as one of the biologically active components of fiber that is responsible in anticancer effect. Phytic acid inhibited tumor growth by induction of apoptotic cell death, cell cycle arrest and differentiation of several human cancer cell lines including colon (Sakamoto et al., 1993), liver (Vucenik et al., 1998a), prostate (Singh et al., 2003; Zi et al., 2000) and breast (Shamsuddin et al., 1996; Vucenik et al., 2005). Since phytic acid is obtained in our everyday diet and ability to quickly absorb from gastrointestinal tract and safe, the continuing investigation has been conducted against this novel biological agent against HepG\textsubscript{2} cell lines.

Herein, we revealed that \textit{in vitro} administration of phytic acid on HepG\textsubscript{2} cells significantly induced growth inhibition and differentiation. Moreover, the anti-proliferative effects and the mechanism intriguing selectivity of phytic acid for normal and malignant cells deserve further investigations. Phytic acid is one of the bioactive compounds that are being intensively studied to evaluate their effects on
health. Phytic acid is a naturally occurring compound that is abundantly found in grains, cereals, nuts, and foods that are high in fiber content (Somasundar et al., 2005). In rice bran, it occurs at 9.5 to 14.5% by weight (Jariwalla, 2001). The effect of phytic acid extracted from rice bran has not been evaluated in hepatocellular carcinoma (HepG2) cell lines; therefore, we hypothesized that phytic acid would significantly inhibit the cell growth rate of HepG2 cell lines.

MATERIALS AND METHODS

Cells and culture conditions
Human hepatocellular carcinoma cell line (HepG2) was purchased from American Type Culture Collection (ATCC) (USA). HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in a humidified atmosphere with 5% CO2.

Sample preparation
In a stabilization process of rice bran, method of Ramezanzadeh et al. (1999) was referred. The purpose of stabilization process was to prevent oxidative rancidity during storage. Total lipid was extracted from rice bran by using hexane regarding method of Hu et al. (2019) was referred. The median inhibitory concentration (IC50) of rice bran phytic acid was 17 µg/ml. For cell viability analysis, phytic acid extracted from rice bran at concentrations of 15, 17 and 19 µg/ml, cells at a density of 1 × 10^4 cells were trypsinized, washed twice with ice-cold PBS, and resuspended in 100 µl of 1 × binding buffer (0.1 M Hepes/NaOH, pH 7.4 and 1.4 M NaCl, 25 mM CaCl2). Then, 5 µl of Annexin V-FITC and 5 µl of propidium iodide were added to each sample and gently vortexed before incubated in the dark for 15 min. Subsequently, 400 µl of 1 × binding buffer was added and the fluorescence of the cells was immediately analyzed by flow cytometry (Beckman Coulter, USA).

Detection of apoptotic cell death by flow cytometry
Annexin V-FITC Apoptosis Detection Kit 1 (BD Bioscience) was used to detect early and late apoptotic activity HepG2 cells after 24, 48 and 72 h of incubation. After treatment of the different forms of phytic acid at concentrations of 15, 17 and 19 µg/ml, cells at a density of 1 × 10^4 cells were trypsinized, washed twice with ice-cold PBS, and resuspended in 100 µl of 1 × binding buffer (0.1 M Hepes/NaOH, pH 7.4 and 1.4 M NaCl, 25 mM CaCl2). Then, 5 µl of Annexin V-FITC and 5 µl of propidium iodide were added to each sample and gently vortexed before incubated in the dark for 15 min. Subsequently, 400 µl of 1 × binding buffer was added and the fluorescence of the cells was immediately analyzed by flow cytometry (Beckman Coulter, USA).

Statistical analysis
Biostatistical analyses were done using the SPSS 18.0 software package. All experiments were repeated at least three times. All data are reported as mean ± SD and statistical significant difference was considered to be present at p<0.05. Post hoc comparison of individual concentration means with the control was completed using the Turkey’s test.

RESULTS

Effect of phytic acid on growth inhibition of HepG2 cells
Firstly, we determined the effect of phytic acid extracted from rice bran on the proliferation of HepG2 cells. Phytic acid induced a dose-dependent decrease in vital formazan dye accumulating cells after 72 h of treatments, ranging from 0 to 100 µg/ml (Figure 1). The mean of IC50 value of rice bran phytic acid was 17 µg/ml. For cell cytotoxicity analysis, phytic acid extracted from rice bran did not show any toxicity towards normal cells, 3T3 with no IC50 was determined (result not shown).

Cell cycle analysis of phytic acid on HepG2 cell
Based on the growth of inhibitory response of phytic acid extracted from rice bran on HepG2 cell lines, we then assessed its effect on cell cycle progression using flow cytometry. The changes in the cell cycle distribution of HepG2 cells after being treated for 24, 48 and 72 h with extracted phytic acid at their respective IC50 ± 2.0. Analysis of the cycle as shown in Figure 2a showed that phytic acid increased the G2/M phase cells due to the
Figure 1. Cellular growth inhibition of extracted phytic acid from rice bran in hepatocellular carcinoma (HepG2). The cell viability was measured by MTT assay after 72 hours culture. Data shown are means ± standard deviations.

Measurement of apoptosis analysis

We determined any possible involvement of apoptosis induction by phytic acid extracted from rice bran that may have contributed in its strong growth inhibitory effect at increasing doses and longer treatment times. The annexin V/PI assay proved phytic acid extracted from rice bran significantly increased total apoptosis of HepG2 cells. Phytic acid also increased the early and late apoptotic HepG2 in a dose and time dependent manner. The total apoptotic cell death significantly increased after 24 h treatment with phytic acid (15 µg/ml) as compared to the control (p<0.05). Phytic acid significantly increased the number of early (93 ± 2.1%) and late apoptotic (58.73 ± 2.3%) HepG2 cells in dose dependent manner (Figures 3a and 4) compared to control only <3% of cell death (p<0.05). In addition, phytic acid also significantly increased the number of early apoptotic (94 ± 2.2%) and late apoptotic (79 ± 2.3%) HepG2 cells in a time dependent manner compared to control <2% of cell death (p<0.05) (Figures 3b and 5).

DISCUSSION

The anticancer effect of phytic acid has been revealed in several studies both in vivo and in vitro (Sakamoto et al., 1993; Vucenik et al., 2004; Somasundar et al., 2005; Matejuk and Shamsuddin, 2010). However, most study did not compare the different sources of phytic acid. Therefore, in this study, effect of phytic acid extracted from rice bran could be observed. Study from Reddy et al. (2000) proved that wheat bran is more effective than corn bran or oat bran, suggesting that the modifying effect of dietary fiber on the production of putative tumor promoters depends on the type of fiber consumed. Phytic acid which is among the components of dietary fiber has been suggested to play a significant role and contributes to inhibit colon cancer (Shamsuddin, 1995).

Various theories have been proposed for the anticancer activity of phytic acid. The most prominent effect of phytic acid has been documented in cancer prevention by controlling tumor growth, progression and metastasis. The anticancer action of phytic acid involved are boosting immunity, antioxidant properties, reducing cell proliferation and inducing differentiation of malignant cells (Matejuk and Shamsuddin, 2010). The major finding of the present study is that phytic acid extracted from rice bran induced growth inhibition, disruption of cell cycle progression and increased apoptosis on HepG2 cells.

Phytic acid extracted from rice bran strongly induced growth of inhibition on HepG2 cell lines in a dose dependent manner, with the IC₅₀ value 17.0 µg/ml after 72 h (Figure 1a). The identifying of least cytotoxic effect of extracted phytic from rice bran acid towards non-tumorigenic cells was conducted on normal cell line, 3T3 (IC₅₀ cannot be determined). The analysis was performed as recommended by US National Institute of Experimental Health Sciences (NIEHS), Iteragency Coordinating Committee in the Validation of Alternative Methods (ICCAM) in order to access basal cytotoxicity...
Figure 2. Effect of phytic acid on cell cycle in HepG2 cells. The distribution of cells in the sub-
G1, G0/G1, S and G2/M phases of the cell cycle in different dosage (a) and different exposure
times (b). Each value is presented as mean ± standard error of mean of triplicate
determinations.
Figure 3. Apoptotic activity, determined by the Annexin V assay, induced early and late apoptosis in HepG$_2$ cell lines when treated with phytic acid in different dosage (A) and different exposure time (B). Each value is presented as mean ± standard error of mean of triplicate determinations.

(NIEHS, 2001). It can be hypothesized that phytic acid normalized the rate of cell growth. Although, the exact mechanism of the cytotoxicity of phytic acid against HepG$_2$ cells is not entirely clear, many possible mechanisms have been proposed for the growth inhibition of phytic acid in cultured cells and animal models. These mechanisms include induction of apoptosis and immunoregulation such as promoted
lymphocytes and IL-2 production by splenocytes (Xu et al., 2007).

Since cell cycle progression is an essential event for cellular growth, we also examined whether the cell growth inhibitory effect by phytic acid and/or mediated through an alteration in a specific phase of the cell cycle progression. In this experiment, flow cytometric analysis of cells (both attached and floating cells) was conducted after staining cells with propidium iodide. The results obtained in the present study provide convincing evidence that phytic acid exerts its effects on cell cycle progression. As shown by the cell cycle analysis by PI staining (Figure 2), there was an increase in cell population in G2/M phase after HepG2 cells were treated with different concentrations of phytic acid extracted from rice bran indicated that phytic acid induced a cell cycle arrest at G2/M phase. Cell cycle arrest in G1 phase of G2/M phase has been demonstrated as one of anticancer mechanisms. The previous study reported by Sherbiny et al. (2001) found that treatment with phytic acid cause G1 cell cycle arrest in mammary cancer cell lines MCF-7 and MDA-MB 231, and in HT-29, a human colon cancer cell line. However, the G1 arrest was not observed in our study when cells were treated with phytic acid, which could be due to the use of different types of cancer cell lines.

Apoptosis is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. A tumor occurs when the balance of cell
proliferation and cell death are broken (Xu et al., 2007). Induction of apoptosis is an effective strategy for cancer therapy (Sun et al., 2004). Induction of apoptosis and cycle arrest are the most common mechanisms studied on the chemotherapeutic drugs in recent years (Zhou et al., 2008). Previous study reported that phytic acid induces apoptosis in vivo and in vitro in prostate, breast, cervical cancer, pancreas, melanoma, Barrett’s adenocarcinoma and KS (Kaposi’s sarcoma) cell lines, involving cleavage of caspase 3, caspase 9 and poly ADP-ribose polymerase (PARP), an apoptotic substrate, in a time an dose-dependent manner (Matejuk and Shamsuddin, 2010).

In this study, the cell treated with extracted phytic acid from rice bran demonstrated typical characteristics of apoptosis. We could observe increases in both early and late apoptotic activity in HepG2 cell lines. The observed increases in early apoptotic activity may indicate that the cells were in static, non-proliferative state, while the cells were in late apoptotic cycle and the cell death was imminent (McFadden et al., 2008). We also incorporated the induction of HepG2 apoptotic cell death by phytic acid extracted from rice bran in a dose and time dependent manner.

Several molecular mechanisms can be suggested for the observed apoptosis effect of phytic acid in HepG2 cell. Agarwal et al. (2003) proved that phytic acid inhibits nuclear kappa B (NFkB) in different models of cancer such as prostate. Nuclear factor kappa B (NFkB) is an early-response gene involved in cell growth and

Figure 5. Apoptosis detection by flow cytometry and fluorescent markers Annexin-V FITC/PI. The experiments were performed after (a) Control (b) 24 h incubation time (c) 48 h incubation time (d) 72 h incubation time. Cell populations in bottom left, bottom right, top right and top left quadrants represented the proportion of viable cells, necrotic cells, late apoptotic cells and early apoptotic respectively. These results were representative of three experiments and at least 10,000 cells were analyzed per experiment.
apoptosis, and has been implicated in various cancers. Whereby, Karmakar et al. (2007) demonstrated that phytic acid down regulates cell survival factors BIRC-2 (baculovirus inhibitor-of-apoptosis repeat containing-2) and telomerase and up-regulates calpain and caspase-3 activities in malignant glioblastoma cells in promoting apoptosis. Further important role in apoptosis is suggested by finding that inositol hexaphosphate kinase-2 is a physiologic mediator of cell death (Nagata et al., 2005). In addition, phytic acid has been shown to regulate pro-apoptotic BCL-2 family of genes (Diallo et al., 2008).

In summary, extracted phytic acid from rice bran significantly decreased growth of hepatocellular carcinoma, HepG2 in vitro without any toxicity effects. Phytic acid proved the antiproliferative effect through altering cell growth and apoptotic activity. These findings suggest that phytic acid has the potential to become an effective adjunct for hepatic cancer. Besides, since the occurrence of phytic acid compound in safe, naturally occurring foods make it an attractive natural therapy for dietary and conventional treatment and possible prevention of hepatocellular carcinoma. Further in vivo and human studies are needed to evaluate safety and clinical utility of this agent in patients with hepatic cancer treatment.

REFERENCES


