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# Low temperature plasma induces angiogenic growth factor via up-regulating hypoxia–inducible factor 1α in human dermal fibroblasts

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ABSTRACT

Numerous studies on the application of low temperature plasma (LTP) have produced impressive results, including antimicrobial, antitumor, and wound healing effects. Although LTP research has branched out to include medical applications, the detailed effects and working mechanisms of LTP on wound healing have not been fully investigated. Here, we investigated the potential effect of inducing growth factor after exposure to LTP and demonstrated the increased expression of angiogenic growth factor mediated by LTP-induced HIF1α expression in primary cultured human dermal fibroblasts. In cell viability assays, fibroblast viability was reduced 6 h and 24 h after LTP treatment for only 5 min, and pre-treating with NAC, a ROS scavenger, prevented cell loss. Fibroblast migration significantly increased at 6 h and 24 h in scratch wound healing assays, the expression of cytokines significantly changed, and regulatory growth factors were induced at 6 h and 24 h after exposure to LTP in RT-PCR or ELISAs. Specifically, LTP treatment significantly induced the expression of HIF1α, an upstream regulator of angiogenesis. Pre-treatment with the inhibitor CAY10585 abolished HIF1α expression and prevented LTP-induced angiogenic growth factor production according to immunoblotting, immunocytochemistry, and ELISA results. Taken together, our results provide information on the molecular mechanism by which LTP application may promote angiogenesis and will aid in developing methods to improve wound healing.



## 1. Introduction

Dielectric barrier discharge (DBD) devices generate low temperature plasma (LTP), which is defined as a partly ionized gas that is produced by electric discharges under atmospheric pressure at room temperature. These LTPs are “cold” because the carrier gas, such as helium, argon, and air, is partially ionized, and therefore, the ions cool down very rapidly [1]. The plasma consists of ions, charged particles, an electric field, and reactive oxygen and nitrogen species (ROS/RNS), which make it biologically active. The direct DBD plasma generates $H_2O_2$, which reduces human fibroblast proliferation and differentiation but does not induce toxicity [2], and the generated nitric oxide (NO) increases mRNA expression of transforming growth factor β (TGF-β) and vascular endothelial growth factor (VEGF) [3]. The indirect plasma irradiation method uses plasma-activated cell-free medium (PAM), which contains approximately 50 μM $H_2O_2$, the main active species in the medium. Under these conditions, activation of the Nrf2 pathway/phase **II** enzymes, such as heme oxygenase 1 (HO-1), protects fibroblasts from ROS [4].

The safety of LTP has been investigated using cell survival assays and by assessing DNA repair capacity in human skin fibroblasts with a μs-pulsed DBD source, and the results indicate that DBD devices are safe for use in therapeutic applications [5]. Low dose

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LTP treatments are not toxic to cells and instead induce proliferation [3,6,7], whereas treatment for longer exposure times has been shown to lead to cell loss [6]. Moreover, LTP treatment was shown to not result in any noticeable side effects or the concomitant activation of pro-inflammatory signaling using gene expression analysis in an in vivo rat skin acute wound model [8].

Cutaneous wound repair is regulated by numerous growth factors, cytokines, and chemokines [9]. In this regard, indeed, the application of LTP supports these requirements during wound repair. LTP applications have been mainly focused on dermatology, such as promoting tissue regeneration and improving infected and inflamed skin diseases [1,5]. Previous studies have demonstrated beneficial effects of LTP on wound healing in vitro and vivo. LTP treatment enhances fibroblast migration, resulting in the closure of gaps in scratch wound healing assays [7,10–12]. Furthermore, after exposure to LTP, fibroblast growth factor-2 (FGF-2), type I collagen (12), fibroblast growth factor-7 (FGF-7) [7], and transforming growth factor-β (TGF-β) [3] increased in fibroblasts, but in HaCaT keratinocytes, there was decreased expression of the gap junction protein connexin 43 (Cx 43), which is important in the regulating wound repair and altered cytoskeletal dynamics [10]. Very recently, treatment of fibroblasts with LTP was shown to induce the epithelial to mesenchymal transition (EMT), which was accompanied by increased slug and TCF8/ZEB1 expression and decreased E-cadherin. This resulted in activating the matrix metalloproteinase (MMP)-9 and urokinase-type plasminogen activator (uPA) system, which degraded various components of the extracellular matrix (ECM), suggesting that LTP treatment is well balanced without resulting in unwanted side effects such as excess scar formation [11]. Animal studies have reported accelerated wound healing by promoting inflammation, re-epithelialization, and contraction [8,10,13].

Fibroblasts are normally present in wounds, from the late inflammatory stage until complete epithelialization has occurred. An important step in the wound healing process is the migration of fibroblasts into the wound bed, where they break down the blood clot, produce collagen and a new extracellular matrix structure for support, and communicate with other cells involved in effective wound healing [14].

Despite the advances in plasma research, many questions remain regarding the effects on cellular physiology and the mechanisms of action of LTP in mammalian cells and tissues during wound healing. Therefore, it is important to elucidate the molecular changes and related mechanisms that correspond to the observed effects of LPT applications. In this present study, the effects of LTP on wound healing and the molecular changes after LTP treatment were investigated in vitro using primary human dermal fibroblasts. Furthermore, we demonstrated that LTP induced the expression of angiogenic growth factor mediated by HIF1α.

## 2. Materials and methods

### 2.1. Primary human dermal fibroblast culture

All cell culture procedures were performed at a clean bench. Human skin biopsies were obtained from the tissue biobank at Hangang Sacred Heart Hospital. Samples were washed with 70% ethanol three times and then placed in cold PBS containing antibiotics and antimycotics (Gibco, Life Technologies, USA). The subcutaneous fat and loose connective tissues were removed using fine tweezers and a scalpel. The tissues were cut into strips that were approximately 3–4 mm in width, and they were then transferred to 50 ml conical tubes containing 10 ml dispase II (1 unit/ml) (Gibco, Life Technologies, USA) solution and kept at 4 °C for 16 h. After digestion, the dermis and epidermis were pulled/peeled using a pair of sterile forceps. The separated dermis was digested with collagenase type IV solution (500 U/ml) at 37 °C for 30 min (Gibco, Life Technologies, USA). The samples were then placed in DMEM containing 10% FBS to inactivate the collagenase, filtered, and centrifuged at 300 × *g* for 5 min. The pellet was resuspended in DMEM with 10% FBS, followed by culture at 37 °C in 5% $CO_2$. Fibroblasts at passage 2–4 were used for all experiments.

### 2.2. LTP device

The LTP system was similar to that previously described [15]. For this study, dielectric barrier discharge remote LTP was used, and the LTP was ejected through two nozzles (20 mm × 1 mm). A secondary ground electrode was placed in the nozzle area to prevent possible arcing, and a tube-like structure was created with a length of about 15 cm. A 5.99-kV sinusoidal voltage with a frequency of 13.0 kHz was applied. The working gas for LTP generation was a mixture of air (50 ccm) and He (5000 ccm). Electric power as measured with the Lissajou figure method was 42 W. The generation temperature was 28 ± 2 °C.

### 2.3. LTP treatment

Fibroblasts were seeded at a density of $1 \times 10^4$ cells per 35 mm Petri dish (Corning, NY. USA). Immediately before LTP exposure, the DMEM was removed, and the cells were covered with 1.2 ml DPBS. The LTP torch was placed at a distance of 3 cm from the Petri dish. The cells were exposed to LTP for 30 s, 1 min, 3 min, or 5 min, after which 2.0 ml of medium was added. Assays were conducted 6 h and/or 24 h after the LTP treatment.

### 2.4. Cell viability assay

Fibroblast viability was assessed using the EZ-Cytox Cell viability assay kit (Dogen, Seoul. Korea). The 96-well cell culture plates were seeded with $5 \times 10^3$ fibroblasts per well (Corning, NY. USA). For one group, 1 h before LTP treatment, 10 mM N-acetyl-L-cysteine (NAC) was added. Immediately before LTP treatment, the DMEM containing 10% FBS was removed, and 100 μl DPBS was added to each well. After LTP treatment for 30 s, 1 min, 3 min, or 5 min, the cells were cultured in 200 μl DMEM, and 6 h or 24 h latter, 10 μl of EZ-Cytox reagent was added to the medium followed by incubation for 1 h at 37 °C. The absorbance measured at 450 nm using a microplate reader (Beckman Coulter 880, USA). The final values were calculated as follows: (sample absorbance − background absorbance = original signal→(original absorbance/control absorbance) × 100 = viability%).

### 2.5. Wound healing assay

Fibroblast migration was assessed with wound healing assays using a culture insert in a 35 mm u-dish (Ibidi GmbH, Germany) according to the manufacturer's instructions. Fibroblasts were seeded at $5 \times 10^3$ cells per culture insert dish. After 24 h, the culture insert was removed, and a cell-free gap or defined wound of 500 ± 50 μl was made. In order to eliminate the impact of cell proliferation during migration, mitomycin C (5 μg/ml, Sigma, USA) was added to the cell culture media [16]. The cells that migrated into the wound area were measured 6 h and 24 h after LTP treatment using a light microscope (IX 70, Olympus, Japan). The untreated fibroblast control was set to 100% and compared with cells treated with LTP for 30 s, 1 min, 3 min, or 5 min. Each analysis was performed in triplicate.