INHIBITION OF CARCINOGENESIS BY SEED AND SOYBEAN MEAL EXTRACT IN COLON OF MICE: APOPTOSIS AND DYSPLASIA

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ABSTRACT

Objective: Colon cancer is a major public health problem. Soybean has demonstrated chemopreventive and anticancer. Here, we have investigated the effect of a standardized seed and soybean meal extract (SE) with content of lunasin, here named grobogan extract (GE) and SE. They are botanical drug substance in experimental models of colon cancer in vivo.

Methods: The effect of GE and SE was examined on the preneoplastic lesions (aberrant crypt foci), polyps and tumors induced by the carcinogenic agent azoxymethane (10 mg/kg) and dextran sodium sulfate 2% as well as in a xenograft model of colon cancer in mice.

Results: GE and SE increased apoptosis (p=0.001). GE (150 mg/kg) has the highest impact level of apoptosis (p=0.009). GE and SE decreased dysplasia (p<0.024). GE (200 mg/kg) has the highest impact level of dysplasia (p<0.002), and SE (200 mg/kg) has the second impact level of dysplasia (p=0.003).

Conclusions: GE and SE inhibition of colon carcinogenesis with increased level of apoptosis and decrease level of dysplasia.

Keywords: Soybean, Lunasin, Colon cancer, Azoxymethane, Dextran sodium sulfate, Apoptosis, Dysplasia.

INTRODUCTION

Colorectal cancer (CRC) represents the third most common cancer in men (746,000 cases, 10% of the total) and the second in women (614,000 cases, 9.1% of the total) worldwide [1]. Almost 55% CRC occur in more developed country but it is the incidence of CRC increased in developing country. It is happened because of change in diet and lifestyle, among other: Increased consumption of red meat and processed foods, decreased consumption of foods containing fiber (fruits and vegetables), increased weight (obesity), low physical activity, smoking and high alcohol intake [1,2].

Pharmacoeconomic studies have highlighted a trend for rising cost associated with CRC, which is linked to the increasing use of targeted biological therapies [3]. Screening strategies are utilized but have not reduced disease incidence or mortality [4]. Furthermore, therapeutic intervention, which is by itself very toxic, may fail to prevent disease progression to metastatic disease [5]. Therefore, there is an interest in both cancer preventive strategies - which include experimentation with safe phytochemical agents - and new curative treatments [6].

Soybean (Glycine max) is an ancient legume consumed worldwide, but most commonly in Asian countries, such as China, Japan, Korea, Taiwan, and Indonesia. The demographic consumption pattern of soybean varies geographically with Asians utilizing (20-80 g/day), mostly in processed forms, such as soybean, soy milk, tofu, tempeh, misso, natto, soybean paste, and soy sauce [7,8] as compared to Western population consuming meagerly (1-3 g/day), mostly in processed forms, such as soy drinks, breakfast cereals, energy bar and soy burger [9].

Epidemiological evidence has demonstrated an association between the consumption of soybean and improved health, particularly as a reduced risk for cardiovascular diseases [10] and cancer, such as breast, prostate, endometrial, lung, and bladder cancer [11-17]. Moreover, a number of animal models support anticancer properties of soy which constituents have been shown to suppress tumor growth in a variety of tissues including skin, bladder, mammary, and prostate [18]. In last decades, studies have isolated and identified an array of biologically active compounds or phytochemicals contained in soybean with cancer preventive effects.

A likely candidate is lunasin, a novel cancer preventive peptide, the efficacy of which has been demonstrated in our laboratory [19-22]. Initially identified in soy, lunasin is also present in barley, a food crop known for its health effects [21]. Lunasin is a unique 43 amino acid soybean peptide that contains at its carboxyl end 9 Asp (D) residues, an Arg-Gly-Asp (RGD) cell adhesion motif, and a predicted helix with structural homology to a conserved region of chromatin-binding proteins. It has been shown to suppress carcinogenesis caused by chemical carcinogens and oncogenes in in vitro models and a mouse model for skin cancer [19-22].

Histone acetylation and deacetylation have been associated with eukaryotic transcriptional regulatory mechanisms [23]. The affinity of lunasin for hypoacetylated chromatin suggests a role in chromatin-binding proteins. It has been shown to suppress carcinogenesis caused by chemical carcinogens and oncogenes in in vitro models and a mouse model for skin cancer [19-22].
In this paper, we used the inhibition of carcinogenesis by lunasin as a bioactivity assay for the first time. These were measured in lunasin extracted from a number of Indonesian soybean varieties and soybean meal through oral administration to measure apoptosis and dysplasia.

**METHODS**

**Plant material and extraction**

Soybean is obtained from Indonesian Legumes and Tuber Crops Research Institute Malang of East Java, Indonesia. Soybean varieties were used in this study is Grobogan because its content highest protein than other varieties (to reach 43.9%). Soybean meal is obtained from local market in Semarang of Central Java, Indonesia.

Before extraction, samples of soybean Grobogan pressed, to separate oil. The residue obtained from pressing process is made of powder with blender. The procedures for maceration were in accordance with previous studies [26,27]. The residue of grobogan seed and soybean meal are macerated with phosphate buffer saline solvent (1:5, 60 minutes) and filtrated twice. Filtrate of grobogan and soybean meal be dried with rotary vapor and kept temperature below 60°C. The result was tested by high performance liquid chromatography to determine the content of lunasin.

**Animal**

Male Swiss-Webster mice (12 weeks old) of ≤20 g body weight, supplied by Agency for Health Research and Development Ministry of Health of the Republic of Indonesia. The mice were maintained under standard laboratory conditions housed in independent ventilation cages (with stainless steel grill top, 48 cm × 35 cm × 20 cm) under hygienic and under controlled humidity conditions (55\%±5\%), light (12 hrs/12 hrs light/dark cycle), and temperature (23±1°C). The mice were allowed free access to food and water. The animals were carefully examined for 1 week prior to use in the experiments, and only healthy animals were chosen. All protocols and surgical procedures were approved by the Institute of Animal Care and Use Committees of Medical Faculty University of Indonesia.

**Induction of colon cancer**

Induction of colon carcinogenesis adopted Kusmardi et al. Mice induced with azoxymethane (AOM) were dissolved in 0.9\% NaCl at a dose of 10 mg/kg body weight by intraperitoneal (i.p.) injection early as one. 1 week after induction of AOM, mice given standard feed and water containing dextran sodium sulfate (DSS) 2\% daily for 1 week [28].

**Experimental groups**

Mice were randomized into nine experimental groups as follows: Normal group, mice oral administration of physiological saline for 4 weeks, without a single i.p. injection AOM and DSS; negative group, mice oral administration of physiological saline for 4 weeks, and received a single i.p. injection AOM and DSS; grobogan treatment group, mice oral administration of grobogan extract (GE) (75, 150, 200 mg/kg, respectively) for 4 weeks, and received a single i.p. injection AOM and DSS; soybean meal treatment group, mice oral administration of soybean meal extract (SE) (75, 150, 200 mg/kg, respectively) for 4 weeks, and received a single i.p. injection AOM and DSS. After 4 weeks treatment, mice of each group were sacrificed to know inhibition of colon carcinogenesis.

**Hematoxylin eosin staining**

A piece of colorectal tissue made paraffin blocks. Paraffin blocks made 4 μm thick slices and mounted on glass objects for HE staining stages as follows: Deparaffinizesection using xylol I, II - III, and the last drops with entelan and covered with a glass lid. Preparations viewed using a light microscope with ×400 magnification. Histological assessment was done in a blinded fashion to avoid bias. Based on H&E staining, histological alterations, such as mucosal ulceration, dysplasia, and carcinoma, were verified by a board-certified pathologist. Carcinoma was defined as a high-grade dysplasia of the colonic mucosa that had invaded beyond the muscularis mucosa and into the submucosa.

**Immunohistochemistry**

For cleaved-caspase 3 immuno-histochemistry analysis, colorectal tissue deriving from mice were fixed in buffered formalin, embedded in paraffin, and cut into 4 μm-thick serial sections. Sections were stained with the cleaved-caspase 3 (1:100 v/v) antibodies. After three 5-minute washes, the secondary antibody was added and the samples were incubated at room temperature for 20 min. The streptavidin-HRP detection system was added and samples were incubated at room temperature. After three 5-minute washes, 50 μl of chromogen was added and the reaction stopped after 1 min in water. Histological analysis colorectal tissues were fixed in 10% formalin. Thin (0.5 mm) paraffinized section were prepared and stained with toluidine blue as and then processed for light microscopy with ×400 magnification.

**Statistical analysis**

All data are presented in mean±standard deviation. Differences in levels among treatment groups were analyzed by ANOVA using SPSS 20.0 statistical package. A p<0.05 was considered significant statistically.

**RESULTS**

Weight body index group of mice with GE and SE is displayed in Table 1. In grobogan group shows GE (150 mg/kg) has the highest weight body index, compared with other weight body index in grobogan treatment group. Weight body index of GE (150 mg/kg) has higer than weight body index of aspirin (control positive). In soybean meal group shows SE (150 mg/kg) has the highest weight body index, compared with other weight body index in soybean meal treatment group. Weight body index of SE (150 mg/kg) has higer than weight body index of aspirin (control positive).

**DISCUSSION**

Effect of giving seed and SE for weight body index as shown in Table 1. This Table 1 shows that weight body index of mice in all of the group treatment at weekly is above 1, which means that seed and SE contribute to weight gain in mice.

Soybean seeds contain to 40% of crude protein and about 20% of fat, and soybean meal characterized higher content of crude protein about

| Table 1: Weight body index group of mice with GE and SE |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Group**       | **weeks**       | **1**           | **2**           | **3**           | **4**           | **5**           | **6**           |
| Normal          | 1.11            | 1.18            | 1.20            | 1.18            | 1.17            | 1.23            |
| Negative        | 1.12            | 1.23            | 1.25            | 1.28            | 1.30            | 1.41            |
| Positive (aspirin) | 1.13       | 1.21            | 1.24            | 1.22            | 1.18            | 1.23            |
| GE (75 mg/kg)   | 1.08            | 1.19            | 1.19            | 1.21            | 1.20            | 1.21            |
| GE (150 mg/kg)  | 1.10            | 1.21            | 1.24            | 1.28            | 1.27            | 1.32            |
| GE (200 mg/kg)  | 1.09            | 1.17            | 1.26            | 1.19            | 1.16            | 1.22            |
| SE (75 mg/kg)   | 1.09            | 1.18            | 1.20            | 1.21            | 1.19            | 1.20            |
| SE (150 mg/kg)  | 1.08            | 1.19            | 1.18            | 1.21            | 1.19            | 1.25            |
| SE (200 mg/kg)  | 1.04            | 1.08            | 1.07            | 1.05            | 1.05            | 1.08            |

GE: Grobogan extract, SE: Soybean meal extract
40–49%. Protein of soybean products characterized much quantity of arginine, cystine, histidine, isoleucin, leucine, lysine, methionine, phenylalanine, theonine, tryptophan and valine [29].

Soybean contains isoflavones. These compounds have got biochemical activity, including treatment of chronic diseases. Studies in humans and animals have shown that the consumption of soybean or soybean-derived products may help to protect against cardiovascular diseases, certain types of cancer, obesity, and diabetes [30]. The ability of isoflavones to prevent these diseases depends on their absorption, metabolism, distribution to target tissues and excretion of waste compounds, that is, their bioavailability. In other study showed that a diet containing soy isoflavones increased the body weight and fat percentage, also the serum concentrations of insulin-like growth factor-1 (IGF-I) and interleukin-6 were increased by high levels of soy isoflavones [31].

Our results indicate that seed and SE provide protection against colon carcinogenesis in male AOM-induced Swiss Webster mice, specifically against earlier stages of colon carcinogenesis. This is evidenced by the higher incidence of apoptosis (Figs. 3 and 5) and the lower incidence of dysplasia (Figs. 4 and 6). In Fig. 3, with ANOVA analysis shows that GE and SE increased apoptosis (p=0.001). GE (150 mg/kg) has the highest impact level of apoptosis (p=0.009). In Fig. 5, it shows that immunohistochemistry to active caspase-3 for apoptosis detection. This figure shows that GE (150 mg/kg) has a same impact level of apoptosis with control positive. In this research, we use aspirin for control positive. Long-term aspirin use reduces the risk of colon cancer [32]. Mechanisms of aspirin for chemoprevention include inhibition of the cyclooxygenase (COX) pathway, or COX-independent mechanisms, for example, the PI3KCA pathway, or therapy-induced senescence of cancer cells [33].

In other study, it shows that the combination of etoricoxib and atorvastatin against DMH-induced colon cancer by increasing the level of antioxidant enzymes. The histopathological this study demonstrated that the combination treatment showed more positive effect in the prevention of colon carcinogenesis by reducing the inflammation, hyperplastic, dysplastic change in colon crypt cells [34]. Etoricoxib specifically binds to and inhibits the enzyme COX-2, resulting in inhibition of the conversion of arachidonic acid into prostaglandins. Inhibition of COX-2 may induce apoptosis and inhibit tumor cell proliferation and angiogenesis [35]. In this study used aspirin, it has same mechanism with etoricoxib. Aspirin exhibit increase level of apoptosis and decrease level of dysplasia in colon crypt cell (Figs. 3 and 4). Apoptosis and dysplasia are indication of carcinogenesis of colon cancer. In this study, adopt colitis-associated colon cancer. AOM and DSS can make chronic inflammatory process may contribute to multistep colon carcinogenesis in the inflamed colon. They include overproduction of reactive oxygen and nitrogen species, overproduction and upregulation of productions and enzymes of arachidonic acid biosynthesis pathway and cytokines, and intestinal immune system dysfunction [36]. Aspirin’s most well-characterized pharmacologic activity is the permanent modification of the prostaglandin endoperoxide synthetase or COX enzymes. These enzymes are rate limiting for the conversion of arachidonic acid to prostaglandins and related eicosanoids. COX-1 isozyme is constitutively expressed in most tissues, whereas growth factors, oncogenes, tumor promoters, and inflammatory cytokines induce the COX-2 isozyme [37]. Treatment with aspirin for colitis-associated
Regulated a set of genes that are necessary for the angiogenesis through the vascular endothelial growth factor [39]. Soybeans down-regulate genes for apoptosis, transformation, metastasis, and induction of angiogenesis in cancer cells, IGF-1R plays an even more critical role because it activates multiple intracellular pathways, mediated by serine/threonine kinase (Akt), yielding growth and enhanced survival.

Soybeans are known a low-risk food for cancer, and correlation of the intake of soybean foods and the morbidity of cancer [38]. Dietary agents known used natural inhibitors of insulin-like growth factor-1 receptor (IGF-1R) for the management of tumors. IGF-1R is a member of the receptor tyrosine kinase superfamily that binds to ligands, activating the phosphorylation of downstream substrates. Signaling through IGF-1R in normal cells leads to the activation of multiple intracellular pathways, mediated by the receptor associated tyrosine kinase domain, PI-3 kinase, and by serine/threonine kinase (Akt), yielding growth and enhanced survival.

In cancer cells, IGF-1R plays an even more critical role because it contributes to the promotion of tumor growth by inhibition of the apoptosis, transformation, metastasis, and induction of angiogenesis through the vascular endothelial growth factor [39]. Soybean down-regulated a set of genes that are necessary for the angiogenesis pathway in LNCaP, PC-3, and DU-145 PCa cells. These genes included platelet-derived endothelial cell growth factor 1, fibroblast growth factor 1 (FGF1), IGF1, PFG receptor 3 (FGFR3), interleukin-1β (IL-1β), IL-6, IL-8, platelet/endothelial cell adhesion molecule (CD31 antigen or PECAM1), CXCL10, and many other molecules that are essential to angiogenesis [40]. That study shown that soybean can prevent colon carcinogenesis by inhibition of IGF-1R.

Lunasin in soybean acts as a surrogate tumor suppressor that tightly binds to deacetylated core histones disrupting the balance between acetylation-deacetylation, which is perceived by the cell as abnormal and leads to cell death [41]. This first mechanism of action involving histone acetylation inhibition is considered as one of the most important epigenetic modifications acting on signal transduction pathways involved in cancer development [42,43]. When the cells are in the steady-state conditions, the core H3 and H4 histones are mostly deacetylated, as a repressed state. When cells were treated with peptide lunasin and well-known deacetylase inhibitor sodium butyrate, histone acetylation was inhibited in C3H10T1/2 fibroblasts and breast cancer MCF-7 cells [44–46]. Furthermore, lunasin has been demonstrated to compete with different histone acetyltransferase enzymes (HATs), such as yGCN5 and PCAF, inhibiting the acetylation and repressing the cell cycle progression [46–49]. Recently, we have reported that lunasin is a potent inhibitor of histones H3 and H4 histone acetylation [49,50]. Lunasin's inhibitory activity was found to be higher than that demonstrated by other compounds, such as anacardic acid and curcumin, which chemopreventive properties have been already reported [51–53]. Studies focused on elucidating lunasin's structure-activity relationship establish that lunasin's sequence is essential for inhibiting H4 acetylation whereas poly-D sequence is the main active sequence responsible for H3 acetylation inhibition [50].

Lunasin has been demonstrated that it causes cytotoxicity in four different human colon cancer cell lines, KM12L4, RKO, HCT-116, and HT-29 cell, with IC50 values of 13.0 μM, 21.6 μM, 26.3 μM and 61.7 μM, respectively [54]. These values suggest that lunasin is most potent killing the highly metastatic KM12L4 colon cancer cells than any other colon cell lines used in this study. Moreover, lunasin was capable to provoke cytotoxic effects on the oxalipatin-resistant variants of this colon cancer cells [55]. Studies on mechanism of action of this peptide have revealed that lunasin causes arrest of cell cycle in G2/M phase and induction of the mitochondrial pathway of apoptosis. The cell cycle arrest was attributed with concomitant increase in the expression of the p21 protein in HT-29 colon cancer cells, while both p21 and p27 protein expressions were up-regulated by lunasin treatment in KM12L4 colon cancer cells [54,56]. Moreover, treatment with lunasin decreased the ratio of Bcl-2: Bax by up-regulating the expression of the pro-apoptotic Bax and downregulating the expression of the anti-apoptotic Bcl-2, also increasing the activity of caspase-3 [55]. This might be attributed to the increase in the expression of the pro-apoptotic form of Bax, which is positively affected by the increase p21 expression and the increase of the expression of cytosolic cytochrome c in KM12L4 cells. It was also demonstrated that treatment with lunasin provokes an increase in the activity of caspase-9 and caspase-3 in both HT-29 and KM12L4 cells [56]. Furthermore, lunasin has been showed to modify the expression of human extracellular matrix and adhesion genes [56]. The Arg-Gly-Asp motif present in the lunasin structure is a recognition site for integrin receptors present in the extracellular matrix (ECM). Integrins are heterodimeric receptors associated with cell adhesion, and cancer metastasis [57]. Treatment of KM12L4 cells with lunasin resulted in the modification on the expression of 62 genes associated with ECM and cell adhesion [56]. These authors also reported that lunasin downregulated the gene expression of collagen type VII α1, integrin α2, matrix metalloproteinase 10, selectin E and integrin α5 by 10.1-, 8.2-, 7.7-, 6.5- and 5.0-fold, respectively, compared to the untreated CRC cells. On the other hand, the expression of collagen decreases dysplasia. Relative amounts of dysplasia were quantified using the Image J software. The values are expressed as the mean±SD, p<0.05; (a) Normal group, (b) negative group, (c) positive group, (d) GE 75 mg/kg, (e) GE 150 mg/kg, (f) GE 200 mg/kg, (g) SE 75 mg/kg, (h) SE 150 mg/kg, (i) SE 200 mg/kg, (magnification ×400)

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type XIV a1 was up-regulated on lunasin treatment by 11.6-fold. These results suggest a potential role of peptide lunasin as an agent to combat metastatic colon cancer particularly in cases where resistance to chemotherapy develops.

In another study, colon cancer liver metastasis is a widely used model to study the effects of different markers and chemotherapy on colon cancer metastasis. Recently, Dias and Gonzalez de Mejia have reported that lunasin acts as chemopreventive agent against this type of metastasis using colon cancer KM12L4 cells directly injected into the spleen of athymic mice [5-4]. Lunasin administered at concentration of 4 mg/kg body weight resulted in a significant inhibition of liver metastasis of colon cancer cells, potentially because of its binding to ε2f1 integrin and subsequent suppression of FAK/ERK/NF-κd signaling. Lunasin was also capable to potentiate the effect of oxicapatin in preventing the outgrowth of metastasis. Moreover, lunasin potentiated the effect of oxalaplatin in modifying expression of proteins involved in apoptosis and metastasis including Bax, Bcl-2, I KKα- and p-p65 [50]. These results suggest that lunasin can be used as a potential integrin antagonist thereby preventing the attachment and extravasation of colon cancer cells leading to its anti-metastatic effect. These results open a new vision about the lunasin used in metastasis that might benefit to prolong the survival of mice with metastatic colon cancer [58].

Effect of giving seed and SE for dysplasia index as shown in Figs. 4 and 6. In Fig. 4 with ANOVA analysis shows that GE and SE decreased dysplasia (p=0.024). GE (200 mg/kg) has the highest impact level of dysplasia (p=0.002) and SE (200 mg/kg) has the second impact level of dysplasia (p=0.003). In Fig. 6, it shows that hematoxylin-eosin staining with dysplasia.

In another study, the effect of dietary supplementation with isoflavone-free soy protein concentrate (SPC) on colon inflammation and carcinogenesis in male, CF-1 mice was investigated in one 10-week and one 20-week study. In both studies, following one-week pretreatment with SPC or casein as the sole source of protein (19% kcal), mice were injected with the colon carcinogen (AOM, 10 mg/kg body wt). One week later, mice received (DSS, 1.5% w/v) as their sole drinking fluid for one week to induce colon-specific inflammation. On days 3 and 7, and weeks 7 and 20 (second study only) after DSS treatment, mice were euthanized and the colons were examined for markers of inflammation and carcinogenesis. All samples were scored using the inflammation index, which measures inflammation area, severity, ulceration and hyperplasia and dysplasia. In both studies, trends in these measures appeared to be highly dependent on sacrifice time-point. With the data from both studies combined, mean polyp multiplicity was significantly reduced in mice fed SPC at week 7 and for the study overall (p=0.001 and p<0.01, respectively). Hyperplasia and dysplasia, inflammation area and total inflammation index score were significantly decreased in the SPC group compared to the control at week 7 (p=0.10, p<0.10 and p<0.05, respectively). At 20 weeks, mice in both the control and SPC-treated groups showed colon tumors. The trend toward reduced tumor burden and multiplicity in mice fed the SPC diet was observed, but the effect was not statistically significant [59].

CONCLUSION

In conclusion, we have shown that GE and SE have beneficial actions in an experimental model of colon cancer. Both of them inhibition of colon carcinogenesis with increased level of apoptosis and decrease level of dysplasia.

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