The Effect of Soybean and Soybean Meal Extract on COX-2 and iNOS Expression in Colon Preneoplasia of Mice Induced by Azoxymethane and Dextran Sodium Sulfate

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Abstract : Several studies shows an increase in colon cancer case which is triggered by lifestyle changes in society such as smoking, obesity, high-fat diet, consumption of burned food and lack of fiber consumption. This study objective is to evaluate the inhibition activities of soybean seed extract and soybean meal extract on COX-2and iNOS expressions in colon preneoplasia of mice induced by azoxymethane and dextran sodium sulfate. Swiss Webster mice are injected intraperitoneally by single dose of 10 mg/kg BW azoxymethane and after 7 days followed by administration of 2% dextran sodium sulfatein their drinking for a week. Both extracts are administered orally in three different doses(75 mg / 20 gBW, 150 mg / 20 gBW and 200 mg / 20 gBW) daily for 4 weeks. Immunohistochemical examination is conducted to see the cells with the expression of COX 2 and iNOSin every 1000 epithelial cells. The result shows both the extracts decreases the expression of COX-2 at dose 150 mg / 20 g body weight and 200 mg / 20 g BW significantly with P <0.05. The iNOS expressionis decreased significantly only by the soybean meal extract at dose 150 mg/20 g BW. The Examination of the extract shows each seed extract and soybean meal containing active compound lunas in 0.623 mg / g extract and 0.823 mg / g extract.
Key words : Soybean, soybean meal, azoxymethane, dextran sodium sulfate, COX-2, iNOS.

Introduction

Colon cancer is the third leading cause of cancer-related deaths in the United States, and in 2015 new patients with colon cancer were estimated as many as 93.090 cases and 39,610 new cases of rectal cancer. Colorectal cancer is a term for cancer that starts in the colon or the rectum, and may also be referred to separately as colon cancer or rectal cancer depends on where the cancer began to emerge[1].

Diet plays an important role in the etiology of certain cancers, especially breast cancer and colon cancer. A high-fat and low fiber diet like that consumed by much of the industrialized countries, increasing the risk of cancers, while a plant-based, rich in whole grains, legumes, fruits and vegetables reducing cancer risk[2].

Chronic inflammation and age areother risk factors associated with the etiology of CRC. Gastrointestinal mucosa forms complex semipermeable barrier between the host antigens and antigen from the outside. Mucosal immune system has the ability to keep the body's immune response against the pathogen. Abnormal mucosal immune response is estimated to result in chronic inflammation such as irritable bowel

syndrome (IBD). IBD in humans is a complex disorder that has been classified into two main forms, ulcerative colitis and Crohn's disease[3].

Inflammation is an important factor in cancer development. Many cancer starts from the location of infection, chronic irritation and inflammation. The tumor microenvironment that is regulated by inflammatory cells is an indispensable component in the neoplastic process, proliferation, survival and migration. Proinflammatory mediators such as ROS can damage DNA and lead to the initiation and progression of tumors[4].

COX-2 is an enzyme involved in the formation of prostaglandins from arachidonic acid and associated with inflammation and tumorigenesis[5,6]. Increased expression of COX-2 mRNA is found in the majority of colorectal cancers as compared to normal mucosa[7].

Several studies of soybean as well as its active ingredients effect on the incidence of colon cancer has been performed. Saponins from soy can inhibit colon cancer in mice by decreasing the number ACFs and activity of β-glucuronidase[8]. Lunasin (peptides contained in soy) shows anti-inflammatory activity against RAW 264.7 cell cultures by reducing the expression of NO and PGE2, iNOS and COX-2[9]. Lunasin can also inhibit the process of carcinogenesis in murine fibroblast cells into cancerous foci through the mechanism of inhibition of histone H3 and H4 acetylation in vivo[10].

Soybean-based foods are widely consumed in Western countries, where soybean is most commonly crushed and the oil is extracted and refined into food-grade oil for frying and cooking. The co-product from this process is a high protein meal, known as soybean meal, mainly used as animal feed[11].

Although lunasin may act on the process of colon carcinogenesis in vitro and in vivo, other soy compound may have some biological effects that interfere with the effect of lunasin. The processing of soy extract is easier and cheaper than obtaining purified lunasin, and soy extract is more stable than lunasin. For these reasons, we chose to use soy extracts in this study.

Here, we investigate the *in vivo* effects of soybean and soybean meal to COX-2 and iNOS expression in colon preneoplasia of mice induced by azoxymethane and dextran sodium sulfate.

Experimental

Animals

Swiss Webster mice aged 8-10 weeks are obtained from National Institute Of Health Research And Development, Ministry of Health of Republic Indonesia, and then maintained and treated in the Laboratory of Experimental Pathology, Department of Anatomic Pathology, Faculty of Medicine, University of Indonesia. Animals are maintained and treated according to the Guide for Care and Use of Laboratory Animals of the Animal Care and Use Committee, and is approved by the Research Ethics Committee of the Faculty of Medicine, University of Indonesia. Mice are maintained in a temperature-controlled conditions at 23 °C and 55% of humidity with a cycle of 12 hours light/dark. Mice are fed standard food in both of the control group and the test group.

Drugs

Azoxymethane (AOM) and Dextrane Sodium Sulfate (DSS)are purchased from Sigma Aldrich.Lunasin standard for HPLC ispurchased from Polypeptide Group (Strasbourg). Acetosalis purchased from Brataco Chemical, Indonesia.

Plant Material

Soybean seed is purchased from Indonesian Legumes and Tuber Crops Research Institute, Malang, Indonesia. The soybean meal ispurchased from local market in Semarang, Indonesia. Soybean seed is pressed with 100-150 atm pressure for 30 minutes at 50°C temperatureto eliminate the oil. The dried slabsfrom this process are blended to get soybean powder. Soybean meal is dried in 50°C temperature oven for 1 hour before extracting process.

Extraction

Soybean powder and soybean meal powder 1250 gram each are macerated with PBS solvent by volume as much as 5 times the weight of the powder (6250 mL) for 60 minutes. Maceration solution is then filtered with gauze filter 3 times. Maceration resulting solution is then dried by evaporator at 50° C to obtain a dry extract.

High-Performance Liquid Chromatographic Analysis of Lunasin in Extract

A Hitachi HPLC system is used forHPLC analysis, consisting programmable sol-vent, UV detector module and WatersXBridge TM C 18.5 μm, diameter 4.6x 150mm column. Extracts are diluted in aquadest and centrifugated at 12.000 rpm for 30 minutes. The clear filtrates are filtered through a 0.22 μm membrane before injection. Briefly, samplesare injectedinto an HPLC equipped with using UV-Vis L-2420 detector (295 nm) with mo-bile phase (5% acetonitrile and 95% aquadest) in a linear gradient for 35 min at 2 ml/min. Lunasinis identified by its retention time using a lunasin standard.

Induction of Colon Preneoplasia by AOM and Dextran DSS

Induction of colon preneoplasia of mouse is done by intraperitoneal injection of AOM dissolved in 0.9% NaCl with dose of 10 mg/kg of body weight. Mice are given standard food and drink mineral water for one week after induction. Furthermore, for the next one-week treatment, the mineral water is replaced by mineral watercontaining 2% of DSS[12,13].Extract are administered orallyin threedifferent doses(75 mg / 20 gBW, 150 mg / 20 gBW and 200 mg / 20 gBW) daily for 4 weeks.

Experimental groups

Code	Group	Treatment	N
(K0)	Normal control	No substance/solvent applied group	3
(A+D)	Negative control	AOM and DSSgroup	3
(A+D+Ac)	Positive control	AOM + DSS + acetosal 150 mg/kg BW	3
(A+D+SBE1)	Soybean extract dose 1	AOM + DSS + Soybean extract 75 mg/20gBW	3
(A+D+SBE2)	Soybean extract dose 2	AOM + DSS + Soybean extract 150 mg/20gBW	3
(A+D+SBE3)	Soybean extract dose 3	AOM + DSS + Soybean extract 200 mg/20gBW	3
(A+D+SBM1)	Soybean meal extract dose 1	AOM + DSS + Soybean meal extract 75 mg/20gBW	3
(A+D+SBM2)	Soybean meal extract dose 2	AOM + DSS + Soybean meal extract 150 mg/20gBW	3
(A+D+SBM3)	Soybean meal extract dose 3	AOM + DSS + Soybean meal extract 200 mg/20gBW	3

Tissue Sample Preparation

Mice are sacrificed by using ether after 6 weeks of carcinogenesis induction by AOM. Colon tissues of mice are taken, then clean from the lumen of the colon with water. Tissue is fixed with 10% of phosphate buffered formalin.

Immunohistochemistry Staining of COX-2

Tissues sample is cut with a thickness of 4 μm for immunohistochemistry staining. After deparaffination and rehydration, specimens aredyed by 0.01 M of citrate buffer (pH 6.0) in microwave for 5 minutes. Specimens are spilled by 3% of hydrogen peroxide to eliminate endogenous peroxide for 5 minutes at room temperature. Specimens are incubated withrabbit polyclonal cyclooxygenase-2 (1:1000 dilution; Cell Abcam Inc., Cambridge, MA) orrabbit polyclonal antibodies of inducible nitric oxide synthase (1:100 dilution; Abcam Inc., Cambridge, MA)in PBS for 2 hours at room temperature in a humidity chamber, followed by overnight incubation at 4°C N-Universal(Dako).Specimens are then incubated with the appropriate secondary antibody for one hour at room temperature, followed by incubation with HRP-conjugated streptavidin for 30 minutes. Proteins is visualized using 3,3'-diaminobenzidine (DAB) for 10 minutes at room temperature. The specimens are added counterstain with Harris hematoxyllin, dehydrated and mounting.

Immunohistochemistry Staining Interpretation

COX-2 expression in the cytoplasm of colon crypt epithel cell is scored semi quantitatively in 5 visual fields with400x magnification. The scoring of expression is defined as: blue epithel=negative, and brown

epithel = positive. The each total expression score is summed, and then the average value is counted. As the positive control of COX-2 staining is used tonsil tissues. For the negative control there is no additional primary antibody in the same tissue specimen.

Statistical Analysis

Two-ways analysis of variance (ANOVA) followed by Post Hoct Testis used to determine the effect of three doses of soybean and soybean meal extract against colon carcinogenesis based on the assessment of the expression of COX-2 and iNOS.. Prior to do ANOVA, the normality of data distribution is tested using the Levene's test, whereas the homogenity of variance tested using the Shapiro Wilk test.

Results and Discussion

By light microscopy, the epithelial cells that expressed COX-2 or iNOSshould be seen in brown colour. Here the samples of the COX-2 immunostain.
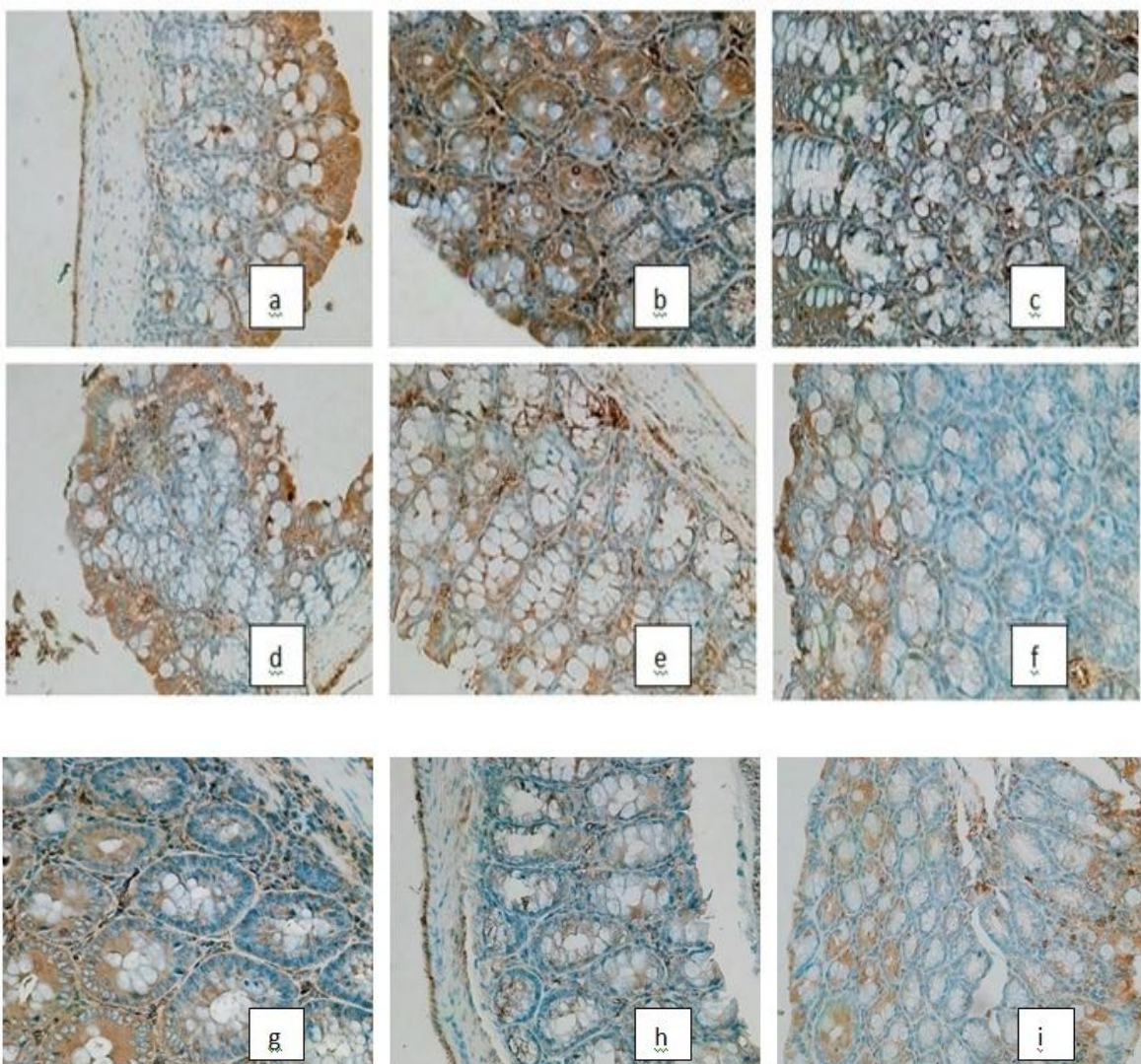

Fig.1. Immunostain of COX-2 antibody in the experimental animal's group(400x Magnification) : (a) Normal control (K0), (b) Negative control (A+DSS), (c) Acetosal control (A+DSS+Asp), (d) Soybean extract dose 1 (A+DSS+SBE1), (e) Soybean extract dose 2 (A+DSS+SBE2), (f) Soybean extract dose 3 (A+DSS+SBE3), (g) Soybean Meal Extract dose 1 (A+DSS+SBM1), (h) Soybean Meal Extract dose 2 (A+DSS+SBM2), (i) Soybean Meal Extract dose 3 (A+DSS+SBM3)
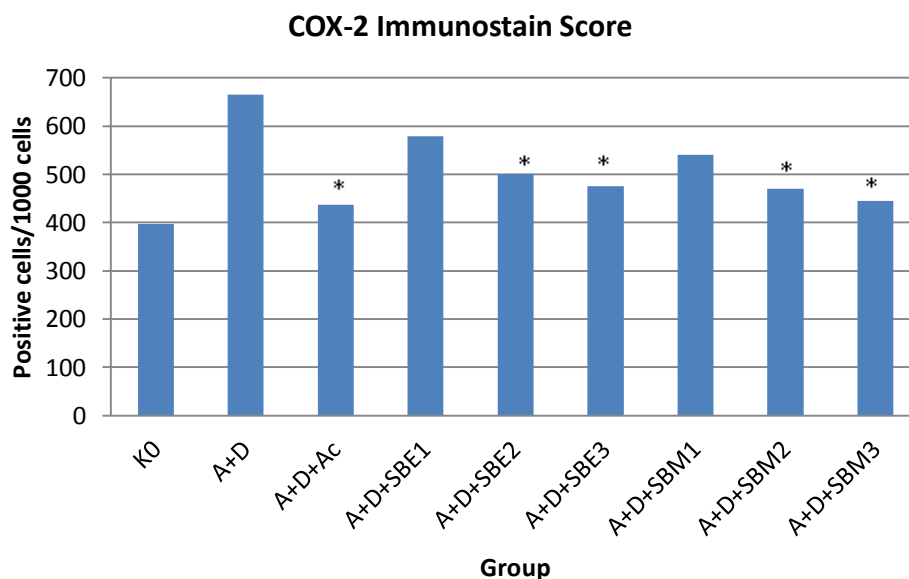
COX-2 Immunostain Score

Fig.2. The activity of soybean and soybean meal extracts on COX-2 expression. COX-2 Immunostain score counted as positive epithelial cell/1000 epithelial cells, K0=Normal control, A+DSS=Negative control, A+DSS+Ac=Acetosal control, A+DSS+SBE1=Soybean extract dose 1, A+DSS+SBE2= Soybean extract dose 2, A+DSS+SBE3=Soybean extract dose 3, A+DSS+SBM1=Soybean Meal Extract dose 1, A+DSS+SBM2 = Soybean Meal Extract dose 2, A+DSS+SBM3=Soybean Meal Extract dose 3. *Significant at p<0.05 compared to negative control.

All groups have a score of positive cells that describe the expression of COX-2 is lower than the negative control, although only the aspirin group, soybean extract dose 2 group, soybean extract dose 3 group, soybean meal extract dose 2 group, and soybean extract dose 3 group are significantly different (P <0.05) with the negative control group and do not differ significantly with the normal group (P> 0.05). Both extracts groups have a tendency to dose-dependent effect although statistically the difference between groups is notalways significant.

Chronic inflammation is often associated with an increased risk of cancer as in the cases of gastrointestinal cancer. Chronic inflammation is a promoter in carcinogenesis by inducing gene mutations, inhibits apoptosis and stimulates angiogenesis and cell proliferation. Inflammation also induce epigenetic changes associated with cancer[14].

A cross-section study in Malaysia using retrospective data over a 2-year period (1999-2000) shows a high proportion of colorectal cancers are found to express COX-2 and a significant number of iNOS production, suggesting that their inhibitors may be potentially useful as chemotherapeutic agents in the management of colorectal cancer[15].

Nimesulide, a COX-2 selective inhibitor, reduces azoxymethane (AOM)-induced aberrant crypt foci (ACF) in rats and colon carcinogenesis in mice as well as formation of intestinal polyps in Min mice. Nitric oxide synthase (NOS) is known to be overexpressed in colon cancers of humans and rats, and a NOS inhibitor, L-N[G]-nitroarginine methyl ester, was found to inhibit the development of AOM-induced ACF in rats. Thus, NOS including iNOS could also be a good target for chemoprevention of colon cancer, as in the COX-2 case[16].

In humans and in animal models, COX-2 expression increases in cases of colorectal cancer compared with normal mucosa[17]. Colorectal cancer in mice induced AOM also increase the expression of COX-2[18,19]. COX-2 is involved in the angiogenic process in mice by inducing vascular endothelial growth factor (VEGF) receptors EP2[20]. Acetosal 150 mg / kg body weight can decrease the expression of COX-2 in colon significantly compared to the negative control group.

Acetosal works by inactivating the cyclooxygenase enzyme. Acetosal will diffuse through the cell membrane, into the COX enzyme's active site, and binds to the arginine residue 120. Then acetosal will acetylate serine residue to prevent the COX enzyme binds to arachidonic acid[21].
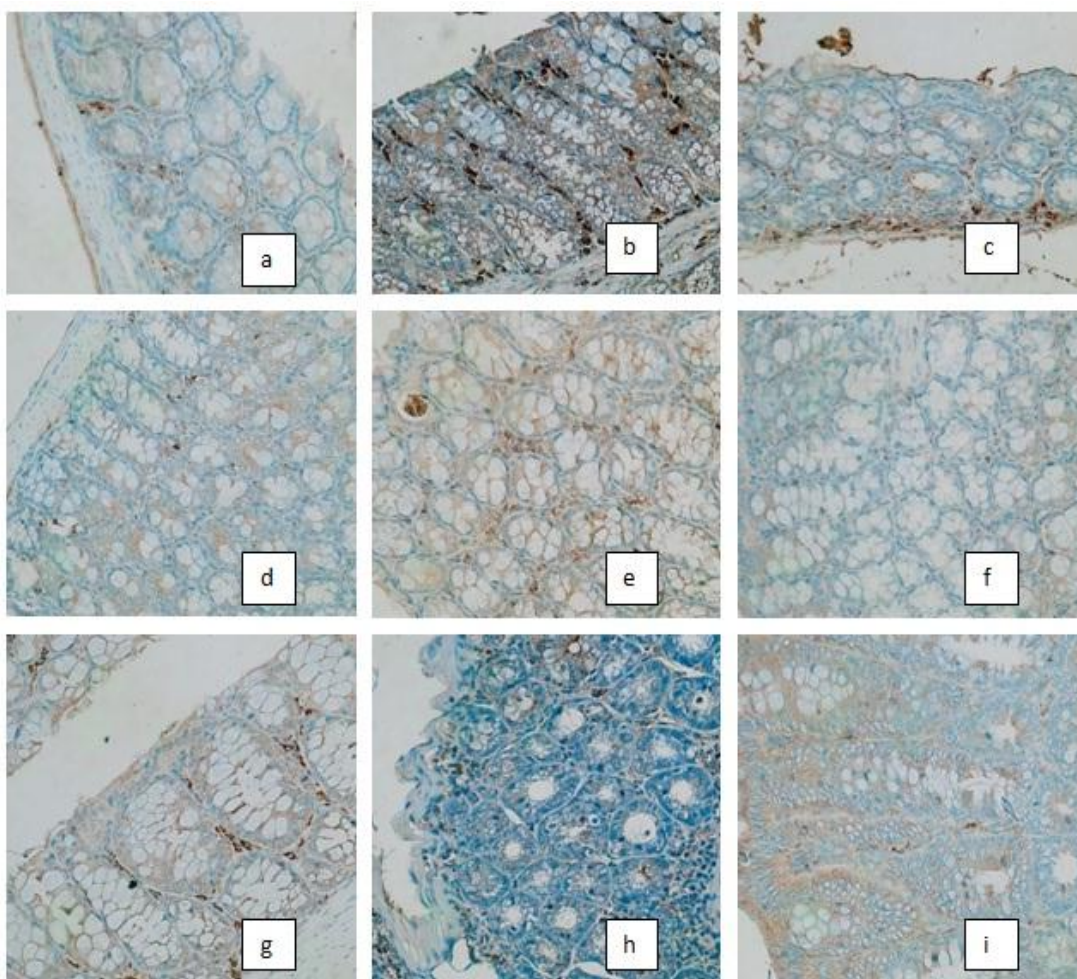

Fig.3. Immunostain of iNOS antibody in the experimental animal's group (400x Magnification) : (a) Normal control (K0), (b) Negative control (A+DSS), (c) Acetosal control (A+DSS+Asp), (d) Soybean extract dose 1 (A+DSS+SBE1), (e) Soybean extract dose 2 (A+DSS+SBE2), (f) Soybean extract dose 3 (A+DSS+SBE3), (g) Soybean Meal Extract dose 1 (A+DSS+SBM1), (h) Soybean Meal Extract dose 2 (A+DSS+SBM2), (i) Soybean Meal Extract dose 3 (A+DSS+SBM3)
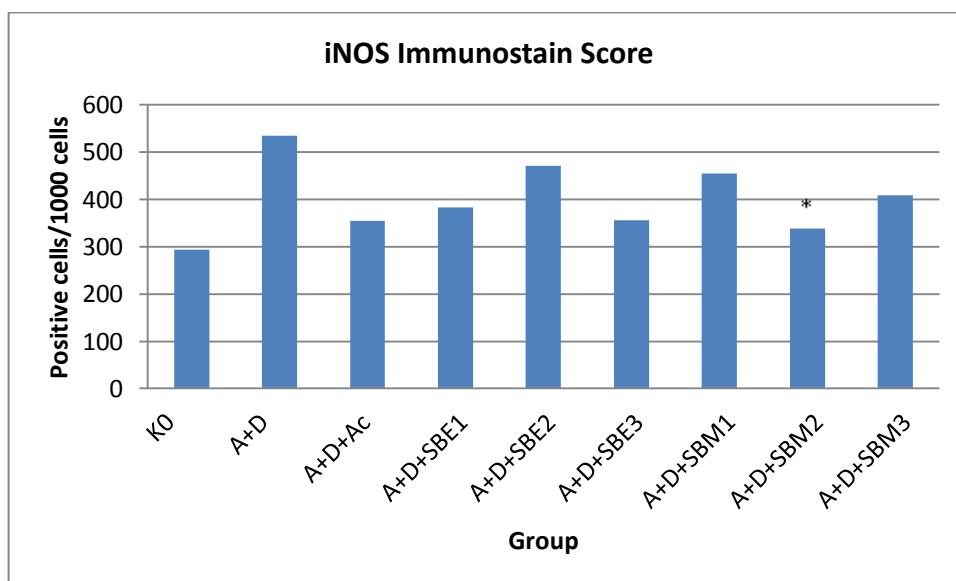
Fig.2. The activity of soybean and soybean meal extracts on iNOS expression. iNOSImmunostain score counted as positive epithelial cell/1000 epithelial cells, K0=Normal control, A+DSS=Negative control, A+DSS+Ac=Acetosal control, A+DSS+SBE1=Soybean extract dose 1, A+DSS+SBE2= Soybean extract dose 2, A+DSS+SBE3=Soybean extract dose 3, A+DSS+SBM1=Soybean Meal Extract dose 1, A+DSS+SBM2= Soybean Meal Extract dose 2, A+DSS+SBM3=Soybean Meal Extract dose 3. *Significant at p<0.05 compared to negative control.

All the three doses of soybean and soybean meal extract can lower the iNOS expression, but only significant at dose 2 soybean meal extract. Both extract groups have a tendency to dose-independent although statisticallythey are not always significant with negative control.

iNOSis involved in chronic inflammatory process, and creates microenvironment triggers for colon cancer development. Expression of iNOS increases in human colorectal adenoma and cancer, also in chemical induced colon tumor in mice[16].

The soybean and soybean meal extract lower the expression of COX-2 and iNOSin the colon of mice induced colon cancer with AOM and DSS. Soybean meal is residue in the manufacture of soybean oil and high in protein. Soybean meal is used as animal feed generally.

As functional food soybean is claimed to reduce the risk of some diseases such as atherosclerosis, osteoporosis and various types of cancer. This activity is associated with the content of isoflavones in soy. Isoflavones have structural similarities to estrogen so it can reduce the risk of hormone-dependent cancer[22].

Besides isoflavones, soybean also contains lunasin, a peptide that consists of 43 amino acids. Lunasin may contribute in preventing inflammation, through inhibition ofCOX-2/PGE2 and iNOS/NO pathways[9], lunasin also inhibits carcinogenesis in murine fibroblast cells induced by chemical carcinogens[10].Soybean and soybean meal extract are used in this study contains lunasin 0.823 mg / g and 0.623 mg / g extract respectively. Although lunasin may act on the process of colon carcinogenesis in vitro and in vivo, other soy compound such as isoflavone may have some biological effects that interfere with the effect of lunasin.

It conclude that soybean and soybean meal extract have inhibitory activity on colon preneoplasia of Swiss Webster mice induced by azoxymethane and dextran sodium sulfate.

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