Non-Thermal Atmospheric Pressure Plasma Enhances Proliferation of Adipose Tissue-Derived Stem Cells via Nitric Oxide-Response Pathways

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Abstract: Our study showed that exposure to non-thermal atmospheric pressure plasma (NTAPP) increased the proliferation of adipose tissue-derived stem cells (ASCs) compared with untreated cells without affecting their stem cell properties. We showed that nitric oxide (NO) is responsible for the enhanced proliferation of ASCs following NTAPP exposure. Altogether, this study suggests that NTAPP would be an efficient tool for the medical application of ASCs.

Key words: non-thermal atmospheric plasma, adipose tissue-derived stem cells, reactive oxygen species, nitric oxide, acceleration of proliferation of ASCs

1. Introduction

Plasma is described as a quasi-neutral mixture of charged particles and radicals in a partially ionized gas. Recently, many studies attempted to take advantage of the low temperature of non-thermal atmospheric pressure plasmas (NTAPPs) for biomedical applications owing to the controllability of plasma chemistry and kinetics [1]. Recently, the clinical applications of NTAPPs have become a very active research area.

In our previous study, we showed that NTAPP exposure selectively induces apoptosis in cancer cells by activating the ROS response system; however, it accelerated the proliferation of normal fibroblast IMR 90 cells and adipose tissue-derived stem cells (ASCs) [2]. NTAPP has also been reported to accelerate wound healing processes by activating the nuclear factor erythroid-related factor 2 (NRF2) signaling pathway in human keratinocyte cell line in vitro [3]. It also has been known to promote re-epithelialization and wound closure by activating keratinocytes and fibroblasts in rats' wound skin [4]. These studies strongly suggested that NTAPP stimulates the proliferation of normal and adult stem cells.

ASCs are mesenchymal stem cells (MSC) that have the potential to differentiate into various cell types such as adipocytes, osteoblasts, chondrocytes, and neurons [5]. ASCs are also capable for self-renewal, which is an important property of stem cells to regenerate damaged tissues [6]. ASCs are relatively easy to isolate from adipose tissues by liposuction and may provide an accessible source of adult stem cells for use in regenerative medicine [7], [8]. However, in general, it is difficult to culture adult stem cells in vitro while ensuring that they maintain their stemness; moreover, adult stem cells undergo rapid senescence in vitro [9].

In this study, we focused on the effect of NTAPP on ACSs and its mechanisms. We showed that NTAPP can enhance the proliferation of ASCs in vitro, thereby supporting the potential applications of NTAPP in the field of regenerative medicine.

2. Results

In order to investigate the proliferative effect of NTAPP on human adipose tissue-derived stem cells (ASCs), we used a helium-based dielectric barrier discharge (DBD) type NTAPP device. The schematics of the experimental setup are shown in Fig. 1A.

To examine whether NTAPP could promote the proliferation of ASCs, we exposed NTAPP to ASCs for a total of 10 times, for 50 sec each time in every hour, and further incubated the cells till 72 after initial NTAPP exposure. Viability of NTAPP-exposed ASCs increased 1.57 fold on an average, compared with that observed with the unexposed control cells (Fig. 1B), suggesting that NTAPP exposure accelerated the proliferation of ASCs.

In order to use NTAPP to accelerate the proliferation of ASCs for applications, the characteristic of ASCs must be maintained after NTAPP exposure. We compared the stemness characteristics of NTAPP-exposed and –unexposed ASCs. CD44 and CD105 were used as positive markers, CD45 was used as a negative marker, and FABP4 was used as a differentiation marker to evaluate the characteristics of ASCs. We exposed the ASCs to NTAPP for a total of 10 times, and incubated the cells for 72 h after first exposure. At the indicated time, we monitored the expression of the markers and observed that CD44 and CD105 continued to be expressed, while CD45 and FABP4 were not expressed. The expression of markers was identical to that in NTAPP-unexposed ASCs (Fig. 1C). These results support that NTAPP exposure does not change the stemness characteristics of ASCs.

It has been reported that most stem cells including human mesenchymal stem cells (hMSCs) are prone to genotoxic damages that eventually lead to cellular senescence when cells proliferate *in vitro* [10]. Thus, we monitored whether ASCs showing increased proliferation following NTAPP exposure underwent cellular senescence. As shown in Fig. 1D, only 12 % of NTAPP-exposed ASCs were positive for

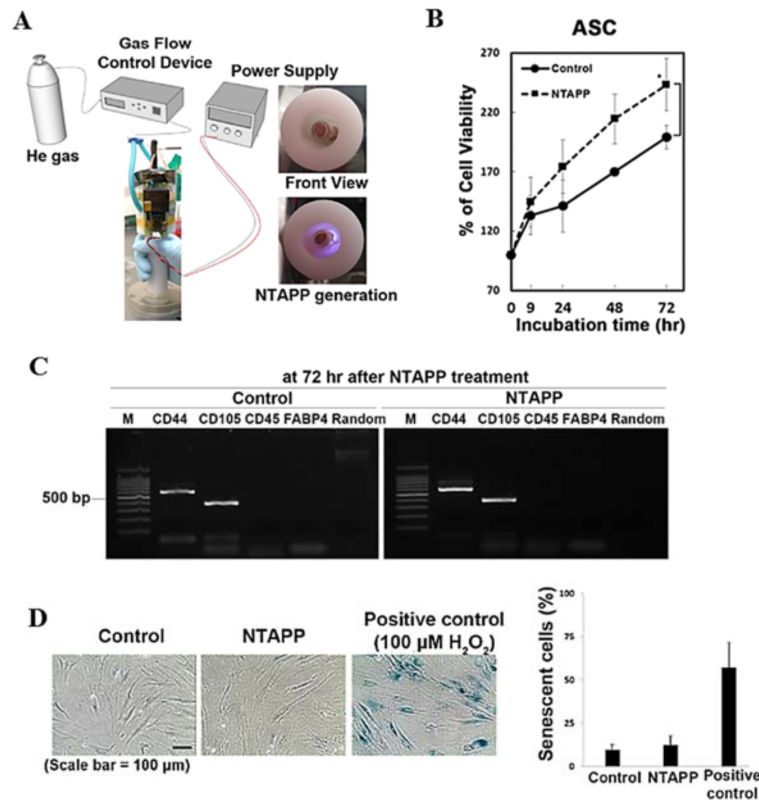

Fig. 1. NTAPP accelerates the proliferation of ASCs, maintaining their stem cell properties.
(A) Schematic description of the NTAPP-generating device used in this study (B) ASCs were exposed to NTAPP for a total of 10 times, for 50 sec in every h, and were further incubated for 72 h from the initial exposure. Cell viability was evaluated at each indicated incubation time-point. (C) Expression of the markers of ASCs was analysed by RT-PCR at 72 h after the first NTAPP exposure and compared to that unexposed control cells. (D) SA-βGal assay was performed to evaluate senescence in ASCs at 72 h after exposure to NTAPP for a total of 10 times. ASCs treated with 100 μM H_2O_2 were used as a positive control. Scale bar, 100 μm. Senescent cells were counted, and the values were expressed as percentages.

β-galactosidase staining, similar to that observed with unexposed ASCs (9 %). These results suggest that NTAPP did not cause cellular senescence in ASCs while it promoted the proliferation of ASCs.

Nitric Oxide (NO) is a well-known second messenger and a key modulator in many physiological functions including cell proliferation [11]. NTAPP generates ROS and RNS; among these species, plasma can easily generate NO from N2 and O2 in the air. Given that NO at a low concentration has been reported to promote cell proliferation through the inhibition of cellular apoptosis [12] and NTAPP exposure is known to promote proliferation in ASCs, we hypothesized that NO might play a role in enhancing the proliferation of ASCs following NTAPP exposure. To examine whether NO generated by NTAPP affects the proliferation of ASCs, we treated the cells with carboxy-PTIO, a NO scavenger, with or without NTAPP exposure. Viability was analyzed after the cells were exposed to NTAPP (control cells were not exposed) in the presence or absence of a NO scavenger in the medium. The viability of NTAPP-exposed cells increased by 199% at 72 h after NTAPP exposure, compared with that at the beginning of incubation (0 h; considered 100%), while the viability of unexposed cells increased only by 148% at 72 h. However, the viability of NTAPP-exposed cells following treatment with carboxy-PTIO was reduced to 170% (Fig. 2A). These observations revealed that NO is mainly responsible for the increased proliferation of NTAPP-exposed ASCs.

To further verify that NO is responsible for the enhanced proliferation of ASCs following NTAPP exposure, we investigated the related cellular pathways of NO-induced cell proliferation. NO is known to be produced by activated nitric oxide synthase (NOS) via the PI-3K/Akt signaling pathway [13] and to induce the mitogen-activated protein kinase (MAPK)/ERK pathway that leads to cell proliferation [14]. Thus, we examined the activation of Akt, and ERK1/2 at 0, 9, and 72 h after the exposure of ASCs to NTAPP. The expression of phospho-Akt was increased in ASCs immediately after NTAPP exposure, which was administered 10 times, but decreased to the normal level at 72 h after the initial NTAPP exposure. Phospho-ERK1/2 was elevated at 72 h (Fig. 2B). These results demonstrated that NTAPP promoted the proliferation of ASCs by

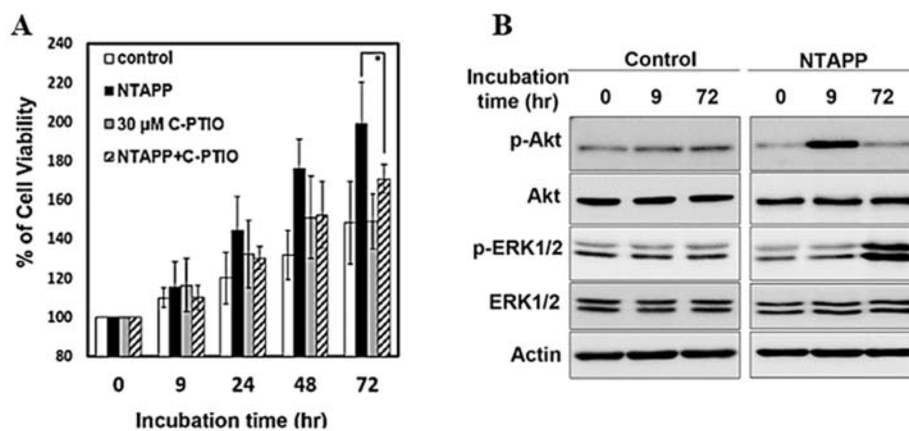

Fig. 2. NO plays a key role in NTAPP-induced proliferation of ASCs.
(A) ASCs pre-treated with culture medium alone (as the negative control) or 30 μM carboxy-PTIO were exposed to NTAPP for a total of 10 times. Cells were totally incubated for 72 h after the initial NTAPP exposure. (B) The expression of Akt, phospho-Akt, ERK1/2, and phospho-ERK1/2 in NTAPP-exposed ASCs was analysed by western blot at 0, 9, and 72 h from the initial exposure. Actin was used as the loading control.

activating the Akt and ERK signaling pathways at different time-points, demonstrating that NTAPP promotes the proliferation of ASCs via NO by activating Akt, and ERK1/2 pathway.

3. Discussion

In recent years, NTAPP has been studied for its clinical applications, especially in cancer therapy and sterilization [15]. While NTAPP has been known to induce apoptosis in various cancer cells [16], its role in the activation of proliferation is not well investigated. In this study, we used a helium-based dielectric barrier discharge (DBD)-type NTAPP device generating multiple intracellular ROS/ RNS, demonstrating that NTAPP promotes the proliferation of ASCs, while maintaining the stem cell characteristics of ASCs. We also showed that nitric oxide (NO) generated from NTAPP plays a key role in NTAPP-induced increased proliferation of ASCs by activating the Akt and ERK1/2 pathways. Collectively, these results strongly suggest that NTAPP can increase the efficiency of ASC culture in vitro, thereby supporting the potential applications of NTAPP in the field of regenerative medicine.

NO acts as an intracellular messenger and regulator in biological functions [17]. Interestingly, different cell fates depend on NO concentrations: low NO concentration promotes cell survival and proliferation in various cells including stem cells [18], while high NO concentration leads to cell cycle arrest and cell death [19]. NO is generated by NTAPP. Our study showed that NO generated by NTAPP plays an important role in inducing the proliferation of ASCs. However, not only NO but also other unknown factors might be involved in the increased proliferation of ASCs following NTAPP exposure because the viability of ASCs following combined treatment with NTAPP and NO scavenger was not recovered to the level in control cells even though viability was reduced, as shown in Fig. 2A. Further studies would be necessary to understand which other components of NTAPP are responsible for the promotion of NTAPP-induced cell proliferation.

As expected from the results in Fig. 2 that show that the increased proliferation of ASCs was mainly attributed to NO, we observed the activation of Akt and ERK1/2 in NTAPP-exposed ASCs. However, their time of activation was different (Fig. 2B). A study of the mechanism underlying the differential regulation of the Akt and ERK signaling pathways would be necessary to understand the mechanism by which NO controls the proliferation of adult stem cells including ASCs.

The results of this study show the potential of NTAPP to be used to control the proliferation of ASCs and suggest a clue as to why NTAPP activates wound healing in tissues. To develop NTAPP as a reliable tool for use in stem cell technology and regeneration, the effect of NTAPP on other stem cells needs to be investigated and further chemical evaluations of NTAPP will be necessary.

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