Non-Thermal Atmospheric Pressure Plasma as an efficient tool to activate the proliferation of most mesoderm-derived adult stem cells *in vitro*

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Abstract: We studied the effect of NTAPP on the proliferation of various adult stem cells and its mechanisms. We showed that exposure to NTAPP increased the proliferation of various mesoderm-derived adult stem cells by ~2 folds, and the expression of well-known pluripotent genes in NTAPP-exposed adult stem cells compared with that of the unexposed cells. Altogether, this study suggests that NTAPP would be an efficient tool to activate the proliferation of various adult stem cells for the medical application of stem cells.

Key words: non-thermal atmospheric plasma, adipose tissue-derived stem cells, bone-marrow-derived stem cells, hematopoietic stem cells, acceleration of proliferation, stem cell therapy

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]. Recently, many studies have recently reported the beneficial outcomes of NTAPP including sterilization and wound healing, suggesting that NTAPP stimulates the proliferation of normal and adult stem cells.

Adult stem cells are multipotent and possess a potential for self-renewal and differentiation into specific cell types, thereby being an applicable tool for stem cell therapy [3-6]. The most well-characterized adult stem cells are mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) derived from the mesoderm layer among three embryonic germ layers [4, 7-9]. These cells are relatively easy to obtain from specific tissues of a patient, do not result in immune-rejection, and present a reduced risk of tumor formation compared to embryonic stem cells. Thus, adult stem cells have become an easily accessible source for biomedical stem cell therapy [10, 11]. However, there are several technical barriers for the use of adult stem cells for regenerative medicine. Only a limited number of adult stem cells can be collected from a patient's tissues [12], and their proliferation is inefficient *in vitro* [13, 14]. Also, it is difficult to maintain the characteristics of stem cells during culture *in vitro*, and they easily undergo senescence [12, 15-17]. Thus, an efficient tool to activate the proliferation of adult stem cells to expand the population *in vitro* is required for cell therapy applications.

In this study, we focused on the effect of NTAPP on most mesoderm-derived adult stem cells and its mechanisms. We showed that NTAPP can enhance the proliferation of ASCs, BM-MSCs and HSCs in vitro while boosting their stemness characteristics, thereby supporting the potential applications of NTAPP in the field of regenerative medicine.

2. Results

To investigate the proliferative effect of NTAPP on ASCs, BM-MSCs and HSCs, 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, BM-MSCs and HSCs, we exposed NTAPP to these cells for a total of 10 times, for 50 sec each time in every hour, and further incubated the cells till each indicated time 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). In addition, we examined the proliferation of BM-MSCs with the exposure of NTAPP in a low-glucose (1 g/L) and basic fibroblast growth factor (b-FGF, 10 ng/ml) medium. NTAPP exposures increased the viability of BM-MSCs compared to that of the unexposed controls by 1.63- and 1.8-fold at 48 h and 96 h, respectively, from the initial exposure of NTAPP (Fig. 1C). Also, when we monitored cell proliferation after NTAPP exposure, the number of HSCs increased by 1.7- and 2-fold at 144 h and 192 h, compared to that of the unexposed control cells at each designated time (Fig. 1D), suggesting that NTAPP exposure accelerated the proliferation of ASCs, BM-MSCs and HSCs.

In order to use NTAPP to accelerate the proliferation of adult stem cells for applications, the characteristic of adult stem cells must be maintained after NTAPP exposure. We first compared the stemness characteristics of NTAPP-

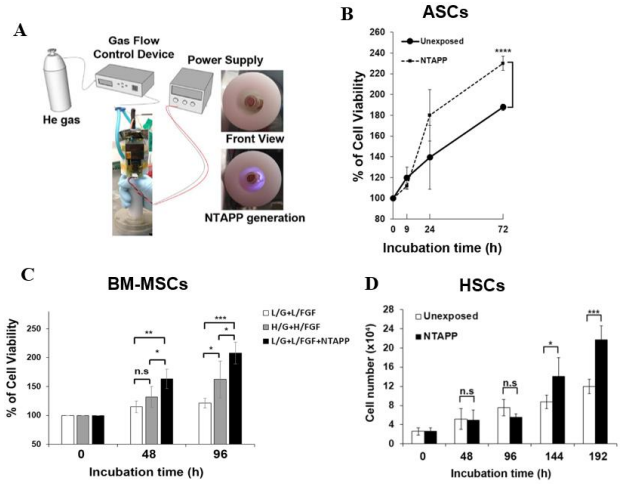

Figure 1. NTAPP accelerates the proliferation of ASCs, BM-MSCs and HSCs

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. 2A).

Recent studies have reported that three transcription factors that play key functions in maintaining the pluripotency of stem cells, Oct4, Sox2 and Nanog, enhance the cell proliferation in various adult stem cells. Because our results showed that NTAPP increased the proliferation of human ASCs, BM-MSCs, and HSCs, we examined whether NTAPP treatment activated the expression of Oct4, Sox2, and Nanog in ASCs. We monitored the expression of Oct4, Sox2, and Nanog in ASCs exposed to NTAPP and incubated for 72 h after the first NTAPP exposure. Peroxisome proliferator-activated receptor gamma (PPARγ) was used as a marker for anti-proliferative differentiation of early adipogenesis. When the mRNA expression of Oct4, Sox2, and Nanog was assessed at 9, 24, and 72 h after the initial NTAPP exposure and compared to that in the unexposed control cells at 0 h, they were continuously augmented with increasing incubation time (Fig. 2B). The expressions of Oct4, Sox2, and Nanog in NTAPP-exposed ASCs were increased by 15-, 13.8-, and 14.8-fold, respectively, at 72 h when compared to that in unexposed cells at the same time point (Fig. 2B). On the other hand, the expression of PPARγ was not changed much by NTAPP exposure and further incubation (Fig. 2B). These results clearly demonstrated that NTAPP maintained the characteristics of ASCs and activated the expression of pluripotent markers Oct4, Sox2, and Nanog in ASCs.

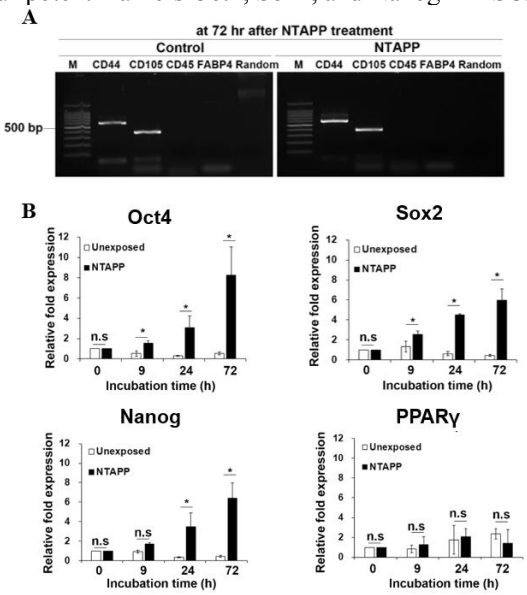

Figure 2. NTAPP maintains the expression of stem cell markers and induces the pluripotent gene markers in ASCs

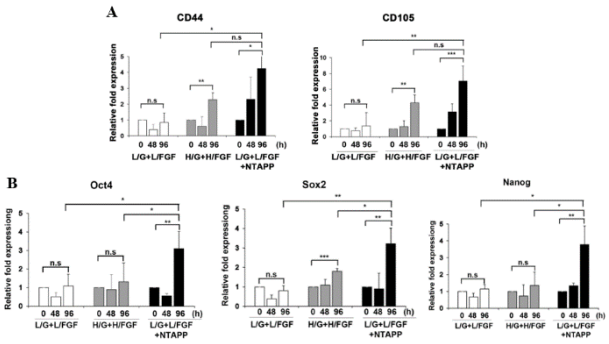

Figure 3. NTAPP induces the expressions of stem-cell specific markers and pluripotency markers in BM-MSCs

We examined the stemness characteristics of NTAPP-treated and -untreated BM-MSCs cultured in a medium with low concentrations of glucose and b-FGF by monitoring the expression of adult stem cell-specific surface markers, CD44 and CD105, after a long-term incubation till 96 h after the initial NTAPP exposure by qPCR. The relative mRNA expressions of CD44 and CD105 increased by 3- and 4-fold, respectively, in NTAPP-exposed BM-MSCs when compared to that of the unexposed controls at 24 h (Fig. 3A). Furthermore, in the 96 h incubation after the initial NTAPP exposure, the relative expression of both CD44 and CD105 by q-PCR was increased 5-fold in NTAPP-exposed cells compared to that in unexposed cells (Fig. 3A). These results demonstrated that NTAPP-exposed BM-MSCs, with low concentrations of glucose and b-FGF, significantly increased the expression of stem cell-specific markers compared to that in unexposed cells.

We also investigated the expressions of Oct4, Sox2, and Nanog in NTAPP-exposed and -unexposed human BM-MSCs cultured in a low concentration of glucose and b-FGF. BM-MSCs were exposed to NTAPP 10 times, for 50 s every h, and incubated for 96 h after the first NTAPP exposure. As observed in ASCs, when compared to that in the NTAPP-exposed BM-MSCs and NTAPP-unexposed control cells at 96 h, the relative mRNA expressions of Oct4, Sox2, and Nanog in NTAPP-exposed cells was increased by 3-, 4-, and 3.3-fold, respectively, on average when compared to unexposed control cells (Fig. 3B). These results demonstrated that NTAPP highly activated the expressions of Oct4, Sox2, and Nanog in BM-MSCs as well.

In order to verify that the NTAPP treatment was as efficient as the treatment of high concentrations of glucose and b-FGF in activating the expression of pluripotent markers in BM-MSCs, we also compared the relative mRNA expressions of Oct4, Sox2, and Nanog in NTAPP-treated BM-MSCs cultured in low concentrations of glucose and b-FGF compared to that in BM-MSCs cultured in high concentrations of glucose and b-FGF. At 96 h after the initial NTAPP exposure, NTAPP-exposed BM-MSCs showed increased relative expressions of Oct4, Sox2, and Nanog by 2.35-, 1.79-, and 2.8-fold, respectively, over unexposed cells cultured in high concentrations of glucose and b-FGF (Fig. 3B). These results strongly suggested that NTAPP treatment was more efficient in activating and maintaining the pluripotency of BM-MSCs compared to high concentrations of glucose and b-FGF.

Nitric Oxide (NO) is a well-known second messenger and a key modulator in many physiological functions including cell proliferation [18]. 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 [19] 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. 4A). These observations revealed that NO is mainly responsible for the increased proliferation of NTAPP-exposed ASCs.

3. Discussion

In recent years, NTAPP has been studied for its clinical applications, especially in cancer therapy and sterilization [20]. While NTAPP has been known to induce apoptosis in various cancer cells [21], 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 and RNS, demonstrating that NTAPP promotes the proliferation of ASCs, BM-MSCs and HSCs. NTAPP not only increased the proliferation but also induced the highly augmented expressions of pluripotency and stem cell-specific surface markers, confirming that NTAPP triggered the stemness characteristics of mesodermal-derived adult stem cells including ASCs and BM-MSCs. Thus, we strongly suggest that NTAPP can be an effective tool to activate the proliferation of various mesoderm-derived adult stem cells while maintaining stemness and pluripotency for stem cell therapy.

Consistent with our results, other research groups have reported that NTAPP increased the proliferation of fibroblasts and keratinocytes for wound healing [22-28]. For example, NTAPP increased the murine fibroblast cell proliferation by activating the NF-κB signaling pathway [22, 23]. NTAPP also activated HaCaT human keratinocyte cell line and the epidermis of mouse skin for skin regeneration *in vivo* [24], and enhanced wound healing in rat models [27, 28]. Thus, this indicated that NTAPP is a general tool for activating the proliferation of not only adult stem cells, but also other cells in tissues, as well for promoting wound healing. Collectively, these results altogether strongly support the potential of NTAPP as a powerful tool to activate the proliferation of various adult stem cells *in vitro* for stem cell therapy and regenerative medicine. Currently, to understand the mechanisms of the activated proliferation of adult stem cells following the NTAPP exposure, we are investigating the whole genome expression profile of NTAPP-exposed ASCs.

4. References

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