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Review article

Comprehensive biomedical applications of low temperature plasmas

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ABSTRACT

The main component of plasma medicine is the use of low-temperature plasma (LTP) as a powerful tool for biomedical applications. LTP generates high reactivity at low temperatures and can be activated with noble gases with molecular mixtures or compressed air. LTP reactive species are quickly produced, and are a remarkably good source of reactive oxygen and nitrogen species including singlet oxygen (O_2), ozone (O_3), hydroxyl radicals (OH), nitrous oxide (NO), and nitrogen dioxide (NO_2). Its low gas temperature and highly reactive non-equilibrium chemistry make it appropriate for the alteration of inorganic surfaces and delicate biological systems. Treatment of oral biofilm-related infections, treatment of wounds and skin diseases, assistance in cancer treatment, treatment of viruses' infections (e.g. herpes simplex), and optimization of implants surfaces are included among the extensive plasma medicine applications. Each of these applications will be discussed in this review article.

1. Introduction

Low-temperature plasma (LTP) applications in biomedical systems are the main element of plasma medicine [1]. This interdisciplinary field of research has been increasingly attracting scientific attention [2]. Physical plasma is generally produced by combining high temperature or electromagnetic field power to a neutral gas so the ionized gaseous substance becomes gradually electrically conductive [3]. LTP jets are currently the concentration of plasma research. LTP jets can generate high reactivity at low gas temperatures and can work with noble gases with molecular mixtures or with compressed air. LTP's low temperature (below 40 °C) and highly reactive non-equilibrium chemistry make it appropriate for the modification of inorganic surfaces in addition to treatment of delicate biological systems [4].

Plasmas produce electromagnetic radiation, including ultra-violet (UV) radiation and light in the visible spectrum, and involves excited gas particles, charged ions, free electrons, free radicals, neutral reactive oxygen and nitrogen species (ROS/RNS), and molecule fragments. LTP is known as the fourth state of matter, along with solid, fluid, and gaseous, considering its unique features when compared to neutral gases [3]. Hence, because of its distinct characteristics, plasma has been used for surface applications in industry [5]. Plasma uses vary from cleaning of microelectronics' surfaces [4], surface activation for enhanced adhesion of paint and glue [4], and to enhance contrast for microscopy images, such as for scanning electron microscopy [6]. The low temperature permits treating polymers, and the design of some plasma equipment allow it to penetrate challenging cavities, which was verified in a stack of polymer discs with openings that shaped a winding cavity [7]. Plasma technology has also been used for the deposit of films containing zinc, using a solution of zinc nitrate hexahydrate salt in water as a source of zinc [8]. The industrial application of plasma processes for medical devices has been extensively demonstrated [9,10]. For example, argon-based non-thermal plasma jet guided by radio frequency (27.12 MHz), which was applied as an antimicrobial therapy on medical devices that were made out of high-temperature susceptible material. This plasma was effective on eradicating *Bacillus atrophaeus* spores and *Escherichia coli* in medical devices surfaces [9], and the best antimicrobial effect was seen when plasma was applied directly to the surface [10].

Among LTP's recognized uses in biomedicine, some efforts have been made to assess its effects on biofilms, which is a dense and complex microbial community surrounded by an adhesive glue-like matrix that enhances resistance to adverse conditions and external stresses [11]. Biofilm organization is known to prevent disease eradication by conventional antimicrobial therapy due to their poor penetration into the biofilm, in addition to the antimicrobial therapy adverse effects related to bacterial resistance and systemic toxicity. However, the antibacterial effects of LTP, which are intermediated by ROS, do not show to increase bacterial resistance when bacteria are continuously exposed to it. These findings indicate that LTP is potentially a good candidate for *in vivo*

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antibiotic treatments [12–14].

Plasmas can reach greater gas-phase chemistry deprived of high gas temperatures [15,16]. It happens because plasmas display much higher electron energies than that of the ions and the neutral species. The energetic electrons go into a collision with the background gas, producing higher dissociation, excitation, and ionization levels. Since ions and neutrals continue quite cold, there is no contact thermal damage caused by the plasma. This feature supports plasma use as a treatment for heat-sensitive materials, for instance biological matter, cells, and tissues [17].

Currently, a large number of research-applied LTP have been developed. Overall, they can be identified as direct and indirect discharge, i.e. dielectric barrier discharge and atmospheric pressure plasma jet, respectively. Furthermore, few devices have been certified for medical use. Among them, kINPen®MED (INP Greifswald/neoplas tools GmbH, Greifswald, Germany), an atmospheric pressure plasma jet; and PlasmaDerm® VU-2010 (CINOGY Technologies GmbH, Duderstadt, Germany), a dielectric barrier discharge source, are both certified by MEDCERT, in Germany, under ISO 13485 [3]. Afterward, SteriPlas (Adtec Ltd., London, United Kingdom) has also been certified as a medical device, being employed as a therapy for chronic and acute wounds, in addition to the reduction of microbial load. However, considering different research groups use different devices, as well as modified therapies, the comparison among their findings is challenging. In fact, due to these differences, determining the long-term security and efficacy of plasma devices in a comparable and standardized way becomes complex. Many organizations have been created to certify technical standardization, including the German Institute for Standardization (DIN); The European Committee for Standardization (CEN); and the International Organization for Standardization (ISO) for plasma standardization [3].

Recently, the United States Food and Drug Administration (FDA) approved cold atmospheric plasma technology for use in a clinical trial for cancer patients. The technology was approved for a phase I clinical trial in 20 patients and is still under development. A multi-institute team established a pen-like electrosurgical scalpel that sprays a jet of LTP for 2–7 min at residual cancerous cells or tissues [18]. The key feature is that the device only targets tumors, leaving contiguous tissue uninjured. These properties were shown by *in vitro*, *in vivo*, and FDA-approved compassionate use cases before the clinical trial [18].

The applications of plasmas in medicine have attracted interest in the medical research communities [11]. Plasma medicine applications include treatment of oral biofilm-related infections [12,13], treatment of wounds and skin diseases [19–21], assistance in cancer treatment [22–24], treatment of viruses infections (e.g. herpes simplex [25]); as well as, but not limited to, optimization of implants surfaces [26–28]. Each of those applications will be discussed in this review article (Fig. 1).

2. Plasma medicine applications

Table 1 contains information of plasma application, type of study and plasma sources of most of research papers discussed in this review.

2.1. LTP therapy in oral biofilm-related diseases

Microorganisms' cells are displayed in two forms: planktonic, also called free-floating relatively independently; or attached to each other and to a host material. This second form can cause the development of a dense community of microorganisms surrounded by an adhesive matrix that, in general, sticks itself to a solid surface. This complex and well-protected community is called biofilm. Biofilms are usually present on solid substrates, but it can also be found in pores interacting with aqueous solutions, as well as floating films on liquid surfaces. Cells in a biofilm are known for closely interacting with each other inside its shielding milieu, showing different phenotypes when compared to the same species free-floating planktonic cells. One of the most remarkable characteristics of these cells, when organized as a biofilm, is their superior resistance to unfavorable conditions and external stresses. Biofilms are known as strong communities, being resilient to chemicals found in detergents and even to antibiotics that are known to affect planktonic cells. Hence, if not controlled, biofilms could characterize as significant health hazards in many situations. Furthermore, biofilms can cause damage to the surfaces they attach to, including corrosion, combining an economical cost in addition to their health risk [29,30].

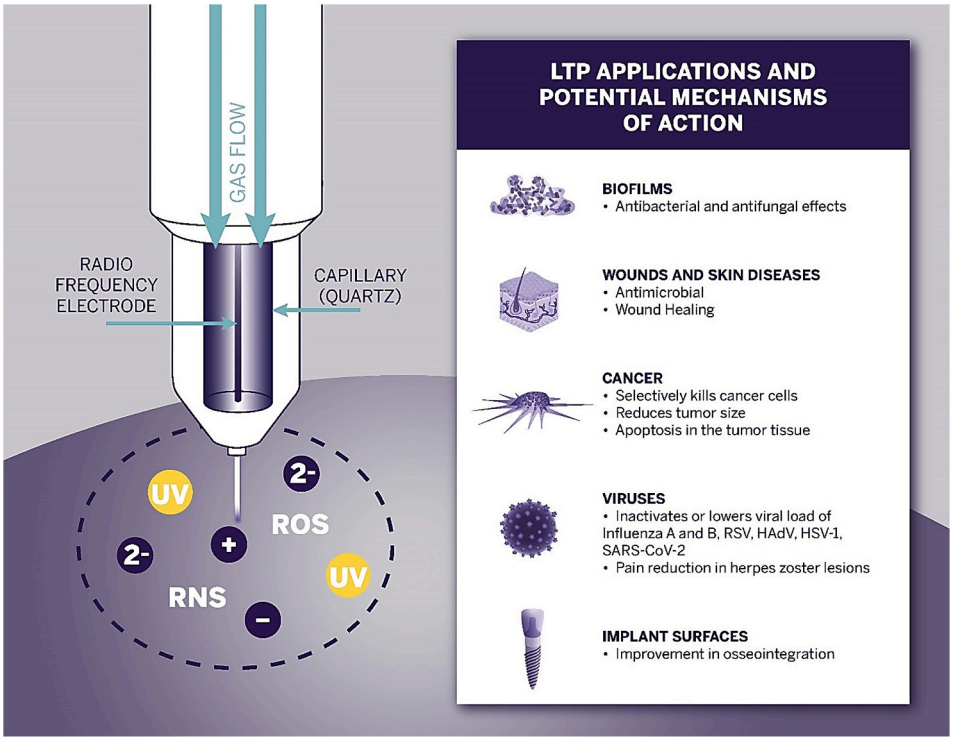

Fig. 1. Plasmas produce electromagnetic radiation, including ultra-violet (UV) radiation and light in the visible spectrum, and involves excited gas particles, charged ions, free electrons, free radicals, neutral reactive oxygen and nitrogen species (ROS/RNS), and molecule fragments. Plasma medicine applications include treatment of oral biofilm-related infections, treatment of wounds and skin diseases, assistance in cancer treatment, treatment of viruses' infections, as well as optimization of implants surfaces. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Plasma application, type of study and plasma sources of research papers discussed in the review.

PLASMA APPLICATION	TYPE OF STUDY	PLASMA SOURCE	REFERENCES
ORAL BIOFILM			
C. albicans + *Staphylococcus aureus*	*In vitro*	Argon-based plasma jet (kINPen®09, neoplas tools GmbH, Germany)	[12]
Enterococcus faecalis	*In vitro*	Argon-based plasma jet (kINPen®MED, neoplas tools GmbH, Germany)	[36,37]
Peri-implantitis microcosm biofilm	*In vitro*	Argon +1% Oxygen based plasma jet (kINPen®08, INP, Germany	[45]
Streptococcus mitis	*In vitro*	Argon-based plasma jet (kINPen®MED, neoplas tools GmbH, Germany)	[46]
Porphyromonas gingivalis	*In vitro*	Argon-based plasma jet (kINPen®09, neoplas tools GmbH, Germany)	[13]
WOUND AND SKIN DISEASES			
Porphyromonas aeruginosa	*In vitro*	Argon based or Argon +1% Oxygen based plasma jet (kINPen®09, neoplas tools, GmbH, Germany	[50]
Staphylococcus epidermidis	*In vitro*		
Chronically infected wounds	*In vivo* (clinical)	Argon-based microwave-driven plasma device (MicroPlaSter alpha and beta, ADTEC Plasma Technology Co. Ltd, Japan/UK)	[51]
Trichophyton rubrum, *Trichophyton interdigitale*, *Microsporon canis*, *C. albicans*	*In vitro*	Argon-based plasma jet (kINPen®09, neoplas tools GmbH, Germany) and Dielectric Barrier Discharge (DBD) plasma device	[49]
Severely contaminated wounds	*In vivo* (clinical)	Argon-based Maxium® electrosurgery unit with maxium® beamer and beam electrode (Gebrüder Martin GmbH + Co. KG)	[19]
Non-infected wounds	*In vivo* (clinical)	Argon-based plasma jet (kINPen®MED, neoplas tools GmbH, Germany)	[52]
Patients with wound healing disorder	*In vivo* (clinical)	Argon-based plasma jet (kINPen®MED, neoplas tools GmbH, Germany)	[53]
Mammalian cell lines related to wound healing	*In vitro*	Argon-based plasma jet (APPJ device)	[20]
Diabetic wounds	*In vivo* (mice model)	Helium-based plasma jet and plasma-activated medium (PAM)	[21]
CANCER THERAPY (DIRECT TREATMENT)			
Lung cancer cell lines Murine melanoma cell lines (B16–F10) Bladder cancer tumor cells (SCaBER)	*In vitro/in vivo* (mice model)	Helium-based plasma jet (lab developed at George Washington University)	[54]
Glioblastoma U87MG	*In vitro/in vivo* (mice model)	Floating Electrode (FE) – DBD plasma device	[55]
Colorectal carcinoma HCT-116	*In vitro*		
Murine neuroblastoma	*In vitro/in vivo* (mice model)	Helium-based plasma jet	[56]
Breast cancer tumor cell lines (4T1)	*In vitro/in vivo* (mice model)	Helium-based Micro (μ) Plasma Jet	[57]
Head and neck cancer cell lines	*In vitro*	Surface Micro Discharging (SMD) plasma device (MiniFlatPlaSter®)	[22]
Head and neck squamous cancer	*In vivo* (clinical)	Argon-based plasma jet (kINPen®MED, neoplas tools GmbH, Germany)	[23]
CANCER THERAPY (INDIRECT TREATMENT)			
Immune modulators, Intracellular ROS production inducers	*In vitro/in vivo*	Nanosecond pulsed dielectric barrier discharge (nsDBD) plasma Nonequilibrium atmospheric pressure Plasma (NEAPP)	[63–66] [68–70]
VIRUSES			
Influenza A and B Respiratory syncytial virus (RSV)	*In vitro* *In vitro*	Nitrogen-based plasma device (BLP-TES, NGK Insulators Ltd., Nagoya, Japan)	[75]
Human adenovirus (HAdV-4, -5, -20, -35, -37, -50)	*In vitro*	Argon-based plasma jet (kINPen®MED, neoplas tools GmbH, Germany)	[77]
Herpes simplex virus type 1 (HSV-1)	*In vitro*	Argon-based plasma jet (kINPen®MED, neoplas tools GmbH, Germany)	[25]
Herpes zoster	*In vivo* (clinical)	Argon-based microwave-driven plasma device (MicroPlaSter alpha and beta, ADTEC Plasma Technology Co. Ltd, Japan/UK)	[79]
IMPLANT SURFACES			
Surface modification and osseointegration	*In vivo* (canine model)	Argon-based plasma jet (kINPen®09, neoplas tools GmbH, Germany)	[26]
Wound healing and osseointegration	*In vivo* (canine model)	Argon-based plasma jet (kINPen	[82]

Biofilms are known, for instance, to play a significant part in the pathogenesis of many oral diseases, one of them being oral candidiasis, which is an opportunistic infection of the oral cavity with a wide range of clinical signs and symptoms, for example, denture stomatitis, pseudomembranous candidiasis, erythematous candidiasis and angular cheilitis [31]. Infections initiated by *Candida* spp. are associated with biofilm formation, and oral yeast infections are mainly caused by *Candida albicans* [32]. Considering there are several challenges for chemical disinfection in dental practice, including the use of sodium hypochlorite for denture cleaning, the effects of plasma treatment as a physical method to disinfect *C. albicans* biofilms has been investigated. Plasma treatment significantly reduced the *C. albicans* colony forming units (CFU) compared to chemical disinfectants (chlorhexidine and sodium hypochlorite) [33]. Likewise, antimicrobial effects of LTP on biofilms of *C. albicans* and *Staphylococcus aureus*, and its safety on an *in vitro* reconstituted oral epithelium were evaluated [12]. Biofilms were submitted to LTP treatment for 1 min. Penicillin G and fluconazole were used as positive controls for *S. aureus* and *C. albicans*, respectively. All

biofilms treated with LTP showed substantial CFU decrease, change in morphology, and decreased viability when compared to control treatments. Additionally, fluorescence microscopy revealed the occurrence of ROS in all biofilms treated with LTP. In the oral epithelium of negative control and plasma group cytotoxicity was low and viability was high. Moreover, histology did not exhibit signs of necrosis or important modification in the plasma-treated epithelium, and it was identified preservation of cell proliferation in the plasma-treated epithelium. Therefore, LTP showed to reduce single- and dual-species of *C. albicans* and *S. aureus* biofilms, with no toxic effects in the reconstituted oral epithelium [12].

Furthermore, decontamination of the root canal system is known to be of the highest relevance in the success or failure of root canal treatment, even though complete sterility is unfeasible [34]. Currently, there is no other solution or material to replace the widespread use of sodium for root canal disinfection. However, much strength has been dedicated to complementing the effects of sodium hypochlorite [35], such as the use of LTP. A study compared the possibility of using an atmospheric pressure plasma jet for endodontic treatment and compared its antimicrobial efficacy with conventional irrigation solutions, such as chlorhexidine digluconate and sodium hypochlorite, against intracanal bacteria *Enterococcus faecalis*. Plasma jet reached greater microbial reduction when compared to chlorhexidine irrigation and a similar reduction compared to sodium hypochlorite [36].

Since LTP has shown promising results in disinfecting *E. faecalis* in root canals, a study analyzed the bactericidal efficiency of LTP in various depths of infected root dentin. Standardized human mandibular premolars root canals were contaminated with *E. faecalis* and incubated for one week. The highest general logarithmic reduction factors were achieved from the combination of chlorhexidine with LTP, followed by LTP alone. Therefore, the adjuvant use of LTP might be beneficial in highly infected root canals to improved disinfection [37].

In addition to root canal and oral candidiasis, oral microbiota substantially affects biofilm formation on recently placed dental implants. It is well established that biofilm-induced inflammation around dental implants plays a dominant role in promoting osteoclast-mediated bone resorption and inhibiting bone formation, leading to net bone loss around implants [38]. This process is referred to as peri-implantitis, a bacteria-initiated infection that to date has no effective treatment [39,40]. Peri-implantitis is a significant oral health issue that can affect healthy and systemically compromised patients and may result in loss of dental implants [41,42]. As many as 45% of patients with dental implants will develop peri-implantitis within a decade and a recent workshop hosted by the American Academy of Periodontology and the European Federation of Periodontology points to this significant problem [43,44]. Peri-implant infections, once established, are likely to progress and lead to implant loss. One of the reasons for the lack of success treating peri-implantitis is frequently that the instrumented implant surface and remaining microbial biofilm slow down or impede re-osseointegration. A human oral biofilm was formed on titanium discs and compared to the treatment with a brush only, 1% oxygen/argon-based plasma, or brushing combined with plasma to disrupt the biofilm. The controls were discs with no biofilm, autoclaved biofilm, or untreated biofilm. Afterward, human osteoblastic cell growth (MG-63) was analyzed after one and 24 h. Biofilm fragments present on the brush and plasma decreased osteoblastic cell development, while the brush + plasma provided a larger area of osteoblastic cells. Brush + plasma was the only treatment in which 5-day cell growth was detected. Based on the results, combining brushing and LTP showed to be a potential strategy for the treatment of peri-implantitis [45].

Since surface cleansing is known to be a challenge for the treatment of peri-implant infection, the bactericidal efficiency of tissue tolerable plasma was investigated on *in vitro Streptococcus mitis* biofilms formed on titanium dental implants. Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) were used to analyze the biofilms. One (1) % sodium hypochlorite was used to rinse the implants as the negative control, and as the positive control, implants were irradiated for 60 s with a diode laser. Treatments were plasma application for 60 or 120 s. Recovered colonies were determined by CFU counting. Further, implants were observed by fluorescence microscopy. The CFU counts on the groups treated with plasma for 60 or 120 s were expressively inferior to the control, and no differences between 60 and 120 s of plasma treatment were detected. Before treatment, CLSM and SEM detected attached bacteria; however, fluorescence microscopy showed that plasma treatment on titanium surfaces exhibit a higher number of dead cells when compared to the controls in the post-treatment [46].

The development of periodontal and peri-implant diseases has been associated with several oral anaerobic-restrict species in the subgingival environment, including black-pigmented *Porphyromonas* species, being *Porphyromonas gingivalis* and *Fusobacterium nucleatum* the main pathogens of this type of infection. Therefore, LTP effects were determined on an anaerobic biofilm of *P. gingivalis* and on how it affects an *in vitro* reconstituted gingival epithelium tissue. LTP treatment for 1 and 3 min was applied to a *P. gingivalis* biofilm grown on titanium discs and to the reconstituted gingival epithelium. The LTP treatments were compared to 0.2% chlorhexidine application for 1 min, with the argon gas-only application for 3 min, and with no treatment application (negative control). LTP application for 1 and 3 min significantly decreased the CFU/mL when compared to the negative control, although they were not as effective as chlorhexidine application for 1 min. As for the gingival epithelium, cytotoxicity was low and viability was high for all the groups. Minor cell damage was observed in the morphologic analysis of gingival epithelium for the plasma groups. Thus, LTP is potentially an effective treatment aiming to reduce *P. gingivalis* biofilm on implant surfaces and is safe for the gingival epithelium. Additionally, it may be related to cell repair [13].

The application of LTP for the treatment of oral infections, mainly to remove biofilms, is a promising field of future application. The unique characteristics of LTP have opened a new era in dental care. It has shown promising results in oral biofilm-related disease control. Plasma dental treatments are painless and drill-less, thereby making them patient-friendly [47]. However, more studies need to be conducted for the clarity in the mechanism of action and its varied application in the dental field.

2.2. LTP to treat wounds and skin diseases

There is evidence showing LTP is an innovative antimicrobial therapy, presenting as an alternative to traditional antibiotic treatments, as well as its beneficial action on wound healing, making it a promising instrument for biomedical uses, including, but not limited to, the management of infections [48]. A large number of studies have described the lethal effects of LTP on microorganisms such as bacteria and fungi, showing its potential as an effective apparatus for disinfection. Skin and nail fungal infections, chronic wounds, and multidrug-resistant pathogens like *S. aureus* (MRSA) are critical therapeutic and economic problems. Therefore, novel therapies for these conditions are highly necessary. The challenge of global rising microbial resistance distresses, comprising various pathogens including methicillin-resistant MRSA, vancomycin-resistant enterococci, and gram-negative species producing beta-lactam hydrolyzing enzymes (extended-spectrum β-lactamases), create the need for alternative antimicrobial treatments. Besides being site-specific, LTP therapy grants a very different mechanism of action when contrasted to traditional antimicrobials, and might function as a substitute for common antibiotic and antiseptic therapies [49].

Knowing that microbial biofilms make wound healing difficult and the antiseptics are not always effective, LTPs from different gases, argon-based and an argon/oxygen-mixture-based plasma, were applied to determine their efficacy on the treatment of biofilms of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* formed *in vitro*. The antiseptic chlorhexidine digluconate was used as the positive control. Biofilms were exposed to LTP for 30, 60, 150, or 300 s and the control group was

treated with 0.1% chlorhexidine solution for 600 s. Biofilms were then dispersed and the recovered CFUs were used to measure inactivation. There was a significant antimicrobial effect when LTP treatment was compared to the untreated control and it was at least as effective as the treatment with chlorhexidine, or even better, depending on the gas, the bacterial strain, and the exposure time [50].

In a prospective randomized controlled study, twenty-four patients with chronically infected wounds received daily treatment with 2 min of argon-based LTP, in addition to the standard wound care. A significant decrease in bacterial load was seen in wounds that were treated with plasma, independent on the bacterial species. In addition, the safety of treatment with argon-based LTP devices has been shown, as well as being painless and effective to reduce bacterial load in chronic wounds [51]. Furthermore, plasma treatment was shown to be effective in damaging microbial, fungal, and parasitic dermal strains and species including *E. coli*, *P. aeruginosa*, *Klebsiella* spp., *S. aureus*, hemolyzing Lancefield Streptococci (group A and B), Proteus group (*Proteus mirabilis, Proteus vulgaris*), *Acinetobacter* spp., *Stenotrophomonas* spp., *E. faecalis*, *C. albicans*, and *S. epidermidis*. *In vitro*, more than 90% of bacteria *Trichophyton rubrum*, *Trichophyton interdigitale*, *Microsporon canis*, and yeast *C. albicans* were killed by 30 s LTP exposure, with no isolate exhibiting resistance [49].

How LTP affects multidrug-resistant pathogens was evaluated concerning non-multidrug-resistant-pathogens in chronic wounds. Eleven patients with 18 severely contaminated wounds were submitted to treatment with an argon-based LTP for 10 s/cm^2 in a single session. It was observed that a single treatment reduced multidrug-resistant microorganisms in all wounds. As proof of principle, it was shown that argon-based low-temperature plasma functions as a strong treatment modality that limited multidrug microbial colonization [19].

Plasma has also been applied in the healing of non-infected, acute (iatrogenic) wounds. For instance, in a clinical study with five individuals, 20 laser lesions were treated with argon-based LTP for 10 s, 30 s, or 3 applications of 10 s, compared to the untreated control. Scar development was observed for 10 days, six months, and one year. In the initial stages of healing, LTP appeared to maintain the inflammation required during tissue recovery. In advanced stages, LTP displayed superior effects regarding evading post-traumatic skin conditions. Plasma treatment exhibited excellent aesthetics throughout scar establishment and no precancerous skin features happened up to one-year [52]. A proof of concept study assessed the clinical effect of LTP exposure in patients presenting wound healing disorders and that had exposed brachial tendons of the radial forearm. In this prospective study, four patients who had suffered radial forearm free flap procedures and faced wound healing disturbance, which ended in exposed flexor tendons, were included. Besides routine wound treatment, all sites were exposed to LTP, which resulted in total wound closure. For all the involved subjects, total wound repair, which was measured by the lack of tendon exposure, was detected within a mean exposure time of 10.1 weeks. Unwanted side effects were not perceived, and there was no inflammation or infection. Therefore, LTP may present a consistent conservative treatment route for complex wound healing disorders [53].

LTP has shown to be promising adjuvant in wound healing, even though its mechanism remains poorly understood. Aiming to understand the effects of LTP on mammalian cells, an argon-based LTP jet was applied to treat *in vitro* cultured L929 murine fibroblasts, as well as BALB/c mice skin wounds. The study showed that when fibroblasts were treated with LTP for 15 s, there was a significant increase in cell proliferation, secretion of epidermal growth factor, and transforming growth factor-βi, production of intracellular ROS, and the percentage of cells in S phase, protein expression of phosphorylated p65 and cyclinD1. However, there was a reduction in protein expression of inhibitor kappa B. The *in vitro* study showed that LTP exposure for 30 s increased the number of fibroblasts and the ability of collagen synthesis, while the exposure for 50 s led to opposed outcomes. This outcome indicates that LTP stimulates wound healing related fibroblast proliferation by inducing the production of ROS, upregulating phosphorylated p65 expression, downregulating inhibitor kappa B expression, and activating the NF-κB signaling pathway, leading to an altered cell cycle progression, demonstrated by the augmented DNA synthesis in S phase [20].

As plasma medicine's applications continue to expand and considering its positive impact in wound healing, a recent *in vitro* and *in vivo* study was conducted to evaluate the LTP's efficacy and safety as a treatment for diabetic wounds. Eight-week-old male db/db mice and C57BL mice were treated with LTP for 90 s and LTP for 180 s, as well as helium gas (flow-control group). Mice were treated and monitored for 2 weeks. Blood samples and skin samples from around the wound were collected. After 14 days of treatment, LTP stimulated the healing of diabetic wounds, which presented closure rates significantly higher in the groups treated with LTP groups (90 and 180 s) in relation to the flow-control group. IL-6, tumor necrosis factor-α, inducible nitric oxide synthase, and superoxide dismutase protein expressions in two LTP groups were significantly reduced, when compared with the control group. However, vascular endothelial growth factor and transforming growth factor-β protein expression groups significantly increased in the two groups treated with LTP. *S*cratch wound-healing *in vitro* assays demonstrated that LTP might effectively guarantee healing in 3 min exposure. Moreover, there was no difference in normal skin histological observations, as well as no difference in the levels of serum alanine transaminase, aspartate aminotransferase, blood urea nitrogen, creatinine, and white blood cells amongst the LTP and control groups. Hence, daily 3 min LTP treatment showed to improve wound healing in diabetic mice by defeating inflammation, reducing oxidative stress, and increasing angiogenesis, engaging various proteins signaling, and LTP therapy showed to be safe for the liver and kidney [21].

LTP has been presented as a favorable and reasonable therapy for a wide range of diseases, however, more studies in the plasma medicine field are needed to further investigate the clinical therapeutic potential of LTP and to entirely comprehend its modes of action. In general, plasma medicine research is growing and providing emergent proof for the use of LTP as a treatment choice for various wounds and skin diseases.

2.3. LTP use for cancer therapy

LTP might have the ability of delivering ROS directly into tissues, demonstrating to be an innovative modality for targeted, or direct cancer therapy. Both *in vitro* and *in vivo* studies using lung cancer cell lines, murine melanoma cells B16–F10, and subcutaneous bladder cancer tumors (SCaBER) cells demonstrated that LTP selectively killed cancer cells with no damage to normal cells; moreover, LTP application expressively reduced tumor size *in vivo*. Furthermore, when LTP was applied ROS metabolism and oxidative stress response genes expression were increased [54].

The *in vitro* antitumor activity of LTP was also evaluated on two human cancer cell lines (glioblastoma U87MG and colorectal carcinoma HCT-116). LTP produced a great quantity of ROS, damaging the DNA damages, which caused a multiphase cell cycle arrest followed by apoptosis induction. Moreover, *in vivo* tests on U87MG bearing mice demonstrated that LTP stimulated a decrease of bioluminescence and tumor volume when evaluated against non-treated mice. Apoptosis induction as well as accumulation of cells in the S phase of the cell cycle were also noticed, indicating a tumor proliferation arrest could have happened [55].

Cold plasma was also studied *in vitro* and *in vivo* murine model for treating neuroblastoma. Mouse neuroblastoma cultures were exposed to LTP for 0, 30, 60, and 120 s and immediately examined for apoptotic and metabolic activity, as well as 24 h and 48 h post-treatment. Tumors measuring 5 mm diameter were subjected to a single transdermal LTP treatment. Then, tumor volume and mouse survival were determined. Plasma treatment reduced metabolic activity, induced apoptosis, and reduced viability of cancer cells, proportionally to both exposure and

post-treatment time. *In vivo*, tumors were also subjected to a single treatment, which resulted in eventually reduced tumor growth. Additionally, survival almost doubled. Hence, neuroblastoma showed to be susceptible to plasma treatment on *in vitro* and in an *in vivo* mouse model of the established tumors [56].

Another study evaluated how micron-sized non-thermal atmospheric pressure plasma applied inside the animal body affects breast cancer tumor. The μ-plasma jet is comprised of micron-sized hollow tube wherein pure helium gas is ionized by high voltage (4 kV) and high frequency (6 kHz). The plasma killing efficacy on cancer cells was primarily determined by treating 4T1 cells and verifying viability using flow cytometry and cell cycle investigation. For examination of the *in vivo* plasma jet effects, BALB/c mice were inoculated with 4T1 cell lines and then the cells were treated subcutaneously for 3 min. The outcomes indicated that μ-plasma efficacy in suppressing tumor growth is similar to that of standard chemotherapy medication. Furthermore, data showed that μ-plasma produces apoptosis in the tumor tissue and enhances the ratio of the apoptotic to anti-apoptotic protein expression [57].

Clinical data shows that head and neck squamous cell cancer takes the position of 7th among the most frequent cancers worldwide [58]. Regardless of the progress of finding new therapies, including monoclonal antibodies, prognosis remains the same for the last decades [59]. In another study, the efficiency of a surface micro discharging plasma device on two head and neck cancer cell lines was evaluated. Effects on cell viability, DNA fragmentation, and apoptosis were tested using MTT assay, alkaline micro-gel electrophoresis (comet assay) and Annexin-V/PI staining. MTT assay demonstrated plasma significantly reduced cell viability for all treatment times (30, 60, 90, 120, and 180 s). Comet assay indicated dose-dependent high DNA fragmentation as one of the key players in the anti-cancer effects of LTP. In contrast, annexin-V/PI staining showed apoptosis induction in plasma-treated head and neck squamous cell cancer lines, but no significant dose-response was observed [22].

Afterward, in order to determine the clinical application of LTP on head and neck squamous cell cancer, patients (n = 12) presenting advanced head and neck cancer and infected ulcerations received local LTP application and subsequent palliative therapy. Four of those patients presented tumor surface response after 2 weeks of intervention. The tumor surface reaction was stated as a flat area with vascular stimulation (type 1) or a contraction of tumor ulceration rims developing recesses covered with scabs, in each case delimited by tumor tissue in evident progress (type 2). Meanwhile, nine patients with similar cancer type were subjected to LTP treatment before radical tumor resection and specimens collected from the tumors were evaluated for apoptotic cells. Apoptotic cells were measurable and appeared more often in tissue areas that were treated with LTP than in untreated areas. Remarkably, no patient showed signs of a higher or stimulated tumor growth due to the effect of LTP [23].

Another clinical study evaluated the effects of LTP treatment on six patients with locally advanced head and neck cancers. Two of those patients presented a remarkably positive response to the treatment with subsequent clear tumor reduction. Whereas the tumor in one of these patients began to regrow leading to the patient's death, the other patient continued receiving treatment, targeting for total remission. Side effects were not detected in two of the patients, while four patients experienced fatigue and dry mouth. Five of the patients had a decrease in odor, perhaps because of disinfection, and four patients presented lower requirement for pain medication. From the six patients at the beginning of the study, five passed away after 1–12 months, which was shown to be unrelated to the LTP application. The role of ROS/RNS, myeloid cells and the immunogenic cell death model of cancer treatment as potential mechanisms of action of LTP treatment was substantially discussed [24].

Besides LTP application for direct cancer therapy, LTP can also be applied for indirect cancer treatment. Indirect treatments comprise plasma-assisted cancer immunotherapy and plasma-activated medium (PAM) therapy [60–62]. Researchers have suggested the use of plasmas as immune modulators for cancer treatment [61,62]. Radiation and some chemotherapeutic medications rise immunogenicity by triggering immunogenic cell death (ICD). Damaged or stressed cancer cells have danger indicator, which are identified as damage-associated molecular pattern (DAMP) molecules. Cancer cells typically induce immunosuppression, which is ICD-associated. DAMP molecules can reactivate anti-cancer immunity by activating dendritic cell maturation and antigen presentation. High-mobility group box 1, ATP, and calreticulin (CRT) are well-known DAMP molecules that are retained inside the cell in the healthy state and released only in response to stress or cell damage. Studies observed that non-thermal plasma treatment induces ICD and stimulates macrophages [63–66]. In the plasma-assisted cancer immunotherapy, LTP treatment stimulates extracellular ATP secretion and increases cell death through ICD-mediated macrophage stimulation. Plasma-generated ROS are main effectors of ICD. LTP produces surface exposure of CRT, and *N*-acetyl cysteine, which is an ROS scavenger, reduces the externalization of CRT. These results propose that intracellular ROS are responsible for plasma-induced CRT production. Tumor necrosis factor–alpha released from plasma-activated macrophages induces tumor cell death [67]. PAM is as a type of cancer chemotherapy, which, in most cases, induces intracellular ROS production following apoptosis of cancer cells [62]. Intraperitoneal injection of PAM/plasma-activated Ringer's lactate repressed the metastasis of gastric, pancreatic and ovarian tumors in a mouse examining peritoneal metastasis [68–70].

However, while LTP has already achieved standard medical care status for wound treatment, only primary data for its potential as a therapy in oncology are available [3]. No resistance to LTP treatment has been reported yet, although the clinical use of LTP requires the improvement of standardized consistent protocols to relate the findings among upcoming clinical trials. Further research is also necessary to define the most effective type of plasma for each variety of cancer [71]. Yet, based on the reported data, LTP remains a promising therapy for cancer treatment.

2.4. LTP to treat viruses' infections

In addition to prior discussed properties, including the fact LTP effects decreasing bacterial load in chronic wounds, possibly promoting wound healing; only a few studies have assessed LTP effects on viruses' infections. Viruses may be transferred straight from one infected person to another, or indirectly through contaminated intermediates, for example, surfaces, objects, food, water, and air. Transmission through contaminated surfaces and aerosols has shown to be of remarkable relevance in the COVID-19 pandemic, which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [72]. The viral capsid is the initial interaction with a host, and for successful recognition of a virus by the host cell receptors, it is essential that their external arrangement is relatively undamaged. When in the host cells, the viral genome takes control of the replication process. Consequently, these two factors are the most significant ones for the assessment of virus inactivation [73].

Management of the respiratory viruses' influenza A and B [74] and respiratory syncytial virus (RSV) [75] have only been executed with the pulsed high-voltage LTP supply. RSV is the most common causal agent of lower respiratory tract infections in infants, being one of the most significant viruses in pediatric patients, mainly because it proliferates simply through contact with contaminated surfaces [76]. Even if 5 min LTP application totally inactivated RSV on et al., glass [75], a portable and more convenient plasma arrangement would be desirable for effective disinfection of, for example, hospital surfaces, whereas the high-voltage plasma devices would be applied only for small objects and tools disinfection (Filipić et al., 2020). Some respiratory viruses are also known to persist stably as aerosols for extended intervals of time, including SARS-CoV-2, and with the aim of diminishing their spread, it

is essential to decontaminate the air, in addition to the surface areas [73].

Besides respiratory viruses', the effects of LTP therapy on human adenoviruses (HAdV) were reported. Adenoviruses involve numerous agents affecting reptiles, birds, fishes, and mammals. There are over 70 categories of HAdV and they can be genetically separated into seven very distinct species (A to G). HAdVs are widespread pathogens known to cause several disease symptoms, dependent on the type. They are typically not deadly and can be self-limiting in immunocompetent patients. These viruses are also well known to affect the upper and lower respiratory tracts and the digestive and urinary tracts, as well as disturbing internal organs, aggravate asthmatic and chronic obstructive pulmonary conditions, cause highly infectious follicular epidemic keratoconjunctivitis or being fatal in immunocompromised patients [76]. Unfortunately, there is a deficiency of specific anti-adenovirus therapy available. To investigate antiviral properties of LTP, human adenovirus types resultant from different species (HAdV −4, −5, −20, −35, −37, −50) were purified and tagged with luciferase. They were then treated with distinct exposures to LTP (no treatment, 30 s, 60 s, 90 s, 120 s, and 150 s). During LTP application, a 15 mm distance from the plasma tip to the medium surface was maintained. After LTP application, the solution containing the virus was transferred to eukaryotic cells and luciferase expression levels were used to determine the viral load. Through LTP treatment, HAdV-5 and HAdV-37 adenovirus driven luciferase expression straight associated with adenovirus transduction efficiencies were lowered. LTP application had no effect on luciferase expression levels for HAdV-4 and HAdV-50 and an increasing effect on luciferase expression levels for HAdV-20 and HAdV-35 was observed. These data show LTP has a type-dependent outcome on adenoviruses and, according to the reported data, it can increase infectivity for certain adenovirus types. Thus, LTP might be a suitable choice as an antiviral treatment, in a virus-type dependent behavior, but further studies should focus on the processes behind this occurrence [77].

Recently, LTP was tested as a suitable therapy for herpes simplex virus type 1 (HSV-1). HSV-1 often expresses as recurrent herpes labialis, however, it is related to cases of encephalitis, conjunctivitis, or herpes neonatorum as a perinatal disease as well. HSV-1 encoding the reporter gene GFP was reproduced. HSV-1 was gradually exposed to LTP; with exposure times ranging from 0 to 150 s. The positive control was the antiviral drug acyclovir. After LTP exposure, the virus suspension was transferred to a standard HSV research cell line (Vero cells), as well as to neuroblastoma cell line SH-SY5Y as a neuronal infection model. The outcomes revealed that LTP had an insignificant antiviral effect on HSV-1 in both Vero- and SH-SY5Y cells at a high dose. Nevertheless, lowering the viral load to 100-fold reduced the number of internalized HSV-1 genomes 3 h post-infection for LTP-exposed viruses. This change was less perceptible concerning GFP expression levels 24 h after infection, which was in strong divergence to the acyclovir-treated (positive control), in which the viral load decreased from 95 to 25%. Briefly, a slight but measurable antiviral effect of LTP on HSV-1 was observed [25].

Herpes zoster virus was also studied in regards to LTP therapy. Herpes zoster is a severe aching infectious skin condition that happens because of the varicella-zoster virus reactivation, with a growing incidence in the elderly population [78]. Severe therapy for pain is typically necessary and post-herpetic neuralgia demanding continuing analgesia is a common problem. The effects of LTP treatments for herpes zoster was evaluated for pain reduction, healing rates, as well as safety. In a prospective randomized placebo-controlled phase II study, 37 herpes zoster patients were treated daily (weekdays) with 5 min of argon-based LTP (active) or 5 min of argon gas (flow-control, placebo), along with a standard therapy regimen. The visual analog scale was used to evaluate pain before and after treatment with LTP or placebo. Three clinicians evaluated digital images of lesions, blinded, and independently, in relation to vesicles, erythema, and general impression. Examination showed in LTP-treated patients presented a significantly greater reduction in pain over the treatment course when compared to controls, as well as an expressively superior median pain reduction immediately after each treatment. Plasma treatment pointed to faster clinical improvement in the first 1–2 days. Therefore, daily 5 min treatments with argon-based LTP was effective, painless, and safe, enhancing initial healing and decreasing severe pain in herpes zoster lesions [79].

The main agreement among the different studies up to the present time is that the ROS and/or RNS production is the main attribute of LTP, which affects viral inactivation, while UV radiation and temperature changes continue as minor contributors or have no effect whatsoever [73]. Studies on how LTP affects viruses are unique, considering that the specific plasma sources for each study are different (for example, dielectric barrier discharge, plasma μjet), with different characteristics (i.e. power, type of gas applied and exposure time). Moreover, the studies contend with the treatment of diverse liquid volumes or types of liquids, different substrates, as well as distinct viruses (surrogates of human viruses, human, animal, and plant viruses). Such a varied range makes a straight comparison of these studies very difficult, making it challenging to define the mechanistic assumptions or any general inactivation considerations [73]. Up to now, studies evaluating the effect of LTP on viruses' infections are scarce, mostly when compared to other diseases, and little is known about the mechanism of the LTP-mediated antiviral action, therefore, further studies with viruses should be performed.

2.5. LTP to optimize implant surfaces

As a complementary medical application, LTP can be used to prime or improve surfaces to turn them medically biocompatible, such as functionalization of implant surfaces. Osseointegration is the direct organizational adaptation of bone to the implant [80], being a topic of distinct relevance to orthopedic surgeons and dentists. In arthroplasty, for instance, surrounding the prosthesis; in trauma, for example, surrounding screws, and in dentistry, around dental implants. Bone anchorage on the implant surface can affect whether the reconstructive surgery will succeed and fail, and surface alterations may enhance the osseointegration of implants [28]. Since it has been established that moderately rough surfaces perform better than turned surfaces [84], current research is aiming at further alterations targeting a possible increase in the bioactivity of the implant. Therefore, some of the contemporary studies have shifted to chemically change slightly rough surfaces, which have been implied to create coactive effects. Moreover, the surface energy is an additional relevant factor participating in the regulation of osteogenesis [81].

Aiming to study the effects of LTP on rough titanium surface dental implants treated or not with argon-based LTP were investigated in an *in vivo* canine model. The surface energy and chemistry were characterized, showing peaks of titanium, calcium, and oxygen for both surfaces, and a 300% improvement in osseointegration was achieved for argon-based LTP treated titanium surfaces when compared to controls after three weeks [26]. However, a considerably slighter size effect of approximately 80% was detected for the argon-based LTP calcium phosphate surfaces relative to their untreated equivalents at the same implantation time [27]. Concerning physicochemical characterization, both studies showed the enhance in surface energy was more significant for the titanium surfaces when compared to the calcium phosphate surfaces, and those relative disparities when argon-based LTP treated and untreated surfaces were compared are the hypothetical cause for the relative changes in osseointegration.

Considering the beneficial results obtained with LTP in dental implants, the osseointegration properties of plasma surface treatment for implants were assessed, in another study, replacing argon for compressed air as the gas source [28]. Physicochemical characterization to evaluate topography, surface energy, and chemical composition was performed. Reduced surface levels of carbon and enhanced titanium and oxygen for titanium surfaces, as well as increased calcium and oxygen for calcium phosphate surfaces were reported. A significant increase in

bone-to-implant contact for atmospheric LTP-treated textured titanium surfaces at 6 weeks was observed, but not at three weeks or for calcium phosphate surfaces. However, no significant differences for bone area fraction occupancy between treated and untreated surfaces for any material at any time point were seen. These results indicate that air-based LTP surface treatment might increase osseointegration of textured titanium surfaces but not calcium phosphate surfaces [28]. Compared with earlier researches, both physicochemical and *in vivo* results showed that air-based LTP treatment enhanced surface energy and assisted earlier osseointegration compared with controls, even though the observed increases were not as important as the ones resulting from the treatment with argon-gas. Nevertheless, further studies optimizing atmospheric pressure plasma parameters and applications are necessary [28].

A recent *in vivo* study determined how plasma surface conditioning interferes with early wound healing and osseointegration. Sixteen dental implants with sandblasted and acid-etched surfaces were treated with LTP before insertion in the frontal bone of four miniature pigs. Bone metabolism was labeled by sequential fluorescence labeling administration, and eight weeks later, bone blocks were collected for radiological, histological, and histomorphometrically assessment. According to the findings, plasma treatment showed to have no effect on the morphology of the implant surface. However, groups treated with LTP exhibited increased bone-to-implant contact ratio, inter-thread, and peri-implant bone density compared to the controls. Interestingly, newly formed bone presented chronological and consistent mineralization, and no undesirable peri-implant side effects were present [82].

In fact, research on the application of LTP on implant surfaces has been shown to modify the surface elemental chemistry, resulting in superior biomechanical fixation and bone formation. However, further studies in prospective clinical trials are needed to substantiate these findings.

3. Conclusion

Plasma medicine applications have been emerging in the last years and can potentially lead to a novel, site-specific, and effective therapy for numerous diseases. Among them, employing LTP for the treatment of oral infections, mainly to remove biofilms, is a promising field. Advantages of LTP over traditional antimicrobial treatments are that LTP can be utilized for site-specific treatment; it provides an almost immediate (seconds) bactericidal response; antimicrobial resistance is less likely to appear because of LTP's multiple mechanisms of action and diversity of active agents [48]; there are minimal side effects [83]. However, regardless of intense research effort through the last years, there is a lack of data for the clinical application of LTP in dentistry to date. In contrast, plasma medicine achieved standard therapeutic status for wound treatment [3] and, recently, the technology was approved for an FDA phase I clinical trial [18].

Research is increasing and providing emergent proof for the use of LTP as a treatment alternative for diverse wounds and skin diseases, but more studies are necessary to further explore to entirely comprehend its mechanisms of action. In terms of LTP's effects in oncology, only preliminary data are available, therefore, further studies using plasma different types of cancer are encouraged. Furthermore, to date, studies evaluating the effect of LTP on viruses are scarce, and little is known about the mechanism of the LTP-mediated antiviral action. Therefore, future studies using plasma to control viruses' infections must be performed, considering the promising results it has been showing in this field. Research on the application of atmospheric plasma on implant surfaces has been shown to modify the surface elemental chemistry, causing higher biomechanical fixation and bone formation. However, additional research in prospective clinical trials is still needed.

What makes LTP therapy unique, though, is that LTP generators are small enough for safe and portable operation in clinical settings, having target-specific therapeutic effects, yet providing sufficient energy to impart meaningful alteration to biomaterials surfaces. Hence, LTP technology has been recently recognized as a powerful tool for biomedicine, considering its diverse plethora of therapeutic benefits.

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