Antitumor Effects of Soybean Hypocotyls and Soybeans on the Mammary Tumor Induction by *N*-Methyl-*n*-nitrosourea in F344 Rats

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Abstract. *Background: Soybeans are reported to have cancer inhibitory effects, probably due to their isoflavones. Soybean hypocotyls are embryo buds of soybeans and contain a higher amount of isoflavones and other factors than soybeans themselves. Materials and Methods: The effects of soybean protein and soybean hypocotyls as diets on the development of N-methyl-n-nitrosourea (MNU) induced tumors were examined in female F344 rats. For this trial, 120 animals were used and at 6 weeks of age, groups of 30 animals were fed diets containing casein, soy protein isolate (SPI), 1.5% soybean hypocotyls and 5% soybean hypocotyls. Three weeks later all the animals except the control animals received a first dose (37.5 mg/kg body weight) of MNU by tail vein injection. At 29 weeks of age the animals received a second MNU dose (50 mg/kg body weight). Testing was performed 42 weeks after the first MNU dose. Results: Analysis of cumulative palpable tumor incidence indicated that final tumor development of the SPI diet group and the hypocotyl diet groups was less than that of the casein diet group. Tumors were detected in one or more sites from 9 out of 24 rats in the casein diet group, 5 of 20 rats in SPI diet group, 6 out of 24 rats in the 1.5% hypocotyl diet group and 6 out of 23 rats in the 5% hypocotyl diet group. Pairwise comparisons indicated that the formation of tumors during the experiment was significantly less rapid in the SPI diet group and the hypocotyl diet groups than the casein group. No difference in tumor promotion was observed between the SPI diet group and the soybean hypocotyl diet groups. Conclusion: Our results suggest that dietary soybeans and soybean hypocotyls are capable of suppressing tumor promotion.*

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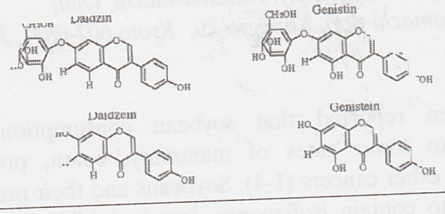
It has been reported that soybean consumption may contribute to lower rates of mammary, colon, prostate, bladder and other cancers (1-4). Soybeans and their products are known to contain isoflavones, trypsin inhibitors, phytic acid and saponin which have an inhibitory effect on cancer development (5). The embryo buds of soybeans (soybean hypocotyls) are a by-product of soybean processed foods and contain higher amount of these components than soybeans themselves. Hypocotyl tea is being produced, which is made by powdering roast soybean hypocotyl. Recently, we showed an inhibitory effect of soybean hypocotyls (raw or roast) on tumor promotion by Epstein-Barr virus activation assay and also by mouse skin tumor promotion test. The inhibitory effect, based on the experiment, might depend on isoflavones (6-7). To our knowledge, no other studies have ever examined the effect of soybean hypocotyls on cancer promotion. To examine the effect of soybean hypocotyls in cancer chemoprevention, we assessed the tumor chemopreventive effects of soybean hypocotyls taken repeatedly over an extended interval, using experiments on animals. The present study was designed to assess the effect of soybeans and soybean hypocotyls on N-methyl-*n*-nitrosourea (MNU) induced carcinogenesis in rats.

Materials and Methods

Animals and animal husbandry. One-hundred and twenty virgin female inbred F344 rats, 4 weeks of age (Yoshitomi Co. Ltd., Fukuoka, Japan.), were maintained on the standard CE-2 diet (Japan Clea Inc., Tokyo, Japan) until 6 weeks of age. Then animals were randomly allocated to one of four different experimental diets. Groups of 30 animals each were placed on diets containing casein (Group 1); soy protein isolate (SPI) (Group 2); SPI and 1.5% roast hypocotyls (Group 3); 5% roast hypocotyls (Group 4). The semipurified diets used in these experiments were based on the recommendations of the American Institute of Nutrition Rodent Diets (8). The concentration of isoflavones in hypocotyls was determined by reversed-phase high-pressure liquid chromatography (6). The animals were maintained on the experimental diets for the duration of the experiment (47 weeks). Three or four

Table I. *Isoflavone content of soybean hypocotyls. Chemical structure of soybean isoflavones, daidzin, daidzein, genistin and genistein measured in this study.*

Isoflavones	Raw hypocotyl	Roast hypocotyl
	(μg/g dry matter)	
Daidzin	7715	4805
Genistin	1342	1180
Daidzein	143	1621
Genistein	34	338
Total	9234	7944



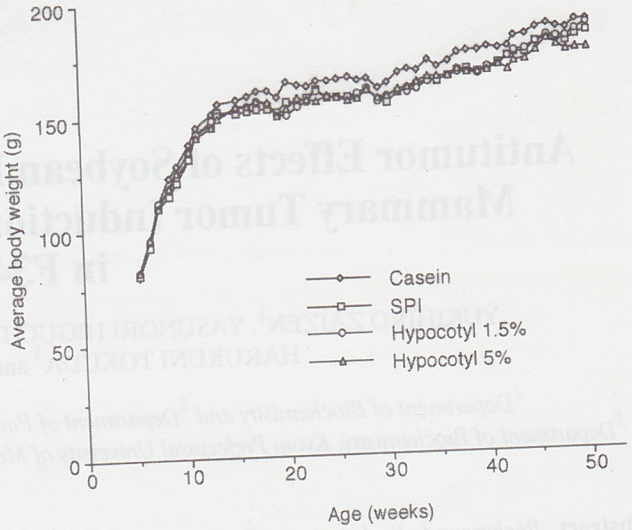


Figure 1. *Change of average group body weights of rats in the experimental period. Error bar of each point was omitted to avoid complexity.*

animals were housed in a polyethylene cage containing hardwood shavings and covered with a filter top. The animal room was controlled for temperature (24 ± 2°C), light (12-hour cycle) and humidity (50%). The diets were administered in powdered form and tap water was provided *ad libitum*. Stainless steel "J" type powder feeders were used to prevent scattering of the food.

Carcinogen administration. At 9 weeks of age 25 of each group at animals received a first dose (37.5 mg/kg body weight) of At MNU (Sigma Co. Ltd., MA. USA) by tail vein injection. At 29 weeks of age the animals received a second MNU dose (50 mg/kg body weight) (9-12). The MNU was dissolved in a few drops of 3% acetic acid and diluted with distilled H_2O to give a solution of MNU at 10 mg/ml, which was administered within 2 hours after preparation. Vehicle treated subgroups consisting 5 animals each, were given the same volume of the dissolving medium without MNU. At weekly intervals, each rat was weighed and the position and number of palpable tumors were recorded.

Autopsy and sectioning. Necropsies were performed on animals which were sacrificed by ether anesthesia upon becoming moribund whilst all surviving animals were anesthetized to be sacrificed at the end of the experiment. The tumors were sectioned and other organs were carefully checked under the naked eye. The tumors and related lesions were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E) and the tumors were classified histologically.

Results

Changes in body weights. Figure 1 shows the changes in average body weight measured throughout the experiment. At the end of the experiment (51 weeks of age), means ± SD was 188.2 ± 7.0 in the casein diet group, 182.9 ± 12.1 in the SPI diet group, 186.7 ± 9.9 in 1.5% hypocotyl diet group and 175.7 ± 8.3 in the 5% hypocotyl diet group. No significant differences among the diets were observed by *t*-test throughout and at the end of the experiment. Table I shows the chemical structure and concentration of isoflavones in soybeans and hypocotyls whitin Table II gives the composition of experimental diets and the concentration of isoflavones in each diet (6).

Tumor incidence. Examination revealed the presence of an indurated mass at mammary gland at axillae and abdomen. Table III shows the MNU induced tumor incidence in animals fed the experimental diets. No tumors were detected in vehicle-treated rats in any dietary groups. All animals in the groups treated with MNU and fed the experimental diets survived until termination of the experiment except 1 rat in the casein group I in the 1.5% hypocotyl diet group, 4 rats in the SPI diet group and 2 rats in the 5% hypocotyl diet group. These animals died within 2 weeks after the first or second carcinogen treatment and were not included in the results. No tumors were detected in these animals. At the end of the experiment (51 weeks of age), definite tumors were detected in one or more sites from 9 out of 24 rats in the casein group and 6 out of 20 rats in the SPI group. Six out of 24 rats in the 1.5% hypocotyl diet group and 6 out of 23 rats in the 5% hypocotyl diet group contracted tumors.

The histological diagnosis of the tumors is also presented in Table III. In this experiment, squamous cell carcinomas (SCCs) in mammary regions were mainly observed, whilst smaller number of SCCs were found in the parotid region and in the oral angle. The mammary gland and salivary gland of the rats might be major target organs of MNU, which induees inflammatory legions and squamous metaplasia and proliferation of metaplastic cells of ducts in the gland. Sections from the tumor mass in the mammary and parotid region revealed identical features. The tumor was composed

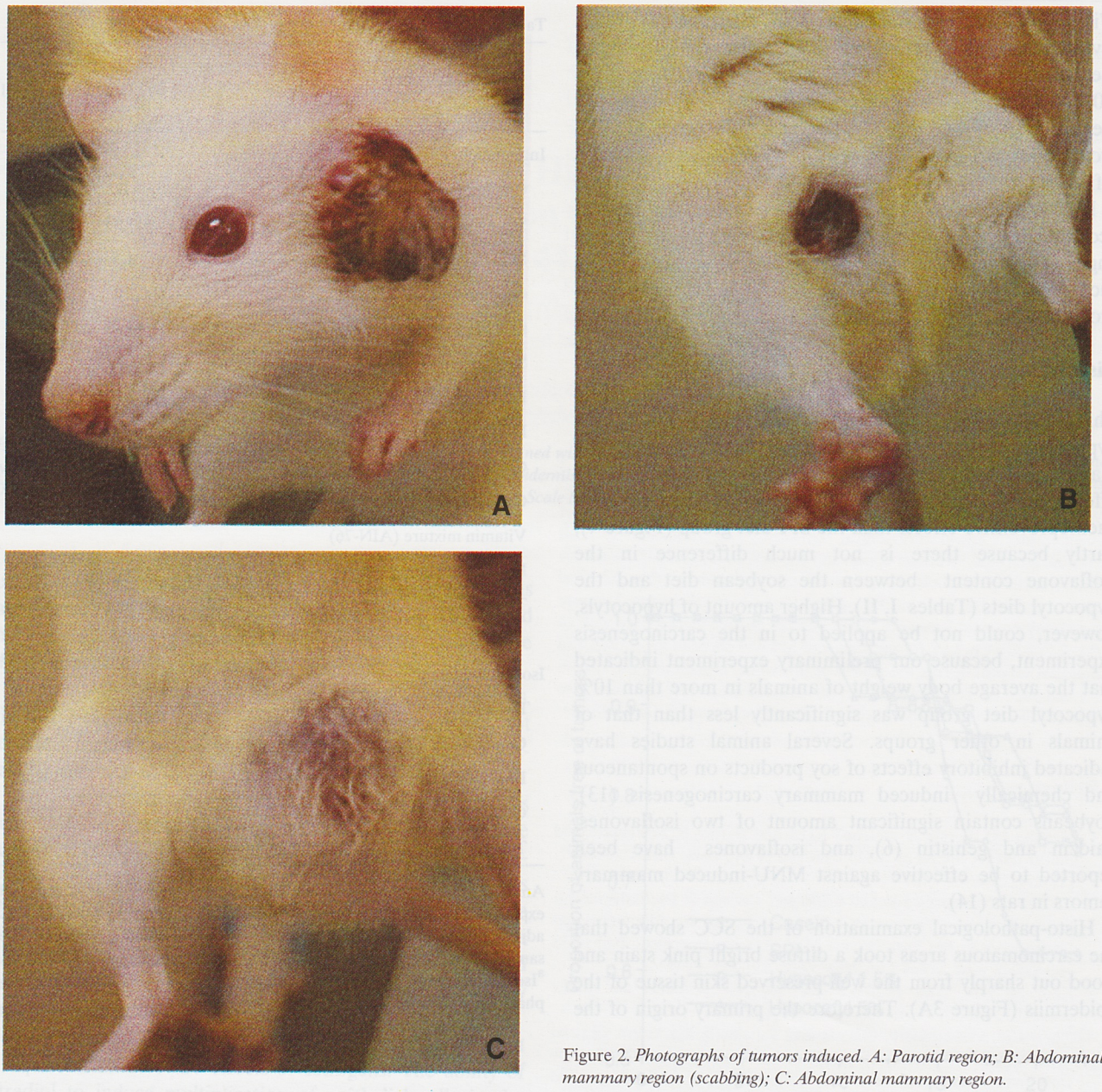


Figure 2. *Photographs of tumors induced. A: Parotid region; B: Abdominal mammary region (scabbing); C: Abdominal mammary region.*

of stratified squamous epithelium covered by scabbing, moderately firm, with its surface ulcerated in places, while cheesy pus was noted inside the tumor. SCCs originated from duct systems grew as dome- shaped nodules (Figure 2) and superficial carcinomas ulcerated to form scabbing and internal lesions necrotized to become abscesses. Two adenocarcinomas (ACs) in the mammary region were found in the casein diet group and one in the SPI diet group. The hypocotyl 1.5% diet group showed an AC in thyroid gland. No AC was found in the 5% hypocotyl diet group. Photomicrographs of SCC and AC stained with H&E are shown in (Figure 3). At the end of the experiment, the incidence of tumors in animals fed the diet containing SPI and hypocotyls was low when compared to the incidence in animals fed the casein diet. Still χ^2 analysis indicated that the differences among the diet groups were not statistically significant (Table III).

The Kaplan-Meier life table curve (Figure 4) revealed that was some difference in the rate of tumor appearance: the highest was in the casein diet group and it was lower in the SPI diet group and the hypocotyls diet groups. From 15 weeks after the second MNU injection, pairwise comparisons by

Wilcoxon's Rank test among the four experimental groups revealed that tumors appeared significantly more rapidly in the casein group than in the 5% hypocotyl diet group ($p < 0.01$), the 1.5% hypocotyl diet group ($p < 0.01$) ; or the SPI diet group ($p < 0.02$). Pairwise comparisons between SPI diet group and hypocotyl diet groups showed no significant difference.

During the course of the experiment, palpable tumors occasionally appeared and then disappeared mostly at oral angles. The number of such animals in each group exhibiting such transient tumors were 3, 3, 11 and 8, respectively, in groups 1-4.

Discussion

This study suggests that the oral administration of soybeans hypocotyls appears to be effective in preventing the mammary tumor, whilst also the use of soybeans is effective. The hypocotyl diet groups did not show stronger chemopreventive effects than the SPI diet group (Figure 4), partly because there is not much difference in the isoflavone content between the soybean diet and the hypocotyl diets (Tables I, II). Higher amount of hypocotyls, however, could not be applied to in the carcinogenesis experiment, because our preliminary experiment indicated that the average body weight of animals in more than 10% hypocotyl diet group was significantly less than that of animals in other groups. Several animal studies have indicated inhibitory effects of soy products on spontaneous and chemically induced mammary carcinogenesis (13). Soybeans contain significant amount of two isoflavones, daidzin and genistin (6), and isoflavones have been reported to be effective against MNU-induced mammary tumors in rats (14).

Histo-pathological examination of the SCC showed that the carcinomatous areas took a diffuse bright pink stain and stood out sharply from the well-preserved skin tissue of the epidermiis (Figure 3A). Therefore the primary origin of the

Table II. *Composition of experimental diets,*

	Group 1 Casein diet	Group2 SPI diet	Group 3 Hypocotyl 1.5%	Group 4 Hypocotyl 5%
Ingredients (g/100 g)				
Casein (86% protein)	20	0	0	0
SPI (78% protein)	0	20	19.25	17.5
Hypocotyl (33% protein)	0	0	1.5	5
α-Comstarch	10	10	10	10
β-Com starch	42	42	41.25	39.5
Sucrose	8	8	8	8
Fat (Com oil)	10	10	10	10
DL-Methionine	0.3	0.3	0.3	0.3
Choline bitartrate	0.2	0.2	0.2	0.2
Cellulose powder	5	5	5	5
Vitamin mixture (AIN-76)				
Mineral mixture (AIN-76)	3.5	3.5	3.5	3.5
Total	100	100	100	100
Isoflavones (μg/g)[a]				
Daidzin	0	111.1	179	337.5
Genistin	0	185.7	196.4	221.5
Daidzein	0	57.6	79.7	131.4
Genistein	0	63.7	66.4	72.7
Total	0	418.2	521.7	763.2

Animals were kept on AIN-76A (American Institute of Nutrition) based experimental diets. The composition of the experimental diets was adjusted so that the animals in all dietary groups would consume the same amount of calories, protein, vitamins, minerals and fiber. [a]Isoflavones were extracted with methanol and separated on reversed-phase high-pressure liquid chromatography.

Table III. *Tumor incidence as a function of dietary group at the end of the experiment: Histological classification of tumors.*

	Number of rats		Number of tumors[a]					
	Rats at risk	Incidence	Total	Mammary			Parotid	Others
Group		(%)		SCC[b]	AC[c]	Others	SCC	
1. Casein	24	9 (37)	9	5	2	0	1	1[d]
2. SPI	20	5 (25)	6	2	1	2[e]	1	0
3. Hypocotyl 1.5%	24	6 (25)	7	4	0	1[f]	1	1[g]
4. Hypocotyl 5%	23	6 (26)	7	3	0	0	2	2[h]

[a] One rat has one or two tumors. [b]Squamous cell carcinoma. [c]Adenocarcinoma. [d]SCC in oral angle. [e]Indistinct. [f]Fibroma. [g]AC in thyroidea. [h]Malignant fibrous histiocytoma in gonadal region, Interstitial increase in both kidneys.

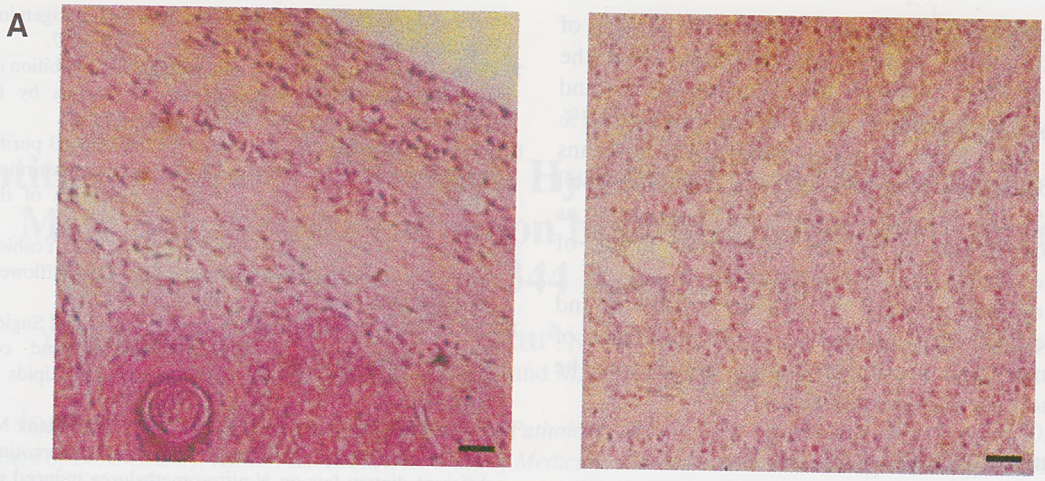


Figure 3. *Microscopic appearance of the mammary carcinoma biopsy stained with H&E. A: SCC. The tumor cells form concentric whorls of keratin and pyknotic nuclei, termed cancer pearls. The tumor cells do not invade the epidermis. B: AC. The carcinoma cells exhibit round nuclei and grow in solid sheets. The ducts of mammary AC resemble those of the normal mammary tissue. Scale bars, 50 μm.*

tumor is the mammary gland where MNU caused squamous metaplasia. Histo-pathological examination of the AC showed that the cells were differentiated and the normal architecture of mammary ducts was preserved (Figure 3B).

Although the animals got transient tumors at oral angles, which appeared not to be malignant neoplasms, it was not necessary for the purpose of this research to count such a self-healing tumor.

Our former study showed that dimethyl sulfoxide extracts from soybean hypocotyls have an inhibitory effect on the expression of Epstein-Barr virus early antigen elicited by 12-*O*- tetradecanoylphorbol-13-acetate (TPA). The effect was stronger than the preparation from soybeans. Moreover, the treatment of mouse skin by the preparation of hypocotyls showed delay of TPA-induced promotion of papillomasl and our data suggest that the anti-tumor activity is mainly attributable to isoflavones (6).

Breast cancers are known to be hormone responsive and it is important to note the effects of estrogens. The ability of estradiol to induce multiplication of epithelial cells in the mammary tissue is thought to be involved (15). In some organs, isoflavones are antiestrogenic, while in other organs, isoflavones are estrogenic. Isoflavones can reduce the effects of estrogens and cancel out the promoting activity of mammary tumor (16).

The risk of breast cancer is high in North America and European countries. In contrast, in most countries of the Pacific basin incidence of breast cancer is five to eight times lower than in the USA. Epidemiologic studies have suggested that soybean consumption may contribute to the lower rate of breast cancer in Asian countries (17). Soybeans contain isoflavones, which have been shown to bind to estrogen receptor and have potent estrogenic activity. Isoflavones seem to prevent cancer through multiple mechanisms. Isoflavones have a suppressive effect on the proliferation of lymphocytes

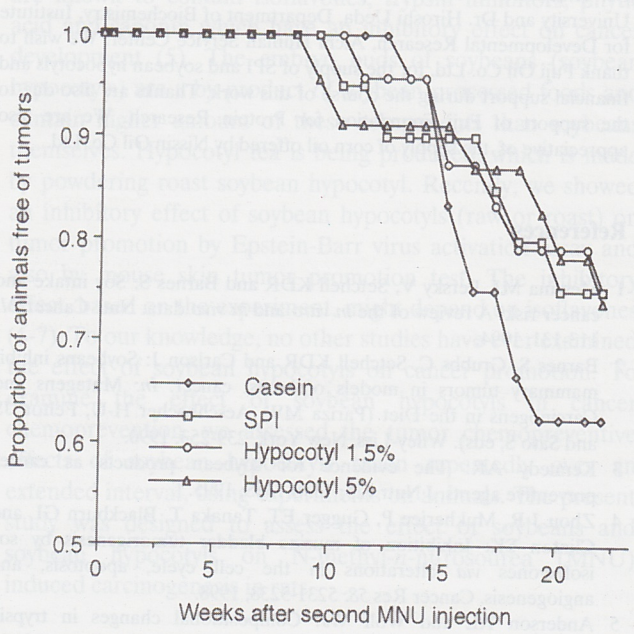


Figure 4. *Kaplan-Meier life table curves for cumulative mammary tumor incidence studied for the diets casein, SPI, 1.5% hypocotyl, and 5% hypocotyl. Life table data includes all palpable tumors (SCC, AC, etc.). Data points represent total palpable tumors, including those that regressed during the experiment. Ordinate = proportion of animals surviving without tumor (1.0 represents 100% tumor-free animals). Abscissa = weeks after second MNU injection.*

and many tumor cell lines in culture and have antiangiogenic activity (4,18,19).

Soybean and its products are known to contain components which are considered to have cancer chemopreventive effects, such as trypsin inhibitors, phytic acid and saponins other than

isoflavones. Soybeans are reported to contain 17-27 mg/g of trypsin inhibitors while, hypocotyls also contain about the same amount. They subject to denaturalization and inactivation by heat. Soybeans contain in the range of 1-2% phytic acid, at which hypocotyl contains only 0.9%. Soybeans contain 0.1-0.5% saponins, whereas the level in the hypocotyl approaches 2% (5). As saponin have been suggested as possible anticarcinogens (20), the synergistic effect of isoflavones and saponin should be entertained.

In this study, the ingestion of soybean hypocotyls and soybeans were shown to be effective in the prevention of mammary tumor. The hypocotyls can be expected to be utilized as cancer chemopreventive diet.

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References

1 Messina MJ, Persky V, Setchell KDR and Barnes S: Soy intake and cancer risk: A review of the *in vitro* and *in vivo* data. Nutr Cancer *21:* 113-131, 1994.

2 Barnes S, Grubbs C, Setchell KDR and Carlson J: Soybeans inhibit mammary tumors in models of breast cancer. *In:* Mutagens and Carcinogens in the Diet (Pariza MW, Aeschbacher H-U, Felton JS and Sato S, eds). Wiley-Liss, New York, 239-253, 1990.

3 Kennedy AR: The evidence for soybean products as cancer preventive agents. J Nutr *125:* 733S-743S, 1995.

4 Zhou J-R, Mukherjee P, Gugger ET, Tanaka T, Blackburn GL and Clinton SK: Inhibition of murine bladder tumorigenesis by soy isoflavones *via* alterations in the cell cyele, apoptosis, and angiogenesis. Cancer Res *58:* 5231-5238, 1998.

5 Anderson RL and Wolf WJ: Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. J Nutr *125:* 581S-588S, 1995.

6 Zaizen Y, Tokuda H, Nishino H and Takeshita M: Inhlbitory effect of soybean hypocotyls on Epstein-Barr virus early antigen induction and skin tumor promotion. Cancer Lett *121:* 53-57, 1997.

7 Constantinou AI, Mehta RG and Vaughan A: Inhibition of *N*-methyl-*n*-nitrosourea-induced mammary tumors in rats by the soybean isoflavones. Anticancer Res *16:* 3293-3298, 1996.

8 Reeves PG, Melsen FH and Fahey GC Jr: AIN-93 purified diets for laboratory rodents: final report of the American institute of nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nut *123:* 1939-1951, 1993.

9 Takeshita M, Ueda H, Shirabe K, Higuchi Y and Yoshida S: Lack of promotion of colon carcinogenesis by high-oleic safflower oil. Cancer *79:* 1487-1493, 1997.

10 Cohen LA, Chen-Backlund J-Y, Sepkovic DW and Sugie S: Effeet of varying proportions of dietary menhaden and corn oil on experimental rat mammary tumor promotion. Lipids *28:* 449-456, 1993.

11 Cohen LA, Thompson DO, Maeura Y, Choi K, Blank ME and Rose DP: Dietary fat and mammary cancer. I. Promoting effects of different dietary fats on *N*-nitrosomethylurea induced rat mammary tumorigenesis. J Natl Cancer Inst *77:* 33-42, 1986.

12 Gullino PM, Pettigrew HM and Grantham FH: N-nitrosomethylurea as mammary gland carcinogen in rats. J Natl Cancer Ins *54:* 401-409, 1975.

13 Herman C, Adlercreutz T, Goldin BR, Gorbach SL, Höckerstedt KAV, Watanabe S, Häämäläinen EK, Markkanen MH, Mäkelä TH, Wähälä KT, Hase TP and Fotsis T: Soybean phytoestrogen intake and cancer risk. J Nutr *125:* 757S-770S, 1995.

14 Gotoh T, Yamada K, Yin H, Ito A, Kataoka T and Dohi K: Chemoprevention of *N*-nitroso-*N*- methylurea-induced rat mammary carcinogenesis by soy foods or bichanin-A. Jpn J Cancer Res *89:* 137-142, 1998.

15 Sellers TA: Genetic factors in the pathogenesis of breast cancer: Their role and relative importance. J Nut *127:* 929S-932S, 1997.

16 Hsieh C-Y, Santell RC, Haslam SZ and Helferich WG, Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo*. Cancer Res *58:* 3833-3838, 1998.

17 Coward L, Barnes NC, Setchell KDR and Barnes S: Genistein, daidzein, and their ß-glycoside conjugates: Antitumor isoflavones in soybean foods from American and Asian diets. J Agric Food Chem *41:* 1961-1967, 1993.

18 Fotsis T, Zhang Y, Pepper MS, Adlercreutz H, Montesano R, Nawrooth PP and Schweigerer L: The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumor growth, Nature *368:* 237-239, 1994.

19 Kondo K, Tsuneizumi K, Watanabe T and Oishi M: Induction of in vitro differentiation of mouse embryonal carcinoma (F9) cells by inhibitors of topoisomerases. Cancer Res *51:* 5398- 5404, 1991.

20 Rao AV and Sung MK: Saponins as anticarcinogens. J Nutr *125:* 717S-724S, 1995.

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