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Cell cycle regulation in human hair follicle dermal papilla cells using nonthermal atmospheric pressure plasma-activated medium

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Abstract

Nonthermal atmospheric pressure (NAP) plasmas have recently been developed and have been used for wound healing, blood coagulation, and cancer therapy. NAP plasmas can induce either cell proliferation or cell death, depending on the dose. Due to their efficacy and application easily, plasma activated mediums (PAMs) have been used in human cells recently.

In atmosphere, NAP plasmas react with molecular content of air such as N_2, O_2, H_2O vapor, etc, and generate a variety of reactive oxygen and nitrogen species. High reactive oxygen species (ROS) levels promote damage of cellular DNA, proteins, and lipids. Such damage can lead to cell-cycle arrest, and cellular death. However, low levels of ROS have been caused an increase in cell cycle progression.

Human skin is arranged in 3 layers, including (from top to bottom) the epidermis (and its appendages), the dermis, and the hypodermis. Human dermal papilla cells (DPCs) are located in the middle or even deep part of the dermis. DPCs play a key role in hair regeneration, and a lot of effort have been made to promote DPC hair formation ability. DPC is increased proliferation, delayed senescence, and enhanced hair by depending on the amount of ROS through the NAP-PAM treatment.

In this study, we used NAP plasmas to the human hair follicle DPCs exposed from 0 to 20 minutes, so we were investigated the effects of PAM on cell proliferation and cell cycle progression. After NAP-PAM treatment for 24 hours, cell cycle was arrested in the G0/G1 phase. The NAP-PAM-treated human hair follicle DPCs recovered gradually after 48 hours of the treatment compared to the untreated cells.

Therefore, this approach offers promising results for further application of NAP-PAM in clinical dermatology. In future, it can be applied clinically in the form of active water that can delay the progression of baldness and alopecia areata.

Abbreviations: DPC = dermal papilla cell, NAP = nonthermal atmospheric pressure, PAM = plasma activated medium, ROS = reactive oxygen species.

Keywords: cell cycle, dermal papilla cell, nonthermal atmospheric plasma, plasma activated medium

1. Introduction

Plasma is the fourth phase of substances, along with solid, liquid, and gas, and occurs widely in nature. Technological advancement has made it possible to sustain plasmas at atmospheric pressure and room temperature.

Thermal and low-pressure plasmas are being used in industry, and innovative technologies for generating nonthermal plasmas at atmospheric pressure have recently been developed.[1] Since nonthermal atmospheric pressure (NAP) functions below 40°C, it can be utilized in several industries, including medicine and biology for applications such as wound healing, blood coagulation, and cancer therapy.[1] NAP plasma can induce either cell proliferation or cell death depending on the dose. For instance, low doses of NAP plasma lead to an increase cell proliferation, whereas high doses of plasma generate large amounts of reactive oxygen species (ROS), resulting in cell cycle arrest and apoptosis.[2,3]

Plasma generates ROS and reactive nitrogen species in cells, both of which have significant impacts on cellular physiology, as several diseases have been associated with increased oxidative stress.[1,4,5] Many antioxidants induce anti-inflammatory, antibacterial, and antitumor activities.

Increased ROS generation causes several dermatological disorders, especially hair loss.[6] To prevent hair loss, many researchers have developed methods of regenerating new hair follicles.[6] Dermal papilla cells (DPCs) play a key role in hair regeneration, so tremendous efforts have been made to increase proliferation of DPCs and promote hair formation ability.[6]

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All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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In our previous study, we examined the effects of NAP-plasma activated medium (PAM) on the functioning of normal human skin melanocytes, including proliferation, cell cycle progression, and melanogenesis.[3] PAM induced cell cycle arrest; however, it did not cause significant cell death. The PAM-induced cell cycle arrest was a transient phenomenon lasting 48 hours or less, and then the cell cycle slowly recovered. Thus, we inferred that PAM treatment at an appropriate concentration caused cell proliferation and cell cycle arrest, which could be recovered after a certain period.

Herein, based on the results of this previous study, we treated human hair follicle DPCs with PAM to ascertain the effects of PAM on other human cells.

2. Materials and methods

This study did not require approval by an institutional review board because all of the materials used in this study were purchased from PromoCell (Heidelberg, Germany).

2.1. Plasma activated medium

We used a microwave plasma device (RADIX-0501; Medipl Co., Ltd., Gyeonggi-do, Korea) to generate NAP plasma. We had also used this device in our previous study.[3] The plasma was generated via microwave power at atmospheric pressure using argon gas, at a temperature below 40°C. The characteristics of the RADIX-0501 were as follows: microwave power of 2.0 W (Mode 1), 2.5 W (Mode 2), and 3.0 W (Mode 3); microwave accuracy of ±2%; gas pressure of 0 to 3 Bar; and gas flow controlled by 0 to 5 standard liters per minute.

Figure 1 shows the emission spectra recorded via optical emission spectroscopy (DM750; Leica, Wetzlar, Germany) using the RADIX-0501 device at mode 3.[3]

The spectra emitted by the excited atoms of the argon feed gas were between 690 and 900 nm. Peaks corresponding to N_2 and O were detected at 337.2 and 777.4 nm, respectively. The hydroxyl band was detected between 307 and 309 nm. The distance between the end of the plasma jet and the upper surface of the medium was fixed at 0.8 cm. In total, 10 mL of medium was suspended in a 10 cm petri dish and was treated with plasma for 0 to 20 minutes. This PAM was applied to the human hair follicle DPCs immediately.

2.2. Cell culture and growth analysis

Normal human hair follicle DPCs were purchased from PromoCell. The DPCs were grown in C-26501 medium (PromoCell). They were subsequently incubated in 10 cm cell-culture dishes (1×10^6 cells/dish) with PAM for 24 or 48 hours. After incubation, the cells were harvested from each dish using trypsin-ethylenediaminetetraacetic acid solution (JBI, Seoul, South Korea) and were washed once with phosphate-buffered saline containing 5% fetal bovine serum. Next, the number of cells was counted using an ADAM-MC cell counter (NanoEnTeK, Seoul, South Korea).[3]

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cell viability was determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using 1×10^4 cells/well in a 96-well plate. Purple formazan crystals were dissolved in dimethyl sulfoxide, and transferred to the 96-well plate (200 μL/well) and absorbance was determined at 570 nm using a microplate reader.[3]

2.4. Cell cycle analysis

Human hair follicle DPCs were incubated in 10 cm cell culture dishes (1×10^6 cells/dish) with PAM for 24 or 48 hours at 37°C in a humidified atmosphere containing 5% CO_2. Untreated DPCs

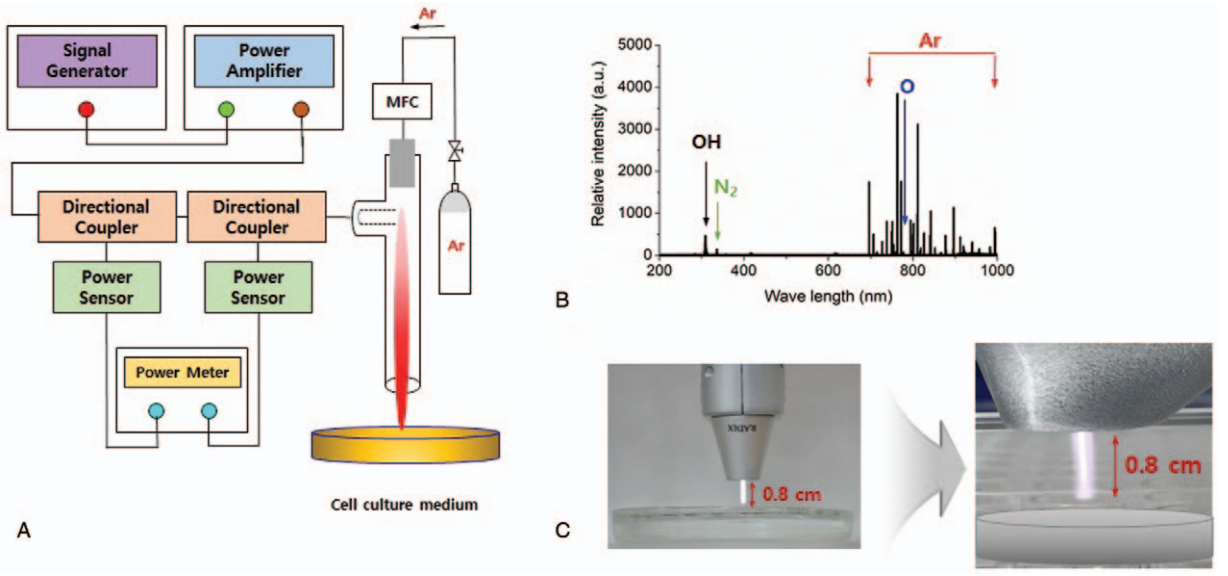

Figure 1. (A) Schematic representation of the NAP plasma generator. (B) Optical emission spectra of the NAP plasma jet in mode 3 during discharge at a wavelength of 200 to 1000 nm. (C) Experimental setup of the NAP plasma jet used to prepare the PAM. NAP = nonthermal atmospheric pressure, PAM = plasma activated medium.

were used to define the base level of apoptosis and cell death. Cells were washed twice with phosphate-buffered saline buffer, treated with 10 μg/mL RNase A, stained with 50 μg/mL propidium iodide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and analyzed using FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and CellQuest 6.0 software.[3]

2.5. Statistics

All data were expressed as mean ± standard deviation. Data were analyzed using 2-tailed Student *t* test (SPSS ver. 13; SPSS Inc., Chicago, IL). *P*-values < .01 were considered as statistically significant.

3. Results

To investigate the effects of PAM on their viability, human hair follicle DPCs were exposed to PAM for 12 minutes, followed by incubation for 48 hours. Figure 2 shows the effect of 12 minute-long PAM treatment on hair follicle DPCs incubated for 24 and 48 hours following the treatment (Fig. 2B and D, respectively). The control cells were incubated for 24 hours (Fig. 2A) and 48 hours (Fig. 2C) with no PAM exposure.

Both groups of cells showed typical hair follicle DPCs morphology and were stably attached to the bottom. Floating or dead cells were rare, with no decrease in cell population. Thus, based on microscopic findings, we confirmed that PAM is not cytopathic.

To understand the mechanism underlying lack of alteration in cell population, we conducted a flow cytometric analysis for investigating the effect of PAM on the cell cycle distribution. After PAM exposure for varying durations ranging from 0 to 12 minutes, hair follicle DPCs were incubated for 24 and 48 hours. After incubation for 24 hours, we observed an increase in the number of cells at the G0/G1 phase in a dose-dependent manner and found that the rate of increase was statistically significant (Fig. 3A and B) (SPSS ver. 13). That was accompanied by a concomitant reduction in the number of cells in the G2/M phase (Fig. 3D). The S phase was obstructed due to PAM treatment (Fig. 3C). After incubation for 48 hours in PAM, the sub-G1 phase increased, but the rate of increase was not statistically significant (Fig. 3A). Cell cycle distribution decreased the G0/G1 phase and increased the G2/M phase (Fig. 3B and D).

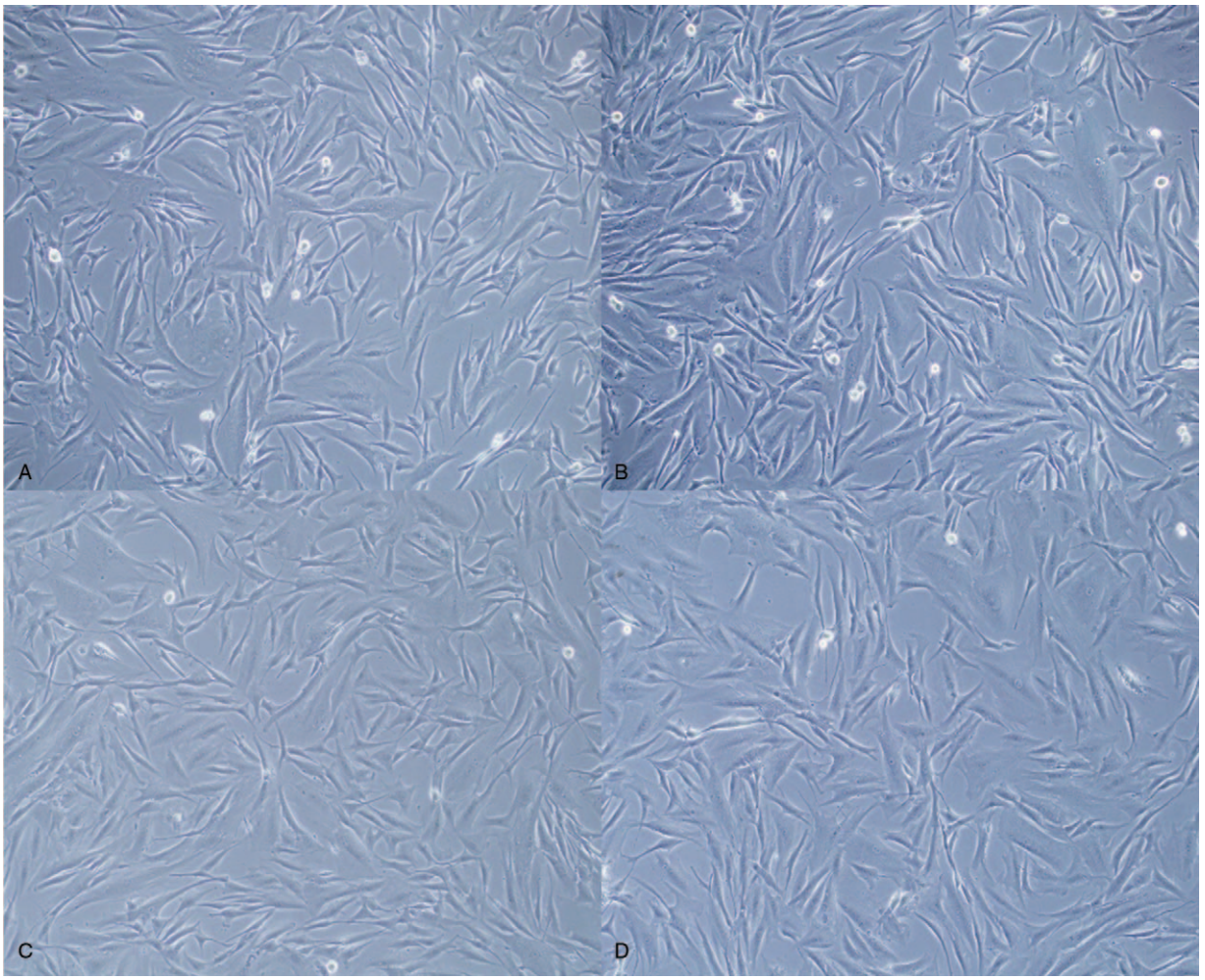

Figure 2. Morphological changes in hair follicle dermal papilla cells treated with PAM for 12 min. (A) The cells were incubated for 24 h with no PAM exposure. (B) After PAM exposure for 12 min, the cells were incubated for 24 h. (C) The cells were incubated for 48 h with no PAM exposure. (D) After PAM exposure for 12 min, the cells were incubated for 48 h. Original magnification ×100. PAM = plasma activated medium.

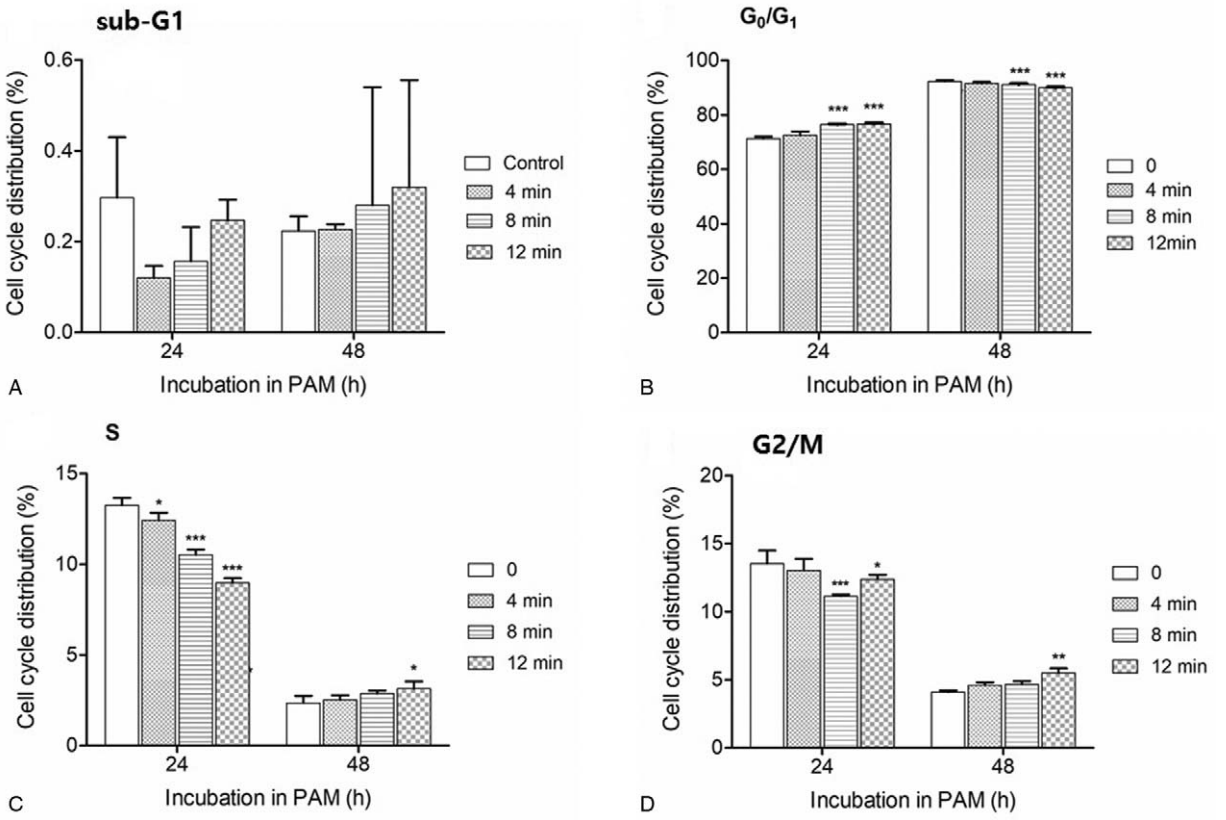

Figure 3. Cell cycle analysis of PAM-treated human hair follicle dermal papilla cells. The cells were incubated with PAM for 24 or 48 h and the cell cycle was examined. The duration of PAM exposure was increased from 0 to 12 min. (A) sub G1 phase. (B) G0/G1 phase. (C) S phase. (D) G2/M phase. The values represent the mean ± SD ($^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$). PAM = plasma activated medium, SD = standard deviation.

These results indicated that the proliferation of hair follicle DPCs was inhibited through an increase in the growth phase along with suppression of DNA synthesis and mitosis. We considered that PAM was not cytopathic due to inhibition of mitosis and arrest of the cell cycle in the G0/G1 phase.

To confirm the cell-cycle switch induced by PAM, hair follicle DPCs were treated with PAM for 8 minutes, following which they were monitored for an extended period of 48 hours. The culture medium was then changed and the cells were incubated for an additional 24 or 48 hours in the new medium that had not been exposed to NAP plasma. Figure 4 shows the results of cell cycle analysis of cell groups incubated for a total of 72 hours (48 hours in PAM + 24 hours in untreated medium) (Fig. 4A and B) and 96 hours (48 hours in PAM + 48 hours in untreated medium) (Fig. 4C and D).

Compared to cells with no exposure to PAM, apoptosis of cells whose cell cycle was arrested in sub-G1 phase was slightly increased, but the difference was not statistically significant (SPSS ver. 13). In the S phase, DNA synthesis was inhibited in the group incubated for 72 hours but increased in the group incubated for 96 hours, indicating that DNA synthesis gradually increased after a certain period following PAM treatment.

After the cells were incubated for 24 hours, the population doubling time (PDT) of cells with no exposure to PAM (control group) was 62.90 hours (Fig. 5).

However, the PDT was delayed after PAM treatment. (PDT after exposure to PAM for 4 minutes to 72.72 hours, 8 minutes to 105.09 hours, and 12 minutes to 98.35 hours). After changing the medium following 48 hours of incubation, PDT of all groups gradually recovered to that observed in control group.

4. Discussion

Cell cycle progression is regulated by various external factors. It has been obtained that ROS may also play an important role in cell cycle progression.[7,8] ROS cause a wide range of adaptive cellular responses to cell cycle arrest, to apoptosis, or to necrosis, dependent on the level of ROS.[7,8] In addition, cell cycle regulation is performed by cyclins and cyclin dependent kinases. Recently, it has become clear that ROS influence the presence and activity of these enzymes and cyclin/cyclin dependent kinases complexes control cell cycle progression.[9]

Cell cycle arrest may be transient or long lasting. With weak stimulation, cell growth and division are inhibited, with an increase in the population of G0/G1 cells.[3] When the intensity of the stimulus reaches the threshold limit, the population of G0/G1 cells decreases and apoptosis increases (sub-G1 phase).[3] A decrease in the population of G0/G1 cells may lead to an increase in number of cells in phases other than sub-G1 phase.[3]

NAP plasma has been widely used for medical purposes such as cancer therapy, wound healing, and blood coagulation. It has been proven effective for immuno-proliferation, cell proliferation, protein stimulation, sterilization, and selective cancer apoptosis.[4,5] These properties of NAP plasma are closely

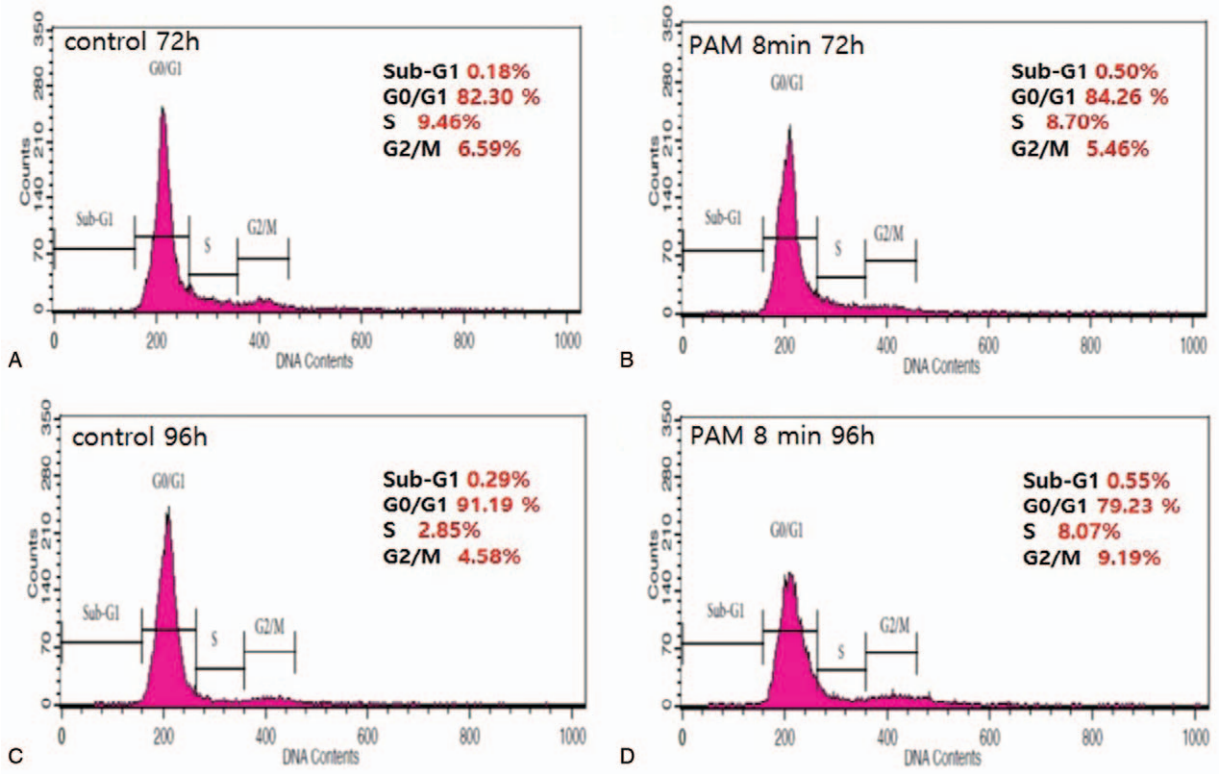

Figure 4. The cells were treated with PAM for 8 min and incubated for an extended period. Cells were incubated in PAM for 48 h and were then incubated in untreated medium for additional 24 h and 48 h, leading to a total incubation period of 72 h and 96 h, respectively. (A) The cells were incubated for 72 h with no exposure to PAM (control). (B) The cells were incubated for 48 h following exposure to PAM for 8 min, and were incubated for additional 24 h with no exposure to PAM. (C) The cells were incubated for 96 h with no exposure to PAM. (D) The cells were incubated for 48 h following exposure to PAM for 8 min, and were incubated for additional 48 h with no exposure to PAM. PAM = plasma activated medium.

related to the generation of ROS. At low levels, ROS stimulate cell proliferation and angiogenesis, whereas high levels of ROS can cause cellular and DNA damage.[7,8]

Hair loss is associated with aging process due to regenerative disorders of hair follicles.[6] Hair follicles are composed of keratinocytes and DPCs.[6] DPCs play a key role in hair neogenesis, and several studies have been conducted on the relationship between ROS generation and DPC growth. ROS generated by NAP plasma affects DPC, causing cell cycle arrest. It depends on the energy generated by the plasma.[3]

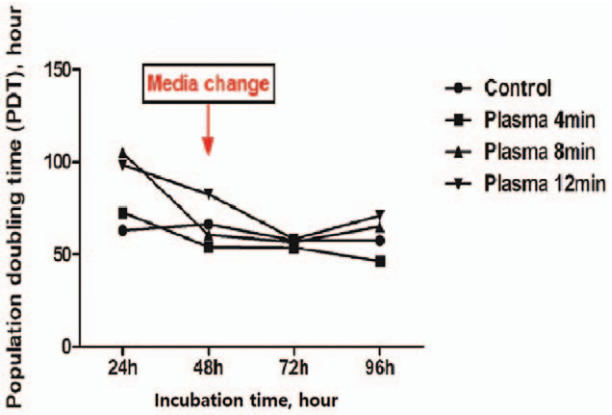

Figure 5. Population doubling time (PDT).

In our previous study, we found that PAM was induced cell cycle arrest after incubating 48 hours in human skin melanocytes, and then the cell cycle was slowly recovered. PAM induced cell cycle arrest through regulation of the G0/G1 and G2/M phases, but it did not cause significant cell death. Furthermore, the cell cycle arrest was a transient phenomenon lasting 48 hours or less, after that the cell cycle slowly recovered. There were indicating that PAM exposure is noncytopathic.[3] In order to determine whether these results could be obtained in other human cells, we replicated the experiment on human hair follicle DPCs. Positive results would mean that NAP-PAM could be applicable to any other field.

In this study, we demonstrated that the cell cycle of human hair follicle DPCs arrested for 48 hours following PAM treatment, and then it recovered slowly. This implies that the level of apoptosis was not significant and lasted briefly. Interestingly, we observed that the cell cycle of cells incubated for 24 hours following PAM treatment for 8 to 12 minutes was inhibited in the G0/G1 phase. However, when the medium was changed after 48 hours of incubation, we observed that mitosis (G2/M) was active, signified by increase in DNA synthesis. We think this phenomenon is indicative of the physiologic homeostasis of the cells. As a result, NAP plasma is not cytopathic and causes only a temporary arrest of the cell cycle.

There are some limitations in our study. At first, we only observed the effect of NAP-PAM on human hair follicle DPCs not performed the detailed mechanism of the oxidation produced by NAP-PAM on human hair follicle DPCs. Further experiments

will have to be conducted to find out the exact mechanism. In second, because NAP-PAM cannot penetrate into the dermis, it may be difficult to use NAP-PAM in hair clinics right now. There has been no evidence yet, and research is in progress, but it is thought that NAP-PAM may affect DPCs by cascade reaction. But if we develop and use equipment that allows plasma liquid to penetrate into the skin, it will not be difficult for clinical use.

Most treatments involve the direct application of plasma to lesions. However, recently it was discovered that PAM induced physiological changes in cells and tissues and an indirect treatments could be a novel approach for temporarily arresting the cell cycle. For this reason, PAM could potentially be used in suppressing the cell cycle in other dermatological fields. It is considered that it can be applied clinically in the form of active water that can delay the progression of baldness and alopecia areata. Since NAP-PAM is noncytopathic, if appropriate concentration and time are applied to DPCs to delay DPCs aging and increase proliferation, hair will grow well through hair follicle transplantation.

5. Conclusions

PAM treatment at an appropriate concentration and duration leads to arrest of cell proliferation and cell cycle, which can be revived after a certain period of time. In other words, the cell cycle can be turned on and off using NAP-PAM treatment without any intracellular toxicity.

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