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# **Cold helium plasma jet does not stimulate collagen remodeling in a 3D human dermal substitute**

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**KEY WORDS:** extracellular matrix, metalloproteinases MMPs, oxidative stress, cold atmospheric plasma (CAP), plasma-activated medium (PAM), skin, wound healing.

**ABBREVIATIONS:** CAP, cold atmospheric plasma; ECM, extracellular matrix; MMP, matrix metalloproteinase; PAM, plasma-activated medium; RONS, reactive oxygen and nitrogen species.

## **ABSTRACT**

Cold Atmospheric Plasma (CAP) is an emerging physical approach displaying encouraging antitumor and wound healing effects both *in vitro* and *in vivo*. In this study, we assessed the potential of direct CAP to remodel skin collagens using an original tissue-engineered human dermal substitute model rich in endogenous extracellular matrix (ECM) covered with 600  $\mu$ l of culture medium and treated with CAP for 30 and 120 seconds. Our results indicated that Reactive Oxygen and Nitrogen Species such as  $H_2O_2$ ,  $NO_3^-$  and  $NO_2^-$  were produced in the medium during treatment. It appeared that in the CAP-treated dermal substitutes 1) cell viability was not altered, 2) pro-collagen I secretion was not modified over 48 h of culture after treatment, 3) global activity of matrix metalloproteinases was not modulated over 48 h after treatment, and 4) no change in hydroxyproline content was observed over 5 days after treatment. In order to confirm the efficiency of our device, we showed that the plasma-activated culture medium induced cell apoptosis and growth delay using a 3D human tumor spheroid model. In conclusion, no effect of direct CAP treatment was monitored on ECM production and degradation, indicating that CAP does not stimulate collagen remodeling at the tissue scale.

## INTRODUCTION

Cold Atmospheric Plasmas (CAPs) are generated by coupling energy from either electric currents, radiofrequencies or microwaves to a gas at atmospheric pressure, resulting in a partially ionized gas. Two main devices are used to produce CAPs: plasma torches and dielectric barrier discharge devices, including rare gas plasma jets [1,2]. CAPs are a source of UV-Vis emission, ions, electrons, radicals and excited atoms and molecules. These species may interact with the ambient air and produce high levels of reduction/oxidation-based molecules [2], particularly reactive oxygen and nitrogen species (RONS) such as nitric oxide ( $^{\circ}\text{NO}$ ), nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) ions, hydroxyl radical ( $^{\circ}\text{OH}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Since RONS are known to be involved in a variety of physiological functions and homeostatic tissue regulation in human [3], and CAPs can be generated at low temperatures not higher than  $40\text{ }^{\circ}\text{C}$ , CAPs raise a growing interest for biomedical applications [4]. Direct exposure to CAP as well as to plasma-activated medium (PAM) is highly effective to inactivate various pathogens, including drug-resistant microorganisms; therefore it is used for sterilization and biological decontamination [5–7]. Other potential CAP and PAM biomedical applications include surface modification of medical implants, management of dental carries and composite restoration, improvement of blood coagulation, treatment of various skin conditions and killing of cancer cells [8] while minimally damaging healthy surrounding cells [9–11].

The selective cytotoxic effect of CAP treatment may be partly explained by the overproduction of RONS in various tumor cell lines compared to their normal counterparts, ensuring a greater sensitivity to the oxidative stress generated by the plasma. This property has been exploited for the specific destruction of tumor cells in breast, cervical, colorectal, lung, pancreatic, prostate, and skin cancers [3,9,10,12,13]. It should be noted that a recent and extensive study on more than 38 healthy and cancerous cell lines studying expression of 33 surface markers demonstrated that the best predictor of sensitivity to plasma treatment is the basal level of metabolic activity [14].

An important potential application of CAP relies on its capacity, in well-defined *in vitro* conditions, to increase proliferation and motility of both cutaneous fibroblasts and keratinocytes [15–19], to promote the production of wound healing-relevant growth factors and cytokines [1,16], and to activate angiogenesis [17]. These effects subsequently lead to stimulation of wound healing and tissue regeneration [1,20–22].

The dermis extracellular matrix (ECM), a complex network of proteins including collagens, proteoglycans, elastin and fibronectin, plays an important role during the wound repair process. ECM ensures a structural bioactive role, providing mechanical support,

activation and modulation of cytokines and growth factor's action, and stimulation of the cells adhesion and migration in the wound bed [23]. It is known that RONS induce ECM remodeling [24,25] and modulate the communication between cells and their surrounding ECM [26]. Since RONS are considered the major mediators of the biological effects of CAPs [3], it has been hypothesized that CAP exposure may induce local RONS-mediated ECM remodeling [26,27].

In the present study, we propose to assess the potential of cold atmospheric helium plasma jet to directly remodel collagens, using a 3D tissue-engineered human reconstructed dermis model [28,29]. This original model offers multiple layers of primary dermal fibroblasts embedded within their endogenous, collagen-rich ECM. We analyzed remodeling of collagens after CAP treatment, through quantification of both pro-collagen I secretion and global collagen content. We also quantified global metalloproteinases (MMPs) activity and MMP-1 content, main actors in maintenance and remodeling of the ECM [30].

## **MATERIAL & METHODS**

**Plasma jet device.** The cold atmospheric helium plasma jet was generated by means of a dielectric barrier discharge homemade device, as described elsewhere [31,32]. Briefly, two aluminum electrodes were wrapped around a quartz tube and connected to a high-voltage monopolar square pulse generator. The characteristics of the applied power supply were fixed at 10 kV voltage, 10 kHz frequency and 1  $\mu$ s pulse duration. Helium gas is delivered through the quartz tube at a controlled flow rate of 3 L/min.

**Cell culture medium exposition to cold atmospheric plasma.** Dulbecco's Modified Eagles Medium (DMEM) without pyruvate (Gibco #41965-039, ThermoFisher scientific, MA, USA) supplemented with 10% of heat inactivated fetal calf serum (Gibco #10270106), 100 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin (Gibco #15140122) was exposed to the helium plasma jet. For that purpose, one well of a 24-well plate containing 600 $\mu$ l of cell culture medium was used and the output of the plasma jet was placed at a distance of 2 cm from the liquid surface. Two different durations of exposition to plasma jet were used: 30 and 120 seconds. Two minutes helium exposure and no exposure were settled as control conditions.

**Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) quantification.** H<sub>2</sub>O<sub>2</sub> concentration in cell culture medium was quantified immediately after exposure to the plasma using Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, #A22188, ThermoFisher scientific) according to manufacturer's protocols. Absorbance at 560 nm was read using a plate reader (Biochrom Asys UVM340, Biochrom Ltd, Cambridge, United Kingdom).

**Nitrite/nitrate (NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>) ions quantification.** NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> ion concentrations in cell culture medium were quantified immediately after plasma jet exposure using a colorimetric Nitrite/Nitrate Assay kit (Sigma#23479, Merck KGaA, Darmstadt, Germany) according to manufacturer's protocols. Absorbance at 540 nm was read using a plate reader (Biochrom Asys UVM340).

**Cell culture.** Primary human dermal fibroblasts previously isolated from a skin biopsy provided by Icelltis (Toulouse, France) were grown in DMEM GlutaMAX (Gibco #10569010) containing 4.5 g/L glucose, 110 mg/L of sodium pyruvate, supplemented with 10% of heat inactivated fetal calf serum (Gibco #10270106), 100 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin (Gibco #15140122), at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere. The medium was renewed 3 times a week. For 3D tumor spheroid experiments, human colorectal carcinoma cell line HCT-116 (ATCC) was cultivated in DMEM without pyruvate (Gibco #41965-039) supplemented with 10% of heat inactivated fetal calf serum (Gibco #10270106), 100 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin (Gibco #15140122) at 37°C and 5% CO<sub>2</sub>

in a humidified atmosphere. The medium was renewed 3 times a week. Cells were regularly tested (every two weeks) for mycoplasma contamination using the MycoAlert Mycoplasma detection kit (Lonza #LT07-118, Basel, Switzerland).

**Reconstruction of a human 3D dermal substitute.** Human dermal tissues were generated following the tissue-engineering self-assembly approach as previously described [33,34]. Cells were cultivated four weeks in cell culture medium supplemented with 50 µg/ml of ascorbic acid (Sigma #A4034). Three days before experiments, cell medium was replaced by DMEM without pyruvate (Gibco #41965-039) supplemented with 10% of heat inactivated fetal calf serum (Gibco #10270106), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Gibco #15140122).

**Tissue exposure to CAP.** Four-weeks-old dermal substitutes in 24-well plates were exposed to the plasma jet for either 30 s or 2 min in presence of 600 µl of fresh complete (10% fetal calf serum + antibiotics) DMEM without pyruvate (Gibco #41965-039). Exposure during 2 min to helium gas at 3 L/min alone and no exposure were settled as control conditions. Evaporation of 20 µl of cell culture medium was quantified (3% of total volume) after 2 min exposure to either plasma or helium alone (data not shown). This volume loss was not compensated during experiments. The distance between plasma jet tube output and samples was set and fixed at 2 cm for every experimental conditions.

**Quantification of tissue hydroxyproline content.** Total hydroxyproline content of dermal substitutes was measured up to five days after cold plasma exposure using the Hydroxyproline Assay Kit (Sigma #MAK008) according to the manufacturer's protocol. The absorbance was read on plate reader Synergy H1 (BioTek, Winooski, VT, USA).

**Quantification of metalloproteinases activity.** Quantification of global MMPs activity in the supernatants was carried out using a SensoLyte 520 Generic MMP Assay Kit \*fluorimetric\* (Anaspec #AS-71158, Fremont, CA, USA) without chemical prior-activation, allowing detecting simultaneously the activity of MMP-1, 2, 3, 7, 8, 9, 12, 13 and 14. Fluorescence ( $\lambda_{exc} = 490 \text{ nm}$ ,  $\lambda_{em} = 520 \text{ nm}$ ) was read on plate reader Synergy H1 (BioTek) in black 96-well plate.

**ELISA quantifications.** Four-weeks-old dermal substitutes were treated and cultivated in 600 µl of cell culture medium. Supernatants were harvested at different time points (6h, 24h, 48h) after cold-plasma exposure and stocked at -80°C to be used within a week. ELISA kits were used strictly according to manufacturer's protocols: human Pro-Collagen I alpha 1 DuoSet ELISA kit (R&D systems, #DY008, Minneapolis, MN, USA), human MMP-1 (ELISA Genie, #HUF100205, Dublin, Ireland) and the absorbance was read using a plate reader Synergy H1 (BioTek).

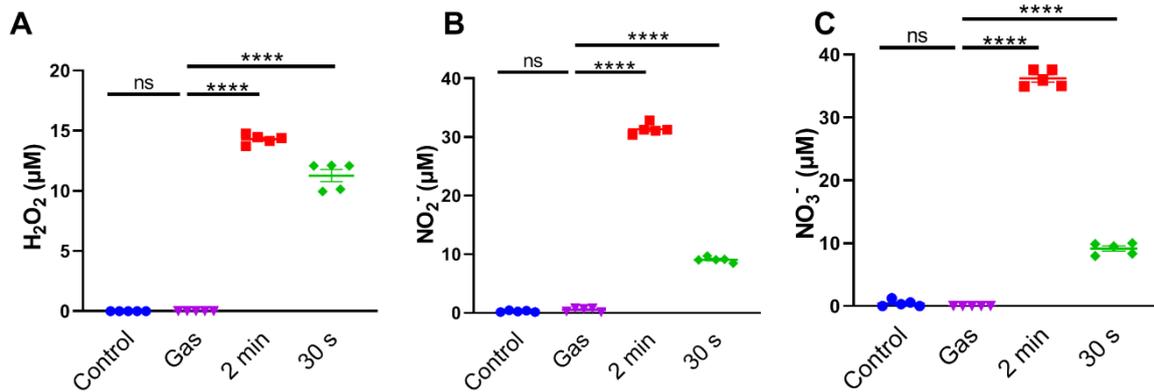
**Spheroid generation and plasma treatment.** 500 HCT-116 cells were seeded in ultralow attachment 96-well plates with a round bottom (Corning, ThermoFisher Scientific) and allowed to grow for 5 days in DMEM without pyruvate (Gibco #41965-039) supplemented with 10% of heat inactivated fetal calf serum (Gibco #10270106), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Gibco #15140122) as previously described [35]. On the day of treatment, 600 µl of fresh complete (10% fetal calf serum + antibiotics) DMEM without pyruvate were exposed to the plasma jet for 30 s or 2 min. Cell culture medium was removed from the wells containing spheroids and replaced by 100 µl of this exposed medium. Exposure of DMEM for 2 min to helium gas alone and no exposure were settled as control conditions.

**Spheroid analysis: growth curve, apoptosis, plasma membrane integrity and intracellular ATP quantification.** Spheroids' growth was followed by taking photographs before treatment and each day after treatment for 4 days, without cell culture medium changing. Spheroids' area was measured using ImageJ software (NIH, Bethesda, MD, USA) as previously described [36–38]. Cell death by apoptosis was visualized in spheroids treated with plasma by adding NucView 488 Caspase-3 Substrate (Biotium #10402, San Francisco, CA, USA) to each well 2h after plasma treatment. Plasma membrane integrity of cells within spheroids was assessed by adding 100 µM of propidium iodide (Sigma-Aldrich #P4170) to each well 24h after plasma treatment. Propidium iodide fluorescence ( $\lambda_{ex} = 493$ ;  $\lambda_{em} = 636$  nm) was detected by fluorescence microscopy and images were analyzed using ImageJ software. Finally, number of viable cells per spheroid 4 days after treatments was determined by intracellular ATP quantification using CellTiter-Glo® 3D Cell Viability Assay (Promega, #G9681) according to the manufacturer's instructions. Luminescence was read using a plate reader Synergy H1 (BioTek).

**Statistical analyses.** Data analysis was performed using GraphPad Prism 8 program (GraphPad Software, Inc., La Jolla, CA, USA) and individual data were plotted and expressed as mean  $\pm$  SEM (standard error to the mean). If not indicated otherwise in the legend of the figures, the data have been statistically analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post-test to compare every condition to the gas alone reference. Statistics are expressed relative to the control condition (0 V/cm). ns, non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

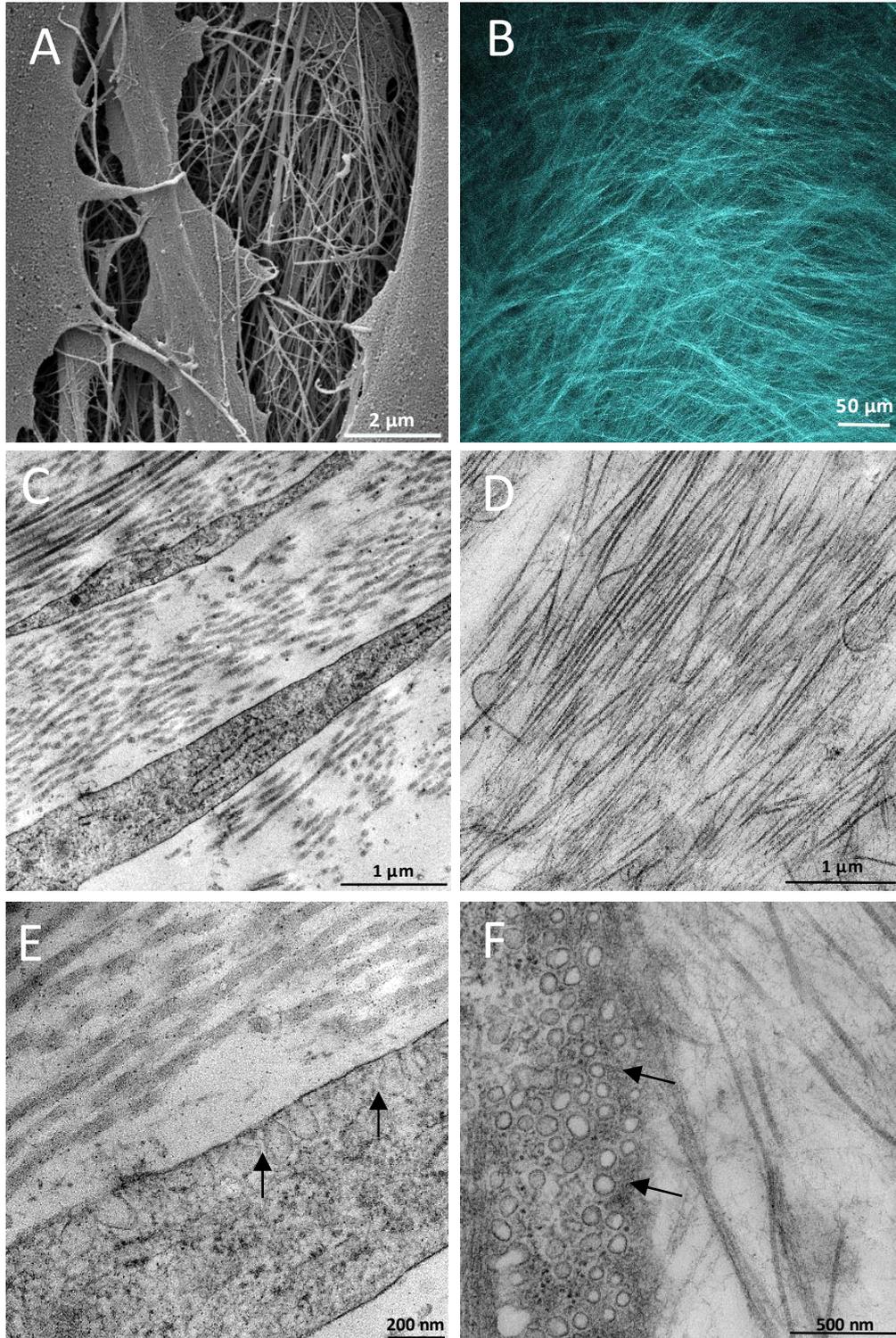
## RESULTS AND DISCUSSION

**Concentrations of RONS increased with the exposure time to helium plasma jet.** During interaction between plasma and liquid, RONS are generated [39,40]: short life-time reactive species such as nitric oxide (NO), hydroxyl radical (OH<sup>•</sup>) or superoxide anion radical (O<sub>2</sub><sup>•-</sup>) which need specific physico-chemical technics like chemical dosimetry or electron paramagnetic resonance spectroscopy to be quantified, and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), nitrites (NO<sub>2</sub><sup>-</sup>) and nitrates (NO<sub>3</sub><sup>-</sup>) which are long-lived RONS. Their long lifetime allows them to be easily quantified in the plasma-treated cell culture medium using commercially available kits. Since pyruvate is known to play a key role in cell response to plasma because of its anti-oxidative properties [41], we used for all experiments pyruvate-free cell culture medium. H<sub>2</sub>O<sub>2</sub>, NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> were quantified in cell culture medium (600µl in a well of a 24-well plate) immediately after exposure to helium plasma jet (Figure 1). H<sub>2</sub>O<sub>2</sub> concentration increased with the exposure time to plasma jet (Figure 1A). Indeed, hydrogen peroxide concentration was  $14.31 \pm 0.17 \mu\text{M}$  and  $11.28 \pm 0.5 \mu\text{M}$  respectively for 120 and 30 s plasma exposure. As for H<sub>2</sub>O<sub>2</sub>, the concentration of nitrite and nitrate anions increases with exposure time to plasma. The nitrite concentration was  $31.36 \pm 0.4 \mu\text{M}$  for 2 min plasma exposure and  $9.1 \pm 0.19 \mu\text{M}$  for 30 s plasma exposure (Figure 1B), and nitrate concentration was  $36.20 \pm 0.58 \mu\text{M}$  for 2 min plasma exposure and  $9.15 \pm 0.41 \mu\text{M}$  for 30 s plasma exposure (Figure 1C). No significant amount of RONS was quantified in both the non-exposed control condition and after 2 min of exposure to helium gas alone. This accumulation of RONS during increasing time of exposure was largely documented in the literature [32,42,43]. We had previously quantified H<sub>2</sub>O<sub>2</sub>, NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> produced using a similar experimental helium plasma jet set up by treating 100 µl of the same cell culture medium in a well of a 96-well plate [44]. Under this configuration, 2 min exposure led to a production of 1.2 mM of H<sub>2</sub>O<sub>2</sub> and 450 µM of NO<sub>2</sub><sup>-</sup>. Since we used the same helium plasma jet device in our previous published study and this one, this underlines the importance of the experimental conditions in the production and maintenance of RONS generated by helium plasma jet.



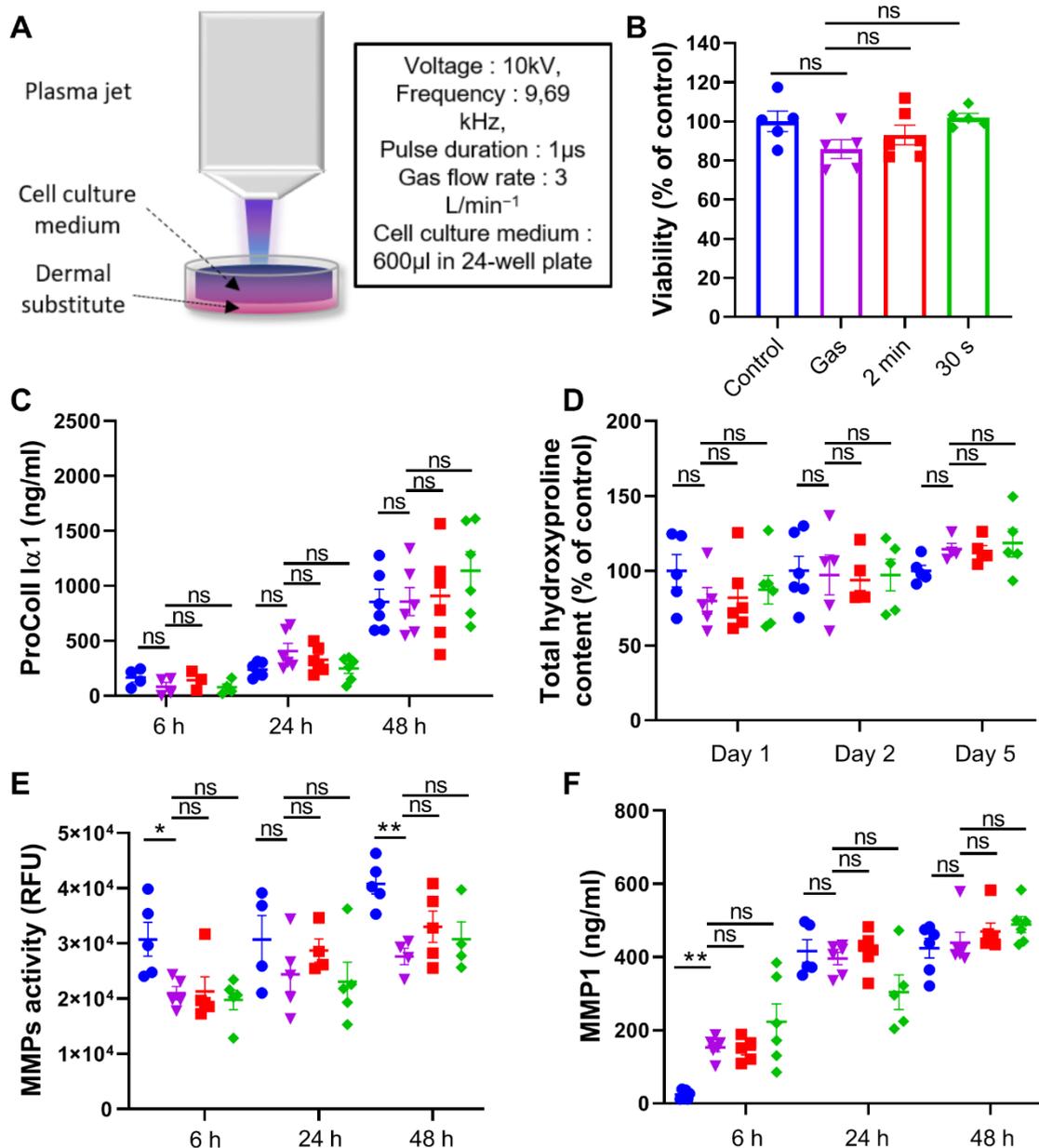
**Figure 1 : RONS concentrations increase with the exposure time to helium plasma jet in pyruvate-free cell culture medium. A. Hydrogen peroxide B. Nitrite anion C. Nitrate anion.** Control condition (●), gas alone 2 min (▼), plasma 2 min (■), plasma 30 s (◆). n=5. \*\*\*\*,  $p < 0.001$ .

**Helium-CAP did not affect dermal collagen content in healthy dermal tissue rich in endogenous extracellular matrix.** To study the direct effect of cold plasma on the ECM remodeling, we used a sophisticated model of human dermis substitute produced by tissue engineering using the self-assembly approach [45]. This human dermal tissue substitute is totally devoid of exogenous material but is rich in endogenous ECM proteins as demonstrated by proteomic analyses [46]. In particular, the fibril-forming collagen types I, III and V are the major components of the extracellular matrix in the model [46], which demonstrates its complex composition and its similarity to the composition of the native dermis in human skin [47]. This endogenous extracellular matrix revealed to be of utmost importance in providing to this dermal tissue model biomechanical properties closed to those of the native dermis [48]. It appears that self-assembled human dermal substitutes are a valuable tool to investigate the effects of CAP on the collagens. The collagen fibers were characterized within the model by complementary microscopy approaches. Electron microscopy and second harmonic generation showed the typical supramolecular assembly of the collagen fibers (Figure 2). As expected [49], transmission electron microscopy underlined that dermal fibroblasts exhibited an elongated shape with a dense endoplasmic reticulum due to high secretory activity, and abundant exocytosis vesicles secreting collagens in the extracellular space, as shown on the transversal and longitudinal sections of the dermal substitute (Figure 2).



**Figure 2: The tissue-engineered human dermal substitute is rich in endogenous collagens.** **A.** Top surface of the dermal substitute observed by scanning electron microscopy **B.** Second Harmonic Generation (SHG) signal (in cyan) detected in fresh dermal substitute by two-photon microscopy. **C.** Transversal and **D.** Longitudinal section of collagens fibers in the dermal substitute. **E.** Transversal and **F.** Longitudinal section of a dermal substitute (arrows indicate exocytosis vesicles in fibroblasts).

To analyze effect of plasma on dermal collagen remodeling, human dermal substitutes were CAP-treated in 600 $\mu$ l of fresh pyruvate-free cell culture medium for either 30 s or 2 min. Note that cell culture medium was not renewed after exposure to CAP, meaning that dermal substitutes were exposed to CAP and then cultivated in the same medium. Exposure during 2 min to helium alone, and no exposure were settled as control conditions. The “gas alone” condition was set as the reference to statistically analyze data in plasma-treated conditions. The experimental set-up is outlined in figure 3A. Firstly, the maintenance of cell viability was checked in dermal substitutes 24 h after CAP-treatment, showing a lack of deleterious effect of the treatment on skin fibroblasts and the sub-lethal range of CAP-treatment (Figure 3B). Then, collagen I being the major dermal collagen [47], procollagen I $\alpha$ 1 secretion was quantified in the supernatant up to 48 h after the treatment. Results showed no statistical differences in procollagen I $\alpha$ 1 secretion compared to the gas alone reference condition over 48 h (Figure 3C). To go further, the global collagen content within the dermal substitute was determined by chemical measurement of a collagen specific amino acid, namely hydroxyproline [50]. The results obtained indicated that CAP-treatment did not affect the global collagen content in the dermal substitute over 4 days after treatment (Figure 3D). MMPs are key players in the remodeling and maintenance of the ECM [51]. MMPs are known to be activated in response to exogenous signals such as low pH, heat, and reactive oxygen species [51,52]. Because plasma jets are recognized to be RONS generators, we quantified global MMPs activity in the supernatants by fluorimetry. Contrary to what was expected and despite the presence of numerous RONS in the CAP treated culture medium (Figure 1), MMPs’ activity was not statistically modified over 48 h by plasma treatment (Figure 3E). To go further, we quantified by ELISA the amount of MMP-1 protein, also named collagenase, which is known to degrade types I, III, and V fibrillar collagens. A transient increase in MMP-1 amount was observed 6 h after the plasma treatments (Figure 3F), independent of RONS and plasma exposure since even the gas alone condition displayed this increase. CAP-treatment induced no statistical difference in MMP-1 concentration over 48 h when compared to the gas alone reference.



**Figure 3: Sub-lethal cold helium plasma jet treatment does not affect collagen content in dermal substitutes.** **A.** Experimental set-up for CAP-treatment of dermal substitutes. **B.** Cell viability in the dermal substitute determined with PrestoBlue assay 24h after cold plasma treatment, ( $n \geq 4$  dermal substitutes) **C.** Dosage by ELISA of secreted pro-collagen I $\alpha$ 1 in supernatants, ( $n \geq 4$  dermal substitutes) **D.** Hydroxyproline content in the whole dermal substitute measured over the time after cold plasma treatment, ( $n \geq 4$  dermal substitutes). **E.** MMPs activity quantified by fluorimetry, ( $n \geq 4$  dermal substitutes). **F.** Expression of MMP-1 quantified by ELISA in dermal substitute supernatants, ( $n \geq 4$  dermal substitutes). Control condition ( $\bullet$ ), gas alone 2 min ( $\blacktriangledown$ ), plasma 2 min ( $\blacksquare$ ), plasma 30 s ( $\blacklozenge$ ).

To the best of our knowledge, a few studies assessed the effect of CAP on ECM components, although this notion is becoming increasingly important, especially in the context of anti-cancer applications *via* the targeting of the tumor microenvironment [26]. CAP treatment of extracted bovine type I collagen, either in dry state [53] or in solution [54], induced its oxidation and structure denaturation in acellular context. CAP surface treatment of acellular 3D dermis substitute enhanced the *in vitro* angiogenesis process and accelerated the neovascularization when transplanted into mouse back skin [55]. In 2D monolayer cell culture, a transient increase in mRNA level of collagen I has been observed 6 h after skin fibroblasts were treated for 2 min with an argon-based plasma jet device while MMP-9 gelatinase protein was undetectable in cell culture supernatant [15], despite a moderate (1.4 fold) induction at mRNA level [17]. Air-based CAP treatment of human osteoblast-like MG63 osteosarcoma cells upregulated the expression of collagen I both at the mRNA and protein level, together with a moderate but statistically significant increase of MMP-1 expression and secretion [56]. Likewise, collagen I gene and protein expression was elevated by treatment of murine dental cementoblasts [57]. CAP treatment of human periodontal ligament cells was also shown to enhance the expression of collagen I and MMP-1 [58]. A study by Schmidt *et al* demonstrated that *in vitro* exposure of murine primary dermal fibroblasts to Argon plasma decreased cell viability but promoted mobility by regulation of transmembrane receptors and actin cytoskeleton [59]. Most of the *in vitro* studies were led using acellular models and cells grown in monolayers, which not fully mimics the dynamic and reciprocal interactions between cells and ECM components. That is why we believe that our *in vitro* 3D human dermal substitute produced by tissue-engineering revealed to be a useful model to better understand the effects of CAP on the ECM remodeling at tissue scale. Further work will be necessary to clarify the effect of each individual mode of CAP generation and decipher the optimal conditions of use.

Published results about *in vivo* plasma exposure and ECM remodeling should be mentioned. One minute argon micro-plasma treatment was shown to promote wound healing in a murine laser-induced partial thickness skin wound and to reduce the protein level of MMP-3 in the wound bed [60]. Using a full-thickness dermal wound in mice ears, Schmidt *et al* demonstrated that argon cold plasma promoted wound closure depending on treatment time, with a better efficacy after only 3 s of exposure [61]. The same group recently demonstrated using the same model that a daily repeated 3 s treatment with argon plasma transiently and significantly increased mRNA and protein levels of collagens COL1A1 and COLIV, and up-

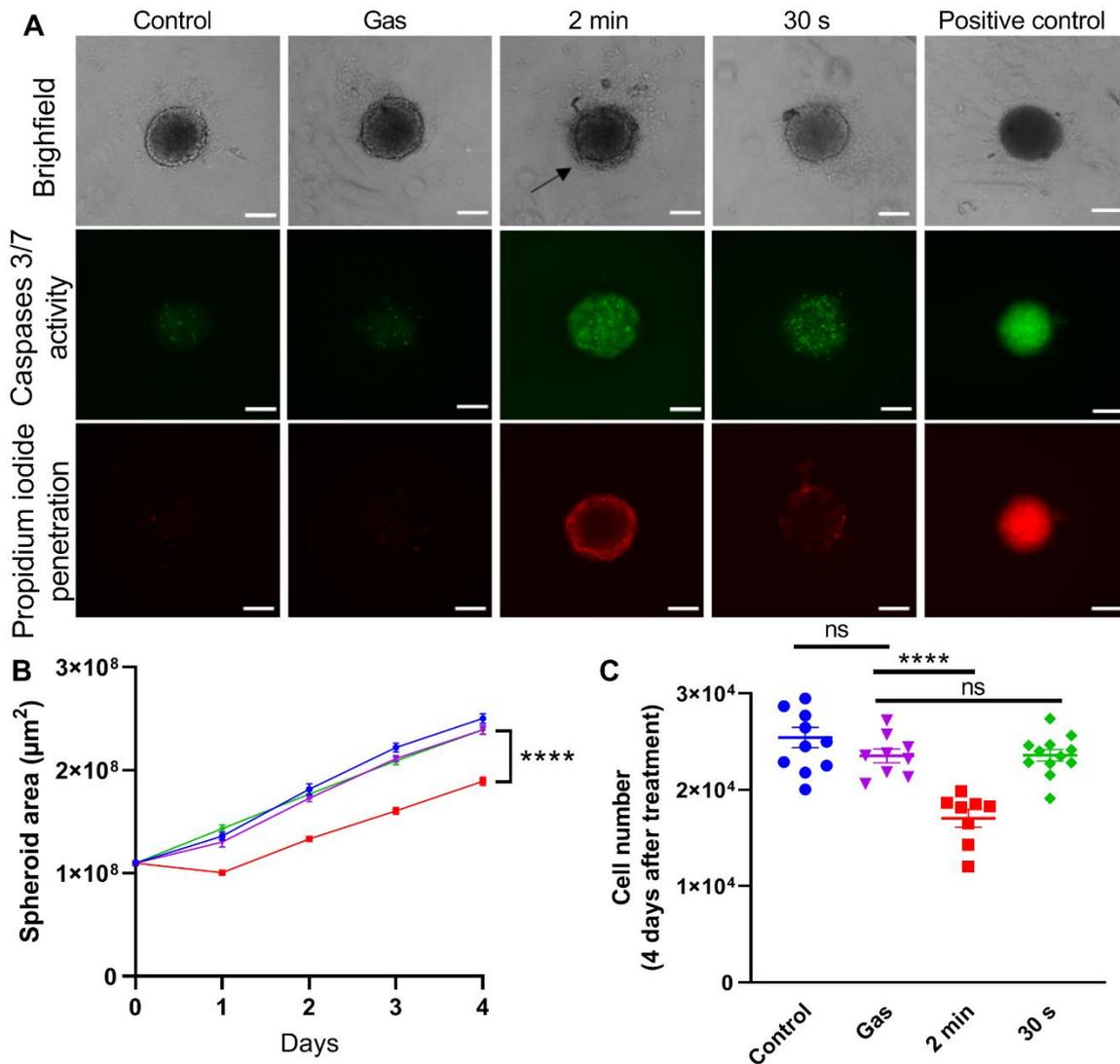
regulated MMP2 and MT1-MMP mRNA expression while TIMP2 mRNA was down-regulated [59].

Finally, Shi *et al.* proposed a dose-dependent effect of CAP where low doses of plasma enhanced fibroblast viability and collagen synthesis while high doses displayed the opposite effects [62]. A reason for the discrepancy between most of these data and our results could be the difference in either the cell types used, the mode of CAP generation (air-, argon- versus helium-generated) or the treatment settings (duration, intensity, etc.). In our experiments, we applied two different times of exposure in order to generate distinct levels of oxidative stress, keeping in mind not to kill the cells meaning staying in the sub-lethal range. Although this threshold theory makes sense, it seemed that our experimental conditions (30 s and 2 min exposure to CAP) did not allow to enter this effective range. Our preliminary tests applying longer time exposure to CAP (i.e 4 min) led to significant cell death induction in the dermal substitute tissue which appeared not optimal to study extracellular matrix remodeling.

In our experimental set up, dermal substitutes were exposed to CAP in 24-well plates while covered by 600 µl of pyruvate-free cell culture medium, and no effects were observed in terms of extracellular matrix remodeling. In the following experiments, we planned to verify the well-documented anticancer effect of PAM generated under quasi-similar conditions. Thus, 600 µl of pyruvate-free culture medium were exposed to CAP in 24-well plates, producing PAM, of which 100 µl were then immediately deposited onto human 3D tumor spheroids. The objective, even if the spheroids exposed to PAM were not exposed to the direct effects of CAP (UV radiation, heating, electric field...) was to verify that in these quasi-similar conditions, the plasma treatment presented efficient therapeutic antitumor effects. The results of these second batch of experiments would allow to reinforce the conclusion that cold helium plasma does not stimulate collagen remodeling in a 3D human dermal substitute.

**Plasma-activated medium induced apoptosis in tumor spheroids.** The antitumor effects of plasma activated media have already been largely demonstrated by others [9,10,12,26] and by ourselves with similar helium plasma jet experimental set up [63,38]. However, the concentration of RONS generated in our experimental set-up was much lower than those usually observed. For this reason, antitumor properties of this plasma treated medium were checked onto a 3D tumor spheroid model composed of human colorectal tumor HCT-116 cells. Spheroids were immersed in plasma-treated or control cell culture medium and their macroscopic aspect was monitored by bright-field microscopy 24 h after treatment (Figure 4A). Spheroid integrity was preserved when exposed over 24 h to medium treated with helium only

(gas alone) and 30s plasma-exposed medium, as compared to the control condition. In contrast, when exposed to medium treated with plasma during 2 min, the spheroids started to disintegrate allowing to distinguish a crown of detached cells surrounding the spheroid core. At this time-point, apoptosis and plasma membrane integrity in the spheroids were monitored looking at caspases 3/7 activity (green) and propidium iodide (red) penetration (Figure 4A), respectively. Spheroids were treated with staurosporine, as a positive control for cell death induction. It clearly appears that plasma treatments induced apoptosis in the spheroids, particularly when these were exposed to 2 min plasma-treated medium and to a lesser extent with 30 s plasma-treated medium. Concomitantly, loss of plasma membrane integrity was observed in the crown of detached cells in the 2 min treatment condition, and marginally in the 30 s treatment condition. Spheroids' growth was then followed during 4 days by taking photographs before treatment and each day after, without medium replacement. Spheroid growth was delayed by 2 min plasma-treated medium only (Figure 4B), with about 30% loss of cell number 4 days after the treatment (Figure 4C). Therefore, we confirmed the *in vitro* anti-tumor effect of plasma activated medium generated with a cold atmospheric helium plasma jet in our experimental conditions. These data showing that PAM, in our experimental conditions, reduced growth of tumor cells, reinforce the conclusion that CAP-treatment does not induce or stimulate ECM remodeling, at least in a 3D human dermal substitute.



**Figure 4 : HCT-116 spheroid growth is delayed by PAM-treatment through apoptosis induction.** **A.** Spheroid macroscopic aspect, apoptosis (Caspases 3/7 activity, green) and loss of plasma membrane integrity (propidium iodide penetration, red) were observed 24h after PAM-treatment. Arrow indicates a crown of detached cells. 1  $\mu$ M of staurosporine was used as a positive control for cell death induction. Scale bar: 200  $\mu$ m. **B.** Spheroids growth over four days after plasma treatment (each growth curve represents mean values  $\pm$  SEM corresponding to  $n \geq 9$  spheroids). Data have been statistically analysed using 2-way ANOVA followed by Dunnett's post-test to compare every condition to the gas alone reference. Control condition (●), gas alone 2min (▼), plasma 2 min (■), plasma 30 s (◆). \*\*\*\*,  $p < 0.001$ . **C.** Number of cells per spheroid measured via intracellular ATP quantification 4 days after plasma treatment, ( $n \geq 9$  spheroids, coming from the same experiment presented in B). Control condition (●), gas alone 2min (▼), plasma 2 min (■), plasma 30 s (◆). \*\*\*\*,  $p < 0.001$ . Same colour code as in B.

## **CONCLUSION**

This study aimed to determine the effect of CAP on skin collagens using an original tissue-engineered human dermal substitute model rich in endogenous extracellular matrix. The type of plasma used in the present study is helium plasma jet based on dielectric barrier discharge configuration generated by pulsed power supply. This work highlights that after helium CAP treatment no major modification in extracellular matrix remodeling occurred at dermal tissue scale, despite the inhibitory effect of the tumor growth of the treatment.

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## **AUTHOR CONTRIBUTIONS**

SG: Investigation, Formal Analysis, Visualization, Validation, Interpretation of data, Writing; AM: Investigation, Formal Analysis, Visualization, Interpretation of data, Writing; PV: Conceptualization, Interpretation of data, Writing; IF: Visualization; MS: Conceptualization, Interpretation of data, Writing; NM: Conceptualization, Interpretation of data, Writing; LG: Conceptualization, Interpretation of data, Writing, Funding acquisition, Supervision.

## **CONFLICTS OF INTEREST**

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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