Hydrogen and Vitamin C Combination Therapy: A Novel Method of Radioprotection

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Article

Hydrogen and Vitamin C Combination Therapy: A Novel Method of Radioprotection

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Abstract: Radiation therapy is employed in treating various types of cancers. However, its benefits may vary depending on the cancer's type and stage, and it can cause serious side effects that negatively impact patient quality of life (QOL). Mitigating these side effects is crucial for improving both the efficacy of cancer treatment and the patient's QOL. In this context, vitamin C and hydrogen have shown promise in reducing the side effects associated with anticancer drugs and radiation therapy. Both have also been suggested to have direct anticancer properties. However, the potential benefits of their combined use in therapy are not well understood. This study explores the hypothesis that a combination of vitamin C and hydrogen can effectively prevent injuries induced by radiation therapy. We examined survival rates in cancer cell lines (MDA-MB231 and GL261) and normal cells (HUVEC) treated with hydrogen, vitamin C, and irradiation. Apoptosis was assessed using the FLICA test, while the antioxidant effects on normal cells were measured through fluorescent detection of reactive oxygen species (ROS). Additionally, we analyzed epithelial-mesenchymal transition (EMT) gene expression using the quantitative PCR (qPCR) technique. In normal cell cultures, adding vitamin C and hydrogen enhanced survival rates and reduced ROS production, demonstrating both radioprotective and antioxidant effects following irradiation. Conversely, cancer cells exhibited decreased survival rates upon the addition of vitamin C and hydrogen, which further declined after irradiation. Treated cancer cells showed signs of apoptosis. The combined treatment with vitamin C and hydrogen also led to reduced caspase activity in viable cells. Additionally, glioblastoma cells subjected to this combination treatment exhibited a decrease in EMT gene expression. Our study indicates that the combined therapy of hydrogen and vitamin C provides radioprotective and antioxidant benefits to normal cells while exerting direct anticancer effects on cancer cells. This combination also enhances the anticancer efficacy of radiation therapy. Significantly, this therapy reduced the radiation-induced EMT signature in the GL261 murine glioma cell line, indicating its potential in reducing treatment resistance and preventing tumor invasion.

Keywords: hydrogen; vitamin C; cancer treatment; radioprotection; HUVEC; MDA-MB-231; GL261; apoptosis; FLICA; EMT gene expression

1. Introduction

Each year, millions of patients receive radiotherapy for cancer treatment. Many of these patients suffer from severe side effects such as mucositis, dermatitis, xerostomia, gastrointestinal disorders,

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bone marrow failure, and Radiation pneumonitis among others. Preventing these side effects can dramatically improve the quality of life (QOL) of patients receiving radiotherapy and decrease medical expenses associated with treatment. However, few radioprotective strategies are currently in use in clinical practice, and the treatment of radiation injury remains problematic. Moreover, radiation exposure from medical procedures is an issue of concern in many countries. In the United States, the use of computed tomography (CT) increased to 70 million scans annually in 2007 [1], and the radiation-related cancer risk is concerning.

Most of the ionizing radiation-induced damage is caused by reactive oxygen species (ROS), especially hydroxyl radicals (·OH) produced by radiolysis of H2O. Antioxidants are believed to be effective for removing ROS [2,3]. The use of antioxidants in the treatment of radiation injury has been studied extensively [2–5]. The common approach is to deliver the antioxidants concurrently; however, tumor protection is a major concern [6]. Antioxidants may facilitate tumor progression and metastasis through supporting the viability and the invasive capacity of cancer cells [7]. Randomized clinical trials demonstrated that the use of antioxidants such as alpha tocopherol and beta carotene as adjuvant therapy might compromise radiation efficacy and is associated with poor tumor control and may increase mortality [8–10].

Amifostine [11,12], the first radioprotective agent approved by the Federal Drug Administration, is associated with toxicities that limit its use and efficacy [13].

Both vitamin C and hydrogen protect from radiation-induced damage [14–29] and function by removing ROS from the human body. They also reduce inflammation in tissues. The two therapies are easy to perform, relatively inexpensive, and most importantly, safe [30–36]. They do not attenuate but sometimes enhance the effect of radiation in cancer treatment [37–39]. Ascorbic acid increases radiation-induced apoptosis in HL60 Human Leukemia Cell lines by activating caspases-3, 8, and 9 [38]. It has also been reported that pharmacological concentrations of ascorbate radiosensitize pancreatic tumor and glioblastoma cells [40–43].

Hydrogen and vitamin C are said to have anticancer effects [40–61]. Schoenfeld et al. recently reported a new mechanism underlying the effect of vitamin C. non-small-cell lung cancer (NSCLC) and glioblastoma (GBM) cells are selectively sensitive to vitamin C because of an altered redox-active iron metabolism [51].

Carosio et al reported that Sodium ascorbate induces apoptosis of neuroblastoma cell lines by inhibiting iron uptake [52].

Hydrogen also has direct and indirect antitumor effects, which could be useful for the treatment of cancer patients. Hydrogen therapy improves overall survival, quality of life, blood parameters, and tumor reduction. In addition, hydrogen attenuates the risk of carcinogenesis induced by radiation.

Therefore, vitamin C and hydrogen may have additional therapeutic effects against cancer. Although the effect of each therapy on various diseases has been studied, the efficacy of combination therapy with vitamin C and hydrogen has not been evaluated to date. The combination of these two therapies could be effective for preventing side effects and improving the QOL of patients receiving radiotherapy.

1.1. Background of the idea of hydrogen vitamin C combination therapy

High-dose intravenous vitamin C infusion is used in many clinics for cancer treatment throughout the world [62,63], but its effectiveness varies from person to person. The recommended dose of vitamin C per infusion is 25 g to 100 g. In the author's clinic (Shimokitazawa Nishiguchi Clinic, Tokyo Japan), there had been cases in which no anti-cancer effect at all was observed with high-dose vitamin C alone.

In many cases, the theory that the anticancer effect of vitamin C increases as the blood concentration rises with increasing dose, does not hold true. Therefore, the author came up with the idea of hydrogen as an adjuvant therapy, to support and enhance the effects of vitamin C. It was the origin of the hydrogen and vitamin C combination therapy, and Miyakawa M has been supporting many cancer patients in her clinic.

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1.2. Hydrogen and Vitamin C Combination Therapy Method

Patients begin hydrogen inhalation 10 minutes prior to vitamin C injection. Patients are treated with high-dose vitamin C injection while inhaling simultaneous hydrogen (99.99% hydrogen gas, 250 ml/min, H2JI1, Doctor's Man, Inc.) with nasal cannula.

To reduce the side effects of radiotherapy, daily infusions are administered after radiotherapy.

When combination therapy was used as supportive care for cancer treatment, the frequency of treatment varied from person to person, from every day, twice a week to once a month, depending on the individual's medical condition and budget.

All patients received standard treatment at the hospital, followed by the hydrogen and vitamin C combination therapy at the clinic.

In addition, patients also performed the hydrogen and vitamin C combination therapy at home on their own as much as possible.

1.3. Home therapy

Not only treated in the clinic, but also patients treated themselves with hydrogen and vitamin C at home. Hydrogen gas inhalers, hydrogen bath generators, and hydrogen water generators were installed in patients' homes, and patients inhale for as long as possible, take hydrogen baths, and drink hydrogen water to literally live a hydrogen-soaked life. In addition, they took hydrogen supplements when they go out or are hospitalized. Hydrogen supplements are those that produce a large amount of hydrogen gas in the intestines. Some patients were only able to incorporate a portion of these hydrogen treatments for financial reasons, so there are individual differences in the way hydrogen is incorporated at home.

For home vitamin C treatment, patients took lipo-capsule vitamin C(Lypo-C). Lypo-C is an ascorbic acid supplement in liposome capsules, which is said to increase blood levels of ascorbic acid to a higher level than regular ascorbic acid supplements. Differences were also observed in the amount of Lipo-C intake per day. These treatments were not covered by insurance, so all were done at their own expense.

1.4. Anti-cancer effects of hydrogen and vitamin C combination therapy

Although individual differences in efficacy were observed, favorable results were obtained in many patients to reduce side effects of not only radiation treatment, but also chemotherapy. It also seemed to have anti-cancer effects. The types of cancers for which efficacy was observed include lung, pancreatic, liver, colorectal, prostate, uterine, breast, and parotid cancers. However, these were all at the level of individual case reports and had not been scientifically proven. Additional studies are necessary before the combination therapy can be routinely applied for radioprotection in the clinic.

1.5. Radioprotection Case

Especially in patients with radiation induced dermatitis, we can visibly confirm the efficacy of this combination therapy. Two patients provided their consent for the publication of their information and photographs.

Case1: 85-year-old male

Malignant parotid carcinoma treated with hydrogen inhalation and vitamin C IV-injection at clinic, and home therapy (hydrogen inhalation and Lypo-C).

The patient received combination therapy at the clinic every day after radiation therapy. The skin redness begins to decrease at 30–40 minutes after the start of treatment (20–30 minutes from the start of vitamin C injection), and patient also reported decreased pain.

Figures 1–3 shows the progress of the patient’s treatment. After surgery for high-grade parotid carcinoma (pathology is conduit carcinoma), stage 4, the patient underwent chemotherapy and radiation therapy at a total dose of 60 Gy for 2 months. After daily irradiation, the patient received hydrogen and vitamin C combination therapy.

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Figure 1 shows the patient before the start of treatment; the skin at the irradiated site is red, swollen, and inflamed. Figure 2 shows the skin 20 minutes after the start of combination therapy, and the redness has decreased to about half of its original size. Figure 3 shows the end of combination therapy, and the redness has almost disappeared.

Remarkably, the patient was able to comfortably complete the two months of radiotherapy without suffering from mouth ulcers, dermatitis, etc., which usually appear as side effects of the treatment.

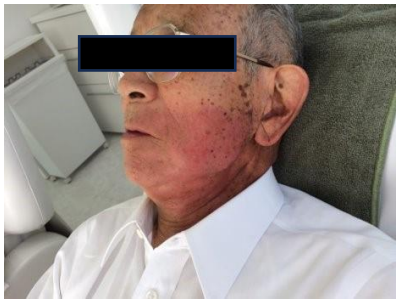

Figure 1. Before combination therapy .

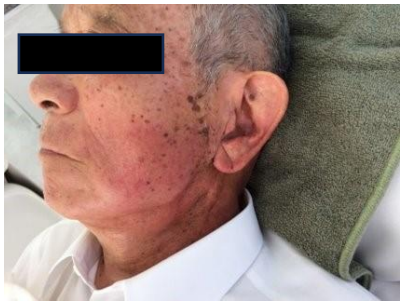

Figure 2. 20 min after the start of combination therapy .

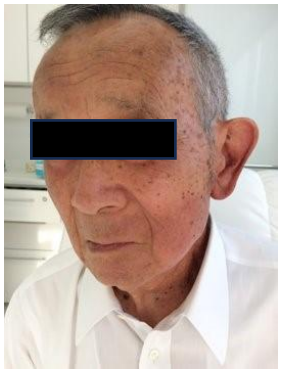

Figure 3. After combination therapy .

Case 2: 47-year-old female

Nasopharyngeal cancer treated with home therapy with Lipo-C, hydrogen inhalation, and hydrogen mist

This is a case of nasopharyngeal cancer treated with radiotherapy for 2 months, a total dose of 70Gy, for 35 times, and anticancer drug therapy. The patient underwent radiation therapy as an outpatient, and daily treatment at home with hydrogen inhalation, and hydrogen mist to the affected area. She also took Lypo-C every day. The skin did not sore at all, and She could complete the radiotherapy with only a little darkening and peeling. His doctor was surprised that under normal circumstances, the skin would have turned red and sore and become inflamed. Figures 4–6 show the

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slightly darkened skin just before the end of the radiation therapy, and Figure 7 shows the skin that had already cleared up one month after the radiation therapy.

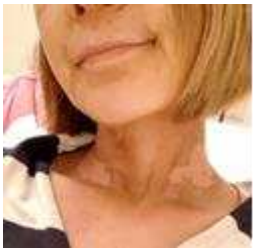

Figure 4. days before end of radiation therapy .

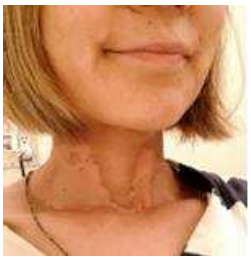

Figure 5. days before end of radiation therapy.

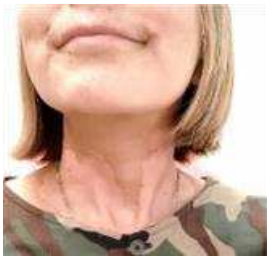

Figure 6. the last day of radiation therapy.

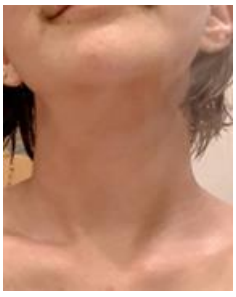

Figure 7. one months after radiation therapy.

Based on these clinical experiences, we hypothesized that the combination therapy of hydrogen and vitamin C would have prevented the radiation induced injury and conducted experiments on the effects of hydrogen and vitamin C on cells.

1.6. The hypothesis

Hydrogen and vitamin C combination therapy is effective to prevent radiation injury.

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2. Material and method

2.1. Cell Lines

HUVEC cells were maintained in Endothelial Cell Growth Medium 2 (PromoCell, Cat. C-22011) supplemented with Growth Medium 2 SupplementMix (PromoCell, Cat. C-39216). MDA-MB-231 was maintained in Dulbecco's modified Eagle's medium (Gibco, Cat. 41965) supplemented with 10% fetal bovine serum (Invitrogen, Cat. 25149-079) together with Antibiotic-Antimycotic (Gibco, Cat. 15240062). The murine glioma model GL261, provided by (NCI-Frederick), was defrosted and expanded in sphere condition (serum-free) containing DMEM/F12-GlutaMAX medium (MilliporeSigma), basic fibroblast growth factor 10 ng/ml, epidermal growth factor 20 ng/ml (Peperotech), B27-supplement 1:50 (Stem cell technology). The cells were kept in culture at 37°C in a humidified 5% CO_2 atmosphere.

2.2. Vitamin C and hydrogen treatment

The methods of administering vitamin C and hydrogen were determined based on previous cell experiment studies [47,48] and preliminary experiments. In preliminary experiments, vitamin C was administered at various concentrations in cell culture medium with reference to previous studies. As MDA-MB231 cells were almost killed at a vitamin C concentration of 2 mM, two concentrations, 0.2 mmol and 1 mM, were selected. At these concentrations, survival was increased after 24 hours for normal cells. In the experiment on EMT gene expression in glioblastoma, the vitamin C concentrations were increased to 5 mM and 10 mM based on the results of previous experiments. For hydrogen, a Pure Hydrogen Gas Generator Model HB-H10 was used for generation of hydrogen gas. The culture medium was placed in a spits (30 ml) and bubbled with 99.99% hydrogen gas at 250 ml per minute for more than 60 minutes to ensure a concentration of more than 1000 ppb when the cells were put into petri dishes.

2.3. Radiation treatment

The irradiation dose was 4 Gy and was performed using Xstrahl (LIFE SCIENCES).

Cells (HEVEC, MDA-MB231, GL261) were added to the culture medium under five condition treatments: 0.2 mmol vitamin C alone, 1 mmol vitamin C alone, hydrogen alone, 0.2 mmol vitamin C + hydrogen, 1 mmol vitamin C + hydrogen. Viability rates were compared to the control (culture medium without addition of vitamin C and hydrogen) as 1. The treatment timing of each cell line was performed at several time point:1) treatment without irradiation, 2) treatment started before irradiation, 3) treatment started after irradiation, and 4) treatment started before and added again after irradiation, and each of the five conditions were applied to all cell lines. The viability rate was observed 24 hours after irradiation (24 hours after addition without irradiation).

Viability rates were measured, using an Invitrogen Countess Automated Cell Counter (Life Technologies), three times each for HUVEC and MDA-MB231 and four times for GL261, and the average value was presented.

2.4. Fluorescent detection of ROS

For determining total ROS (superoxide and hydrogen peroxide) in HUVEC cells that underwent different treatment condition, single cell suspension was prepared from all treatment condition where treatment started either after RT and lasted for 24h, or before and after RT (24h before RT and lasted for 24h after RT). Dihydroethidium (DHE) staining (Invetrogen) was done on viable cells (1mM) for 30min at 37ºC, followed by washing with FACS buffer (PBS+4%FBS). Samples were acquired on a LSR II flow cytometer (Becton Dickinson), then data analyzed using FlowJo 10 (Becton Dickinson)

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2.5. Confirmation of apoptosis

FAM-FLICA® (Fluorescent-Labeled Inhibitor of Caspases Assays) test was used to confirm the extent to which apoptosis actually occurred in MDA-MB231 cells when vitamin C and hydrogen were added and irradiated. The FLICA™ kit can easily measure active caspases from the amount of fluorescence in living cells and distinguishes between apoptosis and necrosis. Instead of using antibodies as in ELISA, the kit utilizes an inhibitor (C-terminally labeled with a red or green fluorescent probe) that is shared by each active caspase. Apoptotic cells fluoresce in red or green. This test was performed for the treatment condition that achieved the highest antitumoral in vitro efficacy depending on viability rate after treatment (hydrogen and vitamin C administered twice before and after irradiation) because the test was performed to confirm what was happening to the cells when the survival rate decreased. It was done with 4 conditions, 1. control (culture medium only), 2. 1 mM vitamin C treatment, 3. Hydrogen treatment, and 4. 1 mM vitamin C and hydrogen combination treatment. For conditions 2, 3, and 4, hydrogen and/or vitamin C were administered before and after irradiation. Photographs were taken of the same specimens at four different locations.

2.6. EMT gene expression

RNA isolation and quantitative real-time PCR.Around 1-1,5x10^6 cultured cells were incubated in 0.7 ml Qiazol (Qiagen) for 5 min at RT. Thereafter, 0.14 ml Chloroform was added and vigorously aspirated and centrifuged at 12000 g for 10 min at 4°C. The aqueous phase was collected and transferred to a new tube, 0.5 ml of 100% Ethanol was added and the mixture was pipetted onto an RNeasy Mini column and centrifuged (12000 g, 15 sec, RT) after which the flow-through was discarded. Then 0.5 ml RPE buffer was added to the column and centrifuged (12000 g, 15 sec, RT). This step was repeated again for 2 min. 20-30 ul of RNase-free water was used to elute the RNA. The RNA concentration was determined using a Nanodrop spectrophotometer and 1µg total RNA was used for cDNA synthesis using the iScriptTM cDNA Synthesis kit, according to manufacturer`s instructions (BioRad). Gene expression analysis was performed using quantitative real-time polymerase chain reaction (qPCR) on a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, USA). The thermoprofile was 95ºC for 15 minutes and 40 cycles of 94ºC for 15 seconds, 55ºC for 20 seconds and 72ºC for 2 minutes. The total PCR volume of 20 microliters consisted of 10 µl 2x QuantiTectTM SYBR® Green PCR master mix (Qiagen), 2 µl 10x QuantiTect Primer Assay (Qiagen) and 20 ng cDNA (2 ng were used for actin) in 96-well optical plates. Extracted DNA samples were analysed in triplicates. The pre-validated QuantiTect Primer Assays were obtained from Qiagen. The gene expression was determined by the method of direct comparison of C T values (C T > 35 was rejected) and relative quantities calculated by the ΔΔCT equation or transformed into linear form. Transcripts were normalized to the quantity of Actin and GAPDH for each condition.

Table 1. primers used in qPCR.

Gene name	Accession number
SNAI1	Hs_SNAI1_1_SG
SNAI2	Hs_SNAI2_1_SG
CDH1	Hs_CDH1_1_SG
TWIST1	Hs_TWIST1_1_SG
TGFB1	Hs_TGFB1_1_SG
STAT3	Hs_STAT3_1_SG
VIM	Hs_VIM_1_SG
CD248	Hs_CD248_1_SG
GAPDH	Hs_GAPDH_1_SG
ACTB	Hs_ACTB_1_SG

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2.7. Statistical Analysis

T-tests and analysis of variance were used to compare survival rates. Statistical analysis was performed using Microsoft Excel for Mac V16.77 or GraohPad Prism V8/9 software (GraphPad Software, San Diego, CA, USA) software.

3. Results

3.1. Comparative experiments of cell viability

Results of the comparison of cell viability are shown in Tables 2–4.

Table 2. Normal cells (HUVEC) .

	control	VC0.2mM	VC1mM	H2	H2+VC0.2mM	H2+VC1mM
Without RD	1	1.31	1.35	1.21	1.03	1.14
Before RD	1	1.37	1.34	1.27	1.31	1.56
After RD	1	1.47	1.47	1.14	1.37	1.52
Before and After RD	1	1.52	1.58	1.09	1.61	1.8*

Table 3. Brest cancer cells (MDA-MB-231).

	control	VC0.2mM	VC1mM	H2	H2+VC0.2mM	H2+VC1mM
Without RD	1	0.94	0.81	0.84	0.87	0.85
Before RD	1	1.09	0.75	0.83	0.71	0.63*
After RD	1	0.74	0.62*	0.81	0.65*	0.44**
Before and After RD	1	0.72*	0.62**	0.76	0.76	0.44 ***

Table 4. Glioblastoma (GL261).

	control	VC0.2mM	VC1mM	H2	H2+VC0.2mM	H2+VC1mM
Without RD	1	0.85	0.72	0.93	0.84	0.54*
Before RD	1	0.89	0.77	0.89	0.8	0.73*
After RD	1	0.86	0.61*	0.83	0.71*	0.71*
Before and After RD	1	0.9	0.77	0.92	0.88	0.76

Survival rate of control group standardized to 1 . Analysis of variance showed a statistically significant difference compared to the control group(*:p<0.05 **:p<0.01, *** ：p<0.001) .

In normal cells, viability after 24 hours of hydrogen and vitamin C treatment increased compared to controls in all five conditions, with or without irradiation. In contrast, the viability rate

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of cancer cells decreased in all five conditions compared to the control, except one column (Table 2 MDA-MB-231, before irradiation, with 0.2mM vitamin C).

Table 2 shows that in the absence of irradiation, the 24-hour viability rates of normal cells tended to increase with the addition of vitamin C or hydrogen alone. In the case of irradiation, the highest viability rate at 24 hours was observed when 1 mM of vitamin C and hydrogen were added to the culture medium (statistically significant only treated before and after irradiation). However, vitamin C tended to increase viability considerably even when used alone. The viability rate tended to be higher when both vitamin C and hydrogen were added at two time points, before and after irradiation. Table 3 shows that in MDA-MB231, both vitamin C and hydrogen tended to suppress the viability rate even when used as sperate treatment. The addition of vitamin C and hydrogen along with irradiation decreased the viability rate in all five conditions, but the addition of 1 mM of vitamin C and hydrogen to the culture medium significantly suppressed the viability rate 24 hours after irradiation.

Table 4 shows that in GL261, the addition of vitamin C and hydrogen to the culture medium suppressed viability after 24 hours.

Under the condition of 1 mM vitamin C plus hydrogen therapy, statistically significant results were obtained in all but the before and after irradiation periods. The lowest viability rate was observed in without irradiation, which can be interpreted as the fact that in highly malignant glioblastomas, half-way anticancer drugs or irradiation may activate surviving cancer cells and increase their proliferation rate.

These results suggest that in normal cells, the combination of 1 mM vitamin C and hydrogen is the most effective radioprotective agent.

Similarly, in cancer cells, the combination of 1 mM vitamin C and hydrogen is likely to enhance the anticancer effect of radiation.

The antioxidant effect of combination therapy in normal cells that underwent radiation therapy (Figure 8).

Normal cells (HUVEC) were used to evaluate the antioxidant effect of combination therapy in response to radiation treatment (4Gys) by measuring ROS production in normal cells. In vitro treatment started, either after radiation treatment, or before and after radiation. Vitamin C was tested at different concentrations: 0,2mM, 1mM, 5mM, 10mM alone or in combination with hydrogen. The combination therapy reduced ROS in normal cells when treatment started after RT directly with best efficacy achieved at H+10mM Vitamin C as shown in Figure 8.

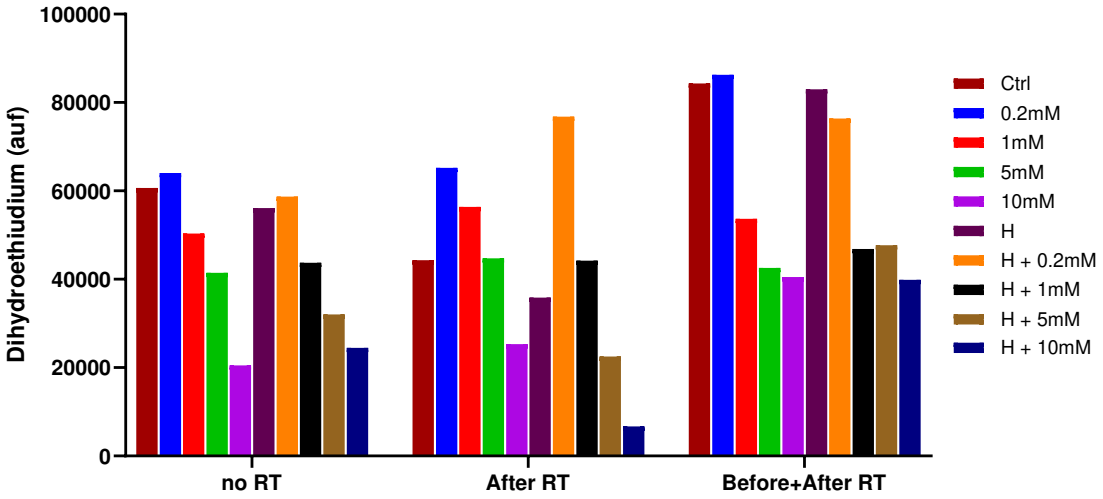

Figure 8. Quantification of ROS production in normal cells (HUVEC) using Dihydroethidium (DHE) fluorescent staining. ROS is reduced in response to combination therapy and to higher extent when treatment started after RT than before and after RT, with highest efficacy seen in high dose 10mM+ H.

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3.2. Confirmation of apoptosis (Figures 9–12)

In MDA-MB231, the FLICA test was used to confirm the extent to which apoptosis actually occurred in the cells when vitamin C and hydrogen were added and irradiated. This test was performed only under the condition of the lowest survival rate (hydrogen and vitamin C administered twice before and after irradiation) because the test was performed to confirm what was happening to the cells when the viability rate decreased. Photographs were taken of the same specimens at four different locations. The four conditions were: 1. control (culture medium only), 2. 1mM vitamin C, 3. hydrogen, and 4. 1mM vitamin C and hydrogen. For conditions 2, 3, and 4, the cells were treated before and after irradiation. Figure 9 shows that little apoptosis occurred in control with only culture medium. Figure 10 shows that administration of vitamin C alone with radiation caused apoptosis at a low rate. Figure 11 shows that overall apoptosis occurred with hydrogen treatment with radiation. Figure 12 shows vitamin C treatment in combination with hydrogen with radiation exposure, apoptosis occurred overall at a much higher rate. The changes in the cells were confirmed by FLICA assay, and apoptosis was observed in cancer cells treated with hydrogen and vitamin C. The results showed that the caspase activity in viable cells was reduced by hydrogen and vitamin C treatment, and that apoptosis occurred in cancer cells treated with hydrogen and vitamin C.

The results of the FLICA test corroborated the experimental results for viability, suggesting that hydrogen and vitamin C combination therapy with radiation had anticancer effects, and hydrogen may have a greater anticancer effect than vitamin C in MDA-MB231.

Apoptosis occurred at the highest rate in the combination of vitamin C and hydrogen with radiation, suggesting that the anti-cancer effect of the combination treatment is high.

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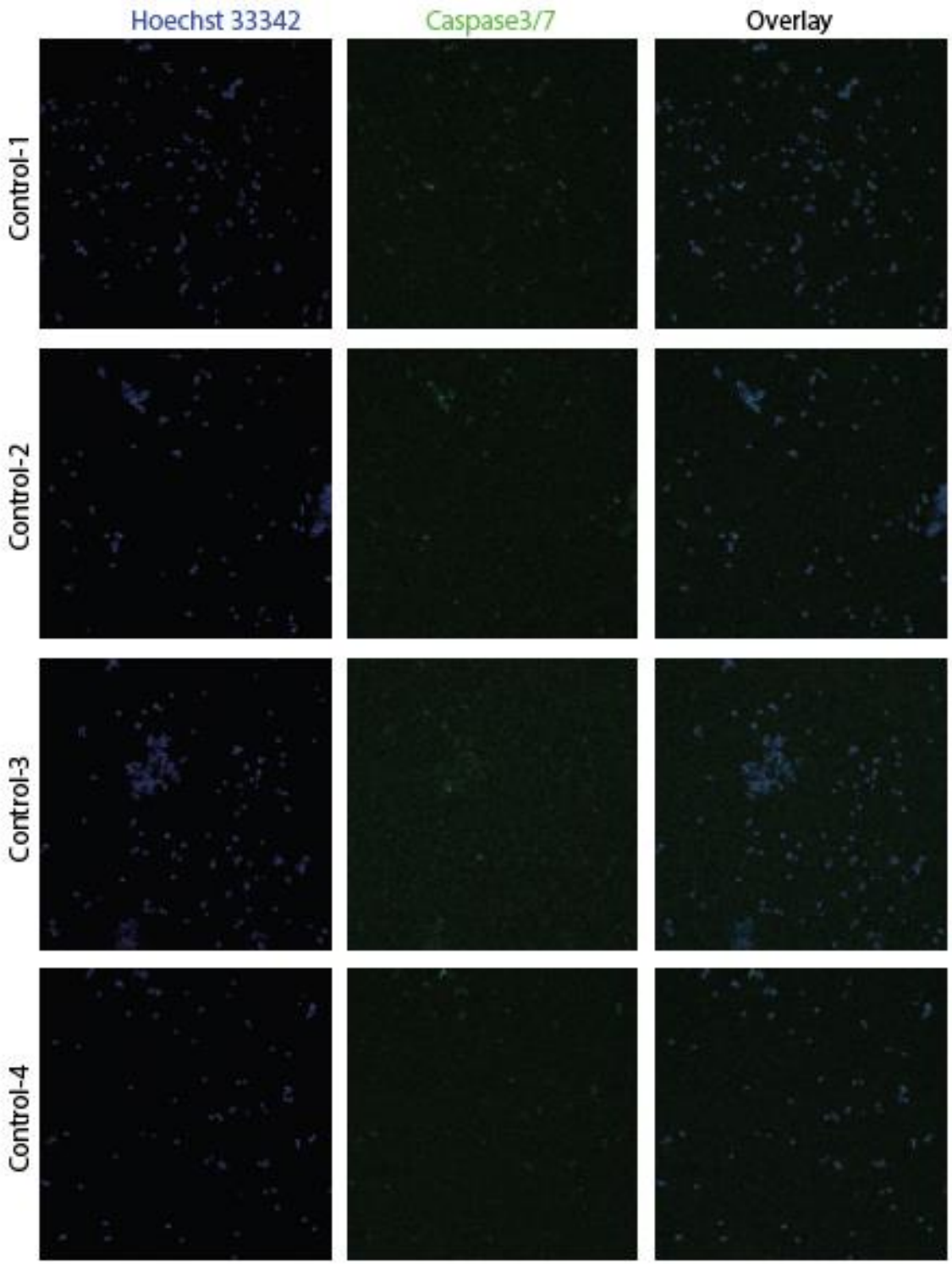

Figure 9. Apoptosis confirmed by FLICA : control apoptosis rarely occurred in control with only culture medium.

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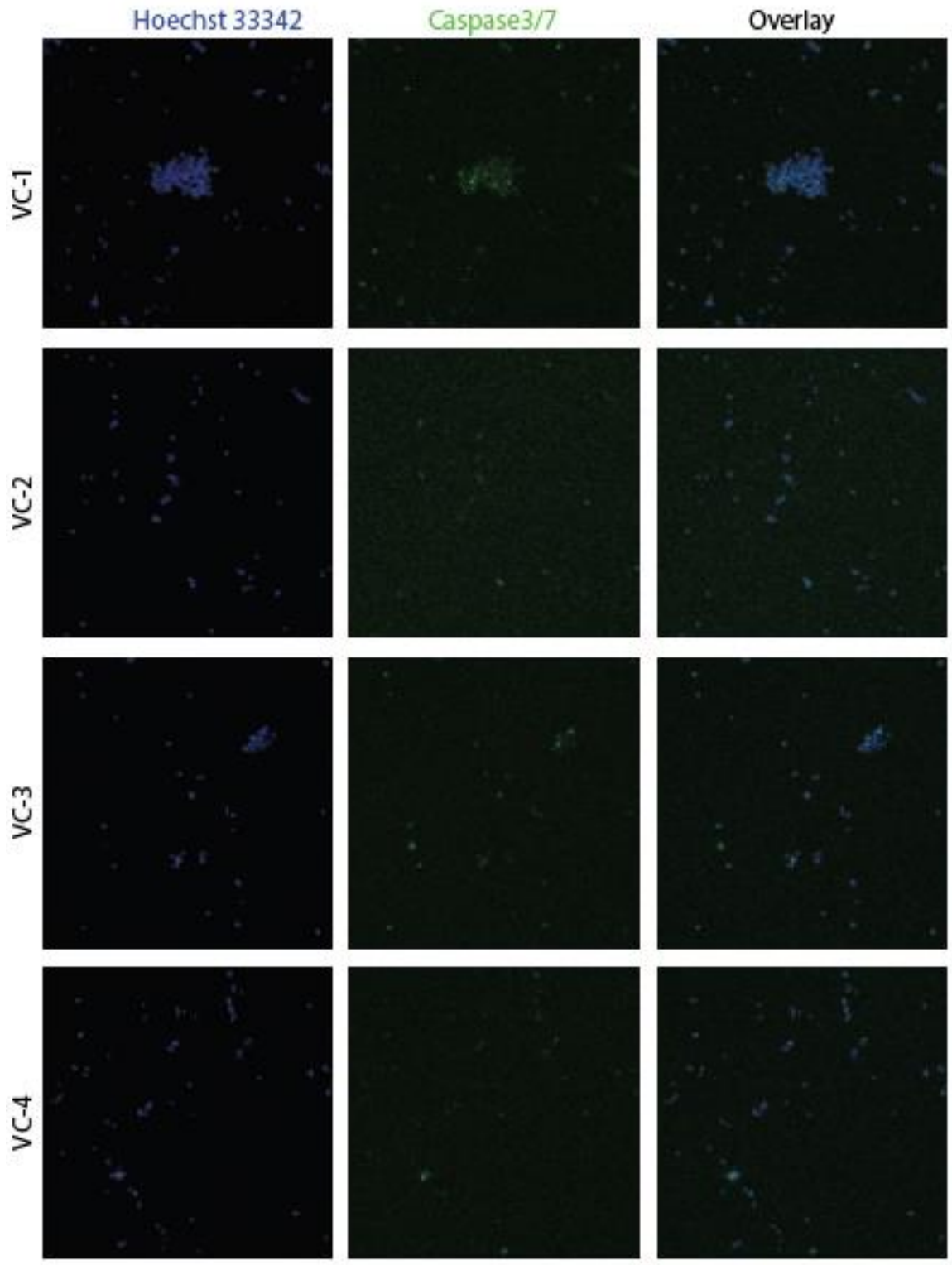

Figure 10. Apoptosis confirmed by FLICA :vitamin C Apoptosis occurred at a low rate.

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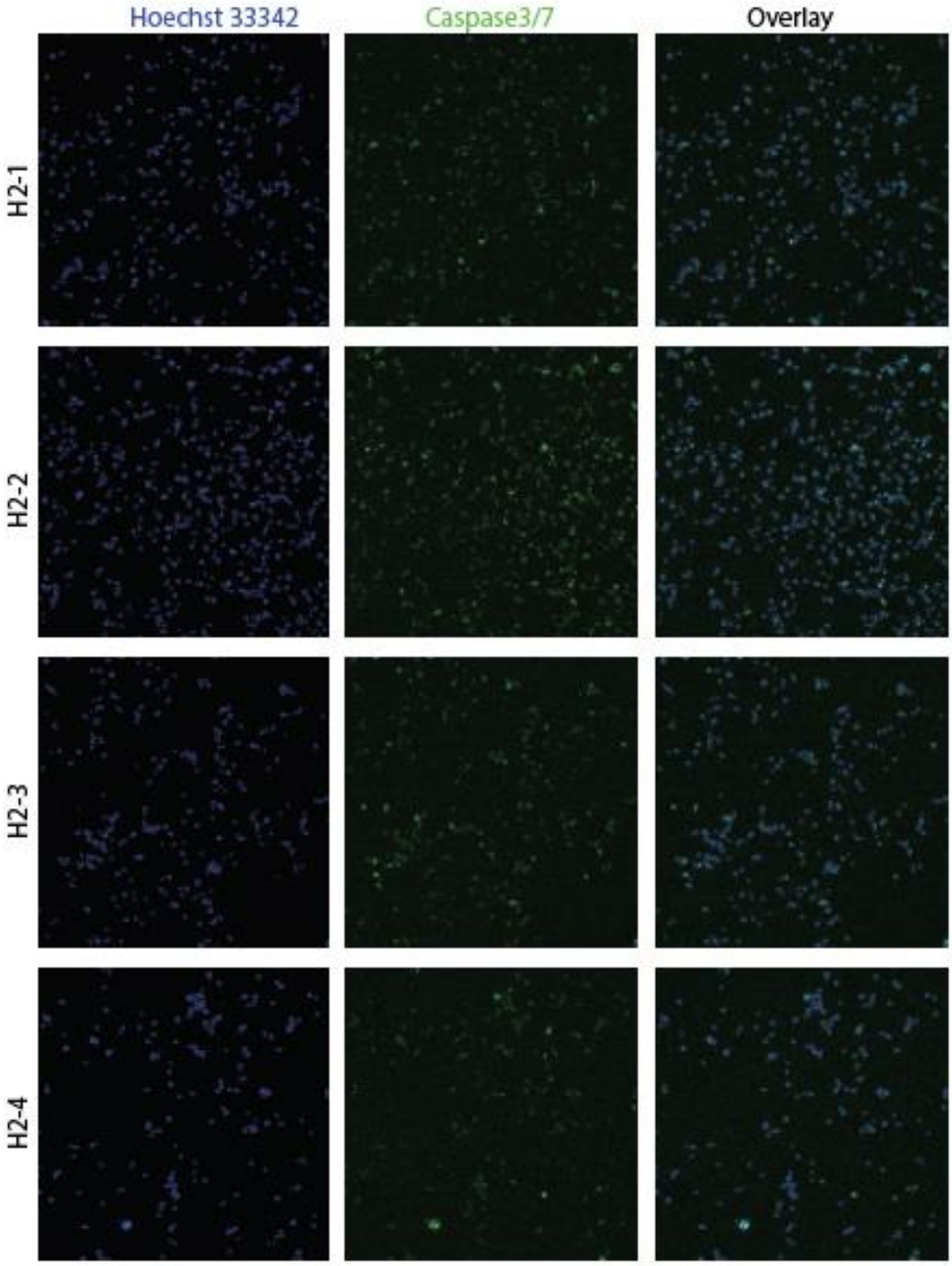

Figure 11. Apoptosis confirmed by FLICA :hydrogen overall apoptosis occurred.

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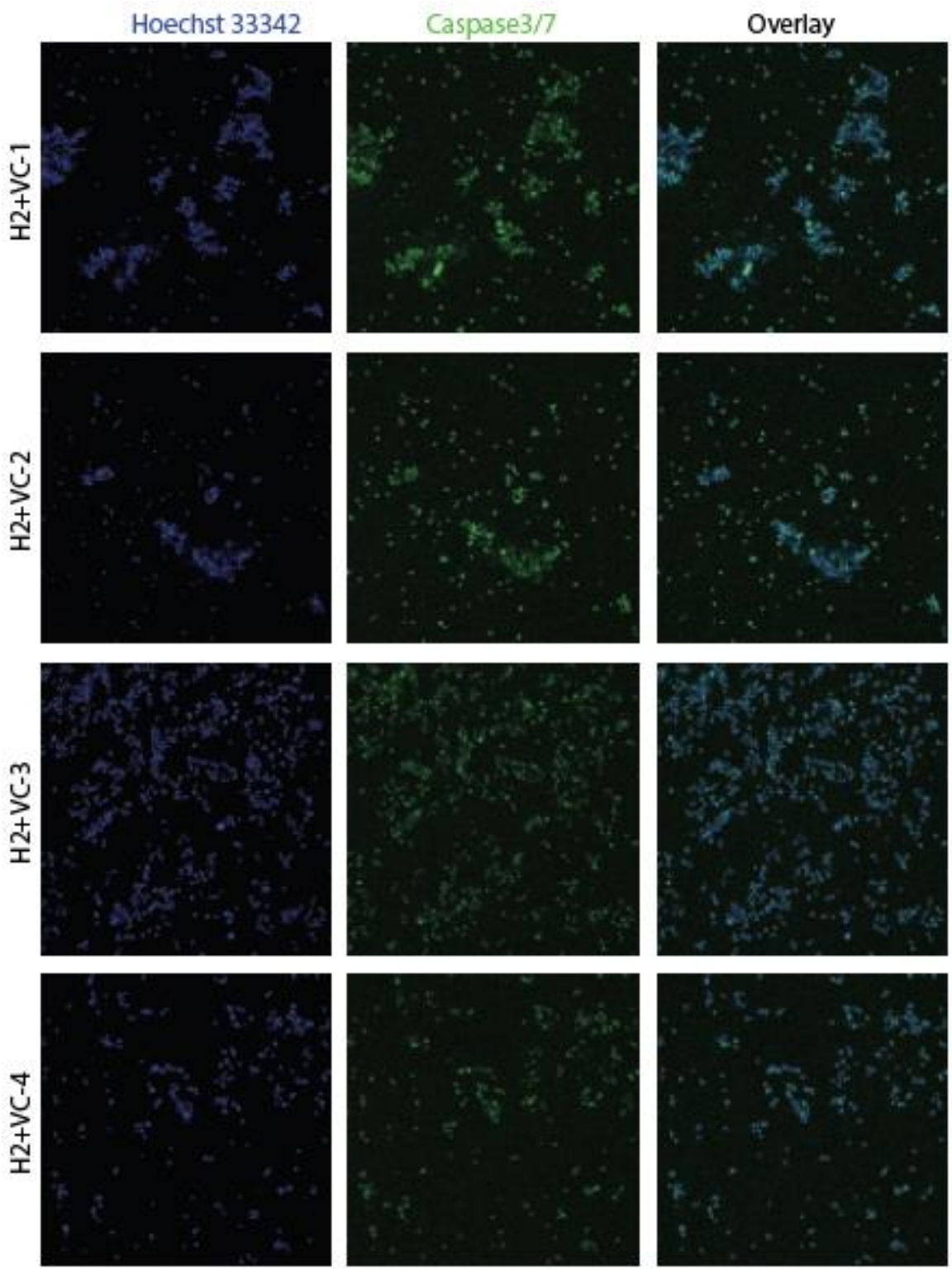

Figure 12. Apoptosis confirmed by FLICA :hydrogen and vitamin C apoptosis occurred overall at a much higher rate.

3.3. The EMT signature in GBM

In high grade glioma, EMT gene signature is associated with therapy resistance [64]. In our work, we treated GL261 glioma cells with Vitamin C and hydrogen. The combination of Vitamin C treatment with hydrogen reduced the gene expression of most EMT gene set (Snai1, Snai2, Twist1, Stat3, TGFB1, Vim) almost by 50%. The expression of cadherin (CDH1) seemed to be less affected

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(Figure 12). The result suggests that hydrogen and vitamin C combination therapy has anti-cancer-effects through reducing EMT signature that usually mediate therapy resistance and tumor invasion.

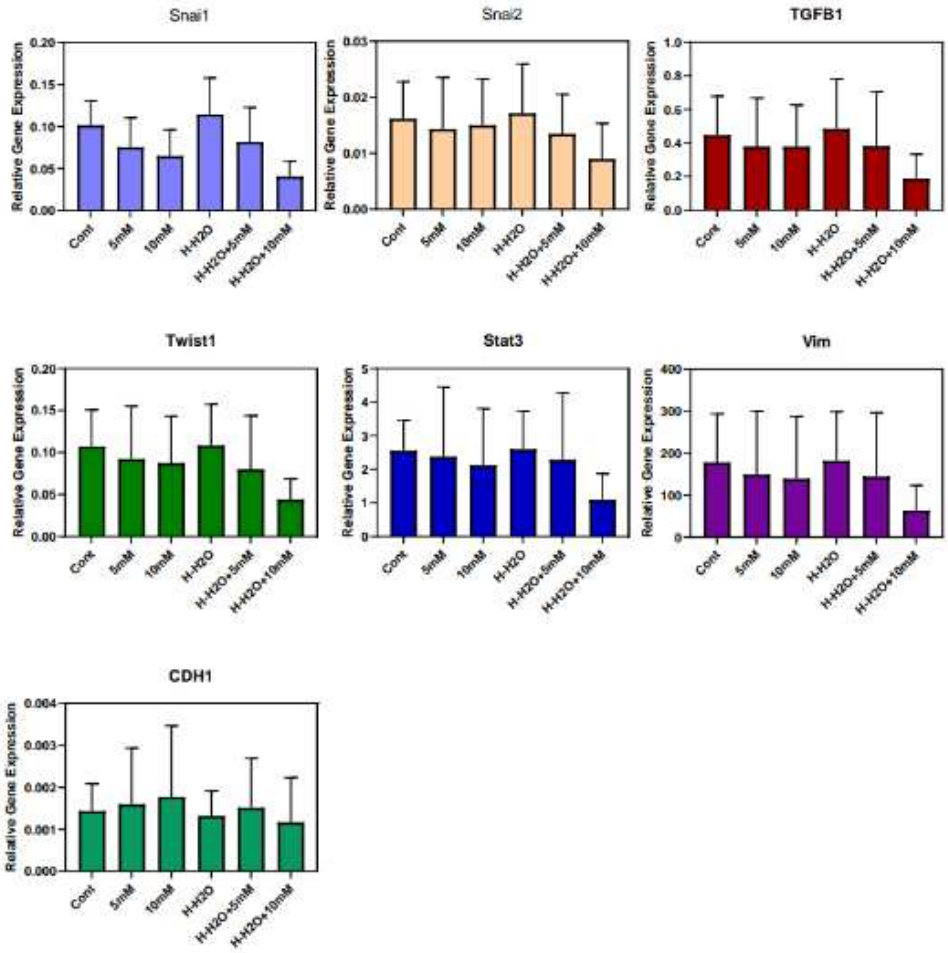

Figure 13. In vitro treatment of GL261 glioma cell line with hydrogen and vitamin C showing the combination therapy reduce EMT gene signature up to 50%, while cadherin (CDH1) expression seemed to be less affected. Gene expression was quantified by qPCR. EMT gee set included Snai1, Snai2, Twist1, Stat3, TGFB1, Vim, CDH1.

Upon radiation treatment, the combination therapy with high concentration of vitamin C achieved the best consistent EMT inhibitory effect (Figure 14). Thus we see that the combination of high dose Vitamin C (10mM) with hydrogen has inhibitory effect on the intrinsic EMT expression in GL261, and RT-induced EMT, suggesting antitumoral effect.

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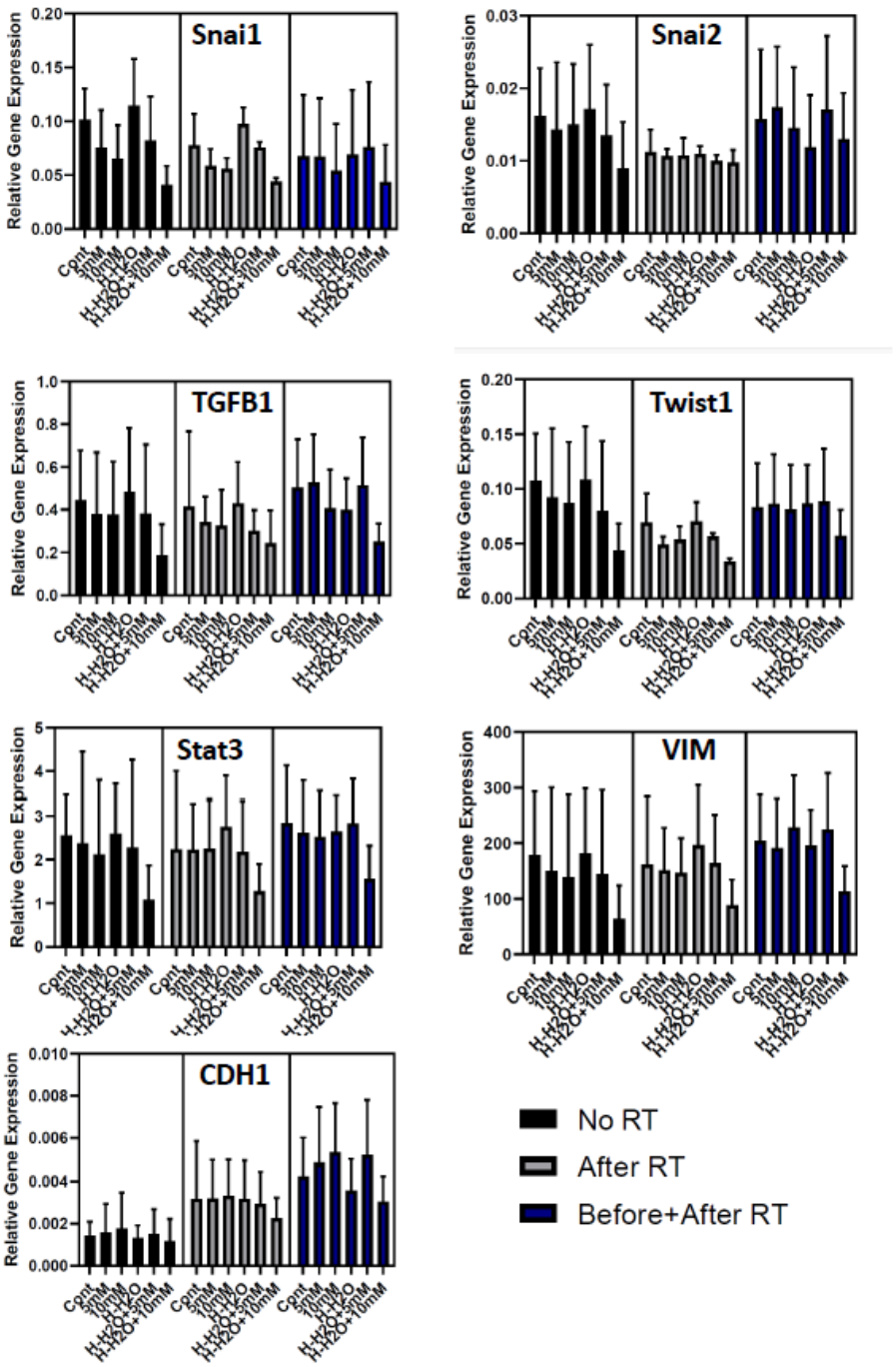

Figure 14. In vitro treatment of GL261 glioma cell line with hydrogen and vitamin C after radiation treatment (After RT) or before and after (Before + After RT) compared to non-radiation treatment (NO-RT). Gene expression was quantified by qPCR relatively to UBC gene expression and calculated using **2^-D ct**. EMT gee set included Snai1, Snai2, Twist1, Stat3, TGFB1, Vim, CDH1.

4. Discussion

4.1. Effects of vitamin C and Hydrogen Peroxide(H_2O_2)

The effectiveness of vitamin C in cancer treatment was reported by Pauling as early as in 1976 [65]. Over the past 50 years, the effectiveness of vitamin C in cancer treatment has been recognized to some extent. Vitamin C is shown to not only decreases radiation-associated complications but has also the potential to kill cancer cells directly. Although, there is no clear evidence based on human

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randomized controlled studies, animal studies and clinical case reports show the preventive effects on radiation-induced injuries [14–16]. This may be attributed to the effect of vitamin C as an antioxidant and the fact that vitamin C has an anti-inflammatory effect and may reduce inflammation in cancer patients [47]. This property could be helpful for preventing radiation injury. Moreover, vitamin C may enhance the efficacy of radiation in the treatment of cancer, as it is suggested to function as a radiosensitizer [40–43]. Importantly, vitamin C is harmless to normal cells, and selectively targets cancer cells [44]. Vitamin C acts as a pro-drug for H2O2 formation and, through this mechanism, kills cancer cells [45]. When vitamin C is sufficiently concentrated in the blood, it is transported outside blood vessels, generating large quantities of hydrogen peroxide (H2O2). Normal, healthy cells are unaffected by H2O2, generating by vitamin C at higher concentrations, as they possess enzymes such as catalase that can neutralize it. Since the cancer cells lack these enzymes, the cellular function is dramatically altered when they take in reactive oxygen and as a consequence of this, cancer cells enter into apoptosis process [66]. Previously, it has been shown by in vitro experiments that increasing vitamin C concentration to the maximum level, up to 20 mM, will kill almost all cancer cells [48]. However, even when intravenous infusions temporarily increased the concentration of vitamin C in the blood, the anticancer effect was often not sufficient. This was thought to be due to some other factors that prevented vitamin C from becoming the standard of care in cancer treatment so far. To overcome this obstacle, the authors considered combining vitamin C with hydrogen. Although hydrogen and vitamin C combination therapy is still not sufficient in terms of anticancer activity, the results of this study suggest that it may be quite effective in terms of radioprotection. Further in vivo experiments are required to confirm the antitumoral efficacy of combining vitamin C and hydrogen.

4.2. The effects of hydrogen

Hydrogen is originally produced by intestinal bacteria in the gut and is naturally present in the body [67]. Previously, it was shown that hydrogen acts as a therapeutic and preventive antioxidant in cultured cells and has cytoprotective effects against oxidative stress [68]. The mechanisms for the effects of hydrogen are also becoming clearer [69]. Hydrogen can rapidly pass through biological membranes and diffuse into the cytosol because of its small size. In addition, hydrogen is soluble in both water and lipids, which contributes to its diffusibility. Hydrogen reaches the cell nucleus and mitochondria, protecting nuclear DNA and mitochondria. In addition, it passes through the blood brain barrier. Hydrogen selectively removes highly active oxidants such as hydroxyl radicals (•OH) induced by radiation and does not react with ROS, which have low reactivity, such as superoxide, H2O2, and CO. ROS with low reactivity play important roles in signal transduction and the immune system [70]. Other antioxidant supplements such as vitamin E also remove all these ROS, which is necessary, and have possibility to increase mortality [8–10]. It means that the fear that antioxidants support tumor cells.

In the past ten years, several studies reported the preventive and therapeutic effects of hydrogen. These studies cover various biological effects against oxidative stress in almost all organs [71–73]. Studies on the treatment of diseases with hydrogen have reported that no side effects at all occur. It became clear that the biological and medical roles of hydrogen are broad, including anti-inflammatory, antiapoptotic, and antiallergic effects. In addition to its efficacy in animal models, many studies examined and reported the efficacy of hydrogen in clinical practice. Furthermore, hydrogen may act as a radioprotector by neutralizing •OH radicals in irradiated tissues. Cyuai et al. showed that the effects of hydrogen are similar to those of Amifostine without side effects, thereby, it can be an ideal radioprotective agent [29]. Yang et al. showed that hydrogen induces apoptosis in endometrial cancer cells via the TNF and NF-κB pathways [56,57]. They also showed that irradiation of HEC1A cells in hydrogen-treated cultures significantly increased the rate of apoptosis compared to normal cultures, indicating that hydrogen is an effective enhancer of radiotherapy. Another study by Liu et al showed that hydrogen is a potential antitumor agent in GBM therapy [58].

4.3. Expected benefits of hydrogen and vitamin C combination therapy

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Under certain conditions, vitamin C plays a role as a pro-oxidant and may induce oxidative stress [74,75]. When vitamin C leads to the production of excess H2O2, the generation of •OH increases and may damage normal cells. In such circumstance, hydrogen can selectively remove •OH and compensate for the effect of vitamin C. Inflammation is associated with cancer metastasis [76,77]. Our data suggests that the combination therapy of Vitamin C with hydrogen reduced ROS level in normal cells supporting the antioxidant role of this treatment.

Our results showed that vitamin C and hydrogen each had anticancer effects on tumor cells and protective effects on normal cells. These effects were further increased when vitamin C and hydrogen were combined. Furthermore, when irradiation was added, the anticancer effect of radiation was enhanced and the radioprotective effect on normal cells was demonstrated. These effects were greater during combination treatment in contrast to treatment with vitamin C and hydrogen alone. Moreover, a higher concentration of vitamin C at 1 mM was more effective than at 0.2 mM. The strongest effects were obtained when hydrogen and vitamin C were added to the cells twice, before and after irradiation. Previously, experiments using mice have shown that using vitamin C before and after irradiation suppresses radiation-induced gastrointestinal damage [14]. Interestingly, our experiments also showed that the addition of hydrogen and vitamin C before and after irradiation was the most effective strategy. However, the viability rate of glioblastoma cells increased after irradiation compared to that without irradiation. This may be due to the fact that irradiation activates the cells and increases the proliferation rate as it is the case of this very malignant tumors in patients. Experiments showed that the hydrogen and vitamin C combination therapy protects normal cells from radiation and impairs cancer cells. It was confirmed that apoptosis occurred in cancer cells after hydrogen and vitamin C combination therapy and irradiation. Furthermore, in glioblastoma, the EMT signature in GBM was reduced in response to combination therapy, which suggests that hydrogen and vitamin C combination therapy has anti-cancer-effects through reducing EMT pathway that play essential role in therapy resistance and tumor invasion.

4.4. Hydrogen and vitamin C combination therapy in health and disease

We aim to routinely administer vitamin C injection and hydrogen inhalation to all patients undergoing radiation therapy. Hydrogen inhalation and high-dose vitamin C injection should be administered before radiation and additional treatment after radiation will boost the effect. The hydrogen and vitamin C combination therapy may also be effective for preventing the complications of brachytherapy in various cancers including prostate cancer, as well as in patients receiving radioiodine therapy for thyroid cancer. In addition, the combination therapy has capacity to reduce the risk of secondary cancer induced by radiation. In addition to cancer patients, individuals exposed to radiation, such as those undergoing upper gastrointestinal tests, CT scans, and those traveling by airplane or space flight, may benefit from vitamin C treatment (orally or via injection according to the situation) and hydrogen (gas inhalation, hydrogen saturated water, hydrogen bath, or hydrogen tablet, according to the situation) before and after the exposure. Regarding medical exposure, the combination therapy will reduce potential radiation-related cancer risks. The proposed combination therapy may also be effective against the risk of continuous radiation exposure in cases such as; individuals living in radiation polluted regions such as Chernobyl and Fukushima in Japan, individuals receiving internal exposure through radiation polluted foods, and individuals working as radiologists or radiation technicians. In summary, the combination therapy is recommended for all people to improve health and prevent diseases by reducing active oxygen.

4.5. Limitations and issues to be solved

This study is an in vitro experiment based on a hypothesis derived from a case. Future clinical studies are needed to confirm the hypothesis. The amount of vitamin C covered by medical insurance is small and far from a sufficient dose in many countries. In addition, there are not so many clinics that offer vitamin C injection, and the procedure is expensive for patients although, oral vitamin C intake via supplements is an alternative. Patients can take vitamin C supplement by themselves to compensate for the needs. Moreover, hydrogen therapy is not covered by insurance. Since hydrogen

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gas generators are relatively expensive and sometimes difficult to purchase for individuals, hydrogen-rich water and hydrogen supplements may play an important role. Both these alternatives, vitamin C and hydrogen supplement are easy to obtain by patients. In addition, the price of high-dose vitamin C injection and hydrogen inhalation may decrease as the demand increases. The widespread use of this therapy should benefit patients, and it would be desirable for health insurance to cover the cost of combination therapy. Unfortunately, most radiology specialists are unaware of the effects of the two therapies. When they consult with their doctors about incorporating hydrogen or vitamin C treatment, they are often saddened to hear that they should not use treatments for which there is little evidence, or that they will not be treated in the hospital if they do so on their own judgment. Doctors are often surprised when patients who receive the combination therapy show only mild complications, but they question the effectiveness of the combination therapy in reducing side effects of radiation. Therefore, reporting the results of combination therapy to a wider audience, especially for radiology specialists, should be raised. Since there is no evidence from randomized controlled trials, it is hoped that trials will be conducted in the future.

5. Conclusion

Combination therapy consisting of high-dose vitamin C injection and hydrogen inhalation may reduce the side effects of radiotherapy by decreasing •OH associated with the reaction of radiation with H2O. Hydrogen facilitates vitamin C absorption and have ability to prevent its excessive oxidative effects. Both therapies are safe and may improve the QOL of patients, as well as decrease medical expenses associated with the treatment of the side effects of radiotherapy. The anti-inflammatory effects of the combination therapy may attenuate radiation injuries and prevent cancer metastasis. The antitumor effects of the combination therapy could also improve survival in cancer patients. Although further studies are needed to confirm the effects of the combination therapy, we believe that the therapy has potential significance for preventing and treating radiation-induced damage in cancer patients. This combination therapy seems to be effective against many conditions, not only in radioprotection, but also in many diseases such as herpes simplex infection, shingles, cystitis, hearing loss, glaucoma, cataracts, renal failure, liver dysfunction, stroke, angina pectoris pain from rheumatoid arthritis, stomatitis, asthma, atopic dermatitis, non-tuberculous mycobacteria, psoriasis, the common cold, pneumonia, lower back pain, stiff shoulder, sunburn, insomnia, and acne, among others. All these diseases are caused by reactive oxygen species and inflammation. The combination of hydrogen and vitamin C is expected to be a savior for many diseases and for health promotion for all people.

6. Patents

A patent for the hydrogen and vitamin C combination therapy is pending from Hosei University, to which M.M. belongs.

Author Contributions: M.M. conceived the study, conducted clinical and cellular experiments, and wrote the paper. S.Z. executed cellular experiments and statistical analyses. J.B. contributed to glioblastoma cell experiments, extracted glioblastoma mRNA, performed radiation experiments on cells, and contributed to writing relevant sections of the paper. CM and KI performed ROS staining and data analysis P.I. handled qPCR and its subsequent data analysis. A.M. provided clinical conceptual guidance and supervised the experiments. A.M. and P.U. oversaw the research.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest statement: The authors declare no conflicts of interest.

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