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Experimental application of cold atmospheric plasma in the treatment of head and neck infections and malignant tumors

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CONTENTS

ABBREVIATIONS

°C	Celsius
APPJ	atmospheric pressure plasma jet
CAP	cold atmospheric plasma
CFU	colony forming units
DBD	dielectric barrier discharge device
ENT	ear, nose and throat
ESBL	extended spectrum beta-lactamase
Gy	Gray
HNSCC	head and neck squamous cell cancer
HPV	human papilloma virus
IC_{50}	half maximal inhibitory concentration
iv	intravenous
KAP	kaltes atmosphärisches Plasma
kHz	kilo Hertz
kV	kilo Volt
LTE	local thermodynamic equilibrium
mg/m^2	milligramm per square meter
mm	millimeter
MRSA	methicillin-resistant Staphylococcus aureus
RCT	radiochemotherapy
RS	reactive species
RT	radiotherapy
s	second(s)
Sek	Sekunde(n)
SMD	surface micro discharge device
TB	Teresa Brunner
URTI	upper respiratory tract infections
UV	ultraviolet

V	volt
w/cm^3	Watt per cubic centimeter

PUBLICATIONS

This cumulative writing is based on the following two original papers:

Sven Becker, Julia L. Zimmermann, Philipp Baumeister, **Teresa F. Brunner**, Tetsuji Shimizu, Yang-Fang Li, Gregor E. Morfill, Ulrich Harréus, Christian Welz (2019): Effects of cold atmospheric plasma (CAP) on bacteria and mucosa of the upper aerodigestive tract, *Auris Nasus Larynx* **46** 294–301
https://doi.org/10.1016/j.anl.2018.07.008

Teresa F. Brunner, Florian A. Probst, Matthias Troeltzsch, Sabina Schwenk-Zieger, Julia L. Zimmermann, Gregor Morfill, Sven Becker, Ulrich Harréus, Christian Welz (2022): Primary cold atmospheric plasma combined with low dose cisplatin as a possible adjuvant combination therapy for HNSCC cells - an *in-vitro* study**,** *Head & Face Medicine*, Volume 18, 21 (2022)

CONTRIBUTION TO PUBLICATIONS INCLUDED IN THIS THESIS

TB (Teresa Brunner) made substantial contributions to acquisition, interpretation of data, statistical analysis and helped carry out manuscript drafting and revision for Publication 1.

TB performed the experiments, the data analysis, the statistical work, and drafted and revised the manuscript for publication 2.

1. INTRODUCTION

1.1. Cold atmospheric plasma

1.1.1. Definition

Matter can be classified into four states, as displayed in Figure 1. These are considered to be solid, liquid, gas and plasma. Plasma can furthermore be categorized into thermal and non-thermal. Thermal plasmas are classified as “hot” plasma and come close to a state of local thermodynamic equilibrium (LTE). Nonthermal plasmas contain neutral atoms and ions at around 21° (room temperature) although electrons are at higher temperature (5000 °C and 105 °C). These are also seen as cold plasmas and usually occur in reactors at low (p < 133 mbar) and atmospheric pressures and can for example be generated through pulse discharge systems or direct (1).

The temperature of CAP is lower than 40°C at the time of application. Methods that can produce CAP are direct, indirect and hybrid sources. Examples of the latter being an Atmospheric Pressure Plasma Jet (APPJ) or Dielectric Barrier Discharge (DBD). Non thermal plasma is generated with noble gases (e.g. Argon, Nitrogen and Helium) or air (2).

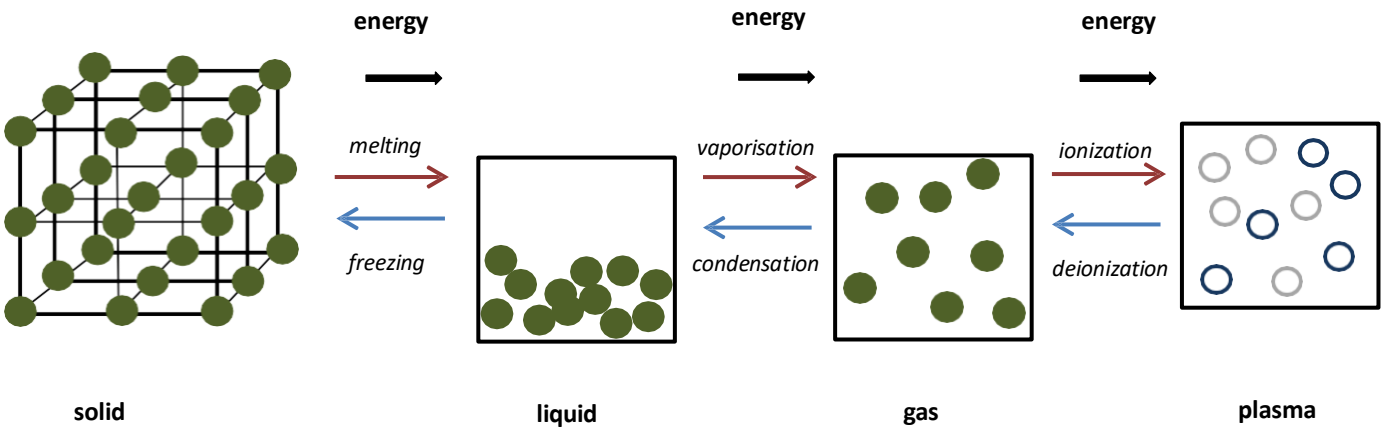

Figure 1 Schematic figure of the four states of matter

1.1.2. Surface Micro Discharge (SMD) Device

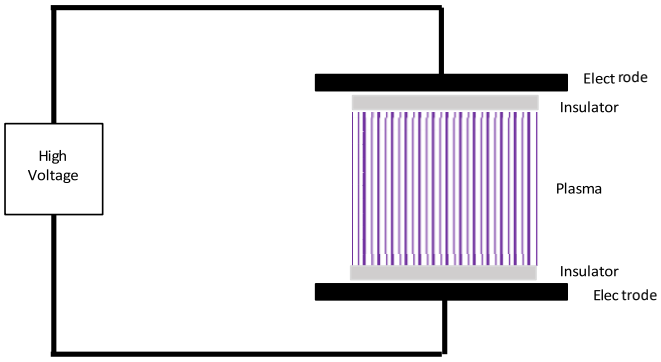

Figure 2 Schematic figure of an example of a planar configuration of a DBD

A DBD is an independent electrical discharge between two electrode configurations containing an insulating material between these (discharge path). Glass, teflon, silicon rubber or ceramics are examples of common insulating (dielectric) materials used. The dielectric barrier causes a self-pulsing plasma operation and this way, the formation of a cold plasma (3). Plasma can be generated through air in a DBD device, hence a carrier gas is not needed for plasma production (4).

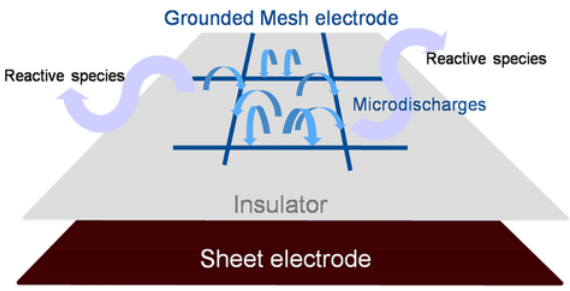

Figure 3 Schematic figure of the plasma production on an SMD electrode (5).

A SMD is a subtype of a DBD plasma source. A grid is the grounded electrode. The discharges are generated between the electrodes and can, because of the grid, spread on the surface of

the dielectric. A mixture of electrons, charged particles, neutral atoms, ROS, RNS and UV radiation are created by partial ionization of the surrounding air by the micro discharges. This way, plasma is generated from the surrounding air and can reach the site of action by diffusion (6)(7). A SMD Device, MiniFlatPlaster®, was used for the experiments in both publications.

1.1.2.1. MiniFlatPlaSter®

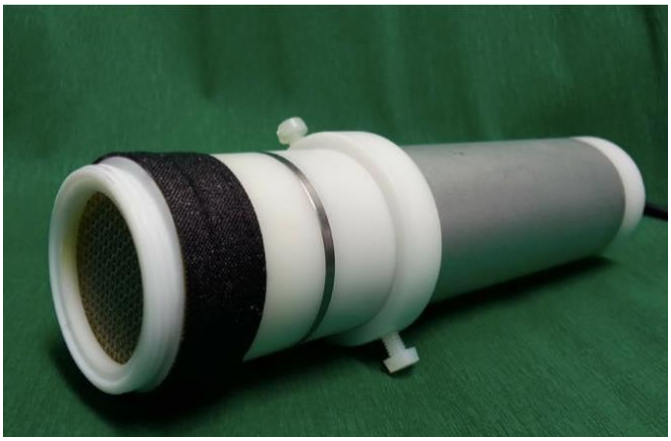

Figure 4 Foto of MiniFlatPlaster ® used in the study (8)

The MiniFlatPlaster® is a portable SMD plasma device with a high voltage battery. Between the copper and stainless steel grid electrodes a glass epoxy is positioned and serves as a dielectric. By transforming the output voltage of about 4-12V to a high frequency voltage and repetition frequency of 7kV and 6.75 kHz respectively, homogeneous CAP is generated at the electrode from the emitting air. The power density is approx. 0,5 W/cm^2 Species such as O_3, NO, NO_2, HNO_3, N_2O_5, N_2O etc. can produce the samples to be treated. Hence, meaning that within the first 20 seconds a high amount of ozone is generated by the plasma, which will be destroyed by the plasma itself if the electrode continues to operate. Longer plasma times will then cause a rise in reactive specie concentration (9)(10).

1.1.3. CAP mechanism

To a large extent, the underlying mechanism of CAP is still not fully known. At the current state of knowledge, chemical as well as physical components of CAP have shown to influence cell biology, especially survival. Chemical constituents involve oxygen- (ROS) and nitrogen-based reactive species (RNS). The leading reagents of these being hydrogen peroxide (H_2O_2) and nitrogen dioxide (NO_2) associated with CAP exposure of cells. Physical components of CAP involve heat, UV-radiation, heat and electromagnetic fields (11).

1.1.4. Effect of CAP on pro- and eucaryotic cells and present medical applications

CAP effects bacterial procaryotic and eukaryotic human and animal cells by the synergy of the above mentioned biologically active plasma components. The mechanism of chemical factors include the alteration of the intracellular redox state and oxidative alteration of certain proteins which are associated with signalling pathways. UV-radiation can be considered as the most important physical factor. The main mechanisms involved for cellular damage are the direct effects and DNA, protein and lipid alterations which are caused (oxidative stress) (12). CAP sources mainly show antimicrobial and cell proliferative effects. Various studies have repeatedly shown its high efficacy against a broad microbial spectrum (13). CAP produced reactive species are of substantial importance in microbial inactivation, followed by ions and UV radiation in descending order (14). *In-vitro* studies have successfully shown CAP's antimicrobial effectiveness on multidrug resistant pathogens and biofilms (15)(16). Using an SMD device *E. coli* and *Candida albican* biofilm were able to be reduced by > 5-$\log_{10}$ CFU (17)(18). However, the efficacy achieved differs in literature and affecting factors include: type of device, gas composition, variation of CAP device characteristics, different exposure distances and times as well as microorganism type and load (14).

In addition to these properties, the growing scientific evidence suggests CAP as an optimistic and encouraging new approach in the treatment of various malignant tumors. The anti-cancer mechanism is said mainly to be associated with chemical factors, especially the rise in ROS and RNS causing DNA damage (11). CAP has shown to potentially inhibit cancer cell migration. An *in-vitro* study has observed that CAP exposure activates β1 integrin causing a decrease in cell migration and an increase in focal adhesion size (19). Additionally, CAP shows a selectivity

towards different cells. Cancers cells showed a higher cell viability decrease after CAP exposure than healthy tissue after the same exposure time (20). CAP seems to be able to selectively target malignant cells by disrupting certain stages during mitosis. Hence, causing DNA damage, cell cycle arrest and apoptosis.

At the same time the toxic effects of CAP on surrounding healthy tissue or tissue attached to microorganisms have proven to be limited.

1.2. Infections of the upper aerodigestive tract (Publication 1)

1.2.1. Overview

The upper respiratory tract includes the nasal passages, the nasal sinuses, the pharynx and the larynx. Upper respiratory tract infections (URTI) and ear nose and throat (ENT) infections therefore include rhinitis, sinusitis, nasopharyngitis, tonsillitis, pharyngitis, and laryngitis. Viruses, bacteria and/or fungi can be the causative organisms in decreasing order of frequency. Especially the viral URTI are one of the most common reasons in developed countries for healthcare professional consultation and absence from work. The rhinovirus is the most common, followed by the influenza virus, adenovirus, enterovirus, and respiratory syncytial virus (21). ENT infections, even though they are mainly viral infections, are still the most common cause for antibiotic prescription (22). However, a viral URTI can also lead to secondary bacterial co-infections (23).

H. influenzae, Staph. Aureus. Strept. pneumoniae and *Strept. Pyogenes* are the most common species for bacterial infection in the URT (24)(25). *H. influenzae* is a gram-negative, aerobic bacteria. It is predominantly present in middle ear infections of infants (26) and as a fatal and severe infection in epiglottitis. URT and lower respiratory tract are commonly colonized by gram-positive bacteria *S. pneumoniae* and *S. pyogenes*. Yearly, worldwide > 600 million people are registered with streptococcal throat infections. *S. aureus, aerobic and gram-positive,* is another critical bacteria with resistance to many antibiotics (27). There are has been an increase in antibiotic resistance and therefore treating multiresistant bacteria, e.g. *methicillin-resistant S. aureus (MRSA)* or extended *spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae*, has become far more common and problematic. This is the one of the biggest clinical challenges.

1.2.2. Therapy options of bacterial infections

ENT and upper airway tract infections are very common. ENT and URT infections are the leading reason for increased antimicrobial resistance and is one of the greatest global health challenges and also an economic issue. Generally, a local treatment of bacterial infections is the primary treatment option with either local disinfection or antibiotics. However, this often fails because of incorrect administration with low tissue concentration or already a developed/existing resistance. The next step would be the use of systemic antibiotics. During the evaluation of the microbiological findings (cultures and antibiograms) a presumptive choice of antibiotic is usually initiated. If necessary the antibiotic therapy is then changed to a targeted therapy against the detected bacteria. Yet again, there is a rather high failure rate because of antimicrobial resistance development or side effects (28). Antibiotic resistance complicates the therapy of bacterial infections and can cause severe complications, sickness and increased mortality rates. Additionally systemic antibiotic treatments can cause numerous over treatments and adverse side effects including gastrointestinal side effects, fungicidal superinfections, and allergic reactions . This can lead to longer treatment times as well as premature treatment terminations.

Novel treatments are needed to eradicate bacteria responsible for ENT infections with less side effects and less risk of acquiring antimicrobial resistance. The antimicrobial feature of CAP could make it a valuable instrument for the local elimination of microorganisms in the ENT and URT. To date, no resistances have been induced and are expected by CAP in bacteria so far (29). Additionally, the exposure of mammalian tissues to CAP generated by an SMD device have not shown any harm or effect (30)(31). Both bacterial load reduction and enhanced wound healing was observed after direct CAP treatment of chronic ulcer wounds (32)(33). Hence, a direct CAP application on ENT infections could be a promising alternative for bacterial eradication without systemic side effects and the risk of further antibiotic resistance.

1.3. Malignant tumors of the head and neck (Publication 2)

1.3.1. Overview

Each year, the prevalence of head and neck cancers worldwide is around 600,000 cases with approximately 350,000 deaths (34)(35). Head and neck malignancies include tumors of salivary glands, oral cavity, pharynx (naso-, oro- and hypopharynx), larynx and the paranasal sinuses (36). The most common (approx. 90%) are squamous cell carcinomas (HNSCC).

Considering the worldwide incidence, HNSCC is the sixth most widespread cancer. HNSCCs are predominantly found in the epithelial lining of the oral cavity, oropharynx, hypopharynx and larynx and have shown a predominant prevalence in males (37). Risk factors leading to HNSC carcinomas are tabacco, alcohol and human papilloma virus (HPV) (38).

The treatment and management of HNSCC is often a challenge because >60 % are already locally advanced tumors at the time of diagnosis (39). The 5 year overall survival rate is approximately 40-50% (40).

The prognosis of the patients with HNSCC, the treatment type and options vary and depend on factors such as risk factors, the molecular properties of the tumor, tumor accessibility, patients' health and preferences and the stage of the cancer at time of treatment. Depending on these factors, HNSCC show a higher or lower successful response to the established therapy modalities.

1.3.2. Therapy options

According to guidelines, first line and preferred therapy for HNSCC is primary surgical resection of the local tumor as well as, depending on CT scan staging results and tumor stage, the surgical removal of the ipsilateral and possibly contralateral cervical lymph nodes. Patients can be treated with a curative radio(chemo)therapy as an almost comparative therapy option. An adjuvant therapy with radiation (RT) or combined radiochemotherapy (RCT) is often indicated, especially in advanced tumor stages. Nevertheless, all these therapy options are often limited and associated with a severe reduction in the patients' quality of life.

Surgery is often difficult to perform due to the size of the tumor and important anatomical structures, tumor accessibility, the patients' risk factors and medical condition. Additionally, cosmetic disfigurement and functional impairment can often have a high impact on the psychosocial environment and quality of life of the patient. All these factors can pose a limiting factor for the degree and possibility of surgery.

RCT is the most common alternate or adjuvant curative treatment modality for HNSCC. Indications and high recommendations for adjuvant RT/RCT include tumor size > 4cm or infiltration > 10mm, invasion of the regional lymph nodes, close margin resection (<5 mm), perineural or vascular invasion (41)(42). On average a dose of 60 Gy is used for HNSCC RT. Due to major advances, RT is high in precision. Nevertheless, severe short and long term side effects are very common. Acute side effects include nausea, hair loss, gingivitis, dysphagia,

and/or lymphedema. Xerostomia, osteonecrosis (especially of the mandible) and fibrosis are examples of late side effects (43)(44). In addition, clinicians and researchers have suggested that even though postoperative RT may decrease the chance of locoregional recurrence, the overall survival could stay unchanged as it may be compensated by a greater risk of distant metastases (45). This causes a high degree of psychological strain and perhaps even a constant need of therapy for the patient as well as premature interruption or even termination of RT. Furthermore, there is a maximum dosage of radiation applicable, hence being another limiting factor for RT.

An additional therapy for locally advanced HNSCC is the administration of cisplatin intravenously. In advanced HNSCC tumor stages RCT and RT + Cetuximab are preferred to solely RT and have shown an increased 5-year survival of ~8% (45)(46)(47) . A standard regimen of 3 doses of chemotherapy cisplatin 20-70mg/m² i.v. are recommended (48). Despite the promising results, the systemic toxicity needs to be addressed. These include renal toxicity, ototoxicity and myelosuppression (49). Moreover, many patients with HNSCC do not experience a response to cisplatin administration. Most patients will even develop an acquired cisplatin-resistance. This can even induce cancer recurrence (50). For many patients chemoresistance and systemic toxicity are restricting factors for the RCT and can result in discontinuation of the anticipated therapy and even cancer relapse.

Post-treatment rehabilitation also has to be taken into account. This can often be a long and strenuous process for the patients.

A direct *in vivo* non-thermal plasma application on HNSCCs would in theory be possible as these can often be accessed directly. Considering the high toxicity of cisplatin, a greater dosage of the systemically applied concentration is not advisable in advanced or relapsed HNSCC. In consequence, CAP and cisplatin combined could be of clinical significance.

HNSCC cells were seen to have the highest cell viability reduction and selectivity towards CAP treatment so far (51). Furthermore, when considering the cell cycle due to the DNA damage caused by CAP in malignant cells there is an accumulation at G2/M phase of cells (52)(53). Even though cisplatin is not a cell cycle specific chemotherapeutic agent, cell cycle-mediated resistances can also be of importance. Based on current literature cells appear highly sensitive to cisplatin in G1-phase. A low sensitivity towards cisplatin has been shown with the beginning

of S-phase (54). Hypothetically, if there is an accumulation of cells at G2/M phase after CAP exposure this could lead to a higher sensitivity towards cisplatin as well as a possible synergistic and/or additive effect of a combination therapy.

According to oncologists chemotherapy has reached a plateau of effectiveness as a primary treatment option even if toxicity can be stabilized or even decreased(55). Therefore, further successful single and combined treatment options are essential in order to improve the prognosis of HNSC cancer and CAP could be a possible promising addition as an adjuvant therapy option for HNSCC in mono- or combination treatment regimes.

2. STUDY OUTLINE

This is an experimental *in-vitro* study to investigate the effectiveness of CAP application for diseases in the head and neck area with limited and problematic therapeutic options. The aims of the study were:

- To examine the bactericidal effect of CAP on frequent ENT bacteria as well as the effect on the surrounding healthy mucosal tissue.
- To examine the cytostatic effect of CAP on common cisplatin resistant and sensitive HNSCC cells. Furthermore, to investigate a possible increase in tumor cell viability reduction with concurrent and/or consecutive combination of low dose cisplatin.

The same SMD device (MiniFlatPlaster®) was used for both publications. CAP application was identical in both publications, the samples were placed horizontally below the electrode, creating a closed volume condition. The device was placed directly onto the well plate and agar plate leaving only the height of the well plate as a space between device and MOCs/HNSC cells. The space between the electrode and agar plate and electrode and cells in well plate were 3mm and 17.5 ± 0.5 mm respectively. The respective controls were treated under the same conditions as the plasma treatments except for the CAP exposure in both publications. CAP treatment times of 30, 60, 90, 120 and 180s were analyzed in both publications. An additional CAP treatment time of 5s was evaluated in publication 1 on healthy mucosal tissue.

The four ENT pathogens used in publication 1 were *H. influenzae*, *Staph. Aureus*. *Strept. pneumoniae* and *Strept. pyogenes*. The CFUs were counted after the set CAP exposure times and compared to the untreated plates. Additionally, cytoplasmic membrane damage and acute cell necrosis was measured by trypan blue staining and Annexin V Fit-C staining respectively after the above mentioned CAP exposure times on healthy mucosal tissue. The healthy mucosal tissue used for the experiment were mucosa samples taken from the oropharynx during tonsillectomy.

In publication 2, a low cisplatin concentration of 2.5µM was used. The concentration was selected based on the dose-response curve. The concentration of 2.5µM of the chemotherapy drug was selected as it only showed a marginal influence on the survival of the different

HNSCC. Thus, meaning only a scarce change in cell viability. This way the impact of a combination therapy on cell viability with less cytotoxicity could be observed. The three different HNSCC cell lines used in publication 2 were Cal 27 FaDu and OSC 19. The cellular responses to mono- and dual therapy with CAP were investigated by trypan blue staining, MTT assay, Annexin V Fit-C staining and comet assay. Hence, measuring cytoplasmic membrane damage, mitochondrial damage, apoptosis and DNA damage respectively.

3. OVERVIEW ON RESULTS AND ACHIEVEMENT

The study revealed an antibacterial as well as an antitumoral effect of CAP in the head and neck region.

To begin with, as described and shown in Publication 1 treatment with CAP showed an increased bacterial reduction with increased CAP exposure. The reduction of the mean amount of viable bacteria by at least 3-$\log_{10}$ was seen as "antimicrobial effective". After 60s of CAP treatment there was at least a 3-$\log_{10}$ reduction (>99% reduction) in the number of CFU in all four different bacteria samples compared to the untreated controls. A difference in load reduction between the different bacteria was seen. *H. influenzae* and *Staphylococcus aureus* showed 3-$\log_{10}$ reduction already after 30s CAP exposure time. Moreover, this publication showed an increased cytotoxic effect with increased CAP exposure on healthy mucosal cells. Nevertheless, CAP exposure of ≤ 60s and below did not imply a significant rise in necrotic cells and viability when compared to the controls (p> 0.0028), therefore only showing minimal cytotoxic effects. CAP exhibited a good bactericidal effect on four of the most frequent ENT bacteria. 60s of treatment time presented a significant antibacterial effect and credibly acute cytotoxic effects on the healthy mucosa.

Furthermore, Publication 2 showed the effectiveness of CAP exposure on three typical HNSCC cells. In all cell lines, CAP had a cytotoxic impact with greater effect at prolonged exposure. 120s of CAP exposure caused a significant decrease of cell survival for all three different HNSCC cells. DNA damage significantly increased after 60s. However, the CAP effect (as monotherapy or combined chemotherapy treatment) differed with respect to the cell line. Results revealed a greater response of more rapid proliferating cells (OSC 19) towards CAP mono-treatment. Overall, additive effects were observed in combination treatments. This effect was especially significant in slower proliferating cells (FaDu and Cal 27). Chemo-resistant Cal 27 showed a considerable lower cell survival by 2.5µM cisplatin treatment directly after CAP exposure, suggesting a possible sensitivity restoration to cisplatin. The study displays the capability of CAP and low dose cisplatin combination as a feasible additional option for an adjuvant therapy of HNSCC.

This cumulative study showed that CAP application could be a clinically relevant single or combined therapeutic approach for various challenging diseases, especially drug resistant diseases, in the head and neck area such as infections and malignant tumors.

4. SUMMARY

In summary, the research which this cumulative dissertation is based on supports the current literature enhancing CAP as a promising therapy tool for medical applications. Non thermal plasma is considered as an ionized gas and the fourth state of matter. It can be generated at atmospheric pressure and room temperature. There are several different types of CAP, in this study an SMD device generating plasma from the surrounding air without any need of gas was used. Chemical and physical components produced by CAP such as ROS, RNS, UV-radiation and heat all contribute to the antimicrobial and antitumoral effect. Both impacts of CAP in the head and neck area were investigated in this study.

The CAP mechanism which seems to be responsible for the microbial and tumor reduction are ROS, RNS, ions, UV radiation and electromagnetic field in descending order. Studies have shown that CAP's antibacterial effectiveness varies depending on bacteria and its cell wall structure, especially its thickness (14)(56). Our findings show a significant reduction of common Gram-positive (*Staph. aureus*, *Strept. pneumoniae*, *and Strept. pyogenes*) and Gram-negative (*H. influenzae*) bacteria found in the upper respiratory tract with a CAP SMD device. However, a difference in susceptibility was also observed. A sufficient antimicrobial effect was already achieved after 30s exposure time for *H. influenzae* and *Staph. Aureus*. *Strept. pneumoniae* and *Strept. pyogenes* showed similar results after a longer exposure time. Additionally, all four different bacterial loads show a proportional increase in reduction with longer CAP exposure periods. These results are in line with several studies using different CAP devices and exposure of different microorganisms (15). A possible reason for this could be more interaction because of more time between the RS and bacteria due to the longer period of exposure (14). CAP exposure times of ≥ 60s cause a decrease of > 99.9 % in the bacterial loads. CAP application show a cytotoxic effect on healthy mucosal tissue with increasing impact at longer exposure times. However, in accordance with other studies CAP exposures of ≤ 60s demonstrate no significant necrosis of healthy mucosa compared to the controls in our study (9)(57).

Furthermore, CAP mono-application shows a significant cell viability reduction within all three different HNSCC cells (chemo sensitive FaDu, chemo sensitive OSC 19 and chemo resistant Cal 27). All three show a different susceptibility towards CAP exposure. 90s of CAP exposure was

needed to reach IC_{50} for OSC 19 and 120s treatment time for FaDu cells. Cal 27 cell line did not reach IC_{50}. After 180s the average amount of viable cells was still 53.1%.

Concurrent low-dose cisplatin application post CAP exposure demonstrates an additive effect in all cell lines, enhancing the hypothesis of CAP and cisplatin acting synergistic within the cell cycle. Furthermore, a hypothetical chemo-sensitivity induction by initial CAP treatment could be shown. Hence, Cal 27 cell line show a higher vulnerability towards treatment with cisplatin by prior CAP application for all exposure times. *Volotskova et al. (2012)* report that CAP exposure increases the number of tumor cells at G2/M phase and a decrease of cells through S phase. Malignant cells have shown to be less sensitive towards cisplatin in S-phase (54).This could be a possible explanation for the additive effect and possible sensitivity induction towards cisplatin. *Soni et al. (2021)* showed *in vitro* and *in vivo* that CAP can sensitize glioblastoma to subsequent temozolomide treatment. They also demonstrated that CAP and temozolomide caused in a downregulation of cell cycle pathway genes (58). Thus, a combination therapy is clearly superior to the respective mono treatment.

CAP application in the head and neck area showed a high antimicrobial and antitumoral effect in common ENT bacteria and different head and neck squamous cancer cells with very limited to no necrosis of healthy mucosal tissue. Additionally, CAP enhanced the therapeutic effectiveness of low-dose cisplatin. Moreover, a possible cisplatin sensitivity restoration in chemo-resistant squamous cell carcinoma cells was generated.

The use of local CAP application on head and neck infections would avoid systemic and local side effects as well as multidrug resistance induced by antibiotics. Furthermore, an immediate treatment without therapy delay due to a pending culture or antibiogram and a broad antimicrobial effect is possible. The antitumoral effect of CAP in the head and neck area suggests a possibility of cisplatin dose reduction decreasing systemic side effects in a CAP and low dose cisplatin combination therapy. Additionally, our findings indicate that CAP can be potentially useful in re-sensitizing chemo resistant cells to cisplatin for the treatment of advanced HNSCC as a promising therapy option.

CAP seems to be a promising additional therapeutic option for clinically challenging and possible drug resistant diseases in the head and neck region.

5. ZUSAMMENFASSUNG

Zusammenfassend zeigen die dieser kumulativen Dissertation zu Grunde liegenden Originalarbeiten eine vielversprechende medizinische Anwendung von KAP im Kopf-Hals Bereich. Plasma ist ein ionisiertes Gas und wird als vierter Aggregatzustand bezeichnet. KAP ist nicht thermisch und wird bei atmosphärischem Druck und Raumtemperatur erzeugt. Die Erzeugung kann durch verschiedene Techniken erfolgen, in dieser Studie wurde ein SMD-Gerät verwendet, das Plasma aus der Umgebungsluft ohne Edelgas hergestellt. KAP besteht aus einer reaktiven Mischung aus Elektronen, Ionen, Stickstoff- und Sauerstoffspezies und UV-Photonen, die alle zu den antimikrobiellen und antitumoralen Eigenschaften beitragen. Beide Wirkungen von KAP im Kopf- und Halsbereich wurden in dieser Studie untersucht.

Die KAP-Mechanismen, die für die Reduktion von Mikroorganismen und maligner Tumorzellen verantwortlich zu sein scheinen, sind Stickstoff- und Sauerstoffspezies, geladene Teilchen, UV-Strahlung und elektrische Felder in absteigender Reihenfolge. Die antimikrobielle Wirksamkeit von KAP varriiert je nach Mikroorganismus und dessen Zellwandstruktur, insbesondere dessen Dicke (13)(41). Unsere Ergebnisse zeigen eine signifikante Reduktion häufiger grampositiver (*Staph. aureus, Strept. pneumoniae und Strept. pyogenes*) und gramnegativer (H. influenzae) Bakterien im oberen Respirationstrakt mit einem KAP-SMD-Gerät. Es wurde auch ein Unterschied in der Empfindlichkeit festgestellt. Eine ausreichende antimikrobielle Wirkung wurde bereits nach 30s Expositionszeit für *H. influenzae* und *Staph. aureus* erreicht. *Strept. pneumoniae* und *Strept. pyogenes* zeigten nach längerer Expositionszeit (60s) ähnliche Ergebnisse. Darüber hinaus zeigen alle vier verschiedenen Bakterienkulturen mit zunehmender Dauer der KAP-Anwendung eine stärkere Reduzierung. Diese Ergebnisse stehen im Einklang mit mehreren Studien, in denen verschiedene KAP-Geräte und die Exposition verschiedener Mikroorganismen verwendet wurde (42). Eine mögliche Erklärung hierfür könnte eine stärkere Interaktion sein, da aufgrund der längeren Expositionsdauer mehr Zeit zwischen den Sauerstoffspezies und den Bakterien vergeht (13). Eine Behandlung mit KAP mit 60Sek. oder länger führte zu einer Bakterienreduktion von > 99,9 %. Die Anwendung von KAP hat eine dosisabhängige zytotoxische Wirkung auf gesundes Schleimhautgewebe, die bei längerer Einwirkungsdauer zunimmt. In Übereinstimmung mit anderen Studien zeigen KAP-

Behandlungszeiten von 60 Sek. und weniger keine signifikante Nekrose von gesundem Schleimhautgewebe im Vergleich zu den Kontrollen (8)(43).

Darüber hinaus konnte *in-vitro* eine signifikante Reduktion der Zellvitalität gegen alle drei verschiedene (chemo-sensitive und chemo-resistente) Kopf-Hals Tumorzelllinien durch eine reine KAP Behandlung mittels einem SMD-Plasmagerät erreicht werden. Alle drei zeigen eine unterschiedliche Anfälligkeit gegenüber KAP-Exposition. IC_{50} Werte zeigten sich unterschiedlich bei den verschiedenen Tumorzelllinien: 90Sek. für OSC 19 und 120Sek. Für FaDu. Nach 180Sek. zeigte die Cal 27 Zellinie noch 53.1% Zellvitalität.

Eine direkte anschließende niedrige dosierte Cisplatin-Applikation nach KAP Behandlung zeigte in-vitro eine zusätzliche Zellvitalitätsreduktion in allen drei Kopf-Hals Tumorzelllinien. Somit zeigte sich eine Kombinationstherapie deutlich überlegen zur jeweiligen Monotherapie. Dies unterstützt die Hypothese von einer synergistischen Wirkung von KAP und Cisplatin innerhalb des Zellzyklus. Zudem konnte *in-vitro* eine mögliche Re-Sensibilisierung Chemotherapie-resistenter Cal 27 Zellen durch eine einmalige KAP Vorbehandlung gezeigt werden, da die Cal 27-Zelllinie eine erhöhte Anfälligkeit für die Behandlung mit Cisplatin durch die vorherige Anwendung von KAP für alle Expositionszeiten aufweist. *Volotskova et al. (2012)* zeigten eine Erhöhung der Anzahl der Tumorzellen in der G2/M-Phase und Reduzierung der Zellanzahlen in der S-Phase nach KAP-Exposition. Maligne Zelle zeigen sich in der S-Phase weniger empfindlich gegenüber Cisplatin (39), was eine mögliche Erklärung für den additiven Effekt und die mögliche Empfindlichkeitsinduktion gegenüber Cisplatin sein könnte. Die *in vitro* und *in vivo* Ergebnisse von *Soni et al. (2021*) zeigten eine Sensibilisierung von Glioblastomen für eine anschließende Temozolomid-Behandlung durch KAP. Zudem zeigte die Studie eine Downregulation bestimmter Zellzyklus-Gene durch KAP und Temozolomid (57). Somit ist eine Kombinationstherapie der jeweiligen Monobehandlung eindeutig überlegen.

Die *in-vitro* KAP-Anwendung im Kopf- und Halsbereich zeigte eine hohe antimikrobielle und antitumorale Wirkung bei gängigen HNO-Bakterien und verschiedenen bösartige Tumorzellen im Kopf- und Halsbereich, wobei das gesunde Schleimhautgewebe nur in sehr geringem Maße bzw. gar keine zytotoxische Wirkung zeigte. Zudem verstärkte KAP die therapeutische Wirksamkeit von niedrig dosiertem Cisplatin und es konnte eine mögliche Wiederherstellung der Cisplatin-Empfindlichkeit bei chemo-resistenten HNSCC-Zellen festgestellt werden.

Die lokale Anwendung von CAP bei Infektionen im Kopf- und Halsbereich könnte systemische und lokale Nebenwirkungen sowie eine durch Antibiotika induzierte Resistenz limitieren. Außerdem ist eine sofortige Behandlung ohne Therapieverzögerung aufgrund eines ausstehenden Antibiogramms und eine breite, vielfältige antimikrobielle Wirkung möglich. Die antitumorale Wirkung von KAP im Kopf- und Halsbereich könnte eine Reduzierung der Cisplatin-Dosis durch einer Kombinationstherapie aus KAP und niedrig dosiertem Cisplatin ermöglichen. Dies würde zur Verringerung von systemischen Nebenwirkungen und höherem Therapieerfolg führen. Darüber hinaus deuten unsere Ergebnisse darauf hin, dass KAP durch Re-Sensibilisierung von chemoresistenten Zellen gegenüber Cisplatin zur Behandlung von fortgeschrittenem Kopf-Hals Tumoren eine vielversprechende Therapieoption darstellen könnte. Physikalisches KAP scheint somit eine vielversprechende Therapieoption bei klinisch herausfordernden und medikamentenresistenten Erkrankungen im Kopf-Hals Bereich zu sein.

6. PUBLICATIONS INCLUDED IN THIS THESIS

6.1 Publication 1

Title:

Effects of cold atmospheric plasma (CAP) on bacteria and mucosa of the upper aerodigestive tract

Authors:

Sven Becker, Julia L. Zimmermann, Philipp Baumeister, Teresa F. Brunner, Tetsuji Shimizu, Yang-Fang Li, Gregor E. Morfill, Ulrich Harréus, Christian Welz

Journal:

Auris Nasus Larynx 46 (2019) 294–301 .

DOI:
10.1016/j.anl.2018.07.008

6.2. Publication 2

Title:

Primary cold atmospheric plasma combined with low dose cisplatin as a possible adjuvant combination therapy for HNSCC cells - an *in-vitro* study

Authors:

Teresa F. Brunner, Florian A. Probst, Matthias Troeltzsch, Sabina Schwenk-Zieger, Julia L. Zimmermann, Gregor Morfill, Sven Becker, Ulrich Harréus, Christian Welz

Journal:
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