

## Water-generated negative air ions activate NK cell and inhibit carcinogenesis in mice

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### Abstract

Negative ions are considered to have potential health benefits, but few studies have examined their effects *in vivo*. We studied water-generated negative ions (WNI) with respect to physical properties as well as immunologic activation and anti-tumor activity (inhibition of carcinogenesis and tumor growth) in mice. Electrically generated negative ions (ENI) served as control. Water-generated negative ions had a long life, significantly enhanced the cytotoxic activity of natural killer cells, and significantly decreased the incidence of cancer and inhibited tumor growth. Anti-tumor effects were attributed to enhancement of natural killer cell activity. The mechanisms and applications of negative ions warrant further investigation.

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### 1. Introduction

#### 1.1. Background of negative ions

Negative ions have attracted considerable attention because of potential health benefits. Many electric appliances have negative-ion functions as an option.

However, few studies have directly examined the effects of negative ions *in vivo*, and scientific evidence is lacking. Studies of negative ions were started in the 1950s [1,2]. Positive ions in the air were found to act on the sympathetic nervous activity and excite nerves, whereas negative ions acted on the parasympathetic nervous system and relaxed nerves. The results of initial studies were criticized because they were not reproducible or supported by firm scientific evidence. However, recent advances in analytical techniques and systems have enabled more rigorous and precise comparisons between positive and negative ions. Data has gradually accumulated, leading to a better understanding of

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the mechanisms underlying the effects of ions on organisms. The present study focused on the *in vivo* effects of negative ions.

### 1.2. Differences between ENI and WNI

Negative ions can be broadly classified into two types: electrically generated negative ions (ENI) and water-generated negative ions (WNI). Electrically generated negative ions are produced in nature by the electrical discharge of thunder, radiant energy, and ultraviolet light. These sources of energy cause electrons to be discharged from air molecules. Discharged electrons combine with oxygen atoms to create negatively charged air ions. Electrically generated negative ions discharge high-voltage electricity and transform particles in the air into negatively charged particles, such as ozone, oxygen radicals, nitrogen oxides, and sulfur oxides [3]. Dust particles in the air are also negatively charged by negative ions, which then adhere to walls and other surfaces. Water-generated negative ions are created by the ionization of water through the Lenard effect [4]. This phenomenon naturally occurs around waterfalls. As water breaks up into small droplets, electrons are arranged on the surface of small water droplets in a dipolar fashion. These electrons combine with oxygen molecules (electron acceptors), which are abundant in air. Negatively charged oxygen molecules combine with several or up to 20 or 30 water molecules, forming negative-ion clusters:  $O_2(H_2O)_n$  [5]. Electrically generated negative ions were first able to be scientifically distinguished from water-generated negative ions in 1998. Subsequent studies by Ryushi et al. [6] reported that electrically generated negative ions significantly increase diastolic blood pressure.

### 1.3. Purpose of this study

Our previous study, showed that manifestations of chronic contact dermatitis of the ears of mice were significantly improved by water-generated negative water ions and significantly worsened by electrically generated negative air ions in a model of atopic dermatitis. We also found that water-generated negative ions increased the activity of natural killer (NK) cells and significantly prolonged survival,

whereas electrically generated negative ions significantly decreased body weight in mice. The present study was designed to examine the physical properties, *in vivo* effects, and anti-tumor activity of water-generated negative ions.

## 2. Subjects and methods

We studied the physical properties, *in vivo* effects, and anti-tumor activity of water-generated negative ions.

### 2.1. Animals and generator of ENI and WNI

Mice were housed in an animal room maintained at a constant temperature ( $24 \pm 2$  °C) and humidity (40–70%) and a regular light-dark cycle. We used a negative water ion generator for home use (Aqurich; Matsushita Ecology Systems Co., Ltd, Aichi, Japan). This appliance sprays negative ions produced by the Lenard effect with air applied at a high flow rate and concentrated steam, thereby producing water-generated negative ions. The water is filtered, and the airflow rate is set at 1800 L/min. The generator was connected to an animal housing rack by means of a hose (2 m in length and 150 mm in diameter). Water-generated negative ions were supplied at a rate of 30 L/min. On the other hand, using an electric appliance, ENI were generated by means of corona discharge.

### 2.2. Physical properties of water-generated negative ions

The lifetime of water-generated negative ions in the air was measured over the course of 300 s by means of an ion measuring apparatus (Ion Tester KST-900; Kobe Denpa Co., Ltd, Hyogo, Japan). As control, the number of electrically generated negative ions was determined.

### 2.3. NK cell activity

#### 2.3.1. Comparison of NK cell activation between water-generated negative ions and electrically generated negative ions

Mice were divided into three groups and were exposed to water-generated negative ions, electrically

generated negative ions, or clean air. The mice were killed after 2 weeks. The spleens were removed, and the residual activity of NK cells was determined.

### 2.3.2. Relation between inhalation time and NK cell activity

Mice were divided into seven groups and were exposed to water-generated negative ions. NK-cell activities were measured in mice that were killed after 1, 2, 4, 8, 12, 24, and 48 h. NK-cell activities were also determined after exposure to water-generated negative ions was discontinued.

### 2.4. Inhibition of carcinogenesis

We examined the effect of water-generated negative ions on carcinogenesis due to methylcholanthrene, a chemical carcinogen. Methylcholanthrene was injected subcutaneously into the backs of mice. The mice were divided into two groups: those exposed to normal air and those exposed to water-generated negative ions. Each group was housed separately and observed for 90 days. Whether cancer developed was examined in each group.

### 2.5. Inhibition of tumor growth

NR-S1 was injected subcutaneously into the backs of 100 mice. The diameters of the resulting subcutaneous tumors were measured bi-directionally once a week. Tumor volume was calculated by the following formula: tumor volume = (major axis  $\times$  minor axis<sup>2</sup>)/2. Seven days after the subcutaneous injection of NR-S1, the mice were divided into five groups ( $n=20$  each), each given the following treatments: TS-1 alone (10 mg/kg of TS-1; Taiho Pharmaceutical Company, Tokyo, Japan); water-generated negative ions alone; TS-1 plus water-generated negative ions; TS-1 plus water-generated negative ions and asialo-GM1 antibodies (Wako Pure Chemical Industries, Ltd, Osaka, Japan); or control. After 5 weeks of observation, 10 mice in each group were killed. Tumor diameters, tumor weights, and body weights were measured. The remaining 10 mice in each group were observed for 72 weeks, and survival was determined.

### 2.6. Cytotoxic activity of NK cells

Immediately after the mice were killed, spleen cells were collected and assessed with the use of conventional <sup>51</sup>Cr-labeled YAC-1 cells. The cytotoxic activity of NK cells was calculated by the following formula: cytotoxic activity (%) = [(experimental release – spontaneous release)/(spontaneous release – experimental release)]  $\times$  100. Total release was calculated using YAC-1 cells labeled with 1% NP-40. Spontaneous release was considered equivalent to 11.5% of total release.

### 2.7. Statistical analysis

Statistical analysis was performed with SPSS software (version 11.0). Significant differences among groups were tested by one-way or two-way analysis of variance. The statistical significance of differences among treatment groups was evaluated by Student's *t*-test. Differences were considered significant when *P* values were less than 0.05.

## 3. Results

### 3.1. Physical properties

The numbers of water-generated negative ions and electrically generated negative ions were measured for 300 s. The lifetime of water-generated negative ions was several minutes, longer than that of electrically generated negative ions (Fig. 1).

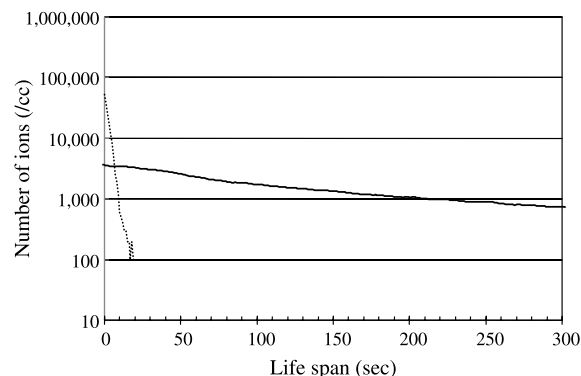


Fig. 1. Life span of WNI and ENI. Life span of WNI (solid line) was several minutes, whereas that of ENI (broken line) was several seconds.

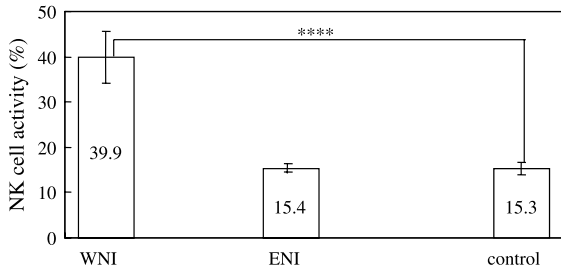


Fig. 2. Comparison of NK cell activity. Mean NK cell activity in mice exposed to WNI was 39.9%, while that of mice exposed to ENI was 15.4% ( $P < 0.0001$ ).

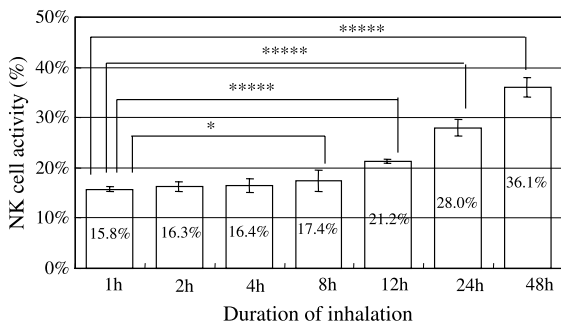


Fig. 3. Relation between NK cell activity and duration of inhalation of WNI. As compared with the value at 1 h (15.8%), the increase in NK cell activity was significant at 8 h (17.4%,  $*P = 0.045$ ) and 12 h (21.2%), 24 h (28.0%), and 48 h (36.1%). ( $**P < 0.0001$ ).

### 3.2. In vivo effects of water-generated negative ions

#### 3.2.1. Comparison of immunologic activation between water-generated negative ions and electrically generated negative ions

The mean cytotoxic activity of NK cells was significantly higher in the mice exposed to water-generated negative ions (39.9%) than in the control group (15.3%). The mean cytotoxic activity of NK cells in the mice exposed to electrically generated negative ions (15.4%) was similar to that in the control group (Fig. 2).

#### 3.2.2. Relation between inhalation time and immunologic activation

The relation between inhalation time and immunologic activation was examined in mice exposed to water-generated negative ions. The cytotoxic activity

of NK cells increased slightly but not significantly to 15.8% after 1 h of exposure to water-generated negative ions. After 8 h of exposure, however, the cytotoxic activity increased significantly to 17.4%. The cytotoxic activity gradually increased to 21.2, 28.0, and 36.1% during continuous exposure to water-generated negative ions (Fig. 3). The cytotoxic activity of NK cells decreased from 36.3 to 23.4% on day 3 after discontinuing exposure to water-generated negative ions and further fell to 16.3% on day 10. These results indicated that the cytotoxic activity of NK cells increased after 8 h of exposure to water-generated negative ions and decreased 3 or more days after discontinuing exposure (Fig. 4).

### 3.3. Antitumor activity

#### 3.3.1. Inhibition of carcinogenesis

Cancer developed in five of the 25 mice exposed to water-generated negative ions and in 22 of the 26 mice exposed to normal air. The cytotoxic activity of NK cells was highest in the cancer-free mice. These

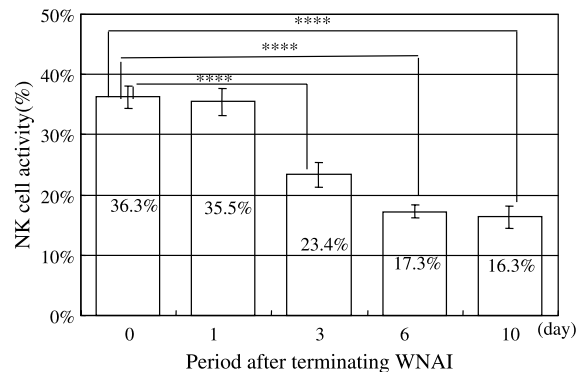


Fig. 4. Relation between NK cell activity and period after terminating inhalation of WNI and NK cell activity. As compared with the value on day 0, NK cell activity was significantly lower on day 3 (23.4%), day 6 (17.3%) and day 10 (16.3%). ( $*P < 0.0001$ ).

Table 1  
Correlation between oncogenesis and exposure to WNI

	Oncogenesis-negative	Oncogenesis-positive
Control	4	22*
NAI	20	5*

\* $P < 0.05$ .

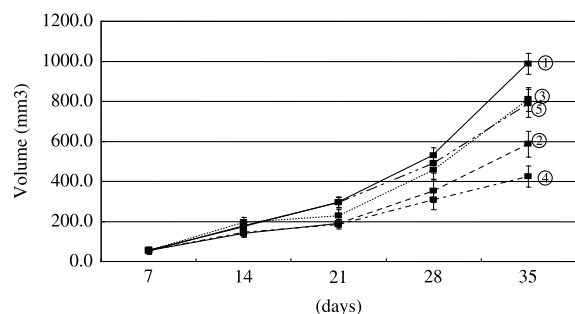


Fig. 5. Tumor volume in the treatment groups. Mean tumor volume in the group treated by TS-1 alone (②) was  $587.0 \pm 65.2 \text{ mm}^3$ . That in the group treated by WNI alone (③) was  $810.1 \pm 59.2 \text{ mm}^3$ . With both TS-1 and WNI (④), mean tumor volume decreased to  $425.5 \pm 52.0 \text{ mm}^3$ . This antitumor effect was reduced by anti-asialo GM-1 antibody (⑤); mean tumor volume in this group was  $789.5 \pm 70.7 \text{ mm}^3$ . Tumor volume in the treated groups were smaller than that in the control group (①),  $987.9 \pm 52.5 \text{ mm}^3$ . ( $P < 0.01$ ). Group: ① control, ② treated by TS-1 alone, ③ treated by WNI alone, ④ treated by TS-1 and WNI, ⑤ treated by TS-1, WNI and anti-asialo GM-1 antibody.

Table 2

Tumor weight, the rate of tumor suppression, body weight, and the rate of increase in body weight in the treatment groups

Group <sup>a</sup>	Tumor weight (g)	Rate of suppression (%) <sup>b</sup>	Body weight (g)	Rate of increase (%) <sup>c</sup>
①	$8.9 \pm 1.2$	0	$19.6 \pm 1.0$	0
②	$5.1 \pm 1.0$	43.7	$23.4 \pm 0.9$	12.0
③	$7.0 \pm 0.7$	21.4	$22.7 \pm 0.8$	11.6
④	$3.9 \pm 0.9$	56.2 <sup>d</sup>	$25.9 \pm 0.8$	32.1 <sup>c</sup>
⑤	$9.6 \pm 1.2$	22.5	$21.2 \pm 1.1$	8.2

<sup>a</sup> ① control, ② treated by TS-1 alone, ③ treated by WNI alone, ④ treated by TS-1 and WNI, ⑤ treated by TS-1 and anti-asialo GM-1 antibody.

<sup>b</sup> Tumor weight ① – Tumor weight / Tumor weight ①

<sup>c</sup> Body weight – Body weight ① / Body weight ①.

<sup>d</sup> Rate of suppression was highest of all groups ( $P < 0.05$ : compared with group ②)

<sup>e</sup> Rate of increase was highest of all groups ( $p < 0.05$ : compared with group ②)

results showed that water-generated negative ions inhibited carcinogenesis (Table 1).

### 3.3.2. Inhibition of tumor growth

After 35 days of treatment, tumor size and weight were lower in the mice given TS-1 plus water-generated negative ions than in the control mice (Fig. 5). Body weight was greatest (Table 2) and

survival was longest in the mice given TS-1 plus water-generated negative ions (Fig. 6). These effects were suppressed by anti-asialo GM1 antibodies, which selectively inhibit the cytotoxic activity of NK cells. These results suggested that water-generated negative ions increased the cytotoxic activity of NK cells, thereby inhibiting tumor growth and improving cachexia.

## 4. Discussion

We obtained three new findings with regard to water-generated negative ions. The first was related to physical properties. The lifetime of water-generated negative ions was longer than that of electrically generated negative ions. Okuyama et al. [7] measured the mobility of air ions produced by different types of generators. Mobility was measured with a cluster

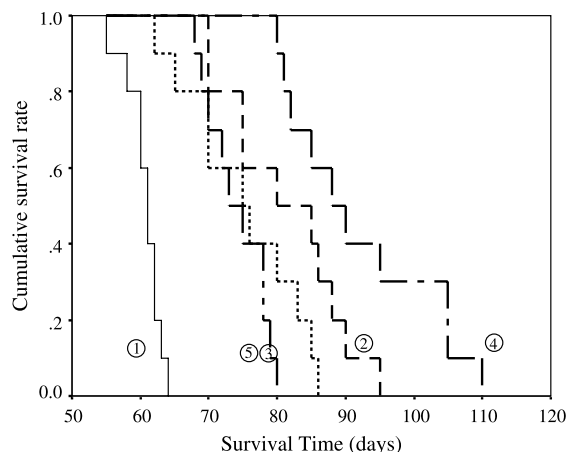


Fig. 6. Cumulative survival rate in the treatment groups. Median survival time in the group treated by TS-1 alone (②) was 85 days. That is the group treated by WNAI alone was (③)76 days. With both TS-1 and WNAI (④), median survival time was prolonged to 90 days. This group survived longest.\* This antitumor effect reduced by anti-asialo GM-1 antibody (⑤), with a median survival time of 75 days. Each treated group survived longer than the control group, 61 days (①). Group: ① control, ② treated by TS-1 alone, ③ treated by WNI alone, ④ treated by TS-1 and WNAI, ⑤ treated by TS-1, WNI and anti-asialo GM-1 antibody. \* $P = 0.0000$ : compared with control group.  $P = 0.0449$ : compared with group treated by TS-1 alone.  $P = 0.0012$ : compared with group treated by WNI alone.  $P = 0.0000$ : compared with group treated by TS-1, WNI and anti-asialo GM-1 antibody.

DMA with a built-in ampere meter. They found that mobility patterns were very similar for ions generated from water by a negative-ion generator and for ions in the air around a natural waterfall. They also found that no water-generated negative ions were smaller than  $10^{-4} \text{ m}^2/\text{V s}$ , whereas the size of the electrically generated negative ions showed a peak distribution at about  $10^{-4} \text{ m}^2/\text{V s}$ . These two types of negative ions were thus shown to clearly differ. These results support the fact that water-generated negative ions are guarded by several to up to 20–30 water molecules and show that large particles move slowly. Okuyama et al. also concluded that large, slowly moving particles have a low probability of colliding with other particles in the air. These findings would explain our results that water-generated negative ions were stable in the air and support the fact that water-generated negative ions existed for several minutes after generation, whereas electrically generated negative ions disappeared within a few seconds. Duan et al. [8] studied the kinetics of inhaled water-generated negative ions or steam produced by a conventional nebulizer, both of which were labeled with  $^3\text{H}$ -thymidine, in mice.  $^3\text{H}$ -thymidine was found in the alveoli of mice that inhaled water-generated negative ions, but not in the alveoli of mice that inhaled steam. These results suggest that water-generated negative ions in inhaled air reach the alveoli via the trachea and are directly absorbed by the blood when gas is exchanged. Yamada et al. [9] studied the effect of water-generated negative ions on erythrocyte aggregation due to hyperlipidemia in mice fed high-cholesterol diets. Iwama [10] reported that negative air ions by water shearing improve erythrocyte deformity and aerobic metabolism.

Exposure to water-generated negative ions was shown to increase the negative charges of the erythrocyte membrane and plasma proteins, thereby increasing interactions among erythrocytes and inhibiting erythrocyte aggregation. There are also many studies of electrically generated negative ions, and the ability of these ions to absorb dust has long been recognized. However, ozone produced at the time of electrical discharge is deleterious to health and can exacerbate atopic dermatitis and elevate adrenaline levels. In our study, electrically generated negative ions had a short life in the air and when inhaled did not

increase NK-cell activity in mice. Therefore, electrically generated negative ions do more harm than benefit.

The second major finding of our study involves immunologic activation. To our knowledge, no previous study has examined the relation between exposure to water-generated negative ions and immunologic activation. We focused on NK-cell activity and studied the relation between exposure to negative water ions and immunologic activation. NK cells are large granular lymphocytes that contain many azurophilic granules in their cytoplasm. These cells have cytotoxic activity against some types of tumor cells and virus-infected cells, without pre-sensitization by antigens or cytokines. We measured the cytotoxic activity of NK cells to evaluate the non-specific activity of the immune system. Kishi et al. [11] reported that NK-cell activity in patients with cancer was significantly lower than that in healthy subjects, and decreased with progression of the clinical stage of disease. Therefore, selection of NK-cell activity as an index of non-specific immune activity may be justified. However, the mechanisms by which NK cells recognize and damage target cells and the receptors of these cells remain unclear. We also did not study the many receptors that control immune recognition. Therefore, the mechanisms by which exposure to water-generated negative ions increases NK-cell activity must await future research.

The third major finding of our study involves anti-tumor activity. To evaluate the anti-tumor activity of water-generated negative ions, we studied inhibition of carcinogenesis and tumor growth. NK cells have long been considered to have a surveillance function against cancer cells, which has been studied previously. The loss of NK-cell activity and suppression of the non-specific immune system in mice increases the incidence of spontaneous cancers and markedly lowers resistance to disease progression. We therefore, studied the relation between water-generated negative ions and carcinogenesis in mice given a subcutaneous injection of methylcholanthrene, a chemical carcinogen. Methylcholanthrene was synthesized from deoxycholic acid, the principal component of bile, by Wieland et al. This aromatic hydrocarbon was shown to be a potent carcinogen by Cook et al. [12]

Ghoneum et al. [13] reported that cancerous nodules developed 60 days after subcutaneous injection of methylcholanthrene. In our study, the incidence of cancer was significantly lower in mice exposed to water-generated negative ions (20%) than in the control group (85%).

To study the anti-tumor effects of water-generated negative ions in tumor-bearing mice, we used a newly developed anti-tumor drug, TS-1, which combines three pharmacological agents, tegafur, 5-chloro-2,4-dihydropyridine, and potassium oxonate, in a ratio of 1:0.4:1. TS-1 plays a key role in the treatment of gastric and other gastrointestinal cancers. In our study, inhibition of tumor growth was minimal in mice exposed to water-generated negative ions alone, but was greatest in mice exposed to water-generated negative ions plus TS-1. The effect was inhibited by concurrent treatment with asialo-GM1 antibodies, which selectively inhibit NK-cell activity. Herberman et al. [14] demonstrated that asialo-GM1 antibodies decrease NK cell activity and thereby augment the growth of transplanted tumors, increase tumor-related mortality, and promote metastasis. Our results suggest that water-generated negative ions enhance NK-cell activity, thereby inhibiting tumor growth in tumor-bearing mice. TS-1 alone significantly prolonged survival in tumor-bearing mice, whereas TS-1 plus water-generated negative ions prolonged survival significantly more. The increase in body weight was significantly greater in mice treated with TS-1 plus water-generated negative ions than in the other groups. These results suggest that a combination of anticancer drug and water-generated negative ions may improve cachexia in tumor-bearing animals. Ades et al. [15] and Nordman et al. [16] showed that chemotherapy with 5-fluorouracil suppressed the immune system and did not increase NK cell activity. Combined treatment with water-generated negative ions and anticancer drugs may therefore be useful in patients with cancer. Kishida et al. [11] used flow cytometry with fluorescent dye to quantify the cytotoxic activity of NK cells in humans. This technique was referred to as the PI (Propidium Iodide)–NK method. Given the effects of water-generated negative ions on NK-cell activity in our study, future investigations should use the PI–NK method to examine the relation between exposure to water-generated negative ions

and the cytotoxic activity of NK cells in patients with cancer.

## 5. Conclusion

We studied physical properties, immunologic activity, and anti-tumor activity of water-generated negative ions. Water-generated negative ions have a long life in air and do not adversely affect the body in vivo. In addition, water-generated negative ions have anti-tumor activity, as demonstrated in mice by inhibition of carcinogenesis, suppression of tumor growth, and improvement of cachexia.

Our study showed that inhaled water-generated negative ions enhance NK cell activity, leading to anti-tumor effects. The mechanisms by which inhaled water-generated negative ions increase NK cell activity and clinical applications of negative ions are topics for future research.

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