原稿

Studies on Biological Activities of Bio-Normalizer

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Abstract

1) Bio-Normalizer reduces lipid peroxide levels in liver and serum.
Bio-Normalizer protects from liver injury induced by oral administration of corn-oil containing lipid peroxide.
2) Bio-Normalizer stimulates epinephrine-induced lipilysis in fat cells. Based on this results, it seems likely that Bio-Normalizer may be suitable for reduction of body fat in obese patients.
3) Bio-Normalizer inhibits toxohormone-L-induced lipolysis. This result suggests that Bio-Normalizer may improve remarkable in the weight loss and the reduction of appetite in cancer patients.
4) Bio-Normalizer enhanced the natural killer cell activity in tumor-bearing mice.

原稿

Introduction:

Bio-Normalizer (BN) is a white, granular, sweet, natural health food commercially sold in Japan and the Philippines. It is made by fermentation of *Carica Papaya* Linn., Pennistum pupureum Schum, Sechium edule Swartz and strains. In 1991, Santiago et al. found that BN was the first fermented food which could scavenge hydroxyl radicals, also inhibit thiobarbituric acid substances (TBARS) inhibiting property *In vivo* using $FeCl_3$-induced epileptic focus in rats. It was also proven that BN was a potent antigenotoxic agent against dimethylnitrosoamine and cyclophosamide. Based on these results, we studied the biological activities of Bio-Normalizer.

Materials:

Bio-Normalizer was obtained from Sun-O International.,(6-2 Kanazono-cho, Gifu). Wistar king strain rats weighing 150g were used. Rats were housed at 22°c ± 2 °c, humidity of 70% ± 5%, given food pellets (Oriental yeats industry) and water *ad libitum*. ICR mice were used for the experiment of natural killer cells activity. Epinephrine was purchased from Sankyo Co., and collagenase from Washington Co., (Freehold, NJ).

Method:

1) Liver injury induced by TBARS;

Corn-oil was subjected to heat treatment at 200 °c for 2 hours, oxygen infusion was carried out during treatment. Lipid peroxide content in corn-oil was found to increase ten folds with this treatment. 3ml of the corn-oil containing 1% of cholestrerol and 1% of cholic acid were administered with a syringe twice a day for 9 days. Blood and liver were collected 1 hour after administration of the corn-oil on the 10th day morning and measured. Total cholesterol, triglyceride and transaminases (GOT, GPT) were measured by the test kit purchased from Wako Pure Chemical co., respectively. Lipid peroxide content was measured as follows. 1 ml of 0.5% thiobarbituric acid solution and water were added with 0.1 ml of serum or liver homogenate (10%), then heated at 100 °c for 1

hour. After the heat treatment, 0.5 ml of 0.1% NHCl and 4ml of n-butanol were added and after mixed, butanol was collected. The value was measured as the absorbance of 532mm, then compared with ethanol solution of maronaldehyde. Liver homogenate was prepared by removal liver of rats added with saline solution. Total cholesterol and triglyceride were measured with the extracts of 10% liver homogenate and chroloform-methanol (2 : 1) mixture of 1 : 10.

2) Epinephrine-induced lipolysis in fat cells;

Male Wistar king strain rats weighing 150 ~ 200 g were used.Fat cells were isolated by the method of Rodbell. 25 μl of epinephrine was added to 50 μl of fat cells (packed volume) , 25 μl of Bio-Normalizer solution and 200 μl of 4% BSA in Hank's buffer, then heated at 37°c for 1 hour. After the treatment, free fatty acids were extracted with the mixing of chloroform, heptanoic acid, methanol (24.5 : 24.5: 1) and Cu-fatty acids base was confirmed. Fatty acids were estimated with the method of Cu-coloring with chelating reagent. On the experiment of effect of BN on toxohormone-L-induced lipolysis in rat fat cells, the ascites fluid after sarcoma 180 cells were inoculated intraperitoneally into male mice was used. The resultant fat cell fraction (50μl packed volume) was incubated for two hours at 37°c in 200 μl of Hank's buffer containing 4% albumin, 25 μl of BN and 25 μl of toxohormone-L solution. After incubation, fatty acids released were estimated.

3) Natural killer activity:

Five-weeks-old, male ICR mice were used. Sarcoma 180 cells($2.5x10^6$ cells) were inoculated subcutaneously to mice. 1 week after inoculation, half of the mice were adminstered orally 0.8 ml of water and others were administered 0.8 ml of Bio-Normalizer solution(50 mg / ml) dissolved with distilled water everyday. After removal of their spleens of the mice, the organs were gently teased by means of dissecting forceps, and allowed to sediment, and the cell suspensions were aspirated and transferred to sterile tube. Then spleenocytes were suspend in Tris-buffered ammonium chloride to lyze red blood cells. Resulting lymphoid cells were washed by

Ginseng improved anorexia of cancer patients. On this result, we investigated toxohormone-L inhibiting components in Ginseng. We found that Saponin Rb_2 and acidic polysaccharide in Ginseng inhibited the action of toxohormone-L and the depression of food intake. As shown in Figure 1, Bio-Normalizer was found to inhibit the toxohormone-L-induced lipolysis. This result suggests the possibility that Bio-Normalizer improves the suppression of appetite in cancer patients. Therefore, it must need further study whether intake of Bio-Normalizer improves weight loss and anorexia on clinical tests.

4. Effect of Bio-Normalizer on natural killer cell activity in tumor-bearing mice;

It is well known that natural killer cells are able to recognize and lyze a wide variety of tumor cells without prior sentization and they play a major role in natural resistance against tumors.

NK activities of tumor-bearing mice were examined after oral administration of Bio-Normalizer at the dose of 40 mg/ mouse/day as shown in Table 11. Among these mice, NK activities of tumor-bearing mice were significantly elevated on 28 days and 34 days after administration as compared to dose of control mice. Whereas, Table 12 shows NK activities of tumor-bearing mice which were administered Bio-Normalizer at the dose of 80 mg/ mouce/day. NK activity of their mice was also significantly elevated as compared to control mice. These results suggest that Bio-Normalizer contains some activating factor of NK activity. It needs further clinical studies whether Bio-Normalizer actually enhances NK activities in cancer patients.

centrifugation in RPMI 1640 medium containing 10% fetal bovine serum (FBS). The chromium release assay was used to evaluate NK cytotoxic activity. 0.4 ml of YAC-1 target cells ($4x10^6$ cells/ ml) suspended in RPMI 1640 medium: 10% FBS were labeled with 100 uCi of sodium chromate (^{51}Cr) for 90 minutes at 37°c, washed 4 times, and re suspendded to 3×10^5 cells / ml. Effector cells from the tumor-bearing mice were adjusted the cell concentration to 1×10^7 cells / ml. 0.15 ml of the cell suspensions were added to each well of the 96-well-plate. Then 0.05 ml of the labeled YAC-1 cells were added to the wells at effector: target ratio of 100: 1. The plate was put in a humidified CO_2 incubator for 4 hours at 37°c. After incubation, the plate was spun down at 1500 rpm for two minutes, and then 0.1 ml of supernatant from each well was taken and the radio activities were counted on a gamma-counter. The percentage of NK cytotoxicity was computed from by following formula.

$$\% \text{ of NK cytotoxicity} = 100 \times \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximal} - \text{cpm spontaneous}}$$

Results and discussion

1. Effect of Bio-Normalizer on lipid metabolism and liver injury

We investigated the followings using rats after oral administration of corn-oil containig lipid peroxides for 10 days.

Bio-Normalizer failed to affect cholesterol and trigryceride contents in liver as shown in Table 1 and 2. On the other hand, lipid peroxide content in liver was significantly reduced by oral administration of BN as shown in Table 3. Average value of lipid peroxide in liver of normal rats was about 5 μmoles/g.

Bio-Normalizer did not affect both serum cholesterol and triglyceride levels as shown in Table 4 and 5. Average value of serum triglyceride is 100 mg/dl. Lipid peroxide content in corn-oil was found to increase ten folds with the treatment, but BN could not reduce serum triglyceride level. It may suggest that BN has no action to serum chylomicron metabolism, such as intensitinal

absorption of triglyceride. Whereas, as shown in Table 6, BN reduced serum lipid peroxide content. Average value of lipid peroxide in sera of normal rats was about 8 nmdes / ml.

Table 7 and 8 show the effect of Bio-Normalizer on serum GOT and GPT. As serum GOT and GPT in normal rats were 90 KU and 30 KU, respectively. Therefore, the corn-oil administration caused elevations of serum GOT and GPT levels, indicating that it induced liver injury. Serum GOT and GPT were found to be reduced by oral administration of Bio-Normalizer. It may suggest that Bio-Normalizer reduces lipid peroxide content in liver and serum and also protects liver injury induced by oral administration of corn-oil.

2. Effect of Bio-Normalizer on epinephrine-induced lipolysis in rat fat cells;

In Table 9, epinephrine(0.1 μg/ml)-induced lipolysis was found to be enhanced by Bio-Normalizer. It was found that Bio-Normalizer itself could not promote lipolysis, but could enhance lipolysis in the presence of epinephrine. The same result was obtained in the presence by 1 μg /ml of epinephrine as shown in Table 10. This action of Bio-Normalizer is so different from Ginseng and Hachimijiougan which can depress lipolysis. Based on this result, it seems likely that Bio-Normalizer may be suitable for reduction of body fat in obese patients. Whereas, Ginseng and Hachimijiougan promote lipogenesis.

3. Effect of Bio-Normalizer on toxohormone-L-induced lipolysis;

Tumor-bearing animals and patients with various neoplasms frequently show a striking depletion of body lipid. This depletion could be related to not only anorexia but also a lipolytic factor. This factor stimulates fat cells and enhances lipolysis. This factor was named toxohormone-L. It was found to be an aspartic acidic protein with N-terminal and a molecular weight of 70,000. Toxohormone-L was present in the ascited fluids of patients with liver cancer, lung cancer, Grawitz's tumor, malignant ovarian tumor, but, it could not be found in non-cancerous fluids. It enhances lipolysis in fat cells and injection of toxohormone-L into the lateral ventricle resulted in significant suppression of food intake. It was said that intake of

Table 1. Effect of Bio-Normalizer on liver cholesterol content

No	Control (mg)	Bio-Normalizer 0.5g/Kg (mg)	Bio-Normalizer 1.0g/Kg (mg)
1	110.2	369.8	359.0
2	196.1	182.3	249.9
3	465.3	280.9	209.9
4	271.2	178.0	153.3
5	113.2	223.6	303.0
6	307.3	136.3	177.8
7	257.8	358.6	289.1
8	238.3	175.4	162.0
mean	244.9	238.1	237.9
SE	40.4	31.3	26.4

Table 2. Effect of Bio-Normalizer on liver triglyceride content

No	Control (mg)	Bio-Normalizer 0.5g/Kg (mg)	Bio-Normalizer 1.0g/Kg (mg)
1	719.1	1532.5	1641.9
2	1083.0	698.0	1288.9
3	1838.9	1322.5	832.6
4	1517.5	918.8	659.2
5	546.9	832.3	977.7
6	1265.1	628.9	786.7
7	1120.2	1515.4	1346.2
8	1240.3	931.4	867.2
mean	1166.4	1047.5	1050.1
SE	145.4	127.0	119.8

Table 3. Effect of Bio-Normalizer on lipid peroxide content in liver

unit: μmoles

No	Control	Bio-Normalizer 0.5g/Kg	Bio-Normalizer 1.0g/Kg
1	19.3	11.6	8.0
2	11.3	9.7	9.2
3	9.1	8.4	7.6
4	19.0	10.6	5.8
5	8.8	5.6	4.9
6	13.2	6.6	8.1
7	9.9	6.4	7.6
8	22.4	10.4	9.7
mean	14.1	8.7	7.6
SE	1.9	0.79	0.57

Table 4. Effect of Bio-Normalizer on serum cholesterol level

unit: mg/dl

No	Control	Bio-Normalizer 0.5g/Kg	Bio-Normalizer 1.0g/Kg
1	92.2	81.9	79.2
2	83.3	92.2	86.0
3	82.6	58.0	76.5
4	115.4	87.4	96.2
5	111.9	90.8	89.4
6	115.4	79.9	66.2
7	74.4	94.2	97.6
8	92.2	90.1	103.8
mean	95.9	84.3	86.9
SE	5.7	4.1	4.4

Table 5. Effect of Bio-Normalizer on serum triglyceride level

unit: mg/dl

No	Control	Bio-Normalizer 0.5g/Kg	Bio-Normalizer 1.0g/Kg
1	1274.4	497.7	1183.7
2	1127.9	676.7	354.7
3	962.8	1554.7	840.7
4	1374.4	1047.7	793.0
5	1525.6	836.0	1212.8
6	352.3	826.7	117.4
7	1055.8	546.5	1207.0
8	944.2	1308.1	994.2
mean	1077.2	911.8	837.9
SE	125.9	131.1	144.9

Table 6. Effect of Bio-Normalizer on serum lipid peroxide level

unit: nmdes/ml

No	Control	Bio-Normalizer 0.5g/Kg	Bio-Normalizer 1.0g/Kg
1	26.5	6.1	15.9
2	7.6	18.9	12.1
3	18.2	5.3	6.8
4	25.0	19.7	9.1
5	15.9	12.1	20.6
6	18.2	6.8	15.0
7	9.1	10.6	6.1
8	41.5	15.9	4.5
mean	20.3	11.9	11.3
SE	3.8	2.0	2.0

Table 7. Effect of Bio-Normalizer on serum GOT level

unit: Karmen U

No	Control	Bio-Normalizer 0.5g/Kg	Bio-Normalizer 1.0g/Kg
1	189.6	122.9	133.3
2	162.5	120.8	127.1
3	135.4	139.6	118.8
4	156.3	127.1	125.0
5	129.2	139.6	125.0
6	131.3	125.0	112.5
7	145.8	125.0	141.7
8	139.6	133.3	129.2
mean	148.7	129.2	126.6
SE	7.2	2.6	3.1

*$p < 0.05$

Table 8. Effect of Bio-Normalizer on serum GPT level

unit: Karmen U

No	Control	Bio-Normalizer 0.5g/Kg	Bio-Normalizer 1.0g/Kg
1	61.7	21.9	55.9
2	66.4	35.5	56.8
3	77.5	16.0	26.9
4	87.0	51.5	33.6
5	35.2	26.5	25.3
6	36.7	28.4	39.2
7	40.4	36.4	23.8
8	38.3	46.0	35.8
mean	55.4	32.8	37.2
SE	7.2	4.2	4.0

*$p < 0.05$

Table 1. NK activity of tumor-bearing mice which were administerd 40 mg/mouse/day of bio-catalizer for 28-36 days.

Duration of Bio-catalizer administration (days)	% of NK cytotoxicity	
	Control(%)	Bio-catalizer(%)
28	-0.1±0.2	1.3±0.3*
34	0.0±0.4	0.8±0.2*
36	9.6±0.6	11.4±0.7
mean	3.2±3.2a)	4.5±3.5a)

Values are expressd as mean±SE (n=8). *:Stasticaly significant at the $p<0.05$ level when compared with control, a):n=3.

Table 2. NK activity of tumor-bearing mice which were administerd 80 mg/mouse/day of bio-catalizer for 14-20 days.

Duration of Bio-catalizer administration (days)	% of NK cytotoxicity	
	Control(%)	Bio-catalizer(%)
14	0.8±0.4	0.7±0.2
17	-0.2±0.3	0.6±0.5
19	4.9±0.5	6.3±0.3*
20	-2.6±0.8	-2.2±0.5a)
mean	0.7±1.6b)	1.4±1.9b)

Values are expressd as mean±SE (n=8). *:Stasticaly significant at the $p<0.05$ level when compared with control, a):n=7, b):n=3.

Fig.1 Effect of Bio-Normalizer on Toxohormone-L-induced Lipolysis in rat fat cells

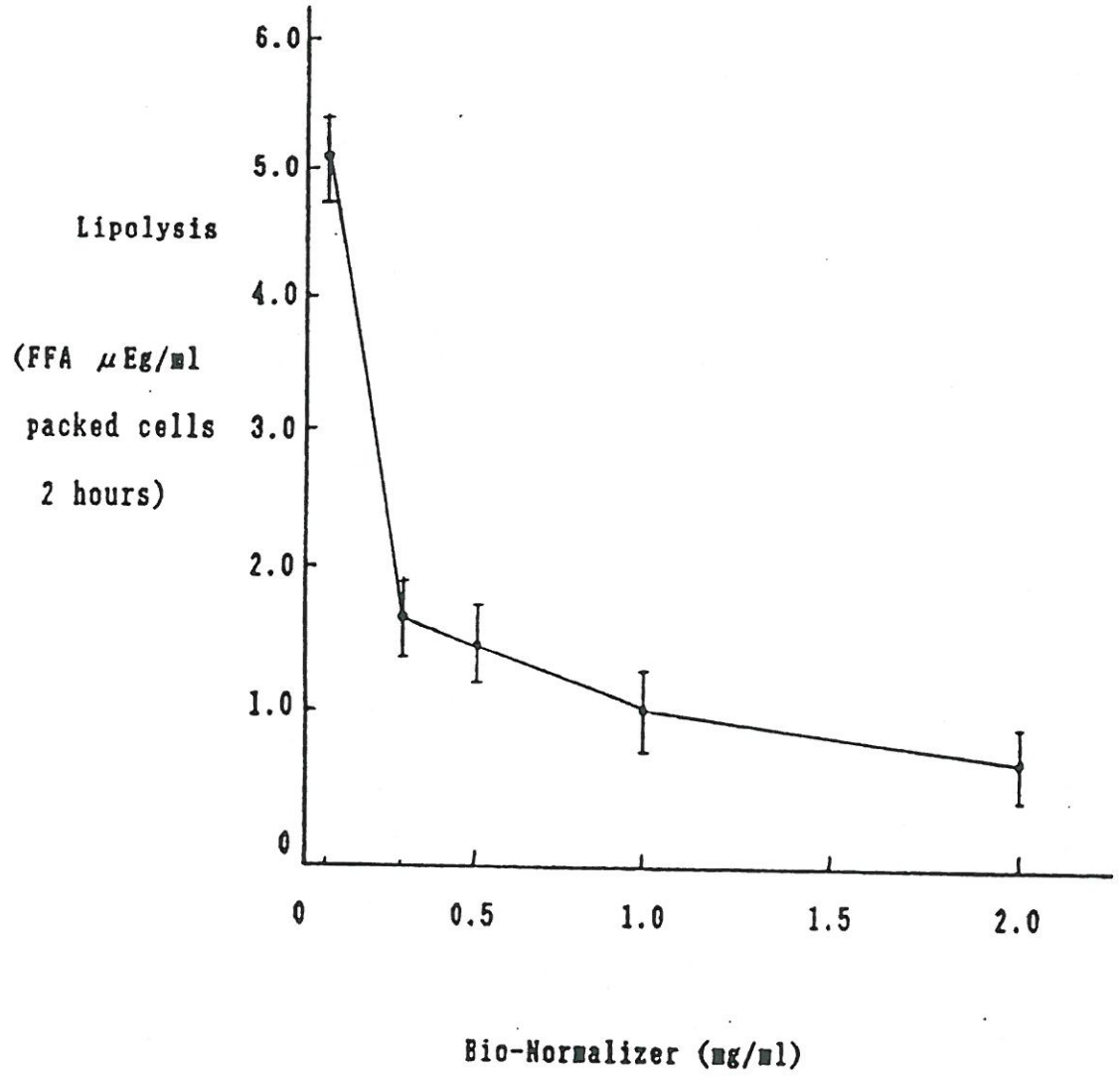

Table 1: Effect of Bio-Normalizer on Epinephrine(0.1 μg/ml)-induced lipolysis

Bio-Normalizer mg/ml	Lipolysis(FFA μEq/ml packed cells/h)	
	(-)Epinephrine	(+)Epinephrine
0	0.033±0.01	8.72±0.28
0.25	-	11.14±0.23
0.5	-	11.33±0.37
1.0	-	11.18±0.26
2.0	0±0	11.27±0.11

(n=3)

Table 2: Effect of Bio-Normalizer on Epinephrine(1.0 μg/ml)-induced lipolysis

Bio-Normalizer mg/ml	Lipolysis(FFA μEq/ml packed cells/h)	
	(-)Epinephrine	(+)Epinephrine
0	0.01±0.01	10.39±0.17
0.25	-	10.94±0.22
0.5	-	11.95±0.21
1.0	-	11.96±0.08
2.0	0±0	12.08±0.17

(n=3)