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Surface treatment with non-thermal humid argon plasma as a treatment for allergic contact dermatitis in a mouse model

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ABSTRACT

Purpose: Cold plasma generated at atmospheric pressure has attracted intense interest in biomedical applications. However, studies of cold plasma in dermatology, and as a possible therapy for non-infectious skin diseases, including burn and wound healing, psoriasis, and allergic contact dermatitis (ACD) *etc.*, are still quite scarce. The present study reports the treatment of ACD *in vivo*, using a non-thermal humid plasma source in a mouse model and shows the latter could be a potential alternative approach for therapy of ACD and other inflammatory skin diseases.

Methods: The model of ACD on the mouse back was induced by a solution of DNFB and treated by argon plasma containing small amounts of either N_2, O_2, or H_2O. The developments of ACD regions were photographed and histopathological analysis by H&E-staining was performed.

Results: The best effect was obtained using humid plasma (H_2O addition), where the ACD symptoms decreased after one or two 1-min plasma treatments. Even for severe ACD with ulcers and crust formation, the humid plasma-treated mice recovered faster than the control group.

Conclusions: The therapeutic ability of the humid argon plasma discharge was proposed to be induced by reactive oxygen species (H_xO_y) transported from the discharge zone, which are adhesive and accumulate on the skin surface, penetrating the subcutis to eliminate inflammation. However, in treatments using plasma with addition of oxygen or nitrogen (without water) the active gaseous species are blocked due to poor adhesion to and penetration into the dry ACD skin, with correspondingly poor treatment effects. The enhanced *in vivo* healing in ACD mice indicate the non-thermal humid plasma could be a potential alternative approach for therapy of ACD and other inflammatory skin diseases.

1. Introduction

Cold plasma discharge generated at atmospheric pressure has attracted wide interest in a range of different healthcare applications, and its medical use has been termed “plasma medicine” [1–3]. Various reactive species are produced within gaseous plasmas such as: free electrons and ions, reactive oxygen species (ROS) and oxygen free radicals, ultraviolet radiation (including vacuum UV) and possibly ozone and reactive nitrogen species (RNS). These reactive species have been reported to carry out bacterial inactivation, blood coagulation, trigger cancer-cell apoptosis, and disrupt mammalian-cell adhesion [4–8]. Proposed medical applications have been proposed in areas such as hospital hygiene, dentistry, wound care, antifungal treatment, anticancer treatment, and possible applications to dermatology for various skin disorders [9–12].

The most important application of cold plasma so far, has been microbial inactivation and ROS/RNS have been shown to be the key factors for this biological effect [4]. Although the underlying

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mechanisms are still not clear, the antimicrobial efficacy is strongly dependent on the type of microorganism, on the environment where the microorganisms are growing, and how the plasma is produced [4,13]. Typically, plasma reacts with the biological substances with which it first comes into contact. In a comparable manner to UV light or antimicrobial blue light, plasma does not penetrate very deeply into tissue, and so can be considered a surface decontamination modality [12,14,15]. This means that the physical and chemical properties of the media the plasma comes into contact with have a significant impact on its treatment efficacy [16]. On the other hand, this surface treatment effect suggests that plasma could be useful in dermatology, as plasma could directly inactivate skin pathogens and stimulate wound healing [11,12]. It has been shown in pre-clinical studies that different bacteria and fungi can be inactivated efficiently in infected wound animal models [9,10,17,18]. However, studies of cold plasma in dermatology, and as a possible therapy for non-infectious skin diseases, including burn and wound healing, psoriasis, and contact allergic dermatitis *etc.*, are still quite scarce [12,19,20].

Contact dermatitis is a common inflammatory disease of the skin with two different manifestations [21]. Perhaps the most common type is irritant dermatitis caused by coming into contact with acids, alkaline materials such as soaps and detergents, fabric softeners, solvents, or other chemicals. This skin reaction most often looks like a burn. The second type is allergic contact dermatitis (ACD). This requires two separate steps, the first being a sensitization step in which a small molecule called a “hapten” penetrates the skin, binds to a skin protein and is recognized as foreign by the adaptive immune system. If a second exposure (elicitation step) to this hapten (or allergen) occurs, then an inflammatory reaction follows, predominantly mediated by T helper cells [21].

ACD severely influences the quality of life of sufferers, due to its complex pathogenesis and tendency to relapse [11]. Conventional treatment consists of avoiding triggers, using moisturizers in cream, lotion, or ointment form, and in severe cases the use of topical or even systemic corticosteroids [22].

The present study reports the treatment of ACD *in vivo,* using a non-thermal plasma source in a mouse model. Different gas compositions of the plasma were investigated. The best therapeutic results were achieved by humid plasma treatment, where obvious improvement was seen after once or two exposures. For severe ACD, mice recovered faster with enhanced skin regeneration compared to the control group.

2. Material and methods

The plasma source used for ACD treatments was a single floating-electrode dielectric barrier discharge driven by a home-made pulse generator. As shown in Fig. 1(a), it consisted of an open-ended wire as the high-voltage electrode inserted into a close-ended quartz tube (D_{in} = 8 mm and D_{out} = 10 mm). These parts were centered within another quartz tube (D_{in} = 10 mm, D_{out} = 14 mm). The distance between the ends of the two tubes was set at 1 mm. Argon mixed with 1% N_2, 1% O_2, or 0.5% H_2O was introduced as a discharge gas to produce various types of plasma species for the dermatitis treatment. The water vapour was introduced by flowing an argon gas channel through a bubbler filled with distilled water (120 mL, height 90 mm). The absolute humidity was tracked by a moisture monitor (LY60P-2X, Fatai Ltd., China). All the gas flows were monitored by a mass flow controller (247D, MKS Instruments Ltd., USA), and the total flow rate was set at 4 standard liter per minute (slm). The outputs of the pulse generator were set at 15 kV of the voltage peak, 100 ns of the pulse width, and 1.0 kHz of the pulse frequency. An 1.0 kΩ resistor was used to limit the discharge current flowing through the treated mouse. Applied voltage and discharge current were monitored by a Tektronix P6015 probe and a Pearson 2877 monitor, respectively.

The model of ACD on the mouse back used in this work was produced by applying a solution of 2,4-dinitrofluorobenzene (DNFB). DNFB is a typical of chemical allergen often used to induce ACD in animal models for study of ACD therapies [21]. All the animal experiments were conducted in accordance with the guidelines and the ethical standards of the Institutional Animal Care and Use Committee of the Third Military Medical University (Chongqing). First, 6–8 weeks old male Balb/c mice (Center of Animal Laboratory, Third Military Medical University, China) were obtained, with body weight around 20 g. The mice were housed for one week, and then were anesthetized by intraperitoneal injection of 200 μL 3.6% chloral hydrate per mouse to allow shaving the abdomen and back. The sensitization step was subsequently performed by uniformly spreading 25 μL 0.5% DNFB solution (prepared by dissolving 5 mg DNFB in 1 mL of a fresh mixture of 4:1 acetone and olive oil) onto the hairless skin region (2 × 2 cm^2) of the mouse abdomen. Then the mice were housed another 5 days. After that anesthetization was performed again for the elicitation step of the dermatitis by spreading a specified volume of 0.2% DNFB solution onto the hairless back region (2.5 × 3.5 cm^2) of each mouse. In the present study we used three different volumes (20 μL, 30 μL, and 50 μL) of DNFB solution as an elicitation to induce ACD with different degrees of severity defined as mild ACD (20 μL), moderate ACD (30 μL), and severe ACD (50 μL).

Two days after the elicitation step, the mice were examined and the skin on the mouse back displayed symptoms of erythema, papulae, blisters and even necrosis (as shown in Figure S1), the ACD model was considered ready for plasma treatment.

The dermatitis area on the mouse back was divided equally into six regions (as shown in Figure S1) and for each region a separate 1-min plasma exposure was performed. The mouse was laid on a grounded-and-movable metal holder during treatment. Except for the control group, all the mice were subjected to 4 treatments (once/day) from day 0 to day 3. The ACD regions were always photographed before treatment. For each experimental condition three mice were treated in each group.

In order to obtain experimental proof of the mechanism, we carried out an experiment with chemiluminescence produced by luminol added to the mouse skin after treatment by the Ar + 0.5%H_2O and Ar + 1%O_2 discharges. Luminol is an organic compound sensitive to oxidation by such species as hydrogen peroxide or singlet oxygen whereupon it emits blue chemiluminescence (~ 425 nm) and has been employed for determination of oxidants or metal ions (like Fe^{2+}) [23,24]. Luminol solution (0.56 mM in Tricine buffer, pH = 9.2) containing 5 mM/L allantion and horseradish peroxidase (> 200 U/mL) was prepared to determine the H_2O_2 concentration. [24] 20 μL luminol solution was dropped individually onto the treated areas immediately after the plasma exposure. Pictures were captured by a Nikon D7000 SL camera with a macro-lens (Nikon 105/2.8 G).

3. Results

Fig. 2 shows representative photographs of the progress of the ACD lesions on the mouse back from day 0 to day 6 after plasma exposure under various gas mixture conditions. In the control group without any plasma treatment, the ACD symptoms started to disappear after 3 days (day 3) and were almost completely gone on day 6 with only local slight scaliness remaining on the skin. On the other hand, the plasma irradiated groups under different gas compositions, showed different healing effects. Groups A and B treated by pure Ar and Ar + 1% O_2 discharges showed similar healing to the control group. However, adverse treatment effects were observed for the ACD mice after exposure to Ar + 1%N_2 and Ar + 1%N_2 + 1%O_2 plasmas, as evidenced by the fact that obvious regions of scaliness still existed on the skin at day 6. These scaly regions almost disappeared after two more days. The best therapeutic effect was obtained in group E treated by the Ar + 0.5% H_2O discharge. The ACD symptoms were obviously better on day 1 after only one plasma exposure. The skin appeared to be smooth without edema and any ulcers started to heal. On day 6 mice had recovered with

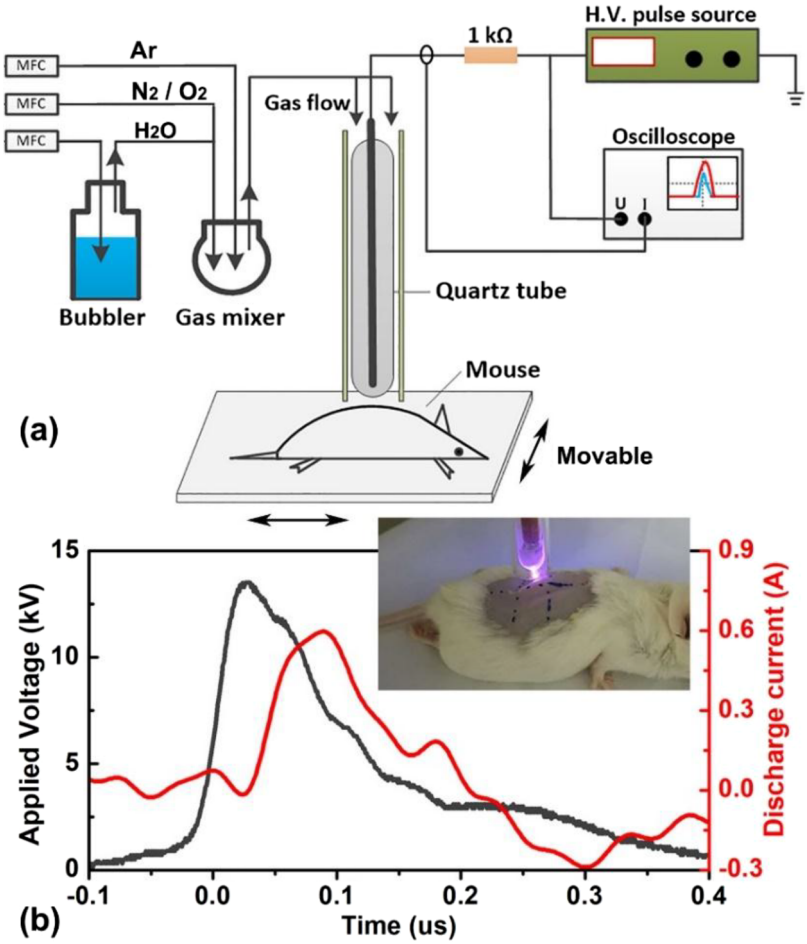


Fig. 1. (a) Experimental schematic of the plasma generator using pulsed argon plasma with different gas admixtures, and (b) Current-voltage characteristics of the Ar + 1% N_2 plasma while treating the area of ACD on the mouse back.

complete ulcer healing and the skin appeared normal coloured without any scaliness.

The UI characteristics were measured and compared to exclude any effects of electrical current flowing on the ACD treatment using different gas-mixture plasmas. The data showed the applied voltage remained constant in Fig. 1(b), but the discharge current varied slightly with different gas compositions (as shown in Figure S2). Maximum or minimum discharge currents were obtained with Ar + 1%N_2 or Ar + 1%O_2 plasmas, with peaks of 596 mA and 500 mA respectively. The current peak was 580 mA for the humid plasma with 0.5% water vapour, slightly lower than that with the Ar + 1%N_2. Taking into account the treatment efficiencies illustrated in Fig. 2, this data suggests the impact of the flowing current through the treated mouse on the ACD healing was not critical. The negligible effects of electrical field or flowing current have been reported as well by others who used plasma to treat mice or even human patients [11,25].

Based on the above encouraging preliminary results, further *in vivo* studies were carried out on mice with more severe ACD disease conditions produced by increasing the amount of the DNFB allergen applied in the elicitation step. These studies were carried out using only the humid argon plasma discharge with another control group consisting of the humid gas flow (GF) alone (with the plasma generation switched off) to assess the effect of humid argon gas on the ACD progression. The corresponding skin images and H&E-staining patterns are presented in Figs. 3 and 4 for the moderate-degree (30 uL DNFB) and severe-degree (50 uL DNFB) ACD mice respectively. As illustrated for the moderate-degree ACD, a similar therapeutic effect was observed on day 1 after only one plasma treatment. Necrotic tissue fell off mostly on day 1 and any ulcers disappeared on day 4, faster than the GF and NT groups. On day 8 the mouse back grew new hair in the affected region of the plasma-treated (PT) group. The H&E-stained images showed abundant hair follicles that had regenerated in the subcutaneous tissue by day 6. The edematous epidermis reduced in thickness to become similar to normal unaffected skin. These observations indicate the inflammation had disappeared by day 6 and ACD was cured in the plasma treated mice. In the GF and NT groups the ACD symptoms had largely been reduced by day 8, but still slight scaliness remained on the skin. The H&E-staining analysis showed the epidermis was still edematous in the GF group on day 6, and no formation of hair follicles was seen in the NT group.

It appeared that the humid gas flow alone had a slight beneficial effect on the healing of ACD lesions compared to the NT group, as some not-fully formed hair follicles were observed in the subcutis. However, compared to the plasma treatment, the humid gas flow alone was clearly inferior for ACD treatment.

With increased severity of the ACD it was observed that the plasma-stimulated healing phase was prolonged. As shown in Fig. 4, the severe-degree ACD mice took 11 days for the dermatitis symptoms to be mostly relieved for the PT group, much longer than for the mild and medium conditions. However, plasma treatment still produced enhanced healing effects on the severe ACD compared to the control and GF groups. On day 11 the latter two groups still displayed obvious necrosis, particularly the NT group. The humid gas flow alone showed a slight benefit to enhance the closure of the severe ulcer on the mouse back, as compared to the untreated group. The necrotic tissue was shed after three more days for the gas-flow treated mouse, while one more week was required

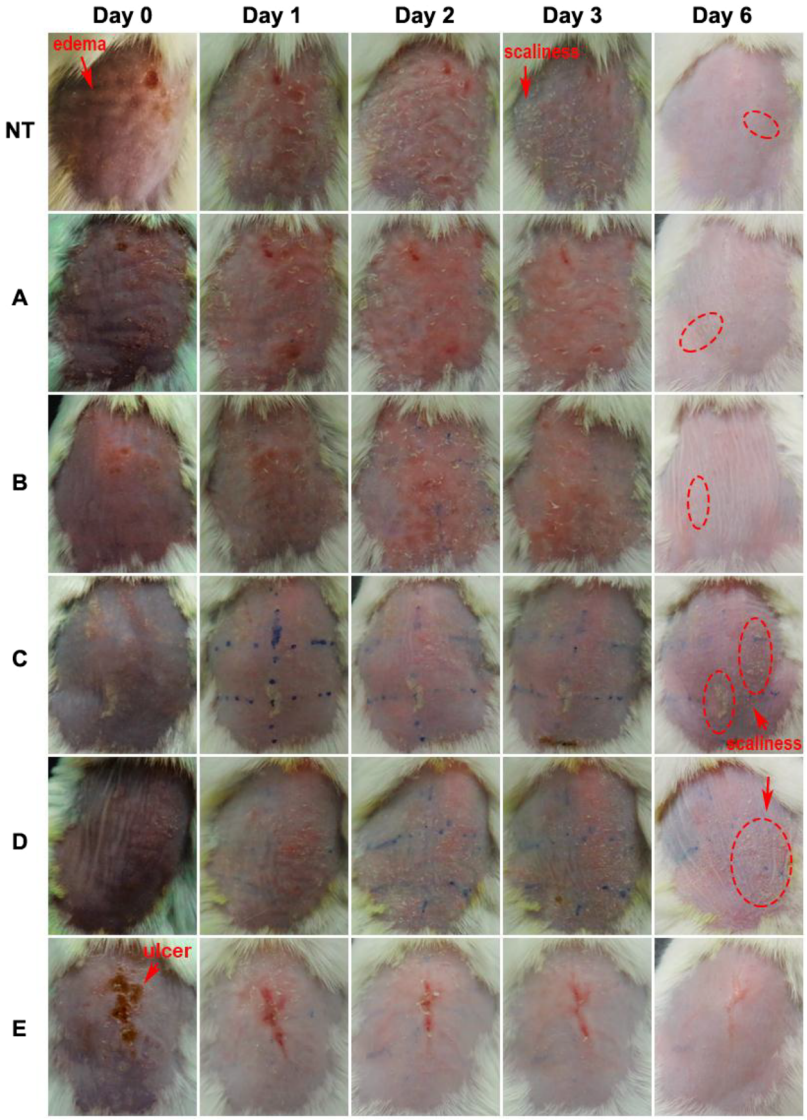


Fig. 2. Images of mild-degree ACD lesions on the mouse back from days 0 to 6 for the non-treated (NT) control group, groups irradiated with plasma gas conditions (A) pure Ar, (B) Ar + 1% O_2, (C) Ar + 1% N_2, (D) Ar + 1% O_2 + 1% N_2, and (E) Ar + 0.5% H_2O. Red circles indicate areas of scaliness remaining on the mouse back at day 6, particularly after plasma treatments in group C and D. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for the NT group.

The thick necrotic tissue formed on the skin in the ACD lesions was considered to play an obstructive role in the healing process even after 4-days (day 0 to day 3) of repeated plasma exposures. As shown in Fig. 4 in all the three groups, necrosis fully or mostly covered the dermatitis lesion in the first 4 days. This dry and hard necrotic tissue significantly prevented the penetration of reactive species (and water molecules) from the gaseous discharge to the skin, not to mention the subcutis. As illustrated by H&E-stained images, severe inflammation still existed in the subcutis on day 3. The necrosis started to partly drop off on day 5, and subsequently on day 8 the plasma-treated mice showed good healing of the ulcer area. In addition to the regenerated epidermis, abundant hair follicles and new collagen bundles were observed on day 11 as illustrated by the H&E-stained images. By contrast the untreated mice still showed inflammation in the subcutis, with thick edematous epidermis. Similar slight improvement in ACD healing was observed using the humid gas flow alone. On day 11 the ulcer healed and inflammation in the subcutis had almost disappeared in the GF group. However, after plasma exposure the ACD mice were cured with much better regenerated skin (normal coloured and smooth) as shown in Fig. 4.

A mechanistic study was carried to detect ROS on the mouse back using a luminol chemiluminescence reaction. Quite weak but still observable blue chemiluminescence was observed on the Ar + 0.5%H_2O plasma treated skin but not on the Ar + 1%O_2 plasma treated skin as shown in Figure S3. The chemiluminescence signal was much more obvious in the dark. This showed that H_2O_2 and other oxidative species were accumulated on the skin surface after 5 min plasma exposure. If the observed chemiluminescence is assumed to be induced by H_2O_2 only, then it was equivalent to a concentration of H_2O_2 of the order of 100 μM, as calibrated by standard H_2O_2 solutions.

4. Discussion

The enhanced healing effects induced by plasma exposures can be attributed to the reactive species transported from the discharge onto the ACD skin. Various reactive species including singlet oxygen ($^1O_2$) and ozone (O_3), reactive nitrogen species or nitrogen oxides N_xO_y (NO, NO_2, N_2O_3, *etc.*), and OH radicals are typically produced if gas admixtures of O_2, N_2, N_2 + O_2, H_2O are added to the argon discharge

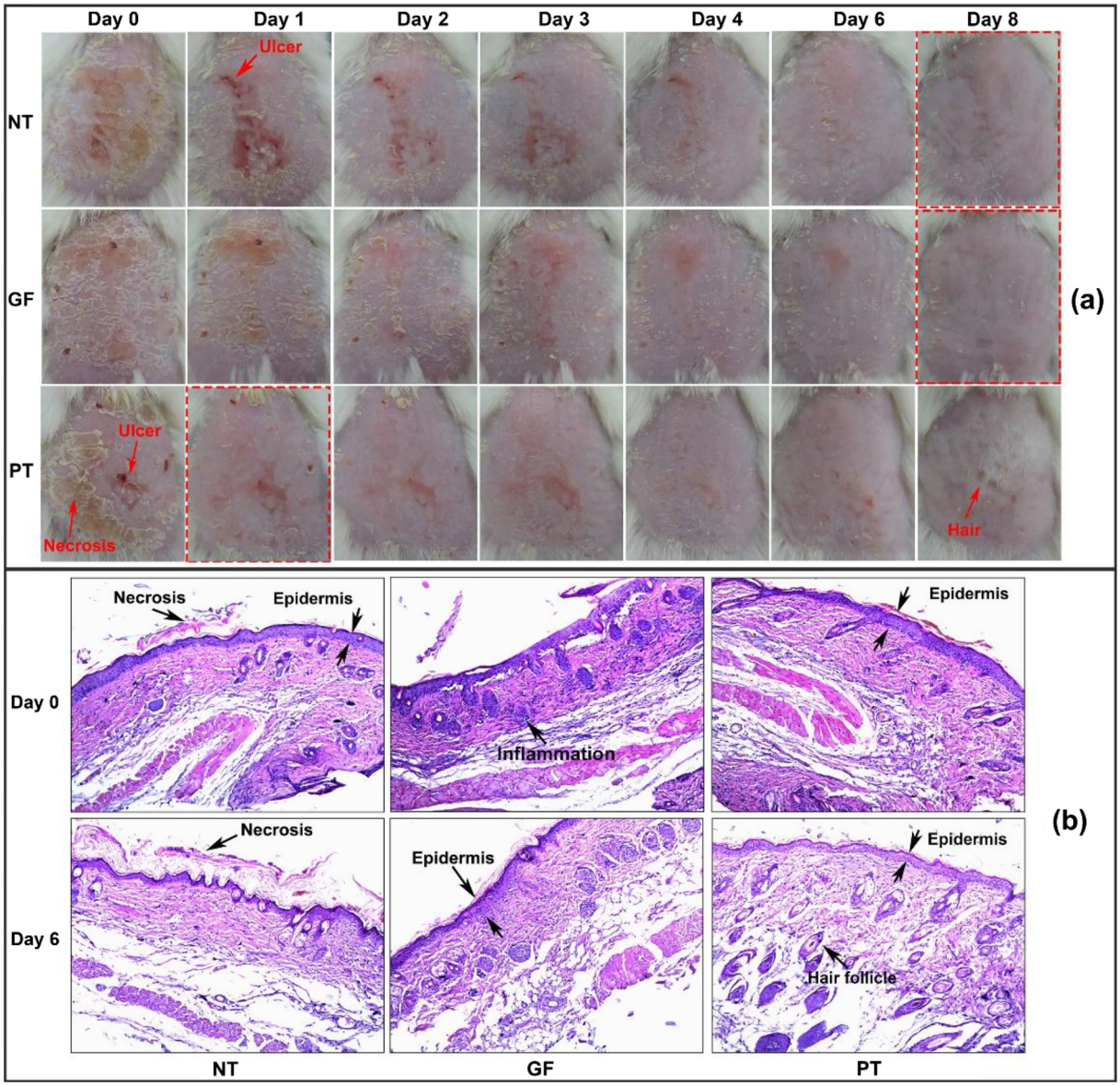


Fig. 3. (a) Moderate-degree ACD regions on mice back obtained from 0 to 8 days in groups of control (NT), gas flow (GF), and Ar + 0.5%H_2O plasma treatment (PT). Red dashed squares mark the day the ACD symptoms had resolved in the three groups. (b) Corresponding H&E staining (400 ×) images on day 0 and 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[4,7,13]. For instance Thi et al. reported that the high NO content in a cold N_2/Ar microplasma stimulated angiogenesis and epithelialization and enhanced the healing of burn wounds in mice [18]. Different treatment effects on ACD in mice were observed here using an argon discharge with different gas impurities. Enhanced skin restoration and regeneration was only achieved by humid plasma-exposure. The presence of moisture can be regarded as an important factor in the plasma treatment; on the one hand it can stimulate the production of reactive H_xO_y species (OH radicals, HO_2, H_2O_2, *etc.*), [26] and on the other hand it can assist the ability of these reactive species to permeate the affected skin and decrease the inflammation within the skin. Furthermore, water molecules can accumulate on the surface of the skin relieving dehydration caused by the AD [21,22]. However, the beneficial effects of moisturization alone are small compared to that of the humid plasma treatment. In the case of plasma treatment, reactive H_xO_y species are likely to be crucial agents resulting in the enhanced healing effects in the ACD mouse model. These active species are typically strong oxidants such as OH radicals that can modulate the T cells and regulate the immune response to decrease the release of inflammatory cytokines particularly those in the interleukin (IL) family [21]. As a result, the unbalanced immune system in the mouse is regulated and the inflammation is relieved, finally promoting the healing of the dermatitis lesions [27]. The therapeutic mechanisms induced by reactive H_xO_y species have been studied in detail in other reports in the field of plasma biomedicine [4,6,7,13,28].

The reactive oxygen or nitrogen species (RONS) produced in other gas-mixture discharges (without the addition of water) to not adhere and do not easily accumulate on the dry skin. Furthermore, the dry necrotic nature of the ACD lesions can act like a permeability barrier blocking these gaseous RONS from passing through the stratum corneum. These active agents are not able to permeate the subcutis to stimulate the immune responses in the same way as the H_xO_y species in humid plasma exposure. Therefore, unsatisfactory treatment effects were observed, as shown in Fig. 2.

The presence of H_2O_2 shown by chemiluminescence indirectly implies that other H_xO_y species like OH and HO_2 radicals could be expected to be formed in the plasma, as these species are important intermediates in the generation of H_2O_2 [29]. These H_xO_y species may

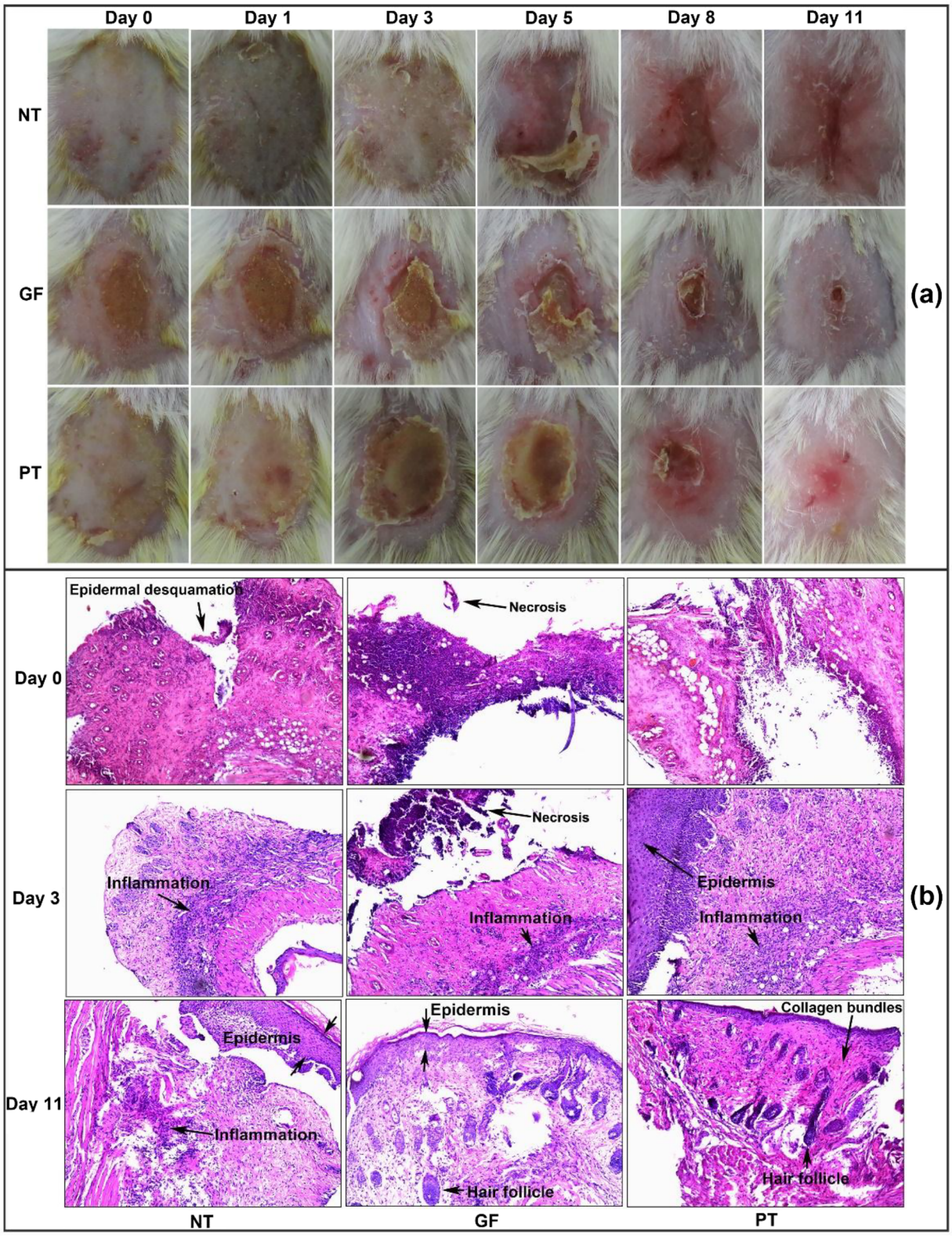


Fig. 4. Images of the severe-degree ACD regions on the mouse back obtained from 0 to 11 days in groups of control (NT), gas flow (GF), and Ar + 0.5%H_2O plasma treatment (PT). (b) Corresponding skin tissues on 0 and 11 day examined by H&E staining (400 ×). H&E-staining images on day 0 are not complete due to the difficulty of cutting the hard-and-dry scabs on the skin, however this did not affect the histopathological analysis of the skin lesions.

permeate the dry skin and diffuse into the subcutaneous inflammation region during or/and after plasma exposures, and stimulate remission of inflammation [14,30,31].

On the other hand, however, no chemiluminescence signal was detected from the region treated by Ar + 1%O_2 discharge, which demonstrates that almost no ROS (including singlet oxygen and ozone) were accumulated on the skin. This supports the above hypothesis that during the dry plasma exposures that contained O_2, N_2, or O_2 + N_2 impurities, poorly-adherent RONS were blocked from the dry skin and were not able to heal the ACD lesions.

In conclusion, the use of a cold argon plasma discharge with various gas impurities was investigated for treatment of ACD in a mouse model. Enhanced therapeutic efficacy against ACD was only obtained if water molecules were admixed in the discharge, and were effective even for severe ACD. The dermatitis symptoms were significantly relieved after a single humid plasma exposure for mild and moderate ACD lesions. For the severe-degree ACD (with ulcers) the healing phase was prolonged, but the mice still recovered at least one week earlier compared to the untreated group. Further study indicated the unsatisfactory effect of admixtures of oxygen or nitrogen could be attributed to poor adhesion and penetration of dry gaseous RONS into the dry skin. Addition of water vapor improved the wettability of the skin and promoted the accumulation and diffusion of reactive H_xO_y species into the subcutis, finally decreasing inflammation and encouraging healing. These preliminary results suggest that humidified atmospheric-pressure cold plasma could be a promising therapeutic alternative in dermatology to treat ACD and other inflammatory skin conditions. Future work should focus on the optimization of the healing effects of plasma on ACD in mice by changing the amount of water vapour in the discharge, and compare cold plasma to traditional medical treatment using corticosteroid ointment.

Ethical statement

All the animal experiments were conducted in accordance with the guidelines and the ethical standards of the Institutional Animal Care and Use Committee of the Third Military Medical University (Chongqing).

Conflict of interest

There are no conflicts of interest.

Author contributions

Qing Xiong, Xia Wang, and Rui Yin designed and supervised the experimental research; Lin Xiong, Qiang Chen, Meng-Xue Zheng, Le Xu, Qing-Hua Huang, performed the experiments; Qing Xiong and Michael R Hamblin wrote the paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cpme.2018.09.002.

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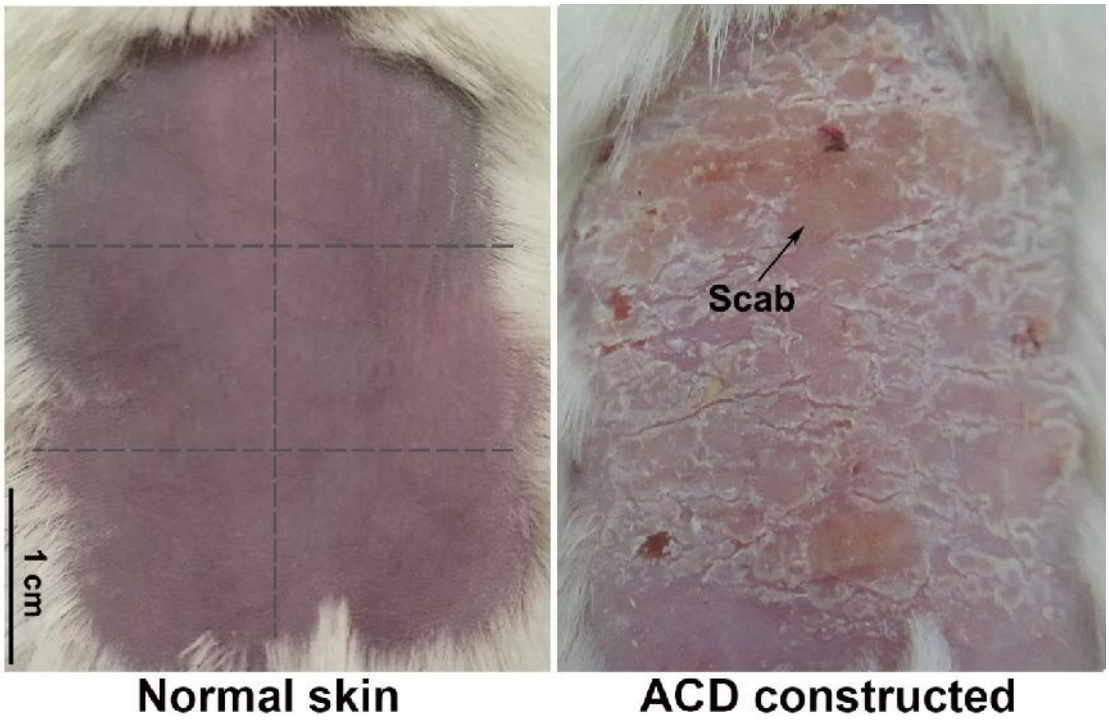


FIGURE S1 Images of skin on the mouse back (left) before and (right) after development of allergic contact dermatitis by application of DNFB hapten. It shows typical ACD symptoms such as scabs formed on the affected skin. The dermatitis area was divided equally into six regions for plasma exposures, as marked similarly by dash lines in the left normal skin image.

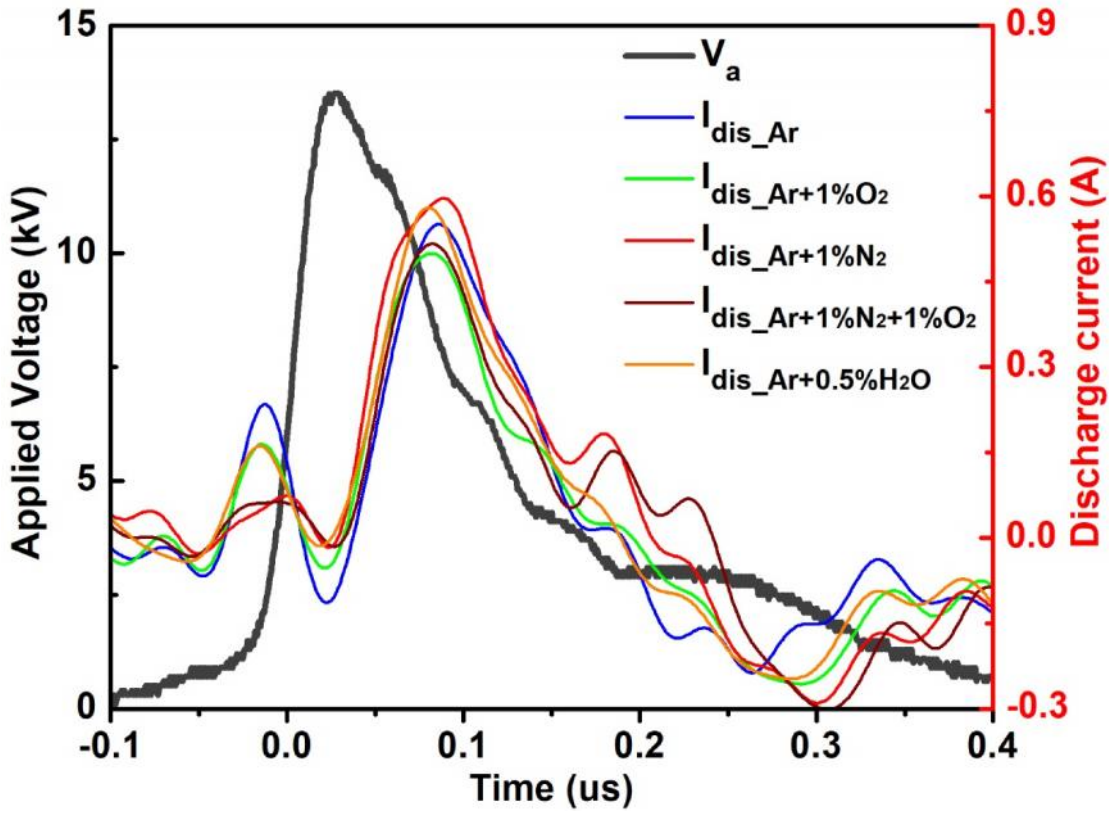


FIGURE S2 The UI characteristics of the argon discharge while treating the ACD area on the mouse back, with addition of 1%N_2, 1%O_2, 1%N_2+1%O_2, and 0.5%H_2O into the background argon gas. The applied voltage was kept constant as different impurities were added.

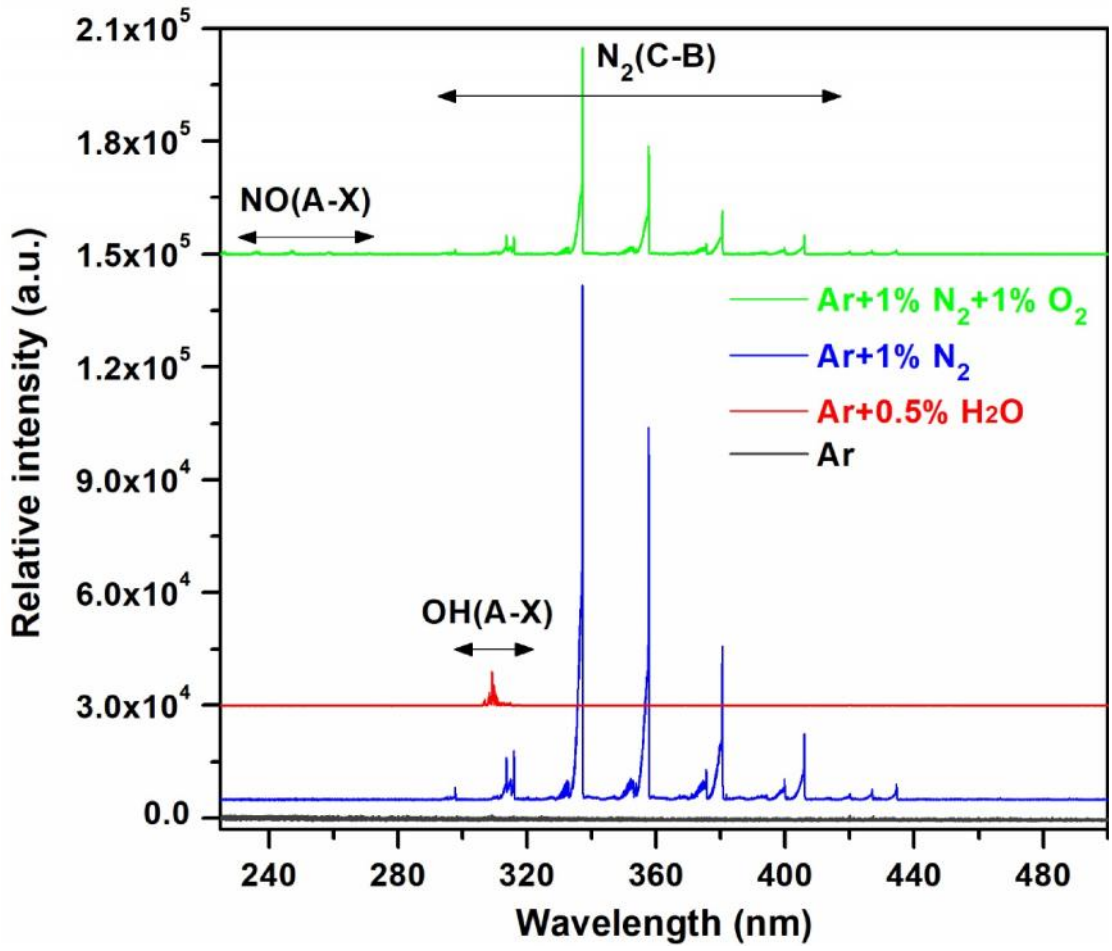


FIGURE S3. Optical emission spectra in the range of 220 to 500 nm from the argon discharge zone while treating the ACD area, with addition of either 0.5% H_2O, 1% N_2, or 1% N_2+1% O_2, respectively. The spectra were detected by a spectrograph system consist of an Andor monochromator (Shamrock 750) with 1200 g/mm grating and a CCD camera (Andor DU416A).