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Virucide Properties of Cold Atmospheric Plasma for Future Clinical Applications

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Cold atmospheric plasma (CAP) has been repeatedly identified to bear powerful microbicidal efficacy on bacteria including multidrug resistant organisms and fungi on non-living surfaces, in biofilms as well as on contaminated and infected tissues. CAP furthermore was found to stimulate wound healing in chronic wounds and exerted anti-neoplastic effects on numerous tumor entities. Thus, CAP represents a promising medical tool for many clinical and therapeutic issues. Studies about CAP effects on virus particles recently were in arrears, but to date increasingly move into the focus of interest. Apparently, CAP treatment is followed by a promising virus inactivation and contributes to tissue regeneration. Here we review the current state of science concerning the so far investigated CAP effects on different virus species and virus-associated disorders. ***J. Med. Virol. 89:952–959, 2017***.

KEY WORDS: cold atmospheric plasma; CAP; virus; virus-associated disorders; virus inactivation

INTRODUCTION

Generation of cold atmospheric plasma (CAP) by various CAP devices is—contrary to expectations—nothing new, as "high frequency therapy" already played an important role in medicine at the beginning of the last century [Daeschlein et al., 2015b]. Numerous studies of the recent years definitely proved that CAP exerts efficacy to a broad microbial spectrum. This includes bacteria of clinical importance such as multidrug resistant species, e.g., methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug resistant Gram negative rods (MRGN), *Pseudomonas aeruginosa* and enterobacteriaceae, divers fungal strains of *Trichophyton spp.*, *Microsporum canis*, and *Candida albicans* [Daeschlein et al., 2011, 2015a; Matthes et al., 2016] as well as biofilms [Koban et al., 2011; Alkawareek et al., 2012]. By reduction of 94% of the regular bacterial skin flora, CAP accomplished sustainable skin disinfection including the follicular reservoir [Lademann et al., 2011]. Moreover, CAP was found to significantly reduce bacterial load in chronic ulcer wounds and to notably improved chronic wound healing [Isbary et al., 2010, 2012; Garcia-Alcantra et al., 2013a; Kramer et al., 2013; Brehmer et al., 2015; Ulrich et al., 2015]. Contemporary, a widely accepted assumption concerning the biological CAP-mediated mechanisms is the occurrence of reactive oxygen and nitrogen species (ROS, RNS) [Bekeschus et al., 2014; Weiss et al., 2015b]. ROS and RNS are only two factors among various biologically reactive particles in the highly reactive-ionized gas, further containing ions, electrons, excited atoms and molecules, free radicals, photons, and electromagnetic fields, subsequently emitting visible ultraviolet, vacuum-ultraviolet, and infra-red radiation [Ahn et al., 2011; Heinlin et al., 2011; Schneider et al., 2011]. CAP treatment of biological tissues and cells becomes feasible due to CAP can be operated at 37–38°C. Beside the considerable and growing evidence about CAP effects in bacteria and fungi, to date the relatively small number of recent studies lead to the assumption that CAP treatment could be of great relevance also for human pathogenic viruses.

Viral infection of eukaryotic cells is a complex mechanism which includes the signaling machinery

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in the intra- as well as the extracellular compartment [Galdiero et al., 2014]. Once a tissue is virus-infected, curative therapy is complex, often multimodal and for the most unsatisfying [Martin-Hirsch et al., 2010; Stojanov and Woo, 2015]. So far, the most effective strategy is the prevention of virus infection by sterilization including virucidal disinfection of medical devices and surfaces [Rutala et al., 2008; Gebel et al., 2013] as well as virucidal disinfection of hands [Kampf and Kramer, 2004] to interrupt cross infections, consequent aseptic technique in patient care as well as propagated education about sexual behavior. Fortunately, for several virus-associated diseases, especially the sexually transmitted human immunodeficiency virus (HIV), the world-wide infection rates are declining. However, a recent report of the Joint United Nations Programme on HIV/AIDS (UNAIDS) estimated that the number of people acquiring HIV increased by 26% for Middle East and North Africa and by alarming 30% in Eastern Europe and Central Asia in the years between 2000 and 2014 [UNAIDS, 2016]. This indicates a decline in prevention behavior in local areas which could result in a turning of the positive virus infection statistics of the recent years for many virus-associated diseases. The supposedly outstanding role of CAP-driven virus inactivation in virus-infected human tissue requires a consequent evaluation and characterization of CAP effects on most of the manifold different virus species and virus-associated disorders.

GENERATION OF CAP-DEVICES AND PHYSICAL PRINCIPALS OF CAP TREATMENT

Physical plasma is defined as a highly reactive ionized gas containing a mix of biologically reactive factors including electrons, ions, free radicals, photons, and electromagnetic fields. The generation of physical plasmas can generally be achieved by strong electric fields, usually associated with the strong emission of heat. Plasma treatment of biological tissues and mucosa becomes feasible because CAP is operating below 40°C [Lademann et al., 2010]. The reason for this was essentially the circumstance that electrons heating up much faster in an electric field compared to ions, resulting ambient temperature plasma [Weiss et al., 2015b]. To date, mainly three classes of differently operating CAP devices are available and accredited for medical application. Dielectric barrier discharge (DBD)—also called direct plasma—is generated by high voltage apply between an insulated electrode and a counter electrode, thus, biological tissue e.g., human skin and mucosa. The DBD setup forms a potent plasma discharge but limits electrical current, which would straight result in heat generation (Fig. 1A).

CAP generation between two inbuilt electrodes and directed evacuation to the target by a carrier gas is utilized in indirect plasma sources. Thereby, the concentration of the reactive CAP components is usually lower than in DBD devices. By bundling the gas flow, the resulting CAP jet enables a highly precise tissue treatment (Fig. 1B).

The combination of DBD and indirect plasma sources led to the development of surface microdischarge (SMD), consisting of an inbuilt power plate, a dielectric plate, and a grounded mesh electrode. This construction forms a homogenous and extensive CAP even out of ambient air.

CAP devices offer a lot of settings for CAP generation by varying the parameters electrical current, voltage, frequency, carrier gases and gas mixes, gas flow, and exposure time. This results in manifold CAP compositions and potential activities on biological material.

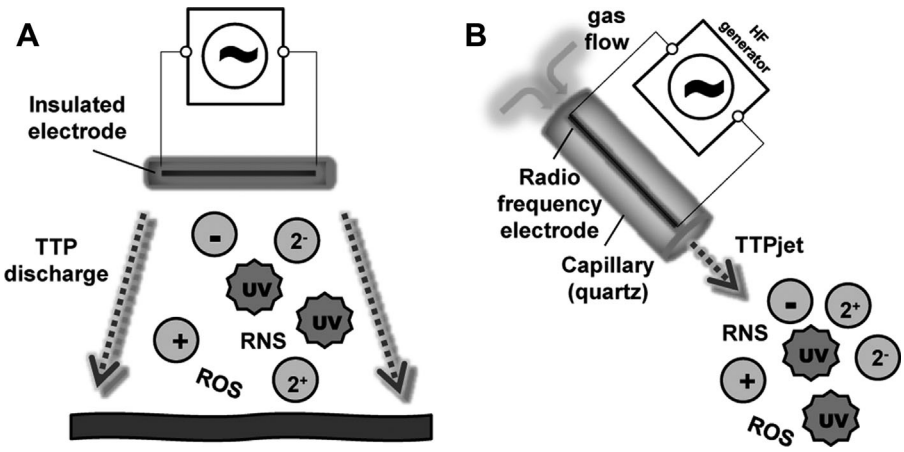

Fig. 1. Cold atmospheric plasma (CAP) generation. (**A**) Direct plasma source: dielectric barrier discharge (DBD). (**B**) Indirect plasma source: plasma jet.

DIRECT CAP EFFECTS ON VIRUS PARTICLES

Despite CAP treatment offered promising and nowadays widely accepted antibacterial and antifungal effects in vitro and in vivo [Isbary et al., 2013b; Brehmer et al., 2015], the investigation of CAP exposure on virus-contaminated materials is still in its infancy. Moreover, possible CAP-dependent alterations on virus replication, progression, and treatment response of virus-related disorders are for the most unknown. CAP treatment increasingly moved into focus of research since it demonstrated to bear important virucidal properties in some— so far distinct—virus species (Table I).

The investigations of CAP effects on different human pathogenic virus species are mainly based on certain very early studies performed in different bacteriophage models. Yasuda et al. [2008, 2010] investigated the early effects of CAP treatment on bacteriophage lambda (λ phage) in two consecutive studies. For the CAP experiments, they applied a DBD CAP device for 20 sec and found a significant virus inactivation due to rapid denaturation of λ phage's proteins. Interestingly, it could be shown that virus DNA was not affected by CAP exposure, hence was not responsible for λ phage's inactivation. Virus proteins, instead, were found to be sensitive to CAP treatment probably through denaturation and/or chemical/physical modification. The assumption that the degradation of viral proteins could be the responsible mechanism of CAP-dependent virus inactivation was supported by Venezia et al. [2008]. In this study, 10 min of CAP treatment caused about 4–6 $\log_{10}$ reductions of viral viability. Again, CAP exposure was followed by denaturation of bacteriophage's proteins (e.g., viral envelop proteins) but subsequently had no impact on CAP-treated isolated phage DNA. Alshraiedeh et al. [2013] used *Escherichia coli* MS2 bacteriophages as surrogate system to evaluate the virucidal efficacy of a CAP jet source operating with varying oxygen concentrations of 0.0–1.0% and 100–99.0% of helium gas, respectively. The application of 100% helium CAP jet was followed by a significant reduction of MS2 bacteriophage's viability at each time point to a maximum of a 4.98 $\log_{10}$ reduction after 9 min of CAP exposure. Interestingly, the authors observed even greater reductions of phage viability when using increasing oxygen concentrations for CAP generation. Recently, Wu et al. [2015] showed a significant CAP-mediated inactivation of airborne as well as waterborne MS2 bacteriophages. Electron microscopy images and SDS-polyacrylamide gel electrophoresis indicated a fragmentation of surface proteins, agarose gel electrophoresis of viral RNA encoding for different surface proteins demonstrated notable RNA damage. This basic research can assume great significance if it succeeds to transfer CAP treatment on human pathogenic viruses.

Human Adenovirus

A study by Zimmermann et al. [2011] showed very impressive virus inactivation of CAP-exposed human adenovirus (hAV). Adenoviruses are dsDNA viruses lacking a virus envelope. However, due to the protein capsid, hAV is characterized by environmental stability and a low susceptibility to physical and chemical noxa (e.g., heat, changes in pH, biocides). Generally, hAV infection causes mild respiratory and gastrointestinal symptoms beside serious keratoconjunctivitis often associated with epidemic spread. CAP treatment with a SMD device resulted an over three log viral inactivation after 240 sec. Additionally, by CAP treatment of hAV coding for the fusion protein of eGFP and firefly luciferase before infection of CMS-5 cells they could show that both the infectivity as well as the replication of the viruses were potently suppressed. The group mainly attributed the observed CAP effects to the generation of RNS, including its various intermediates and adducts, which were already shown to interact with DNA synthesis and repair, protein expression and regulation as well as

TABLE I. CAP effects on virus species

	Virus name	CAP method	CAP effect	Reference
1	Bacteriophage	DBD	Virus inactivation; protein denaturation	Yasuda et al. [2008, 2010], Venezia et al. [2008]
			Protein fragmentation; RNA damage	Wu et al. [2015]
2	Human adenovirus	SMD	Virus inactivation; infectivity ↓, replication ↓	Zimmermann et al. [2011]
3	Human herpes simplex virus	PJ	/	Brun et al. [2012]
4	Human respiratory syncytial virus	BCOP	Virus inactivation	Terrier et al. [2009]
5	Type A influenza virus	BCOP	Virus inactivation	Terrier et al. [2009]
6	Human parainfluenza virus type 3	BCOP	Virus inactivation	Terrier et al. [2009]
7	Feline calicivirus	PJ	Virus inactivation	Aboubakr et al. [2015]
8	Human norovirus	SMD	Virus load reduction	Ahlfeld et al. [2015]

DBD, dielectric barrier discharge; SMD, surface micro-discharge; PJ, plasma jet; BCOP, biozone cold oxygen plasma.

immunogenic pathways [Bogdan, 2001]. Most interestingly RNS were already shown to inhibit the replication of numerous RNA and DNA viruses [Reiss and Komatsu, 1998].

Human Herpes Simplex Virus

Brun et al. [2012] puplished very interesting data concerning the impact of CAP on human herpes simplex virus (hHSV) 1 infected Vero cells. For this, they measured the cytopathic efficacy of hHSV-1 after 5 min CAP treatment using a plasma jet with helium as carrier gas. Surprisingly, CAP treated hHSV-1 infected cells exhibited the same cytopathic repercussions as were observed in helium control-treated cells, thus, showed to be insensitive to CAP treatment. As hAV, which was shown to be highly sensitive to CAP treatment, hHSV-1 is a dsDNA virus. A substantial difference between hAV and hHSV-1 is that hAV is lacking a virus envelope, a circumstance which, besides, is the reason for the low efficacy of many antiinfectives [Kampf and Kramer, 2004]. Therefore, the expression of a shielding virus envelope could be the main factor for viral insensitivity to CAP exposure. Future studies will have to proof if envelope expression is a reproducible factor for CAP resistance in other virus species as well.

Human Respiratory Viruses

Terrier et al. [2009] obtained first data concerning the efficacy of CAP-driven inactivation of the airborne viruses human respiratory syncytial virus (hRSV), human parainfluenza virus type 3 (hPIV-3), and a type A influenza virus (IV-A). Whereas hRSV and hPIV-3 are leading causes for acute lower respiratory tract illness, human influenza viruses are primarily responsible for fatal upper respiratory tract infections and pandemics [Cox and Subbarao, 2000; Durbin and Karron, 2003; WHO, 2009; CDC, 2014b]. To assess CAP's efficacy for airborne virus decontamination, high virus concentrations were nebulized and the pre- and post-CAP infectious virus titers were compared [Terrier et al., 2009]. Usual CAP was generated by the use of oxygen as carrier gas and moreover, a so called "Biozone cold oxygen plasma" (BCOP) generated by high energy deep-UV light represented by an effective radiation spectrum between 180 and 270 nm. Despite the authors could show a significant loss of virus particles, it was possible to detect small amounts of infectious virus particles. However, the study demonstrated a virus inactivation efficiency of at least 99.0% for every virus species. Notably, the application of BCOP accomplished a loss of infectious particles of 99.98% (hRSV) and more than 99.99% (hPIV-3 and IV-A).

Human Norovirus

To date, the gastroenteritis causing human norovirus (hNV) underwent special attention as a possible target for CAP-mediated surface and food decontamination. The members of the *Caliciviridae* family are ssRNA viruses and are expected to be the most frequent triggers of acute non-bacterial gastroenteritis worldwide, associated with considerable financial burdens [Frankhauser et al., 2002; Lopman et al., 2004; Green, 2007; Bitler et al., 2013; CDC, 2014a].

Due to the lack of a suitable cell culture system for analysis of hNV biology, investigations have to be performed in surrogate virus models so far [Duizer et al., 2004; Dawson et al., 2005].

Recently, Aboubakr et al. [2015] investigated the effects of CAP treatment on feline calicivirus (FCV), another established model for human norovirus. FCV is a member of the *caliciviridae* family and so is hNV. A total of 120 sec of CAP exposure generated by an argon plasma jet device inactivated more than 99.99% of FCV.

Ahlfeld et al. [2015] waived the usage of surrogate culture systems for human norovirus by CAP treatment of fecal samples derived from a norovirus outbreak in a German military facility. The foodborne outbreak including the symptoms nausea, diarrhea, and circulatory disorders was attributed to human norovirus genotype GII.4. For experiments, a surface microdischarge (SMD)-based CAP device was applicated on suspended norovirus faeces samples for 0.5–15 min. CAP treatment was followed by up to 1.69 $\log_{10}$ reductions of viral load. The results thereby crucially depended on the extent of the initial virus load and the use of whether unmodified fecal- or phosphate-buffered saline (PBS)-diluted norovirus samples.

DIRECT CAP EFFECTS ON VIRUS INFECTION-ASSOCIATED DISORDERS

Besides the treatment of an acute virus infection by virus inactivation or virus reduction, the relief of directly virus associated and post-infectious symptoms are attributes of great importance today. Probably the most prominent factor accompanied with reduced quality of life is pain. Further uncomfortable virus-related symptoms, especially when located on mucosal tissue can be pruritus, increased sensitivity, and tightness of the skin. Recently, CAP demonstrated the ability to control virus-related and virus-exacerbated syndromes and pain.

These CAP effects have been well studied particularly in case of Herpes zoster (HZ) which is a reactivation of latent *alphaherpesvirinae* species varicella-zoster virus (VZV) infection, the pathogen of the chickenpox. The disease is clinically characterized by painful dermatome-associated unilateral vesicular eruption due to the dissemination of VZV along the corresponding sensory nerves. A redoubtable complication of HZ is the development of post-zoster neuralgia (PZN), which is defined as persistence of pain for more than 4 weeks after healing [Archer and Eedy, 2010; Cohen, 2013]. Isbary et al. [2014] investigated

CAP treatment as an innovative technology to reduce HZ-derived pain in a prospective randomized placebo-controlled clinical trial with 19 CAP and 18 control-treated patients. For active CAP treatment of HZ lesions, a microwave-based CAP jet device was used. In this study, an average of 4.7 5-min CAP applications were performed for each patient additionally to standard HZ treatment. Notably, significantly more patients improved in pain and reported a greater immediate median pain reduction when being CAP treated, compared to the control-treated group. In the control group significantly more patients described a pain exacerbation immediately after argon control treatment. Moreover and in contrast to the control group, CAP treatment reduced the frequency of persisting pain about 20% after 2 weeks and nearly about 50% after 4 weeks of follow-up. CAP-treated patients showed ingestion of lower overall paracetamol dosages and besides, the clinical manifestation of HZ erythema and vesicles resolved more quickly in the actively CAP-treated group. The results are consistent with a previous pain study by Isbary and Shimizu [2013a]. Thereby, CAP treatment potently diminished the severe pain in a 49-year-old man after cholesteatoma surgery and subsequently reduced the development of chronic external auditory canal infection with a mixed bacterial spectrum. A total of 43 5-min CAP applications during a period of 105 days significantly reduced pain sensation on a visual analog scale, limited analgesics requirement to zero and was well tolerated with no side effects.

POTENTIAL APPLICATIONS OF CAP TREATMENT AND ITS MOMENTOUSNESS FOR ONCOVIRUS-RELATED CANCER PREVENTION AND THERAPY

Virus infections, particularly of mucosal tissue, are widely accepted in triggering the development of intraepithelial neoplasia and invasive cancer. Cancerogenic viruses (so called oncoviruses), according to recent studies, are responsible for up to 15% of cancers [Martin and Gutkind, 2008]. This includes Epstein-Barr-Virus (EBV), human herpesvirus 8 (HHV 8), human T-lymphotropic virus 1 (HTLV 1), hepatitis virus B (HBV) and C (HCV) as well as different serotypes of human papillomavirus (HPV). Despite a diverse spectrum of antiviral treatment approaches, including broad-spectrum agents and targeting of virus-driven oncogenic signaling pathways, current therapy options are promising but remain unreliable and infection prevention with vaccines, as already applied for human papilloma virus, may currently be the only way to effectively reduce the incidence of the disease [Villa et al., 2006]. Thus, the detection of virus-related epithelial lesions often requires radical surgical and physical elimination [Stojanov and Woo, 2015]. These procedures, however, are often associated with high costs, side-effects, discomfort, and pain, and exert a recurrence rate of over 20% [Lacey et al., 2013; Rosales et al., 2014]. Recently, a phase I/II clinical trial investigated the effect of intralesional vaccination of HPV-induced CIN with recombinant bovine vaccinia virus "vaccinia virus Ankara (MVA) E2" and demonstrated significant decreases to complete eliminations of virus-induced precancerous lesions [Corona Gutierrez et al., 2004; Rosales et al., 2014]. This example impressively demonstrates the benefit and especially the need for innovative therapies for mucosal virus elimination and treatment of virus-related precancerous lesions.

Lately, also CAP treatment showed notable decreases of tumor growth and tumor masses of various cancer entities. In 2010, CAP treatment of colon cancer cells resulted in a significant decrease in cancer cell proliferation, migration, and invasion [Kim et al., 2010a]. Similar results were obtained for murine melanoma cells, which, furthermore, showed 2.5 times higher apoptosis rates than CAP-treated non-tumor fibroblasts [Kim et al., 2010c]. Further studies in human melanoma cells demonstrated CAP-driven DNA damage and increase of pro-apoptotic proteins [Arndt et al., 2013]. Kim et al. [2011] sufficiently induced apoptosis in murine lung carcinoma cells by a flexible optical fiber-based CAP device offering new possibilities of CAP application. CAP, moreover, exerted strong anti-cancer effects due to induction of apoptosis in pancreatic and head and neck cancer cells and furthermore by activation of pro-apoptotic redox signaling in prostate cancer cells [Partecke et al., 2012; Weiss et al., 2015a,b]. Analogously to the selective inactivation of tumor cells by ROS [Ishaq et al., 2014], it seems possible that virus-infected cells are more sensitive against increasing ROS levels compared to non-infected cells which are able to adjust their metabolic pathways. This may enable controlled elimination of virus-infected cells.

Additional important arguments for CAP tissue treatment are (i) its low invasiveness, (ii) the absence of treatment associated pain or discomfort, and (iii) the overall brilliant tolerance and acceptance in patients [Isbary et al., 2013c, 2014; Heinlin et al., 2013; Metelmann et al., 2015]. A study by Partecke et al. [2012] estimated CAP-induced apoptosis only in the uppermost cell layers with a depth of effective tissue penetration of a maximum of 60 μm [Partecke et al., 2012]. Thus, the small tissue penetration of CAP may explain the overall good tissue tolerance and the almost absence of pain during CAP application. However, it is the most limiting part for CAP applications which seem to be confined to topical treatment indications.

In 2012, Wu et al. investigated thresholds for CAP-induced damage on intact and wounded porcine skin using a DBD CAP device [Wu et al., 2013]. They concluded that CAP treatment is possible for up to 15 min at CAP power of 0.17 W/cm^2 without causing any microscopic tissue damage. At a level of 0.31 W/cm^2 CAP power, the limit of safe CAP treatment was

0.5–1 min. Notably, 2–3 min of CAP treatment at the same power level caused full-thickness burns and epidermal damage.

Moreover, a number of in vitro studies demonstrated DNA damage and genotoxicity following CAP treatment of mammalian cells [Kim et al., 2010b; Ptasinska et al., 2010; Kalghatgi and Azizkhan-Clifford, 2011; Garcia-Alcantra et al., 2013b; Morales-Ramirez et al., 2013]. Kluge et al. [2016] recently CAP-treated fertilized chicken eggs inner membranes. Thereby, even with a treatment duration 5- to 10-fold above the limit which is currently recommended for chronic wound treatment they found no genotoxic or mutagenic effects. Henceforth, many new insights can be expected in this field of physical plasma investigation.

CONCLUSION

Until today, CAP treatment was correlated with significant virus reduction and inactivation by only a handful of studies. Moreover, preliminary in vivo studies revealed no side-effects and risks for CAP-treated patients but rather evidenced a low invasiveness and simplicity of CAP application as well as improved wound healing and inhibition of pain and inflammation.

The combination of broad antiviral and antitumor efficacy could make CAP a suitable tool for treatment of virus infection and virus-related cancers. It is conceivable that particularly "high risk" HPV serotypes 16 and 18 associated cervical, vulvar, vaginal, and penile intraepithelial neoplasia could be prevented or at least the further progression to an invasive carcinoma could be decelerated. CAP technique could be a highly specific, simple, time-, and cost-effective tool in clinical virology and oncology. Notwithstanding the immense medical and health economical capability of CAP-mediated mucosal virus eradication further studies in this field are still vacant. Here, quite a lot of new insights can be expected over the next few years.

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