Effects and applications of ozonated water on tumors

腫瘍に対するオゾン水の影響およびその応用

The United Graduate School of Veterinary Science

Yamaguchi University

Kohei KURODA

March 2018

Doctor's Thesis

The United Graduate School of Veterinary Science

Yamaguchi University

Effects and applications of ozonated water on tumors

腫瘍に対するオゾン水の影響およびその応用

Kohei KURODA

March 2018

Laboratory of Veterinary Imaging diagnosis,

Department of Veterinary Clinical Medicine,

Tottori University

CONTENTS

Page No.

CHAPTER Ⅱ

The Indirect Effects on Tumor and Applications of Ozonated Water

GENERAL INTRODUCTION

Ozone is an active form of oxygen consisting of three oxygen atoms that is generated from diatomic oxygen by ultraviolet light and high voltage. Ozone gas is clear, colorless, and smells slightly like grass. Moreover, it is very unstable and decomposes into diatomic oxygen within a few hours in air, and has a very short half-life of approximately 30 min in water (Viebahn-Haensler, et al., 2007). Because ozone decomposes and returns to oxygen, it does not remain in the environment. In addition, it has strong oxidative effect and is applied to sterilization, deodorization, decolorization.

Ozone therapy has been receiving increasing attention in Europe in recent years. The primary form of ozone therapy is major autohemotherapy (MAH). In MAH, patient blood is mixed with ozone gas before being retransfused. It has been known for its beneficial effects in cardiovascular diseases, infections, cancer, rheumatoid arthritis, and osteoarthritis (Clavo, et al., 2004). Additionally, MAH in patients with cancer may improve tumor hypoxia and enhance sensitivity to both chemotherapy and radiation therapy (Clavo, et al., 2004). In a clinical setting, a combination of radiation therapy and MAH or rectal administration of an ozone/oxygen gas

mixture achieved the same effects as a combination of radiation therapy and chemotherapy in preventing progression of head and neck cancer(Clavo, et al., 2004) .

While ozone gas therapy has many advantages, it has not been widely used in medical settings. It seems that the reason is largely due to risk of ozone. Ozone gas is toxic to the respiratory tract of the lung (Bhalla, 1999; Pryor, 1995). The limitation of ozone concentration is established in each country. In Japan, the Japan Society for Occupational Health has established an acceptable concentration of ozone in the working environment as 0.1 ppm.

On the other hand, although ozone water is used industrially, it is not common in the medical field. However, ozonated water which means the water dissolved ozone gas has no reported side effects, and it is much easier to handle than ozone gas. Despite these advantages, however, ozonated water has not found widespread application in medicine because it is difficult to obtain an exact concentration of ozone in water. Recently, a device has been developed that can produce ozonated water to an exact concentration (Azuma et al., 2014). In this report, we studied about Effects and applications of ozonated water on tumors in order to establish the new ozone therapy using ozonated water for tumor.

CHAPTER I

The Safety and Direct Effects on Tumor of Ozonated Water

ABSTRACT

Ozonated water is easier to handle than ozone gas. However, there have been no previous reports on the biological effects of ozonated water. We conducted a study on the safety of ozonated water and its anti-tumor effects using a tumor-bearing mouse model and normal controls. Local administration of ozonated water (208 μM) was not associated with any detrimental effects in normal tissues. On the other hand, local administration of ozonated water (20.8, 41.6, 104, or 208 μM) directly into the tumor tissue induced necrosis and inhibited proliferation of tumor cells. There was no significant difference in the number of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL)-positive cells following administration of ozonated water. The size of the necrotic areas was dependent on the concentration of ozonated water. These results indicate that ozonated water does not affect normal tissue and damages only the tumor tissue by selectively inducing necrosis. There is a possibility that it exerts through the production of reaction oxygen

species (ROS). In addition, the induction of necrosis rather than apoptosis is very useful in tumor immunity. Based on these results, we believe that administration of ozonated water is a safe and potentially simple adjunct or alternative to existing antineoplastic treatments.

INTRODUCTION

Ozone is an active form of oxygen consisting of three oxygen atoms that is generated from diatomic oxygen by ultraviolet light and high voltage. Ozone gas is clear, colorless, and smells slightly like grass. Moreover, it is very unstable and decomposes into diatomic oxygen within a few hours in air, and has a very short half-life of approximately 30 min in water (Viebahn-Haensler, et al., 2007). Ozone therapy has been receiving increasing attention in Europe in recent years. In this regard, major autohemotherapy (MAH), the main form of ozone therapy, is known for its beneficial effects as adjunct therapy for cardiovascular disease, infection, cancer, rheumatoid arthritis, and osteoarthritis (Nogales, et al., 2008). In MAH, the patient's blood is mixed with ozone gas before being retransfused. A previous report indicated that MAH in patients with cancer may improve tumor hypoxia and enhance sensitivity to both chemotherapy and radiation therapy (Clavo, et al., 2004). In a clinical setting, a combination of

radiation therapy and MAH or rectal administration of an ozone/oxygen gas mixture achieved the same effects as a combination of radiation therapy and chemotherapy in preventing progression of head and neck cancer (Clavo, et al., 2004). However, there have been no reports on the direct effects of ozone gas on tumor cells in vivo, and to the best of our knowledge, only the in vitro effects have been studied (Bucci, et al. , 2006; Sweet, et al., 1980).

While ozone gas therapy has many advantages, it has not been widely used in medical settings since ozone gas is toxic to the respiratory tract of the lung (Bhalla, 1999; Pryor et al., 1995). In contrast, ozonated water which means the water dissolved ozone gas has no reported side effects, and it is much easier to handle than ozone gas. Despite these advantages, however, ozonated water has not found widespread application in medicine because it is difficult to obtain an exact concentration of ozone in water. Recently, a device has been developed that can produce ozonated water to an exact concentration (Azuma et al., 2014). We made use of this device to study the safety and anti-tumor effects of ozonated water through the administration of ozonated water to normal and tumor-bearing mice.

MATERIALS & METHODS

Preparation of Ozonated Water

The device producing ozonated water was provided by Sakuragawa Pump Co., Ltd., (Osaka, Japan). This device can produce ozonated water from O_2 and water and can regulate the concentration of the dissolved O_3. In all experiments, ozonated water was administered within 10 min after production.

Animals

BALB/c mice (4–5 week-old, female) were purchased from CLEA Japan, Inc., (Osaka, Japan). All mice were maintained under conventional conditions. The study was carried out according to Tottori University animal experiment rule. The use of these animals and the procedures undertaken were approved by the Animal Research Committee of Tottori University (Permit Number: 13T4). All treatments were performed under anesthesia induced by inhalation of 3%–5% isoflurane and all efforts were made to minimize suffering.

Preparation of the Tumor-Bearing Mouse Model

Mouse cell line derived from mouse rectal cancer, Colon 26 (RCB2657), was obtained from by the RIKEN BioResource Center (BRC) through the National Bio-Resource Project of the MEXT (Ibaraki, Japan). The tumor-bearing mouse model was prepared as previously described, with slight modification (Nitta et al., 2013). In brief, 1×10^6 colon 26 cells (1×10^7 cells/mL) were subcutaneously injected into the dorsal regions in BALB/c mice. Mice whose tumors grew to a diameter of 7–10 mm were used in the experiments.

Effects of Administration of Ozonated Water into Normal Tissue

Mice were randomized into three groups: intraperitoneal administration (IP) group, subcutaneous administration (SC) group, and intramuscular administration (IM) group. In the IP and SC groups, 1 mL of 208 μM ozonated water was administered under anesthesia induced by inhalation of 3%–5% isoflurane. In the IM group, 0.1 mL of 208 μM ozonated water was administered. Ozonated water was administered for three days. Twenty-four hours after the last administration, all mice were euthanized by cervical dislocation under anesthesia induced by inhalation of 3%–5% isoflurane. Each organ, as well as samples of subcutaneous tissue and muscle, were observed macroscopically and fixed in 10% buffered formalin.

Concentration and Time-Dependent Effects of Ozonated Water after Local Injection in Tumor Tissue

Mice were randomized into six groups: non-treatment (NT) group; local administration of sterile distilled water (solvent control) into the tumor tissue (C); local administration of 20.8 μM ozonated water into the tumor tissue (O_3-1); local administration of 41.6 μM ozonated water into the tumor tissue (O_3-2); local administration of 104 μM ozonated water into the tumor tissue (O_3-3) (n = 3 in each group); and local administration of 208 μM ozonated water into the tumor tissue (O_3-4) (n = 9). One milliliter of sterile distilled or ozonated water was administered under anesthesia induced by inhalation of 3%–5% isoflurane. In the NT, C, O_3-1, O_3-2, and O_3-3 groups, all mice were euthanized by cervical dislocation and the tumors were resected 24 h after administration. In the O_3-4 group, all mice were euthanized by cervical dislocation and the tumors were resected one, three, and seven days after administration (O_3-4/1 day, O_3-4/3 day and O_3-4/7 day groups, each n = 3). The tumors were fixed in 10% buffered formalin.

The Effects of Local Administration of Ozonated Water on Tumor Growth

Mice were randomized into three groups: NT group (n = 5); local administration of sterile distilled water (solvent control) into the tumor tissue (C, n = 5); and local administration of ozonated water into the tumor tissue (O_3, n = 6). The volumes of the tumor tissues were calculated as follows: (mediastinum × transverse line × depth × π)/6 (mm^3). After calculations of the tumor volumes, distilled water or 208 μM of ozonated water was locally administered into the tumor tissues (0.2 mL/head, day 1). After three days (day 4), a second local injection of distilled water or 208 μM ozonated water was given. On day 7, mice were euthanized by cervical dislocation under anesthesia induced by inhalation of 3%–5% isoflurane. Based on the tumor volumes on days 1 and 7, the tumor growth rates were calculated as follows: (tumor volume on day 7 − tumor volume on day 1)/7 (mm^3/day). The tumors were fixed in 10% buffered formalin.

Histological Evaluation and Image Analysis

Thin sections (5 μm) were cut from each sample for histological observation and stained with hematoxylin and eosin. Each section was examined microscopically and quantitative digital morphometric analysis of necrotic areas was performed. The color

wavelengths of the copied images were transformed into digital readings using the Lumina Vision software program (Mitani Corporation, Tokyo, Japan), which allows for quantification of the various color wavelengths with pixels as the unit of measurement. The percentage of necrosis in the tumor tissues was calculated by dividing the total pixel area of the necrotic area by the total pixel area corresponding to the total tumor tissue in the field of view (Nitta et al., 2013).

Apoptosis of the tumor tissues was investigated using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) staining. Staining was performed according to previously described methods (Azuma et al., 2012). Each section was examined microscopically and the number of TUNEL-positive cells in each tumor was calculated. Cell counts were calculated in five fields, excluding necrotic regions, at 400× magnification using five mice in each group. The mean score of 25 fields was considered the number of TUNEL-positive cells in each group.

Statistical Analysis

Data were expressed as the mean ± standard error (SE). Statistical analyses were first performed using one-way analysis of variance (ANOVA) and compared using the Tukey–Kramer test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effects of Administration of Ozonated Water into Normal Tissue

In the intraperitoneal administration (IP) group, no abnormalities such as edema of the organs or ascites were observed macroscopically (Figure 1A). No abnormalities were observed in any of the examined organs (liver, spleen, kidney, and small intestine) on histopathological examination (Figure 2A–D). In the subcutaneous administration (SC) group, no subcutaneous abnormalities were observed macroscopically (Figure 1B). In the intramuscular administration (IM) group, no abnormalities such as claudication were observed in any mice. No abnormalities were observed on histopathological examination either (Figure 2E).

Concentration and Time-Dependent Effects of Ozonated Water after Local Injection in Tumor Tissue

Necrosis was observed around the injection site in all groups except for the non-treatment (NT) group. In the O_3-1, O_3-2, and O_3-3 groups, nuclei were not observed in the tumor cells in the centermost areas of necrosis. Pyknotic nuclei were abundant at the edges of the necrotic region (Figure 3). In the O_3-3 group, the area of necrosis was particularly broad and the area in which nuclei were not observed in the tumor cells was larger compared to the O_3-1 and O_3-2 groups (Figure 3E). In the O_3-4/1 day, O_3-4/3 day, and O_3-4/7 day groups, necrosis in the tumor tissues was observed to about the same extent as that in the other ozonated water groups (Figure 4). However, viable tumor cells were observed in the necrotic area in the O_3-4/7 day group (Figure 4E). In the NT and local administration of sterile distilled water (solvent control) into the tumor tissue (C) groups, many pyknotic nuclei were seen in the necrotic areas (Figure 5). In the NT group, pyknotic nuclei were more frequent in the centermost regions of tumor tissue compared with other groups (Figure 5A, B).

The Effects of Local Administration of Ozonated Water on Tumor Growth

The tumor growth rate in the O_3 group (9.9 ± 2.7 mm^3/day) was significantly decreased compared to that of the NT group (55.3 ± 16.0 mm^3/day) ($p < 0.05$, Figure 6). The tumor growth rate in the C group was 32.9 ± 8.1 mm^3/day. No significant difference was seen between the O_3 and C groups. However, a tendency was observed toward decreased rates of tumor growth in the O_3 group compared to the C group.

The area of tumor necrosis was significantly increased in the O_3 group ((26.0% ± 5.2%)/field), compared to that in the NT group ((8.3% ± 1.5%)/field) ($p < 0.05$, Figure 7). Meanwhile, the area of tumor necrosis in the C group was (18.5% ± 2.6%)/field, with no significant difference from that in the O_3 group. However, a tendency toward increased tumor necrosis was seen in the O_3 group compared to the C group. The number of TUNEL-positive cells in the three groups did not significantly differ (Table 1).

DISCUSSION

It has been shown that ozonated water has antibacterial effect(Restaino, et al., 1995; Scott et al., 1963). Ozmen et al. reported that “ozonated saline was effective as irrigation for

treating experimental peritonitis rats" (1993). To the best of our knowledge, the side-effects of ozonated water have not yet been reported. In the present study, it was found that ozonated water did not affect normal tissue. Moreover, ozonated water was found to inhibit tumor growth through the induction of necrosis of tumor cells. During the experimented period, no detrimental effects were observed following local administration of ozonated water. These results indicate that administration of ozonated water has low toxicity and is safe and very useful in anti-tumor treatment. However, these results are only indicative of a short-term effect. Therefore, further information regarding the potential long-term effects of ozonated water is needed.

Necrosis of the tumor cells was s following local injection of ozonated water directly into the tumor tissues. Moreover, the tumor growth rate was significantly decreased and there was a significant increase in the tumor necrotic area. However, there were no significant differences among the three groups in the number of TUNEL-positive cells. These results indicate that local administration of ozonated water does not induce apoptosis, although it does act directly on the tumor cells. It has been reported that ozone gas directly inhibits neoplastic cell growth in the neuroblastoma SK-N-SH cell line in vitro by modulating the cell cycle (Bucci

et al., 2006). However, to the best of our knowledge, no previous studies have investigated the direct effects of ozone on tumor tissue in vivo. This report is the first regarding the direct effects of ozone on tumor tissue.

In this study, the tendency that the tumor growth rate is decreased and the area of tumor necrosis is increased was observed in C group. However, these data were not significantly compared to that in NT group. We think that it was induced by physical injury when water and/or osmotic pressure was injected.

Many tumor cells have few antioxidant materials, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, in comparison with normal cells (Ahmad et al., 2005). Therefore, the tumor cells are greatly affected by reactive oxygen species (ROS). It is well known that in a variety of anticancer agents, such as adriamycin and cis-diamminedichloroplatinum (II), ROS play important roles in the cytotoxic effects on tumor cells (Alegria, et al., 1989; Sasada et al., 1996). High-dose ascorbic acid therapy controls the growth of aggressive tumors by producing ROS (Chen et al., 2008). ROS can interact with numerous cellular components, including DNA, lipids, and proteins (Bayr, 2005). The consequences of these interactions include losses of cell integrity, enzyme function, and genomic stability (Gille,

et al., 1994; Halliwell, 1999). When ozone is administered in vivo, it is well known that ROS such as hydrogen peroxide (H_2O_2) are generated (V Bocci et al., 1998). Therefore, the anti-tumor mechanism of ozonated water is likely to also be the cell injury caused by ROS. It will be necessary to verify the association between ROS and anti-tumor effects of ozonated water using ROS inhibitors.

The necrotic cell releases danger signals such as deoxyribonucleic acid (DNA), high mobility group box 1 protein (HMGB1), and uric acid (Gallucci, et al., 2001; Ghaemi-Oskouie, et al. 2011; Scaffidi, et al., 2002). On the other hand, the apoptotic cell does not release these signals (Gallucci, et al., 2001; Matzinger, 2002). An antigen-presenting cell (APC), particularly the dendritic cell (DC), matures following stimulation by cytokines and danger signals, after uptake of the tumor antigen. In conditions in which danger signals are lacking, APCs cannot mature and activate T cells. Therefore, the growth of tumors may be promoted under the non-existence of danger signals. In a previous report, a decrease in metastasis and an extension in the duration of survival were observed in a mouse tumor necrosis model compared to a mouse tumor apoptosis model (Sabel, et al., 2010). Therefore, the induction of necrosis rather than apoptosis by administration of ozonated water is very useful in tumor immunity.

It has been reported that the production of several cytokines including interferon (IFN)-α, IFN-β, and IFN-γ, interleukin (IL)-1, IL-2, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor was induced after human blood, heparinized platelet-rich plasma, and Ficoll-purified blood mononuclear cells were ozonated(Bocci, et al., 1990; Bocci, et al., 2007; Paulesu, et al., 1991; Valacchi, et al., 1999). Previous reports have indicated that cytokines inhibit both tumor growth and metastasis(Aulitzky et al., 1994; Bonnem, 1991; T. D. Brown et al., 1993; Kaplan et al., 1998; Richtsmeier, et al., 1990). Therefore, cytokines may also participate in the growth restraint of tumors. Further studies on the kinetics of these cytokines following administration of ozonated water will be needed to determine their roles in the antineoplastic activity of ozonated water.

Importantly, the size of the necrotic area in the current study was dependent on the concentration of the ozonated water. Concentration-dependent growth inhibition has been reported in vitro in human cancer cells from lung, breast, and uterine tumors following treatment with ozone gas (Sweet et al., 1980). Herein, when the observation time was extended, viable tumor cells were observed in the necrotic area in the O_3-4/168 h group. This result

indicates that a single administration of ozonated water cannot achieve complete destruction of tumor cells, and thus repeated administration is required.

In this study, it is suggested that ozonated water has an anti-tumor effect. There is a possibility that it is exerted through the production of ROS. Moreover, ozonated water has low toxicity and is safe and easy to handle. On the other hand, ozone gas is dangerous and not easy to handle. This is because ozone gas is toxic to the respiratory tract of the lung(Bhalla, 1999; Pryor et al., 1995). Administration of ozonated water is a safe and potentially simple adjunct or alternative to existing antineoplastic treatments.

FIGURES & TABLE

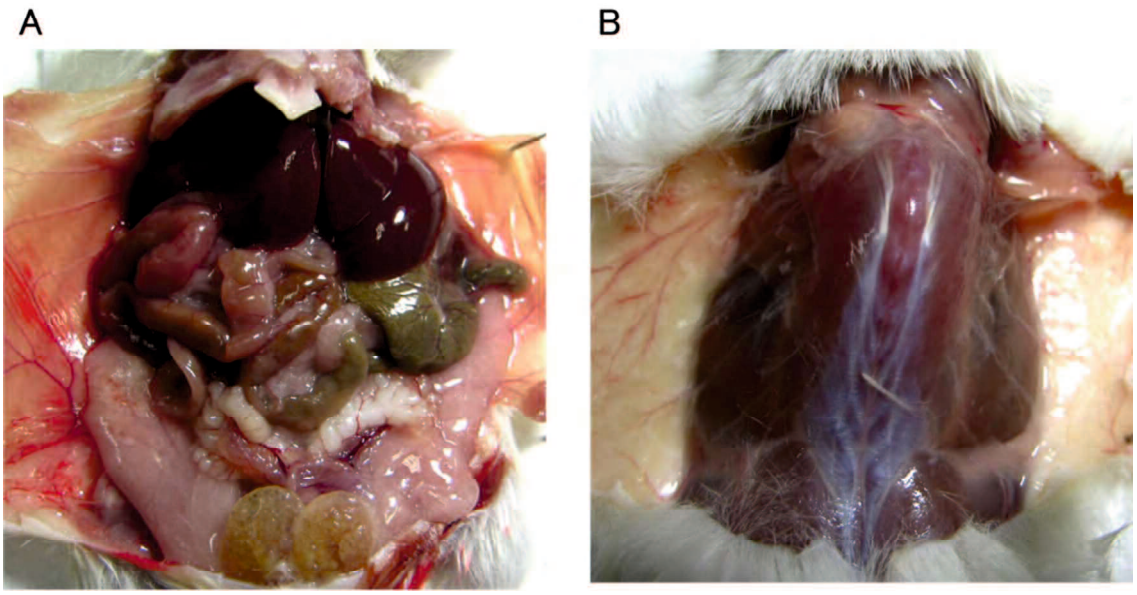

Figure 1. Macroscopic images of the organs in the abdominal cavity (**A**) and subcutaneous tissue (**B**). No abnormalities such as edema of the organs or ascites were observed.

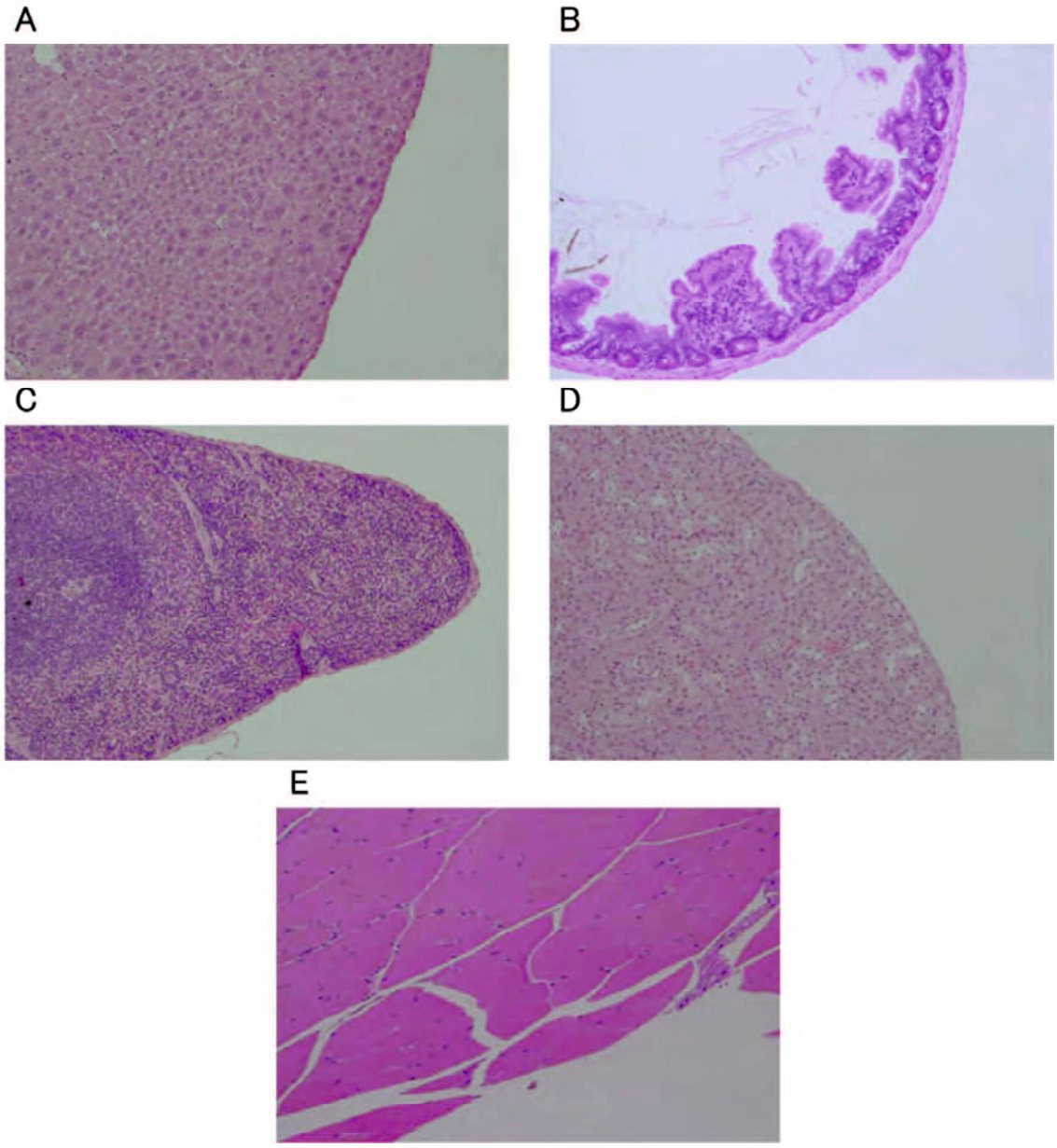

Figure 2. Histological images of normal tissues of the liver (**A**), small intestine (**B**), spleen (**C**), kidney, (**D**) and muscle (**E**) 24 h after the last administration of ozonated water. All sections were observed (100×) after staining with hematoxylin and eosin.

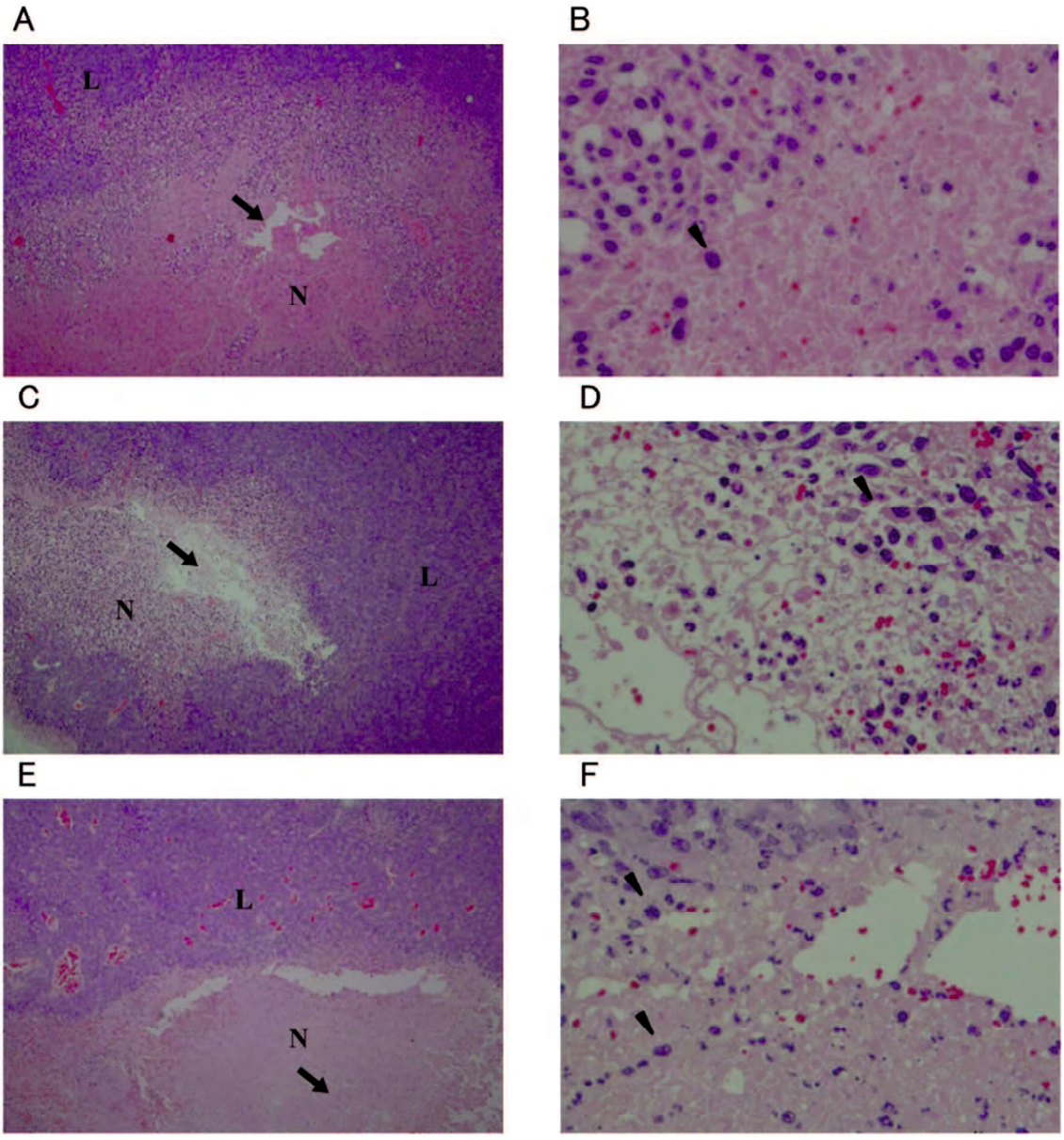

Figure 3. Histological images of tumors 1 day after administration of 20.8, 41.6, or 104 μM ozonated water. Low magnification (40×): (**A**), (**C**) and (**E**). High magnification (400×): (**B**), (**D**) and (**F**). The concentration of ozonated water was 20.8 μM (**A**,**B**), 41.6 μM (**C**,**D**), or 104 μM (**E**,**F**). All sections were stained with hematoxylin and eosin. N: necrotic area; L: viable tumor area; arrow: site of injection; arrowhead: pyknotic nuclei.

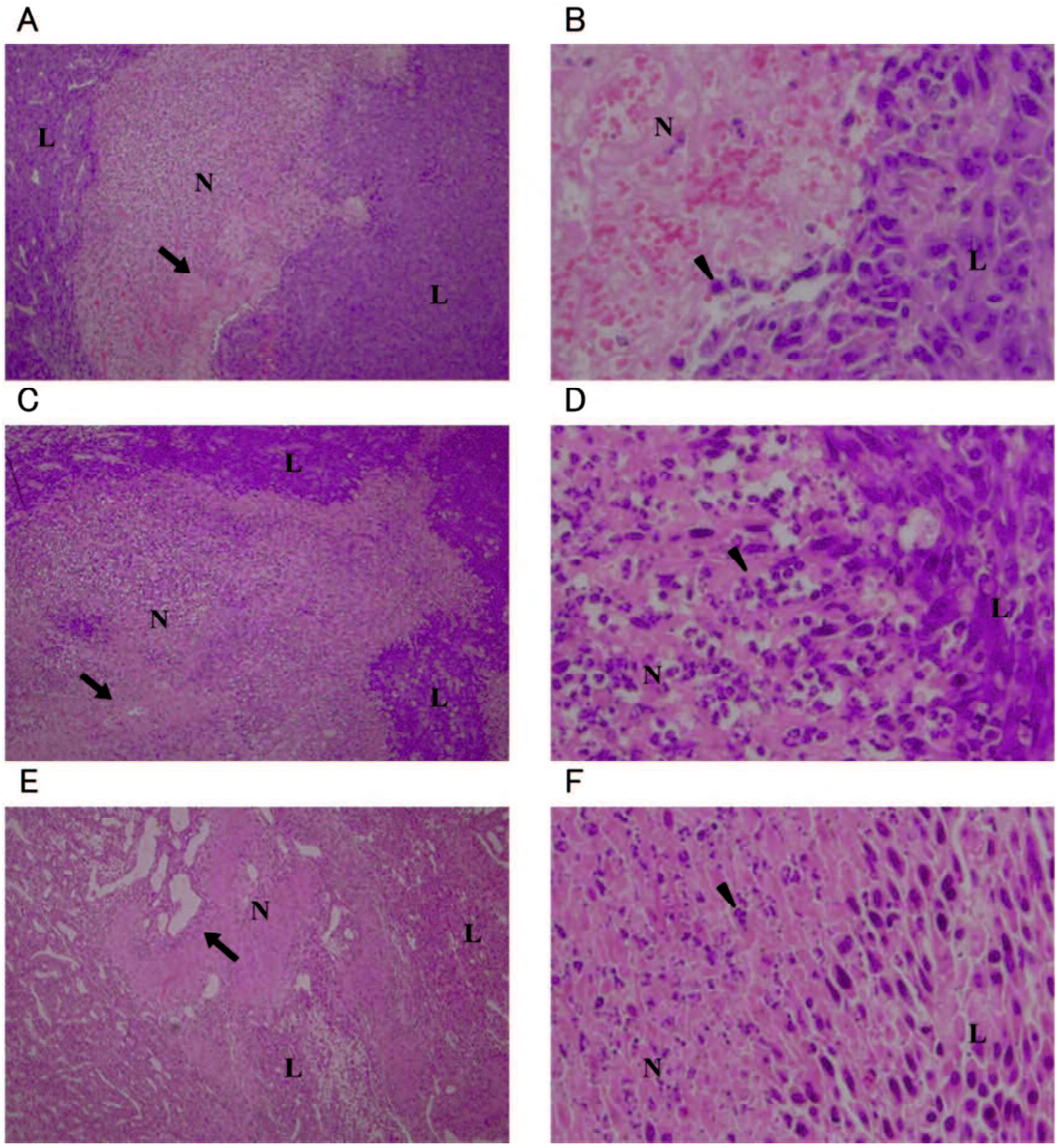

Figure 4. Histological images of tumors after administration of 208 μM ozonated water. the O_3-4/1 day group is (**A**) and (**B**). O_3-4/3 day group is (**C**) and (**D**). O_3-4/7 day group is (**E**) and (**F**). Low magnification (40×): (**A**), (**C**), and (**E**). High magnification (400×): (**B**), (**D**) and (**F**). All

sections were stained with hematoxylin and eosin. N: necrotic area; L: viable tumor area; arrow: site of injection; arrowhead: pyknotic nuclei.

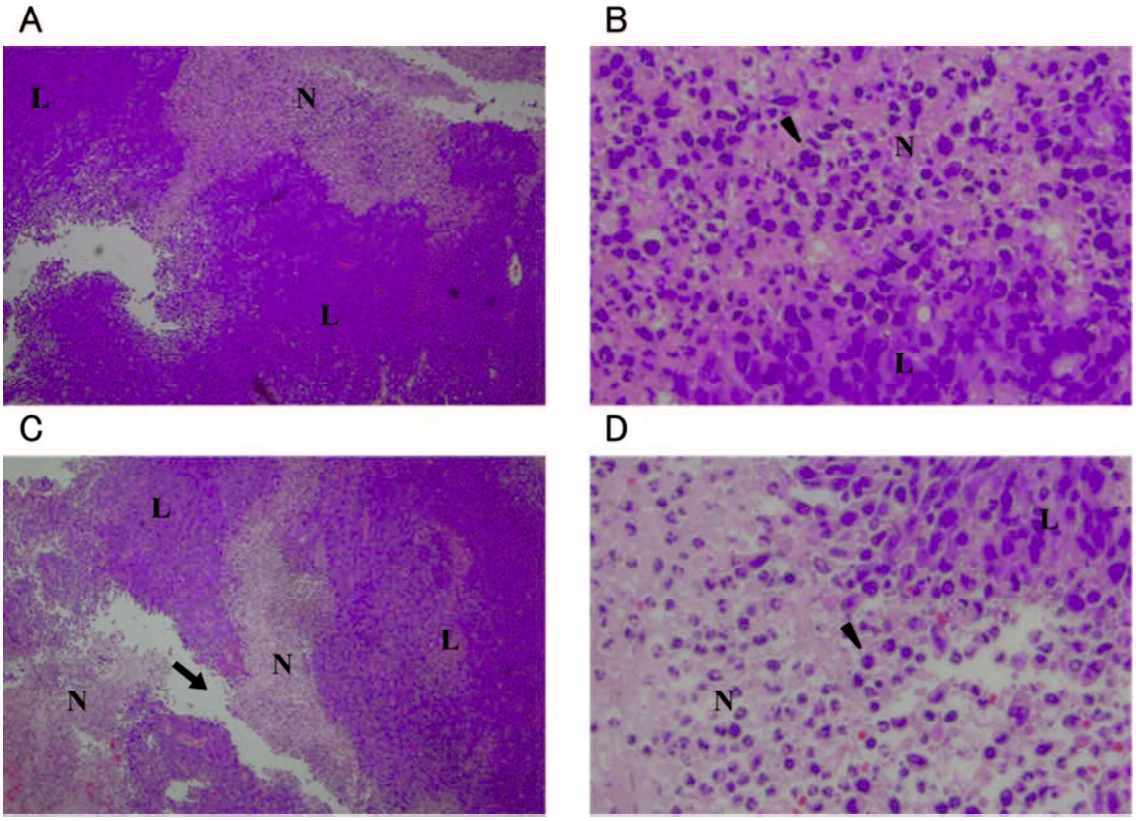

Figure 5. Histological images of tumors that were either untreated or administered sterile distilled water, after 24 h. Non-treated (**A**, **B**); treated with sterile distilled water (**C**,**D**). Low magnification (40×): (**A**) and (**C**); High magnification (400×): (**B**) and (**D**). All sections were stained with hematoxylin and eosin. N: necrotic area; L: viable tumor area; arrow: site of injection; arrowhead: pyknotic nuclei.

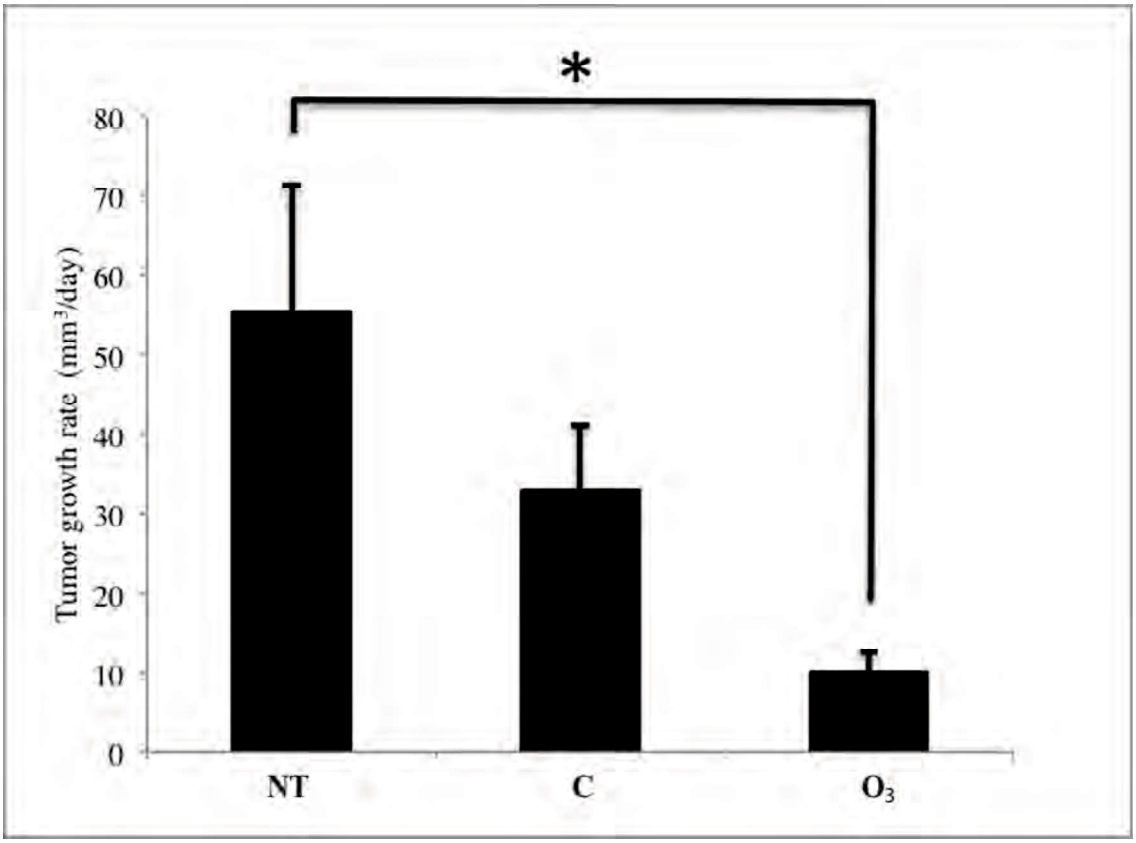

Figure 6. The effect of local administration of ozonated water on rates of tumor growth. The data represent the mean ± SE; n=5 in the NT and C groups, n=6 in the O_3 group. * $p < 0.05$ compared to the NT group by using the Tukey–Kramer test. NT: non-treatment group; C: local administration of sterile distilled water group; O_3: local administration of ozonated water group.

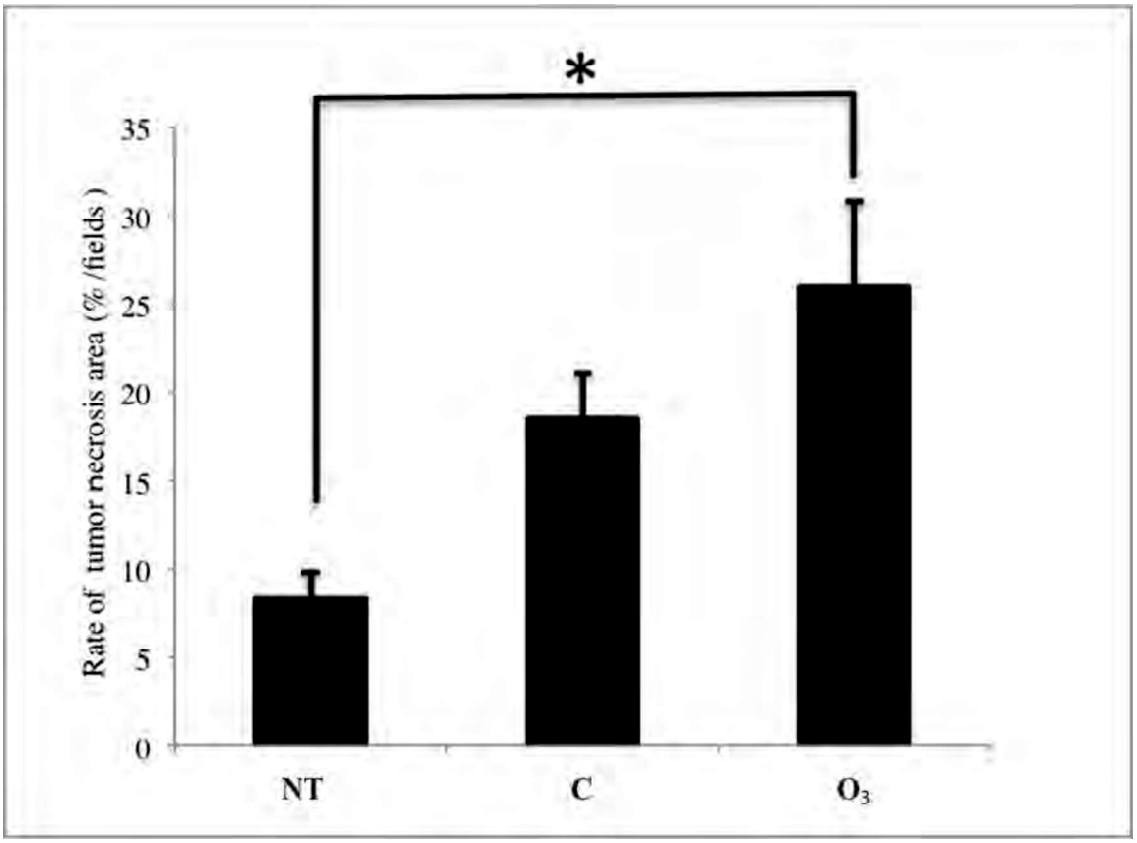

Figure 7. The effect of local administration of ozonated water on size of areas of tumor necrosis. The data represent the mean ± SE; $n = 5$ in the NT and C groups, $n = 6$ in the O_3 group. * $p < 0.05$ compared to the NT group by using the Tukey–Kramer test. NT: non-treatment group; C: local administration of sterile distilled water group; O_3: local administration of ozonated water group.

Table 1. The number of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) positive cells in tumors.

	NT	**C**	**O_3**
TUNEL positive cells (cells/field)	7.92 ± 0.63	9.24 ± 0.61	9.34 ± 1.02

The data represent the mean ± SE; n = 5 in the NT and C groups, n = 6 in the O_3 group. NT: non-treatment group; C: local administration of sterile distilled water group; O_3: local administration of ozonated water group.

CHAPTER II

The Indirect Effects on Tumor and Applications of Ozonated Water

ABSTRACT

Tumor hypoxia is a severe problem affecting tumor therapy because it reduces the sensitivity of chemotherapy and radiation therapy. Ozone has been known to improve peripheral blood perfusion and oxygen partial pressure. We studied the effect of ozonated water on tumor hypoxia, alone and in combination with an antitumor drug. After intraperitoneal administration of ozonated water to colon-26 (C26)-bearing mice, the Hoechst33342-positive area and the intratumoral oxygen partial pressure significantly increased. The tumor growth rate was more suppressed when ozonated water was combined with cisplatin (CDDP) than with CDDP alone. The number of Ki-67-positive cells significantly decreased, while that of TUNEL-positive cells significantly increased. This study showed that ozonated water increases intratumoral blood perfusion and improves tumor hypoxia. In addition, ozonated water increased the therapeutic effect of CDDP. These findings as well as previous reports suggest that tumor growth is suppressed on treatment with ozonated water because the amount of CDDP reaching the tumor

is increased when the intratumoral blood perfusion is increased because of ozonated water. Thus, the administration of ozonated water could be a new therapeutic approach to solve current concerns plaguing antitumor treatment.

INTRODUCTION

Ozone is an active form of oxygen, consisting of three oxygen atoms generated from diatomic oxygen by ultraviolet light and high voltage. Ozone gas is clear, colorless, and smells slightly like grass (Viebahn-Haensler, et al., 2007). Ozone therapy has been receiving increasing attention in Europe and is known for its beneficial effects in reperfusion injury, infections, and cancer (Nogales et al., 2008).

Ozonated water is the liquid form generated when ozone is dissolved in saline, which is much easier to handle than the gaseous form. The safety and direct antitumor effect of ozonated water have been previously described (Kuroda et al., 2015).

The primary form of ozone therapy is major autohemotherapy (MAH). In MAH, patient blood is mixed with ozone gas before being retransfused. It has been known for its

beneficial effects in cardiovascular diseases, infections, cancer, rheumatoid arthritis, and osteoarthritis (Clavo, et al., 2004).

Ozone gas is known to improve peripheral blood perfusion. Ozone therapy in tumor patients improves intratumoral oxygen partial pressure (Clavo, et al., 2004). Tumor hypoxia is a serious concern affecting tumor therapy because it reduces the sensitivity of chemotherapy and radiation therapy (Bertout, et al., 2008; Gardner et al., 2001; Semenza, 2003; Teicher, 1994). Therefore, it is expected to improve the sensitivity of antitumor therapy. In clinical settings, the combination of radiation therapy and MAH or rectal administration of ozone gas/oxygen gas mixture has helped achieve the same effects as those obtained by using a combination of radiation therapy and chemotherapy in preventing the progression of head and neck cancer (Clavo, et al., 2004). Reports related to chemotherapy mostly discuss side effects; few reports discuss therapeutic effects (Borrego et al. 2004; Borrego et al. 2006; González et al. 2004). To the best of our knowledge, no other study has discussed ozone therapy using ozonated water. Therefore, a new approach using ozonated water was examined to solve concerns around antitumor treatment. In this report, we decided to study the effect of ozonated water on tumor hypoxia, alone and in combination with an antitumor drug.

MATERIALS & METHODS

Preparation of Ozonated Water

The device producing ozonated water was provided by Sakuragawa Pump Co., Ltd., (Osaka, Japan). This device can produce ozonated water from O_2 and water and can regulate the concentration of dissolved ozone. In all experiments, the concentration of ozonated water was 208 μM and it was administered within 10 min of production.

Animals

BALB/c mice (4 to 5-week-old, female) were purchased from CLEA Japan, Inc., (Osaka, Japan). All mice were maintained under conventional conditions. The study was performed according to the rules put down by the Tottori University. The use of these animals and the procedures undertaken was approved by the Animal Research Committee of Tottori University. All treatments were performed under anesthesia, induced by the inhalation of 3–5% isoflurane; all efforts were made to minimize suffering.

Preparation of the Tumor-Bearing Mouse Model

The mouse cell line derived from the Colon 26 (RCB2657), the mouse colorectal cancer model, was obtained from the RIKEN BioResource Center (BRC) through the National Bio-Resource Project of the MEXT (Ibaraki, Japan). The tumor-bearing mouse model was prepared as described previously, with slight modification (Nitta et al., 2013). In brief, 1×10^6 cells (1×10^7 cells/mL) were subcutaneously injected into the dorsal region of BALB/c mice. Mice whose tumors grew to a diameter of 7–10 mm were included in the study.

Measurement of Intratumoral Oxygen Partial Pressure

Mice were randomized into two groups: sterile saline (S) group and ozonated water (O_3) group (n = 5 per group). Oxygen and indifferent electrodes (Bio Research Co., Nagoya, Japan) were inserted into the center of the tumor. After intraperitoneal administration of sterile saline or ozonated water (0.2 mL/head), intratumoral oxygen partial pressure was measured by polarography (Bio Research Co.), every 10 min immediately after administration up to 150 min.

Measurement of Blood Gas

Blood samples were collected after administration of sterile saline or ozonated water, and blood gas was measured using at 0, 10, 30, 60, and 120 min after administration.

Evaluation of Intratumoral Blood Perfusion

Eighty minutes after administration of sterile saline or ozonated water, 30 mg/kg Hoechst33342 (H33342) dissolved in phosphate-buffered saline (PBS) was administrated intravenously. After 4 min, the mice were euthanized by cervical dislocation under anesthesia induced by the inhalation of 3–5% isoflurane. The tumors were embedded in an optimal cutting temperature (OCT) compound. The cryostat sections (10 mm) were fixed in acetone for 5 min at 4℃. These sections were analyzed by fluorescence microscopy. The percentage of positive areas in the tumor tissues was calculated by dividing the total pixel area of the positive areas by the total pixel area corresponding to the entire tumor tissue in the field of view. The mean scores for 25 fields were used as the percentages of positive areas per group.

Effect of Ozonated Water and Cisplatin (CDDP) in Combination, on Colon-26 Mice

For this experiment, the mice were randomized into six groups (n = 8 per group): sterile saline (S) group, ozonated water (O_3) group, sterile saline and 5.0 mg/kg CDDP group (S-CDDP5.0), ozonated water and 5.0 mg/kg CDDP group (O_3-CDDP5.0), ozonated water and 3.4 mg/kg CDDP group (O_3-CDDP3.4), and ozonated water and 1.7 mg/kg CDDP group (O_3-CDDP1.7). The volume of these tumor tissues was calculated as follows: (mediastinum × transverse line × depth × π)/6 (mm^3). Then, CDDP (5.0, 3.4, 1.7 mg/kg) and saline or ozonated water (0.2 mL/head) were intraperitoneally administered to the S-CDDP5.0, O_3-CDDP5.0, O_3-CDDP3.4, and O_3-CDDP1.7 groups. In the S and O_3 groups, only sterile saline or ozonated water was administered. On day 7, the volumes of the tumor tissues were calculated and the mice were euthanized by cervical dislocation under anesthesia as described before. Based on the tumor volumes on days 1 and 7, the tumor growth rates were calculated as follows: (tumor volume on day 7 − tumor volume on day 1)/7 (mm^3/day). The tumors were fixed in 10% buffered formalin.

Ki-67 Staining

Tissue sections (3 μm) obtained on glass slides were deparaffinized, washed with ethanol and water, and soaked in PBS. The sections were autoclaved with 0.01M citrate buffer (pH 6.0) for 15 min (121˚C). The sections were then washed with PBS and incubated with the rabbit polyclonal anti-Ki-67 antibody (1:50, E0468; Dako, Glostrup, Denmark) for 30 min at room temperature. After washing with PBS, the sections were incubated with rat anti-IgG antibody (1:100, sc-372; Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature. The slides were washed with PBS and stained using the ABC method (PK-4000; Vector Laboratories, Inc.) for 30 min. Cell counts in 25 random fields were calculated at a magnification of 400× by using five mice from each group.

TUNEL Staining

Tissue sections (3 μm) obtained on glass slides were deparaffinized, washed with ethanol and water, and soaked in PBS. TUNEL staining was performed using the in-situ Apoptosis Detection kit (Takara Bio, Inc., Shiga, Japan), according to the manufacturer's instructions. Cell counts were calculated as described in the previous subsection.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD) or standard error (SE). Statistical analyses were first performed using F- test or analysis of variance (ANOVA) and compared using the Student's t- test or Tukey–Kramer test. $p < 0.05$ indicated statistical significance.

RESULTS

Effect on Blood Gas and Intratumoral Oxygen Partial Pressure

Before and after the administration of O_3 or sterile saline, no apparent change was noted in the blood gas levels (Table 1). No significant difference was noted between the O_3 and S groups.

In the S group, there was no apparent change in the intratumoral oxygen partial pressure. However, the partial pressure in the O_3 group significantly increased compared to that in the S group at 20 min after administration (Fig. 1). It peaked at 50 min after administration and gradually decreased afterward. At 130 min after administration, no significant difference was noted between the O_3 and S groups.

Effect on Intratumoral Blood Perfusion

The intratumoral blood perfusion in the O_3 group increased compared to that in the S group (Fig. 2). The H33342-positive area was 12.3% ± 3.2% in the S group and 25.6% ± 4.6% in the O_3 group. There was a significant difference between the two groups ($p < 0.05$).

Effect of Ozonated Water and CDDP in Combination

Tumor growth was significantly suppressed in the O_3-CDDP5.0 group compared to that in the S and O_3 groups ($p < 0.05$) (Fig. 3). A statistically significant difference was not observed between the S-CDDP5.0 and O_3-CDDP5.0 groups. However, tumor growth in the O_3-CDDP5.0 group tended to be suppressed, compared to that in the S-CDDP5.0 group. In the O_3-CDDP3.4 group, despite reducing the concentration of the antitumor drug, tumor growth suppression was observed, which was comparable to that observed in the S-CDDP group.

In the O_3-CDDP5.0 group, the number of Ki-67-positive cells significantly decreased, compared to that in the other groups (Fig. 4). The number of Ki-67-positive cells in the O_3-CDDP3.4 and S-CDDP groups significantly decreased, compared to that in the S, O_3,

and O_3-CDDP1.7 groups. There was no statistically significant difference between the S-CDDP5.0 and O_3-CDDP3.4 groups.

In the O_3-CDDP5.0 group, the number of TUNEL-positive cells significantly increased, compared to that of the other groups (Fig. 5). The number of TUNEL-positive cells in the O_3-CDDP3.4 group significantly increased, compared to that in the S and O_3 groups. No statistically significant difference was observed among the S-CDDP5.0, O_3-CDDP3.4, and O_3-CDDP1.7 groups.

DISCUSSION

In this study, the intratumoral blood perfusion was found to have increased and the intratumoral oxygen partial pressure was found to have improved after intraperitoneal administration of ozonated water. To the best of our knowledge, such effects of ozonated water have not been reported thus far. Further, when ozonated water was used in combination with CDDP, the antitumor effect of CDDP was enhanced. Most studies related to chemotherapy discuss side effects; very few reports discuss therapeutic effects(Borrego, et al. 2004 and 2006;

González, et al. 2004). The findings of this study can be considered extremely important evidence with respect to the use of ozonated water.

When the ozonated water was intraperitoneally administered, no apparent change was noted in the blood gas levels. However, the intratumoral oxygen partial pressure significantly increased. These results indicate that ozonated water increases intratumoral oxygen partial pressure without affecting blood gas levels. Ozone gas is known to improve peripheral blood perfusion owing to its vasodilating effect (Dutka, 1998; Mukhina, et al., 2005). When the extent of intratumoral blood perfusion was evaluated by H33342, a significant increase was noted in the O_3 group. Tumor hypoxia generally progresses because of reduction in the intratumoral blood perfusion caused by an imbalance between the tumor growth rate and tumor blood vessel formation rate (J. M. Brown, et al., 2004; Lunt, et al., 2009). This result indicates that ozonated water as well as ozone gas increase peripheral blood perfusion. In addition, it indicated that the increase in the intratumoral oxygen partial pressure might be due to the increase in intratumoral blood perfusion. Oxidation as well as activation of antioxidant enzymes are caused by the administration of ozone. When active oxygen is removed by superoxide dismutase (SOD), hydrogen peroxide (H_2O_2) is produced. H_2O_2 is involved in vasodilation as an

endothelium-derived hyperpolarizing factor (EDHF), one of the endothelium-derived vasorelaxation factors (Takaki et al., 2008). Therefore, it has been thought that ozone extends the peripheral vessels by means of H_2O_2 and increases peripheral blood perfusion (Mukhina et al., 2005). Ozonated water is likely to increase blood perfusion by a similar mechanism. However, the underlying mechanism needs to be studied further. In addition, the changes in peripheral blood perfusion in normal tissues warrant further study.

Tumor hypoxia is a serious problem for tumor treatment because it reduces the sensitivity to chemical, radiation, and photodynamic therapy (Bertout et al., 2008; J. M. Brown, et al., 2004; Graeber et al., 1996; Semenza, 2003; Teicher, 1994). The reasons for resistance to chemotherapy include cell cycle arrest (Amellemet al., 1994; Gardner et al., 2001), acquisition of antiapoptotic activity by inhibition of apoptosis-inducing proteins such as Bid and Bax (Erler et al., 2004; Mayes et al., 2011), and reduction in the amount of drug reaching the tumor because of decreased blood flow (Teicher, 1994).

The rate of tumor growth in the O_3-CDDP5.0 group tended to decrease compared to that in the S-CDDP5.0 group. In addition, the number of Ki-67-positive cells significantly decreased and the number of TUNEL-positive cells significantly increased in the O_3-CDDP5.0

group compared to that in the S-CDDP5.0 group. CDDP exerts an antitumor effect by inducing apoptosis and suppressing tumor growth by inhibiting deoxyribonucleic acid (DNA) synthesis(Kartalou, et al., 2001). Therefore, the results of Ki-67 and TUNEL staining suggested that the effect of CDDP was enhanced by the administration of ozonated water. The antitumor effect of CDDP depends on the amount of CDDP reaching the site of tumor rather than the proliferation activity of tumor cells (Drewinko, et al., 1973; Takahashi, 1982). These findings as well as previous reports suggest that tumor growth is suppressed on treatment with ozonated water because the amount of CDDP reaching the tumor is increased when the intratumoral blood perfusion is increased because of ozonated water. We plan to measure the concentration of intratumoral antitumor drug in the future and investigate in detail.

In recent years, it has been reported that the inhibition of apoptosis-inducing protein is associated with resistance to platinum-based drug preparations (Erler et al., 2004). However, it is unknown whether those proteins are involved in the resistance mechanism. It might not affect protein expression because the increase in oxygen partial pressure observed in this study occurred only 2 h after the administration of ozonated water. In order to clarify the mechanism, the genes and proteins related to hypoxia and apoptosis need to be studied further.

The O_3-CDDP5.0 group showed tumor growth suppression, to almost the same extent as that observed in the S-CDDP5.0 group. This suggests that it is possible to reduce the required drug concentration, while maintaining the antitumor effect, by using ozonated water and general chemotherapy in combination. We intend to conduct detailed studies on other drugs and tumor types in the future.

This study showed that ozonated water increases intratumoral blood perfusion and improves intratumoral oxygen partial pressure. In addition, tumor growth was more suppressed when ozonated water and CDDP therapy were combined. Thus, the administration of ozonated water could be a new approach to solve current concerns around antitumor treatment, such as tumor hypoxia and drug resistance of tumors.

FIGURES & TABLE

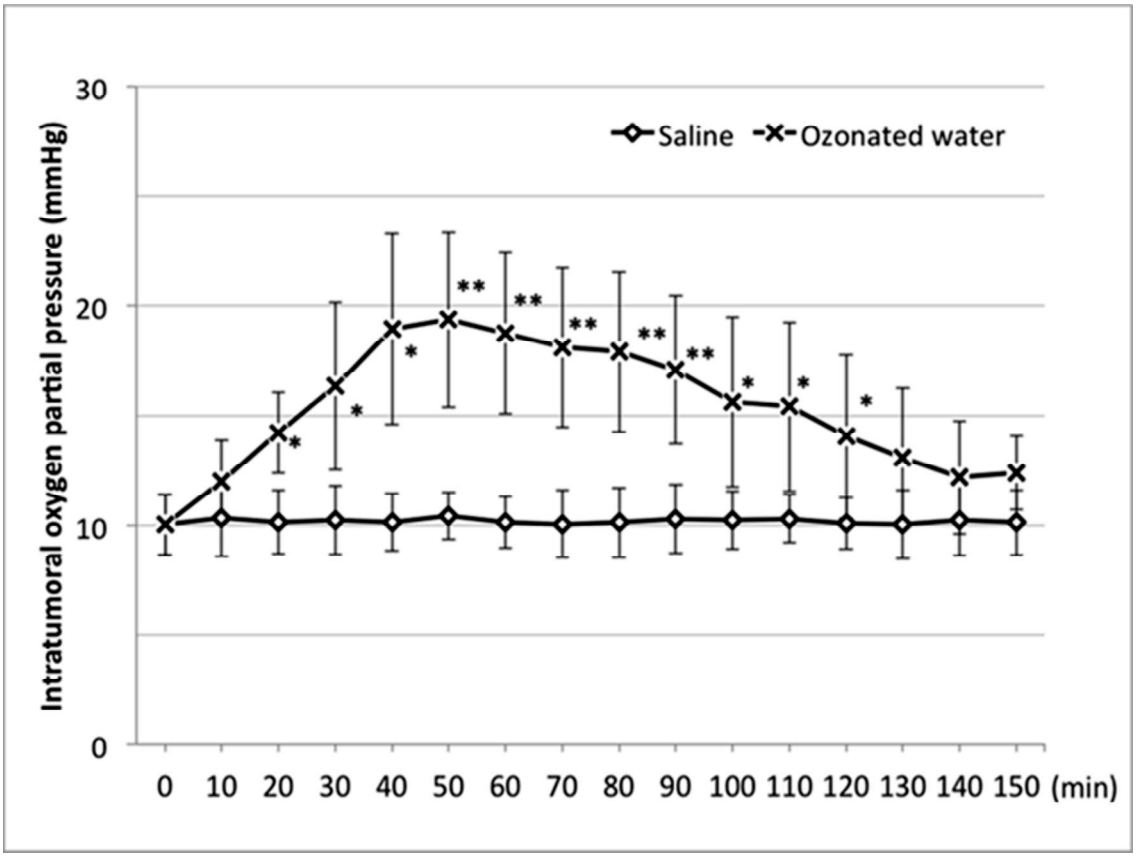

Figure 1. Effect of ozonated water on the intratumoral oxygen partial pressure. This graph shows the change in the intratumoral oxygen partial pressure after intraperitoneal administration of ozonated water or saline. “Diamond” indicates the group administered sterile saline (S) and “cross” indicates the group administered ozonated water (O_3). Data are shown as the mean ± standard deviation (SD). *$p < 0.05$ or **$p < 0.01$ (obtained by using t-test) indicates significant difference.

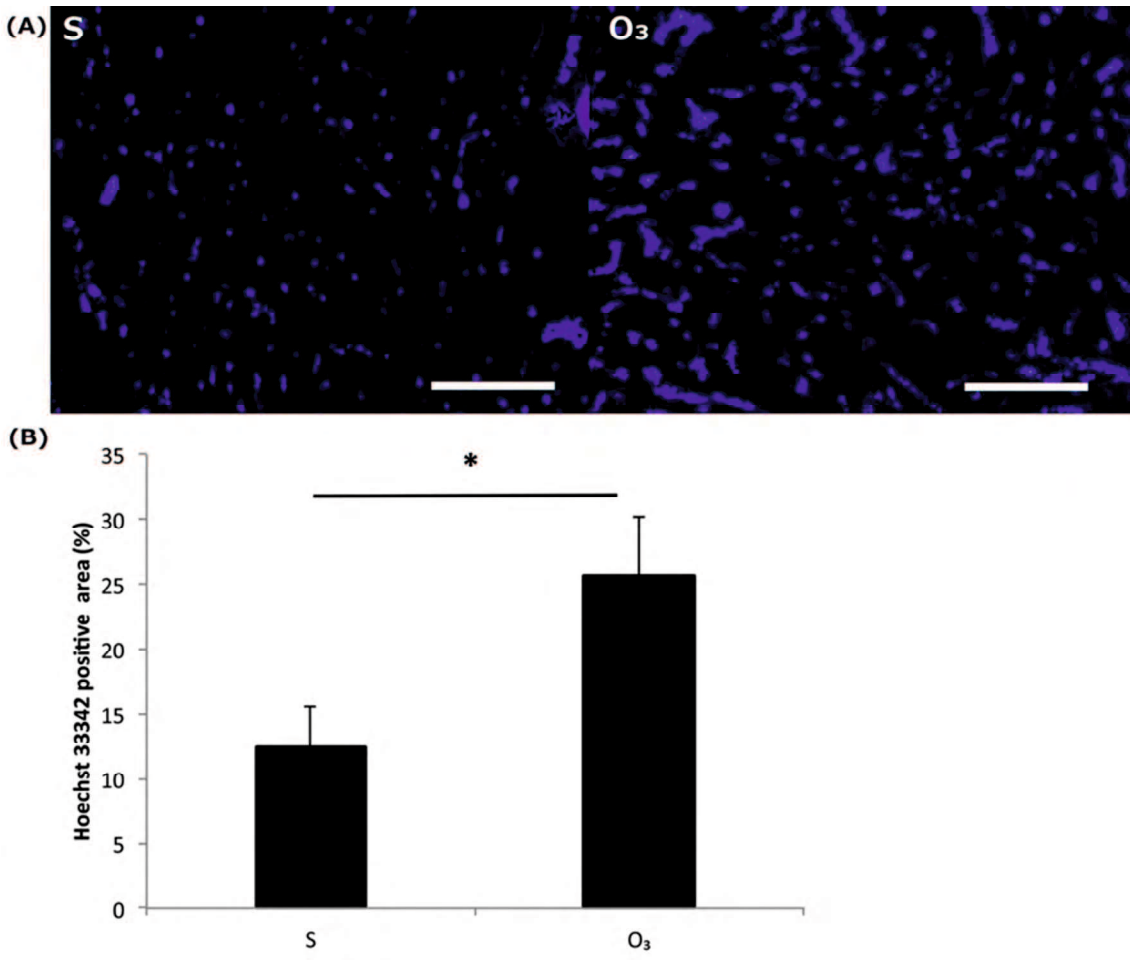

Figure 2. Effect of ozonated water on intratumoral blood perfusion. (A) H33342-positive area in each group: sterile saline (S) and ozonated water (O_3) groups. Scale bar: 50 μm. (B) Quantitative value of the H33342-positive area. Data are shown as the mean ± standard deviation (SD). *$p < 0.05$, obtained by using t-test, indicates significant difference.

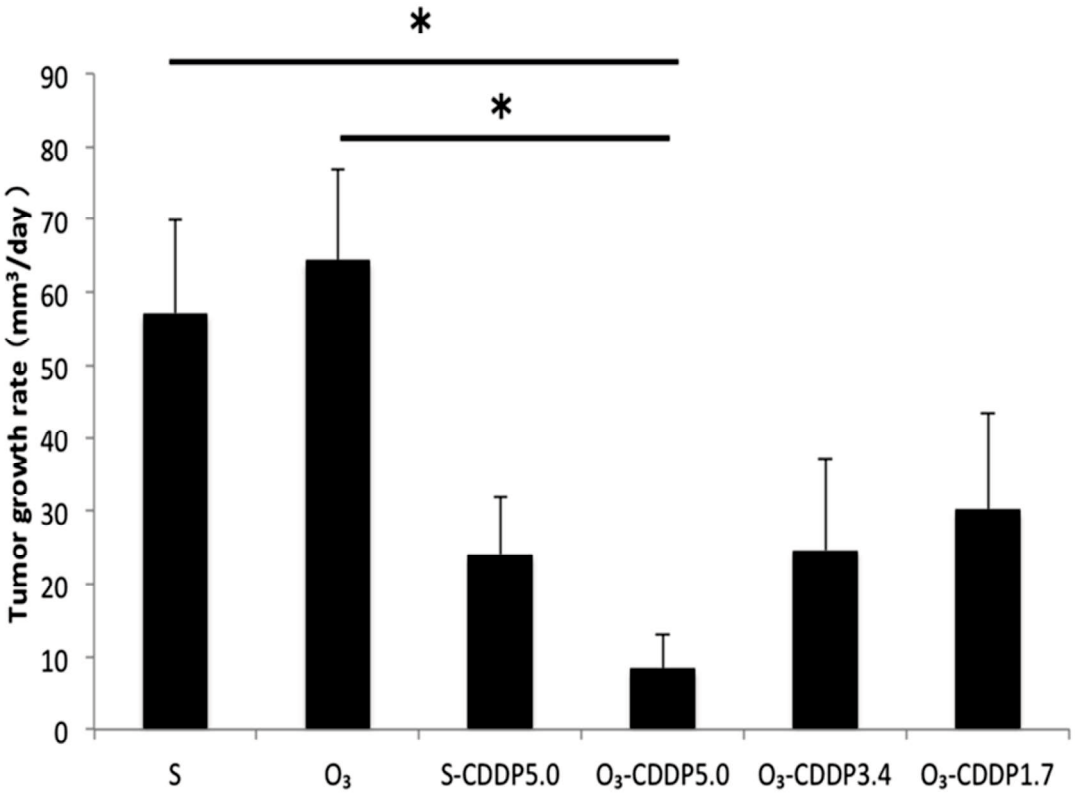

Figure 3. The effect combining ozonated water with CDDP on tumor growth rate. The graph shows the tumor growth rate for each group: sterile saline administration (S) group, and ozonated water administration (O_3) group, administration of sterile saline and 5.0mg/kg CDDP group (S-CDDP5.0), administration of ozonated water and 5.0mg/kg CDDP group (O_3-CDDP5.0), administration of ozonated water and 3.4mg/kg CDDP group (O_3-CDDP3.4), administration of ozonated water and 1.7mg/kg CDDP group (O_3-CDDP1.7). The data show the mean ± SE. Significant differences are shown by * $p < 0.05$ using Tukey–Kramer test.

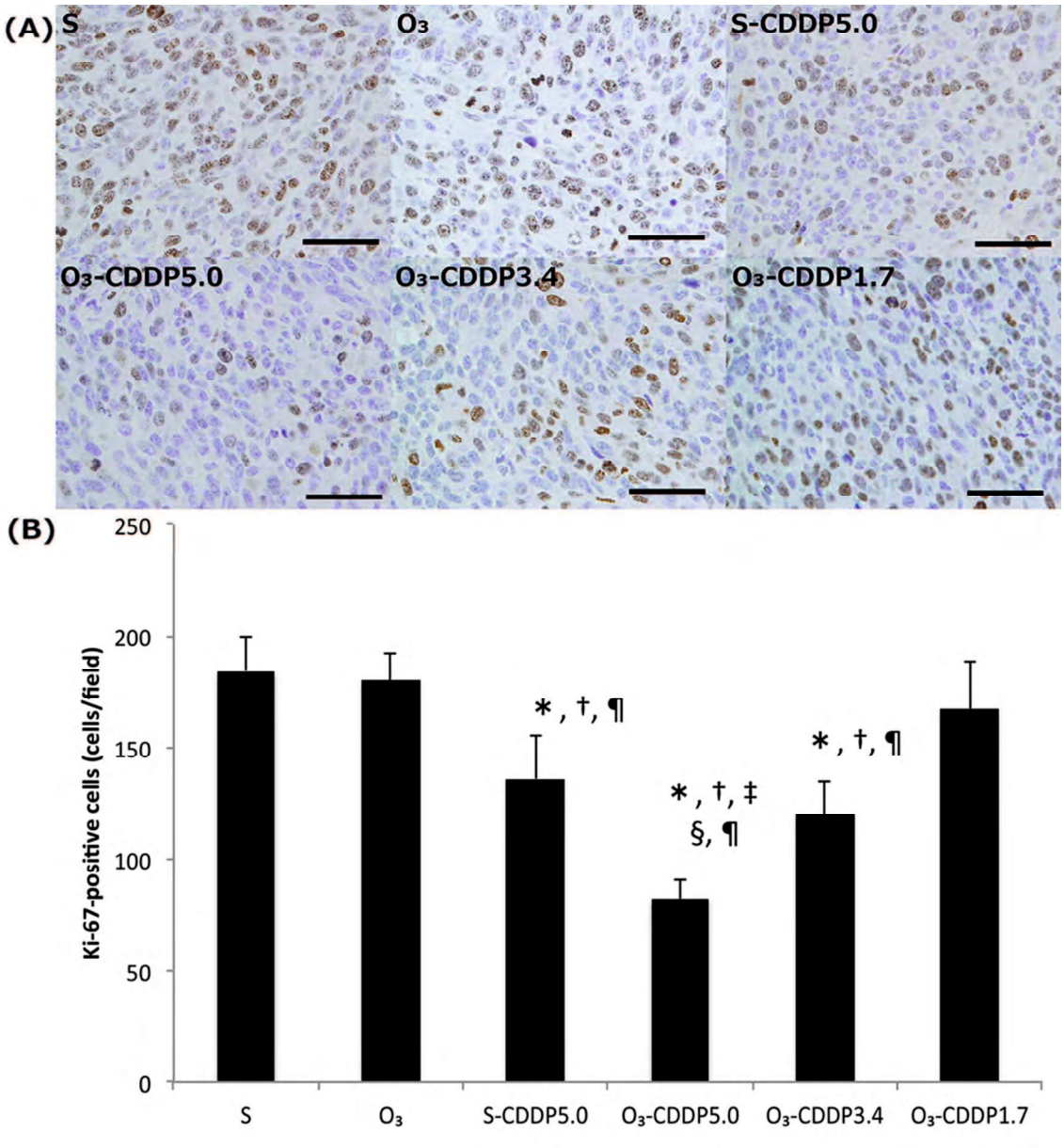

Figure 4. The effect combining ozonated water with CDDP in Ki-67 stainig. (A) The graph image shows the Ki-67 positive area for each group: sterile saline administration (S) group, and ozonated water administration (O_3) group, administration of sterile saline and 5.0mg/kg CDDP group (S-CDDP5.0), administration of ozonated water and 5.0mg/kg CDDP group (O_3-CDDP5.0), administration of ozonated water and 3.4mg/kg CDDP group (O_3-CDDP3.4),

administration of ozonated water and 1.7mg/kg CDDP group (O_3-CDDP1.7). Scale bar: 500 μm. (B) The graph shows the number of the Ki-67 positive cells. The data show the mean ± SE. Significant differences are shown by $p < 0.01$ (*: vs S, †: vs O_3, ‡: vs S-CDDP5.0, §: vs O_3-CDDP3.4, ¶: vs O_3-CDDP1.7) using Tukey–Kramer test.

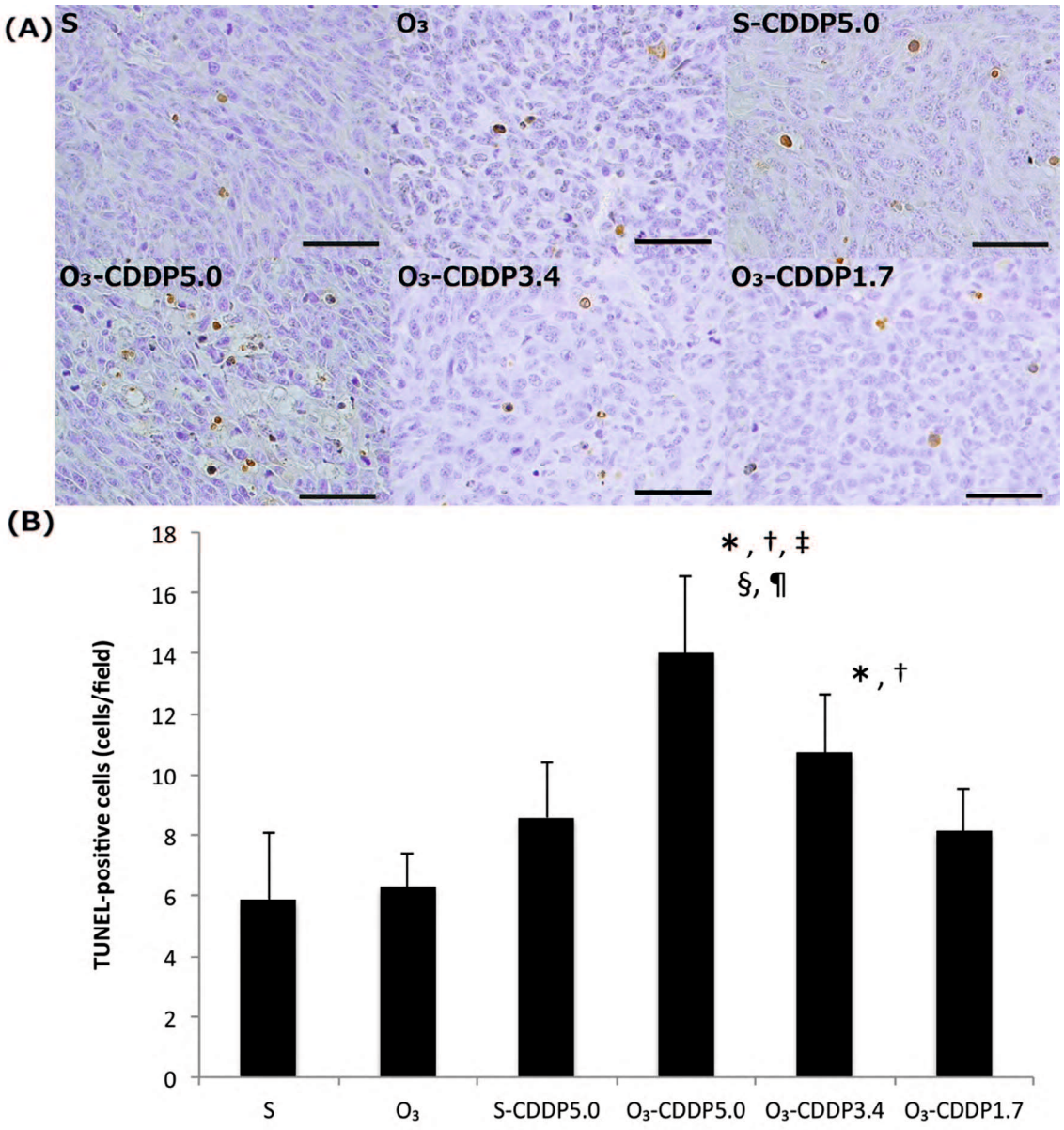

Figure 5. The effect combining ozonated water with CDDP in TUNEL stainig. (A) The images show the TUNEL positive area for each group: sterile saline administration (S) group, and ozonated water administration (O_3) group, administration of sterile saline and 5.0mg/kg CDDP group (S-CDDP5.0), administration of ozonated water and 5.0mg/kg CDDP group (O_3-CDDP5.0), administration of ozonated water and 3.4mg/kg CDDP group (O_3-CDDP3.4),

administration of ozonated water and 1.7mg/kg CDDP group (O_3-CDDP1.7). Scale bar: 500 μm. (B) The graph shows the number of the TUNEL positive cells. The data show the mean ± SE. Significant differences are shown by $p<0.01$ (*: vs S, †: vs O_3, ‡: vs S-CDDP5.0, ¶: vs O_3-CDDP1.7) or $p<0.05$ (§: vs O_3-CDDP3.4) using Tukey–Kramer test.

Table I. Effect of ozonated water on blood gas.

O_3

Time (min)	pH	pCO_2 (mmHg)	pO_2 (mmHg)	HCO_3 (mmol/l)	sO_2 (%)
0	7.335 ± 0.03	38.9 ± 4.5	56 ± 4.7	20.7 ± 1.2	87 ± 4.6
10	7.276 ± 0.04	38.6 ± 4.9	53 ± 4.3	20 ± 0.6	85 ± 4.8
30	7.277 ± 0.03	38.1 ± 5.0	59 ± 5.2	19 ± 1.0	87 ± 5.8
60	7.339 ± 0.02	36.9 ± 4.3	54 ± 4.6	21 ± 0.8	86 ± 3.8
120	7.323 ± 0.03	42.4 ± 4.5	64 ± 4.8	23 ± 1.3	90 ± 5.2

S

Time (min)	pH	pCO_2 (mmHg)	pO_2 (mmHg)	HCO_3 (mmol/l)	sO_2 (%)
0	7.347 ± 0.03	39.2 ± 6.7	57.3 ± 5.4	19.6 ± 1.3	83 ± 3.9
10	7.336 ± 0.02	39.7 ± 3.7	46 ± 6.3	19.9 ± 0.5	78.6 ± 5.3
30	7.324 ± 0.04	39.3 ± 4.3	55 ± 4.6	20.5 ± 0.7	86 ± 4.6
60	7.382 ± 0.03	35.2 ± 5.4	51 ± 4.2	20.5 ± 0.6	85 ± 5.3
120	7.229 ± 0.02	47.9 ± 4.4	58 ± 4.2	23.5 ± 0.8	87 ± 4.9

The data show the mean ± SE. pCO_2: carbon dioxide partial pressure, pO_2: oxygen partial pressure, HCO_3: Bicarbonate ion, SO_2: oxygen saturation.

CONCLUSION

In chapter Ⅰ, it is suggested that ozonated water has a direct anti-tumor effect. There is a possibility that it is exerted through the production of ROS. Moreover, ozonated water has low toxicity and is safe and easy to handle. Administration of ozonated water is a safe and potentially simple adjunct or alternative to existing antineoplastic treatments.

In chapter Ⅱ, ozonated water increases intratumoral blood perfusion and improves intratumoral oxygen partial pressure. In addition, tumor growth was more suppressed when ozonated water and CDDP therapy were combined. Thus, the administration of ozonated water could be a new approach to solve current concerns around antitumor treatment, such as tumor hypoxia and drug resistance of tumors.

In this experiment, the safety of ozonated water was demonstrated and a possibility of new ozone therapy using ozonated water was suggested. By using ozone, it is possible to increase the choices of current tumor therapy and increase the therapeutic effect. Further investigation will be made and further clinical application is expected.

REFERENCE

Ahmad, I. M., Aykin-Burns, N., Sim, J. E., Walsh, S. A., Higashikubo, R., Buettner, G. R., Venkataraman, S., Mackey, M. A. Flanagan, S. W., Oberley, L. W., & Spitz, D. R. (2005). Mitochondrial O2*- and H2O2 mediate glucose deprivation-induced stress in human cancer cells. *The Journal of Biological Chemistry*, *280*(6), 4254–63.

Alegria, A. E., Samuni, A., Mitchell, J. B., Riesz, P., & Russo, A. (1989). Free radicals induced by adriamycin-sensitive and adriamycin-resistant cells: a spin-trapping study. *Biochemistry*, *28*(21), 8653–8.

Amellem, O., Löffler, M., & Pettersen, E. O. (1994). Regulation of cell proliferation under extreme and moderate hypoxia: the role of pyrimidine (deoxy)nucleotides. *British Journal of Cancer*, *70*(5), 857–66.

Aulitzky, W. ., Lerche, J., Thews, A., Lüttichau, I., Jacobi, N., Herold, M., Aulitzky, W., Peschel, C., Stöckle, M., Steinbach, F., & Huber, C. (1994). Low-dose γ-interferon therapy is

ineffective in renal cell carcinoma patients with large tumour burden. *European Journal of Cancer*, *30*(7), 940–945.

Azuma, K., Ishihara, T., Nakamoto, H., Amaha, T., Osaki, T., Tsuka, T., Imagawa, Tomohiro Minami, S., Takashima, O., Ifuku, S., Morimoto, M., Saimoto, H., Kawamoto, H., & Okamoto, Y. (2012). Effects of oral administration of fucoidan extracted from Cladosiphon okamuranus on tumor growth and survival time in a tumor-bearing mouse model. *Marine Drugs*,10(10), 2337-48.

Azuma, K., Mori, T., Kawamoto, K., Kuroda, K., Tsuka, T., Imagawa, T., Osaki, T., Itoh, F., Minami, S., & Okamoto, Y. (2014). Anti‐inflammatory effects of ozonated water in an experimental mouse model. *Biomedical Reports*, *2*(5), 671–674.

Bayr, H. (2005). Reactive oxygen species. *Critical Care Medicine*, *33*(Suppl), S498–S501.

Bertout, J. A., Patel, S. A., & Simon, M. C. (2008). The impact of O2 availability on human cancer. *Nature Reviews Cancer*, *8*(12), 967–975.

Bhalla, D. K. (1999). Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. *Journal of Toxicology and Environmental Health. Part B, Critical Reviews*, *2*(1), 31–86.

Bocci, V., Aldinucci, C., Mosci, F., Carraro, F., & Valacchi, G. (2007). Ozonation of human blood induces a remarkable upregulation of heme oxygenase-1 and heat stress protein-70. *Mediators of Inflammation*, *2007*, 26785.

Bocci, V., & Paulesu, L. (1990). Studies on the biological effects of ozone 1. Induction of interferon gamma on human leucocytes. *Haematologica*, *75*(6), 510–5.

Bocci, V., Valacchi, G., Corradeschi, F., Aldinucci, C., Silvestri, S., Paccagnini, E., & Gerli, R. (1998). Studies on the biological effects of ozone: 7. Generation of reactive oxygen species

(ROS) after exposure of human blood to ozone. *Journal of Biological Regulators and Homeostatic Agents*, *12*(3), 67–75.

Bonnem, E. M. (1991). Alpha interferon: The potential drug of adjuvant therapy: Past achievements and future challenges. *European Journal of Cancer and Clinical Oncology*, *27*, S2–S6.

Borrego, A., Zamora, Z. B., González, R., Romay, C., Menéndez, S., Hernández, F., Berlanga, J., & Montero, T. (2006). Ozone/Oxygen Mixture Modifies the Subcellular Redistribution of Bax Protein in Renal Tissue from Rats Treated with Cisplatin. *Archives of Medical Research*, *37*(6), 717–722.

Borrego, A., Zamora, Z. B., González, R., Romay, C., Menéndez, S., Hernández, F., Montero, T., & Rojas, E. (2004). Protection by ozone preconditioning is mediated by the antioxidant system in cisplatin-induced nephrotoxicity in rats. *Mediators of Inflammation*, *13*(1), 13–19.

Brown, J. M., & Wilson, W. R. (2004). Exploiting tumour hypoxia in cancer treatment. *Nature Reviews Cancer*, *4*(6), 437–447.

Brown, T. D., Goodman, P. J., Fleming, T., Macdonald, J. S., Craig, J. B., & Einstein, A. B. (1993). Phase II trial of amonafide in advanced colorectal cancer: a SouthWest Oncology Group study. *Anti-Cancer Drugs*, *4*(1), 49–50.

Bucci, B., Cannizzaro, A., Brunetti, E., & Martinelli, M. (2006). Ozone Treatment Inhibits Proliferation in Human Neuroblastoma SK-N-SH Cells, 85–92.

Chen, Q., Espey, M. G., Sun, A. Y., Pooput, C., Kirk, K. L., Krishna, M. C., Khosh, D. B., Drisko, J., & Levine, M. (2008). Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(32), 11105–9.

Clavo, B., Pérez, J. L., López, L., Suárez, G., Lloret, M., Rodríguez, V., Macías, D., Santana, M., Hernández, M. A., Martín-Oliva, R., & Robaina, F. (2004). Ozone Therapy for Tumor Oxygenation: a Pilot Study. *Evidence-Based Complementary and Alternative Medicine : eCAM*, *1*(1), 93–98.

Clavo, B., Ruiz, A., Lloret, M., López, L., Suárez, G., Macías, D., Rodríguez, V., Hernández, M. A., Martín-Oliva, R., Quintero, S., Cuyás, J. M., & Robaina, F. (2004). Adjuvant Ozonetherapy in Advanced Head and Neck Tumors: A Comparative Study. *Evidence-Based Complementary and Alternative Medicine : eCAM*, *1*(3), 321–325.

Drewinko, B., Brown, B. W., & Gottlieb, J. A. (1973). The effect of cis-diamminedichloroplatinum (II) on cultured human lymphoma cells and its therapeutic implications. *Cancer Research*, *33*(12), 3091–5.

Dutka, M., & Adamczak, M. (1998). Vasorelaxant activity of ozone-in vitro studies. *Advances in Clinical and Experimental Medicine*, *4*(7), 391–398.

Erler, J. T., Cawthorne, C. J., Williams, K. J., Koritzinsky, M., Wouters, B. G., Wilson, C., Miller, C., Demonacos, C., Stratford, I. J., & Dive, C. (2004). Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms and contributes to drug resistance. *Molecular and Cellular Biology*, *24*(7), 2875–89.

Gallucci, S., & Matzinger, P. (2001). Danger signals: SOS to the immune system. *Current Opinion in Immunology*, *13*(1), 114–119.

Gardner, L. B., Li, Q., Park, M. S., Flanagan, W. M., Semenza, G. L., & Dang, C. V. (2001). Hypoxia Inhibits G1/S Transition through Regulation of p27 Expression. *Journal of Biological Chemistry*, *276*(11), 7919–7926.

Ghaemi-Oskouie, F., & Shi, Y. (2011). The role of uric acid as an endogenous danger signal in immunity and inflammation. *Current Rheumatology Reports*, *13*(2), 160–6.

Gille, J. J. P., & van Berkel, C. G. M. (1994). Mutagenicity of metabolic oxygen radicals in mammalian cell cultures. *Carcinogenesis*, *15*(12), 2695–2699.

González, R., Borrego, A., Zamora, Z., Romay, C., Hernández, F., Menéndez, S., Montero, T., & Rojas, E. (2004). Reversion by ozone treatment of acute nephrotoxicity induced by cisplatin in rats. *Mediators of Inflammation*, *13*(5–6), 307–12.

Graeber, T. G., Osmanian, C., Jacks, T., Housman, D. E., Koch, C. J., Lowe, S. W., & Giaccia, A. J. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature*, *379*(6560), 88–91.

Halliwell, B. (1999). Oxygen and nitrogen are pro-carcinogens. Damage to DNA by reactive oxygen, chlorine and nitrogen species: measurement, mechanism and the effects of nutrition. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *443*(1–2), 37–52.

Kaplan, D. H., Shankaran, V., Dighe, A. S., Stockert, E., Aguet, M., Old, L. J., & Schreiber, R. D. (1998). Demonstration of an interferon -dependent tumor surveillance system in immunocompetent mice. *Proceedings of the National Academy of Sciences*, *95*(13), 7556–7561.

Kartalou, M., & Essigmann, J. M. (2001). Mechanisms of resistance to cisplatin. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *478*(1), 23–43.

Kuroda, K., Azuma, K., Mori, T., Kawamoto, K., Murahata, Y., Tsuka, T., Osaki, T., Ito, N., Imagawa, T., Itoh, F., & Okamoto, Y. (2015). The Safety and Anti-Tumor Effects of Ozonated Water in Vivo. *International Journal of Molecular Sciences*, *16*(10), 25108–25120.

Lunt, S. J., Chaudary, N., & Hill, R. P. (2009). The tumor microenvironment and metastatic disease. *Clinical & Experimental Metastasis*, *26*(1), 19–34.

Matzinger, P. (2002). The danger model: a renewed sense of self. *Science (New York, N.Y.)*, *296*(5566), 301–5.

Mayes, P. A., Dolloff, N. G., Daniel, C. J., Liu, J. J., Hart, L. S., Kuribayashi, K., Allen, J. E., Jee, D. I. H., Dorsey, J. F., Liu, Y. Y., Dicker, D. T., Brown, J. M., Furth, E. E., Klein, P. S., Sears, R. C., & El-Deiry, W. S. (2011). Overcoming hypoxia-induced apoptotic resistance through combinatorial inhibition of GSK-3β and CDK1. *Cancer Research*, *71*(15), 5265–75.

Mukhina, I., Dudina, E., Yakovleva, E., & Zhemarina, N. (2005). The dose-dependent effect of ozonated physiological solution on arterial vasodilation. *IOA 17th World Ozone*.

Nitta, M., Azuma, K., Hata, K., Takahashi, S., Ogiwara, K., Tsuka, T., Imagawa, T., Yokoe, I., Osaki, T., Minami, S., & Okamoto, Y. (2013). Systemic and local injections of lupeol inhibit tumor growth in a melanoma-bearing mouse model. *Biomedical Reports*, *1*(4), 641–645.

Nogales, C. G., Ferrari, P. H., Kantorovich, E. O., & Lage-Marques, J. L. (2008). Ozone therapy in medicine and dentistry. *The Journal of Contemporary Dental Practice*, *9*(4), 75–84.

Ozmen, V., Thomas, W. O., Healy, J. T., Fish, J. M., Chambers, R., Tacchi, E., Nichols, R. L., Flint, L M., & Ferrara, J. J. (1993). Irrigation of the abdominal cavity in the treatment of experimentally induced microbial peritonitis: efficacy of ozonated saline. *The American Surgeon*, *59*(5), 297–303.

Paulesu, L., Luzzi, E., & Bocci, V. (1991). Studies on the biological effects of ozone: 2. Induction of tumor necrosis factor (TNF-alpha) on human leucocytes. *Lymphokine and Cytokine Research*, *10*(5), 409–12.

Pryor, W. A., Squadrito, G. L., & Friedman, M. (1995). The cascade mechanism to explain ozone toxicity: The role of lipid ozonation products. *Free Radical Biology and Medicine*, *19*(6), 935–941.

Restaino, L., Frampton, E. W., Hemphill, J. B., & Palnikar, P. (1995). Efficacy of ozonated water against various food-related microorganisms. *Applied and Environmental Microbiology*, *61*(9), 3471–5.

Richtsmeier, W. J., Koch, W. M., McGuire, W. P., Poole, M. E., & Chang, E. H. (1990). Phase I-II Study of Advanced Head and Neck Squamous Cell Carcinoma Patients Treated With Recombinant Human Interferon Gamma. *Archives of Otolaryngology - Head and Neck Surgery*, *116*(11), 1271–1277.

Sabel, M. S., Su, G., Griffith, K. A., & Chang, A. E. (2010). Rate of freeze alters the immunologic response after cryoablation of breast cancer. *Annals of Surgical Oncology*, *17*(4), 1187–93.

Sasada, T., Iwata, S., Sato, N., Kitaoka, Y., Hirota, K., Nakamura, K., Nishiyama, A., Taniguchi, Y., Takabayashi, A., & Yodoi, J. (1996). Redox control of resistance to cis-diamminedichloroplatinum (II) (CDDP): protective effect of human thioredoxin against CDDP-induced cytotoxicity. *The Journal of Clinical Investigation*, *97*(10), 2268–76.

Scaffidi, P., Misteli, T., & Bianchi, M. E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*, *418*(6894), 191–5.

Scott, D. B., & Lesher, E. C. (1963). Effect of Ozone on Survival and Permeability of Escherichia Coli. *Journal of Bacteriology*, *85*(October), 567–576.

Semenza, G. L. (2003). Targeting HIF-1 for cancer therapy. *Nature Reviews Cancer*, *3*(10), 721–732.

Sweet, F., Kao, M., Lee, S., Hagar, W. W., & Sweet, W. W. (1980). Ozone selectively inhibits growth of human cancer cells. *Science*, *209*(4459), 931–933.

Takahashi, K. (1982). The effect of cis-DICHLORODIAMMINEPLATINUM (II) on tumor growth and progress in the cell cycle. *Cancer & Chemotherapy*, *9*(4), 624–631.

Takaki, A., Morikawa, K., Tsutsui, M., Murayama, Y., Tekes, E., Yamagishi, H., Ohashi, J., Yada, T., Yanagihara, N., & Shimokawa, H. (2008). Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice. *The Journal of Experimental Medicine*, *205*(9), 2053–2063.

Teicher, B. A. (1994). Hypoxia and drug resistance. *Cancer and Metastasis Reviews*, *13*(2), 139–168.

Valacchi, G., & Bocci, V. (1999). Studies on the biological effects of ozone: 10. Release of factors from ozonated human platelets. *Mediators of Inflammation*, *8*(4–5), 205–9.

Viebahn-Haensler, R., & Lee, A. (2007). *The use of ozone in medicine*. Iffezheim: ODREI-Publishers, 1- 30.

ACKNOWLEDGMENT

I wish to pay sincere acknowledgement to Professor Tomohiro Imagawa, Department of Veterinary Clinical Medicine, Joint School of Veterinary Medicine, Tottori University, for his supervising and encouragement during the present investigation.

I also wish to thank Professor Yasuho Taura, Joint Faculty of Veterinary Medicine, Yamaguchi University; Professor Yoshiharu Okamoto, Department of Veterinary Clinical Medicine, Joint School of Veterinary Medicine, Tottori University, for their helpful suggestion as co-supervisor.

I am grateful to Associated Professor Norihiko Ito, Tottori University; Associated Professor Takeshi Tsuka, Tottori University; Associated Professor Tomohiro Osaki, Tottori University; Assistant Professor Kazuo Azuma. Tottori University; Assistant Professor Yusuke Murahata, Tottori University.

Finally, I wish to express special thanks to my family, especially to my wife Moegi for their hearty supports.

PUBLICATION

The contents of this thesis were published in the following journals.

Chapter I

Kohei Kuroda, Kazuo Azuma, Takuro Mori, Kinya Kawamoto, Yusuke Murahata, Takeshi Tsuka, Tomohiro Osaki, Norihiko Ito, Tomohiro Imagawa, Fumio Itoh and Yoshiharu Okamoto. 2015. "The Safety and Anti-Tumor Effects of Ozonated Water in Vivo." *International Journal of Molecular Sciences,* 16 (10): 25108–20.

Chapter II

Kohei Kuroda, Masamiti Yamashita, Yusuke Murahata, Kazuo Azuma, Tomohiro Osaki, Takeshi Tsuka, Norihiko Ito, Tomohiro Imagawa and Yoshiharu Okamoto. "Use of ozonated water as a new therapeutic approach to solve current concerns around antitumor treatment." *Experimental and Therapeutic Medicine*, In press.