Original Article

# Radioprotection and antitumor effects of mechanism on AHCC (Active Hexose Correlated Compound)

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Abstract: Some natural products are able to inhibit radiation effects and exert an antitumor effect with fewer adverse reactions; however, their antitumor effects are less than those of widely-used synthetic drugs. AHCC (Active Hexose Correlated Compound) is a natural material that has been attracting attention, and we extracted this material with water and investigated the effect of continuous AHCC administration on radioactivity-induced reduction of hemocytes, in addition to the antioxidant and or effects of AHCC. Following a 1-week adjustment period, AHCC was administered intraperitoneally to male ICR mice at a dose of 250mg/kg every other day for 2weeks. Following administration, 2Gy whole-body irradiation was performed and the counts of leukocytes, lymphocytes, and granulocytes and monocytes in the peripheral blood were determined 1, 3, 7, 15 and 30days after irradiation. These cells were considered since they are closely associated with immunity to radioactivity. In a second experiment, AHCC was similarly administered to the mice for 2weeks after a 1-week adjustment period, and 2Gy whole-body irradiation was performed. The antioxidant effects in hemocytes were then investigated using 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH), a radical generator. In a third experiment, $1 \times 10^6$ Sarcoma-180 cells were inoculated into the right thigh of mice, which were divided into four groups: control, AHCC-treated, 6Gy partial irradiated and AHCC-treated + 6Gy partial irradiated groups, and changes in tumor size were measured for 20days. Statistical analysis was conducted using ANOVA for multiple groups. In the three experiments, administration of AHCC inhibited the reduction of hemocytes caused by whole-body irradiation, showed antioxidant effects against radioactivity, and inhibited tumor growth, respectively. In conclusion, our data suggest that the antioxidant effect of AHCC inhibits hemocyte reduction caused by whole-body irradiation and enhances immunological inhibition of tumor growth.

Keyword: Radiation protection, anti-tumor effect, SOD, Lymphocyte, *AHCC*

## Introduction

Active hexose correlated compound (AHCC), an extract prepared from mycelia of the Basidiomycete mushroom (Lentinula edodes), contains oligosaccharides, amino acids, lipids, and minerals[1]. About 74% of AHCC are composed of oligosaccharides, which are enriched in low molecular weight acetylated alpha-1, 4-glucans. Gamma delta T cells is that the cell population with a normal different type than the T cells of the T cell receptor on the cell surface.

The biological effects of AHCC have been attributed to its glucan fraction[2, 3]. AHCC is well tolerated and is largely free of adverse effects. It has been shown that AHCC has a positive effect on the immune system of humans[2] and rodents[3-5]. Oral administration of AHCC has been shown to enhance NK activity and gd T cell expansion in human and rodents with malignances[3, 6]. In the trinitrobenzenesulfonic acid model of colitis in rats, AHCC acts as a prebiotic and is antiinflammatory[7].

The majority of patients with advanced hepatocellular carcinoma (AHCC) survive no longer than 6 mo from the day of initial diagnosis[8]. It was reported that improvement of implanted drug delivery systems has made it possible to administer repeated hepatic arterial infusion of anticancer agents to patients with AHCC and that hepatic arterial infusion therapy not only improved survival but also the quality of life (QOL)[9].

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Continuous local arterial infusion of 5-fluorouracil (5-FU) and cisplatin (CDDP) using an infuser pump and an implanted reservoir has been shown to prolong the survival of patients with severe advanced AHCC[9-11]. Leucovorin is a biochemical modulator of 5-FU[12-14]. A randomized study showed that the regimen using CDDP, 5-FU, and leucovorin (LV) was significantly better than that of the low-dose CDDP and 5-FU alone[15]. According to the above study, it is recovered from the immune activity and chemotherapy of side effects by AHCC administration. Thus, the radiation in this study, the mechanism of recovery by the immune depression and radiotherapy of side effects would be similar.

However, there were differences of the response rate and the regimen used in these studies, such as 250 mg of 5-FU for 5 or 24 h. For staging of HCC, Cancer of the Liver Italian Program (CLIP) score[16] has been reported to be very useful[18, 19]. However, it was also reported that the stratification ability and prognostic power of the Japan Integrated Staging (JIS) score[20] were superior to those of the CLIP score for staging of AHCC[21]. Accordingly, this study was performed to evaluate antitumor effect and radioprotection effect. This time, we report that effects of blood cell and antioxidant for whole-body irradiation of mice and influence of Sarcoma-180 antitumor *in vivo* using AHCC.

## Material and Methods

AHCC (Imuno Medci®) was dissolved in distilled water. Mice were orally administered with AHCC (200 mg/kg) by gavage every other day for 1 week before infection and at day 1 and 3 postinfection in a 200 mL volume (total administration dose with AHCC (6000 mL/kg)). Control mice received 200mL distilled water. Similar doses of AHCC were used previously based on body weight and did not produce toxic effects in mice.

### *Animals*

Five-week-old male ICR mice with a mean weight of 18-20 g were purchased from Japan SLC Inc. and kept under standard conditions (room temperature 22 ± 3 ℃, humidity 60%) with free access to food (CA-1, Japan Clare, Inc.) and drinking water. The mice were acclimated to the breeding and experimental environment for 1 week prior to the experiments.

### *Irradiation*

X-ray irradiation was administered to each mouse using an X-ray generator designed for animal use (Phillips, Inc.). The table was rotated at a constant speed so that these mice were irradiated evenly. The conditions for irradiation were: source voltage, 200 kV; rate of radiation, 0.35 Gy/min; and supplemental filter, 0.1 mm Cu + 1 mm Al.

### *Measurement of peripheral blood cell count*

Mice were divided in 4 groups, control, AHCC, irradiation alone and combined with AHCC and irradiation. After acclimation, control and irradiation alone group of mice were i.p. injected with saline, AHCC and combined with AHCC and irradiation group of mice were injected with AHCC 2 weeks of every other day. Irradiation groups had 2 Gy whole body irradiation and 10 $\mu$l of peripheral blood was collected with capillary tube from tail vein then counted with an automated blood cell counter (Celltac-$\alpha$ MEK-6318, Nippon Koden Inc.). The numbers of peripheral leukocytes, lymphocytes, granulocytes and monocytes, which all have relatively high sensitivity to radiation and primary cells of the immune system, were counted. Measurements were done at 1 day before irradiation and at 1 day, 3 days, 7 days, 15 days and 30 days after irradiation.

### *Antioxidant effect*

### *Measurement of SOD activity in the serum*

After acclimation, mice were divided in 4 groups same as the experiment of blood cell count. AHCC was injected to mice for 2 weeks. In this study, we studied by orally administration AHCC. After 2 Gy whole-body irradiation, whole blood was collected from mouse hearts by puncture with 23-G needle under anesthesia, mixed with heparin, and either

centrifuged（10 min at 1200 rpm 2 ℃）to separate blood plasma. Using SOD Activity Detection Kit Wako Pure Chemical Industries, Ltd., for the measurement of SOD activity, we have measured the activity of SOD in serum by reduction method NBT.

***Measurement of AHCC orally administration or combined with X-irradiation on tumor growth***

After acclimation, mice were divided in 4groups same as the experiment of blood cell count. AHCC was orally administrated to mice for 2weeks then $1 \times 10^6$ Sarcoma-180cells were injected into the right femoral region. When tumor grew to 10 mm in diameter, irradiation groups were treated with 3times of 2Gy irradiation every other day and all groups of tumor diameters were measured with a caliper for 20 days. Tumor volumes were calculated using the formula: $V=ab^2/2$, where "a" was the shortest tumor diameter and "b" was the longest tumor diameter measured.

***Survival ratio of methods by radiation***

Survival rate calculation is carried out for 11days at intervals of every days, the calculation method was performed by the following equation. We used 8Gy x-irradiation on total body irradiation. The mice were irradiated while rotating to put in acrylic of gauge.

Survival rate（%）=（number of breeding / number of survivors）× 100

***Statistical analysis***

Experimental values are given as mean ± standard error of the mean（SE）. All experiments of statistical analysis were performed using a parametric ANOVA test among the groups to determine significant difference.

**Results**

***Radioprotective effect and immune-enhancing effect***

***Lymphocyte count and leukocytes count of irradiation group***

We were shown in Fig.1 the mean and standard error values of the measured results for the irradiated group leukocytes count. Compared with the 2Gy group, the day before irradiation AHCC administration+ 2Gy group, 3 hours after irradiation, significant decrease suppression was observed 15-30 days after irradiation, early recovery was observed.

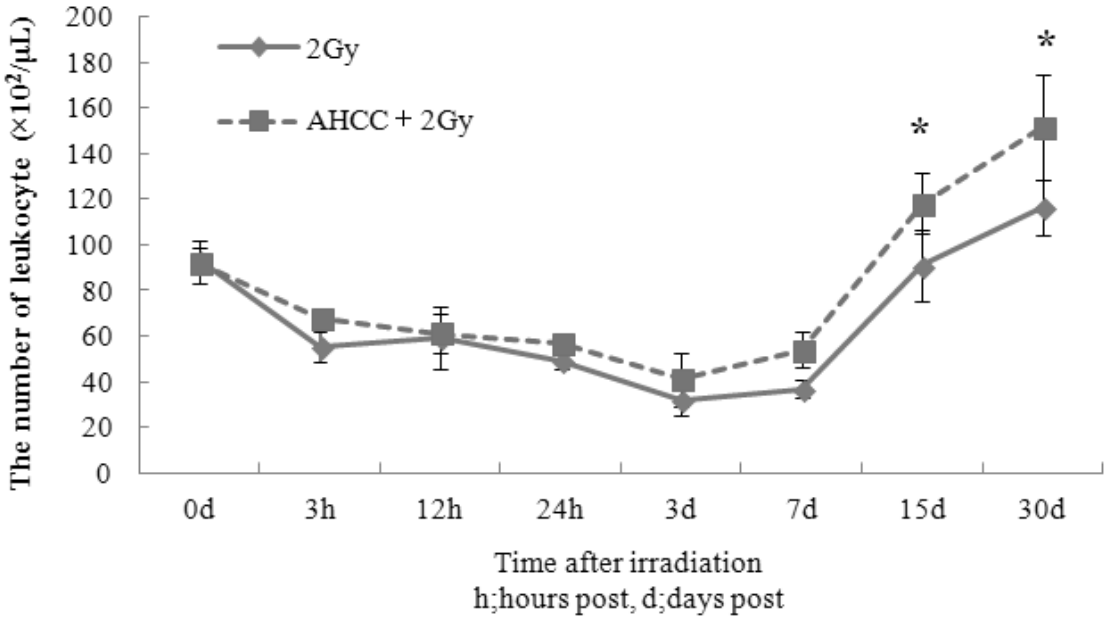


Fig.1. Leukocyte counts on different days after irradiation in mice of different groups. The number of leukocyte was calculated from the pre-irradiation values taken as 100%. The bars represent standard deviation.* Statistically significant ($p<0.05$) from the 2Gy group.

We were shown in Fig.2 the mean and standard error values of the measured results for the irradiated group lymphocyte count. Lymphocyte count was also a tendency similar to that of the leukocytes.

***SOD mimicking activity***

Average value of the measurement results of anti-oxidation activity and I were shown in Fig.4 the standard error. SOD-like activity was observed with *AHCC* dose group compared to the control group.

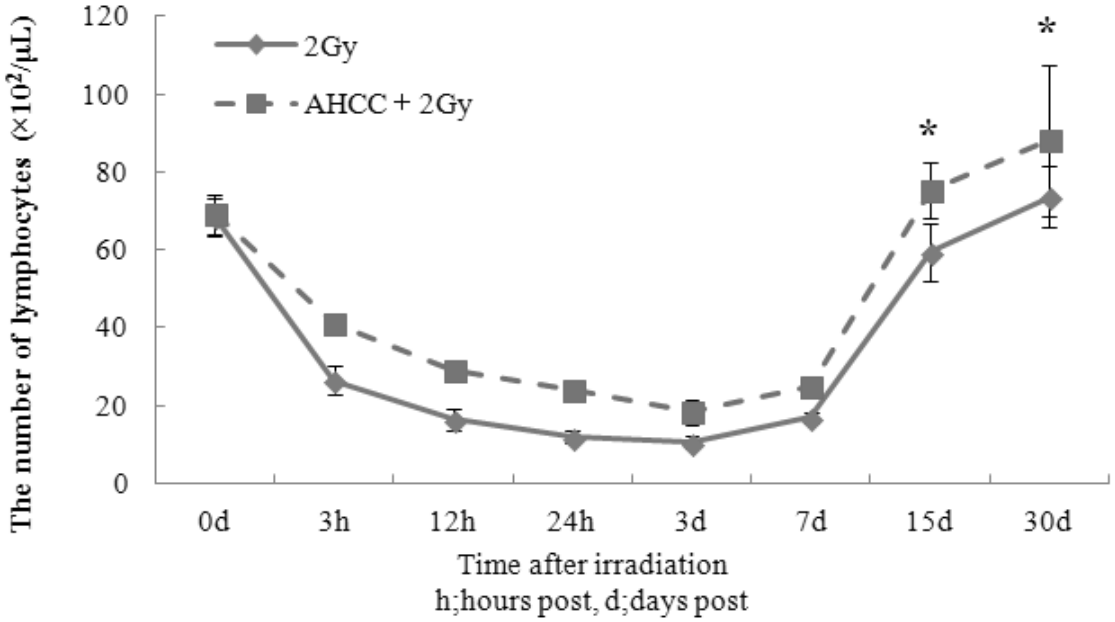


Fig.2. Lymphocyte counts on different days after irradiation in mice of different groups. The number of leukocyte was calculated from the pre-irradiation values taken as 100%. The bars represent standard deviation.* Statistically significant ($p<0.05$) from the 2Gy group.

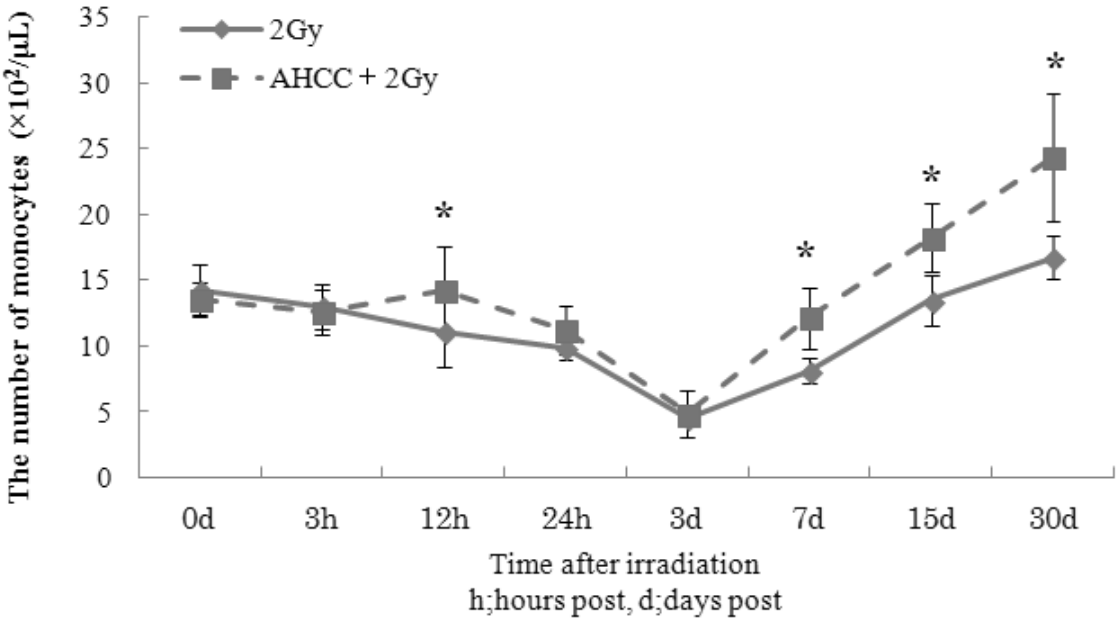


Fig.3. Monocytes counts on different days after irradiation in mice of different groups. The number of monocytes was calculated from the pre-irradiation values taken as 100%. The bars represent standard deviation.* Statistically significant ($p<0.05$) from the 2Gy group.

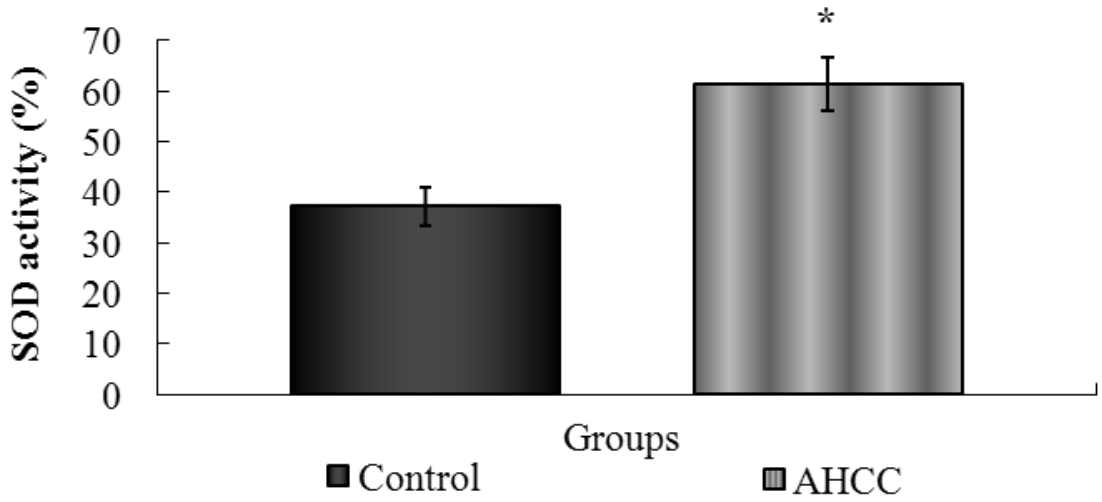


Fig.4. Anti-oxidation activity measurement by the SOD method. * Statistically significant (P<0.05) from the control group and *AHCC* group.

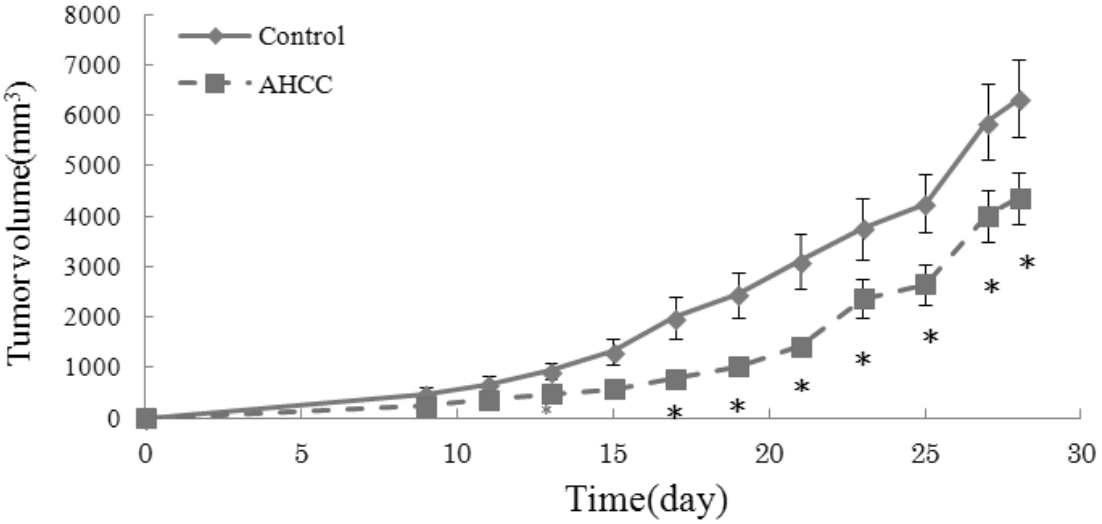


Fig.5. Effect of *AHCC* on the tumor growth in mice inoculated with SCC-7 (Squamous Cell Carcinoma−7). Groups of ten mice each were subjected to each treatment. Results represent means ± S.D.* Statistically significant (P<0.05) from the control group.

***Anticancer effect***

***Tumor volume of the non−irradiated group***

It is shown in Fig.5 tumor volume calculated. For *AHCC* group, significant growth inhibition was observed up to −30 days after 15 days as compared to the control group.

***Tumor volume of the irradiated group***

It is shown in Fig.6 tumor volume calculated. *AHCC* +6 Gy group growth significant suppression of cancer was observed in up to −30 days after 5 days measurements on all compared to +6 Gy group Control.

***Tumor weight of the non−irradiated group***

When *AHCC* was administered for 30 consecutive days to squamous cell carcinoma−7−bearing mice, the suppressive ratio was 14.1 %（P< 0.01）(Fig.7).

***Tumor weight of the irradiated group***

When *AHCC* was administered for 30 consecutive days to squamous cell carcinoma−7−bearing mice, the suppressive ratio was 20.6 %（P< 0.01）(Fig.8).

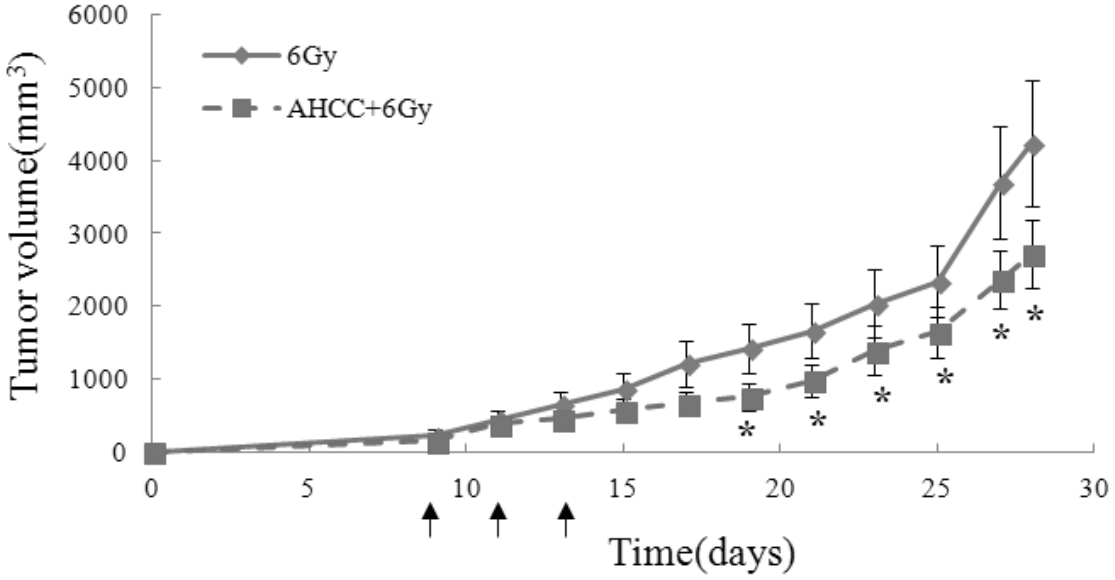


Fig.6. Effect of *AHCC* on the tumor growth in mice inoculated with SCC-7 (Squamous Cell Carcinoma−7). Groups of ten mice each were subjected to each treatment. Results represent means ± S.D.* Statistically significant (P<0.05) from the control group. ↑ irradiated 2Gy.

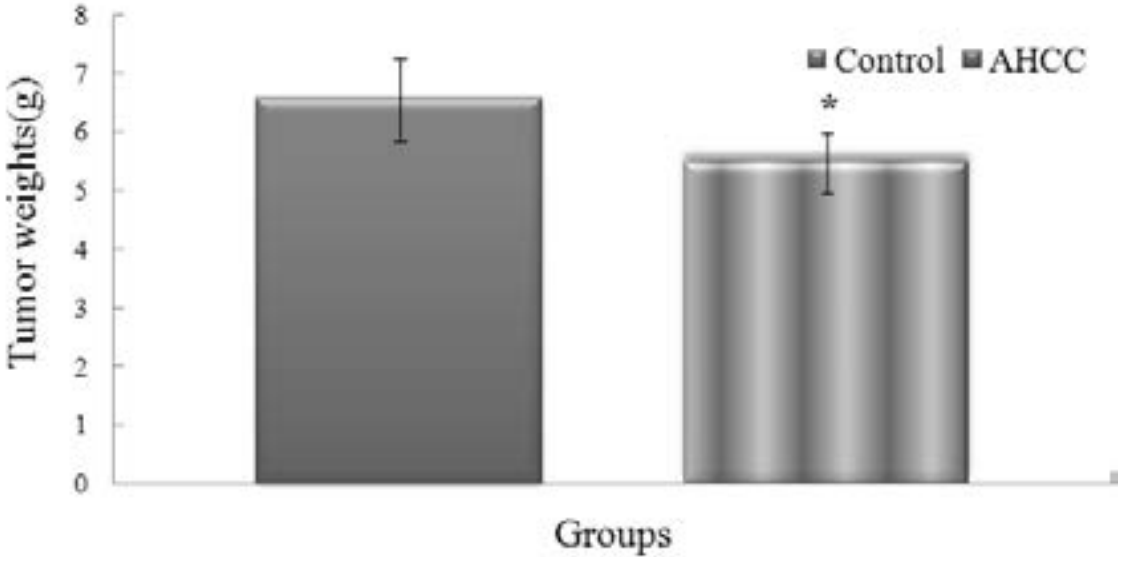


Fig.7. Effect of *AHCC* on the tumor weights in mice inoculated with SCC-7 (Squamous Cell Carcinoma−7). Groups of ten mice each were subjected to each treatment. Results represent means ± S.D.* Statistically significant (P<0.05) from the control group.

***Survival ratio***

The survival of irradiated mice is summarized in Fig.9. All of the animals in the irradiated 8Gy group died from the 5th day to the 7th day following irradiation. *AHCC* injected intraperitoneally prolonged the survival of mice; as shown in the Fig.9 60% of mice still survived on the 11th day at the both doses of 200mg/kg. There were 10 animals in each experimental group. Survival rate calculation is carried out for 11days at intervals of every days, the calculation method was performed by the following equation. 8Gy x−irradiation was used on total body irradiation.

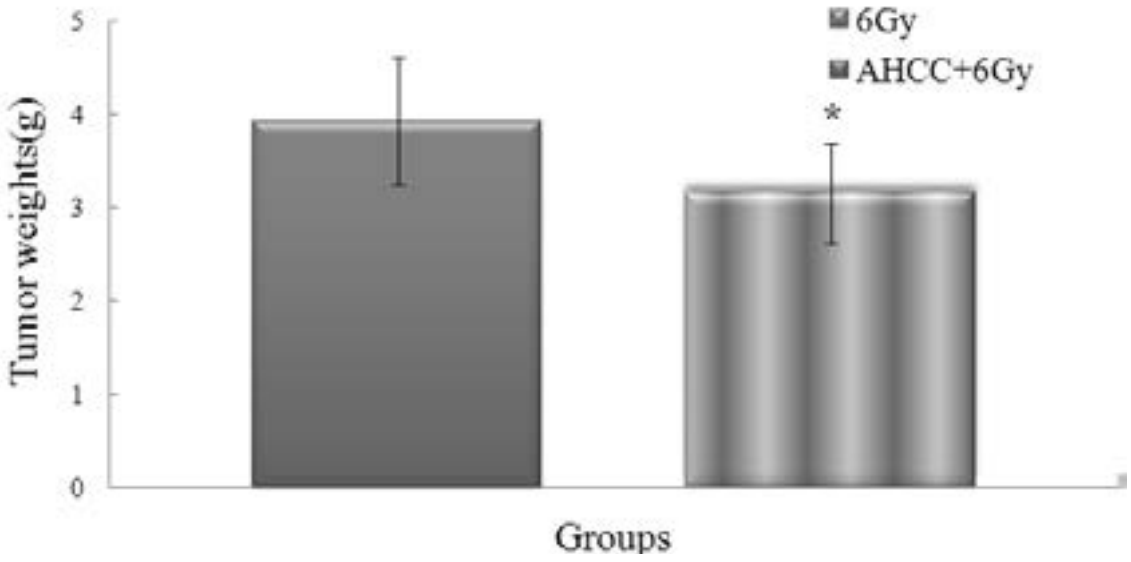


Fig.8. Effect of *AHCC* on the tumor weights in mice inoculated with SCC-7 (Squamous Cell Carcinoma-7). Groups of ten mice each were subjected to each treatment. Results represent means ± S.D.* Statistically significant ($P<0.05$) from the control group.

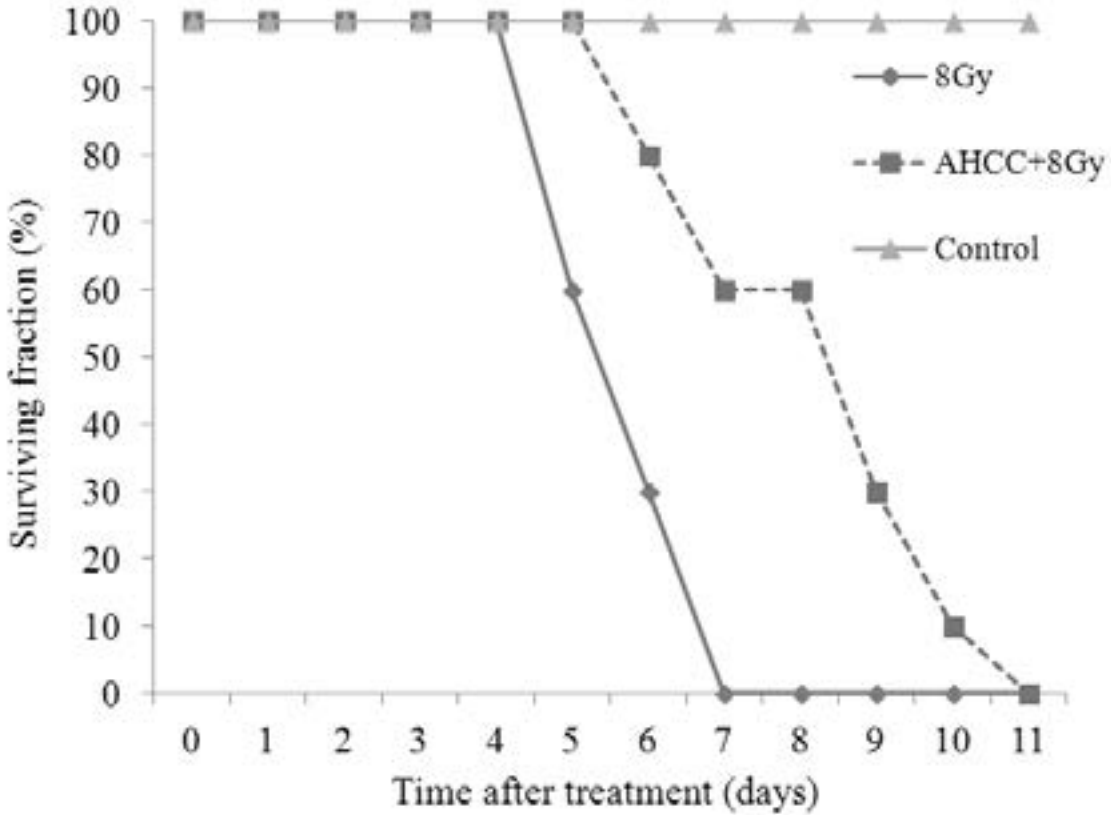


Fig.9. Effect of *AHCC* on the mortality of mice exposed to whole body radiation. There were 10 animals in each experimental group. Survival rate calculation is carried out for 11days at intervals of every days, the calculation method was performed by the following equation. We used 8Gy x-irradiation on total body irradiation.

**Discussion**

Whole-body irradiation is known to cause free radical-induced DNA damage and carcinogenesis, and to compromise the immune system due to its effect on leukocytes[22]. To evaluate the effects of AHCC on irradiation-induced damage, we investigated changes

of hemocytes in the peripheral blood, inhibition of oxidation induced by AAPH (2, 2'-Azobis (2-amidinopropane) dihydrochloride), a radical generator, and changes in tumor growth of Sarcoma-180 following AHCC administration. The effects of irradiation and AHCC on hemocytes (i.e., lymphocytes, monocytes and leukocytes), which are directly associated with the immune system, is shown with time in Fig.1. to Fig.3. The direct immunization said that is natural immunity. This is a system to eliminate such as cancer cells born in the pathogens and the body that you enter the body without specific opponent.

Administration of AHCC prior to irradiation increased the hemocyte count significantly. Hemocytes were markedly reduced by irradiation, but AHCC administration inhibited this reduction and increased the minimum level. Nada *et al*. have reported that single intraperitoneal administration of AHCC at a dose of 50 mg/kg significantly increases leukocytes 3 and 7 days after administration, with a concomitant increase in the weight of the spleen[23], and also reported that leukocyte count and spleen weight increased after repeated oral administration of AHCC at a dose of 50 mg/kg 5, 10 and 15 days before determination[24, 25]. Kimoto *et al*. showed both *in vivo* and *in vitro* inhibitory effects of artepillin C, which is a component of AHCC, on the growth of various tumors, and reported that cytotoxicity and adjuvanticity increased in lymphocytes[26].

Hydroxyl radical (-OH) and superoxide radical ($O2^-$) are free radicals that are induced by irradiation. The OH radical is known to oxidize lipids to peroxy radicals (ROO-) and to increase lipid peroxide levels[22]. AAPH, a soluble azo compound, is a free radical initiator that generates radicals through simultaneous thermolysis, which results in oxygen inclusion into peroxy radicals[27].

Reoxygenation is a phenomenon seen in cancer cells when you are doing the radiotherapy. Typically, among normal cells but not hypoxic cells and anoxic cells present, it is present some cancer cells.

These radicals can then abstract a hydrogen atom from various molecules, combining with the hydrogen to stabilize themselves while inducing a radical chain reaction in the substances undergoing hydrogen loss. In this study, under conditions with and without irradiation, AHCC inhibited AAPH-induced peroxy radical formation. Nagai *et al*. have reported the antioxidant and radical scavenging effects of AHCC on DPPH and hydroxyl radicals[28], and Cos *et al*. showed similar effects for a caffeic acid ester extracted from AHCC[29]. El-Ghazaly *et al*. have reported that AHCC suppresses inflammation caused by whole-body irradiation[30], and also showed that superoxide dismutase (SOD) activity is increased in hemocytes following AHCC treatment of both irradiated and non-irradiated rats. SOD inhibits oxygen toxicity by catalytic scavenging of free radicals that would otherwise cause tissue damage[31]. Hence, AHCC is a natural antioxidant that enhances SOD activity and inhibits production of oxides from lipid oxides[32-36] showed the $O_2$-scavenging effect of AHCC at super critical state (40 ℃, 350 atm pressure), and suggested that AHCC contains vitamin C[37]. Collectively, these results suggest that AHCC has both an antioxidant effect and a free-radical scavenging effect.

From Fig.5 to Fig.8 shows the tendency for inhibition growth of the Sarcoma 180 tumor by combined administration of AHCC and irradiation. Similar antitumor effects have been reported previously; hence, Rodrigo *et al*. showed an effect of AHCC on tumorigenesis of colorectal cancer, and showed that AHCC regulates DNA damage, using a Comet assay[38]. It has also been reported that AHCC and its components, including flavonoids, aromatic carboxylic acids and esters, prevent oncogenesis[39, 40]. Matsuno *et al*. showed that PRF-1 extracted from AHCC has toxicity for human hepatocellular carcinoma[41], and Kimoto *et al*. showed that AHCC suppresses FeNTA-induced renal adenocarcinoma in CD-1 and ddY mice[42]. Clinical treatment methods using AHCC have yet to be clearly established, but many pathological studies have shown biological effects of AHCC[43]. It has been reported that leukocytes and $CD8^+$ and $CD4^+$ cells are significantly

increased in AHCC-treated mice[44], and AHCC and its components have been shown to have mobility and bactericidal properties *in vivo* and *in vitro*, while enhancing tumorigenic activity and producing factors activating IL-1, TNF and lymphocytes in mammals [44]. It is well known that irradiation of tumors suppresses tumor growth: irradiation has a direct effect on tumor DNA, thereby suppressing cell growth and inducing apoptosis[45], and can delay tumor growth by an oxygenic effect (reoxygenation) on tumor blood vessels[46]. The combined antitumor effects of AHCC and irradiation-inhibited tumor growth appear to be stronger than the effects of each treatment alone. In conclusion, prior administration of AHCC enhances the immune systems and suppresses irradiation-induced damage to hemocytes in the peripheral blood. AHCC also shows an antioxidant effect in reducing irradiation-induced free-radical damage, and can inhibit the growth of sarcoma cells, with increased inhibition occurring in combination with irradiation.

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