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Original Article

Antitumor Effects of Water Extracts of *Panax notoginseng* on NCI-H460 Tumor Regression Model

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Objective: This study aimed to investigate the antitumor effects of water extracts of Panax notoginseng (WEPN) in NCI-H460 human lung cancer cell xenografted nude mice.

Materials and Methods: We cultured NCI-H460 cell lines and xenografted them to nude mice. The mice were divided into 3 groups; positive control group, NCI-H460+150 ㎎/㎏ WEPN-treated group, and NCI-H460+300 ㎎/㎏ WEPN-treated group. They had been raised and treated in 28 days. We checked their body weight and tumor weight and volumes twice a week and their absolute organ weight and microhistological observation at the final day. We also calculated their tumor inhibition rate (I.R.), mean survival time and percent increase in life span (% ILS).

Results: Body weight of WEPN (300 ㎎/㎏) treated mice tended to slightly greater increase than those of the positive control group, but had no significance. Tumor volume (measurement with a caliper) of WEPN-treated mice tended to be lower than that of the positive control group. Inhibition rate (I.R.) of the WEPN group decreased more than the positive control group, but had no significance. Results of tumor weights and volume (plethysmography) had no significance. Mean survival time and percent increase in life span (% ILS) in the WEPN 300 ㎎/㎏ treatment group were higher than those of any other group ($p<0.05$). In absolute organ weights, the WEPN (150-300 ㎎/㎏) treatment group decreased liver weights ($p<0.05$). Liver tissue of mice treated with WEPN (300 ㎎/㎏) did not show any specific lesions.

Conclusion: We suggest that WEPN may have potential as a growth inhibitor of solid tumors induced by NCI-H460 without any side effects. However, this study has limitations in proving anti-tumor effects of WEPN, so further studies to overcome those limitations will be needed.

Key Words : *Panax notoginseng*, NCI-H460, lung cancer, antitumor, *in vivo*

Introduction

Ginseng is a widely-consumed medicinal herb worldwide. Over thirteen different species of ginseng have been identified[1-2]. *Panax notoginseng* Burk. F.H. Chen (Araliaceae) (*Sanqi* or *Tianqi* in Chinese) is a highly valuable and important herb in oriental traditional medicine for its therapeutic abilities to stop hemorrhaging and to influence blood circulation. It is also the most common drug used to treat chronic liver disease in Korea[2-7].

The main root of *Panax notoginseng*, called *notoginseng*, is known to have many pharmacological activities such as anti-inflammatory and anti-tumor effects, and the functional balance of the immune system[8-9]. Recently, many studies have indicated that *Panax notoginseng* extracts inhibit the growth and metastasis of various cancer cells *in vitro* and *in vivo*. The inhibitory effects of *Panax notoginseng* extracts are associated with cell cycle arrest and the

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stimulation of apoptotic cell death[9-16]. However, the precise mechanisms of apoptotic cell death are largely unknown in human cancer cells.

The present study attempts to evaluated the anti-tumor activity of water extracts of *Panax notoginseng* (WEPN) for NCI-H460 human lung cancer cells (NCI-H460 cells) with *in vivo* nude mouse xenograft model.

Materials and Methods

1. Plant material, cell line and culture conditions

Panax notoginseng was supplied by Daejeon University Oriental Hospital (Daejeon, Korea). WEPN was prepared in the following manner: Distilled water at 90°C was added to dry root (5 ㎖) and the temperature was maintained for 10h. The mixture was allowed to cool at room temperature, and was then filtered with Whatman filter paper (pore size; 11㎛) and lyophilized. The yield of the lyophilized residue corresponded to 4.5% (wt/wt) of the original dry root weight and the extracts powder was dissolved directly in distilled water.

NCI-H460 cells were from the Korean Cell Line Bank (Seoul, Korea). The cells were grown in the RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 100 U/㎖ penicillin and 100 mg/㎖ streptomycin[17].

2. Experimental animal

Balb/c nude mice (male, 9~11 weeks n=21) weighing 21-25 g were purchased from Japan SLC, Inc (SLC Inc., Shizuoka, Japan) and were housed under specific pathogen-free conditions according to the guidelines of the Chungbuk National University Animal Care and Use Committee.

The animal room was controlled for constant temperature (22±2℃), humidity (50±10%) and periodic light (12h light/dark cycle). All laboratory feed pellets and beddings were autoclaved.

3. Experimental design

The tumor regression model on nude mouse has been successfully applied to evaluate common anti-tumor activity, so this model was used to evaluate the suppression of WEPN on solid tumors. When the tumor volume reached 100 mm^3, the nude mice xenografted with tumor fragments were randomly distributed into three groups containing seven mice each: positive control group (administered only distilled water), NCI-H460+150 ㎎/㎏ WEPN-treated group, and NCI-H460+300 ㎎/㎏ WEPN-treated group. WEPN was orally administrated every day for four weeks.

4. Cell preparation

NCI-H460 cells were cultured in 260 ㎖ tissue culture flasks in Eagle's minimum essential medium (EMEM) containing 100 U/㎖ penicillin and 10% (vol/vol) heat-inactivated fetal calf serum in an incubator with 95% (vol/vol) air and 5% (vol/vol) CO_2 at 37℃. When the cells became confluent, they were washed twice with Hank's balanced salt solution (HBSS), trypsinized with 0.25% (wt/vol) trypsin in HBSS and washed twice with fresh culture medium[18].

5. Xenografts

NCI-H460 cells corresponding to 1 x 106 cells/mouse in 0.1 ㎖ HBSS were injected subcutaneously into the flank of mice using a 26-gauge needle. After 14-16 days observation, apparent solid tumor mass was removed from 3 out of 5 mice inoculated with NCI-H460 cells. Tumor fragments (3×3×3 mm) were made by trimming with a knife and xenografted into the flanks of new mice using a trocar.

The suppressive effect of anticancer agents on solid tumors was evaluated in a tumor-regression model. In brief, from the day tumor volume reached

100 mm^3, WEPN (water or 150 ㎎/㎏ or 300 ㎎/㎏) were administrated orally to the mice xenografted with tumor fragments, every day, for 28 days.

6. Changes in tumor volume

The changes in the size of tumor mass were recorded twice a week by measuring with a digital caliper. That is, the largest and smallest diameters were measured in each mouse and the tumor volume was estimated according to the following formula[17].

V (mean tumor volume) = $(A*B^2)/2$,

where V is the tumor volume in mm^3, and A and B are the largest and smallest tumor diameters in mm, respectively. Based on the regression of tumor volume, the antitumor activities of treatment were expressed by inhibition rate.

I.R. (Inhibition Rate)(%) = [(CV-TV)/TV] × 100,

where CV and TV are tumor volumes in positive control and treatment groups, respectively. In addition, the tumor weights were measured at the final day after sacrifice of animals and removal of the tumor mass.

7. Mean survival time and percent increase in life span

To evaluate the life span of mice xenografted with NCI-H460 tumor fragments, survival time was estimated at the day when the tumor volume reached 1,000 mm^3 as described previously[19-20] and % increase in life span (%ILS) was calculated according to the equation:

%ILS (increase in life span) = [(T-C)/C] × 100,

where C and T are mean survival days of mice in positive control and treatment groups, respectively[19-20].

8. Statistical analysis

The significance of difference between the means of positive control and treatment groups was analyzed using one-way analysis of variance (ANOVA) followed by a Dunnett's t-test correction, paired t-test and linear regression analysis. Statistical significance was determined at the level of $p<0.05$ or $p<0.01$.

Results

1. Changes in body weights

Changes in body weights of each group are shown

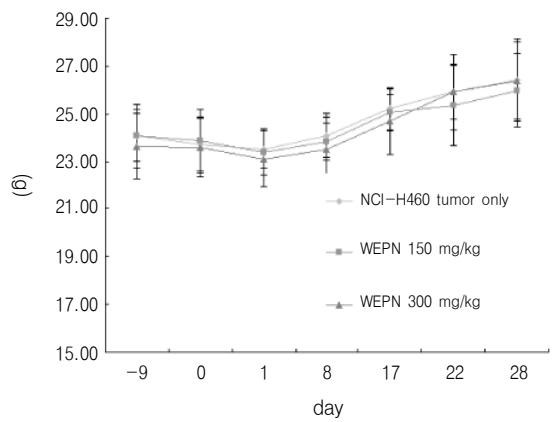

Fig. 1. Changes in body weights in nude mice bearing NCI-H460 cell solid tumor.

After nude mice with NCI-H460 cell-transplanted tumors were treated with a daily dose of WEPN (150-300 ㎎/㎏) for 28 days. The body weights of nude mice in the positive control, NCI-H460 cell alone (◆, n=7), WEPN 150 ㎎/㎏ (■, n=7), and WEPN 300 ㎎/㎏ (▲ n=7) were measured twice a week.

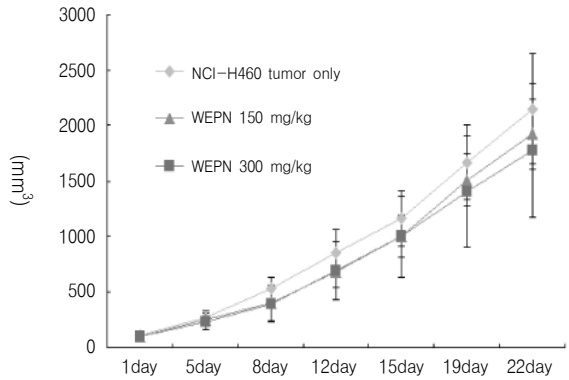

Fig. 2. Time-course of increase in tumor volumes in NCI-H460 cells-bearing mice treated with WEPN.

Mice xenografted with tumor fragments were treated with anticancer agents at the day when tumor mass reached 100 mm^3. The lengths and widths of solid tumor in the positive control, NCI-H460 cell alone (◆, n=7), WEPN 150 ㎎/㎏ (■, n=7) and WEPN 300 ㎎/㎏ (▲ n=7) groups were measured twice a week and tumor volumes were evaluated.

in Fig. 1. The mean body weights of the WEPN treatment mice showed a value of 23.70-25.47 g in WEPN (150 ㎎/㎏) treated and 23.76-26.79 g in WEPN (300 ㎎/㎏) treated groups, of which the body weights in WEPN-treated group (300 ㎎/㎏) were slightly more increased than those of positive control group (24.36-26.45 g). However, there was not obse-rved statistical significance between WEPN-treated groups and the positive control group.

2. Changes in tumor volume (measurement with a calipers)

Treatment with WEPN (150-300 ㎎/㎏) inhibited the growth of NCI-H460 cell-transplanted tumors compared to the values of the positive control group (Fig. 2). At day 22, the mean tumor volumes of the WEPN (300 ㎎/㎏) treated group were lower than those of the positive control group, with value of 2150.81 mm^3-1782.09 mm^3.

3. Inhibition rate (I.R.) on tumor volume

I.R. (%) data of each group are shown in Table 1. From the day 8 to 22, I.R. (%) showed slow decrease tendency in a dose-dependent manner (WEPN 150 ㎎/㎏ I.R. 87.28-83.78% < WEPN 300 ㎎/㎏ I.R. 84.42-82.86%). Compared to the positive control group, the I.R. of WEPN treatment groups decreased, but no significant differences were observed (Table 1).

4. Tumor weights and volume (plethysmography)

Final tumor weights and volumes of each group

Table 1. Inhibition rate (I.R.) on tumor volumes of NCI-H460 tumor-bearing mice (%)

	1 day	5 day	8 day	12 day	15 day	19 day	22 day
NCI-H460 tumor only	100	100	100	100	100	100	100
WEPN 150 ㎎/㎏	95.14	104.47	87.28	87.49	88.54	87.54	83.78
WEPN 300 ㎎/㎏	94.38	93.76	83.42	77.28	84.51	83.45	82.86

Table 2. Tumor weights and volume in mice xenografted with NCI-H460 cells on the final day

	NCI-H460 tumor only	WEPN 150 ㎎/㎏	WEPN 300 ㎎/㎏
Tumor weight (g)	3.05 ± 0.79	2.58 ±0.57	2.79 ± 0.70
Tumor volume (cm^3)	3.38 ± 1.18	3.30 ± 0.92	3.75 ± 0.99

Table 3. Percent increase in life span (%ILS) of NCI-H460 tumor-bearing mice

Treatment	Mean survival time (day)	% ILS
NCI-H460 tumor only	18.43±2.23	0
WEPN 150 ㎎/㎏	19.29±2.56	4.66
WEPN 300 ㎎/㎏	22.00±3.51*	19.37

Significant difference from positive control (NCI-H460 cell only) group at $p<0.05$.

are shown Table 2. Tumor weight and volume of the positive control group (NCI-H460 only) was 3.05 ± 0.79 g and 3.38 ± 1.18 cm^3 at the final day 22. Tumor weight and volume of the WEPN (150 ㎎/㎏) group were 2.58 ± 0.57 g and 3.30 ± 0.92 cm^3, respectively. Tumor weight and volume of the WEPN (300 ㎎/㎏) treatment group were 2.79 ± 0.70 g and 3.75 ± 0.99 cm^3, respectively. Compared to the positive control group, tumor weights of the WEPN treatment groups decreased, but no significant differences were observed (Table 2).

5. Mean survival time and percent increase of life span (% ILS)

Mean survival time and percent increase in life span are shown in Table 3. The positive control group (NCI-H460 only) survived for 18.43±2.23 days. Mean survival time and percent increase of life span in the low WEPN treatment group (150 ㎎/㎏) was extended to 19.29±2.56 days and 4.66 %ILS, respectively. The mean survival time and percent increase of life span in the high WEPN treatment group (300 ㎎/㎏) was 22.00±3.51 days and 19.37 %ILS, respectively. %ILS increased in a dose-dependent manner. In particular, the high WEPN treatment group (300 ㎎/㎏) showed statistically significant difference compared to the positive control (NCI-H460 cell alone) group.

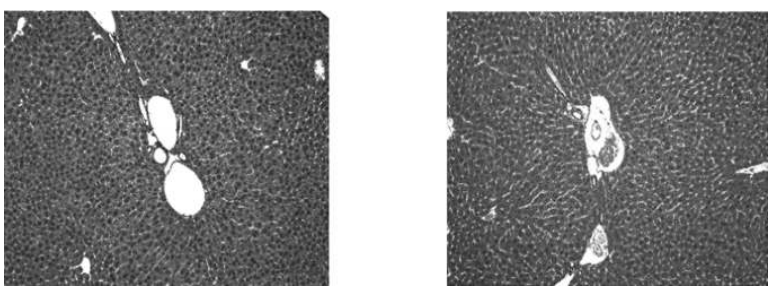

Fig. 3. Light microscopic picture of liver of NCI-H460 cells-bearing mice with positive control, NCI-H460 cell only (A) and WEPN 300 ㎎/㎏ (B).

Light microscopic histopathological examination of liver tissue of mice treated with WEPN did not show any specific lesions compared to positive control.

Table 4. Organ weights at the final day of NCI-H460 tumor-bearing mice

Group		body weight (g)	Absolute organ weights			
			epididymis (g)		kidney (g)	
			L	R	L	R
NCI-H460 tumor only	M	26.429	0.036	0.033	0.268	0.266
	SD	1.149	0.006	0.005	0.034	0.035
WEPN 150 mg/kg	M	25.934	0.047	0.039	0.251	0.248
	SD	1.276	0.025	0.007	0.025	0.030
WEPN 300 mg/kg	M	26.478	0.043	0.074	0.249	0.255
	SD	1.579	0.006	0.090	0.020	0.018

Group		Absolute organ weights					
		liver (g)	spleen (g)	heart (g)	lung (g)	testis (g)	
						L	R
NCI-H460 tumor only	M	1.869	0.455	0.158	0.218	0.091	0.091
	SD	0.169	0.154	0.017	0.041	0.006	0.005
WEPN 150 mg/kg	M	1.689	0.461	0.171	0.228	0.094	0.092
	SD	0.120	0.092	0.024	0.017	0.017	0.018
WEPN 300 mg/kg	M	1.749	0.451	0.169	0.233	0.094	0.096
	SD	0.131	0.088	0.024	0.026	0.010	0.011

Group		Weight (g)	Relative organ weights			
			epididymis (g)		kidney (g)	
			L	R	L	R
NCI-H460 tumor only	M	26.429	0.131	0.120	0.991	1.027
	SD	1.712	0.022	0.020	0.105	0.123
WEPN 150 mg/kg	M	25.934	0.176	0.146	0.976	0.937
	SD	1.444	0.088	0.021	0.074	0.068
WEPN 300 mg/kg	M	26.478	0.159	0.246	0.957	0.980
	SD	1.540	0.023	0.281	0.085	0.078

Group		Relative organ weights					
		liver (g)	spleen (g)	heart (g)	lung (g)	testis (g)	
						L	R
NCI-H460 tumor only	M	7.140	1.655	0.586	0.762	0.326	0.327
	SD	0.455	0.688	0.051	0.116	0.029	0.033
WEPN 150 mg/kg	M	6.697*	1.773	0.678	0.872	0.356	0.309
	SD	0.291	0.294	0.065	0.089	0.042	0.124
WEPN 300 mg/kg	M	6.609*	1.676	0.663	0.877	0.361	0.350
	SD	0.254	0.300	0.067	0.085	0.041	0.049

Data were presented as average ± standard deviation from triplicate experiment. * $p<0.05$

6. Histopathological observation by light microscopy

Absolute and relative organ weights of kidneys, liver, spleen, heart and lungs are shown in Table 4. In particular, WEPN (150-300 ㎎/㎏) treatment groups markedly decreased relative liver weights with statistical significance ($p<0.05$). However, light microscopic histopathological examination showed that liver tissue of mice treated with WEPN (300 ㎎/㎏) did not show any specific lesions compared to liver tissue of NCI-H460 cells-bearing mice with positive control (Fig. 3).

Discussion

Recent studies have reported that treatment with extracts of *Panax notoginseng* can cause the accumulation of cells in G1 or S phase of cell cycle and apoptosis[11,13-14]. These suggest that the inhibitory effects of the extracts on the growth occur through the blockage of G1 or S phase and that these cells do not enter the G_2 phase. While some cell killing mechanism of these extracts has been suggested [10-11,14-15,21], little is known about their effects on the growth of human lung cancer cells.

This study was focused to elucidate the mechanism related to cell death by determining whether WEPN treatment inhibits the growth of NCI-H460 cell-transplanted tumors *in vivo*. Human tumor xenografts in immunodeficient animal models provide a means to evaluate potential anti-tumor drugs in preclinical studies and are applicable for studying many different types of human malignancies[22].

Thus, we also conducted experiments on nude mice with NCI-H460 cell-transplanted tumors over 28 days under the treatment of WEPN (150-300 ㎎/㎏). For example, the time-dependent changes of tumor volume were measured by a digital caliper, and the volumes of removed tumors were measured by a plethysmometer on the final day. All volumes of applied cell-transplanted tumors were suppressed by increasing doses, however, no group showed any statistically significant results.

The results showed that WEPN inhibited dosage-dependently the growth of NCI-H460 cell-transplanted tumors compared to the positive control. We selected the results of day 22 for calculation since the death rate exceeded 50% at day 25. At the day 22, numerical values of solid tumor showed 1782.09 mm^3 (300 ㎎/㎏ WEPN), 1801.87 mm^3 (150 ㎎/㎏ WEPN) and 2150.81 mm^3 (positive control group), respectively (Fig. 2). Compared to the positive control, the experimental groups showed tumor growth inhibition from day 8 to 22 (WEPN 150 ㎎/㎏ I.R. 88.54-83.78% < WEPN 300 ㎎/㎏ I.R. 84.51±77.28%) in a dose-dependent manner. However, the difference wasn't significant (Table 1). At the final day, tumor weights showed 2.793±0.700 g (300 ㎎/㎏ WEPN), 2.577±0.567 g (150 ㎎/㎏ WEPN) and 3.046±0.793 g (positive control group) (Table 2).

Mean survival time and the rate of increasing life span of the low dosage WEPN (150 ㎎/㎏) treatment group was 19.29±2.56 days and 4.66%ILS, respectively. The high dosage WEPN (300 ㎎/㎏) treatment group showed 22.00±3.51 days and 19.37%ILS ($p<0.05$, compared with positive control group). The positive control group (NCI-H460 tumor alone) survived only for 18.4±32.23 days (Table 3). Thus the high WEPN treatment group (300 ㎎/㎏ WEPN) showed significant difference from the positive control ($p<0.05$).

In relative organ weights, the WEPN (150-300 ㎎/㎏) treatment groups decreased markedly in liver weights ($p<0.05$). However, light microscopic histopathological examination of the liver tissue of mice treated with WEPN (300 ㎎/㎏) did not show any specific lesions compared to that of the NCI-H460 cells-bearing mice as positive control (Table 4, Fig. 3). The study results showed that WEPN may have potential as a growth inhibitor of solid tumors induced by NCI-H460 without any side effects.

In summary, WEPN treatment increased survival time and decreased tumor volume in mice. But this study has limitations as following to prove anti-tumor effects of WEPN. First, the scale of this study is too small. Second, the results of this study about tumor volume and weight showed no significance. Further studies to overcome those limitations will be needed.

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