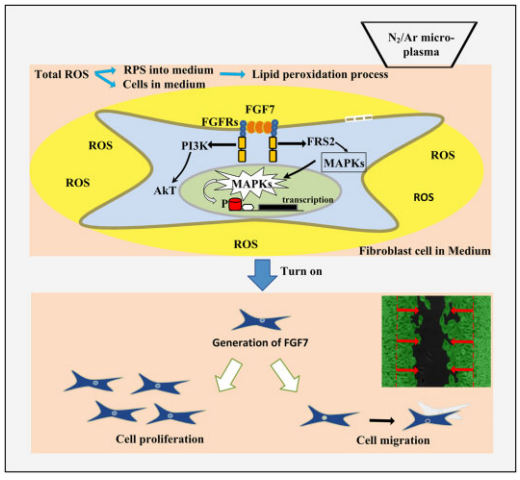
Increased Fibroblast Cell Proliferation and Migration Using Atmospheric N_2/Ar Micro-Plasma for the Stimulated Release of Fibroblast Growth Factor-7

Minh-Hien Thi Ngo, Jiunn-Der Liao,* Pei-Lin Shao, Chih-Chang Weng, Chen-Young Chang

In situ assessment of cell functions on N2/Ar micro-plasma exposed fibroblast cells is examined to better understand the effect of atmospheric low-dose plasma treatment. The cells number increased threefolds for plasma exposure time of 5 or 10 s, followed by cell culture for 48 h. The cell coverage rate rose 20% for the same plasma exposure time, followed by cell culture for 6 or 12 h. 0.5% N_2/Ar micro-plasma exposure can particularly be used to achieve the stimulated release of FGF7 and subsequent enhancement of cells proliferation and migration. This work thus provides a potential of using micro-plasma system for cells related studies and the improvement of cutaneous wound healing.



1. Introduction

Several different methods have recently been applied to promote cell proliferation, cell migration, and, presumably, wound healing.[1,6,7] For example, low dose laser therapy with a narrow spectral width in the range of the red and near infrared spectrum (600–1 000 nm) has been applied to stimulate cell functions via an increase in mitochondrial production, and activation of lymphocytes and mast cells with the proliferation of fibroblasts, and other cells then being observed.[2] Nitrogen oxide (NO) species alone or in combination with UV-radiation has also been shown to affect cell viability.[3] The effects of reactive oxygen species (ROS) on cell proliferation and cell apoptosis are correlated.[4] Cell permeabilization is caused by non-thermal plasma,[5a,b] and related phenomena were also found when endothial cells were exposed to dielectric-barrier-discharge (DBD) plasma with the results suggesting that cell proliferation in such cases is mostly related to the induced release of fibroblast growth factor-2.[6] However, some drawbacks to applying these methods have been reported, such as the cost of the laser equipment and the

M.-H. T. Ngo, Prof. J.-D. Liao, Dr. P.-L. Shao, Dr. C.-Y. Chang
Department of Materials Science and Engineering, National Cheng Kung University, 1 University Road, Tainan 70101, Taiwan
E-mail: jdliao@mail.ncku.edu.tw
Prof. J.-D. Liao
Medical Device Innovation Center, National Cheng Kung University, 1 University Road, Tainan 70101, Taiwan
Dr. C.-C. Weng
Industrial Technology Research Institute, Sec. 4, Chung Hsing Road, Chutung, Hsin Chu, Taiwan

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Minh-Hien Thi Ngo is currently pursuing her Ph.D. degree at Department of Materials Science and Engineering (MSE), National Cheng-Kung University (NCKU), Tainan, Taiwan. She received her M.S. degree in Mechanical Engineering at Southern Taiwan University of Technology, Tainan, Taiwan in 2008. Her research is focused on non-thermal micro-plasma in biomedical applications, especially for stimulating cell functions and healing burn wounds.

Jiunn-Der Liao is currently the Distinguished Professor at Department of MSE, NCKU, Tainan, Taiwan. He obtained his B.S. degree at the same department, NCKU in 1984, M.S. degrees at K.U. Leuven (Belgium) in 1990 and 1991, respectively, and Ph.D. degree at ENS Mines (France) in 1994. He also worked at University of Heidelberg (Germany) as a research fellow from 1995 to 1996 and Chung Yuan Christian University (CYCU, Taiwan) as Associate Professor from 1996 to 2002. His current research interests are focused upon: (1) mechanics of biomaterials, e.g., tissue engineering, scaffold materials, cell–surface interactions, nano-indentation, mechanical transduction; (2) plasma chemistry and plasma processing, e.g., plasma generation, plasma diagnoses, plasma physics, and chemistry, metal vapor vacuum arc, plasma ion immersion; (3) nano-fabrication and nano-characterization, e.g., focused ion beam based nano-fabrication, micro-contact imprinting, synchrotron-based and laboratory-based high resolution analyses.

Pei-Lin Shao obtained her M.S. degree in basic medical sciences from Kaohsiung Medical University (KMU, Kaohsiung, Taiwan) in 2003. She obtained her Ph.D. in Institute of Medicine also from KMU in 2011. Her Ph.D. topic was focused on the role of shock wave therapy in attenuating intima injury-induced vascular remodeling. Her current research topic is focused on the advanced wound management.

Chih-Chiang Weng obtained his B.S. and M.S. degrees in Biomedical Engineering from CYCU in 1999 and 2001, respectively. He obtained his Ph.D. in MSE from NCKU in 2008. The research topic of his Ph.D. degree was focused on the fabrication low-temperature plasma or micro-plasma system for various biomedical and micro-electronics applications. His previous paper was selected by Plasma Process and Polymers as the cover page (April, 2013). In addition, he is familiar with synchrotron-based facilities and plasma diagnostic techniques.

Chen-Young Chang obtained his B.S. and M.S. degrees at Department MSE from NCKU in 2011 and 2013, respectively. The M.S. thesis was focused on the blood coagulation process under non-thermal Ar micro-plasma exposure.

need to use a large-area DBD-type plasma. A number of potential side effects, although minor, have also been reported with regard to applying reactive plasma species (RPS) directly on living cells.[7]

Non-thermal atmospheric micro-plasma devices with an operational heat close to body temperature have received considerable attention due to their great potential for a variety of biomedical applications, such as heat-free bacteria inactivation and sterilization,[8] in vivo and in vitro blood coagulation,[1b] acute and chronic wound healing,[9] and regeneration of damaged tissues.[10] When N, O-containing plasma species are exposed to target substances, neutral atoms and molecules and active short- or long-lived species, including O_3, NO, OH radicals, singlet O_2 $^1\Delta g$, and super oxide radicals[1b,11] will presumably play major roles in the interactions that occur at the plasma/target interface.

A general in vitro model is often used when examining how to improve the cutaneous wound healing process using a micro-plasma system. When human skin is damaged, the body initiates chemical and biological processes that help to repair the area that has been exposed to a trauma.[12] The normal mammalian in response to injury occurs in three overlapping but distinct stages: inflammation (which causes the removal of bacteria), re-epithelialization (by the processes of angiogenesis, collagen deposition, epithelialization, and wound contraction), and recovery with the remodeling phase.[13] Fibroblast cells play a guiding role in the second phase of wound healing that occurs 2–10 d after injury, and is usually characterized by cellular proliferation and migration of different cell types.[12] Fibroblast cells are presumed to secrete fibroblast growth factor-7 (FGF7), a novel member of the FGFs family that binds exclusively to a splice variant of the FGF receptor, a transmembrane protein tyrosine kinase receptor, that is present on epithelial cells.[14] The FGF7 expression at the wound site is restricted to dermal fibroblasts cells, and thus it is hypothesized to act in a paracrine manner, stimulate keratinocytes for cell proliferation and migration, and thereby effect wound reepithelialization.[14b]

In this study, plasma plume temperature and RPS in N_2/Ar micro-plasma were first measured, and then the characterized micro-plasma was applied to fibroblast cells (L929) in a medium. Several techniques were used for the assessment of cell functions after plasma exposure cells, as follows: mitochondrial function (MTS assay) for cell proliferation, ROS detection, lactate dehydrogenase assay (LDH assay) for the sub-lethal effect, a quantitative immunoassay technique such as enzyme-link immunosorbent assay (ELISA) for the release of FGF7, and cell coverage model in vitro for the tendency of accelerating cells migration. The importance of this work is that it aims to interpret the role of RPS, and thereafter ROS, in a cell-containing medium for the stimulation of fibroblast cells in

association with the release of FGF7. A possible mechanism for re-epithelialization at the molecular level will be proposed based on the results of this work, thus helping to explain the process of cutaneous wound healing.

2. Experimental Section

2.1. Fibroblast Cell (L929) Culture

Fibroblast cells derived from an immortalized mouse fibroblast cell line were preserved in alpha modified Eagle's medium (α-MEM) with 10% horse serum (Gibco, Invitrogen, CA, USA) and 10 ml 10 000-U ml^{-1} penicillin – 10 000 μg ml^{-1} streptomycin (Sigma, St. Louis, MO). Before the experiments, fibroblast cells were washed with phosphate buffered saline (PBS), and detached with trypsin (Gibco, Invitrogen).

For the MTS assay, ROS detection, LDH assay, and ELISA, fibroblast cells were seeded near confluence (8 000 cells/well = 2.7×10^5 cells/ml) on 48-well plates (Nunc, Thermal Scientific, Denmark). The cells were then cultured in a complete medium, maintained at 37 °C under 5% CO_2 incubator for 24 h, and transferred to the low concentration serum (4% horse serum) for 48 h. In the cell coverage model, fibroblast cells were placed consistently in a 24-well plate (Nunc, Thermal Scientific), with 7.5×10^5 cells/ml in a complete medium, and maintained at 37 °C under 5% CO_2 for 24 h. Fibroblast cells with two different cell densities were prepared for the various experiments, and these are denoted as "the test cells."

2.2. Micro-Plasma System and Plasma Plume Temperature

A custom-made micro-plasma jet source was driven by a radio-frequency power supply of 13.56 MHz (ENI ACG-3B, MKS Instruments, Inc., Rochester, NY, USA) with a matching device (ACG-3B, ENI Corp., Rochester, USA). This jet source was a capillary electrode through which the additive N_2 or O_2 gas was injected. The micro-plasma device used in this work as illustrated in Figure S1 in the Supporting Information, contained a quartz tube as the gas channel and a dielectric layer with an outer diameter of 2 mm. At the center of the quartz tube, a stainless steel capillary tube (with a diameter of 0.2 mm, fixed by a perforated teflon fitting) was used as the inner electrode as well as the N_2 or O_2 feeding tube. A copper chip was used as the outer electrode, and this was connected to a generator.

Plasma plume temperature was estimated using a fiber optic thermometer (Luxtron 812, Santa Clara, USA). The fiber was placed on an *X*–*Y* coordinated table and the distance from the fiber to micro-plasma jet nozzle was ≈12 mm. The temperature was measured in a range of applied powers from 15 to 25 W with a flow rate of 5 slm Ar. To produce N, O-containing radials in an excited phase, 0.1, 0.5, and 2% (2.5, 12.5, and 100 sccm) of N_2 or O_2 were poured into Ar plasma in the capillary tube.

2.3. RPS Measurement

RPS such as NO, OH, O were analyzed using optical emission spectroscopy (OES, SpectraPro 2300i, Acton Research Corp., MA, USA). The optical emission spectra were taken along the axis of micro-plasma jet and recorded in the range of 200–1 100 nm. The emitted light was then focused by optical fibers into the entrance slit of single monocromater (SpectraPro 2300i, Acton Ltd, MA, USA) equipped with a CCD detector (1 340 × 100 pixels). The resolution of the collected spectra was 1 200 grooves per millimeter with the slit width of ≈0.1 nm. Two gratings, 200–500 (1 200 g mm^{-1}) and 500–1 100 nm (1 200 g mm^{-1}), were utilized to estimate the composition of RPS before interacting with the test cells.

2.4. Cell Proliferation and Cell Coverage Tests (In Vitro Model)

The test-cell viability with and without plasma exposure was assessed via MTS assay (CellTiter 96 AQueous One Solution proliferation, Promega). In the experiments, plasma-exposed cells were cultured for 24 or 48 h (two groups). The reagent AQueous One solution (≈60 μl) was directly added to the culture wells containing both groups of cells. After 3 h incubation at 37 °C under 5% CO_2, the solution (≈100 μl) in each culture well was transferred to 96-well plates (Nunc, Thermal Scientific). The absorbance at 492 nm for the solution in 96-well plates as measured with a standard microplate reader (Multiskan EX Labsystems, Finland). The quantity of formazan product in association with the intensity of absorbance was directly proportional to the number of cultured living cells.

The migration ability of the plasma-exposed cells was assessed. The test cells were first exposed to N_2/Ar micro-plasma for 5, 10, or 15 s. The gaps filled by the test cells and plasma-exposed cells were observed using an optical microscope and evaluated with the WIMASIS image analysis software. Cell coverage tests were carried out every 3 h until the gaps were fully covered.

2.5. ROS in Plasma-Exposed Medium

RPS interacting with the medium containing the plasma-exposed cells was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA, Cayman chemicals). Before the experiments, DCHF-DA was directly dissolved in 0.1 M Na_2CO_3 (or 5 mg ml^{-1}, Sigma), and then immediately diluted with PBS (pH 7.2). The product was stored on ice until use. Fresh DCHF was prepared for each experiment and used the same day. Before plasma exposure, the as-prepared product (≈25 μM) was added in each well of 48-well plates and the test cells were then exposed to N_2/Ar micro-plasma for 5, 10, and 15 s. In the experiments, the medium containing the plasma-exposed cells maintained its pH value in the range of 8.67 ± 0.01 ($n = 6$). Three kinds of cells (≈100 μl for each), the test cells, the test cells with H_2O_2 (the positive control), and the test cells exposed to N_2/Ar micro-plasma, were transferred to 96-well plates. After 5, 30, 60 min incubation at 37 °C under 5% CO_2, the absorbance at 500 nm for these samples was analyzed using a microplate reader (Tecan, Infinite 200 PRO; Tecan Group Ltd., Mannedorf, Switzerland).

2.6. LDH Leakage

LDH leakage was measured under the same conditions of N_2/Ar micro-plasma exposure and gas flow as in the cell proliferation

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assay, cell coverage, and ROS. A cytotoxicity detection kit (Roche Applied Science, Mannheim, Germany) was utilized for LDH leakage analysis. LDH was quantitatively measured by colorimetric assay, which calculates the amount of a stable cytosolic enzyme that is released upon cell lysis. The released LDH in culture supernatants was measured with a 30-min coupled enzymatic assay that might result in the conversion of a tetrazolium salt into a red formazan product. Note that the intensity of the resulting color is proportional to the number of lysed cells.

Plasma-exposed cells were then cultured for 24 h. The cell membrane was broken by 0.1% triton X-100, and then incubated at room temperature for 5 min. The solution (≈100 μl) was then transferred to 96-well plates in the dark and stored at 37 °C under 5% CO_2 for a duration of 10–50 min. The absorbance at 492 nm for the solution in 96-well plates as measured with a standard microplate reader. The formula, LDH leakage = supernatant/(supernatant + cell lysate), was used for this calculation.

2.7. Release of FGF7

The release of FGF7 was measured under the same conditions of N_2/Ar micro-plasma exposure and gas flow as for the assessments of cell proliferation, cell coverage, ROS, and LDH leakage. Mouse keratinocyte growth factor kit (FGF7) was obtained from Cusabio Biotechnology (Newark, DE, USA). The quantitative sandwich enzyme immunoassay technique was employed. The release of FGF7 from the plasma-exposed cells was measured in the collected medium 3 or 24 h after N_2/Ar micro-plasma exposure and gas flow. The antibody specific for FGF7 was pre-coated onto a microplate, and then 100 μl of samples were added into the wells, while the release of FGF7 could be bonded by the immobilized antibody. The as-tested microplate was incubated for 2 h at 37 °C under 5% CO_2.

After the removal of the unbonded substances, 100 μl biotin-conjugated antibody specific for FGF7 was consequently added to the wells and incubated for 1 h. After washing, 100 μl avidin conjugated horseradish peroxidase (HRP) was added to the well and continuously incubated for 1 h. After the removal of the unbonded avidin-enzyme reagent, 90 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added to the wells and incubated for 15–30 min. Afterwards, 50 μl stop solution was added to the wells, and the plate was finally read using a microplate reader at the absorbance of 492 nm for 5 min. The commercial software package "Curve Expert 1.3" was used to make a standard curve, and then the data was linearized by plotting the log of the FGF7 concentration versus the log of the optical density (OD), and the best fitting line was then determined by regression analysis.

2.8. Statistical Analysis

All experimental data were normally distributed and expressed as the mean ± SD. Data were analyzed by Student's *t*-test and Tukey's test to establish significance between data points. The values of $p \leq 0.05$ (*) and $p \leq 0.01$ (**) were considered statistically significant.

3. Results and Discussion

3.1. Results

3.1.1. Micro-Plasma Diagnosis

In Figure 1a,b, the apparent plasma plume temperatures were measured as a function of the supplied power with the addition of N_2 or O_2 (in percent). Note that in plama physics, the term "plasma temperature" may be correlated with electron temperature, and thus the velocity of the excited electrons in plasma.[15] When a relatively low power supply (e.g., 15, 16, or 17 W) was applied to the micro-plasma, it exhibited an average temperature below 40 °C (see the lines marked in the Figure 1a,b). In these cases, the cells are presumably subjected to very minor effects due to the heat. In addition to the supplied power, the plasma plume temperature was changeable by varying the percentage of N_2 or O_2 that was added to the Ar plasma. With the same supplied power, an increase in the N_2 or O_2 content tended

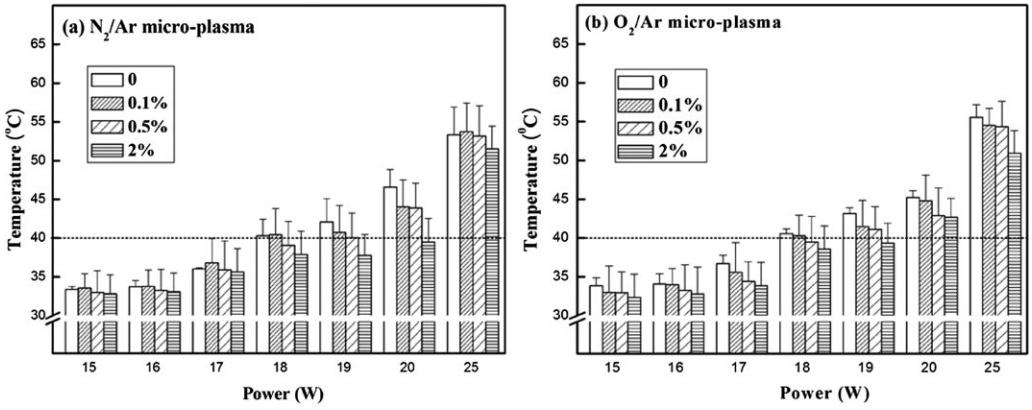

Figure 1. Plasma plume temperatures were measured with varied percentages (0.1, 0.5, and 2%) of (a) N_2 or (b) O_2 addition. Low supplied power, e.g., 15, 16, or 17 W, maintained an average temperature below 40 °C. Error bars indicate the standard error of the mean for $n = 6$ independent experiments.

to significantly decrease the average temperature, in particular for relatively high supplied powers. Plasma composition thus has an impact on the plasma plume temperature. In the following study, a supplied power of 17 W was used and a distance of ≈12 mm between the target substance and micro-plasma jet nozzle was fixed in order to maintain the desired temperature.

In Figure 2a,c, taking the optical emission spectra of 0.5% N_2/Ar and 0.5% O_2/Ar micro-plasma as examples, the presence of NO (237 nm), OH (306 nm), Ar–I (750 nm), and O (777 nm) is obvious. Note that only the relative intensities for each plasma species can be compared. In Figure 2b,d, the emission intensities of NO, OH, O, and Ar–I all vary with the addition of N_2 or O_2 (0.1, 0.5, and 2%, respectively) to the Ar plasma. The intensity of each plasma species from pure Ar plasma (0% N_2 or O_2) was taken as the reference (100%). In Figure 2b, the NO level increased significantly with the addition of N_2, whereas the emission intensities of OH and O decreased. In Figure 2d, when the gas added was changed to O_2, the NO and OH levels were decreased significantly, whereas the emission intensity of O increased significantly. These decreases in NO and OH with the addition of O_2 are most probably related to the discharge characteristics and the electron energy distribution function.[15] Note that the generation of OH species is usually associated with the moisture that is present under atmospheric conditions. In the following study, in order to assess the reactivity of the plasma composition, 0.5% N_2/Ar micro-plasma was generated using a supplied power of 17 W.

3.1.2. Stimulation of Fibroblast Cell Proliferation and Migration

Cell proliferation tests for the plasma-exposed cells (Figure 3a) with respect to the test cells (0 s) and those under gas flow (Figure 3b) were first carried out. The proliferation of plasma-exposed cells (5, 10, or 15 s) was significantly enhanced ($p < 0.05$ or 0.01) after incubation for 24 or 48 h, as compared with untreated cells, with the increase being around threefold.

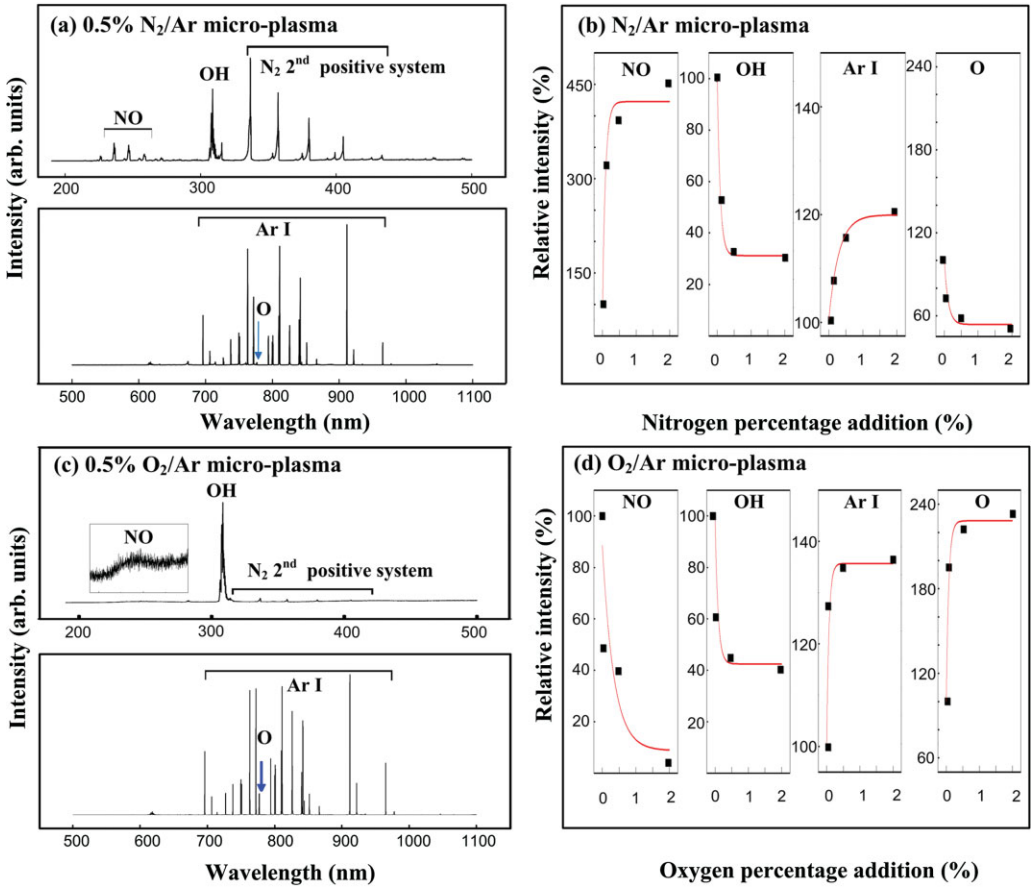

Figure 2. Optical emission spectra and varied intensities of reactive species in plasma: a) optical emission spectrum for 0.5% N_2/Ar micro-plasma; b) relative intensity of individual reactive species with respect to N_2 addition in Ar plasma; c) optical emission spectrum for 0.5% O_2/Ar micro-plasma; (b) relative intensity of individual reactive species with respect to O_2 addition in Ar plasma. The relative intensities of individual reactive species were obtained by the mean of $n = 6$ independent experiments.

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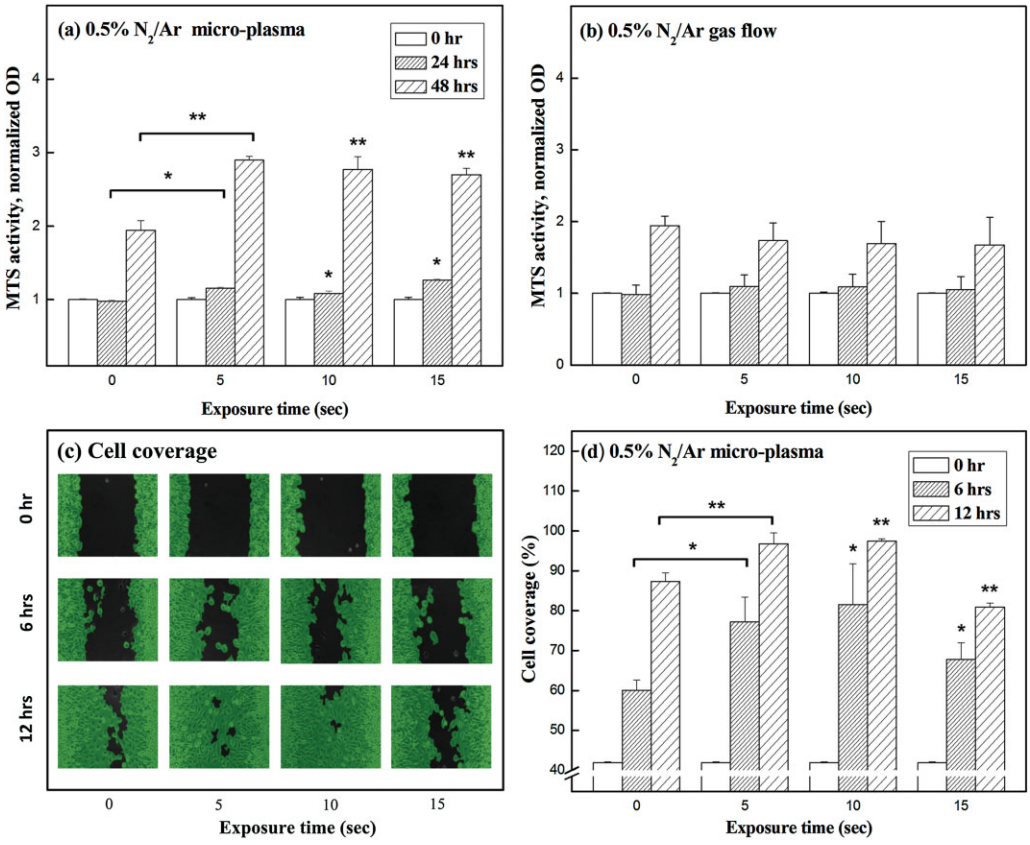

Figure 3. a) The proliferation of the stimulated test cells after plasma exposure for 5, 10, or 15 s and incubation for 24 or 48 h, as compared to (b) the control group. All values were normalized to the values obtained with the control group. Error bars indicate the standard error of the mean for $n = 6$ independent experiments. c) The progression of cell migration or coverage, 6 and 12 h after plasma exposure times of 5, 10, and 15 s, were recorded with an optical microscope and d) plotted for statistical analyses. Error bars indicate the standard error of the mean for $n = 6$ independent experiments.

Figure 3c shows images taken during the cell coverage tests for the plasma-exposed cells after incubation for 6 or 12 h. Figure 3d further examines the cells coverage rates in association with the cell migration ability. Significant increases in cell migration were found for the plasma-exposed cells (5, 10, or 15 s) after incubation for 6 or 12 h ($p < 0.05$ or 0.01), as compared with the untreated ones. For example, there was an ≈80% increase in the cell coverage rate (≈60% increase for the untreated cells) for the test cells after 10 s plasma exposure and 6 h incubation. For a similar plasma exposure time and 12 h incubation, an ≈98% increase of cells coverage rate (≈87% increase for the untreated cells) was estimated.

3.1.3. ROS in the Plasma-Exposed Cell-Containing Medium

Figure 4a shows the ROS levels in the plasma-exposed cell-containing mediumafter 5, 30, and 60 min incubation. Significant increases were found for the medium after plasma exposure for 5, 10, or 15 s. In addition, the ROS levels in the medium roughly increased along with the plasma exposure time, as also compared with the medium under gas flow (Supporting Information, Figure S3).

Figure 4b shows the influence of ROS levels in the medium with respect to LDH leakage from the plasma-exposed cells. After incubation for 24 h, the level of LDH leakage from the plasma-exposed cells increased along with the plasma exposure time, as also compared with the medium under gas flow. These results corresponded to the changes in ROS levels in the plasma-exposed cell-containing medium.

3.1.4. Release of FGF7 in the Plasma-Exposed Cell-Containing Medium

Figure 5 shows the release of FGF7 from the plasma-exposed cells, compared with the test cells under the same gas-flow time. Figure 5a showed the results after plasma exposure and incubation for 3 h, the release of FGF7 from the plasma-exposed cells increased along with the plasma exposure time, as also compared with the

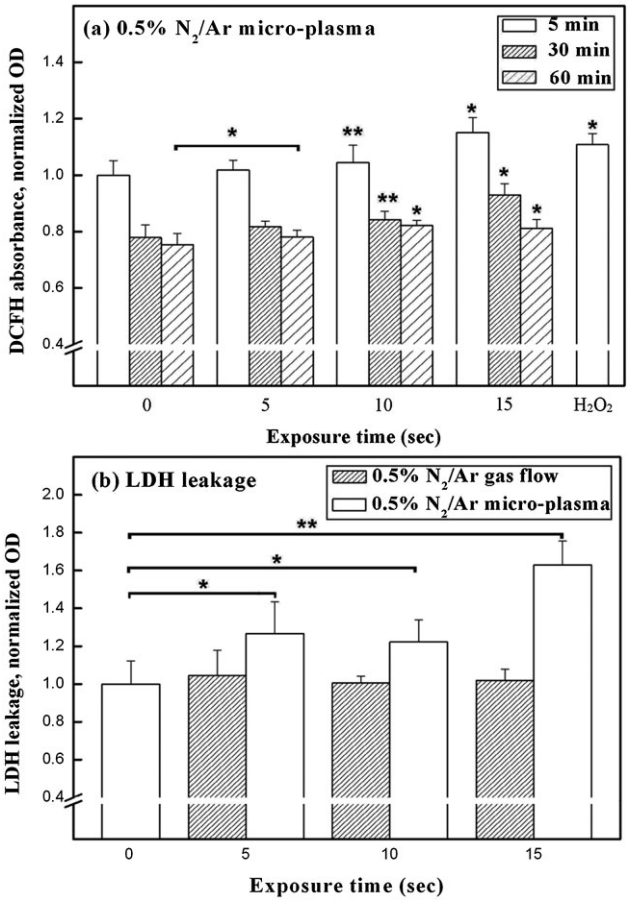

Figure 4. The (a) ROS content in the stimulated test cells medium, followed by culturing for 5, 30, and 60 min, respectively, and (b) LDH leakage with respect to plasma exposure times of 5, 10, and 15 s, all compared to the control group, were plotted for statistical analyses. All values were normalized to the values obtained with the control group. Error bars indicate the standard error of the mean for $n = 6$ independent experiments.

medium under gas flow. These results also corresponded to the changes in ROS levels in the plasma-exposed cells-containing medium. Figure 5b showed the results after plasma exposure and incubation for 24 h, the release of FGF7 from the plasma-exposed cells (5, 10, or 15 s) was significantly different to that seen with the untreated cells ($p < 0.05$) and the cells after incubation for 3 h ($p < 0.01$), and these results corresponded to the changes in LDH leakage in the plasma-exposed cells-containing medium. There is thus a clear incubation time-dependent effect on the release of FGF7 from the plasma-exposed cells.

4. Discussion

The results reported above indicate that RPS, and thus the ROS levels in the medium, have significant effects on the living cells. The probable mechanism underlying these effects is illustrated in Figure 6. In the first stage, as RPS (i.e., marked as I-1) is introduced into the cell-containing medium, the ROS level in the medium (i.e., marked as I-2) around the test cells increases due to the inclusion of RPS and ROS from the test cells themselves. For an optimized 0.5% N_2/Ar micro-plasma, RPS contains NO and other OH, Ar I, and O species, which partially contribute to the ROS contents in the medium.

In the second stage, the test cells in the medium surrounded by ROS simultaneously raise the level of LDH leakage and cause lipid peroxidation to occur (i.e., marked as II-1). For an optimized 0.5% N_2/Ar micro-plasma, after incubation for 24 h, the level of LDH leakage from the plasma-exposed cells increases along with the plasma exposure time, which indicates that the ROS level increases in the plasma-exposed cell-containing medium. It is very likely that the test cells are affected by the interactions between ROS and the various components in the medium,

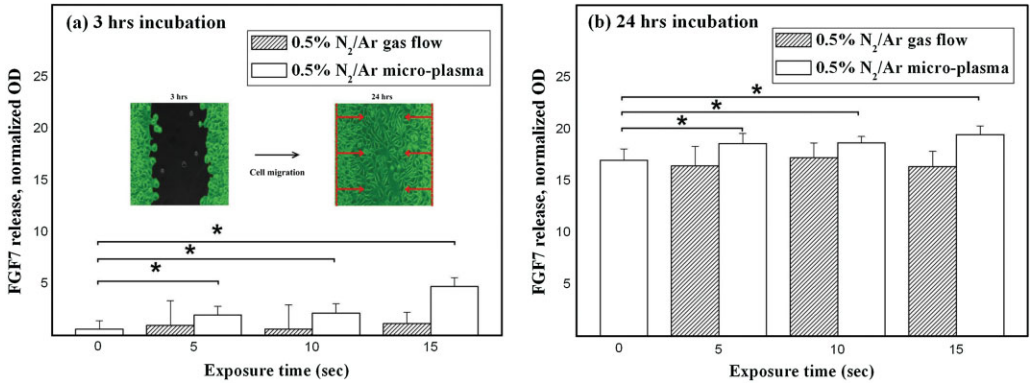

Figure 5. The release of FGF7 from the stimulated test cells followed by culturing: a) for 3 h and b) for 24 h with respect to plasma exposure times of 5, 10, and 15 s were plotted for statistical analyses. All values were normalized and compared to those obtained with the control group. Error bars indicate the standard error of the mean for $n = 6$ independent experiments.

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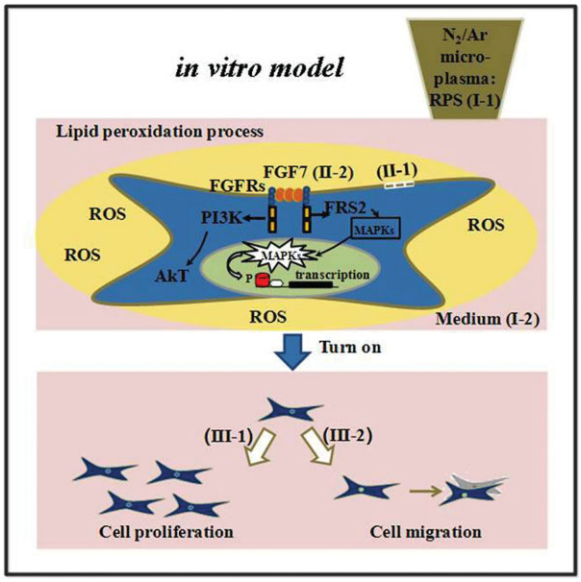

Figure 6. The in vitro model based on this study and inferences from the literature. Three stages were proposed: I-1 for RPS, I-2 for medium, II-1 for LDH leakage, II-2 for FGF7 release, III-1 for cell proiferation, and III-2 for cell migration.

such as amino acids and proteins, salts, phenol indicator, and vitamins, which leads to the production of long living reactive organic hydroperoxides.[4] As they increase with plasma exposure time, these hydroperoxides may induce lipid peroxidation and have sub-lethal effects on the membranes of test cells. The effects of LDH leakage are due to the creation of pores in the cell membrane; however, a cell repairing process may lead to the release of FGF7 secreted from the stimulated test cells (marked as II-2). In this study, both the release of FGF7 and the leakage of LDH occur from the stimulated or damaged test cells. At an early stage of stimulation, there are insignificant differences in the LDH levels,[6] and thus, the plasma exposed cell-containing medium after incubation for 24 h is of particular interest. The sub-lethal membrane damage they have sustained is gradually repaired, without harming the functions of the fibroblast cells. In addition, significant LDH leakage tends to promote the cells' subsequent functions, such as viability and differentiation.[6,16]

Furthermore, FGF7 is a novel member of the fibroblast growth factor (FGFs) family, which has various biological functions both in vivo and in vitro.[14a] FGFs are able to enhance the proliferation, migration, and survival of many different cell types through binding into and activation of FGF receptors (FGFRs).[14a,17] As also illustrated in Figure 6, the activated FGFRs tend to stimulate the phosphoinositide 3-kinase-protein kinase B (PI3K/AkT) and the FGFR substrate 2-mitogen activated protein kinases (FRS2-MAPK) pathway. In particular, the activated MAPKs, which include extracellular signal-regulated kinases (ERKs), p38, and Jun-terminal kinases (JNKs), subsequently regulate the activity of downstream kinases and translocate to the nucleus, where transcription factors are phosphorylated (P), and thus target genes are regulated.[18] In the third stage, cell proliferation (marked as III-1) and migration (marked as III-2) are modulated. From the in vitro model, the ROS levels in the cell-containing medium have a major role in the functions of the stimulated test cells.

This possible mechanism in the in vitro model may be applicable to in vivo conditions. An in vivo model for subcutaneous tissue repair is proposed and described in Figure S4 in Supporting Information. We hypothesize that under the effect of RPS and intracellular ROS, the stimulated test cells will release FGF7. FGF7 then transduces their signals through four high-affinity transmembrane protein tyrosines kinases, which synthesize from epithelial cells. This signaling pathway will stimulate keratinocyte in epidermis migration to the wound bed, which help to regulate the wound re-epithelialization process. It is therefore anticipated that further development of N_2/Ar micro-plasma applied to skin tissues may provide a feasible method for the improvement of cutaneous wound healing.

5. Conclusion

N_2/Ar micro-plasma with optimized parameters, i.e., a supplied power of 17 W for an average temperature below 40 °C, a working distance of 12 mm, and 0.5% N_2 addition to Ar plasma to obtain RPS, was applied to a fibroblast cell-containing medium. Both plasma composition and its exposure time had effects on the total ROS surrounding the test cells. After incubation for 24 h, the level of LDH leakage or the stimulation of the lipid peroxidation process from the plasma-exposed cells significantly increased along with the plasma exposure time. The sub-lethal membrane damage was gradually repaired by incubation time-dependent release of FGF7 from the stimulated test cells. As a result, the cells' subsequent functions, such as viability, proliferation, and migration were influenced by plasma composition, its exposure time, and incubation time. In particular, 0.5% N_2/Ar micro-plasma with an exposure time of 5 or 10 s on fibroblast cells was suggested for realizing the stimulated cell functions. A possible mechanism based upon in vitro model was thereafter proposed. This preliminary result open a potential of using N_2/Ar micro-plasma for cells related studies and is promising with regard to the improvement of cutaneous wound healing.

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