Original Research

Liquid-type non-thermal atmospheric plasma ameliorates vocal fold scarring by modulating vocal fold fibroblast

Ho-Ryun Won[1,2], Eun Hye Song[2], Jong Eun Won[3], Hye Young Lee[3], Sung Un Kang[2], Yoo Seob Shin[2,3,*] and Chul-Ho Kim[2,3,*]

[1]Department of Otolaryngology-Head and Neck Surgery, Chungnam National University Hospital, Daejeon 35015, Republic of Korea; [2]Department of Otolaryngology, Ajou University School of Medicine, Suwon 16499, Republic of Korea; [3]Department of Molecular Science & Technology, Ajou University, Suwon 16499, Republic of Korea

*These authors contributed equally to the paper as corresponding author.

Corresponding author: Yoo Seob Shin. Email: ysshinmd@ajou.ac.kr

Impact statement

Voice disorder has a significant impact on life quality, and one of the major causes of this voice disorder is vocal fold scarring. Therefore, various approaches have been tried to treat for voice disorder. However, no method has satisfied all requirements until now. Plasma medicine, which involves the medical application of plasma, is a rapidly developing field. We have confirmed that liquid-type plasma improved vocal fold scarring by mobilizing and activating vocal fold fibroblast. In conclusion, liquid-type plasma is a potential therapeutic agent for promoting vocal fold scarring through simple injection and it may be an alternative therapeutic agent for the current situation to treat voice disorder.

Abstract

Injection laryngoplasty is a widely used therapeutic option for drug delivery into vocal folds (VFs). Efficient injectable materials are urgently needed for treating intractable VF disease. Liquid-type non-thermal atmospheric plasma (LTP) has been found to be useful for various biological applications, including in regenerative medicine. We evaluated the effects of LTP on VF regeneration. Migration and matrix metalloproteinase-2 expression of lipopolysaccharide (LPS)-treated human vocal fold-derived mesenchymal stem cells (VF-MSCs) were enhanced by LTP treatment. LTP treatment not only ameliorated nuclear factor-κB and interleukin-6 activation, induced by LPS treatment, but also the increased manifestation of α-smooth muscle actin and fibronectin, induced by transforming growth factor-ß. In a rabbit VF scarring animal model, histological analyses showed increased hyaluronic acid deposition and decreased collagen accumulation after LTP injection. Videokymographic analysis showed more improved vibrations in LTP-treated VF mucosa compared to those in non-treated group. In conclusion, LTP treatment enhanced the recruitment and activation of VF-MSCs. Regulated extracellular matrix (ECM) synthesis and eventual functional improvement of scarred VFs were observed upon LTP treatment. The results of this study suggest that LTP injection can enhance wound healing and improve functional remodeling following VF injury.

Keywords: Extracellular matrix, lipopolysaccharide, mesenchymal stem cell, vocal fold fibroblast, wound healing

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Introduction

The most important factor in the trauma of the vocal folds (VFs) and postoperative recovery is effective regeneration of the VF mucosa. Improper regeneration of the VF mucosa leads to VF scarring, which causes deformities in the VF edges or stiffness of the vibrating structure.[1] One cause of VF scarring is the production of an extracellular matrix (ECM) containing excess collagen by myofibroblasts.[2] These mature myofibroblasts can be transformed from human vocal fold fibroblasts (hVFFs), which have properties similar to human mesenchymal stem cells (MSCs). Thus, favorable differentiation of vocal fold-derived mesenchymal stem cells (VF-MSCs) is important for regenerating high-quality VF mucosa after injury.[3–5] However, there is no treatment modality that promotes efficient ECM accumulation through modulation of VF-MSCs to regenerate the VF mucosa.

The human body has the potential to use its own cells, which reside in a quiescent state, for tissue regeneration.[6] Upon acute injury by trauma or infection, through various

chemotactic or mechanical factors, varying numbers of host stem cells are recruited to the newly injured tissue site.[7] The quiescent host-residing stem cells can be activated and self-renew and differentiate to build new tissue in response to tissue injury. Application of external stimuli to mobilize, recruit, and activate endogenous stromal cells for recovery may be an alternative therapeutic option for VF regeneration. Bioactive agents may be useful for favorable VF regeneration without having cells use the innate regenerative properties of the body.

Plasma is a mixture of electrons, ions, and energetic photons in the form of ionized gas that is generated when the gas is further energized.[8] Numerous studies have shown the *in vivo* effects of non-thermal atmospheric plasma (NTP) including sterilization, anti-cancer, anti-inflammation, and tissue regeneration.[9–11] The medical use of this plasma, plasma medicine, is a promising discipline. Plasma can be stored in the liquid form as non-thermal plasma-treated solution (LTP) for easier clinical application than the gaseous form of plasma. LTP shows good permeability through the epidermis compared to gas-type plasma and is easily injected. We hypothesized that LTP can recruit and activate VF-MSCs, leading to favorable ECM restoration and eventual VF regeneration.

In this study, the therapeutic mechanism and effectiveness of LTP for favorable regeneration of VF were investigated. The *in vitro* effects of LTP were evaluated using VF-MSCs isolated from normal human VFs. Furthermore, we evaluated the *in vivo* effects of LTP on damaged VFs using a rabbit animal model. We have confirmed through this study that LTP induces effective regeneration of VFs mucosa through modulation of VF-MSCs.

Materials and methods

Cell culture

Human VFFs were isolated from a normal human VF (contralateral, uninvolved VF of a glottic cancer patient who underwent total laryngectomy), as previously described.[12] Human tissue sampling was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-BMR-SMP-17–194). All procedures were conducted on the basis of guidelines and regulations of the Institutional Review Board of Ajou University Hospital. The study participant provided informed consent before participating. Briefly, the cells obtained from the patient were cultured in Dulbecco's Modified Eagle's Medium-high glucose (DMEM-high glucose, GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 1× penicillin-streptomycin (GIBCO), 1× nonessential amino acid (NEAA, Sigma-Aldrich, St. Louis, MO, USA), and 0.05% trypsin-EDTA (GIBCO) under 37°C with 5% CO_2 conditions.

Fluoresce-activated cell sorting analysis

Single cell suspensions prepared by collecting VF-MSC were stained and analyzed within 24 h. Samples were stained with anti-CD29, anti-CD44, anti-CD90, anti-CD45, and anti-HLA-DR antibodies conjugated to fluorescence markers (BD Pharmingen, San Diego, CA, USA). Afterwards, the stained cells were evaluated on a flow cytometer (FACS Calibur, BD Biosciences, San Diego, CA, USA) and analyzed using FlowJo software (Ashland, OR, USA).

Design of liquid-type non-thermal atmospheric pressure plasma (LTP)

The non-thermal plasma system was designed based on previous studies using nitrogen as the carrier gas.[9] The NTP treatment time was 30 and 60 s per mL. As shown in Figure 1, the specifications for the power supply were 7–8 kV and the average frequency was 15 kHz.

Cell viability assay (MTT assay)

The effect of LTP treatment on cell viability was assessed using MTT (3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma-Aldrich, St. Louis, MO, USA) assay.[9] Briefly summarized, human VF-MSCs were cultured in a 96-well culture plates at a density of 5×10^3 cells/well. The medium was treated with LPS (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 5 μg/mL. Additionally, the cells were treated with LTP. The viability of human VF-MSCs was calculated as a standardized percentage compared with control cells.

Scratch wound healing analysis

For wound healing analysis, human VF-MSCs were seeded and cultured on six-well culture plates with a density of 5×10^5 cells/well. Scratch wound healing analysis was performed in the same methods as the previous study.[10] Washing was carried out to remove the fragments of cells that had fallen off after the monolayer was scratched with a sterile blue pipette tip. The remnant cells were treated with LTP and LPS after incubation at 37°C for 24 h, and the cells were evaluated under a microscope. The evaluation of

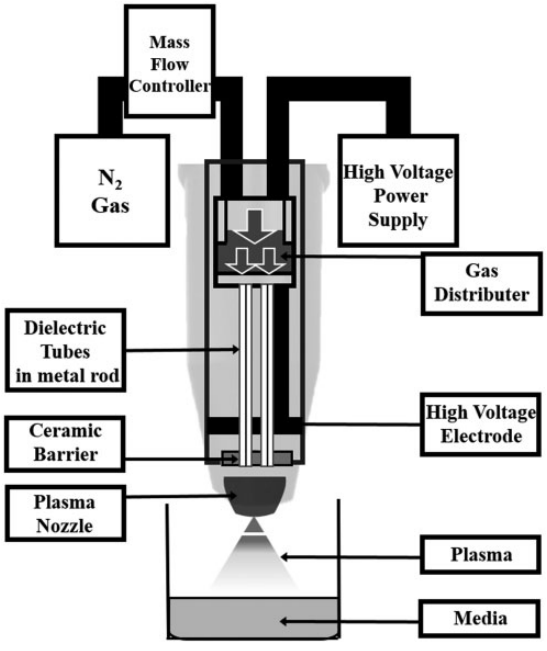

Figure 1. Liquid-type non-thermal atmospheric plasma generation system.

scratch wound healing analysis used the value of mean denude zone. The mean denude zone was calculated by analyzing the denude zone as a percentage of the area where scratches were performed.

Quantitative real-time PCR

In human VF-MSCs treated with LTP and LPS, the total RNA was extracted using TRIzol® reagent (GIBCO). For cDNA synthesis, total RNA (1 μg) was mixed with 10 μL of ReverTrace qPCR RT (Toyobo Co., Ltd., Osaka, Japan) in accordance with the manufacturer's manual. The target genes were analyzed by One-Step Real-Time PCR using StepOnePlusTM (Applied Biosystems, Foster City, CA, USA). Primers used in the experiments were as follows: matrix metalloproteinases-2 (MMP-2) Forward, 5′-TTCAG CTCTGGGATGACCTT-3′, MMP-2 Reverse, 5′- CAAGGTG CTGGCTGAGTAGATC-3′; matrix metalloproteinases-9 (MMP-9) Forward, 5′- TTGACAGCGACAAGAAGTGG-3′, MMP-9 Reverse 5′-GCCATTCACGTCGTCCTTAT-3′; interleukin-6 (IL-6) Forward 5′-CTCTTCAGAACGAATTG ACAAAC-3′, IL-6 Reverse 5′-CAGTGCCTCTTTGCTGCTT T-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Forward 5′-AGGGCTGCTTTTAACTCTGGT-3′, GAPDH Reverse 5′-CCCCACTTGATTTTGGAGGGA-3′.

Western blotting

The VF-MSCs were immersed in RIPA buffer on ice for 30 min. The composition of the RIPA buffer is as follows: 150 mM NaCl, 1.0% Nonidet-P 40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tri (pH 8.0), complete EDTA-free protease inhibitor, and PhoSTOP (Roche Molecular Biochemicals, Basel, Switzerland).[10] At 4°C, centrifugation was performed for 20 min at 14,000 $\times$g to isolate the protein. The proteins were electrophoresed on 10% polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (Pall Corporation, Port Washington, NY, USA). Next, the transferred membrane was blocked with 5% skim milk. Then, primary antibodies against NF-κB, phospho-NF-κB, AKT, phospho-AKT, IκB, phospho-IκB, and GAPDH (1:1000; Cell Signaling Technology, Danvers, MA, USA) were added and incubated overnight. The next day, after washing the membrane, the secondary antibody (anti-rabbit IgG or anti-mouse IgG, 1:2000, Cell Signaling Technology, Danvers, MA, USA) was mixed and then incubated. The results were verified using the ECL Western Blot Kit (GE Healthcare, Little Calfont, UK).

Reverse transcription-PCR

The total RNA of VF-MSCs was extracted in the same method as described above and amplified using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). RNA primers sequences used in the experiments were as follows: ColA1 Forward, 5′-CCTGGATGCCARCAAAGTCT-3′; ColA1 Reverse, 5′-CGCCATACTCGAACTGGAAT-3′; Fibronectin Forward, 5′-CCTGAAGCCTGGTGTGGTAT-3′, Fibronectin Reverse 5′-AGTGGCCACAAGAGGAGAA A-3′; α-SMA Forward 5′-ACTGGGACGACATGGAAAAG-3′, α-SMA Reverse 5′-TACATGGCTGGGACATTGAA-3′; 18s Forward 5′-CACGGACAGGATTGACAGATT-3′, 18s Reverse 5′-CGAATGGGGTTCAACGGGTT-3′.

Animal model and surgical techniques

Fourteen healthy New Zealand white rabbits were used in the experiment and weighed approximately 3 kg. The rabbits were adapted for 7 days at 21 $\pm$ 1°C, 50 $\pm$ 5% humidity, and 12 h brightness cycle conditions. The animals also had free access to food and water. Animal experimental protocols and procedures were approved by the Committee for Ethics in Animal Experiments of the Ajou University School of Medicine (IACUC number: 2017–0023). Anesthesia was performed by intramuscular (i.m.) injection with tiletamine (8.0 mg/kg; Virbac Ltd., Carros, France) and zolazepam (8.0 mg/kg; Virbac Ltd., Carros, France). Fourteen rabbits were divided randomly into two groups of seven rabbits each: (1) LPS (7.5 μL/mL) only group and (2) LPS plus LTP group. We used a 4 mm rigid endoscope (Karl Storz, Tuttlingen, Germany) to observe the VF and made scars consistently in each VF with micro-scissors. After that, applying a 25 gauge long needle, 0.1 mL of LPS and LPS–LTP were injected into the deep layers of the lamina propria of the left VF. The other side of VF was preserved as a control.

Endoscopic and histologic analysis

Visual structural changes and regeneration of VFs were observed using 4 mm rigid endoscope, and observations were conducted six weeks after LTP or LPS-LTP was injected. After conducting an endoscopic examination, all rabbits were sacrificed. The larynx, including VFs, was excised and the specimens were fixed in formalin solution (Sigma-Aldrich, St. Louis, MO, USA) overnight. The prepared paraffin-embedded samples were sectioned continuously at a thickness of 6 μm. Next, the H&E, Masson's trichrome, and Alcian blue stain was performed and then observed and analyzed using a light microscope.

Immunohistochemical analysis

Immunohistochemistry was performed on embedded VF tissue sections. For immunohistochemical staining of collagen type I and type III, the sections were stained with anti-collagen type I (1:500, Abcam, Cambridge, UK) and type III antibody (1:250, Abcam, Cambridge, UK) overnight at room temperature. After washing with PBS three times, the sections were stained with secondary antibody, according to the manufacturer's manual (GBI Labs, Mukilteo, WA, USA). Images were analyzed using a light microscope.

Videokymographic analysis

Videokymographic analysis was performed on the basis of previous study.[13] To summarized briefly, after excision of the larynx with VFs, the supraglottic structure was removed to facilitate observation of VFs. Next, the arytenoid cartilage was sutured to allow both VFs to be closed. The nozzle was connected to the trachea remaining in the larynx, and the VF vibration was induced after the airflow generated by the air flow generator passed through the VFs.

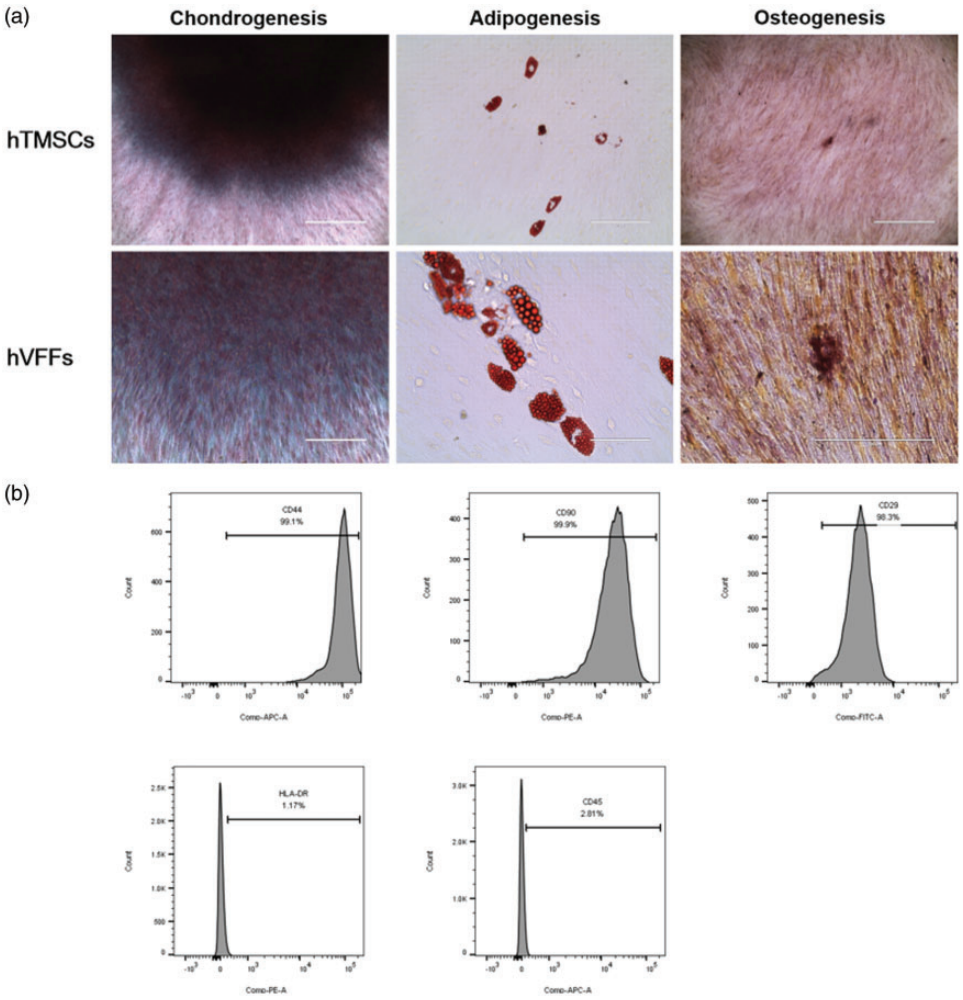

Figure 2. Human VF-MSCs have multipotent differentiation capacity such as MSC. (a) Human VF-MSCs show multi-lineage differentiation potential of chondrogenesis, adipogenesis, and osteogenesis as well as human T-MSCs. The first row represents the differentiation of human T-MSCs and second row represents the differentiation of human VF-MSCs; (b) Human VF-MSCs expressed surface markers such as MSCs. FACS analysis revealed surface markers expressed in human VF-MSCs. The cells were positive for MSC markers, CD-44, CD-90, and CD-29, but negative for the hematopoietic markers, CD-45 and HLA-DR. hVFFs: human vocal fold fibroblasts; hT-MSCs: human tonsil-derived mesenchymal stem cells. (A color version of this figure is available in the online journal.)

Vibration of the VF mucosa was observed and recorded with a high-speed digital imaging system (NX4-S2, Integrated Design Tools, Tallahassee, FL, USA) at 5000 frames per second (fps) under moisturized conditions. All experiments were performed three times, and the results were averaged. The kymograph was produced by Metamorph® NX image software automatically. The results were analyzed by measuring the amplitude of the mucosal vibration from the center of the open phase and the close phase of the VFs on the kymography and compared with the amplitude of the untreated VF mucosa.

Statistical analysis

All experiments were performed three times and analyzed the data. The parameters are expressed as the means ± standard deviations. We performed one-way analysis of variance (ANOVA) following Mann–Whitney U test. All statistical analyses were performed using SPSS 20.0 statistical software (IBM SPSS, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Results

Human VF-MSCs showed multi-potency of differentiation into various tissues

Human VF-MSCs were isolated from normal human VFs (contralateral, uninvolved VF of a glottic cancer patient who underwent total laryngectomy) and cultured to confirm the multi-potency of differentiation. After the sixth passage, the cells were compared with human tonsil-derived mesenchymal stem cells (T-MSCs). After incubation under the same culture conditions as human T-MSCs, human VF-MSCs showed multi-potency differentiation for chondrogenesis, adipogenesis, and osteogenesis (Figure 2(a)). For morphological analysis, cartilaginous ECM was stained red with Safranin O after one week of culture with chondrogenic medium in both groups. The formation of cytoplasmic lipid droplets was detected by Oil Red O staining and the mineralized matrix stained with Alizarin Red S was clearly observed in both groups (Figure 2(a)). Surface markers expressed in human VF-MSCs were identified by fluoresce-activated cell sorting

(FACS) analysis. MSC markers, such as CD-44, CD-90, and CD-29, were expressed in human VF-MSCs, while hematopoietic markers, such as CD-45 and HLA-DR, were not expressed (Figure 2(b)). As a result, human VF-MSCs showed the same multipotent differentiation capacity as human T-MSCs.

LTP does not induce cytotoxicity in VF-MSCs and increases cell viability

To mimic the inflammatory conditions of VF injury, the following experiments were designed to treat with LTP after LPS treatment.[14] First, 30 or 60 s of LTP with/without LPS showed no toxic effects on the viability of human VF-MSCs. No significant cytotoxicity was observed in LPS-only-treated human VF-MSCs. The cell viability of 30 s of LTP treatment on human VF-MSCs was significantly increased in both the LTP-only-treated and LPS plus LTP-treated groups (Figure 3). LTP treatment significantly increased the proliferation of human VF-MSCs under both non- and pro-inflammatory conditions.

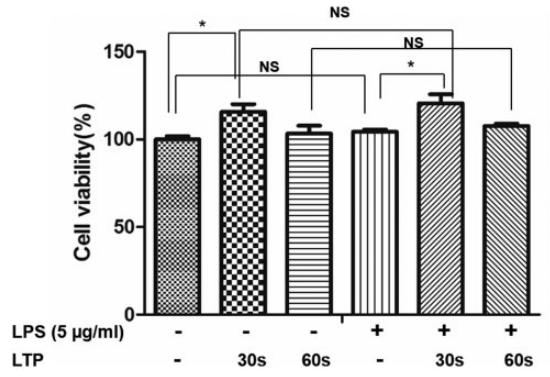

Figure 3. LTP does not induce cytotoxicity in human VF-MSCs and proliferation is increased. Cell viability of human VF-MSCs was confirmed by MTT assay after NTP and LPS treatment. LTP was generated by processing NTP directly on the medium for 30 s and 60 s. Cytotoxicity was not observed after LPS and LTP treatment. Regardless of LPS treatment, viability of the cells increased significantly after LTP treatment (*$P < 0.05$). LTP: Liquid-type non-thermal atmospheric plasma; LPS: lipopolysaccharide.

LTP increases migration of VF-MSCs and upregulates expression of MMP-2/MMP-9

The effects of LTP on the migrations of VF-MSCs were evaluated by the scratch wound healing assay. LTP enhanced the migration abilities of human VF-MSCs, and the decrease in migration due to LPS treatment was successfully restored by LTP treatment (Figure 4(a)). The denuded zone of the scratch area was measured and quantified as shown in Figure 4(b). In both the LTP-only and LPS plus LTP-treated groups, LTP treatment significantly increased the migration of human VF-MSCs compared to that of the control group (Figure 4(b)).

We next determined the activity of MMP-2/MMP-9 in VF-MSCs treated with LPS with or without LTP by real-time PCR. MMP-2/MMP-9 play important function in wound healing and induce cell migration. MMP-2 and MMP-9 were generally increased during LPS and LTP treatment, and the increase in MMP-2 by LTP treatment was significant ($P < 0.05$, Figure 4(c)). In conclusion, LTP increased the mobilization of LPS-treated human VF-MSCs, which is associated with activation of MMP-2/MMP-9.

LTP reduces fibrosis-related molecules and inhibits transformation of VF-MSCs into myofibroblasts

NF-κB plays important roles in regulating inflammatory mediators.[15] NF-κB is expressed in fibroblasts stimulated by LPS and is involved in the transformation of fibroblasts into myofibroblasts, leading to fibrosis.[16] NF-κB is activated by pro-inflammatory cytokines, such as IL-6, which play a pro-fibrotic role and are regulated by the phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K)/AKT pathway. As shown in Figure 5(a), increased NF-κB phosphorylation by the LPS-induced inflammatory reaction was successfully reduced by LTP treatment in human VF-MSCs. The expression of phospho-AKT following LPS treatment was also decreased by LTP treatment (Figure 5(a)). The increased mRNA level of IL-6 by LPS treatment was significantly reduced by LTP treatment (Figure 5(b)).

Once transformed into myofibroblasts, VF-MSCs are involved in VF scarring by the accumulation of irregularly arranged collagen bundles. This process is typically induced by transforming growth factor β1 (TGF-β1).[17] Reverse transcription-PCR showed that expression of α-SMA and fibronectin, which are markers of myofibroblasts, increased on TGF-β1 treatment in human VF-MSCs and successfully decreased on LTP treatment (Figure 5(c)). In contrast, the expression of ColA1, another myofibroblast marker, decreased slightly. Taken together, LTP may reduce VF scarring by reducing fibrosis and inflammation in human VF-MSCs.

LTP modulates ECM deposition of VF mucosa and promotes recovery of VF function *in vivo*

Animal experiments were performed to evaluate the effect of LTP in VF regeneration *in vivo*. With micro-scissors, a full-thickness wound was made on the left side of the rabbit VF under endoscopic guidance (Figure 6(a)). Next, LPS was injected at the same site, and the experimental group was further injected with LTP, while the control group remained untreated (Figure 6(b)). After six weeks of treatment, VFs from both groups were examined by endoscopy to evaluate structural changes and regeneration at the injected sites after anesthetization. After endoscopic analysis, the animals were sacrificed. The irregular margin and fibrotic scarring of VFs were observed in the LPS-only injected group (Figure 6(c)), while less prominent scarring was observed in the LTP-treated group (Figure 6(d)).

Hematoxylin and eosin (H&E) staining revealed irregularly arranged lamina propria of VFs in the LPS-only treated group compared to that in the LTP-treated group (Figure 7(a), upper row). In Masson's trichrome staining, collagen is stained dark blue. Denser collagen deposition was detected in the LPS-only treated groups than that in the LTP-treated group. In the LTP group, collagen deposition

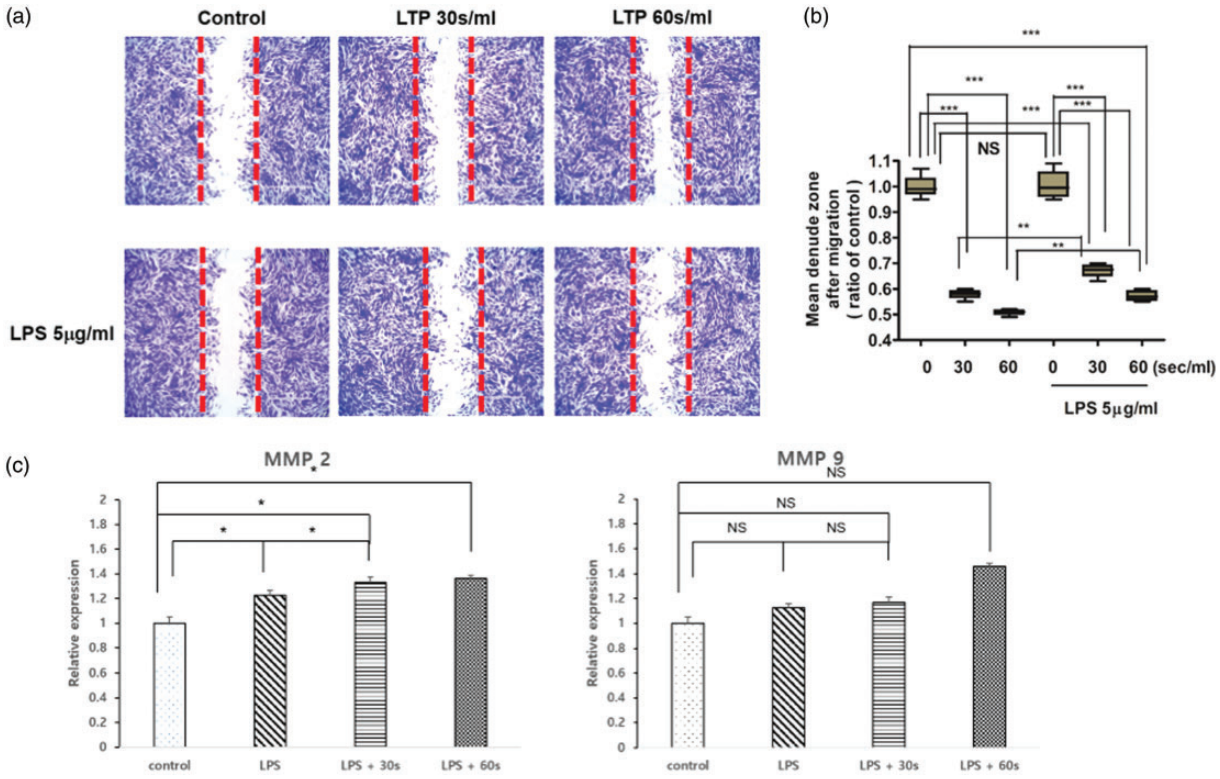

Figure 4. LTP increases migration of human VF-MSCs and upregulates expression of MMP-2/MMP-9. (a) LTP increased human VF-MSCs migration. Human VF-MSCs were treated LTP with or without LPS. Human VF-MSCs were cultured in six-well plates and performed to scratch wound healing assay; (b) The degree of migration was quantified as the mean denuded zone. Denuded zone values were calculated as the ratio of the average area of the denuded zone to the scratch area in the control group. After LTP treatment, the area of the denuded zone decreased significantly (**$P < 0.01$; ***$P < 0.001$); (c) LTP increased mRNA expression of MMP-2/MMP-9. MMP-2/MMP-9 mRNA level was confirmed by real-time PCR. MMP-2 expression alone was significantly increased (*$P < 0.05$). LTP: Liquid-type non-thermal atmospheric plasma; LPS: lipopolysaccharide. (A color version of this figure is available in the online journal.)

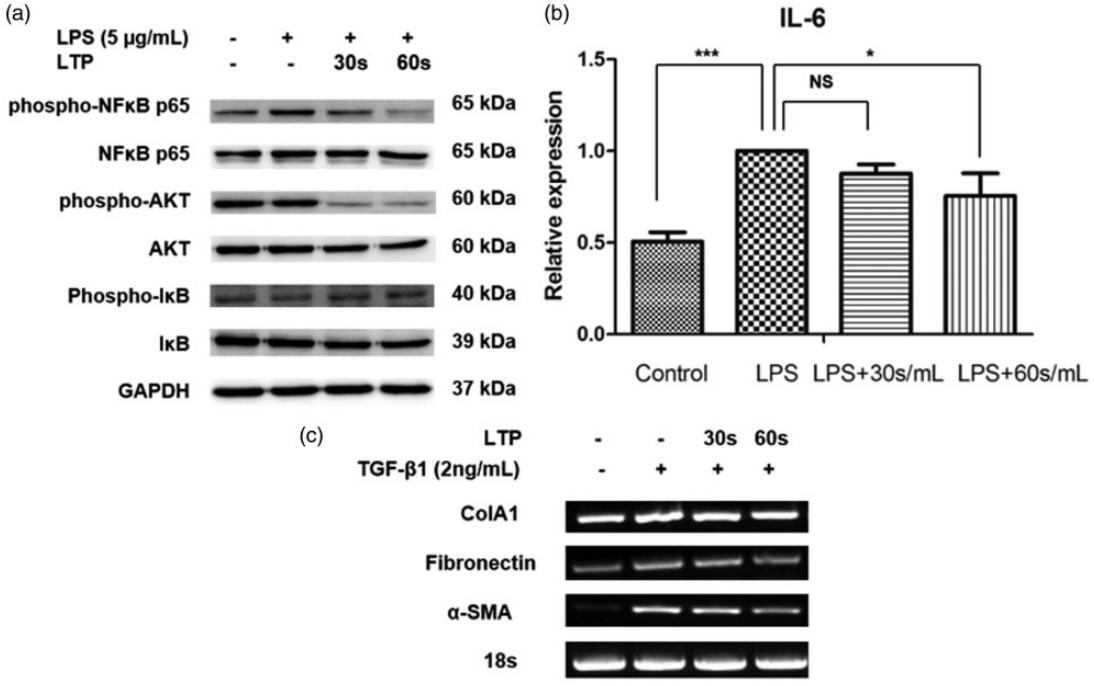

Figure 5. LTP reduces fibrosis-related molecules and inhibits transformation of human VF-MSCs into myofibroblasts. (a) Expression of fibrosis related proteins was evaluated by Western blotting. The amount of protein expression of phospho-NF-κB and phospho-AKT increases with LPS. Increased expression was reduced by LTP over treatment time; (b) LTP decreased the relative expression of IL-6 over treatment time (*$P < 0.05$; ***$P < 0.001$); (c) ColA1, fibronectin and α-SMA mRNA levels were measured by reverse transcription PCR. Fibronectin and α-SMA, which showed increased expression after LPS treatment, decreased after LTP treatment. LTP: Liquid-type non-thermal atmospheric plasma; LPS: lipopolysaccharide.

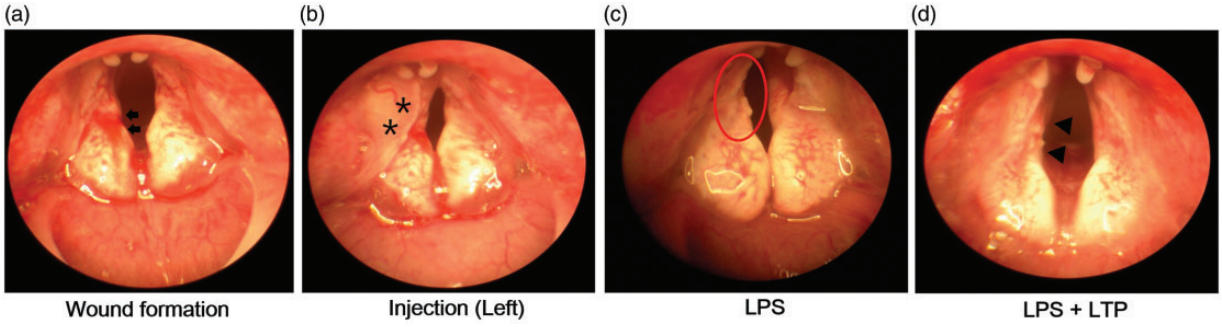

Figure 6. Endoscopic view of an experimental rabbit VFs. (a) A micro-scissor was used to create a wound on the left VF, and VF mucosa was congested compared to the mucosa of the control (black arrows); (b) After injection of the material, the lamina propria swelled (asterisks); (c) (D) VF mucosa edge was irregularly regenerated after six weeks of LPS-alone treatment compared to that in the control (red circle). The VF mucosa edge treated with LTP was irregular than that of the control (black arrowheads). LTP: Liquid-type non-thermal atmospheric plasma; LPS: lipopolysaccharide. (A color version of this figure is available in the online journal.)

was similar as in the undamaged left VFs (Figure 7(a), mid row). In Alcian blue staining, blue staining indicates hyaluronic acid (HA) deposition. HA deposition was greater in the LTP-treated group than in the LPS-only treated group (Figure 7(a), lower row).

In the LPS-only treated group, the deposition of densely tangled collagen type I was observed in the lamina propria, whereas in the LTP-treated group, deposition was decreased and showed to be linear tendency (Figure 7(b), upper row). The deposition of collagen type III in the lamina propria did not differ between groups, but collagen alignment was better in the LTP-treated group than in the LPS-only-treated group (Figure 7(b), lower row).

Videokymographic analysis was performed to determine the degree of functional recovery of the regenerated VF mucosa. In the LPS-only group, the amplitudes of the VF mucosal wave were severely decreased compared to those of the contralateral undamaged normal VF mucosa, while the amplitudes of LTP-treated VFs were similar to those of the normal VF (Figure 8(a)). These results indicate that the stiffness of the VF mucosa was increased by VF scarring, and LTP treatment ameliorated VF scarring. The left (L)/right (R) ratio was measured by kymography. The L/R ratio was significantly higher in LTP-treated VFs than in LPS-only VFs (Figure 8(b)).

Discussion

MSCs are pluripotent cells that are rarely found *in vivo*.[18] Many studies worldwide have examined the potential therapeutic effects of MSCs in various fields over the past three decades.[19] Currently, the consensus standards developed to define MSCs are as follows. MSCs are adherent under standard culture conditions and specific surface antigens must be expressed. MSCs also have multipotent differentiation capabilities.[19,20] Therefore, MSCs can be transformed into cells to form bone, cartilage, muscle, fat, and other connective tissues according to the stimulus or culture conditions.[21–23] These MSCs were originally isolated from the bone marrow but have recently been isolated from most tissues of the body, such as fat, bone, and muscle. The MSCs isolated from these various tissues have similar biological characteristics, differentiation potential, and immunological properties.[24] Similarly, hVFFs can be isolated from the lamina propria, which is a connective tissue layer composed of VFs, and have also been reported to have characteristics of hMSCs including regenerative potential.[25] In this study, we confirmed the multipotent and regenerative nature of human VF-MSCs, such as hMSCs.

Voice disorders are a common disease that affects 3–9% of Americans each year.[25] These voice disorders have a serious deteriorating impacts on the patient's life quality.[25] The most common cause of voice disorders is VF scarring after VF injury such as surgery, infection, inflammation, or trauma, resulting in deformation of the VF edge, thinning of the viscoelastic layer of the lamina propria, increasing stiffness of the vibration structure and eventual glottic incompetence.[25] Although VF scarring is an important problem, a definite treatment for it has not yet been established. Therefore, various treatment methods have been studied to improve the composition of the ECM, which is the main cause of VF scarring. Among these treatments, injection of various possible therapeutic agents by injection laryngoplasty has been actively tried and implemented.[1] However, no material has shown satisfactory clinical outcomes. Therefore, many studies on the therapeutic effect of MSCs have been conducted, and the application of VF treatment using MSCs extracted from other tissues or embryonic stem cells has been examined.[26,27] However, the therapeutic effects of MSCs extracted from other sites are controversial, as gene expression signatures may differ.[25] MSCs can be extracted from various tissues in the human body. In addition, MSCs migrate to damaged tissues, where they can interfere the release of pro-inflammatory cytokines. VFFs are known to have properties of these MSCs.[6] However, few studies have been performed to stimulate favorable VF mucosa regeneration by modulating such multipotent fibroblasts present in VFs. Previous studies showed that LTP plays an effective role in epidermal wound healing and muscle regeneration through several mechanisms.[8,9] Therefore, we predicted that LTP can recruit and activate human VF-MSCs, leading to favorable ECM restoration and eventual VF regeneration.

In this study, LTP significantly increased the migration of LPS-treated VF-MSCs by activating MMP-2. NF-κB and

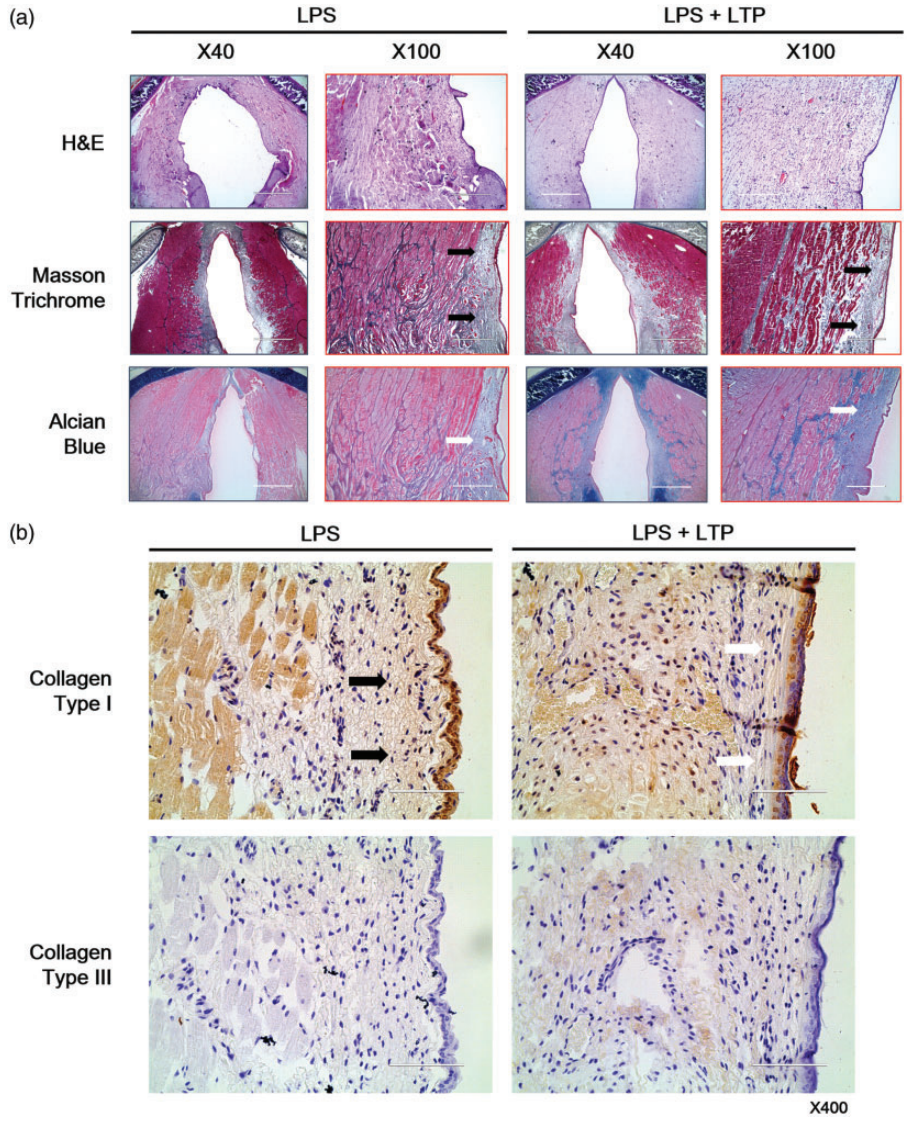

Figure 7. LTP modulates ECM deposition. (a) H&E staining of VFs after LPS-only and LPS plus LTP-treatment. LTP was generated by processing NTP directly on the medium for 60 s. The lamina propria of VF mucosa in the LTP-treated group showed a uniform pattern compared to LPS-only treated group (upper row). In Masson's trichrome staining, collagen was stained as dark blue. Collagen deposition increased in the LTP-treated group compared to that in the control, but the degree of deposition was less than that in the LPS-only treated group (mid row, black arrows). In Alcian blue staining, blue stain indicates HA. HA deposition increased in the LTP-treated group (lower row, white arrows); (b) Immunohistochemical analysis results of collagen types I and III. The degree of deposition was similar, but the arrangement of collagen deposits in the LTP-treated group was uniform (white arrows) compared to LPS-only treated group (black arrows). LTP: Liquid-type non-thermal atmospheric plasma; LPS: lipopolysaccharide. (A color version of this figure is available in the online journal.)

IL-6 activation by the LPS-induced inflammatory response was successfully decreased by LTP treatment. α-SMA and fibronectin expression by TGF-β-induced fibrosis was also ameliorated by LTP treatment. Injection of LTP in a rabbit VF scarring animal model improved ECM restoration according to histological analysis and mucosal vibration according to functional analysis.

The migration of VF-MSCs was significantly increased by LTP treatment without specific cytotoxicity. Increased cell mobility during wound healing is an important process for effective regeneration. MMP has the function of degrading and metabolizing the aggregation of ECM components such as collagen, fibronectin, elastin, and cellular debris after wound formation.[28,29] Thus, MMP is a protease that plays an important factor in tissue remodeling and cell migration by modifying the ECM.[30] LTP increased the expression of these MMPs. As a result, when VF-MSCs were treated with LTP, cell mobilization was increased possibly by increasing the expression of MMP, which may have increased cell recruitment. Further studies on the influence of migration pathways, such as cell adhesion molecules, are needed to determine the precise mechanisms.

When injury occurs in the VFs, fibroblasts and myofibroblasts become aggregated. ECM components are then synthesized, and the microenvironment is reconstituted, resulting in the healing process.[31] These fibroblasts and myofibroblasts differentiate in human VF-MSCs according to signals.[2] Deformation of the ECM by myofibroblasts and excessive accumulation of collagen increases the stiffness of VFs, resulting in chronic voice disorder.[32] LTP reduced the

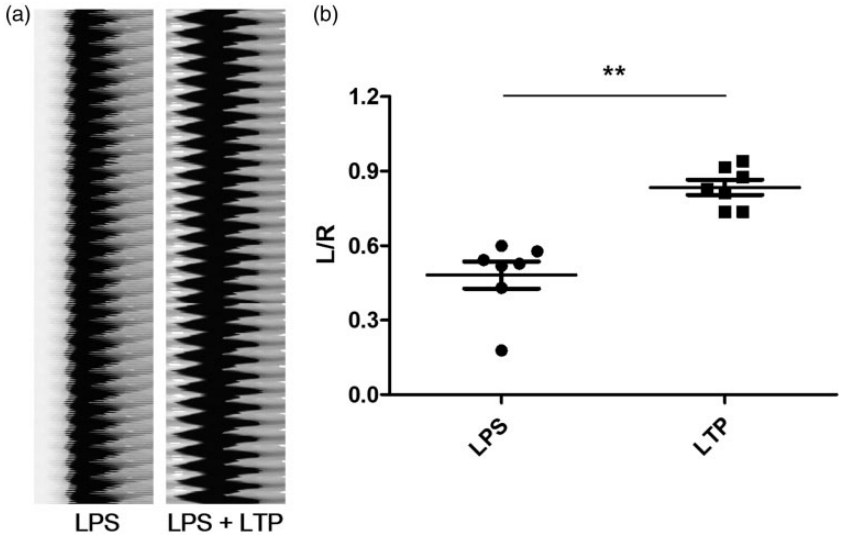

Figure 8. LTP promotes recovery of VFs function. (a) VF recovery was assessed by measurement of mucosal wave. Representative kymographic images are presented from the LPS-only and LPS plus LTP-treated group; (b) The amplitude ratios of mucosal waves in treated VFs relative to normal contralateral VFs were determined. Mucosal regeneration was effective when the L/R ratio was close to 1. In the LPS plus LTP-treated group, the mucosal wave was significantly similar to the opposite side (**$P < 0.001$). LTP: Liquid-type non-thermal atmospheric plasma; LPS: lipopolysaccharide.

expression of cytokines and proteins associated with LPS-induced fibrosis, such as IL-6 and NF-κB, as well as decreased VF scarring by reducing the differentiation of VF-MSCs into myofibroblasts. It is challenging to identify pathways that inhibit NF-κB-related signaling induced by LTP and block the differentiation of fibroblasts into myofibroblasts.

In vitro experiments demonstrated the favorable effects of LTP on the mobilization and recruitment of VF-MSCs. To confirm the effect of mobilization and cell recruitment, we performed *in vivo* experiments for six weeks and confirmed the significant results of promoting favorable VF mucosa healing. Furthermore, immunohistochemical analysis and videokymography confirmed that LTP treatment led to favorable ECM remodeling and eventual functional improvement represented by the improved vibration of VFs.

A lot of tissue engineering studies on VF regeneration are currently underway. These studies have revealed the microstructure and physiology of VFs.[27] The potential endogenous cell sources for VF regeneration include side population cells, stellate cells, and VFFs.[27] Especially, as already known through other studies, side population cells are considered to contain a large numbers of stem cells. The presence of side population cells in the VFs was reported in 2007 by Yamashita *et al*.[33] Therefore, side population cells play an important function in early regeneration of VF wound.[34] One limitation of this study is that the effect of LTP on VFF was evaluated among several endogenous cells involved in VF regeneration. Therefore, further experiments will be needed to evaluate the effect of LTP on the various cells involved in regeneration, such as side population cells, and it will be necessary to analyze the integrated mechanism involved in VF regeneration.

LTP increased *in vitro* VF-MSC migration and reduced inflammation and fibrosis. In a rabbit animal model, LTP improved *in vivo* VF scarring by mobilizing and activating VF-MSCs, leading to favorable ECM restoration. This suggests that LTP is a potential therapeutic agent for promoting VF scarring through simple injection using the characteristics of the solution at the time of injury. Thus, LTP may be an alternative therapeutic agent for the current situation to treat voice disorders, for which there is no definite treatment.

Authors' contributions: HRW analyzed data and wrote the paper; SUK performed research and analyzed data; EHS performed research and analyzed data; JEW performed research. HYL performed research and analyzed data. YSS analyzed data and wrote the manuscript. CHK planned and led this research. YSS and CHK contributed equally to this paper as corresponding author. All authors reviewed the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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ORCID iD

Yoo Seob Shin https://orcid.org/0000-0002-2007-1100

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