Biological solutions activated by cold plasma at atmospheric pressure (PAM) : a potentially new therapeutic approach for skin wound healing

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RESUME

Cold atmospheric pressure plasmas, which are partially ionized gases, are used for a variety of biomedical applications, such as the decontamination of surgical instruments. Recently, an alternative method has been developed that is easier to implement: the use of cold plasma-activated media (PAM). PAM treatment of skin wounds appears to promote healing. Thanks to the reactive oxygen and nitrogen species they contain, PAMs stimulate the migration of keratinocytes and fibroblasts, as well as angiogenesis, essential mechanisms for wound healing, while inhibiting bacterial proliferation. To overcome the limitations of traditional *in vitro* and *in vivo* models of skin wound healing, we developed a standardized *ex vivo* model using burned human skin explants, that preserves full skin architecture and enables detailed analysis of wound healing processes in a human-specific context. This reproducible superficial burn model allowed us to monitor wound healing over time, paving the way to assess and understand the molecular effects of PAM on wound repair.

Key words

Cold atmospheric plasma; plasma-activated media; RONS; skin wound healing; chronic wounds; cellular migration

1. INTRODUCTION

Skin wound healing is a physiological process characterized by a precise sequence of events requiring the coordination of the different cell types involved in the restoration of the skin barrier integrity. The process of skin wound healing comprises four successive yet overlapping phases: hemostasis, inflammation, proliferation and remodeling[1]. A wound that does not heal within 6 weeks is considered as chronic. Chronic wounds are skin lesions that do not progress through the various phases of healing, and often remain in an inflammatory phase, preventing epithelial cells from closing the epidermis and exposing it to the risk of infection[2]. In addition, the presence of infection due to colonization by bacterial species such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* is an important factor in chronicity.

In 2012, approximately 650,000 individuals in France were estimated to be affected by chronic wounds, with a higher prevalence observed among the elderly[3]. Chronic wounds are associated with a high level of morbidity and a significant impact on patients' quality of life. Such wounds require multiple, sometimes extensive interventions, which can result in amputation. The increasing prevalence of chronic wounds as populations' age and the resulting rising in healthcare costs represents a major public health challenge. New treatments for these wounds are therefore needed. Several studies have shown that cold plasma generated at atmospheric pressure (CAP) has beneficial effects on wound healing, making it a potential new therapeutic approach[4].

Plasmas are partially or totally ionized gases composed of electrons, ions and electrically neutral species that occur naturally at very high temperatures or are generated by the continuous application of thermal, electrical or electromagnetic energy. Although plasma is electrically neutral on a macroscopic scale, due to the presence of particles carrying positive and negative electrical charges, it is a conductive, highly reactive medium that can be influenced by electrostatic and electromagnetic fields and forces. During plasma generation, various elements can be produced such as light (ultraviolet, visible and infrared rays), thermal radiation, electromagnetic fields, ions and reactive oxygen and nitrogen species (RONS)[5].

Plasma can be generated at temperatures as low as 40°C and at atmospheric pressure, and thus is called CAP. Recently, CAP has been proposed for use in biomedical applications[6]. Its low temperature makes it compatible with tissue viability. CAP is mainly used for decontamination, as it inhibits the proliferation of several bacterial species. At high doses, plasma may have an anti-cancer effect, as it specifically increases apoptosis of cancer cells *in vitro* and inhibits their migration. Since plasma can act on the skin and has healing properties, direct treatment with CAP has been proposed for chronic wounds. Treatment with CAP accelerates skin wound healing in preclinical *in vivo* models in rats and mice. Clinical trials have also demonstrated the therapeutic potential of CAP in chronic human wounds.

However, CAP treatment can have adverse effects at high doses, and it is difficult to treat large surfaces uniformly due to the limited diameter of the plasma plume.

Recently, an alternative method to direct CAP treatment has been proposed: the use of plasma-activated biological solutions, known as plasma-activated media (PAM) or plasma-activated liquids (PAL). Various biological media can be activated, such as cell culture medium, PBS or water. The advantage of this method is that it eliminates the harmful elements produced by plasma, but retains the ions and RONS[7]. To study the effects of PAM on wound healing, various activated media were applied *in vitro* to the cutaneous cell types involved, such as keratinocytes, fibroblasts or endothelial cells, and *in vivo* in preclinical models[8].

The biological effects of PAM are highly dose-dependent, influenced by variables such as device type, plasma exposure duration, and application intensity. Excessive plasma doses increase reactive RONS generation, potentially shifting effects from therapeutic to cytotoxic. Accurate quantification of these species is essential to elucidate PAM's biological impact. While conventional colorimetric and fluorometric assays are commonly used, advanced methods like ion chromatography and machine learning-based oxidation potential modeling offer improved precision, particularly in complex media. Machine learning also enables prediction of reactive species levels based on plasma parameters such as voltage, gas flow, oxygen content, and humidity[10].

2. EFFECTS OF PAM TO IMPROVE WOUND HEALING

PAM may act throughout the entire wound healing process, notably on the various cellular and molecular players involved[9].

2.1. Cellular players

The difficulty in interpreting published results concerning PAM treatment of keratinocytes, fibroblasts and endothelial cells lies in the plasma parameters, media activation time (and therefore the dose delivered), the gas used (air, argon or helium), and the cell type used (lines or primary cells). For example, treatments with media activated for too long (high dose) have detrimental effects on cells, leading to cell cycle arrest and induction of apoptosis. On the other hand, treatment with activated media for short periods of time appears to have beneficial effects, in particular by stimulating cell migration. Conflicting results have been published regarding the viability of human keratinocytes after treatment with PAM. For Lee *et al.*, PAM (water activated with air plasma jet; treatment for 24 hours) was not cytotoxic, did not affect proliferation, but increased migration of the HaCaT cell line and primary keratinocytes[11]. However, another study showed that PAM treatment (culture medium activated with helium plasma jet for 60 to 180 sec) did not alter the migration of HaCaT keratinocytes. PAM also promotes angiogenesis, which is essential for healing. It has been shown to stimulate the proliferation of human umbilical vein endothelial cells (HUVECs) as well as the formation of endothelial tubes.

2.2. Molecular players

Growth factors, particularly vascular endothelial growth factor (VEGF), are essential for stimulating endothelial cell expansion. Studies have demonstrated that PAM can enhance VEGF production both *in vitro* and *in vivo*.

Other molecules, such as matrix metalloproteinases (MMPs), participate in tissue remodeling and facilitate stromal cell migration by degrading extracellular matrix components. The treatment of HaCaT, HUVECs and BEAS-2B (a human bronchial epithelial cell line) cells with PAM has been shown to induce an increase in both the expression and the activity of MMP-2 and MMP-9.

Chronic wounds often remain in an inflammatory state that does not allow the proliferative phase to occur. The inhibitory effect of PAM on inflammation was supported by decreased levels of proinflammatory cytokines, including interleukin (IL)-1, IL-6 and tumor necrosis factor α (TNF-α), and increased levels of IL-10 following the treatment of LPS-activated M1 macrophages. A comparable modulation of cytokine expression was observed *in vivo* in the wounded skin of mice treated with water-activated plasma.

2.3. Mechanism of action

It has been suggested that the RONS generated by CAP are the primary mediators of its effects on living cells[12]. Indeed, RONS mediate cell adhesion and migration, as well as the immune response, by acting on various signaling pathways. PAM treatment of HaCaT keratinocytes has been observed to induce an increase in intracellular and mitochondrial RONS. The addition of a ROS chelator resulted in a return to normal cell migration and normal expression of molecular players involved in this process, including MMP-2, integrin β1, p-FAK and p-paxillin. As previously stated, these are increased by PAM. Thus, it appears that the RONS produced by PAM are responsible for stimulating cell migration.

Despite these advances, understanding of the mechanisms involved in the beneficial effects of PAM on skin wound healing remains incomplete.

3. ESTABLISHING A RELIABLE HUMAN SKIN INJURY MODEL TO INVESTIGATE PLASMA-INDUCED EFFECTS ON WOUND HEALING

To study PAM effects on wound healing, the major models used are cell cultures in monolayers and animals. However, *in vitro* models lack of tissue complexity, with no vascularization and immunity, and *in vivo* models, while valuable for replicating complex physiological processes, are limited by interspecies differences, ethical constraints, high costs, biological variability, and challenges in accurately modeling chronic human wound conditions. *Ex vivo* models serve as an intermediate between *in vitro* and *in vivo* systems. These models retain the full skin architecture and viability for extended periods under organotypic culture, allowing the analysis of key wound healing processes such as keratinocyte migration, extracellular matrix remodeling, and inflammatory mediator production in a human-specific, near-physiological setting.

We aimed to develop a standardized and reproducible model of skin injury and wound healing using *ex vivo* cultures of human skin explants to investigate the effects of PAM on wound healing.

To achieve this, initial efforts focused on optimizing the culture conditions. Explants obtained from abdominal skin samples of adult healthy volunteers undergoing abdominal surgery could be maintained in culture at the air-liquid interface for up to seven days without inducing apoptosis using a 50/50 (v/v) mix of Dulbecco's Modified Eagle Medium and Epilife as the culture medium.

Subsequently, we established a reproducible protocol to generate a standardized burn model. A thermal injury was inflicted to the skin samples using a metallic homemade device

(5 cm long x 2 mm wide) heated in a controlled manner, followed by mechanical removal of the damaged epidermis. Biopsy punches along the burned area were realized and then cultured individually (Figure 1B). The resulting wounds were highly uniform and reproducible across samples (Figure 1A and B) with a 2321 ± 322 µm wide. This procedure consistently produced superficial second-degree burns, characterized by complete epidermis removal while preserving the underlying dermis and the dermo-epidermal junction (DEJ). Integrity of the DEJ was confirmed via immunohistochemical staining for collagen IV (Figure 1C), a structural marker of the basement membrane.

Wound healing dynamics were monitored over time during 4 days (Figure 1D). Keratinocyte migration onto the wound bed began as early as day 1, forming a re-epithelialization tongue. By day 4, wound closure was observed, followed by epidermal stratification.

To further characterize the model, the expression of key markers of keratinocyte proliferation and differentiation was observed. Keratin 6 (K6), which is typically upregulated during the wound healing process, was detected within a few days post-injury (Figure 2A). Ki67, a marker of cellular proliferation and keratin 10 (K10), a marker of epidermal early differentiation, were also expressed (Figure 2B and C), confirming the activation of regenerative processes in the explants. Corneodesmosin (CDSN), a marker of the granular layer of the epidermis, was expressed mainly at the edges of the wound (Figure 2D), because the epidermis has started to differentiate. However, another marker of the uppermost epidermal layers, β2-defensin (BD-2), was not expressed in the neo-epidermis (Figure 2E), indicating insufficient epidermal differentiation. Extending the culture period of the explants to 7 days might have permitted the formation of a fully stratified epidermis, enabling the expression of late differentiation markers such as BD-2.

Having successfully established and thoroughly characterized a wound healing model, it is now possible to investigate the effects of plasma treatment. To this end, a comparison between untreated skin explants and those exposed to PAM will be realized, in order to confirm that plasma application accelerates the wound healing process. This will subsequently enable exploration of the cellular and/or molecular mechanisms underlying this effect, with particular attention to the role of RONS generated within the PAM.

4. CONCLUSIONS

Chronic wounds represent a major public health concern, given the growing number of individuals affected by this condition as a consequence of population aging. Conventional treatments have proven ineffective in some cases, underscoring the urgent need for novel technologies to address this issue. Indeed, a number of *in vitro* and *in vivo* studies have demonstrated that the activation of liquids by cold plasma at atmospheric pressure exhibits healing properties. In particular, these activated media have been demonstrated to inhibit bacterial proliferation and stimulate angiogenesis and cell migration in a number of skin cell types, including keratinocytes and fibroblasts. PAM treatments act at the molecular level, stimulating the production of growth factors or enzymes such as MMPs, which are involved in the healing process. Additionally, they act on proteins involved in the formation and modification of the actin cytoskeleton. These actions would be mediated by the RONS generated in the media following plasma activation. PAM treatments may also have applications for other skin pathologies, such as inflammatory diseases and cancers. This is due to their capacity to reduce the production of proinflammatory cytokines in allergic pathologies and induce apoptosis in cancer cells. However, the challenge inherent to PAM treatments lies in the plasma parameters, activation times, and gas used, as evidenced by the conflicting findings regarding the impact of activation duration. While treatments with solutions activated for short periods have been shown to improve wound healing, treatments with solutions activated for extended periods result in a decrease in cell viability and in apoptosis. The use of PAM appears to have many potential applications in dermatology, in particular to promote wound healing. Further studies are required to understand the mechanisms of action at the molecular level. To address these challenges, we established a reliable human skin injury model to investigate the effects of plasma on wound healing. This standardized and reproducible model enables consistent wound generation, providing a validated model to elucidate the molecular mechanisms involved in PAM-mediated enhancement of the healing process.

5. REFERENCES

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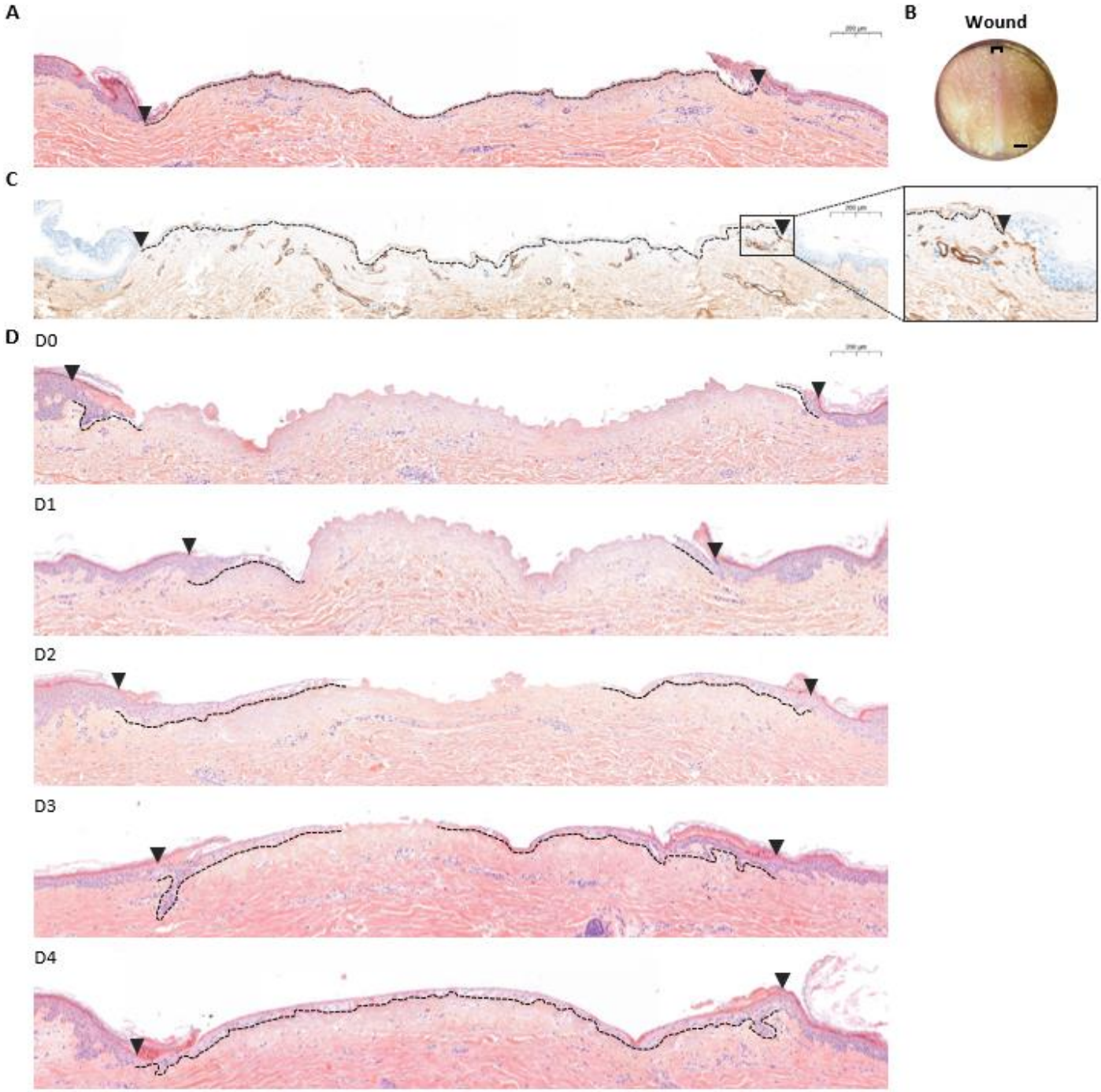

Figure 1. The standardized superficial second-degree burn wound closed in 4 days

(A) Representative hematoxylin and eosin (H&E) staining images of the burn wound immediately after induction. Black arrows indicate the wound edges, and the dotted line marks the wound bed. Scale bar = 200 µm. **(B)** Macroscopic image of an 8 mm in diameter skin explant featuring a precisely defined 2 mm-wide linear burn. Scale bar = 2 mm. **(C)** Representative immunohistochemical staining for collagen IV (ochre color) in the wound area immediately after burn induction. Black arrows indicate the wound edges, and the dotted line marks the dermo-epidermal junction within the wound bed. Scale bar = 200 µm; inset zoom = 20 µm. **(D)** Representative hematoxylin and eosin (H&E) staining images of the burn wound over time from day 0 (D0) to day 4 (D4). Black arrows indicate the wound edges, and the dotted line marks the progression of the keratinocyte tongue on the wound bed. Scale bar = 200 µm.

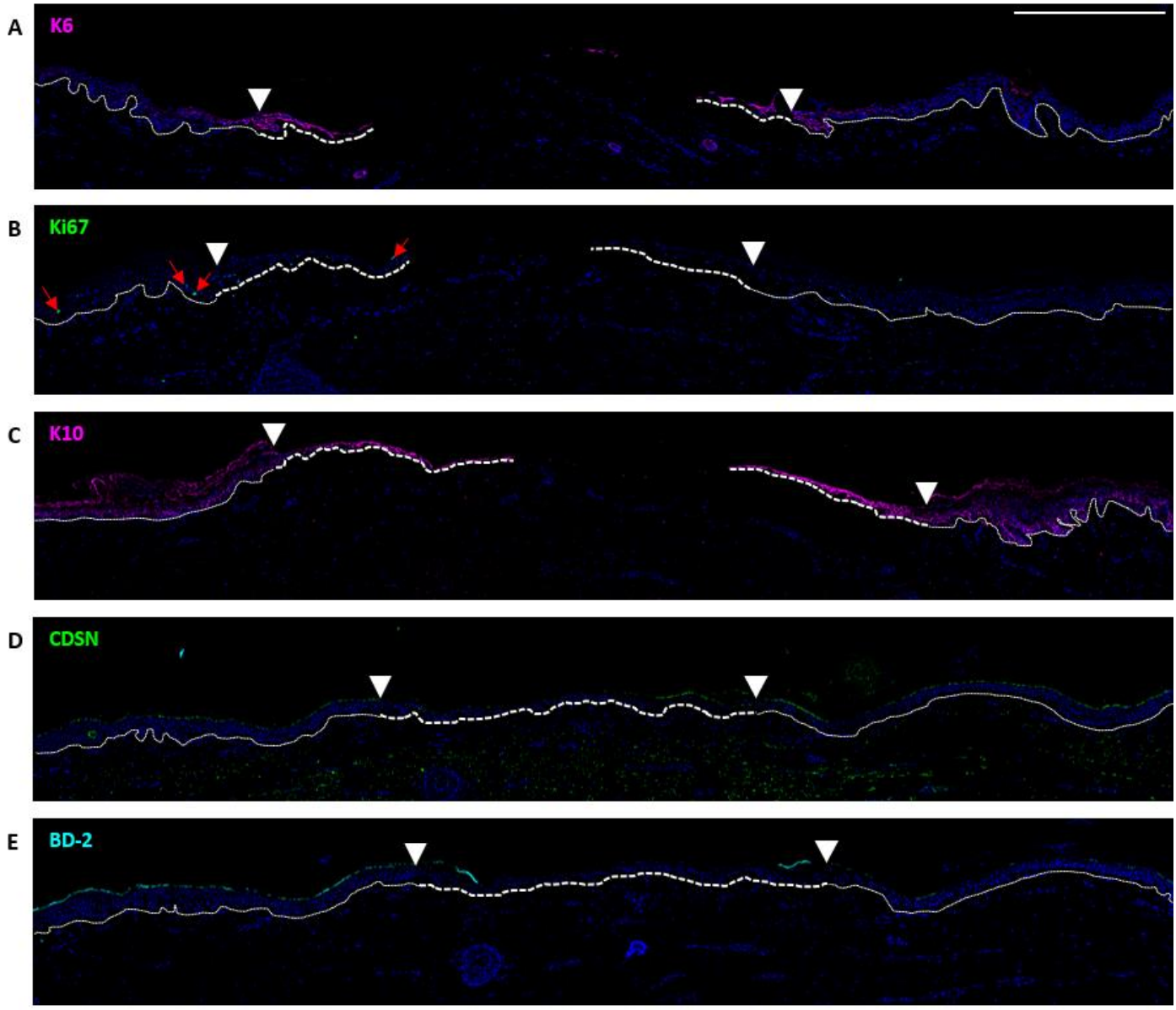

Figure 2. The wound healing model expressed keratinocyte differentiation and proliferation makers

(A) Indirect immunofluorescence staining of K6 (magenta) at day 2. White arrows indicate the wound edges, the thick dotted line marks the reepithelization tongue, the thin dotted line marks the dermo-epidermal junction. Scale bar = 500 µm. **(B)** Indirect immunofluorescence staining of Ki67 (green) at day 3. White arrows indicate the wound edges, the thick dotted line marks the reepithelization tongue, the thin dotted line marks the dermo-epidermal junction. Red arrows indicate positive cells for Ki67. **(C)** Indirect immunofluorescence staining of K10 (magenta) at day 2. White arrows indicate the wound edges, the thick dotted line marks the reepithelization tongue, the thin dotted line marks the dermo-epidermal junction. **(D)** Indirect immunofluorescence staining of CDSN (green) at day 4. White arrows indicate the wound edges, the thick dotted line marks the reepithelization tongue, the thin dotted line marks the dermo-epidermal junction. **(E)** Indirect immunofluorescence staining of BD-2 (cyan) at day 4. White arrows indicate the wound edges, the thick dotted line marks the reepithelization tongue, the thin dotted line marks the dermo-epidermal junction.