Non-thermal plasma specifically kills oral squamous cell carcinoma cells in a catalytic Fe(II)-dependent manner

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Oral cancer accounts for ~2% of all cancers worldwide, and therapeutic intervention is closely associated with quality of life. Here, we evaluated the effects of non-thermal plasma on oral squamous cell carcinoma cells with special reference to catalytic Fe(II). Non-thermal plasma exerted a specific killing effect on oral squamous cell carcinoma cells in comparison to fibroblasts. Furthermore, the effect was dependent on the amounts of catalytic Fe(II), present especially in lysosomes. After non-thermal plasma application, lipid peroxidation occurred and peroxides and mitochondrial superoxide were generated. Cancer cell death by non-thermal plasma was promoted dose-dependently by prior application of ferric ammonium citrate and prevented by desferrioxamine, suggesting the association of ferroptosis. Potential involvement of apoptosis was also observed with positive terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and annexin V results. Non-thermal plasma exposure significantly suppressed the migratory, invasive and colony-forming abilities of squamous cell carcinoma cells. The oral cavity is easily observable; therefore, non-thermal plasma can be directly applied to the oral cavity to kill oral squamous cell carcinoma without damaging fibroblasts. In conclusion, non-thermal plasma treatment is a potential therapeutic option for oral cancer.

Key Words: non-thermal plasma, oral squamous cell carcinoma, catalytic ferrous iron, apoptosis, ferroptosis

Oral cancer accounts for approximately 2% of all cancers worldwide, but the proportion has gradually increased in some countries.2–4 As oral cancer develops and infiltrates into the surrounding tissues with accompanying necrosis, it causes severe pain, nerve damage and facial deformation and is directly linked to a decrease in quality of life. Therefore, early detection and treatment are very important, as in other cancers. In addition, monitoring for locoregional recurrence and distant metastasis after surgery is also important.

Plasma is the fourth physical state beyond the normal solid/liquid/gas phases and is a mixture of gas, radicals, electrons, cations, anions, and ultraviolet (UV) radiation.5,6 Non-thermal plasma (NTP) represents an apparently contradictory condition with high energy but at near-body temperature, which was realized through engineering in the 1990’s. NTP is currently applied in preclinical experiments in a variety of fields, especially in the medical field.7–10 Studies have shown that NTP acts specifically on cancer cells, leading to cell death, and therefore represents a notable new potential cancer treatment.11–14 NTP has the same temperature as body temperature, and many chemical species/agents have been detected with NTP exposure, including ·OH, H2O2, O2−, nitric oxide, electrons and UV radiation.15 Sufficient evidence demonstrates that NTP promotes wound healing and possibly kills cancer cells.16–18 The theoretical basis for NTP use in cancer treatment is that cancer cells are oxidatively stressed in general, and the Fenton reaction occurs after NTP exposure.19

We have thus far conducted various research projects on the relationship between excess iron and carcinogenesis20–23 and recently showed that iron chelation or phlebotomy by reducing local iron can suppress mesothelial carcinogenesis.24 A phlebotomy intervention trial in humans led to not only a decreased cancer incidence but also significantly decreased cancer mortality;25 thus, a close relationship exists between cancer and iron. Although the effect of NTP on oral cancer cells has previously been reported,26–30 research has not been performed to explore the relationship between oral cancer cells and iron metabolism. Thus, the objective of this study was to evaluate the effect of NTP on oral cancer cells and its association with catalytic Fe(II).

Materials and Methods

Cell lines and reagents. We used seven human oral squamous cell carcinoma cell lines (SAS, Ca9-22, HSC-2, HSC-3, HSC-4, Sa3 and Ho-1-u-1) and two human fibroblast cell lines (HS-K and IMR-90-SV) in this study. The oral squamous cell carcinoma cells were all obtained from the Riken Cell Bank (Ibaraki, Japan). The fibroblast cell lines were also obtained from the Riken Cell Bank and were used as a control. All the cells were grown in RPMI-1640 medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France) and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA), hereafter referred to as complete medium, and 0.25% trypsin (Nacalai Tesque, Kyoto, Japan) was used to collect cells. The cells were maintained at 37°C in a humidified incubator with 5% CO2.

Non-thermal plasma (NTP). NTP was generated by the following well-established procedure17 using a Habahiro (wide orifice) instrument (from Prof. M. Hori, Plasma Nanotechnology...
Research Center, Nagoya University, Nagoya, Japan) by applying 10 kV from a 60-Hz commercial power supply, using 2 electrodes that were 20 mm apart. The NTP had an ultrahigh electron density (approximately 2 x 10^16 cm^-3) and an O density of approximately 4 x 10^15 cm^-3, as previously reported.\(^{31,33}\) Argon (Ar) was used as the gas to generate NTP and was implemented at a flow rate of 2 L/min. The distance between the plasma source and the samples was about 15 cm. In all the experiments, cells in complete media were treated with NTP for 30–120 s at 25°C and then returned to 37°C for various incubation periods. For studies using 96-well plates, a cover was placed to expose only one well, as previously reported.\(^{31}\) As a relevant control, studies were performed using Ar alone under the same experimental conditions.

**Cell viability assay.** Nine kinds of cell lines were cultured in 96-well plates (Thermo Fisher Scientific, Waltham, MA) at 5,000 cells/well for 24 h at 37°C. NTP exposure (30, 60, 90, and 120 s at 25°C) or Ar gas only was applied as a control to the cells, which were then cultured for 24 h at 37°C. Absorbance was measured at 450 nm using the WST-8 cell proliferation assay (Nacalai Tesque) and the POWERSCAN 4 microtiter plate reader (BioTek, Winooski, VT) to determine the viability of the cells after applying NTP.

**Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) analyses and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.** We selected two squamous cell carcinoma cell lines (SAS and Ca9-22) that were highly sensitive to NTP and one non-tumorous fibroblast line (IMR-90-SV). In the NTP group and the Ar alone group, a total of 1.5 x 10^5 cells/well were seeded in a 60-mm dish (Corning, Corning, NY) and cultured for 24 h. NTP or Ar alone was applied, and the cells were incubated for an additional 24 h. After incubation, we used the Annexin V-FITC Apoposis Detection Kit according to the manufacturer’s protocol (Nacalai Tesque) and evaluated cell death using the Gallios flow cytometer (Beckman Coulter, Brea, CA). Furthermore, we performed a TUNEL assay. In the NTP group and the Ar alone group, a total of 5,000 cells/well were seeded in a 96-well plate (Thermo Fisher Scientific) and cultured for 24 h. NTP or Ar alone was applied, and the cells were incubated for an additional 24 h. After incubation, we used the In Situ Cell Death Detection Kit, Fluorescein to observe apoptosis microscopically according to the manufacturer’s protocol (Roche Applied Science, Mannheim, Germany) and observed the staining results, using the BZ9000 microscope (Keyence, Osaka, Japan).

**Quantitation and localization of catalytic Fe(II).** SiRhoNox-1, which is a catalytic Fe(II)-specific probe, was synthesized and characterized as previously described\(^{32}\) and was adjusted to 1 μM in RPMI-1640 without phenol red (Wako). Then, 5 x 10^4 cells/well were seeded in a 60-mm dish and cultured for 24 h. After incubation, the cells were stained with SiRhoNox-1 (1 μM) for 30 min and washed with phosphate-buffered saline (PBS) (Wako). The fluorescence intensity of each cell was evaluated using a Gallios flow cytometer. Subsequently, 2.5 x 10^5 cells were plated into glass-bottom dishes (Matsumai, Osaka, Japan) and incubated overnight. Then, the cells were stained with SiRhoNox-1 (1 μM), MitoTracker Green FM (50 nM: Thermo Fisher Scientific) and ER-Tracker Blue-White DPX (200 nM: Thermo Fisher Scientific) in RPMI (FBS–, ANTI–). Then, NTP or Ar alone was applied for 60 s, followed by incubation for 30 min. We stained the cells with MitoSOX (5 μM: Thermo Fisher Scientific) and a chloromethyl derivative of 2',7'-dichlorodihydrofluorescein (DCF) (10 μM: Thermo Fisher Scientific) in either RPMI-1640 without phenol red for 10 min or BODIPY 581/591 C11 (BODIPY) (2 μM: Thermo Fisher Scientific) in RPMI-1640 without phenol red for 15 min. After washing twice with PBS, we evaluated ROS, using the Gallios flow cytometer.

**Scratch wound-healing assay.** Cells were seeded in 35-mm dishes (Iwaki, Shizuoka, Japan) and cultured to 100% confluence. Scratches were made with 200-μl pipette tips, and the cells were photographed with a phase contrast microscope. Then, the Ar alone group and the NTP group were examined (60s). After 24 h, we observed the cells with a phase contrast microscope.

**Transwell migration assay.** In the NTP group and the Ar alone group, a total of 1.0 x 10^5 cells/well were seeded in a 60-mm dish and cultured for 24 h. NTP or Ar alone was applied, and the cells were incubated for 30 min. Then, a cell culture insert (Corning) was placed in a 24-well plate, and cells were seeded at 5.0 x 10^4 cells/200 μl in the same well. After 24 h, the cells were stained with hematoxylin and counted.

**Western blotting.** For the treatments with NTP and Ar alone, a total of 1.0 x 10^6 cells/well were seeded in a 60-mm dish and cultured for 24 h. After incubation, NTP or Ar alone was applied, and the cells were incubated for 30 min. Then, Matrigel matrix (Corning) was added to a cell culture insert, which was placed in a 24-well plate, and the cells were seeded at 5.0 x 10^4 cells/200 μl, as previously reported.\(^{33}\) After 24 h, the cells were stained with hematoxylin and counted.

**Soft agar colony formation assay.** In the NTP group and the Ar alone group, a total of 1.0 x 10^6 cells/well were seeded in 6-well plates (Corning) and cultured for 24 h. After incubation, NTP or Ar alone was applied, and the cells were incubated for 30 min. During incubation, we prepared agar, using agar Noble (Becton, Dickinson and Company, Franklin Lakes, NJ). We poured 2.5 ml of 0.75% agar into the 6-well plate as bottom agar. After confirming that the bottom agar had solidified, we collected...
cells and poured 1.5 ml of 0.36% agar into the 6-well plate as top agar which was mixed in each well. We incubated the cells for 1 or 2 weeks and counted the number of colonies.

**Statistical analysis.** Statistical analysis was performed using Student’s t test and one-way analysis of variance with GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). The data are expressed as the means ± SE of the mean (n = 3–6) unless otherwise specified. All experiments were performed in triplicate (*p<0.05, **p<0.01, ***p<0.001; ns, not significant).

**Results**

The killing effect of NTP is specific to squamous cell carcinoma cells. We performed a cell viability assay to evaluate the effect of NTP and compared differences in the effect between fibroblasts and squamous cell carcinoma cells. The results showed that the viability of HS-K and IMR-SV-90 cells did not significantly decrease upon NTP application until 120 s. However, the cell viability of the squamous cell carcinoma cell lines began to rapidly and significantly decrease with increasing NTP application time (Fig. 1A). SAS, HSC-3 and HO-1-u-1 cells are poorly differentiated cancer cells, whereas the other squamous cell carcinoma cells are well differentiated. The average application time leading to a significant decrease in cell survival was 100 s and 75 s, respectively, for each differentiation group. The exposure time until cell death by NTP for the well-differentiated cancer cells tended to be shorter than that for the poorly differentiated cancer cells. To evaluate cell death type of oral squamous cell carcinoma, we measured Annexin V-FITC and propidium iodide (PI) staining. We found that apoptosis occurred after NTP application to SAS cells.
and Ca9-22 cells (Fig. 1B). In addition, according to the TUNEL assay, we also observed apoptotic cells in the NTP group (Fig. 1C).

**Squamous cell carcinoma cells harbor more catalytic Fe(II) than fibroblast cells.** To study the association between Fe and NTP cytotoxicity, we measured catalytic Fe(II) in individual cells using SiRhoNox-1. We observed that the Fe level in IMR-SV-90 fibroblast cells was lower than those in SAS and Ca9-22 cancer cells; however, no difference in the iron levels were observed between SAS and Ca9-22 cells according to the flow cytometric analysis (Fig. 2A). Consequently, to evaluate the localization of catalytic Fe(II), we observed the cells, using confocal microscopy. This examination revealed that the intensity of catalytic Fe(II) in the lysosomes of IMR-SV-90 cells was lower than that in SAS and Ca9-22 lysosomes (Fig. 2B). Furthermore, we found that the intensity of catalytic Fe(II) in lysosomes of cancer cells was higher than that in the mitochondria and endoplasmic reticulum (ER).

**The killing effects of NTP depend on the amounts of catalytic Fe(II).** We examined differences in the killing effect of NTP by modifying the intracellular iron level in each cell. SAS and Ca9-22 cells were loaded by FAC, and then the catalytic...
Fe(II) levels increased in both cell types. Conversely, when DFO was applied, the catalytic Fe(II) levels decreased in both cell types (Fig. 3A). Subsequently, we evaluated the effect of iron modification at NTP application. The results revealed that the viability of SAS and Ca9-22 cells significantly decreased as the applied concentration of FAC increased (Fig. 3B). Conversely, the viability of SAS and Ca9-22 cells significantly increased as the applied concentration of DFO increased (Fig. 3C).

**Discussion**

Here, we studied the effects of NTP exposure on oral squamous cell carcinoma for the first time, with a focus on catalytic Fe(II). A distinct difference in the effects of NTP was observed between the oral cancer cells and non-tumor cells. We found a proportional association between the killing effect of NTP exposure and the amounts of catalytic Fe(II) in oral squamous cell carcinoma cells in iron modification experiments.

Oxidative stress via excess iron has been associated with carcinogenesis, and cancer cells are oxidatively stressed in general. In a previous experiment, we showed that cancer cells have more catalytic Fe(II) through maturation of HL-60 leukemia cells to macrophage-like cells. Here we revealed that abundance of catalytic Fe(II) was also true for the oral squamous cell carcinoma cells, with a higher fraction in the lysosomes than in the mitochondria and ER. In this experiment, we did not find a difference in the levels of catalytic Fe(II) between well-differentiated and poorly-differentiated oral cancer cells. However, we believe that this finding is the basis for the differential effect of NTP between tumor and non-tumor cells.

Regarding the type of cell death induced by NTP, we obtained a few evidences supporting apoptosis; namely, increased annexin V with flow cytometric analysis and TUNEL positivity. However, simultaneously, we observed iron-dependent lipid peroxidation and mitochondrial superoxide during cell death by the use of three different fluorescent probes, thus suggesting the mixed presence of ferroptotic process. In the case of mesothelioma cells exposed to NTP, we observed activation of the autophagic pathway and lipid peroxidation, suggesting ferroptosis. On the contrary, ovarian cancer and melanoma skin cancer cells mainly showed apoptosis upon NTP exposure. Here we revealed that abundance of catalytic Fe(II) was also true for the oral squamous cell carcinoma cells, with a higher fraction in the lysosomes than in the mitochondria and ER. In this experiment, we did not find a difference in the levels of catalytic Fe(II) between well-differentiated and poorly-differentiated oral cancer cells. However, we believe that this finding is the basis for the differential effect of NTP between tumor and non-tumor cells.

Due to the accessibility of the oral cavity, clinical application of NTP is easier at this site compared to other organs, except for the skin. The depth of penetration with direct NTP exposure is ~1 mm. We also showed that NTP treatment of oral cancer cells...
significantly impairs tumor migration and invasion, which may at least result from suppressed MMP-2 expression. As clinical applications, NTP can be used for preoperative progression control and irradiation of intraoperative margins rather than curative treatment. NTP treatment may be suitable for preventing recurrence after surgery. In general, a 10-mm safety margin is used in oral surgery. However, if this large margin can be reduced with NTP exposure, then functional loss can be reduced, resulting in cosmetic benefits and better quality of life. Notably, NTP exposure is substantially less harmful to normal tissues, as shown by the findings in fibroblasts, and can further promote wound healing.\(^{(18,37)}\) We believe that NTP exposure as an additional therapy is feasible until radiation therapy and chemotherapy are completed. Preclinical in vivo experiments are in progress to study the merits of NTP exposure.

In conclusion, NTP exposure specifically killed oral squamous carcinoma cells compared to fibroblasts, which was dependent on ample catalytic Fe(II). The type of cell death was mixed apoptosis and ferroptosis.

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**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| Ar           | argon       |
| BODIPY       | BODIPY 581/591 C11 |
| DCF          | chloromethyl derivative of 2',7'-dichlorodihydrofluorescein |
| DFO          | desferrioxamine (desferal) |
| ER           | endoplasmic reticulum |
| FAC          | ferric ammonium citrate |
| FITC         | fluorescein isothiocyanate |
| FBS          | fetal bovine serum |
| MMP-2        | matrix metalloproteinase-2 |
| NTP          | non-thermal plasma |
| PBS          | phosphate-buffered saline |

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**Fig. 4.** Non-thermal plasma results in reactive oxygen species (ROS) generation. ROS generation was detected using MitoSOX (5 μM), a chloromethyl derivative of 2',7'-dichlorodihydrofluorescein (DCF) (10 μM) and BODIPY 581/591 C11 (BODIPY) (2 μM) by flow cytometry. All signals significantly increased. Error bars represent the standard error of the mean. *p<0.05, **p<0.01, ***p<0.001; ns, not significant.
Fig. 5. Non-thermal plasma suppresses the migration and invasion abilities of squamous cell carcinoma cells. (A) Wound-healing assay at 0 and 24 h after treatment. (B, C) Transwell migration and invasion assays at 24 h after treatment. Five representative fields of cells in each group were captured and analyzed. Scale bar = 500 μm. (D) Colony formation assay. Four representative fields of cells in each group were captured and analyzed. Scale bar = 500 μm. Error bars represent the standard error of the mean. *p<0.05, **p<0.01, ***p<0.001; ns, not significant.
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