EFFECT OF RED GINSENG ON NATURAL KILLER CELL ACTIVITY IN MICE WITH LUNG ADENOMA INDUCED BY URETHAN AND BENZO(A)PYRENE

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ABSTRACT

It was previously reported that red ginseng extract inhibited carcinogenesis by urethan, DMBA and aflatoxin B_1 (Cancer Detection and Prevention, 6: 515-525, 1983). In an attempt to investigate the mechanism of the anticarcinogenic effect of ginseng, we assayed natural killer (N.K) activity in mice treated with urethan and benzo (a)pyrene.

In our experiment newly born Swiss Webster mice, less than 24 hrs. old, were given a single subcutaneous injection of 1mg of ure-than and 40μg of benzo(a)pyrene. The mice had been administered with ginseng since weaning, and sacrificed at various intervals. Major organs were examined both, with the naked eye and microscopically. N.K. activity of spleen cells was analyzed in a 12-hour ^{51}Cr release assay against YAC-1 cells.

Administration of ginseng resulted in an increase of N.K. activity by 18% at 4 weeks, 20% (P < 0.05) at 6, 29% (P < 0.05) at 12, and 13% at 24 following a single injection of urethan. At the same time, significantly lower incidences of lung adenoma were noted at 6 weeks (50%) and 12 weeks (27%) following the administration of ginseng to urethan-injected mice. This result indicates that the enhancement of N.K. activity by ginseng makes a contribution to its anticarcinogenic effect.

On the hand, N.K. activity was suppressed by benzo(a)pyrene during the time span of this experiment and it almost returned to the level of controls following the adminsitration of ginseng. However, the lung adenoma induced by benzo-(a)pyrene began to occur at 48 weeks in which N.K. activity had naturally declined to a very low level in all experimental mice, and administration of ginseng did not decrease the incidence. In explanation of this result, we might propose that the recovery of the N.K. activity by ginseng had little effect on the incidence of lung adenoma because of the long latent period of carcinogenesis by benzo(a)pyrene.

In conclusion, these results suggest that the anticarcinogenic effect of ginseng in urethan-treated mice may be related to the augmentation of N.K. activity.

INTRODUCTION

Ginseng is one of the most popular natural tonics used in oriental countries. Its diverse pharmacological effects on the human body have been the targets of research for many years, and a number of investigators continue to study these

effects from various approaches. Brekhman and Dardymov (1) suggested that the effect of ginseng might be due to its capacity to increase non-specific resistance of the organism.

The immune system may play an important role in the hostrelated aspect of resistance to carcinogenesis. In general, there have been numerous indications of increased oncogenesis in immunosuppressed experimental animals or patients. However, there have also been a number of objections raised against the theory of immune surveillance (2, 3). Most of the apparent contradictions to this theory actually have been related to the postulated central role of T-cells in the antitumor defense system (4). Therefore, increasing attention has been directed toward alternative host defense mechanism, particularly natural antitumor resistance by lymphocytes or macrophages (5-7). Natural Killer (N.K.) activity appears to be associated with a subpopulation of normal lymphocytes capable of spontaneously lysing certain tumor and normal cell targets (8). There is now considerable evidence supporting the role of N.K. cells in vivo resistance to tumor growth in mice (9-13), and it has been hypothesized that N.K. activity is a primary mechanism of immune surveillance (8, 14). Although N.K. activity has been studied extensively in humans and other animals, its regulation is poorly understood. Interferon and substances capable of inducing interferon enhance N.K. activity (15-17), while a variety of other immunopharmacological agents inhibit N.K. activity (18-21).

We previously reported that ginseng had anticarcinogenic effect on lung tumor induced by DMBA urethan, and aflatoxin B (22). Ginseng extract inhibited the incidence and also the proliferation of tumors induced by carcinogens when orally adminsitered In vivo (22). In addition, urethan and DMBA have been found to strongly depress N.K. activity during the latent period before tumor development (23, 40). In this regard, it was of interest to determine whether the anticarcinogenic effect of ginseng might be accompanied by resistance to the ability of the agent to depress N.K. activity. The study reported herein examines the relationship be-

tween the effect of ginseng on N.K. activity and anticarcinogenic resistance.

MATERIALS AND METHODS

Experimental Animals: Non-inbred Swiss Webster mice were obtained from NCI (National Cancer Institute, NIH, U.S.A.), and bred at random inter se. All mice were housed in a controlled room with food and water ad libitum. Food was given as solid pellets prescribed by NIH-7 open formula (28).

Ginseng Extract: Korean red ginseng extract powder, spray-dried, was obtained from Office of Monopoly, Seoul, Republic of Korea. It was dissolved in tap water at concentration of lmg/ml, and administered to mice ad libitum instead of water from weaning to sacrifice.

Chemical Carcinogens: Newly born mice, less than 24 hours old, were injected subcutaneously in the subscapular region with 0.02ml of the suspension, containing lmg of urethan (Fisher Scientific Co.) in 1% aqueous gelation (29,30). In the case of benzo(a)pyrene (Sigma Chemical Co.), mice were given a single injection with 40 μg in 0.02ml of 1% aqueous gelatin via the same route as for urethan (31). The suspension of benzo(a)pyrene was prepared by procedures in (29) and promptly used after preparation.

The weaning rates of mice injected with urethan and benzo(a)pyrene are summarized in Table 1. The weaning rates of all experimental groups were above 75% and almost equal.

Controls: There were three controls in this experiment as follows.
1) Untreated control mice were given solid pellets and tap water libitum.
2) Ginseng control mice were given solid pellets and the solution of ginseng extract.
3) Vehicle control mice were injected with 0.02ml of 1% aqueous gelatin (Difco Lab.) in subscapular region within 24 hrs. after birth.

Naked Eye and Microscopical Examination: Experimental mice were sacrificed at 4, 6, 12, 24, and 48 weeks after birth. Their various organs were examined; lung, heart, salivary gland, liver,

Table 1. Weaning rate in Swiss Webster mice treated with urethan and benzo(a)pyrene.

Treatment of mice	Dose and route	Vehicle	No. mice	No. Survivors at weaning	% Survivors at weaning
Untreated	———	———	1880	1509	80
1% Gelatin	0.02ml x 1,S.C.	H_2O	889	743	84
Urethan	1mg/0.02ml x 1,S.C.	1% gelatin	1883	1412	75
B(a)P	40μg/0.02ml x 1,S.C.	1% gelatin	1757	1463	83

thymus, pancreas, spleen, kidney, brain, pituitary gland, testis, and ovary. The organs and tumors were stained by hematoxylineosin for microscopical observation. Spleen was used for assay of N.K. activity.

Preparation of Effector Cells: At different times following carcinogen treatment, 30 mice from every experimental group were sacrificed, and 10 spleens were pooled in each petri-dish containing 20ml cold Hank's balanced salt solution (HBSS. Grand Island Biological Co.). After being washed twice with HBSS, spleen cell suspensions were prepared in cold HBSS by gentle teasing of the organ with forceps, and aspirating it through 10ml pipet. After allowing the tissue debris to sediment for 5 minutes at ice-bath, the cell suspensions in HBSS were layered on Ficoll-Hypaque solution (specific gravity 1.078) and centrifuged at 400 x G for 30 minutes at 18-20°C. The mononuclear cell band was harvested, and washed 3 times with HBSS. All cells were resuspended to desired concentration in complete medium. Complete medium is RPMI 1640 medium (Grand Island Biological Co.) supplemented with 10% heat-inactivated fetal bovine serum (Grand Island Biological Co.), 100 unit of penicillin, 100μg of streptomycin, and 2mM of fresh glutamine (32-34).

Preparation of Target Cells: YAC-1 cell line, a cell line of Moloney virus-induced lymphoma of A/Sn origin, was used as target cells (35). This cell line was obtained from the Albert Einstein Hospital, and maintained in complete medium. The target cells were labelled by incubating 1 x 10^7 cells in 1ml of medium with 100μ Ci of Na$_2$ ^{51}CrO$_4$ (Specific activity 283.58 mCi/mg, 1mCi/ml ; New England Nuclear) at 37°C water bath for

1hr. with occasional shaking. The labelled cells were washed 3 times with HBSS supplemented with 5% fetal bovine serum (5% FBS-HBSS), and adjusted to the desired concentration (2 x 10^5 cells/ml).

Assay for Natural Killer (N.K.) Activity: A 12-hr ^{51}Cr-release assay method was used for N.K. activity, as described by Herberman et al. (36). Desired concentrations of effector cells were mixed with labelled target cells in 1ml per culture tube (Falcon, 2058) in triplicate, and incubated for hrs. at 37°C. Most experiments were performed with E:T ratios of 100:1, 50:1, and 25:1 The tubes were centrifuged for 10 minutes at 500 x G at 4°C, and 500 μl of the supernatants were collected. Their radioactivities were measured in a well-type γ-counter (Aloka Universal Scaler, Japan).

Calculation of Natural Killer Activity: Spontaneous release (SR) was defined as the counts per minute (cpm) released from targets incubated with medium alone, and maximum release (MR) was determined as the cpm in the supernatants after lysis of the target with 1% Triton X-100. The formula used to calculate the % specific ^{51}Cr-release was;

$$\% \text{ Specific release} = \frac{\text{cpm Experimental - cpm SR}}{\text{cpm MR} - \text{cpm SR}} \times 100$$

Throughout the experiments, MR were higher than 95% of total isotope uptake, SR were less than 10%.

Statistical analysis of each experiment was done with the use of X^2-test and t-test for lung adenoma incidence and N.K. activity, respectively.

RESULTS

Several studies have demonstrated that ginseng inhibits carcinogenesis by various chemical carcinogens. In this regard, we assayed N.K. activity in mice treated with urethan and benzo-(a)pyrene in order to investigate the mechanism of anticarcinogenic effect of ginseng, and obtained the results as follows:

N.K. activity in the mice was highest at 6 weeks and completely depressed at 48 weeks of age. Its kinetics were similar in all control and experimental groups (Fig. 1 and Fig. 2). The incidence of tumors occurring in the mice has been carefully followed in controls during the time span of this experiment. Lung adenomas have been found in about 10% of control mice of more than 12 weeks of age but have not been seen prior to this time.

1. Enhancement of N.K. activity by ginseng and its correlation with lung adenoma incidence in mice injected with urethan.

Table 2 shows the N.K. activity of spleen cells of mice in each group at designated time points following urethan treatment. Profound and sustained suppression of N.K. activity was found in mice treated with urethan alone. At 12 weeks following its treatment, N.K. activity was decreased by half as compared with untreated control. Administration of ginseng to urethan-treated mice resulted in enhancement of N.K. activity from 4 to 24 weeks. N.K. activity showed an increase of 18% at 4 weeks, 20% (P < 0.05) at 4, 29% (P < 0.05) at 12 and 13% at 24 as compared with urethan treatment alone.

The incidence of lung adenoma was also examined at the same time points following urethan treatment, too (Table 3). In urethan-treated group, the lung adenoma was induced in all mice at 12 weeks following urethan teatment. In contrast, it was not until 24 weeks following urethan teatment. In contrast, it was not until 24 weeks following urethan treatment that lung adenoma was induced in almost all the mice in the ginseng combined with urethan-treated group.
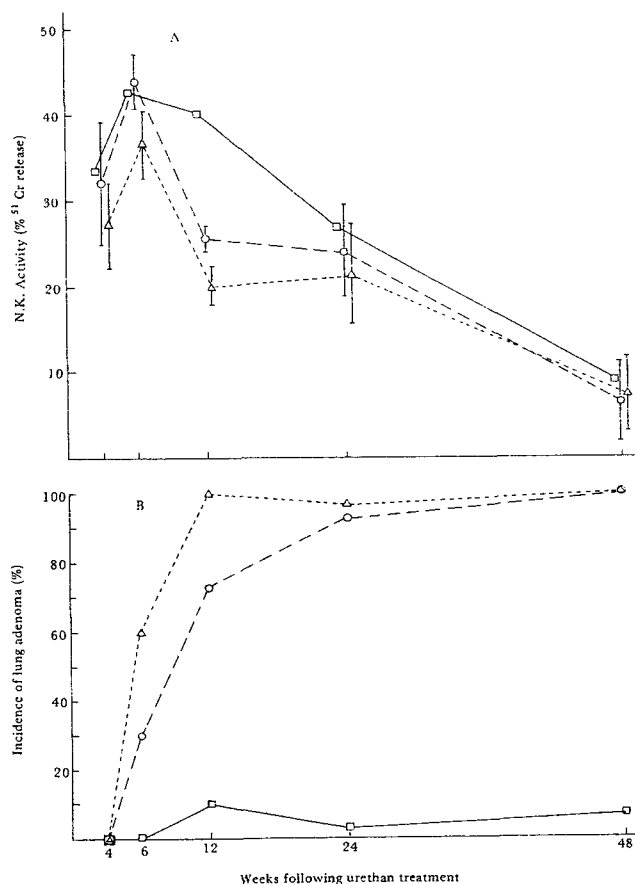

Fig. 1. Enhancement of N.K. activity by ginseng and its correlation with lung adenoma incidence in mice injected with urethan.
Panal A and B represent the effect of ginseng on N.K. activity of spleen cells and incidence of lung adenoma in mice injected with urethan, respectively. Squares designate untreated control mice, triangles show urethan-injected mice, and circles represent ginseng combined with urethan-injected mice.

The incidence of lung adenoma in the ginseng combined with urethantreated group showed a significant decrease of 50% (P < 0.05) and 27% (P < 0.05) at 6 and 12 weeks respectively, following urethan treatment as compared with that of the urethan-treated group. Thus it appeared that administration of ginseng caused the late occurrence of lung adenoma in urethan-treated mice.

2. Effect of ginseng on N.K. activity and lung adenoma incidence in mice treated with benzo(a)pyrene.

This experiment was performed to investigate the effect of ginseng on N.K. activity and the

incidence of lung adenoma in mice which were previously treated with benzo(a)pyrene. N.K. activity and incidence of lung adenoma in each group were monitored at designated time points following benzo(a)pyrene treatment. The results were summarized in Table 4 and Table 5.
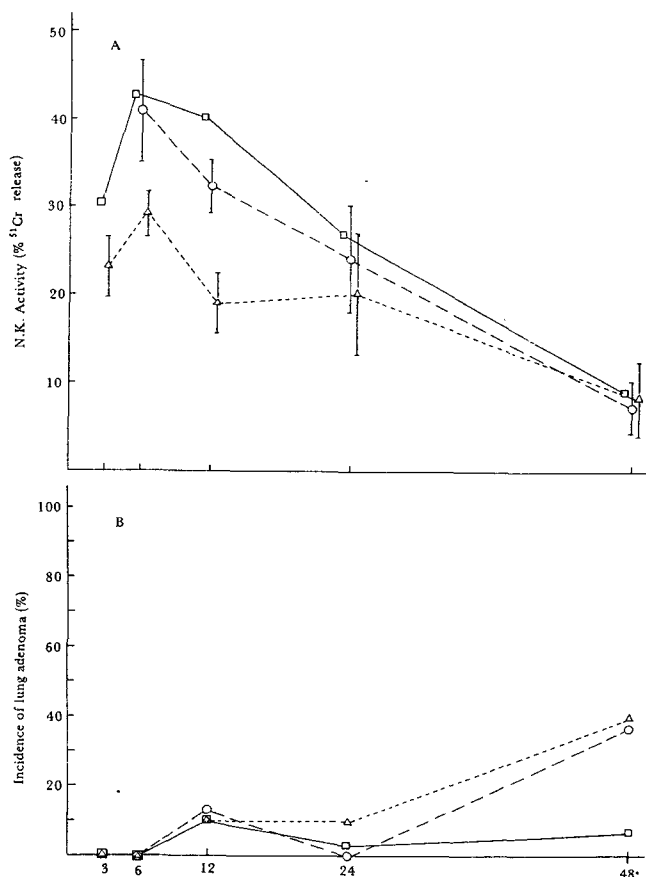

Fig. 2. Time course of the effect of ginseng on N.K. activity and lung adenoma incidence in mice injected with benzo(a)pyrene.
Panal A and B represent the effect of ginseng on N.K. activity of spleen cells and incidence of lung adenoma in mice inejcted with benzo(a)-pyrene, respectively. Squares designate untreated control mice, triangles show benzo(a)pyrene-injected mice, and circles represent ginseng combined with benzo(a)pyrene-injected mice.

There was a profound reduction of N.K. activity in benzo(a)pyrene-treated mice as compared with that of untreated mice from 3 to 24 weeks following its treatment. Administration of ginseng to benzo(a)pyrene-treated mice resulted in an increase of N.K. activity by 39% (P < 0.05) at 6 weeks, 69% (P < 0.05) at 12 and 20% at 24

as compared with benzo(a)pyrene treatment alone.

The lung adenoma incidence was less than 10% both in benzo(a)pyrene and ginseng combined with benzo(a)pyrene group by 24 weeks following benzo(a)pyrene treatment, and the incidence was similar to the spontaneous in control mice. The lung adenoma induced by benzo(a)pyrene began to occur at 48 weeks in which N.K. activity in all experimental mice had naturally declined to very low level. The incidence of lung adenoma at 48 weeks was 40% and 37% in benzo(a)pyrene and ginseng combined with benzo(a)pyrene group, respectively.

DISCUSSION

The results of the present study indicate for the first time that the long-term oral administration of red ginseng extract augments N.K. activity and, furthermore, that the augmentation correlates with its anticarcinogenic effect in urethan-treated mice, As already shown in other reports (24,40), suppression of N.K. activity by urethan was also detected in this mouse (Fig. 1). At 4 weeks following urethan treatment, N.K. activity decreased by 18% and returned to the level of the untreated control group by red ginseng oral administration, in which lung adenoma was not induced yet. Administration of ginseng to urethan-treated mice resulted in a significant increase of N.K. activity as well as a significant inhibition of incidence of lung adenoma at 6 and 12 weeks following urethan treatment (Table 2 & Table 3). These results indicate a positive correlation between augmentation of N.K. activity and inhibition of urethan-induced lung carcinogensis by ginseng and support the hypothesis that N.K. cells play a role in resistance to urethan-induced lung carcinogenesis (24).

On the other hand, this experimental result that the administration of ginseng augmented N.K. activity but did not decrease the incidence of lung adenoma in benzo(a)pyrene-treated mice might be due to the long latent period of carcinogenesis by benzo(a)pyrene. As shown in

31

Table 2. Effect of ginseng on the N.K. activity of spleen cells in mice treated with urethan.

Treatment of mice	No. of mice	N.K activity[a] following treatment(%)				
		3 weeks	6 weeks	12 weeks	24 weeks	48 weeks
Untreated	50	33.6 ± 3.8	42.6 ± 0.8	40.1 ± 3.0	27.0 ± 5.7	9.1 ± 5.0
1% Gelatin	30	not - tested	40.1 ± 1.0	35.7 ± 5.5	26.9 ± 8.3	7.8 ± 5.0
Ginseng	30	not - tested	44.5 ± 0.2	41.3 ± 4.6	28.4 ± 6.7	11.2 ± 7.8
Urethan	30	27.3 ± 5.0	36.6 ± 4.0	20.1 ± 2.1[c]	21.6 ± 5.8	7.5 ± 4.5
Urethan + Ginseng	30	32.1 ± 7.2	43.9 ± 3 1[b]	25.8 ± 1.3[d]	24.3 ± 5.9	6.8 ± 5.0

a : Mean ± S.D. of three pools. 10 spleens of mice were pooled and tested three times. E:T ratio = 25:1, similar results were obtained with other effector cell: target cell ratio.
b, d : Significantly different (P < 0.05, t - test) from urethan-treated group.
c : Significantly different (P < 0.05, t - test) from untreated group.

Table 3. Effect of ginseng on the incidence of lung adenoma in mice treated with urethan.

Treatment of mice	No. of mice	lung adenoma incidence following treatment(%)				
		4 weeks	6 weeks	12 weeks	24 weeks	48 weeks
Untreated	30	0/30(0)	0/30(0)	3/30(10)	1/30(3)	2/30(7)
1% Gelatin	30	not-tested	0/30(0)	3/30(10)	0/30(0)	1/30(3)
Ginseng	30	not-tested	0/30(0)	1/30(3)	0/30(0)	3/30(10)
Urethan	30	0/30(0)	18/30(60)	30/30(100)	(29/30(97)	30/30(100)
Urethan+ Ginseng	30	0/30(0)	9/30(30)[a]	22/30(73)[b]	28/30(93)	30/30(100)

a, b ; Significantly different (P < 0.05, X^2-test) from urethan-treated group.

Table 4. Effect of ginseng on the N.K. activity of spleen cells in mice treated with benzo(a)pyrene.

Treatment of mice	No. of mice	N.K. activity[a] following treatment				
		3 weeks	6 weeks	12 weeks	24 weeks	48 weeks
Untreated	30	30.4 ± 10.5	42.6 ± 0.8	40.1 ± 3.0	27.0 ± 5.7	9.1 ± 5.0
1% Gelatin	30	32.9 ± 6.0	40.1 ± 1.0	35.7 ± 5.5	26.9 ± 8.3	7.8 ± 5.0
Ginseng	30	not-tested	44.5 ± 0.2	41.3 ± 4.6	28.4 ± 6.7	11.2 ± 7.8
B(a)P	30	23.2 ± 3.4	29.3 ± 2.4[b]	19.1 ± 3.4[d]	20.3 ± 6.9	8.3 ± 4.3
B(a)P + Ginseng	30	not-tested	40.9 ± 5.8[c]	32.4 ± 3.0[e]	24.2 ± 6.1	7.4 ± 3.0

a : Mean ± S.D. of three pools. 10 spleens of mice were pooled and tested three times; E:T ratio = 25:1. Similar results were obtained with other effector cell: target cell ratio.
b, d : Significantly different (P < 0.05, t - test) from untreated group.
c, e : Significantly different (P < 0.05, t - test) from B(a) P treated group.

Table 5. Effect of ginseng on the incidence of lung adenoma in mice treated with benzo(a)pyrene.

Treatment of mice	No. of mice	lung adonoma following treatment(%)				
		3 weeks	6 weeks	12 weeks	24 weeks	48 weeks
Untreated	30	0/30(0)	0/30(0)	3/30(10)	1/30(3)	2/30(7)
1% Gelatin	30	0/30(0)	0/30(0)	3/30(10)	0/30(0)	1/30(3)
Ginseng	30	not-tested	0/30(0)	1/30(3)	0/30(0)	3/30(10)
B(a)P	30	0/30(0)	0/30(0)	3/30(10)	3/30(10)	12/30(40)[a]
B(a)P + Ginseng	30	not-tested	0/30(0)	4/30(13)	0/30(0)	11/30(37)

a ; Significantly different $(P < 0.05, X^2$-test) from untreated group.

Fig. 2, benzo(a)pyrene significantly decreased N.K. activity from 6 to 24 weeks following its treatment as compared with the notreatment. The decreased N.K. activity was almost recovered by ginseng administration. The N.K. activity in the mice was highest from 6 to 12 weeks after birth and, furthermore, the administration of ginseng most markedly enhanced the N.K. activity at that time in benzo(a)pyrene-treated mice. However, the lung adenoma induced by benzo(a)pyrene began to occur at 48 weeks after birth when the N.K. activity had naturally declined in all experimental mice, to a level too low to be affected by ginseng. Benzo(a)pyrene is found in tobacco smoke and the ruban atmosphere (41-43). The increasing sociological impact of environmental carcinogens is most clearly revealed in international epidemiological studies. It appears that the majority of cancers are initiated by external agents. Therefore, the enhancing effect of ginseng on the N. K. activity suppressed by benzo(a)pyrene might give some practical benefits for prevention of carcinogenesis by other agents.

In conclusion, the results support the idea that the anticarcinogenic effect of ginseng in mice treated with urethan may be related, at least in part, to its ability to enhance the N.K. activity of the host.

Rueckert: Do you know the specification of the extract you used, such as the content of ginsenoside?

Yun: I used the ginseng extract which was provided by the Office of Monopoly. It was water extract, so it may contain ginsenoside and other water-extractable material.

Rueckert: Do you know the content of ginsenoside?

Yun: I don't know the ginsenoside content. It was the crude total-water extract of ginseng.

Rueckert: But, was the content of ginsenoside standardized and is there any information?

Yun: No, it was not standardized on the level of ginsenoside.

Rueckert: Did you use it by injection or oral administration?

Yun: We orally administered the ginseng extract dissolved in water.

홍삼이 Urethan 및 Benzo(a)pyrene에 의하여 폐선종이 유발된 마우스에서 Natural Killer 세포활성도에 미치는 영향

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홍삼의 항발암작용 기전을 규명하기 위한 목적의

일환으로 홍삼추출물이 urethan 및 benzo(a)pyrene 을 투여한 마우스에서 natural killer(N.K) 세포활성 도 및 폐선종 발생에 미치는 영향을 발암물질 처리 후 48주동안 검색하였다.

N.K 세포 활성도는 urethan 및 benzo(a)pyrene 의 처리에 의하여 현저히 저하되었다. 이와 같은 N.K 세포활성도의 저하는 상기 발암물질 투여후 4주부터 나타나 24주까지 계속되었으며 홍삼투여에 의하여 정 상대조군의 수준으로 되돌아 왔다.

동시에 urethan에 의해 6주시부터 유발되기 시작한 폐선종 역시 홍삼투여에 의해 그 발생빈도가 현저히 억제되었다. 반면 benzo(a)pyrene에 의해 유발된 폐 선종은 48주시에 나타나기 시작하였는데 이 시기는 N.K 세포 활성도가 자연적으로 너무 낮은 수준으로 떨어져 있어 홍삼의 영향을 받을 수 없는 시기였으며 홍삼투여에 의해 폐선종의 발생빈도 역시 억제되지 않았다.

결론적으로 저자들은 본 연구를 통하여 홍삼에 의 한 항발암 효과는 N.K 세포활성도의 증대와 관련되 어 있음을 알 수 있었다.

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