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The Anticancer Effect of *Inonotus obliquus* Pilat (Chaga) Processed by Nanomill Technology *In vivo*

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나노밀 가공된 차가버섯의 항암효과

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ABSTRACT

Extracts and fractions of *Inonotus obliquus* (Chaga in Russia) have been known to have various biological activities, including antimutagenic, anticancer, antioxidative, and immunostimulating effects. This study was performed to confirm anticancer effect of 10% superfine Chaga mushroom processed by nano-mill technology on C57BL/6 mice. Chaga particles belonged in the size of 1 μm was about 40% after nanomill processing according to the volume distribution. As the result of subcutaneous injection of B16BL6 melanoma cells to the mice, the tumor volume ($p < 0.001$) and tumor weight ($p < 0.01$) was significantly decreased in the experimental (NCh) group as compared with control (C) group and the tumor growth inhibitory rate was 29.2%. On examination of survival rate after intraperitoneal injection of B16BL6 melanoma cells, the mean survival time per a mouse was 17.7 and 26.0 days in C and NCh group respectively. The survival rate of NCh group was 40% when that of C group was 0% at the 35th day. On the result of examination to confirm histological toxicity by Chaga superfine particles, both groups did not show any morphological and pathological changes in the small and large intestine under the light microscope. These results suggest that feeding of superfine Chaga produced by nanomill technique has a tumor growth inhibitory effect *in vivo.*

Keywords : *Inonotus obliquus*, Superfine particle, Nanomill, Anticancer effect

INTRODUCTION

Medicinal plants having anticancer effect are found all over the world. The study on the cancer treatment and prevention are on going in these medicinal plants due to their outstanding anticancer effect with minimal toxicity and side

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effect. Among them is the *Inonotus obliquus* (persoon) Pilat (in Russia it's called Chage, in Japan it's called kabanoa-natake or Chaga). During 16th century in Russia and in the western part of Siberia the decoction of Chaga mushroom (Chaga) were known as nontoxic and was used as folk remedy to treat cancer and digestive disorder (Reid, 1976; Saar, 1991; Wasser, 2002). Chaga an example of the white-rot fungus, parasites on stem and stocks of the birch tree, alder tree, and rowan tree, it is usually distributed in cold and humid northern hemisphere at more than 45 degrees north latitude like Russia, Siberia, Finland, Norway, Ukraine, and Hokkaido in Japan. The polyphenolic compounds, triterpenoid, steroid, polysaccharides, peptide extracted from the Chaga has biologically active substances including the anti-oxidative (Babitskaia et al., 2000; Cui et al., 2005; Lee et al., 2007; Nakajima et al., 2007), anti-inflammatory and anti-nociceptive (Park et al., 2005; Kim et al., 2007), immunostimulating (Kim et al., 2005), anti-cancer (Kim et al., 2006; Park et al., 2006; Nakata et al., 2007) and hepatoprotective (Zjawiony, 2004) effect. The Chaga can be differentiated to brownish anterior fruiting body and the blackish exterior slerotium, both sides has higher antioxidant effect compared to the other medicinal mushroom (Nakajima et al., 2007).

The Chaga extract has anti-cancer materials directly reacting to the carcinogenesis and cancer growth, but it also display indirect anti-cancer effect through anti-oxidative effect and immunostimulation (Wasser, 2002; Valko et al., 2006; Federico et al., 2007). The oxidative stress caused by the excess active oxygen will cause oxidoreductase imbalance of cellular signaling pathway and dictates DNA mutation to trigger carcinogenesis on which the antioxidant effect will give help in cancer prevention (Valko et al., 2006; Federico et al., 2007). The immunostimulation of the Chaga has a effect on the suppression of cancer proliferation and transition (Wasser, 2002). Hence the biologically active substance included in the Chaga could effect independently and to each other to cause synergism.

Up to present the anti-cancer effect of the Chaga was usually studied with the extraction or fraction with the water or the organic solvent. However this study the sclerotium of the Chaga were pulverized into superfine power by nanomill processing and given to the test animal to observe the anti-cancer effect. Recently the application of nanotechnology in the future developing industry such as telecommunication, computer, life science, medical, and environmental has produced different product were commercialized, and the research and development are on going freely. However the nanofood technology a nanotechnology applied to the food is still at the initial stage and research and development is still on going in focus to the cosmetics, medicine and bioindustries. The technique applied to the nanofood involves the bottom-up approach processing the smaller size into bigger size and the top-down approach pulverizing the bigger materials into level of nano. On the base of the property, bottom-up technique is generally used for the wet process, and top-down technique for the dry process. The electron uses nucleation or the chemical reaction within the solution thus have higher production but difficult in safety precaution. On the other hand the latter has uneven size and shape of nano particles, it has weakness on the change of physical properties; however it has strong points in the effectiveness by increasing the surface area with small amount, increases the function of the raw material, increases the efficiency in the body, and is safe compared with the wet processing. However at present due to the specificity of the dehydrated vegetal materials it has technical limitation to overcome 1 μm pulverizing barrier.

This study observed the anti-cancer effect of the feed containing the sclerotium Chaga powder processed into superfine powder, 10% Chaga food were given to the C57BL/6 mouse, histological examination of the small and large intestines are undergone to identify the safety of superfine particles in the digestive system.

MATERIALS AND METHODS

1. Experimental animal

The experimental animal was a 4～6 weeks old C57BL/6 mouse, the mouse were provided by the Jungang Lab. Animal Inc. Seoul, Korea and acclimated for one week with common feeds and enough water. The experiment animals were divided into two groups. The control group (C; n=10) were fed with general murine food (Samyang feeds Inc., Korea), and the experimental group (Nch; n=10) were fed with the experimental food with 10% Chaga processed in nanomill technique. Water supplied to both experimental groups was distilled with inverse osmotic pressure water purifier and was given spontaneously.

2. Pulverization of Chaga mushroom and particle analysis

The sclerotium of Russian Chaga mushroom was made into superfine powder by nanomill technology. Nanomill process of Chaga was done by Turbolyzer HKP-05 (Korea Energhy Technology Ltd., Chuncheon, Korea) made appro-

priately for pulverizing the dehydrated biological specimens, centrifugal and drag force were used to classify. The making procedures are all done in low temperature to minimize the denaturation of the raw materials due to high temperature. The cooling system of the mill chamber was processed with mill chamber cooler (R-22) using nitrogen gas. The distribution map and the particle size of the Chaga processed to superfine powder is measured with the Beckman Coulter LS-230 (Beckman coulter Inc., USA) which uses the light scattering principle.

3. Experimental food

The feeds of the experimental group were made with the mixture of 10% ratio of superfine chaga powder processed in nanomill and common food for mouse (Samyang feed Inc.) and produced into the size of commercial food and dried on room temperature.

4. Cell line and cell culture

B16BL6 melanoma cells from Korean Cell Line Bank (Seoul, Korea) were used to induce the growth of tumor in the C57BL/6 mouse. Modified egle's medium (MEM) (Gibco, Carlsbad, USA) were used as the culture medium and, added 10% fetal bovine serum, 100 U/mL penicillin and 100 g/mL streptomycin. The cell was cultured in the condition with 95% humidity, 37°C temperature and 5% CO_2, culture solution was changed every 2∼3 days.

5. Tumor volume and weight

For examination of the tumor volume and tumor weight, all mice's were inoculated with 1×106 B16BL6 melanoma cells in 100 μL phosphate-buffered saline (PBS) intrasubcutanously from the abdomen, and the three orthogonal tumor diameters (D1, D2 and D3) were measured at intervals of 5 days during 25 days with calipers. The volume (V) of tumors was calculated using the formula: $V=D1\times D2\times D3\times\pi/6$) (Plotnikov et al., 2004). The weight of tumor was measured after the harvest of tumors from mice at the termination of experiment. The volume of food intake was measured everyday at the same time during the experimental period. At the last day of experiment, body weight of mice was measured.

6. Survival rate and observation survival time

In order to observe the survival rate and time, all mouse of C and NCh groups respectively were inoculated intrasubcutanously with 1×106 B16BL6 melanoma cells in 100 μL. The control group was fed with the commercial feeds (Mouse E.P., Superfeed Co. Ltd., Korea) and the experimental group fed with the feeds containing 10% superfine Chaga powder and observed the number of surviving mouse everyday. The experiment was ended on the day that one of the groups survival rate is 0% and then calculated the survival rate (%) of the group. The survival time on each animal were indicated in mean score.

7. Histological examination

For the histological examination the small and large intestines are harvested from all the animals from experimental group inoculated with the tumor cells intraperitonealy and fixed in 10% formalin for 24 hours. It was washed in running water for 12 hours, dehydrated with alcohol, and cleared with xylene by routine method. It was cut into 2 μm thick and it was dyed with hematoxylin-eosin (Sigma Co. Ltd., USA) and observed with light microscope (Olympus, USA).

8. Statistical analysis

The results were expressed as the mean±standard deviation (SD). ANOVA (non-parametric) test by Graph Pad Prism 4.0 package (GraphPad Software, Inc., USA) was used for the statistical analysis. $P<0.05$ was considered statistically significant.

RESULTS

1. Particle analysis and Electron microscope observation

By particle size analyzer on volume distribution, mean

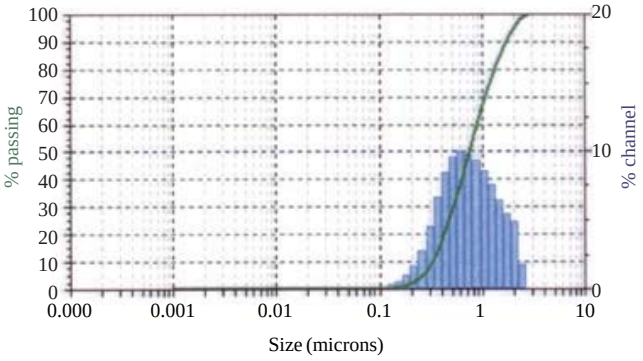

Fig. 1. Particle size analysis in volume of nano-mill processed Chaga powder.

volume was 0.893±0.858 μm, the smallest particle was 121.5 nm, and the particles included within 1 μm were about 70% (Fig. 1). The observation of superfine Chaga particles with the scanning electron microscopy has even size, shape and surface compared with the conventionally processed powder (Fig. 2).

2. Food intake and body weight

The feed intake was measured at same time for 25 days after inoculating the 1×106 B16BL6 melanoma in 100 μL suspension into the abdominal subcutaneous layer of C57BL /6 mouse, and the body weight was measured after sacrificing the experiment animal on the termination of the experiment. As the result the food intake of the control group was 4.2±1.0 g, and 4.9±1.0 g for the experimental group, the body weight for the control group was 27.6±1.3 g, and 29.3±1.6 g for the experimental group. There were no sig-

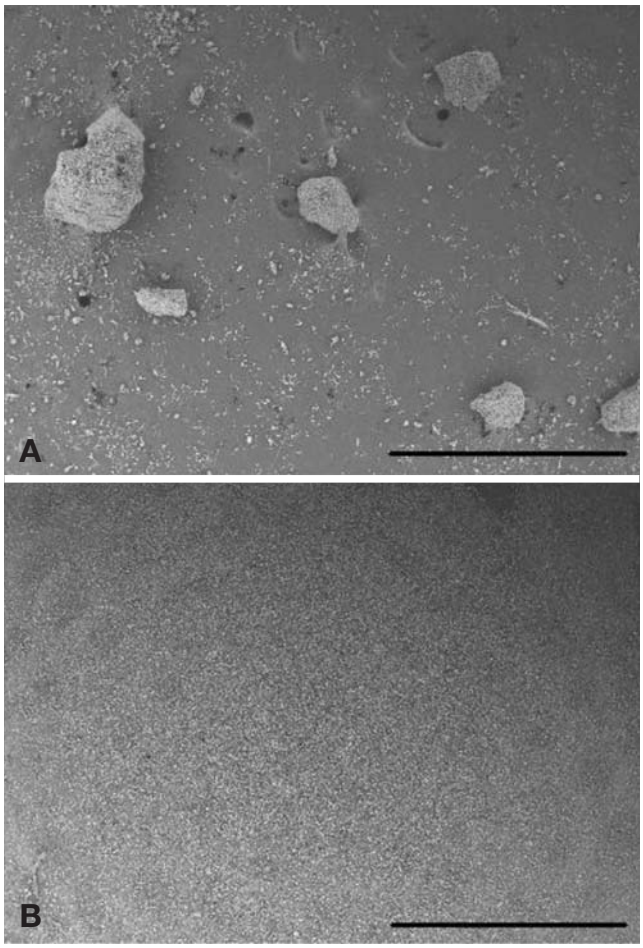

Fig. 2. Scanning electron micrography of Chaga powder (A) conventional mill processed and (B) nano-mill processed (scale bar=1 mm).

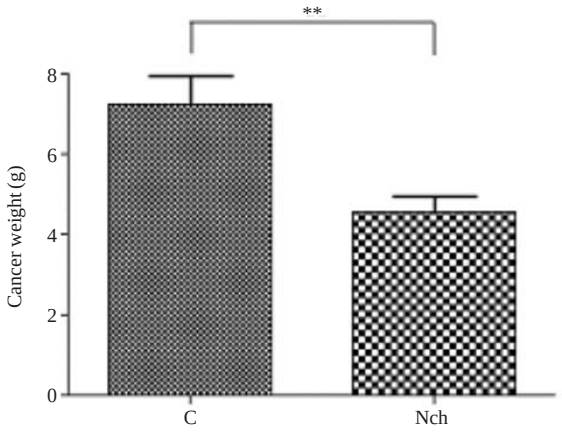

Fig. 3. Tumor weight at the 25th day after intra-subcutaneous injection of melanoma cells. Values are the mean±SD, ** $p < 0.01$.

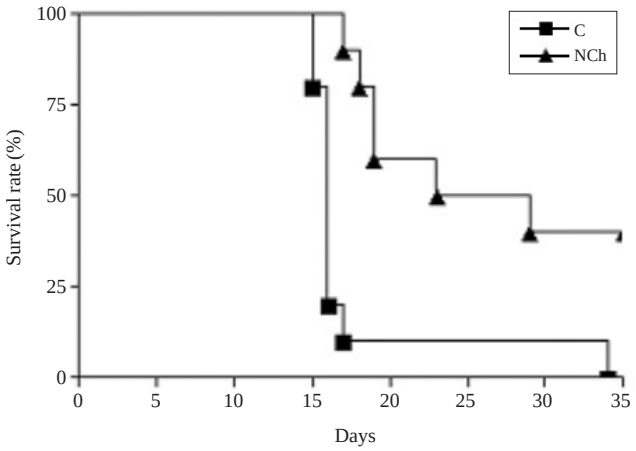

Fig. 4. Survival rate after intraperitoneal injection of B16BL6 melanoma cells to C57BL/6 mice for 35 days. It showed significant difference on the 35th day with 0% survival rate on the control group and 40% survival rate in the experimental group.

Table 1. Tumor volume measured at an interval of 5 days for 25 days after Intra-subcutaneous injection of melanoma cells

Group	Days				
	5	10	15	20	25
C	0	620.2±267.9	2,039.4±951.2	3,973.7±1,693.3	11,828.1±1,492.1
NCh	0	652.7±195.6	1,565.1±442.9	3,178.2±982.3	8,377.6±785.6[a]***

Values are the mean±S.D. [a]***$P < 0.001$ C group vs. NCh group at 25th day

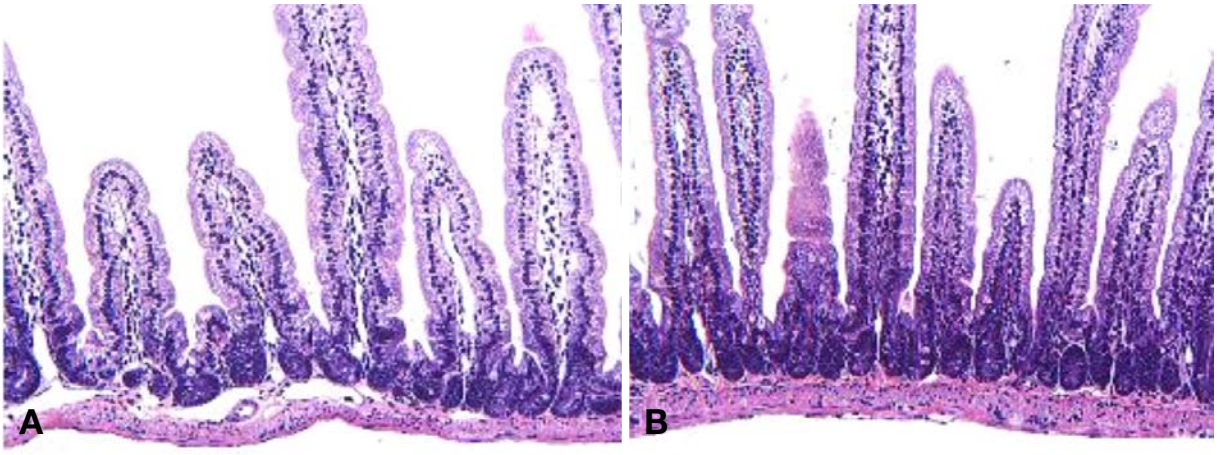

Fig. 5. Light micrographs of small intestine (×200). H-E stain. A: C group, B: NCh group. There was no significant difference in histomorphological and histopathological change in small intestines compared with the normal group.

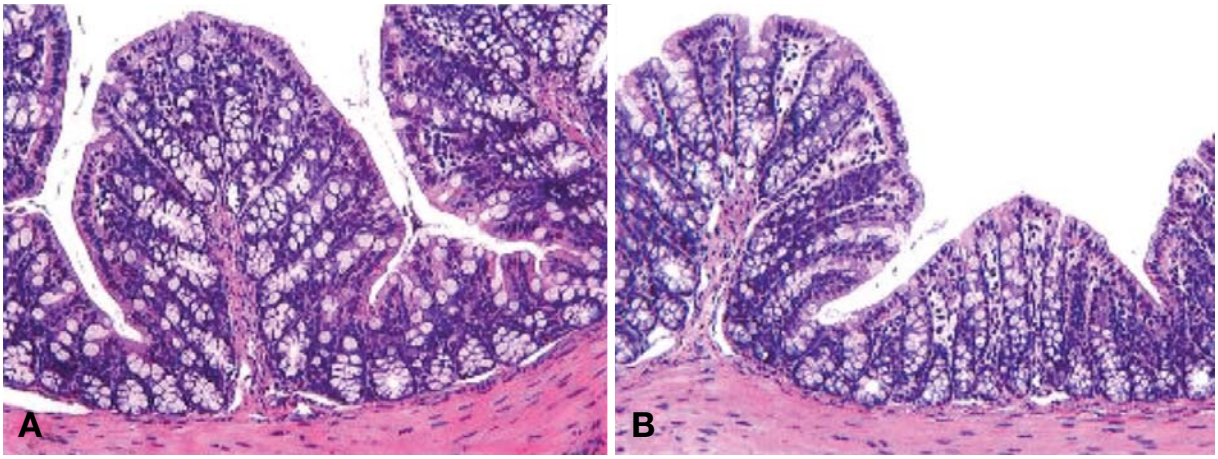

Fig. 6. Light micrographs of large intestine (×200). H-E stain. A: C group, B: NCh group. There was no significant difference in histomorphological and histopathological change in large intestines compared with the normal group.

nificant differences in both food intake and body weight of mice in the NCh and C groups.

3. Volume and weight of tumor

Of the 20 C57BL/6 mice that were injected intrasubcutaneously with 1×106 B16BL6 melanoma cells in 100 μL PBS, all of mice developed detectable tumor masses and during the experimental period. The average tumor weight of the NCh group was significantly lower ($p < 0.01$) than that of the C group at the termination of this experiment (Fig. 3). Also, the average tumor volume of the NCh group was much lower ($p < 0.001$) than that of the C group (Table 1). The tumor inhibit rate was 29.2%.

4. Observation on survival rate

The results of the survival rate after intraperitoneal injection with 1×106 B16BL6 melanoma cells in 100 μL PBS to the C57BL/6 mouse, it showed significant difference on the 35th day with 0% survival rate on the control group and 40% survival rate in the experimental group (Fig. 4).

5. Histological examination

The This experiment was performed to observe the effect of superfine Chaga powder processed by the nanomill technique in small and large intestines while passing through the digestive tract. As the result there was no significant difference in histomorphological and histopathological change in

small and large intestines compared with the normal group. The villi, enterocyte, intestinal mucosa was normal and pathological findings on infiltration of inflammatory cell, cell necrosis, and fibrosis were negative (Figs. 5-6).

DISCUSSION

The anti-cancer effect of Chaga mushroom extract were proved recently by many researchers, and the research on biological activation is on going with emphasis on extracts by water or solvents and fractional matter. The polysaccharides that is one of the most studied biological response modifiers (BRMs) has β-glucans's greater water solubility character that suppress the carcinogenesis through immunoregulation, has direct cancer suppressing effect, and prevents transition of cancer (Wasser, 2002; Zjawiony, 2004). The polysaccharides are abundantly present in medicinal mushrooms; the researchers reported that the endo-polysaccharide segregated from the cultured Chaga mycelium trigger immune reaction and directly affects anti-cancer effect (Kim et al., 2005; Kim et al., 2006). Park et al. (2006) reported that Chaga extract may act as a natural anticancer product by preventing the inhibition of gap junctional intercellular communication through the inactivation of ERK1/2 and p38 MAP kinase. Moreover the Chaga extract is known to involve in the cell cycle. Jarosz et al. (1990) mentioned that the Chaga extract suppress the mitosis of M, G1 and G2 level of cultured HeLa cell which is uterine cancer cell. Youn et al. (2008) reported that Chaga extract leads G0/G1 arrest and apoptosis in human hepatoma HepG2 cells. 3,4-Dihydroxybenzalacetone a type of polyphenol extracted from Chaga suppressed the expression of TNF-induced and NF-κB-regulated proliferative, antiapoptotic, and metastatic gene products (Sung et al., 2008). Also, inotodiol a type of triterpenoid has potent anti-tumor promoting activity *in vivo* (Nakata et al., 2007). Aside from those, polyphenols such as inonoblins and phelligridins (Lee et al., 2007), melanin complex (Babitskaia et al., 2000), triterpenoids and steroids has strong antioxidative effect to suppress the carcinogenesis.

This experiment showed the anticancer effect of nanomill processed Chaga after oral ingestion to melanoma-induced mice; the result indicated that it had greater effect on cancer cell growth suppression and survival rate compared with the control group fed with the conventional feeds. Generally, it has high possibility that when the particle size of the food material decreases into less than 1 μm the specific physiochemical characteristic of the food material will change partially, and the bioavailability in the body may increase due to the increase in activation of the functional components, which is predicted to have greater effect with less amount. However, this study was focused on the anticancer effect of only superfine Chaga by top-down technique. Thus comparative study between the fine Chaga processed by conventional pulverizing technique and superfine Chaga by nanomill technique will be necessary to confirm the difference of anti-cancer effect according to the change of particle size.

Up to now the research on the anticancer effect of Chaga were made through extracts using solvents such as water, ethanol and methanol or specific components isolated from the extract. These types of extracting process is accompanied with high pressure and high temperature, which has possibility to break or loss the essential component. Moreover the type of the extracted component may change due to the solvent used, temperature and time. Therefore the utilization of Chaga into superfine powder can complement these problems, and it has strength to increase bioavailability of insoluble or effective components. There are different varieties of biological active substances in the Chaga; these types of materials may be affected by the extraction and processing. Specially, during the processing of Chaga into superfine powder the physical friction will cause high temperature. This high temperature may denature the raw materials, therefore the cooling system has the technical importance to preserve the essential components. In this experiment, the cooling system using nitrogen gas in the mill chamber of pulverizer was applied, which maintained the temperature of the mill chamber low and minimized the denaturation of the materials.

First of all to use the Chaga as a food it must verify it safety in the body. Shashkina et al. (2006) had reported that different Chaga preparation such as tea, decoction, tincture, powder and extract had no toxicity in the body, and they had no counter indication for medicinal usage. However there is no report on the food safety of whole Chaga processed to superfine powder when taken orally in a large amount. To observe the histological safety of Chaga superfine powder taken orally passing through in direct contact with digestive tract, small and large intestines were observed with the light microscope after feeding the experiment animal with solid foods made from 10% superfine powder of Chaga. As the result, pathological findings such as inflammation and necrosis of small and large intestines, deterioration and disruption of mucosa were not observed, in which

it is verified that the mass consumption of the superfine Chaga powder is non-hazardous to the digestive tissue. These results are able due to the particle size of the superfine Chaga powder was not that big to reach effect on the tissues, or it did not cause cell deposition and toxicity while in contact with the digestive tissue. However, other researchers reported that macrocorpuscular particles such as starch granule, cellulose particle, charcoal, silicate crystal, latex particle within the micrometer size range were transferred from the wall of the gastrointestinal tract by the intercellular persorption mechanism to the secondary organ of body such as kidney, spleen, bone marrow, liver, heart, lung, placenta through lymphatic system and the portal system. These persorpted particles were eliminated after staying for a certain period but showed temporary embolization of vessels in Bowman's capsule or in the pulmonary alveoli. (Volkheimer, 1974; Florence, 1995). These results indicate that insoluble Chaga superfine particles may cause histological affect to the several organs of body. Therefore histological and biochemical study related in the persorption of superfine particles is more necessary for food safety of Chaga.

As conclusion the superfine Chaga powder processed in nanomill technology indicated remarkable cancer suppressing effect and high survival rate on the animal experimentation *in vivo* and also had verified that the Chaga particle did not affect the digestive tissues. This result reveals that the superfine Chaga powder processed by nanomill technology has effect in cancer prophylaxis and chemotherapy. The nanomill technology is expected to increase the activation and maintenance of biological components when applied to medicinal-use of plants and increase bioavailability in the body. Also this experiment showed the histological safety of gastrointestinal tract contacted directly with Chaga superfine powder. However further study is necessary to compare the effects of various types of Chaga preparations and to confirm the food safety of superfine particles.

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＜국문초록＞

차가버섯(*Inonotus obliquus*)의 추출물과 분획들은 항돌연변이, 항암, 항산화 및 면역자극 효과 등을 비롯하여 다양한 생물학적 활성을 갖는 것으로 알려져 있다. 본 연구는 나노분쇄 기법으로 제작된 10% 차가사료를 C57BL/6 생쥐에게 식이한 후 항암효과를 확인하기 위해 시행되었다. 부피평균에 의한 입도분석에 따르면 1 μm 내에 포함되는 초미세 차가버섯 입자는 전체의 40%인 것으로 나타났다. 생쥐에게 B16BL6 흑색종세포를 피하로 주사한 결과, 종양의 부피($p < 0.001$)와 무게($p < 0.01$)는 초미세 차가버섯을 식이한 실험군(Nch)이 일반사료를 먹인 대조군(C)에 비해 현저히 감소하였고, 종양성장 억제율은 29.2%를 나타내었다. 흑색종 세포를 복강 내로 주사한 후 생존율을 관찰한 결과, 마리 당 평균 생존기간은 대조군과 실험군이 각각 17.7일과 26.0일이었다. 대조군이 모두 사망한 날인 35일 째 실험군의 생존율은 40%를 보여주었다. 초미세 차가입자가 소화기관에 문제를 유발시킬 수 있는 가능성 때문에 안전성을 확인하기 위해 소장과 대장을 조직학적으로 관찰한 결과 두 군 모두 형태학적 또는 병리학적 변화는 관찰되지 않았다.