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Cold Atmospheric Plasma: A New Therapeutic Modality in The Treatment of Wound

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Abstract

Cold atmospheric plasma is an emerging field in medicine, especially in dermatology with promising applications for skin aesthetics, wound healing, skin malignancies, and the treatment of various skin diseases (e.g., psoriasis, atopic dermatitis, scleroderma, etc.). Among them, the most studied is the role of plasma in wound healing. This paper reviews three aspects of the mechanisms by which cold atmospheric plasma promotes wound healing, details the effects of cold atmospheric plasma on skin cells, skin tissues, and on trauma-loaded microorganisms, and provides a brief analysis on the biosafety of cold atmospheric plasma.

Keywords

Cold atmospheric plasma; Skin cells; Skin tissue; Microorganism; Biosafety.

1. INTRODUCTION

Plasma is a substance that exists in nature at the same level as solids, liquids and gases and can be produced under natural or artificial conditions. It is an objective system consisting of a large number of positively charged ions and negatively charged electrons, as well as some other neutral particles (atoms and molecules) that have not been ionized[1] . Plasmas can be broadly classified into two main categories: High temperature plasma, and Non high temperature plasma. High temperature plasma are mostly observed in nature such as thunderstorm lightning, Due to its high temperature and unstable physical properties, it is difficult for researchers to conduct deeper scientific research on it. Non high temperature plasma is further divided into cold high-pressure plasma and cold atmospheric plasma, both of which produce temperatures close to the temperature of the human skin; the former requires operation under vacuum, which results in relatively high generation costs and tends to produce excessive discharge, while the latter can operate at atmospheric pressure and has a relatively mild discharge pattern, making it more suitable for application on cells, animals and tissues[2] . Most of what is studied in modern medicine is cold atmospheric plasma (CAP). CAP generates a large amount of active substances such as reactive oxygen(ROS), reactive nitrogen(RNS), ultraviolet light, electric field, heat, etc. through electrical discharge. CAP devices for laboratory and clinical use are divided into three main categories: those based on direct discharge (such as DBD); those based on indirect discharge (sunch as plasma jets, plasma pens etc.); and hybrid plasma devices, but are currently used only in experimental applications[3-5] . CAP can also be divided into direct and indirect plasma in terms of the way it is used. In direct plasma applications, cells are exposed to plasma discharges in vitro and animal models and human tissues are exposed to discharges in vivo. Whereas plasma activated medium or solution, applied to cells, tissues or animals is called indirect plasma. Plasma devices used in clinical and laboratory settings have been repeatedly validated by national and international research groups and no significant side

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effects have been found[6-9] . However, it is important to standardize the treatment modality and strictly control the treatment dose of CAP to reduce the potential risks during use.

Wound healing is a complex and coordinated process, which can be broadly divided into four phases: the hemostatic phase, the inflammatory phase, the proliferation phase with the formation of vascular and granulation tissue, and the re-epithelialization phase with tissue remodeling[10] . The hemostatic phase is characterized by vasoconstriction, platelet aggregation, and activation of the complement system. This is followed by the migration of neutrophils and macrophages to the wound surface, initiating the inflammatory response[11] . The inflammatory phase is characterized by increased skin temperature, redness and pain. At the end of the inflammatory phase, the proliferative phase is entered, which requires the involvement of a large number of cells, growth factors, and enzymes to achieve neovascularization and granulation tissue production[12] . It eventually enters the remodeling phase, which can last for one year or more, where the abundant type III collagen is gradually replaced by type I collagen with the involvement of multiple cells, transforming factors, and matrix metalloproteinases(MMPs) to form a mature scar[13] .

Skin is the largest organ of the body and the first line of defense against external irritants. When the integrity of the skin is compromised, various acute and chronic wounds will be formed. The existing wound treatment methods include debridement of necrotic tissue, sterilization, wound dressings and compression bandages[14] . Antibiotic drugs are the most commonly used topical antiseptics which can kill microorganisms on the surface of wounds, However, repeated use will increase the risk of antibiotic resistance, resulting in less than optimal final treatment outcomes. Debridement refers to the removal of necrotic tissue and cleaning of the wound to achieve healing effect, but it is easy to cause damage to normal tissues at the same time, as well as pain may lead to intolerance of partial patients[15，16] . Wound dressings are also a common method to treat wounds, with a wide variety of dressings ranging from simple sterile bandages to complex stem cell engineered dressings, but most wounds need to be changed every 1-3 days in the early stages of formation, which is not very convenient for remote areas. The remaining novel treatment modalities such as hyperbaric oxygen and nanomaterials are being developed, but their relatively high cost is difficult to ignore[17,18] . Although there are enough modalities available to treat trauma, there is still a huge resistance to treatment and an urgent need to introduce new alternatives.

As an emerging field of research, CAP has proven its great potential in wound healing with its multi-pathway mechanism of action; CAP can reduce the microbial load of wounds and restart the stalled healing process[19,20] ; induce proliferation and migration of keratinocytes[21-24] , modulate the inflammatory response, enhance angiogenesis and collagen deposition, and accelerate re-epithelialization[25-27] . Because of its lower cost, ease of use, wide range of action, and good biocompatibility, CAP has been investigated for a variety of medical applications, such as dermatology, dentistry, plastic surgery, and regenerative medicine. Although several groups at home and abroad have demonstrated that CAP is safe and harmless to cells and tissues, the therapeutic dose of CAP is difficult to be precisely controlled in clinical applications, which urgently needs to be effectively addressed. However, the great potential shown by CAP in the wound healing process makes it promising as a new modality for treating wounds.

2. EFFECT OF CAP ON SKIN CELLS

Among the national and international studies, several in vitro studies focused on the effect of CAP on non-diseased skin cells（Table 1）. Most of these research teams chose human primary keratinocytes as a cell model. Keratinocytes are the most predominant cell type involved in constituting the epidermis and enable wound repair by continuously proliferating and

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migrating directionally into the wound bed during skin regeneration. The researchers found that CAP not only induces cell proliferation and migration, but their expression at the protein and gene level is also affected accordingly.

In 2013, Arndt et al. used MicroPlaSter to study Primary human dermal fibroblasts in vitro. They found that in the presence of CAP, keratinocytes migrated faster and proliferation was not significantly affected; while at the molecular and genetic levels, the expression of interleukin-6 (IL-6), interleukin-8 (IL-8) and transforming growth factor (TGF) were found to be significantly up-regulated. Among them, TGF is an important transforming factor that coordinates wound healing and is involved in various activities such as cell proliferation and migration, angiogenesis, and tissue remodeling, suggesting that CAP may be involved in regulating the inflammatory phase and proliferative process during wound healing by affecting TGF expression. Additionally, CAP treatment accelerated the synthesis of α-SMA and type I collagen, both of which are important components involved in extracellular matrix synthesis[21] .

Primary human epidermal keratinocyte was also studied in vitro by Cui et al. They discovered that CAP induced the expression of angiogenic factors in keratinocytes in a HIF-1a-dependent manner. At the cellular level, the viability of keratinocytes after CAP treatment was first measured, and the migration ability of the cells was examined by scratch assay. To avoid the cell migration process from being affected by proliferation, mitomycin C was used to inhibit cell proliferation. The results showed that CAP treatment did not reduce the viability of keratinocytes and significantly affected cell migration. At the protein and gene level, the expression of Ang-1, Ang-2, VEGF-A, HB-EGF, FGF-2, FGF-10, and PDGF were found to be significantly induced to be elevated. Subsequently, they further confirmed that HIF-1a levels were also elevated by CAP induction, but after using CAY10585 (an inhibitor of HIF-1a accumulation and transcriptional activity), they found that HIF-1a expression induced by CAP was suppressed; also the expression of VEGF-A, Ang-1, and Ang-2 was suppressed, too. From the results, it was seen that the changes of VEGF-A, Ang-1, Ang-2 and HIF-1a were parallel, so they speculated that CAP may affect the expression of angiogenic factors in keratinocytes by regulating HIF-1a. HIF-1a may be an upstream regulator of cell migration[22] .

In another study, human keratinocytes were treated with He/Ar-CAPJ for 15s and a significant increase in cell proliferation and migration was observed. It was further confirmed at the molecular level that CAP decreased E-cadherin expression as well as affected the increased expression of N-cadherin, p-ERK, cyclin D1 and Cdk2. This suggests that CAP can have an impact on the proliferation and migration of keratinocytes by regulating epithelial-mesenchymal transition (EMT) and cell cycle progression[23] .

Similarly, Schmidt et al. pointed out that the nuclear factor erythrocyte-associated factor 2 (Nfr2) and phase II enzyme pathway components are key controllers in the regulation of keratinocyte behavior. They pointed out that CAP discharge produces a large amount of ROS, but this does not affect the viability of cells. In subsequent studies, they first identified differentially expressed gene profiles by transcriptome microarrays, followed by functional enrichment analysis and further identified the role of Nfr2 targets by siRNA silencing of genes, revealing that NRF2 is an important switch for sensing oxidative stress events[24] .

In addition to keratinocytes, the effects of CAP on various cell lines such as bronchial epithelial cells, osteoblasts, endothelial cells, and cortical astrocyte have also been studied by others, showing favourable promotion of cell proliferation, migration, and differentiation. However, repeated CAP treatment seems to have a negative effect on osteoblast proliferation, which may be related to the CAP dose; at the molecular and genetic level, appropriate doses of CAP show positive effects on cellular involvement in wound healing[28-31] .

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Table 1. The effect of CAP on skin cells.

Theme	Cell type	Cellular level	Molecular and Gene level	Refs
CAP triggers key events at the cellular level that are important for wound healing.	Primary human dermal fibroblasts	Cells migrated faster and proliferation was not significantly affected;	Increased expression of IL-6, IL-8, TGF-1, TGF-2,α-SMA, type I collagen.	[21]
CAP increases the production of angiogenic cytokines in a HIF-1α dependent manner in keratinocytes.	Human keratinocytes	CAP treatment did not reduce the viability of keratinocytes and significantly affected cell migration.	Promote the expression of Ang-1, Ang-2, VEGF-A, FGF-10, PDGF, HIF-1α.	[22]
CAP affects keratinocyte proliferation and migration by regulating EMT and cell cycle.	Human keratinocytes	Keratinocyte proliferation and migration were significantly increased.	The expression of E-cadherin decreased, while N-cadherin, p-ERK, cyclin D1 and Cdk2 increased.	[23]
Nfr2 and phase II enzyme pathway components are key controllers in the regulation of keratinocyte behavior.	Human keratinocytes	CAP produces a large amount of ROS, but this does not affect the viability of cells.	Non-thermal Plasma Activates Phase II Enzymes through NRF2 Pathway to Scavenge ROS.	[24]

3. EFFECT OF CAP ON SKIN TISSUE

The cellular effects induced by CAP were also reflected on the skin tissue. Researchers observed the effect of CAP treatment on skin tissue by hematoxylin-eosin(HE) staining, Masson trichrome staining and immunohistochemistry (IHC) analysis(Table 2). In a diabetic rat trauma model, histomorphological alterations were observed in CAP-treated wounds compared to controls, with the most significant differences in the epidermis. This was mainly reflected by faster proliferation of fibroblasts, more neovascularization, earlier epidermal formation, and thicker collagen deposition in dermis and keratin layer on skin surface. At the same moment of observation, the epidermal layers of CAP-treated diabetic rats were more clearly defined and collagen fibers were more closely arranged. In addition, a significantly higher number of positive cells for TGF-β was observed in the CAP group compared to the control group[25] .

In another study, also in a diabetic mouse trauma model, two CAP groups with different treatment doses were set up. Compared to the control, the trauma IHC analysis in both CAP groups showed the protein expression of interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), and superoxide dismutase (SOD) were significantly decreased, while the protein expression of vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) were protein expression was significantly increased. The results were validated at the genetic level by mRNA and were consistent. Therefore, it is concluded that CAP improves wound healing in diabetic mice by inhibiting inflammation, reducing oxidative stress, and enhancing angiogenesis[26] .

Similarly, in another study by Guo operated, HE analysis of skin tissue on the 14th day of trauma formation revealed reduced inflammatory cell infiltration, faster regenerative

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epithelialization process and more intact skin structure in the CAP-treated group; Masson trichrome staining showed that CAP treatment increased collagen deposition, and IHC staining confirmed a significant increase in the number of CD31 and TGF-β-positive cells, which demonstrated that CAP promotes traumatic neovascularization and extracellular matrix formation[27] . In conclusion, these in vivo studies support the promising use of CAP in trauma treatment.

Table 2. The effect of CAP on skin tissue.

Disease	Test model	Synopsis	Refs
Diabetic wound	rat	CAP treatment accelerated cell proliferation and neovascularization.	[25]
Diabetic wound	mice	CAP treatment can inhibit wound inflammation, reduce oxidative stress, and promote angiogenesis, which involves a variety of protein signals.	[26]
Acute wound, diabetic wound	rat	CAP treatment significantly reduced inflammation and enhanced re-epithelialization, fibroblast proliferation, deposition of collagen, neovascularization, and expression of TGF-β, CD31.	[27]
Infective diabetic wound	rat	CAP treatment resulted in regeneration, collagen deposition, and neovescularization of the epidermis and dermis, and no wound contraction, scar formation, or tissue thermal damage was found.	[19]

4. CAP REDUCES TRAUMATIC MICROBIAL LOAD

The healing process of wounds with large amounts of protein and tissue fluid exudation provides a positive environment for microorganisms to survive. Therefore, it is prone to accompany infections. Staphylococcus aureus is the most common bacterium involved in skin infections, including methicillin-resistant Staphylococcus aureus (MRSA)[32-34] . Other microorganisms associated with skin infections are Pseudomonas aeruginosa, Escherichia coli, Acinetobacter baumannii, and coagulase-negative staphylococci (CNS), including Staphylococcus epidermidis and Staphylococcus lutens[35,36] . And biofilm formation leads to more complex infections, creating a great challenge for pathogen removal and further increasing the difficulty of wound healing[37,38] .

Boekema et al. used a dielectric blocking discharge (DBD) as plasma source in vitro treatment of S. aureus for 2 min resulted in a 4 log reduction in viable cells[39] ; in another study, Guo et al. demonstrated that reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by CAP were important factors in the inactivation of S. aureus and noted that CAP treatment restored MRSA susceptibility to antibiotics. CAP can be used synergistically with antibiotics to control traumatic infections and improve the efficacy[40] .

It has also been demonstrated that CAP can cause cell death in E. coli by disrupting the integrity of the E. coli plasma membrane[41] ; similar studies have been conducted on P. aeruginosa. Dijksteel et al. found that exposure of P. aeruginosa to CAP for 2 min resulted in complete bacterial inactivation[42] , and Wang et al. also demonstrated that CAP killed multidrug-resistant Pseudomonas by significantly increasing intracellular ROS levels [43] .

Of concern is the need to be alert to the increased risk of fungal infections under conditions of adequate antibiotic use. Choi et al. first observed the inhibitory effect of CAP on Candida albicans in vitro and further demonstrated the accelerated wound healing effect of CAP in a

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diabetic rat model infected with Candida albicans[19] . In addition to Candida albicans, Klämpfl et al. also demonstrated a significant effect of CAP on reducing spore survival[20] .

In a recent study, Khosravi et al. used air-sourced DBD plasma devices for inactivation of S. aureus and E. coli biofilms. The results showed that the biofilms of Staphylococcus aureus and Escherichia coli were damaged by up to 70% and 85% respectively after 4min of exposure to CAP[44] . In Handorf's study, after 60 s of CAP treatment, the three-dimensional structure of biofilms was disrupted and the morphology of Candida albicans cells changed significantly, especially with more pronounced inactivation of cells at the edges of biofilms[45] . CAP showed conspicuous inactivation of several bacteria commonly found on skin surfaces as well as of fungi, spores and biofilms, which provided a positive impact on wound healing(Table 3).

Table 3. CAP inactivates microorganisms.

Microorganism	Synopsis	Refs
S. aureus	In vitro treatment of S.aureus with CAP for 2 min reduced in a 4log reduction in viable cell.	[39]
MRSA	CAP produces large amounts of ROS and RNS to inactivate MRSA and restore MRSA susceptibility to antibiotics.	[40]
E. coli	CAP causes cell death by disrupting the integrity of the E. coli plasma membrane.	[41]
P. aeruginosa	In vitro treatment of Pseudomonas aeruginosa for 2min resulted in cell death.	[42]
Multidrug-resistant Pseudomonas	CAP inactivates multidrug-resistant Pseudomonas by increasing intracellular ROS concentration.	[43]
Candida albicans	In vitro treatment of Candida albicans with CAP resulted in reduced cell numbers.	[19]
spore	CAP reduces spore survival.	[20]
S. aureus and E. coli biofilms	After 4min exposure to CAP, biofilms of Staphylococcus aureus and Escherichia coli were destroyed by up to 70% and 85%, respectively.	[44]
Candida albicans biofilm	CAP treatment resulted in the destruction of the three-dimensional structure of the biofilm and the morphological changes of the cells, especially the inactivation of the cells at the edge of the biofilm.	[45]

5. BIOSAFETY OF CAP

Although CAP shows promising applications in the wound healing process, CAP can only accelerate the healing process through direct contact with the wound, so the biosafety of CAP is crucial. Wende et al. used different test systems to treat several cell lines with CAP to detect mutagenicity. It showed that appropriate doses of CAP treatment did not increase the genotoxicity of the cells[6] ; in another study, Xu et al. used a plasma-activated solution to treat trauma on the back of mice, and at the end of the treatment, serum was collected for biochemical assays and vital organs (heart, liver, spleen, lungs, and kidneys) were dissected for histological examination. Biochemical examinations confirmed that CAP had no effect on liver and kidney function, lipids, blood glucose and antioxidants (total superoxide dismutase) in mice, and histological examinations also confirmed that there were no significant abnormal changes in the tissue structure and cellular composition of important organs in mice[7] . Evert repeatedly exposed the oral mucosa of mice to CAP for up to 1 year and showed that the oral mucosa of mice was well tolerated to CAP and did not lead to non-invasive lesions or squamous cell carcinoma (SCC) of the mucosa; and the size of precancerous lesions or SCC was significantly reduced in mice treated with CAP, which even suggests that CAP exposure is beneficial in inhibiting the development of precancerous lesions and malignancies[8] . In another study by

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Schmidt et al., they used an argon-sourced plasma jet device for repeated treatment of wounds in mice for 2 weeks and followed up for 12 months after the treatment.. The results showed that the wounds firstly showed a similar appearance in healing to the control animals without excessive scarring; secondly, serum biochemical tests ruled out the development of pathology or excessive inflammation; and finally, the occurrence of malignant tumors in various vital organs was ruled out by imaging[9].

6. CONCLUSION

CAP can support wound healing by stimulating the proliferation and migration of epidermis-associated cells, activating or inhibiting relevant signaling pathways to reduce the inflammatory response, stimulating neovascularization and collagen deposition, and effectively reducing the microbial load on the wound surface. CAP is a new treatment with broad application prospects. It is not only used for the treatment of acute or chronic wounds, but also shows beneficial therapeutic effects in other skin diseases, malignant tumors and regenerative medicine. However, the quantification of CAP doses is still not clearly addressed protocols, and the standardization of treatment modalities for different types of wounds and skin diseases is very important and urgently needed. Studies by different teams at home and abroad have provided important reference values for this purpose, but further exploration and discoveries are still needed in the future.

7. FINANCIAL & COMPETING INTERESTS DISCLOSURE

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