Nutritional Sciences 9(1), February, 2006, pp.14-19

Effects of Dietary Peroxidizability Index Values on Hepatic TBARS and Antioxidant Enzyme Activity in 7,12-dimethylbenz[α]anthracene-treated Rats

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Breast cancer may be the consequence of free radical damage, which is partially caused by the excessive intake of dietary fat and imbalances in antioxidant scavenger systems. In this experiment, we examined the effects of dietary peroxidizability index (PI) values on hepatic thiobarbituric acid reaction substances (TBARS) and antioxidant enzyme activities in rats treated with 7,12-dimethylbenz[α]anthracene (DMBA). Female Sprague-Dawley rats were used and 7,12-DMBA (20 mg/kg body weight) was gastrically intubated at seven weeks of age in order to induce mammary tumors (MT). The levels of dietary PI were 36, 81, 126 and 217 (LPI, MLPI, MHPI and HPI), while dietary polyunsaturated/saturated fatty acids ratio was maintained at the same level (1.0). Fat used in the experiment was mixed with soybean oil, corn oil, palm oil, perilla oil, sesame oil, fish oil, and beef tallow. Experimental diets were given for the following 20 weeks. We measured tumor numbers and weights, and then assayed the hepatic TBARS levels and antioxidant enzyme activities such as superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione-*S*-transferase (GST) and glutathione reductase (GR). The incidence of MT was the lowest in the MHPI group. The hepatic TBARS level was significantly raised with increasing dietary PI value. The hepatic SOD and GR activities were differed significantly by dietary PI value. The hepatic SOD activity was negatively correlated with dietary PI value and GR activity was the highest in the rats fed the MHPI diet. When the dietary P/S ratio is kept at 1.0, adequate dietary PI value (PI value of 126) may reduce the incidence and growth of MT, but this benefit may be lost with higher dietary PI value. These results suggest that the awareness of dietary PI values may help to decrease breast cancer incidence and growth.

Key words: Breast cancer, Peroxidizability index, TBARS, Antioxidant enzyme, Dimethylbenz[α]anthracene

Received November 18, 2005; Revised January 5, 2006; Accepted January 12, 2006

INTRODUCTION

Reactive oxygen species (ROS) in living organisms are generated during phagocytosis, redox reactions of xenobiotics, and enzymatic reactions catalyzed by oxidases, cyclooxygenases, lipooxygenases, dehydrogenases and peroxidases, as well as reductases.[1,2] Living organisms protect themselves against ROS with antioxidative defense systems, which are responsible for establishing the balance between the generation and removal of ROS.[1] The defense systems against ROS include enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), and non-enzymatic antioxidants.[3] Cancer is one of the diseases whose etiopathology is connected with ROS.[2,4-7] Both *in vitro* and *in vivo* experiments showed that ROS participate in carcinogenesis.[8]

Evidence for the involvement of ROS in cancer development is based on the following:(i) the increase of lipid peroxidation products, (ii) the decrease in antioxidative enzyme activities and in the concentrations of non-enzymatic antioxidants and (iii) the positive influence of antioxidants in cancer prevention and treatment.[2]

The etiology of breast cancer, although controversial and still unclear, may include the involvement of environmental factors, sex hormones, and genetic factors as well as immunological factors.[2,9,10] Environmental factors, especially diet, may be associated with the tumorigenesis of various organs, such as the breast, colon, oral cavity, and pancreas.[9-11] Several studies have shown that the incidence of breast cancer and the number of tumors are correlated with the intake of total fats and animal fats.[9,12] Also, high polyunsaturated/saturated fatty acid (P/S) and unsaturated/saturated fatty acid (U/S) ratios in the diet decrease the incidence of breast cancer.[10] Concerning

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the types of fatty acids, it has been reported that n-3 fatty acids attenuate tumor initiation, promotion and progression and that n-9 fatty acids are considered promoters, whereas the role of n-6 fatty acids remains controversial.[9]

Studies of breast cancer have been carried out in relation to dietary fat.[9,12,13] However, few studies have examined the relationship between breast cancer and dietary peroxidizability index (PI) values. Hence, in this study, we investigated the effects of different dietary PI values, under the control of a fixed P/S ratio on mammary tumorigenesis, hepatic thiobarbituric acid reaction substances (TBARS) level and hepatic enzyme activities in rats treated with dimethylbenz [α]anthracene (DMBA).

MATERIALS AND METHODS

1. Experimental Diets

The experimental diets were prepared using a modified American Institute of Nutrition-93 Growth (AIN-93G) diet as shown in Table 1.[14] The dietary fat was mixed with soybean oil (CJ, Korea), corn oil (CJ), palm oil (Lottesamkang, Korea), perilla oil (directly extracted from perilla), sesame oil (Ottugi, Korea), fish oil (Dongwon, Korea) and beef tallow (Lottesamkang). In the previous study, the fatty acid composition of seven fat sources was analyzed by gas chromatography (HP 6890, Hewlett Packard Co., USA).[15,16] Based on our previous results, we calculated peroxidizability index (PI) values when the P/S ratio was maintained at the same level (1.0). When the seven fats were mixed and the P/S ratio was maintained at 1.0, the lowest PI value was 36 and the highest PI value was 217. The average of the lowest PI value and the highest PI value was 126 and the average of 36 and 126 was 81, which was chosen to be the desirable range in our previous study.[15,16] Table 2 shows the dietary fatty acid compositions according to PI values in the experimental diets. These diets were stored in weekly portions at -20 ℃ under nitrogen gas until used. Also, the residual diets were discarded daily.

Table 1. Composition of the experimental diet

(g/kg die)

	LP[2]	MLPI	MHPI	HPI
Casein	150	150	150	150
Corn starch	500	500	500	500
Sucrose	100	100	100	100
Fat	150	150	150	150
Soybean oil	*30.7*	*23.1*	*15.5*	*0.3*
Corn oil	*15.1*	*11.3*	*7.7*	*0.5*
Palm oil	*30.0*	*22.9*	*15.7*	*1.4*
Perilla oil	*3.0*	*2.3*	*1.6*	*0.3*
Sesame oil	*27.0*	*20.4*	*13.7*	*0.5*
Fish oil	*0.8*	*31.3*	*62.0*	*123.0*
Beef tallow	*43.4*	*38.7*	*33.8*	*24.0*
DL-methionine	3	3	3	3
Choline Chloride	2	2	2	2
α-Cellulose	50	50	50	50
Vitamin mixture[1]	10	10	10	10
Mineral mixture[1]	35	35	35	35

1) Vitamin mixture and mineral mixture are based on American Institute of Nutrition-93 Growth diet.[14]
2) LPI, low peroxidizability index (PI value of 36); MLPI, mid-low peroxidizability index (PI value of 81); MHPI, mid-high peroxidizability index (PI value of 126); HPI, high peroxidizability index (PI value of 217)

Table 2. Fatty acid composition of experimental diets

(weight %)

Fatty acid	Diets[2]			
	LPI	MLPI	MHPI	HPI
C14:0	1.16[1]	1.80	2.43	3.70
C14:1	0.19	0.17	0.15	0.11
C16:0	21.72	21.36	21.00	20.28
C16:1	1.00	4.45	7.91	14.82
C18:0	7.71	7.59	7.47	7.24
C18:1(n9)	35.17	31.60	28.03	20.90
C18:2(n6)	27.95	21.54	15.13	2.31
C18:3(n3)	2.98	2.43	1.88	0.78
C20:4(n6)	-	0.38	0.75	1.50
C20:5(n3)	0.03	1.13	2.23	4.44
C22:6(n3)	0.14	5.70	11.27	22.39
Σn6 PUFA	27.96	21.92	15.89	3.81
Σn3 PUFA	3.15	9.26	15.38	27.61
n6/n3 ratio	8.88	2.37	1.03	0.14
P/S ratio	1.00	1.00	1.00	1.00
Peroxidizability index[3]	36	81	126	217

1) Values are % of total fatty acid methyl esters.
2) LPI, low peroxidizability index (PI value of 36); MLPI, mid-low peroxidizability index (PI value of 81); MHPI, mid-high peroxidizability index (PI value of 126); HPI, high peroxidizability index (PI value of 217)
3) Peroxidizability index is calculated as follows: peroxidizability index= (% monoenoic acid×0.025)+(% dienoic acid×1)+(% trienoic acid×2)+(% tetraenoic acid×4)+(% pentaenoic acid×6)+(% hexaenoic acid×8).[33]

2. Animals and Tumor Induction

The experiment was carried out on 6-week-old female Sprague-Dawley rats, each weighing 180 ~ 200 g. The rats were adapted to their surroundings for one week before they were fed the experimental diets. The rats were then divided into four groups (n=11), using a randomized complete block design. Then, in order to induce mammary carcinomas, each rat received a single intragastric administration of DMBA (Sigma-Aldrich, Korea) of 20 mg per kg body weight at seven weeks of age.[17] Because DMBA is a fat-soluble compound that induces mammary cancer quite specifically in young female rats, we dissolved

20 mg of DMBA per milliliter of corn oil.[9,17,18] For 20 weeks after the carcinogen administration, the experimental groups were fed the four different experimental diets. Water and food were provided *ad libitum*. The rats were housed in suspended stainless steel mesh cages and kept in an environmentally-controlled room at 22±2 ℃, relative humidity of 50±10% and automatic 12-h light/dark cycle. All animals were cared for in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

3. Measurements of Tumor Numbers and Weights

After anesthetization with diethyl ether, the tumors were excised from the rats and the tumor numbers and weights were measured.

4. Liver Tissue Assays

Sample preparation

The rats were slightly anesthetized with diethyl ether after overnight fasting. The livers were promptly excised and rinsed with cold isotonic saline. The liver tissues were frozen at -70 ℃ until analyzed. Liver sample was mixed in a 1:10 (W/V) ratio with phosphate buffer (PB, 10 mM, pH 7.4) and homogenized under cold conditions. This homogenate was sonicated for 30 sec under an ice bath (GE 50, Sonics & Materials Inc., CT, USA) and used to measure thiobarbituric acid reactive substance (TBARS). This homogenate was centrifuged at 20,000 ×g at 4 ℃ for 30 min in an ultracentrifuge (Optima™ TL, Beckman Coulter, USA) before being used for superoxide dismutase (SOD) and catalase (CAT) samples. In addition, liver sample was diluted to 1:20 (W/V) ratio with PB (50 mM in 0.25 mM sucrose -0.5 mM EDTA, pH 7.4) and homogenized under cold conditions. This homogenate was sonicated for 30 sec under an ice bath and subjected to two centrifugation steps before being used for glutathione peroxidase (GSH-Px), glutathione-*S*-transferase (GST), and glutathione reductase (GR) samples. The homogenate was centrifuged at 10,000×g at 4 ℃ for 20 min in an ultracentrifuge (Optima™ TL, Beckman Coulter). Then, the supernatant was re-centrifuged at 100,000×g at 4 ℃ for 1 hr (Optima™ TL, Beckman Coulter). The final supernatant was used as an enzyme sample.

TBARS and antioxidant enzyme assays

The hepatic TBARS level was measured by a modification of the method used by Fraga *et al.*.[19] The absorbance of the final supernatant was determined at 534 nm in a spectrophotometer (DU 600, Beckman Coulter, USA). Thiobarbituric acid 1,1,3,3-tetramethoxypropane (Aldrich Chemical, Korea) was used as a TBARS standard. SOD (*EC* 1.15.1.1) activity was measured by Marklund's method using pyrogallol.[20] The prepared enzyme sample was mixed by 50 mM tris-acetate buffer (pH 8.26). After the addition of 24 mM pyrogallol (in 10 mM degassing acetic acid), the colored chromogen formed by the autoxidation of pyrogallol under alkali conditions was monitored by spectrophotometer at 420 nm for 3 min. CAT (*EC* 1.11.1.6) activity was measured by the disappearance rate of H_2O_2 and monitored spectrophotometrically at 240 nm, according to the method of Aebi[21] and Claiborne.[22] GSH-Px activity was measured according to the method of Flohe and Gunzler.[23] The activity of GST was measured according to Warholm *et al.*, using 1-chloro-2,4-dinitrobenzene (CDNB, Sigma Aldrich) as a substrate.[24] GR activity was determined by the method of Carlberg and Mannervik.[25] The enzyme activity was determined by the disappearance of NADPH at 340 nm.[25] Protein determination was performed according to Bradford, using bovine serum albumin (Sigma Aldrich) as a standard.[26]

5. Statistical Analysis

For statistical analysis, the SPSS/PC computer program (Statistical Package for Social Science 12.0) was used. Data were expressed as mean±S.E.. The differences among mean values were assessed by one-way analysis of variance (ANOVA) coupled with Duncan's multiple range test; a *p*-value less than 0.05 was considered significant.

RESULTS

1. Effects of Dietary PI Values on Mammary Tumor Incidence, Numbers, and Weights

Mammary tumor (MT) incidence was defined as the percentage of MT-bearing rats out of the total rats. The MT incidence was the lowest in rats fed the MHPI (PI

Table 3. Effects of dietary PI values on mammary tumor (MT) incidence, number and weights in rats

	Groups[2]			
	LPI	MLPI	MHPI	HPI
MT incidence (%) (MT-bearing rats/total rats)	82 (9/11)	73 (8/11)	64 (7/11)	82 (9/11)
Average number of MT per tumor bearing rat (numbers)	2.67±0.52[1]	2.63±0.60	1.43±0.29	2.33±0.50
Average weight of MT per tumor bearing rat (g)	8.21±2.30	10.23±3.60	6.53±2.50	8.89±1.87

1) The average number of MT per tumor-bearing rat and the average weight of MT were expressed as the mean±S.E. (n=11).
These were not significantly different among four groups by one-way ANOVA.
2) LPI, low peroxidizability index (PI value of 36); MLPI, mid-low peroxidizability index (PI value of 81); MHPI, mid-high peroxidizability index (PI value of 126); HPI, high peroxidizability index (PI value of 217)

value of 126) diet. The average number of MT per tumor-bearing rat tended to be lower in the MHPI group, but the difference was not significant. Also, the average weight of MT per tumor-bearing rat was not statistically significant, but had a tendency to be lower in the MHPI group than in any other group.

2. Effects of Dietary PI Values on Hepatic TBARS Levels and Antioxidant Enzyme Activities

Table 4 indicates the effects of dietary PI values on hepatic thiobarbituric acid reactive substances (TBARS) levels and antioxidant enzyme activities in rats. The hepatic TBARS level was increased significantly with increasing dietary PI value when the dietary P/S ratio was maintained at the same level. The hepatic superoxide dismutase (SOD), glutathione-*S*-transferase (GST) and glutathione reductase (GR) activities were significantly different by dietary PI values. SOD activity was the highest in the LPI (PI value of 36) group and GST activity was the lowest in the MLPI (PI value of 81) group. Also, GR activity was the highest in rats fed the MHPI diet. However, hepatic catalase and glutathione peroxidase activities did not differ significantly in dietary PI values.

Table 4. Effects of dietary PI values on hepatic TBARS levels and antioxidant enzyme activities in rats

	Group[2)]			
	LPI	MLPI	MHPI	HPI
TBARS[3)] (ng/mg protein)	67.19±4.85$^{a1)}$	69.23±9.73^{a}	120.29±10.22^{b}	181.79±17.60^{c}
SOD (Unit/mg protein)	19.91±0.90^{b}	17.08±1.80^{ab}	13.82± 0.80^{a}	14.00± 1.25^{a}
CAT (Unit/mg protein)	4.63±0.23	5.26±0.54	5.08± 0.52	5.72± 0.43
GSH-Px (Unit/mg protein)	2.94±0.22	2.53±0.10	3.09± 0.24	2.58± 0.22
GST (Unit/mg protein)	21.93±1.90^{b}	16.19±1.39^{a}	24.36± 1.70^{b}	22.21± 2.38^{b}
GR (Unit/mg protein)	1.16±0.06^{ab}	1.09±0.04^{a}	1.36± 0.07^{c}	1.32± 0.09^{bc}

1) Values are mean±S.E. (n=11).
Values in the same row with different superscripts (a, b and c) are significantly different at p<0.05 by one-way ANOVA and Duncan's multiple range test.
2) LPI, low peroxidizability index (PI value of 36); MLPI, mid-low peroxidizability index (PI value of 81); MHPI, mid-high peroxidizability index (PI value of 126); HPI, high peroxidizability index (PI value of 217)
3) TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; GST, glutathione-*S*-transferase; GR, glutathione reductase

3. Correlation Coefficients Between Dietary PI Values and Various Parameters of Mammary Tumor Rats

In DMBA-treated rats, dietary PI value was positively correlated with the hepatic TBARS level (r=0.765, p=0.001) and GR activity (r=0.369, p=0.018). Also, dietary PI value was negatively correlated with the hepatic SOD activity (r=-0.526, p=0.001; Table 5).

Table 5. Correlation coefficients between dietary PI values and various parameters in mammary tumor rats

	Dietary PI values	
	r[1)]	p
MT incidence (MT-bearing rats/total rats)	-0.023	0.880
Average number of MT per tumor bearing rat (numbers)	-0.158	0.380
Average weight of MT per tumor bearing rat (g)	-0.017	0.926
TBARS[2)] (ng/mg protein)	0.765*	0.001
SOD (Unit/mg protein)	-0.526*	0.001
CAT (Unit/mg protein)	0.237	0.135
GSH-Px (Unit/mg protein)	-0.092	0.574
GST (Unit/mg protein)	0.169	0.304
GR (Unit/mg protein)	0.369*	0.018

1) Correlation coefficients (r) were obtained by linear regression of dietary PI value vs. mammary tumor parameters and hepatic various parameters.
2) TBARS, thiobarbituric acid reactive substance; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; GST, glutathione-*S*-transferase; GR, glutathione reductase
* Significance at p<0.05.

4. Correlation Coefficients between Mammary Tumor Parameters and Antioxidant Enzyme Activities

The average number of MT per tumor-bearing rat was negatively correlated with GST activity (r=-0.437, p=0.016). Also, the average weight of MT per tumor-bearing rat was negatively correlated with GST (r=-0.520, p=0.003) and GR (r=-0.374, p=0.032) activities.

Table 6. Correlation coefficients between mammary tumor parameters and antioxidant enzyme activities

	MT incCidence		Average number of MT per tumor-bearing rat		Average weight of MT per tumor-bearing rat	
	r[1)]	p	r	p	r	p
SOD[2)] (Unit/mg protein)	-0.195	0.216	0.042	0.824	-0.346	0.057
CAT (Unit/mg protein)	-0.418*	0.007	-0.004	0.982	-0.136	0.456
GSH-Px (Unit/mg protein)	-0.134	0.408	-0.230	0.221	-0.053	0.781
GST (Unit/mg protein)	-0.227	0.165	-0.437*	0.016	-0.520*	0.003
GR (Unit/mg protein)	0.021	0.896	0.277	0.119	-0.374*	0.032

1) Correlation coefficients (r) were obtained by linear regression of mammary tumor parameters vs. hepatic enzyme parameters.
2) SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; GST, glutathione-S-transferase; GR, glutathione reductase
* Significance at p<0.05.

DISCUSSION

In this study, mammary tumor (MT) incidence was the lowest in the MHPI group, peroxidizability index (PI)

value of 126. The average number of MT per tumor-bearing rat tended to be low in rats fed the MHPI diet, but was not significantly different among the four groups. Some studies have shown that diets are involved in the promotion rather than initiation stage of carcinogenesis. In particular, the tumor-promoting capability of a diet depends on the type of fatty acids included.[3] However, others have shown that dietary lipids influence breast cancer development at several stages, and that the key factors in this relationship are the amounts and types of fats.[27,28] For example, diets containing high proportions of n-6, primarily linoleic acid (18:2n-6), have greater enhancing effects than high saturated fat diets.[13,28] In contrast, high levels of n-3 polyunsaturated fatty acids (PUFA) inhibit tumor growth.[13,28] Thus, fish oils that contain n-3 fatty acids show an inhibiting effect on experimental mammary carcinogenesis.[3] As shown in Table 2, n-3 PUFA contents of the diet were increased with increasing dietary PI values in this study. As shown in Table 5, MT parameters were not correlated with dietary PI values. Accordingly, MT incidence and the average number of MT did not differ significantly by n-3 PUFA or by dietary PI values when the dietary P/S ratio was maintained at 1.0. However, the MT parameters tended to be the lowest in the MHPI group. This result suggests that a moderate dietary PI value (PI value of 126) may reduce the incidence and growth of MT, but this benefit may be lost at higher dietary PI values.

Oxidative stress resulting from the imbalance between pro-oxidants and anti-oxidants seems to play an important role in breast carcinogenesis.[29,30] There are conflicting reports regarding the effects of the tissue level of TBARS and the activity of superoxide dismutase (SOD) in breast cancer.[30] Hu *et al.*[31] reported that hepatic TBARS level is increased with increasing fish oil intake. As mentioned above, the total n-3 PUFA content in diets is increased with the increase of PI values when the dietary P/S ratio is kept at the same level. In our results, the hepatic TBARS level was increased with the increase in dietary PI values, which shows a similar pattern to that of fish oil content. It seems that the increase in the hepatic TBARS level is due to the increase in dietary PI value as well as n-3 PUFA. This conclusion is clearly supported by the positive relationship between hepatic TBARS level and dietary PI value shown in Table 5.

In our results, the hepatic SOD, GST and GR activities were differed significantly by dietary PI values. However, dietary PI value was negatively correlated with SOD activity and positively correlated with GR activity, and GST activity was not correlated with dietary PI value. SOD, CAT and GSH-Px are considered as primary antioxidant enzymes, since they are involved in the direct elimination of reactive oxygen metabolites.[29,32] They can also act as anticarcinogens and inhibitors at the initiation and promotion/transformation stages of carcinogenesis.[32] As shown in Table 6, MT incidence was negatively correlated with hepatic CAT activity. The average number of MT per tumor-bearing rat was negatively correlated with GST activity. Also, the average weight of MT per tumor-bearing rat was negatively correlated with GST and GR activities. Accordingly, in this study, it seems that CAT is involved in the initiation stage of MT, as indicated by MT incidence. Additionally, it seems that GST and GR are related to the promotion stage of MT, as signified by tumor number and tumor weight (growth). The dietary PI value did not directly affect MT parameters. However, dietary PI value had a positive relationship to GR and GR had a negative correlation to the average weight of MT per tumor-bearing rat. Accordingly, dietary PI value indirectly affected the average weight of MT per tumor-bearing rat through GR activity. Additional studies are required to support these results.

In conclusion, based on this study, we are not able to recommend the most efficient dietary PI value for reducing MT parameters. However, an adequate dietary PI value (PI value of 126) may reduce the incidence and growth of MT and this advantage may be lost with higher dietary PI values. Also, our results suggest that dietary PI value has an indirect influence on MT parameters through GR activity in DMBA-treated rats. Accordingly, the awareness of dietary PI values should be considered as a requirement for decreasing breast cancer incidence and growth.

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