Wound healing using cold plasmas for: in vitro and in vivo study

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Abstract:
This study investigates both in vitro and in vivo effects of direct and indirect plasma treatment and their influence on wound healing. The effect of plasma jet treatment on cellular viability, migration and proliferation were studied using keratinocytes, fibroblasts and endothelial cells. A murine model is used to study burn and excisional wound healing, as well as collagen production ad angiogenesis.

Keywords: Cold atmospheric plasma, dermatology, wound healing, skin graft.

1. General

The skin, largest human organ, is composed of three layers: epidermis, dermis and subcutaneous fat tissue. Cutaneous injury repair involves complex biological processes, classically divided into three stages namely, inflammation, new tissue formation and remodeling. Inflammation occurs directly after tissue damage. Immune cells are recruited to remove non viable tissue and prevent infection. Homeostasis is achieved with the formation of platelet plug followed by a fibrin matrix. New tissue formation, the second stage of wound repair, is characterized by cellular proliferation and migration. Thus, keratinocytes in the epidermis, fibroblasts in the dermis and endothelials cells with the formation of new bloods vessels (angiogenesis) proliferate and migrate in the wound bed. In the later part of this stage, fibroblasts differentiate in myofibroblasts, contractile cells which assist in wound contracture. The third stage of wound healing is remodeling. Once the injury is repaired cells accumulated in the wound bed exit or undergo apoptosis and the extra-cellular matrix is remodeled [1]. The application of CAP in biomedical research has increased in the last ten years in several domains such as infection-related disease in dermatology [2-5].

In this study, *in vitro* experiments were first performed on three cells lines: keratinocytes, endothelial cells and fibroblasts. An optimum treatment time to ensure that skin cells remain viable to promote their migration *in vitro* was found. Three different types of treatment were compared: direct treatment of cells in media, indirect treatment using freshly prepared Plasma activated Media AM and treatment using chemicals. The chemicals used are long life RONS produced by plasma in liquids: H_2O_2, NO_2^- and NO_3^-. Furthermore, an *in vivo* trial was also conducted using a model a murine full-thickness excisional wound and as well third degree burn grafted with allogeneic skin

2. Experimental set-up

Plasma source: The plasma source used is a 3D printed plasma jet made in polylactic acid (PLA)-Graphite (resistivity ρ=44mΩ.m from Proto-Pasta) and a needle electrode is placed inside. The gas used is helium from (Air Liquide, Paris, France). Helium flows in a capillary made in the middle of the 3D-printed structure using a total flow rate of 500 sccm. The capillary has a length of 60mm and a diameter of 3mm. A 32 kV 50Hz power supply is used. In vivo, self-adhesive electrode WhiteSensor (Ambu, Ballerup, Denmark) placed near the treated area ensured the link to the capacitor for the power measurement (Figure 1).

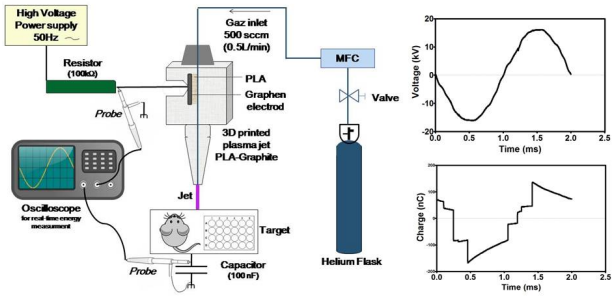

Figure 1: Schematic diagram of cold atmospheric plasma (CAP) generating system. (A) Voltage of the power supply measured by a high-voltage probe (B) Charge measured at the terminal of the measuring capacitor (Cm=105nF). 2ml of PBS exposed to the plasma source.

In vitro experiments: Adherent cells were cultured in confluent monolayers. Cells were seeded in a 24-well plate to be treated. Wells were filled with 2 ml of fresh media and the distance between the liquid surface and the plasma jet's nozzle was fixed at 5mm. The voltage was set at 32kV during the treatment. Treatment was direct (cells were treated in the media) or indirect using plasma activated media (PAM). In this case, the media was treated in the same conditions and immediately put on the cells. Cells were treated for 1, 2 or 3 minutes with the plasma jet. Cells were treated once a day over 24 or 48 hours. All experiments were performed in triplicates

In Vivo experiments: For the acute wound experiments, female Balb/C mice between 6 and 10 weeks of age (18-22g) were used (Janvier, Genest-St-Isle, France). All animals experiments were approved by the relevant animal ethics committee. Mice were anesthetized using 4% isofluorane and hair was removed from the dorsum of the mouse using hair clippers and depilatory cream. A full-thickness wound measuring 6 mm in diameter was

induced on the dorsum surface of mice using a biopsy punch (Dutscher, Brumat, France). Experiments were carried out using six mice per groups. For the burn wound experiments, a third degree burn (80°C for 20 seconds) was created on the dorsum of the recipient mouse, the burn was left for 24 hours, and a 15 mm diameter circle over the burn site was excised prior to skin grafting. In all instances skin graft refers to the use of a full-thickness skin graft fashioned from donor mouse tail. Donor tail skin grafts (15 mm) were laid over the excised burn wounds. The skin grafts were then secured with a Leukosan adhesive surgical glue purchased from BSN medical (Quickbornstraße, Hamburg, Germany).

3. Results

3.1 Production of RONS by the plasma jet

The following table shows values of deposited energy and production of H_2O_2, NO_2^- and NO_3^- in 2 ml treated PBS. The level of NO in media could not be measured. Thus, it was assumed that NO produced by our plasma was below the detection range (5nM) of the method.

	24-well plate		
	1min	2min	3min
Energy (J)	4,3	9,1	13
Energy density (J/cm3)	2,2	4,5	6,5
H2O2 (µM)	8	14	23
NO2- (µM)	1,2	2	2,6
NO3- (µM)	0,5	0,65	1,4

Table 1 : Energy calculated from the power measurement and concentration of H_2O_2, NO_2^- and NO_3^- in 2ml of media treated by CAP in a 24-well plate. The energy is represented for two different biological targets: (helium flow = 500sccm, height = 5mm, peak to peak voltage = 32kV) as a function of treatment time. Each value is the average of 3 different treatments with SEM

3.2 Toxicity on cells

Firstly, the toxicity of our plasma device treatment (1, 2 or 3 minutes) on keratinocytes, endothelials cells and primary fibroblasts was tested. Two tests were performed to check the cytotoxic effects of plasma treatment: a LIVE/DEAD assay which gave us the percentage of dead cells and a WST-1 assay which enable quantification of cells proliferation, viability and cytotoxicity. Cells were treated every 24 hours and the percentage of mortality was measured 24 hours of 48 hours after the first treatment. Indirect treatment from 30 seconds to 3 minutes had no toxic effects on skin cells or didn't affect significantly their proliferation during the observation period of up to 48 hours. Direct treatment, as indirect treatment, had no effect on cells proliferation. However a direct treatment of 3 minutes led to an increase of the percentage of dead cells. Furthermore, endothelial cells were the most fragile cells among the three studied with an increased of cell mortality by 70%. Direct treatment was more damageable for the cells than indirect treatment. Thus, long live species in PAM as H_2O_2 had no effect on cell viability. To verify this assumption, cells viability was measured after a treatment using H_2O_2 up to 150µM equivalent to 10 minutes of plasma treatment, equivalent to 10 minutes of plasma treatment. Treatment with H_2O_2 did not induce cell toxicity. Thus, plasma toxicity on cells after treatment longer than 3 minutes might be due to oxidative stress from short-life ROS or electromagnetic field.

3.3. Effect on cell migration

Then the effect of the plasma treatment on cell migration was evaluated using scratch assay. Cells were seeded in a 24-well plate (250 000 cells/well). Cells were allowed to grow to 90% confluence. Then, a scratch was mechanically done in the middle of the wells using a 1mL tip. The long-axial of the tip should always be perpendicular to the bottom of the well. The resulting gap distance therefore equals to the outer diameter of the end of the tip. After scratching, the well was washed with fresh medium to remove detached cells. The cells were treated in 2 ml of fresh media one (T24) or two time (T0 and T24 hours). The first treatment was performed immediately after the scratch. 3 types of treatments were processed for this experiment: direct treatment, indirect treatment (PAM) and a treatment with H_2O_2, NO_2^- and NO_3^- at a concentration equivalent to 1minute of plasma treatment. Photos of the scratch were taken 0,24 and 48 hours after the first scratch. The gap distance was evaluated using ImageJ. Results were represented as the mean percentage of the original wound at T0. Migration of keratinocytes, fibroblasts and endothelials cells in the wound bed is a key process which aids timely wound closure. The effect of direct and indirect CAP treatment on migration of these three skin cells was studied using a scratch assay (figure 2). Cells treated directly by plasma during an optimized duration were migrated faster. Thus, with keratinocytes and endothelials cells, 1 and 2 minutes of treatment led to a faster scratch closure 24 and 48 hours after the scratch. At 24 hours, the surface of the scratch in the treated keratinocytes wells was 70% smaller than in the control. For endothelials cells, after 24 hours, the surface of the scratch in the treated wells was about 50% smaller compared to the control. For the primary fibroblasts, only direct treatments during 1 minute had a significant positive effect on their migration with a reduction of the scratch surface of 25%. Contrary to direct treatment, PAM (indirect treatment) had no effect on the migration of all cell types whatever the treatment time. Thus, the set of long-live species composing the PAM didn't induce cell migration in this range of concentration. However, each species may have a positive or negative effect resulting in an auto-compensation. In order to answer this question, the same scratch assay treating cells was performed using H_2O_2, NO_2^- and NO_3^-. Concentrations used were equivalent to what is produced

by 1 minute of plasma treatment in 2 ml of PBS (*cf*. table 1).

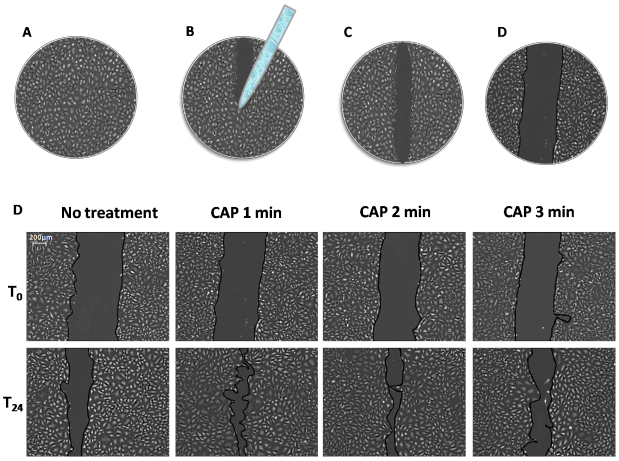

Figure 2: *In Vitro* scratch wound healing assay method. (A) The cells are seeded in a 24-well plate and allowed to grow to 90% confluence. (B) A scratch is mechanically done in the middle of the well with a 1ml tip. (C): Treatment is done 0 and 24 hours after the scratch has been done. (D) Photos are taken 0, 24 and 48 hours after the scratch. At the different time point, the gap area was evaluated with ImageJ (E) Representative images: effect of a direct plasma treatment on HMVEC migration. The scale bar is the same for all the images.

For all studied cells, 1 min of treatment with plasma led to an acceleration of their migration rate. These species were tested separately and mixed (the treatment is called mix). Chemical composition of the mix (chemical compounds dissolved in water) is similar to that of PAM. It was found that none of these long-live species, alone or together had an effect on cells migration. These results taken together could mean that short-live species and possibly the pulsed electric field were responsible for boosting the scratch wound closures for the three cell lines studied since only direct treatment impacts migration.

3.4 In vivo wound healing

Direct plasma treatment did not accelerate the wound closure during the 3 days of wound size measurement. Direct plasma treatments of 10 seconds (one or three time) and 30 second (one time) had no significant positive or negative effects on the wound closure. However, direct treatments of 30 seconds repeated every 48 hours significantly decreased the wound closure rate one week after the wound induction. All the wound specimens were subjected to histological analysis at day 7 histological size of the wound measured after H&E staining. There was no significant difference between histological wound sizes of day 7 wound treated with PAM compared to wound treated with PBS. Thus, neither direct treatment, neither indirect treatment was efficacious for the acceleration of wound healing in this model of full-thickness wound.

Excised and grafted wounds treated with either CAP (voltage set at 32 kV), topical application of NO donor (1.5 mg) or transdermal application of NO donor (1.25 mg) showed no signs of transplant rejection. On the contrary, clear signs of graft healing were observed at day 7 after burn induction. In contract, wounds treated with NO synthase inhibitor (L-NAME) showed signs of delayed healing. Histological dermal wound gape was determined by measuring the distance between the dermal margin of the burn wound and dermal margin of transplanted skin graft. Histological wound gape was significantly smaller in wounds treated with CAP, topical and transdermal NO donor compared to time-matched helium treated controls.

4. References

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