INTRODUCTION

Colorectal cancer (CRC) is the third most common type of cancer in men and the second most common type of cancer in women, contributing to 9.7% of all cancer worldwide [1, 2]. In 2012, 7,466,000 men and 6,144,000 women were diagnosed CRC, and 694,000 people died from CRC [2]. Its incidence is correlated with genetic factors and lifestyle, such as an increased intake of red and processed meat, a high-fat diet, a decreased intake of high-fiber food (e.g., vegetables and fruits), high alcohol intake, and smoking. This can lead to chronic inflammation, which plays a major role in CRC pathogenesis [3, 4].

The high prevalence of cancer and cost of its treatment have amplified the importance of CRC screening and the promotion of a healthy lifestyle. Although the incidence of CRC decreased by 34% from 2000 to 2014 in the <50 age group, there was an increase of 13% from 2000 to 2014 in the >50 age group during the same period [5]. In addition, current cancer therapies, such as surgery, radiation, chemotherapy, and immunotherapy are far from flawless; radiation and chemotherapy have a low therapeutic index and high toxicity, and sometimes, tumor cells can grow back. Meanwhile, immunotherapy shows toxicity and autoimmunity, and its effects quickly disappear. Therefore, innovative therapeutic strategies are needed to reduce the risk of cancer and modify tumors by utilizing knowledge in tumor biology and tumor-host interactions [6].

In recent years, peptides and proteins in foods are gaining attention as a cancer adjuvant therapy. Unlike drug molecules, peptides have a higher affinity and specificity to a target organ, a lower toxicity, and better penetration in tissue [7]. Therefore, there is a possibility of using proteins and peptides as cancer prevention methods in the phases of initiation, promotion, and progression [8]. One interesting peptide for this purpose is lunasin, a peptide consisting of 43 amino acids isolated from soybeans. One of its anticancer mechanisms is its anti-inflammatory activity, which it does by suppressing nuclear factor-kappa beta (NF-κB) [9]. NF-κB activation increases inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α, which are proven in cancer pathogenesis. In an in vitro study, the administration of lunasin for 24 h on inflammatory-induced macrophage using lipopolysaccharide (LPS) showed that lunasin significantly reduced IL-6 and TNF-α, as well as reactive oxygen species (ROS) [10].

Until now, there has been no in vivo research focused on the immune system in cancer carcinogenesis that observes TNF-α expression after the administration of lunasin from soybean extract. Therefore, in this paper, we use animal models that were induced with colorectal carcinogenesis using dextran sodium sulfate (DSS) and azoxymethane (AOM). Lunasin was administered orally to measure the expression of TNF-α on colonic epithelial cells.

MATERIALS AND METHODS

Lunasin extraction

Grobogan soybean variety, obtained from Indonesia Legumes and Tubers Crops Research Institute in Malang, East Java, Indonesia, was used in this study because it is one of the superior varieties and has the highest protein content. The soybean was pressed to separate its oil and obtain the residue, which is in the form of a powder. The residue was macerated with phosphate buffer saline (1:5) for 60 min, then filtrated thrice. The procedure of maceration was based on that of previous studies [11, 12]. The filtrate was dried in an evaporator at 50 °C. The concentration of lunasin in soybean extract was determined using high-performance liquid chromatography (HPLC).

Animals

Thirty male Swiss Webster mice (12 wk old and 25 g body weight) were supplied by the Agency for Health Research and Development, Ministry of Health, of the Republic of Indonesia. The mice were acclimatized for one week in the Pathological Anatomy Laboratory of the Faculty of Medicine, University of Indonesia,
under controlled temperature (25 °C), humidity (55%), and light (12 h/12 h light/dark cycle), and with free access to food and drink. Their physical condition was examined, and only the healthy ones were chosen for use in the experiment.

Colorectal carcinogenesis induction

The method for the induction of colorectal carcinogenesis was adopted from Kusmardi et al. [13]. Mice were given a single intraperitoneal (i.p.) injection of 10 mg/kgBW azoxymethane (AOM), diluted in 0.9% NaCl, early in week one. Throughout the second week, the mice were given food and drink containing dextran sodium sulfate (DSS) at 2%, daily.

Experimental group

Mice were randomly allocated to six experimental groups, as follows: the normal group, in which mice did not receive AOM/DSS but only received oral administration of physiological saline for 4 w; the negative control group, in which mice were given AOM/DSS followed by oral administration of physiological saline for 4 w; the positive control group, in which mice were given AOM/DSS, followed by oral administration of 150 mg/kgBW aspirin for 4 w; and the lunasin-rich soybean extract (LSE) treatment group, in which mice received AOM/DSS, followed by the oral administration of LSE (250, 300, or 230 mg/kg BW) for 4 w (fig. 1). At the end of the week, all the mice were sacrificed.

Immunohistochemistry

Distal colorectal tissue obtained from the mice was washed with water and fixed in buffered formalin 10%. The tissue was embedded in paraffin block, then cut into 4 µm-thick sections. Sections were incubated with primary (anti-TNF-α) antibodies (1:100 v/v) at room temperature for one hour. After a 10-minute wash, the samples were incubated with secondary antibodies at room temperature for 15 min. TrekAvidin-HRP was added to the samples and incubated at room temperature. After two 15-minute washes, chromogen (diamino benzidine) was added to the samples, and the reaction took 30 seconds. The samples were washed with water, soaked in lithium carbonate solutions for one minute, and washed with water again. They were then dehydrated with ethanol.

Quantification of TNF-α expression

The samples were observed under light microscope (400X magnification), and five fields of view were randomly taken of each of the samples. Epithelial cells that did not express TNF-α in their cytoplasm appeared blue (negative), while cells that expressed TNF-α appeared brown (positive). The intensity of this brown color was differentiated into three classes: high, moderate, and low positive (fig. 2). The number of cells in each group was transformed into a percentage (%). The index of TNF-α expression was calculated using optical density score, as follows: (4*%high positive)+(3*%moderate positive)+(2*%low positive)+(1*%negative).

RESULTS

The more TNF-α was expressed in the cytoplasm of colonic epithelial cells, the greater intensity of the brown color that appeared after immunohistochemical staining was. It was seen that the greatest intensity of brown color occurred in the negative control group, while the lowest intensity was found in the normal and LSE 350 mg/kg BW groups. In the LSE group, TNF-α expression was decreased progressively, as shown in D, E, and F of fig. 3.
Fig. 3: Expression of TNF-α in colonic epithelial cells after immunohistochemical staining (400X magnification). Normal (A); negative control (B); positive control (C); LSE-250 (D); LSE-300 (E); LSE-350 (F).

A statistically significant difference was seen in the index expression of TNF-α between the normal and negative control (p=0.009), LSE-250 (p=0.028), and LSE-300 (p=0.009) groups; between the negative control and positive control (p=0.009), LSE-300 (p=0.016), and LSE-350 (p=0.009) groups; between the positive control and LSE-300 (p=0.028) group; between the LSE-250 and LSE-350 (p=0.047) groups; and between the LSE-300 and LSE-350 (p=0.009) groups.

Fig. 4: Mean index expression of TNF-α (p=0.001); *p<0.05 is statistically significant
DISCUSSION

Colorectal carcinogenesis with AOM/DSS

Chronic inflammation, as occurs in inflammatory bowel disease (IBD), has a role in carcinogenesis [14, 15]. Okaya et al. [15] simulated colorectal carcinogenesis as it occurs in humans by injecting mice once with 7.4 mg/kgBW of AOM and giving them a drink containing 3% DSS for four cycles to trigger chronic ulcerative colitis. Several colon mucosal tumors, the majority of which were seen in the left colon (descending, sigmoid, and rectum) and transverse colon, were found in the AOM/DSS group. AOM demonstrates genotoxic (i.e., mutagenic) activity which is ideal to initiate carcinogenesis. Meanwhile, DSS causes chronic inflammation (colitis). Thus, both AOM and DSS play a role in the initiation and progression of carcinogenesis in animal models [16]. In this study, we attempted to mimic colorectal carcinogenesis with AOM/DSS as well, but with the dosage and method adopted by Kusmardi et al. [17].

Cytokines are produced due to carcinogen stimuli, infections, and inflammation. Among cytokines, TNF-α is the main mediator of inflammation [14]. The focus of this study was to assess TNF-α expression in colonic epithelial cells. TNF-α is a pro-inflammatory cytokine not only produced by inflammatory cells but also by other cells, including epithelial cells [18]. The results of this study indicate that there are significant differences in TNF-α expression between the normal and AOM/DSS-induced (negative control) groups. This suggests that AOM/DSS successfully creates an inflammatory condition so that TNF-α increases, as evidenced by the dark brown epithelial cells that were observed.

The activation of the NF-κB pathway can explain why the expression of TNF-α increases during inflammation. NF-κB is a transcription factor found in cytoplasm that plays a role in inflammation and carcinogenesis. In unstimulated conditions, NF-κB is in an inactive state because it binds to the protein inhibitor kB (IκB) [14, 18]. NF-κB can be activated if IκB becomes dissociated when there are stimuli, such as IL-1, TNF-α, LPS, DNA damage, double-stranded RNA (RNAs), cyclic adenosine monophosphate (cAMP), toll-like receptor (TLR) due to tissue damaged by microorganisms, hypoxia, and solid tumors. NF-κB regulates genes associated with inflammation, one of which is TNF-α, as well as proliferation; angiogenesis; survival; and tumor promotion and metastasis [14, 18]. Rogler et al. [19]. Observed the results of the biopsy of human intestinal mucosa that was undergoing inflammation and found that activated NF-κB was not only found in macrophages but also in epithelial cells.

Although not given AOM/DSS, the colonic epithelial cytoplasm in the normal group was not completely blue (indicating a negative expression of TNF-α) but instead tended to be light brown in intensity. Considering that NF-κB can be activated by microorganisms through TLR, it is possible that microbiota (bacteria) in the mice’s colon stimulates the activation of NF-κB so that TNF-α is formed in small amounts.

Aspirin therapy in colorectal carcinogenesis models

This study used a dose of 150 mg/kgBW of aspirin, according to previous studies by Amalia et al. [17]. In this study, no significant differences in TNF-α expression were seen between the aspirin (positive control) group and the normal group, but a significant difference was seen between the positive control group and the negative control group. This suggests that aspirin is reduced in the amount of TNF-α in epithelial cells that had undergone colorectal carcinogenesis. Moreover, the TNF-α expression was similar to that of the normal group.

Some studies have proven the relationship between aspirin use and a reduced risk of cancer. Low-dose aspirin (i.e., 81–160 mg) can reduce the risk of developing an adenoma [20]. However, the mechanism by which aspirin prevents CRC is still unknown. There are several possible mechanisms, including cyclooxygenase (COX) dependent pathways and COX-independent pathways. In COX-dependent pathways, aspirin and its metabolites (sodium salicylate) can permanently inactivate COX-1 and COX-2, resulting in apoptosis and the inhibition of angiogenesis. Meanwhile, in COX-independent pathways, aspirin or salicylates inhibit the activity of IκB kinase (IκK) to prevent NF-κB activation, both in vivo and in vitro [20, 21]. As a result of NF-κB inhibition, TNF-α expression decreased in the positive control group.

LSE therapy in colorectal carcinogenesis model

After mice had undergone colorectal carcinogenesis, mice were treated with LSE at three different doses (250, 300, and 350 mg/kgBW). These doses were determined based on previous studies by Amalia et al. [17]. Which showed that soybean extracts at doses of 150 and 200 mg/kgBW were able to increase apoptosis and significantly reduce dysplasia in the colon against negative controls. Soybeans were chosen because they have the highest lunasin content among other sources, at about 0.5–8.1 mg/g [22]. Lunasin content in this study was 0.823 mg/g of soybean extract, which falls within the projected range.

The LSE-250 group did not show significant differences in TNF-α expression with negative controls. However, at higher doses (i.e., LSE-300 and LSE-350), significant differences with negative controls were observed. The higher the LSE doses was, the lower the TNF-α expression was, indicating a dose-dependent effect. A study by Hernández-Ledesma et al. [10] proved that the inhibitory effect of TNF-α on RAW 64.7 macrophage cells, in which inflammation was induced using LPS, was greater as the dose of lunasin increased. At 200 μM lunasin, TNF-α decreased by 23%. Previous studies by de Mejia et al. [23], showed that lunasin derived from soybeans can inhibit the inflammatory process through the NF-κB pathway.

LSE-350 reduced the TNF-α expression significantly when compared to the negative control group but insignificantly when compared to the normal group. The result of immunohistochemical staining between these two groups was almost identical. This indicates that LSE-350 mg/kgBW can restore TNF-α expression to resemble that of the normal group.

Although the results of LSE therapy showed the restoration of TNF-α expression, this result does not rule out the contribution of other contents in the soybean extract, which could also work as an anti-inflammatory, such as isoflavones. Isoflavones have been known for their use in the treatment of hormone-related cancers, such as breast, cervical, and prostate cancers, by inducing apoptosis through mitochondrial pathways and NF-κB [9].

CONCLUSION

LSE inhibits TNF-α expression in the colonic epithelium of Swiss Webster mice in which carcinogenesis has been induced with AOM/DSS, with significant inhibition at doses of 300 and 350 mg/kgBW. The inhibition of TNF-α expression increases as the dose of LSE increases (i.e., in a dose-dependent manner). At a dose of 350 mg/kgBW, TNF-α expression is very similar to that of the normal group.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

There are no conflicts of interest to declare

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