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Original Article

EFFECT OF LUNASIN-ENRICHED SOY EXTRACT ON HISTONE DEACETYLASE EXPRESSION IN DISTAL COLON EPITHELIAL CELLS FROM AOM/DSS-INDUCED MICE

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ABSTRACT

Objective: Lunasin peptide, with its chemopreventive and chemotherapeutic abilities, is known to affect carcinogenesis via epigenetic regulation involving histone acetylation. This study investigated lunasin, which can be found in soy, and its effects towards histone deacetylase (HDAC] expression in a mouse model of carcinogenesis.

Methods: Thirty Swiss Webster mice were grouped into normal, positive control, negative control, and experimental groups. Except for the normal group, mice underwent carcinogenesis induction through azoxymethane (AOM] and dextran sodium sulfate (DSS] injection. Experimental mice received lunasin-enriched soy extract at a dosage of 250 mg/kg BW (kilogram body weight), 300 mg/kg BW, and 350 mg/kg BW for 4 w. Distal colon samples were stained by using immunohistochemistry (IHC]. HDAC expression was measured by IHC optical density score.

Results: Average HDAC expression was 202.4% in the normal group, 239.3% in the negative control, 175.25% in the positive control, 202.03% at 250 mg/kg BW dose, 219.53% at 300 mg/kg, and 166.68% at 350 mg/kg BW. There was no significant difference between HDAC expression at 250 mg/kg BW and 300 mg/kg BW soy extract. However, at 350 mg/kg BW soy extract there were significant changes in HDAC expression.

Conclusion: lunasin in soy extract at a 350 mg/kg BW dose can decrease HDAC expression in a colorectal cancer carcinogenesis model.

Keywords: Colorectal cancer, Epigenetics, Histone deacetylase enzyme, Lunasin, Soy

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INTRODUCTION

Globally, colorectal cancer is the third most prevalent cancer in men, with 746,000 cases diagnosed annually, and the second most prevalent in women (614,000 cases). Approximately 55% of colorectal cancer cases occur in developing countries [1]. In Indonesia, colorectal cancer incidence is 12.8 cases for every 100,000 adults [2]. Colorectal cancer is the fourth highest cause of mortality with almost 700,000 deaths in 2012, and the third most frequently diagnosed malignancy, with annual new cases totaling around 1.4 million. It is estimated that global colorectal cancer burden will increase by approximately 60% to 2.2 million new cases and 1.1 million deaths by 2030 [3].

There are multiple options for colorectal cancer treatment. If the cancer is resectable, surgery and radiofrequency ablation can be undertaken alongside cytotoxic therapies such as radiation and chemotherapy, as well as other alternatives such as immunotherapy. However, the majority of colorectal cancer cases cannot be resected and radiofrequency ablation has a high rate of local tumor recurrence. Moreover, radiation and chemotherapy have a low therapeutic index, high toxicity, and various side effects. Inflammation involving multiple immune cells is associated with tumorigenesis and is thus a target of immunotherapy, but this modality is still associated with toxicity. Innovative strategies in adjuvant therapy to improve the effects of conventional therapies are still required [4-6].

Other therapeutic options currently being researched are nutritional therapies. Soy consumption is associated with a reduction in cancer risk of approximately 21% in women [7]. Soy contains a protein called lunasin, which consists of 43 amino acids.[8] A study by Mejia *et al.* found that lunasin concentrations in tested substances including soy flour and soy protein concentrate varied between 0.1–1.33 g/100 g of flour [9]. Multiple studies have demonstrated that lunasin has a chemopreventive capability by inhibiting cell transformation and increasing chemotherapeutic response in various cancer types [10]. In HT-29 colon cancer cells, lunasin can induce apoptosis through the mitochondrial pathway and pro-apoptotic clusterin expression [11]. Its sensitivity has been shown in animal models of lung cancer and melanoma, and is thought to relate to integrin signaling and histone acetylation changes. In colorectal cancer cells, lunasin has cytotoxic effects associated with integrin expression and is suggested to have the potential to prevent metastasis. Lunasin also has epigenetic mechanisms by inhibiting H3 and H4 histone acetylation and functioning as a tumor suppressor [12-14]. Studies have shown that lunasin can affect cancer through multiple mechanisms, but research regarding the effects of lunasin on cell acetylation changes in colon cancer have yet to be performed.

Based on the current challenges in colorectal cancer therapies and the lunasin research undertaken to date, this study aimed to determine whether lunasin-enriched soy extract can induce changes in histone deacetylase (HDAC) expression in an animal model of colon cancer induced by azoxymethane (AOM) and dextran sodium sulfate (DSS). It is hoped that lunasin has the potential to increase the efficacy of colorectal cancer therapy. Data obtained from this study can be used for further research of lunasin effects in colon cancer.

MATERIALS AND METHODS

Research animals

Swiss Webster mice were obtained from the Animal Laboratory of Research and Development, Department of Health Ministry, Jakarta. Thirty-one male mice of±25 g weight and±12 w of age were used. Using the Federer formula, the minimum number of mice was calculated to be 24. Mice were sampled randomly and divided into six groups: normal, negative control, positive control, and 250 mg/kg BW, 300 mg/kg BW, and 350 kgBW of lunasin-enriched soy extract.

Soy extraction and lunasin analysis

The Grobogan variety of soy from Bogor, Indonesia underwent a pressing process for 30 min at 100–150 atm pressure and 50 °C to separate the oil. The resulting material was blended to soy flour. A total of 1250 g of the flour was macerated for 60 min in 6250 ml of

phosphate buffer saline (PBS). The maceration solute was filtered three times with filtration paper, and the filtrate was dried in an evaporator at 50 °C, resulting in a dry extract. The quantity of lunasin in the extract was analyzed by high-performance liquid chromatographic (HPLC) using a WatersXBridge TM C from Agilent Technologies at 18.5 µm with a UV detector a and column of 4.6 × 150 mm in diameter.

Mouse experiments

Mice underwent adaptation for one week in the laboratory prior to the start of the experiments. The physical health of the mice was confirmed before carcinogenesis induction. Mouse experiments were performed according to the Guide for the Care and Use of Laboratory Animals and the Animal Care and Use Committee. Mice received ad libitum standard food and drink and were maintained in a 12h light/dark cycle at a controlled temperature of 25oC at 55% humidity.

The carcinogenesis induction protocol was adapted from Kusmardi *et al.* [15] Mice received azoxymethane (AOM) diluted in 0.9% NaCl through intraperitoneal (ip) injection of a single dose of 10 mg/kg BW in the first week of the experiment; the normal group received an injection of 0.9% NaCl. One week after AOM administration, mice received food and drink containing 2% dextran sodium sulfate (DSS) daily for a week.

After carcinogenesis induction with AOM+DSS, mice were divided into six groups and treated accordingly for four weeks. The normal and negative control group received 0.9% NaCl, the positive control group received 150 mg/kg BW aspirin, and the experimental group received oral lunasin therapy with 250 mg/kg BW, 300 mg/kg BW, or 350 mg/kg BW of soy extract. The process of carcinogenesis induction followed by administration of lunasin therapy was conducted over a total of six weeks. Mice were anesthetized with 75–100 mg/kg of ketamine and 10 mg/kg xylazine with a 23–25 G syringe and needle, and then killed by neck dislocation and colon tissue was excised. The tissue was washed in water and fixed with 10% formalin before embedding in paraffin medium. Sections of 4 µm thickness were then cut. Immunohistochemistry (IHC) with primary anti-HDAC antibody was undertaken and sections were counterstained with hematoxylin-eosin.

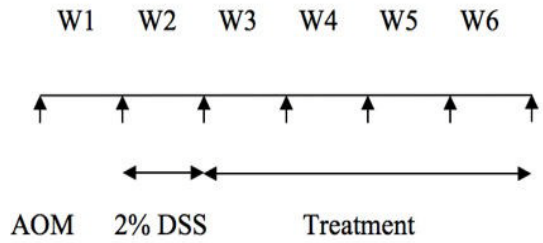

Fig. 1: Experimental procedure in mice

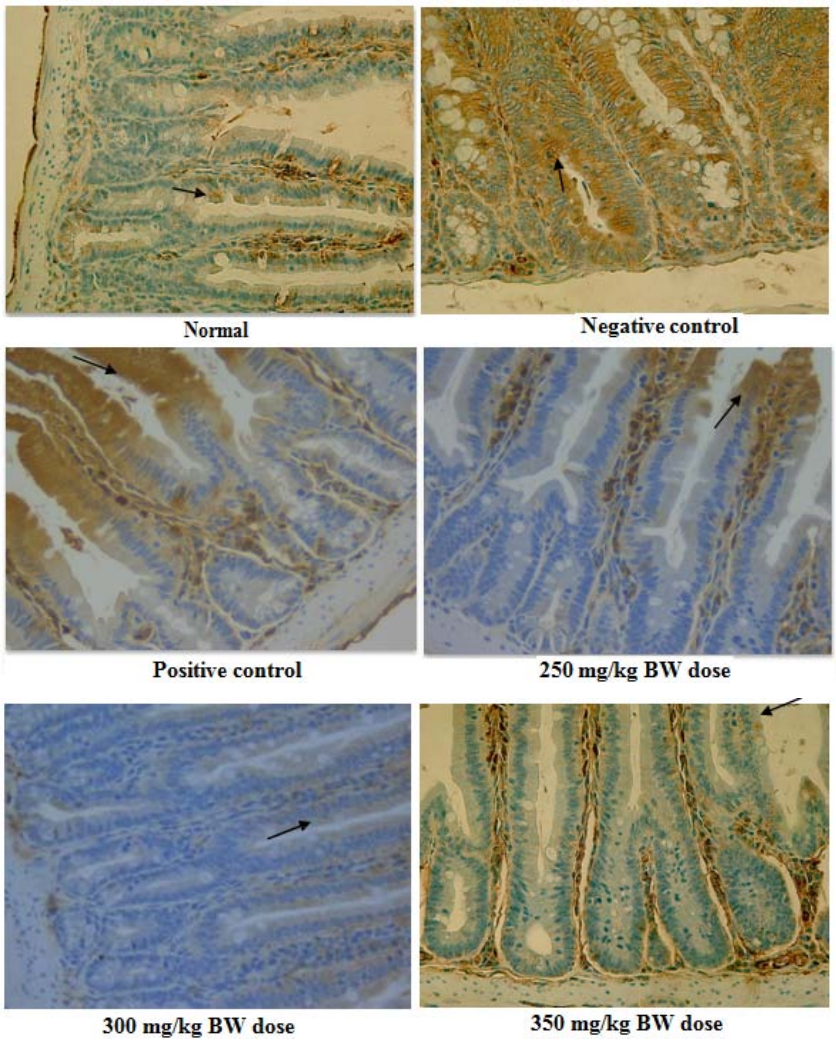

Fig. 2: IHC of mouse distal colon. Arrows indicate HDAC expressionKruskal Wallis test revealed there were statistically significant differences between two or more groups (*P*=0.031), table 2 shows the *P*-values of mann whitney test between the groups, and fig. 2 details the median, minimum, and maximum values of HDAC expression, revealing a significant differences between the groups

Data interpretation

Slides were observed at ×400 by light microscopy (Leica and Olympus). Each slide was imaged in five random visual fields. Total positive cell count (brown color) and negative cell count (blue color) were obtained with Image J software. Cells with positive staining in the nucleus and/or cytoplasm were marked with a dark blue dot for high positivity, a light blue dot for moderate positivity, and a green dot for weak positivity. Negative cells were marked with a purple dot. Cell count results are shown as a percentage based on IHC optical density score. [16]

100

HP, high positivity; MP, moderate positivity; WP, week positivity; N Negative

Statistical analysis

Data were analyzed using SPSS (Statistical Package for Social Sciences) for Mac 24.0 version. Kruskal Wallis non-parametric test followed by Mann Whitney test was applied to identify differences between two groups. Significant differences are indicated at *P*-values of<0.05.

RESULTS

HDAC expression was quantified as a percentage. Based on the Shapiro-Wilk normality test, data distribution was not normal (*P*=0.005). HDAC expression is shown as median, minimum, and maximum values in table 1.

Table 1: Median, minimum, and maximum values of HDAC expression

Group	n	Median (Minimum-Maximum)
Normal	4	180,645 (156,44–183,21)
Negative control	5	217,200 (197,88–318,55)
Positive control	4	163,605 (149,03–224,76)
250 mg/kgBW dose	4	200,000 (187,89–220,23)
300 mg/kgBW dose	5	184,86 (159,04–292,61)
350 mg/kgBW dose	4	165,250 (147,02–189,21)

IHC of mouse distal colon epithelial cells is shown in fig. 2. Morphological changes and high HDAC expression were observed in the negative control group.

Table 2: Mann-whitney *post-hoc* test results p value

Group		N	NC	PC	D250	D300	D350
1.	N	-					
2.	NC	0,014*	-				
3.	PC	0,386	0,086	-			
4.	D250	0,021*	0,221	0,248	-		
5.	D300	0,086	0,347	0,142	0,624	-	
6.	D350	0,564	0,014*	0,773	0,043*	0,142	-

**P*<0.05; N, normal, NC, negative control, NP, positive control; D250, 250 mg/kg BW; D300, 300 mg/kg BW; D350, 350 mg/kg BW.

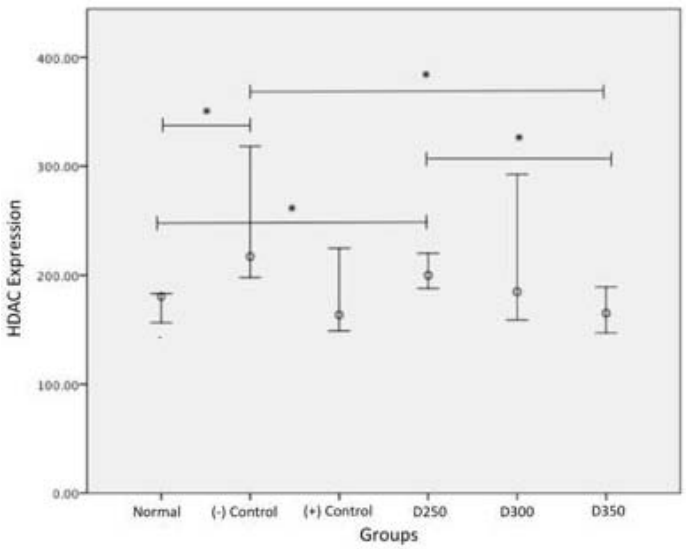

Fig. 2: Median, minimum, and maximum values of HDAC expression. *P<0.05

DISCUSSION

Class 1 HDAC enzymes, such as HDAC1, HDAC 2, HDAC 3, and HDAC 8, and class 2 HDAC enzymes such as HDAC 4 are found in the normal colon and intestine, especially in proliferating crypts. The abundance of HDAC expression in crypts is consistent with the role of HDACs in maintaining cell proliferation and survival and inhibiting differentiation [17]. This study used an animal model to study the effects of lunasin-enriched soy extract in colorectal cancer. We chose male Swiss Webster mice because of their size and practicality. Male mice were chosen to eliminate the effects of the estrous cycle and to obtain more homogenous data.

In this study, HDAC expression was demonstrated by IHC staining. Brown staining indicated HDAC expression and blue counterstaining was undertaken with hematoxylin-eosin. The highest levels of HDAC

expression were observed in the negative control group, and the lowest levels were identified in the group that received 350 mg/kg BW soy extract. HDAC expression decreased with increases in soy extract dose, as demonstrated by stronger brown staining in tissue that was exposed to low doses of soy extract. Besides the negative control and experimental groups, the normal and positive control groups also expressed HDAC. Morphological changes such as hyperplasia were identified in the negative control group, but not in the positive control group.

Cancer induction in mice using azoxymethane (AOM) and dextran sodium sulfate (DSS) was successful, but with significant differences between the normal and negative control groups. Both agents work synergistically to initiate carcinogenesis in mice. As its 1,2 dimethylhydrazine (DMH) metabolite, AOM is a carcinogenic substance with high potency and good stability. After metabolization in the body, AOM is catalyzed to the mutagenic agent methylazoxymethanol (MAM), which can function through various signaling pathways such as TGF-β, β-catenin, and K-RAS. The inflammatory agent DSS shortens latency time in in colorectal cancer induction in a dose-dependent manner. Inflammation has been known to be related to cancer, and DSS is a nongenotoxic carcinogen. In colorectal cancer, risk increases with the degree of inflammation and disease duration [18].

HDAC expression between the normal and positive control groups was not statistically significant, and the average expression in the positive control group was lower compared with the normal group. In normal colon tissue, HDAC enzyme was expressed but expression increased during carcinogenesis. Treatment with lunasin-enriched soy extract resulted in lower HDAC expression in the positive control group compared with the normal group, but this decrease was not statistically significant.

There was no significant difference between the positive control group and the negative control group. In an *in vivo* study in mice, Guo *et al.* observed that 0.02% aspirin in AOM/DSS-induced mice was capable of downregulating HDAC protein expression and activity. It also lowered acetylation levels at histone H3 lysine 27. Aspirin is thought to exerti its effects through an epigenetic mechanism, but further research is required to understand the mechanism by which aspirin modifies histone acetylation and HDAC activity [19]. Meanwhile, an *in vitro* study by Sonnemann *et al.* in ovarian cancer cells revealed that aspirin had no effect on histone acetylation regardless of whether HDAC inhibitor was present. They found no significant changes in HDAC expression following administration of aspirin in an animal model of colorectal cancer carcinogenesis [20]. Compared with the study by Guo *et al.* using 0.02% aspirin, Sonnemann *et al.* used a dose of 0.015% [19]. Thus, aspirin levels may not be enough to induce significant differences in HDAC expression.

Lunasin-enriched soy extract was obtained from the Grobogan variety of soy. Grobogan was chosen because it matures faster than other varieties, with larger beans of 18 g/100 beans, and has a high protein content [21]. In Indonesia, soy is easily obtained and is consumed by much of the population in a variety of ways. Lunasin content in Grobogan soy extract was analyzed by HPLC and the concentration of lunasin was found to be 0.838 mg/g soy extract. Hernandez-Ledesma *et al.* found that, compared with other plants such barley, wheat and rye, soy (*Glycine max)* had the highest lunasin content, with concentrations ranging 0.5–8.1 mg/g [22]. In soy, protein concentration is related to nutrient distribution. Soy with low protein content contains high amounts of isoflavone and saponin, but lower levels of minerals and bioactive peptides such as lunasin [23].

To observe the effects of lunasin-enriched soy extract in mice with colorectal cancer, the experimental groups were compared with a negative control group. Significant differences were identified at the highest dose of 350 mg/kg BW. Expression of HDAC at 250 mg/kg BW and 300 mg/kg BW was not significant. A dosage of 350 mg/kg BW soy extract significantly reduced HDAC expression but this was not found at lower doses. Upregulation of HDAC is associated with abnormal histone acetylation, which causes dysfunctional gene transcription regulation during cancer progression. Decreases in histone acetylation have been implicated in colorectal cancer, such as H3ac, H4ac, H4K16ac, and H3K18ac [24].

Further research using doses higher than 350 mg/kg BW is required to determine whether decreases in HDAC expression results in dose-dependent effects and whether these changes are linear. To date, there are no other studies comparing lunasin with HDAC expression in colorectal cancer, and thus the dose and observed effects of lunasin cannot be compared.

Statistical analysis revealed significant differences between the experimental groups at 250 mg/kg BW and 350 mg/kg BW. The group that received 300 mg/kg BW dose did not demonstrate significant changes in HDAC expression compared with 250 mg/kg BW and 350 mg/kg BW. This could infer that the dose difference between 250 mg/kg BW and 350 mg/kg BW was sufficient, but that the 300 mg/kg BW dose was too close to the doses of 250 and 350 mg/kg BW. Future research should use larger intervals between the doses in experimental groups.

Lunasin has epigenetic roles in disrupting cancer formation. The protein goes into the cell nucleus and competes with histone acetylase (HAT) enzyme to bind the deacetylated core of histones H3 and H4. Inhibition levels of lunasin correlates with its concentration. The affinity of lunasin towards hypoacetylated chromatin and its inhibitory effect towards histone acetylation is explained by the E1A–Rb–HDAC model. Lunasin kills cell undergoing selective changes by interfering with histone acetylation–deacetylation dynamics during cell transformation. Tumor suppressor protein, Rb, interacts with E2F promoter to recruit HDAC, maintaining deacetylation of the histone core. When oncoprotein E1A releases the binding between Rb and HDAC, the deacetylated histone core is exposed to acetylation by HAT. This initiates lunasin, which competes with HATs such as P300/CBP-associated factor (PCAF) and yeast transcriptional coactivator GCN5 (yGCN5) to stop transcription and halt cell cycle progression. Disruption in the acetylation–deacetylation balance is perceived as abnormal by cells, causing apoptosis.

The mechanism of lunasin competition with HAT is not fully understood. It is thought that the inhibitory effect of lunasin involves ionic binding of lunasin with negatively charged poly-D in deacetylated histones [8, 22, 25].

CONCLUSION

Administration of lunasin-enriched soy extract at 350 mg/kg BW can significantly lower HDAC expression in a colorectal carcinogenesis animal model. Further studies using higher lunasin concentrations and a larger dosing range are required to verify whether lunasin exerts dose-dependent effects and if these effects are linear.

ETHICAL APPROVAL

This study was approved by the FKUI-RSCM Medical Research Ethics Committee, with ethical approval letter number 181/UN2. F1/ETIK/2016.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

No conflict of interest is declared

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