Research Article

Journal of Nutritional Biology

Changes in Ki-67 and Bcl-2 Expression in Rat Gastric Mucosa Secondary to Soybean Feeding: An Assessment of Proliferative and Apoptotic Activity of Flavonoids

Oya N.Sivrikoz^{1*}, Sibel D.Kececi², Fatma S.Pehlivan³ and Salahattin M.Sanal⁴

¹Ozel frozen patoloji lab, Alsancak, Izmir, Turkey

²Department of Pathology, Merkezefendi Devlet Hastanesi, Manisa, Izmir, Turkey

³Department of Pathology, Özel Tinaztepe Hastanesi, Ahmet Piristina Bulvari, Tinaztepe/ Buca, Izmir, Turkey

⁴Division of Oncology, Özel Tinaztepe Hastanesi, Ahmet Piristina Bulvari Tinaztepe/ Buca, Izmir, Turkey

***Correspondence:** Oya N Sivrikoz, Ozel frosen patoloji laboratuvarı, Alsancak, Izmir, Turkey, Tel: +9 (553) 6364188; E-mail: onsoral@yahoo.com

Rec date: Apr 08, 2018; Acc date: June 07, 2018; Pub date: June 12, 2018

Abstract

Background: Gastric cancer is a common malignancy and nutritional factors are believed to play an important role in its development. Isoflavones in soybean products are an important nutritional source, especially in some Asian Countries, having protective role against development of gastric cancer. In our study, we have prepared soybean extracts via different methodologies and assessed their apoptotic role and proliferation index in gastric mucosa of the rodents.

Methodology: We have fed 2 months old 64 rats for a period of 1 month with soybean extracts prepared by 3 different methodologies and in 6 different concentrations. Subsequently, the rodents were subjected to euthanasia and their gastric mucosal linings were processed accordingly for H & E, Bcl-2, and Ki 67 staining. Thereafter, the specimens were evaluated under light microscopy.

Results: In all the experimental groups fed with soybean extracts, weak staining was observed with Bcl-2, more significantly in the group of rodents fed with 100 mg/kg and 200 mg/kg ethanol extract as well as in the group fed with high ethyl acetate extract ($p < 0.05$). There was no difference between the groups with respect to Ki 67 staining and inflammation rate ($p > 0.05$).

Conclusions: We have discovered that soybean extracts and specifically genistin and daidzein induces apoptosis at high concentrations. This observation makes us believe that soybeans have a protective role in the development of gastric cancer.

Introduction

Gastric cancer is the fourth commonly seen malignancy and second leading cause of death among cancer victims in the world. While it is seen more frequently in Japan and Korea, it is less common in the western world [1,2]. The difference in geographic distribution is probably related to varying forms of diet, genetic composition and prevalence of helicobacter infection in the population [1]. Even though the exact pathogenesis of gastric cancer is

not well understood, changes in balance between the apoptosis and proliferation of the gastric epithelial lining is considered to be the leading cause for carcinogenesis.

Contrary to food with high sodium content that increases the risk of gastric cancer, there is published information suggesting that intake of fresh vegetables, fruits, and food made out of soybean may have protective effect [2,3]. Furthermore, soybean products have

been shown to carry anti-inflammatory, anti-diabetic, hypoglycemic, anti-mutagenic, and anti-viral properties.

A group of investigators in Japan demonstrated that high consumption of non-fermented soybean products have inverse relationship with gastric cancer; whereas, fermented soybean products have no such relationship [3,4]. Similar observations, investigating black soybean extracts were reported by others. Black soybean (*Glycine max* (L.) Max) is one of the principal food source, especially in Asian countries. Besides being easily available and affordable, it has high nutritional value due to isoflavones, proteins, fatty acids, carbohydrates, and fiber content [2,5,6]. The most important isoflavones existing in black soybean extracts are genistein, daidzein, and glycitein [2]. Daidzein is an isoflavone that has been consumed and studied the most. It has been demonstrated that daidzein interrupts the cell cycle at G1 and G2/M phase in human mammalian cancer cells [7]. It also increases the biological activity of caspase-9, downregulates the bcl-2/Bax expression that is responsible for apoptosis, thus restoring apoptosis and decreasing the cyclin D expression [5]. Apoptosis is important for normal development of cells, suppression of oncogenesis, and hence plays a crucial role in maintaining normal health of the human body. Furthermore, investigators also demonstrated that genistein has anti-carcinogenic effect and by increasing the expression of tumor suppressor gene, PTEN in gastric epithelium and consequently interrupting the cell cycle at G2/M phase [8].

The purpose of this study is to investigate the anti-carcinogenic role of isoflavones, prepared by different techniques and delivered in different doses. Accordingly, we explored the effect of soya-bean extracts on gastric mucosa in female rats, specifically their influence in inflammation, cell proliferation, and apoptosis, prepared by different techniques. We chose Ki-67 antibody to measure proliferation index of cells and Bcl-2 level to assess apoptosis. Ki 67 is a macromolecule expressed in all cycles (G1, S, G2, and M) of actively dividing cells. It is not expressed in cells at G0 phase and cells that have differentiated [9]. Bcl-2 is the first discovered and well known anti-apoptotic gene that is located in human chromosome 18 [10]. Bcl-2 activity in cells plays a critical role in anti-apoptotic activity, thus decline in the level of Bcl-2 restores apoptosis in cells.

Animal experimentations pertaining to this study were conducted at Afyon Kocatepe University with the approval of Ethics Committee and in accordance with the guidelines regarding animal experimentations in

research laboratories.

Materials and Methods

Glycine max plant produced in Turkey, has been dried and treated with ethyl acetate, ethanol, and N-hexane in order to obtain the extracts. Later, these extracts were subjected to preliminary tests to measure their biological activities. High Performance Liquid Chromatography (HPLC) was utilized since it is demonstrated to be the most effective and reliable technology, clarifying origins of plants, producing plant based medicines, and determining standards in making new plant based medicines. By means of this technology, all the extracts produced for this study have undergone testing and thereby isoflavone contents standardized.

Carboxymethyl Cellulose (CMC) solution has been used to dilute the extracts fed to rodents. Sixty four, 2 months old female Sprague-Dawley rodents were separated into 8 experimental groups. Throughout the whole experiment, the rodents were kept in an environment at 21 degrees of centigrade and 50% moisture, 12 hours of light alternating with 12 hours of darkness. Every 2 days, the cages where the rodents were kept were cleaned, fresh water and food supplied. For a period of one month, the rodents were fed with soy-bean extracts delivered by oral gavage.

The experimental groups were classified as follows

Group A was the control group that did not contain CMC. Group B was the control group that received 0.5% CMC only, no extract. In Group C, the rodents were fed at a dose of 100 mg/kg with n-Hexane extract. In Group D, the rodents were fed at a dose of 200 mg/kg with n-Hexane extract. In Group E, the rodents were fed at a dose of 100 mg/kg with ethyl acetate extract. In Group F, the rodents were fed at a dose of 200 mg/kg with ethyl acetate extract. In Group G, the rodents were fed at a dose of 100 mg/kg with ethanol extract. In Group H, the rodents were fed at a dose of 200 mg/kg with ethanol extract.

After one month of experimentation, the rats were subjected to euthanasia by cervical dislocation under general anesthesia. Doublet tissue samples, measuring 1 centimeter in diameter were obtained from gastric antrum of each rodent, preserved in 10% formalin solution.

Pathological evaluation

Tissue samples were kept in 10% formalin solution for routine processing. They were subsequently embedded in paraffin and three samples of tissue were cut in 4 micrometer thickness. One of the samples was stained with H&E and the other two samples were stained for Ki 67 and Bcl-2 with the help of Dako Autostainer 48 Link (Dako, Denmark) after they had been placed on positively charged glass slides. Each sample was analyzed independently by 2 pathologists under light microscopy.

Inflammatory response was evaluated by measuring the degree of lymphocytic infiltration in a dense area, under high power field. More than 21 lymphocytes count was assigned as “strong response” (Figure 1A); less than 21 lymphocytes as “weak response” (Figure 1B).

Figure 1A: Strong inflammatory response in rat gastric mucosa (H&E X 200).

The interpretation of immunohistochemical staining was made by counting 100 cells from a densely stained area on a slide. For the interpretation of Ki 67, nuclear staining was considered positive. 1+ was assigned for 1% to 5% staining (Figure 2A), 2+ for more than 6% staining (Figure 2B). Similarly, for Bcl-2, cytoplasmic staining was considered positive. 0 was assigned for absence of staining (Figure 3A), 1+ for 1% to 5% staining, and 2+ for more than 6% staining (Figure 3B).

Preparation of soybean extracts

25 gr of soybean was extracted twice at room temperature by shaking for 48 hours in 500 ml of n-Hexane. Similarly, 500 ml of 70% ethanol, containing 0.1% acetic acid was employed to obtain ethanol extract after 48 hours of shaking. The ethanol extracts were dried in a vacuum desiccator under reduced pressure and concentrated by using rotavapor at 40°C.

To obtain an ethyl acetate extract, 25 gr of soya-bean was extracted twice at room temperature by magnetic stirring with 500 ml of ethyl acetate for 48 hours. The ethyl acetate extracts were dried in a vacuum desiccator under reduced pressure and concentrated by using rotavapor at 40°C.

For HPLC analysis, all the extracts were standardized in accordance with their composition. Table 1 lists the composition of extracts and isoflavones.

Figure 1B: Weak inflammatory response in rat gastric mucosa (H&E X 200).

Statistical methods

We employed SPSS 22.0 software program (IBM Corporation, Armonk, New York, USA) for the analysis of results. The results were analyzed by Kruskal-Wallis H test through Monte Carlo Simulation Technique. For Post Hoc analysis, nonparametric posthoc test (Miller 1966) was utilized. In order to analyze the relationship between the

results we used the Spearman's rho test and to analyze the relationship between different categorical results with each other, Phearson Chi-Square and linear-by-linear Association tests through Monte Carlo Simulation Technique. In tables, quantitative results were expressed as mean values (Maximum-Minimum). Categorical results were expressed as numbers (n) and percentage (%). The results were analyzed in 95% confidence limit and was considered significant if it was less than 0.05.

Figure 2A: 1+ Ki67 staining in rat gastric glands (X200).

Figure 2B: 2+ Ki 67 staining in rat gastric glands (X200).

Figure 3A: Negative Bcl-2 staining in rat gastric glands (X 200).

Figure 3B: 2+ positive Bcl-2 staining in rat gastric glands (X200).

Results

When inflammatory response was evaluated separately in all the groups, weak inflammatory response was more common in all the groups, including the control groups

($p < 0.05$). In C, H, E groups, inflammatory response was weak in all the rodents. Only ingroup G, inflammatory response was significantly stronger than group C, E, and H ($p < 0.05$). Nonetheless, when control groups (A and B) were compared with the rest of the experimental groups, there was no meaningful statistical difference.

As a whole, when relationship between inflammatory response and Ki 67 was analyzed, independent of each experimental group, there was a positive correlation between inflammatory response and Ki 67 positivity ($p < 0.05$).When such relationship was analyzed separately for each group, only in group G, there was a meaningful relationship between inflammatory response and Ki 67 staining ($p < 0.05$).

No statistically significant difference was observed when Ki 67 staining characteristics were analyzed between each group. Similarly, there was no meaningful relationship between Ki 67 and Bcl-2 between each group and as a whole.

We have discovered that Bcl-2 staining was 87% (1+ and 2+) in group A and 100% in group B. There was no statistical difference between group A and B. In all the groups the staining was weak when compared to control groups. Among rodents in groups G and H, there was no staining in 75% and in group F there was no staining in 62.5%. Similarly, no +2 staining was observed in any of the three groups.

With respect to Bcl-2 staining characteristics, there was statistically meaningful difference between group A and groups F, G, and H, as well as between group B and groups E, F, G, and H($p < 0.05$) (Table 2). While there was statistically meaningful difference between group D and the experimental groups G and H, there was no such relationship with the remaining groups.

Discussion

Gastric cancer draws worldwide attention. Thus far, the studies indicate that diet, helicobacter pylori infection, and chronic gastritis play an important role in its pathogenesis [2,11]. Several studies demonstrated that nitrites added to acidified solutions show antimicrobial

activity against biological agents such as helicobacter, yeast and enterobacter species [12].

Phenolic compounds carry benzene ring in its structure. Neutral phenolic compound, isoflavone isthe principal compound in soybean extracts.

Ferreira et al., demonstrated that when soyaextracts, rich in phenolics were mixed with acidified nitrites, their antibacterial efficacy was potentiated [13]. More importantly, endogenous nitrate production is increased in the stomach during helicobacter pylori infection. This in turndecreases the gastric vitamin C concentration and leads to overproduction of N-nitroso compounds [14].

Toyoizumi et al, demonstrated that rodents when fed with potentially genotoxic and mutagenic NaNO₂ (a precursor of N-nitroso) and soybeans, DNA damage induced by free radicals were less, in high isoflavone concentrations. Thus, this study has demonstrated that non- react isoflavones at high concentrations have the ability to clear free radicals in stomach. In studies where daidzein, genistein, and NaNO₂ were given individually, no lymphocytic infiltration was induced [14]. Similarly, in our study the inflammatory response was less prominentin rest of all the groups. Nonetheless, the reason for profound inflammatory response in the group where 100 mg/kg ethanol extract was fed, may well be due to the damage induced by ethanol itself in the gastric mucosal lining. Conversely, the reason why inflammatory response was less in rodents fed with 200 mg/kg ethanol extract may be related to the counter protective effect of isoflavones against the adverse effect of ethanol. This contradictory result may be a reason for renewed interest to repeat the study ingreater number of rodents.

Nat et al., reported that fermented soybean paste, Doejang, had inhibitory effect on oxidative stress and inflammation in rat fat tissue, restoring dysregulated genes related to excess adiposity [15]. Similarly, in our study the inflammatory response was in general low except in group of rodents fed with 100 mg/kg ethanol extract, where the inflammatory response was higher.

We have not clarified the pathogenesis of gastric cancer; however, when the balance between apoptosis

Table 1: Extracts and their respective isoflavone concentrations.

Soybean extracts	Total isoflavone concentration	daidzin	genistin	daidzein	genistein
n-hexane	27 mg/25g	40%	56%	2%	2%
Ethyl acecate extract	48 mg/25g	37%	58%	2%	3%
Ethanol extract	52 mg/25g	36%	59%	2%	3%

Table 2: Distribution of Bcl-2 staining in each group ($p < 0.001$).

Groups	No detectable staining	% 1-5 positive staining	%5 and over positive staining
	n(%)	n (%)	n(%)
A	1(12,5)	4 (50,0)	3 (37,5)
B	0 (0,0)	5 (62,5)	3 (37,5)
C	2 (25,0)	5 (62,5)	1 (12,5)
D	2 (25,0)	4 (50,0)	2 (25,0)
E	4 (50,0)	3 (37,5)	1 (12,5)
F	5 (62,5)	3 (37,5)	0 (0,0)
G	6 (75,0)	2 (25,0)	0 (0,0)
H	6 (75,0)	2 (25,0)	0 (0,0)

and proliferation is disrupted, paving its way for the development of cancer. Furthermore, the generation of oncogenes and suppression of tumor suppressor genes may transform the premalignant lesions to malignancy. Normally Ki 67 is positive in the majority of cells localized in the regenerating zone of the stomach glands. In one of the studies performed by Docea et al, they discovered that Ki 67 proliferation index was higher in less differentiated, more invasive, and higher stage tumors [16]. In our study we have discovered that the rodents fed with soybean extracts, had lower Ki 67 index compared to the control groups. Notwithstanding, the reason for difference not being statistically significant may be related to inadequate number of rodents we analyzed.

The anti-inflammatory and anti-oxidant effects of soybean have been verified by a good number of studies [13-15,17]. Kumar et al. reported the anti-ulcerative effect of Glycine max in aspirin induced gastric ulceration in rodents [18]. Furthermore, the presence of a positive correlation between Ki 67 and cell proliferation in our study, may explain the reducing effect of soybean on inflammation with resultant decline in proliferation index. Tang et al., demonstrated that daidzein increased the pace of apoptosis through down regulation of bcl-2/bax [7]. Similarly, Zou et al., also demonstrated that black soybean induced apoptosis by changing the ratio of bcl-2 and bax [2].

In our study, we have demonstrated that rodents fed with soybean extracts had less bcl-2 staining intensity. Specifically, the group fed with ethanol extract and higher isoflavone concentration showed lesser bcl-2 staining intensity, supporting the observation that apoptosis was induced. High concentrations of genistin and daidzin in ethyl acetate extracts yielded the same conclusion.

The absence of any significant relationship between
J Nutri Bio, 4(2): 196-202 (2018)

bcl-2 staining characteristics and n-Hexane extract feeding of the rodents indicated that n-Hexane had no effect on apoptosis as opposed to other extracts, tested. One reason may be secondary to low concentration of isoflavones in n-Hexane extracts compared to the other group.

In conclusion, we have demonstrated that soybean has anti-inflammatory, anti-proliferative, and pro-apoptotic effects; nonetheless, in ethyl acetate and ethanol extracts with high concentration of isoflavones, the apoptotic effect was more pronounced. We believe that human beings when fed with such extracts as part of their daily dietary intake, will experience a protection against the development of gastric cancer. Additionally, in patients diagnosed with gastric cancer, such a diet, in addition to modern cancer treatment strategies, will have a positive impact on them to survive longer and experience better prognosis. Furthermore, we hope that this study will justify further investigation in larger number of rodents.

Conflict of Interest

All the authors listed above acknowledge no potential conflict of interest relevant to this study.

Author Contributions

Sivriköz ON planned, directed the research and wrote the manuscript. Sivriköz ON, Keceli SD and Pehlivan FS performed and evaluated the histopathology. Sanal SM is a senior professional member and he guided us on the design of study as well as writing the manuscript.

References

1. Kweon S-S, Shu X-O, Xiang Y, Cai H, Yang G, Ji B-T, et al. Intake of specific nonfermented soy foods may be inversely associated with risk of distal gastric cancer in a Chinese population. J Nutr. 2013 Nov;143(11):1736-

1742.

2. Zou Y, Chang SKC. Effect of black soybean extract on the suppression of the proliferation of human AGS gastric cancer cells via the induction of apoptosis. *J Agric Food Chem*. 2011 May 11;59(9):4597-4605.
3. Wada K, Tsuji M, Tamura T, Konishi K, Kawachi T, Hori A, et al. Soy isoflavone intake and stomach cancer risk in Japan: From the Takayama study. *International Journal of Cancer*. 137(4):885-892.
4. Kim J, Kang M, Lee J-S, Inoue M, Sasazuki S, Tsugane S. Fermented and non-fermented soy food consumption and gastric cancer in Japanese and Korean populations: a meta-analysis of observational studies. *Cancer Sci*. 2011 Jan;102(1):231-244.
5. Uyar B, Sivriköz ON, Özdemir U, Dasbasi T, Sacar H. Histological investigation of the effect of soybean (*Glycine max*) extracts on the collagen layer and estrogen receptors in the skin of female rats. *Clinics (Sao Paulo)*. 2014 Dec;69(12):854-861.
6. Le Marchand L. Cancer preventive effects of flavonoids--a review. *Biomed Pharmacother*. 2002 Aug;56(6):296-301.
7. Tang S, Hu J, Meng Q, Dong X, Wang K, Qi Y, et al. Daidzein induced apoptosis via down-regulation of Bcl-2/Bax and triggering of the mitochondrial pathway in BGC-823 cells. *Cell Biochem Biophys*. 2013 Mar;65(2):197-202.
8. Liu Y-L, Zhang G-Q, Yang Y, Zhang C-Y, Fu R-X, Yang Y-M. Genistein induces G2/M arrest in gastric cancer cells by increasing the tumor suppressor PTEN expression. *Nutr Cancer*. 2013;65(7):1034-1041.
9. Tanaka R, Tainaka M, Ota T, Mizuguchi N, Kato H, Urabe S, et al. Accurate determination of S-phase fraction in proliferative cells by dual fluorescence and peroxidase immunohistochemistry with 5-bromo-2'-deoxyuridine (BrdU) and Ki67 antibodies. *J Histochem Cytochem*. 2011 Aug;59(8):791-798.
10. N. Sivriköz O, Kandiloglu G. The Effects of Cyclin D1 and Bcl-2 Expression on Aggressive Behavior in Basal Cell and Basosquamous Carcinoma. *Iran J Pathol*. 2015;10(3):185-191.
11. Cao D-Z, Sun W-H, Ou X-L, Yu Q, Yu T, Zhang Y-Z, et al. Effects of folic acid on epithelial apoptosis and expression of Bcl-2 and p53 in premalignant gastric lesions. *World J Gastroenterol*. 2005 Mar 21;11(11):1571-1576.
12. PHENOLIC COMPOUNDS AND ANTINUTRITIONAL COMPONENTS IN SOYBEAN AND SOYBEAN PRODUCTS [Pamukkale Univ Muh Bilim Derg] [Internet]. [cited 2018 Jun 5]. Available from: <http://pajes.pau.edu.tr/eng/jvi.asp?pdire=pajes&plng=eng&un=PAJES-26235>
13. Ferreira PG, Lima MASS, Bernedo-Navarro RA, Conceição RA, Linhares E, Sawaya ACHF, et al. Stimulation of acidic reduction of nitrite to nitric oxide by soybean phenolics: possible relevance to gastrointestinal host defense. *J Agric Food Chem*. 2011 May 25;59(10):5609-5616.
14. Toyozumi T, Sekiguchi H, Takabayashi F, Deguchi Y, Masuda S, Kinae N. Induction effect of coadministration of soybean isoflavones and sodium nitrite on DNA damage in mouse stomach. *Food Chem Toxicol*. 2010 Oct;48(10):2585-2591.
15. Nam YR, Won SB, Chung Y-S, Kwak CS, Kwon YH. Inhibitory effects of Doenjang, Korean traditional fermented soybean paste, on oxidative stress and inflammation in adipose tissue of mice fed a high-fat diet. *Nutr Res Pract*. 2015 Jun;9(3):235-241.
16. Docea AO, Mitrut P, Grigore D, Pirici D, Calina DC, Gofita E. Immunohistochemical expression of TGF beta (TGF- β), TGF beta receptor 1 (TGFBR1), and Ki67 in intestinal variant of gastric adenocarcinomas. *Rom J Morphol Embryol*. 2012;53(3 Suppl):683-692.
17. Ko K-P, Park SK, Yang JJ, Ma SH, Gwack J, Shin A, et al. Intake of soy products and other foods and gastric cancer risk: a prospective study. *J Epidemiol*. 2013 Sep 5;23(5):337-343.
18. Kumar D, Hegde HV, Patil PA, Roy S, Kholkute SD. Antiulcer activity of water soaked *Glycine max* L. grains in aspirin induced model of gastric ulcer in Wistar rats. *J Ayurveda Integr Med*. 2013;4(3):134-137.