Clinical Plasma Medicine and Head and Neck Cancer –
Key points of clinical studies

Inaugural-Dissertation

zur

Erlangung des akademischen Grades

Doktor der Zahnmedizin

(Dr. med. dent.)

der

Universitätsmedizin

der

Universität Greifswald

2018

vorgelegt von: Matthias W. Schuster
geboren am: 15.09.1977
in: Hamburg
Dekan: Prof. Dr. rer. nat. Max P. Baur
1. Gutachter: Prof. Dr. Michael Jünger
2. Gutachter: Prof. Dr. Thomas von Woedtke
Ort, Raum: Greifswald, Seminarraum Universitätsmedizin
Tag der Disputation: 08.01.2019
Table of Content

List of abbreviations

1. Background .................................................................................................................. 5

2. Scientific Introduction .................................................................................................. 7

3. Aim ............................................................................................................................... 8

4. Key points of study design ........................................................................................... 9
   4.1 Type of plasma source ............................................................................................. 9
   4.2 Type of cancer ........................................................................................................ 9
   4.3 Study Hypothesis .................................................................................................... 10
      4.3.1 Decontamination of infected tumor surfaces .................................................. 10
      4.3.2 Reduction of tumor growth .............................................................................. 10
      4.3.3 Patient benefit ................................................................................................ 11
      4.3.4 Side effects and complications ........................................................................ 12
   4.4 Resulting study protocol - Cold atmospheric plasma trial ....................................... 13

5. Discussion and outlook ................................................................................................ 14

6. Conclusion .................................................................................................................... 15

Literature ............................................................................................................................. 17

Annex ................................................................................................................................ 28
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBM</td>
<td>Evidence-based medicine</td>
</tr>
<tr>
<td>CAP</td>
<td>Cold atmospheric pressure plasma</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>PI3K/Akt pathway</td>
<td>Phosphoinositide 3-kinas pathway</td>
</tr>
</tbody>
</table>
1. Background

Evidence-based Medicine

The treatment of a patient suffering from cancer is, of course, a personal and individual mission; however, considering the doctor’s responsibility and by coincidental agreement with health authorities, medical care and research may sensibly follow the rules of evidence-based medicine (EBM), as defined by the U.S. Agency for Health Care Policy and Research 1992, whenever possible. There is a rigid hierarchy in this system (Figure 1): introducing a new concept of cancer therapy that requires full recognition has to start with (a) simulation studies and investigations in cell cultures and animals, leading in summary to a (b) consent of experts (EBM-level IV) that gives way to (c) clinical pilot studies (EBM-level III) and further proceeds to (d) non-randomized studies (EBM-level II). EBM-level I of an innovative cancer treatment concept is reached with successfully evaluated (e) randomized clinical studies (EBM-level Ib), concluded by the positive outcome of a (f) structured review of several randomized clinical trials as EBM-level Ia.

![The pyramid of evidence-based medicine (EBM).](image)

**FIGURE 1** The pyramid of evidence-based medicine (EBM).

*SR=*systematic review; *RCT=*randomized controlled trials

First objective of clinical studies

The main goal of cancer treatment is the healing of a patient, which will be achieved by the total extinction of the tumor and no evidence of disease for at least 5 years, and is referred to as curative treatment. Logically, the first objective of EBM-targeted clinical cancer studies...
is the removal of all tumor cells that are able to generate new tumor cells, known as tumor stem cells.

**Second objective of clinical studies**

In case of failure of healing, treatment is at least aiming at relief from the health problems of the patient caused by cancer, referred to as palliative treatment. Typical health problems of cancer of the oral cavity, face, or neck are pain, fatigue, mental burden, loss of weight, shortness of breath, swallowing difficulties and infection of tumor ulcerations. Consequently, the second objective in clinical cancer research is alleviation and easing of these severe complaints.

**EBM in head and neck cancer**

With regards to curatively intended treatment of head and neck cancer, and especially squamous cell carcinoma of the oral cavity, the total removal of tumor bulk and inclusion of potent stem cells by radical surgery is currently the first-line therapy and EBM-level Ia. Second-line treatment and EBM-level Ia, likewise, includes radiotherapy attacking tumor stem cells locally in the area under radiation. Third-line treatment as well as EBM-level Ia is chemotherapy attacking tumor stem cells wherever they occur and whenever they build up distant metastasis. To select one of these established therapy options or combine them has to comply with many features, mainly with the specific individual conditions of the tumor and the state of health of the patient.

**Cold atmospheric pressure physical plasma as a new option in cancer**

Referring to the role of the single stem cell in cancer disease and as a target in tumor treatment Fridman und co-workers in 2007 have published a landmark paper for new options in cancer therapy [1], and Keidar and co-workers introduced cold atmospheric pressure physical plasma (CAP) that is selectively effective in these cancer cells as a new option in curative as well as palliative cancer therapy [2].

Since then, a tremendous amount of cell studies and animal investigations has been performed as listed at first, by Schlegel and co-workers [3], reporting the effectiveness of plasma in cancer cell lines, cultivated human tumor cells, human tumor specimen freshly explanted from patients, animal model tumors, or animals with transplanted human tumor stem cells. There is consent among experts, referred to as EBM-level IV, that in experimental settings, plasma kills various types of cancer with major clinical concern. Now plasma

2. Scientific Introduction

Anti-tumor effect of CAP in a variety of cancer types
The development of sources providing physical plasma at body temperature and under atmospheric pressure has opened the use of plasma components for medical purposes. From the clinical point of view the approval of the first plasma sources for medical treatment purposes in 2013 has been based upon their well-known effectiveness not only to stimulate tissue regeneration but also against microbial pathogens. Cold atmospheric pressure plasma (CAP) has shown to be effective in cancer as well. The antitumor of effect of CAP has also been demonstrated in various types of cancer cell lines in vitro, such as melanoma, glioblastoma, lung cancer, colon cancer, pancreatic cancer, breast cancer, leukemia, thyroid cancer, and head and neck cancer. Several studies in experimental animal models have underlined this notion using e.g. melanoma, colon cancer, pancreatic cancer, endometrial adenocarcinoma, and ovarian cancer cells [3]. Clinical observations have been added recently focusing colorectal cancer and head and neck cancer [4, 16-19].

Understanding the effectiveness of CAP in cancer cells
The single cancer stem cell is the therapeutic target, when treating malignant tumors with curative intention, as this is the source of clonal tumor growth, metastasis, recurrent cancer and development of cancer disease [7]. The evidence to date suggests that CAP has a significant apoptotic effect on cancer cells [3, 8, 9]. Triggering reactive oxygen and nitrogen species to derange the redox balance and redox signaling of cancer stem cells is considered as a key pathway for understanding CAP-induced apoptosis, since the survival and proliferation signaling network is one of the most essential signaling networks. Two major signaling pathways within this network are the Phosphoinositide 3-kinase (PI3K)-AKT signaling pathway and the rat-associated sarcoma (RAS)-Mitogen-activated Protein Kinase (MAPK) signaling pathway [10, 11]. Activation of this signaling network often seen in cancer
patients leads to induction of cell growth and inhibition of apoptosis. CAP treatment hinders both pathways [12] and induces apoptosis of tumor cells due to downregulation of the survival and proliferation signaling network [13, 14].

3. Aim

The therapeutic target when treating malignant tumors with curative intention is the cancer stem cell as this is the source of clonal tumor growth, metastasis, recurrent tumor and development of cancer disease [7]. The evidence to date suggests that CAP has a direct significant effect on cancer cells of several tumor lines and tumor models in vitro [3]. Moreover, immunocompetence seems to be induced by CAP as an indirect effect [15].

According to the discussions started at the 2nd International Workshop on Plasma for Cancer Treatment held at Nagoya University March 16-17, 2015, our knowledge of plasma medicine in cancer patients stands to benefit from clinical trials.

Clinical trials with the aim to investigate the outcome of CAP application in cancer patients need a prospective, randomized, multi-center protocol with a result assessment that is blinded and evaluated remotely. The primary objective of such study protocols might be to look for total extinction of the tumor and no evidence of disease for at least 5 years, referred to as curative treatment success. More appropriate for the time being seems to be the secondary objective, to look for relief from the health problems of the patients caused by cancer as palliative treatment. [Referring to pages 33-40: “Clinical experience with cold plasma in the treatment of locally advanced head and neck cancer”, HR Metelmann, C Seebauer, V Miller, … , M Schuster et al. Clin Plasma Med 9: 6-13, 2018]

The aim of this paper is to present and discuss a draft of a clinical trial protocol in CAP-treatment of cancer patients.
4. Key points of study design

There are several aspects that have to be considered as key points of a protocol.

4.1. Type of plasma source

CAP can be generated by different methods [20]. This leads to a variety of plasma sources and medical devices with different types of architecture, working gases, discharge, energy yield and geometric factors. Due to the need of adequate sample sizes and truly comparable results especially in multi-center studies, it is important to standardize the study protocol by defining and agreeing upon a plasma source to be commonly used, that has been well studied in pre-clinical trials. Moreover the plasma source has to be suitable for use on clinical features of the tumor under investigation and the instrument itself should be easy to handle from the surgeon’s point of view. In the case of head and neck cancer, presenting mainly open ulcerations with massive bacterial contamination, medical devices in the shape of plasma jets like kINPen MED (neoplas tools GmbH, Greifswald, Germany) are preferable because of their lancet-like plasma tip that offers easy access to the extremely uneven surfaces of this kind of tumor, especially when occurring intra-orally.

4.2. Type of cancer

Head and neck cancer cells are of confirmed sensibility to CAP [21-24], and also bladder cancer [2], brain tumor [12, 25-27], breast cancer [28, 29], cervical cancer [30-33], colorectal cancer [34-38], gastric cancer [39], leukemia [40, 41], liver cancer [42], lung cancer [43-46], malignant melanoma [1, 47-52], ovarian cancer [9, 53, 54], pancreatic cancer [55, 56], prostate cancer [57] and thyroid cancer [58, 59].

In comparison, for clinical trials squamous cell carcinoma samples of the head and neck area offer several advantages: they are open, visible, solid surfaces which are easy to reach and easy to observe clinically over a significant period of time. And they may not be as difficult as other cancers to harvest specimens periodically from the same source if needed for direct analysis of effects and follow up therapy. Moreover, there are already some case reports for CAP treatment of this type of cancer previously published that can be used for scientific estimation and comparison [4].
4.3. Study Hypothesis

The main intention of the first clinical study programs and for the time being should be to confirm or refute recent findings that lead to three promising study topics: the application of CAP is reducing the microbial contamination of infected tumor surfaces and the growth of cancer and thereby is of benefit for the cancer patient suffering from head and neck squamous cell carcinoma.

4.3.1. Decontamination of infected tumor surfaces

A huge number of data based upon clinical trials of supported wound healing by CAP [60-67] gives broad evidence to CAP’s ability to reduce the bacterial load of cancer ulcerations, particularly by decreasing the number of anaerobe species, but a total removal of bacteria was not yet achieved by CAP. New trials are needed to determine the microbiological aspects, specifically to differentiate between detectable pathogenic species and the extent of measurable reduction that can be achieved with CAP. This knowledge will help increase the efficiency of decontamination.

4.3.2. Reduction of tumor growth

A study protocol needs to yield visible information that CAP can affect cells in clinical head and neck cancer as known for cell lines [21-24] and is causing apoptotic cell kill in head and neck cancer tissue as well. In a case of cancer response visible effects at the tumor surface became obvious 2 weeks after CAP application and appeared in 2 different types. There was no clinical observation of stimulated tumor growth in all of the patients. From the clinical point of therapeutically applicability these findings are limited by study protocol restraints, however the small number of case reports so far published or presented is in line with the first impression of these results [4, 17, 68]. Concerning the different efficacy of CAP in different tumor patients, it is not surprising and maybe simply due to the biological idiotypes and clinical presentations unique to each individual patient. VonHoff and his group have mentioned clonal evolution and therapeutic resistance in solid tumors [69]: Tumors frequently arise as a result of an acquired genomic instability and the subsequent evolution
of neoplastic populations with variable genomes. That might be due to response or no-response to CAP, too. Another limitation of the analysis is the unknown cellular and histological background of tumor surface changes. Apoptotic cell kill is obvious, however, some of the adverse effects of CAP treatment published recently [4] have to be interpreted as stroma reactions, i.e. bleeding and erythema, not directly to be related to cancer stem cell behavior [70]. There is no evidence for CAP influence on cancer stem cells exclusively, but a particular thought, that apoptotic cell kill might be a necessity for tumor regression starting with tumor surface change, seems to be not a sufficient premise. From a clinical point, this is a matter of minor concern, however, as long as tumor growth in general might be reduced by CAP.

Tumor surfaces exposed to CAP in vivo do not show instant local destruction, but only a partial remission after two weeks. Visible effects of CAP (e.g. flattening and arresting of granulomatous proliferations) were observed on treated tissues, but the effects were irregular where some parts remained in a progressive disease state. Many more standardized trials are needed to prove if CAP has a reproducible anti-cancer effect and clarify why there may have previously been an inhomogeneous cellular response. [3, 4, 16-19] [Referring to pages 41-48: “Visible tumor surface response to physical plasma and apoptotic cell kill in head and neck cancer”, M Schuster, C Seebauer, R Rutkowski, A Hauschild et al. J Cranio-Maxillo-Facial Surg 44:1445-52, 2016]

4.3.3. Patient benefit

Clinical experience with head and neck cancer shows that patients appreciate treatment with CAP at least in palliative care. By decreasing the load of anaerobic bacteria, CAP also reduces the typical fetid odor of cancer ulcerations and even might support the microcirculation of tissue. Furthermore, many patients submitted fewer requests for pain medication when treated with CAP. The reported amount of pain reduction varied between patients and usually faded out when CAP-treatment was completed. Further research would help to individualize the prescription of drugs by differentiating between responding and non-responding patients. Based on patient surveys and more general knowledge about patient acceptance [2, 71], CAP-therapy could become a well-accepted adjunct to standard anti-

4.3.4. Side effects and complications

Available pre-clinical and clinical data in general show CAP as a well-tolerated treatment, causing no severe side effects or complications. The question of why the application of CAP, the effectiveness in terms of decontamination, stimulation the healing of chronic wounds or reducing growth of tumor cells, presents mostly no side effects or discomfort is calling for further clinical data and biological interpretation. From the beginning, safety of plasma medicine was an important issue in research. With the increasing knowledge about details of plasma-cell and plasma-tissue interactions including reports on plasma impact on DNA integrity of cells in vitro, at first the risk of potential genotoxic effects of CAP was in the focus, and the basic insight that biological plasma effects are mainly based on the activity of reactive oxygen and nitrogen species (ROS, RNS) [72]. Because these ROS and RNS are the same as occur in regular physiological and biochemical processes in the body, mammalian cells have mechanisms to save from excess levels of ROS and RNS [73]. It was demonstrated that such antioxidative protection mechanisms are up-regulated in cells in response to plasma treatment [74]. Further detailed investigations could demonstrate that detrimental plasma effects on cells result either in cellular repair processes or in induction of programmed cell death (apoptosis) [75]. Meanwhile, several studies using well-established and accepted experimental procedures have proven that CAP treatment does not cause increased risk for genotoxicity [76-78]. This was supported by first clinical experience [63]. However, besides this inevitable exclusion of such fundamental side effects, it is also necessary to monitor and learn about acute and short-term side effects of CAP treatment. [Referring to pages 55-61: “Side effects in cold plasma treatment of advanced oral cancer – Clinical data and biological interpretation”, M Schuster, R Rutkowski, A Hauschild, R Shojaei et al. Clin Plasma Med 10:9-15, 2018]
4.4. Resulting study protocol - Cold atmospheric plasma trial

Concluding the key points of study design Tab.1 gives an example of a trial synopsis.

*Table 1: Trial synopsis*

<table>
<thead>
<tr>
<th>Title and Short title</th>
<th>Clinical phase I trial of palliative treatment with CAP applied to infected tumor surfaces of patients suffering from locally advanced oral cavity carcinoma. (OncoTher-Clin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project phase</td>
<td>Phase I</td>
</tr>
<tr>
<td>Indication</td>
<td>Indication for CAP treatment includes decontamination of cancer ulcerations to reduce pain and need of potent drugs, to reduce the socially isolating odor and by this to add to the benefit of patients under palliative treatment.</td>
</tr>
<tr>
<td>Study design</td>
<td>Clinically explorative single-arm, -randomized, open, multicentric study</td>
</tr>
<tr>
<td>Primary objective</td>
<td>Reduction of microbial burden of cancer ulcerations by application of CAP</td>
</tr>
<tr>
<td>Secondary objective</td>
<td>Reduction of tumor following local CAP application</td>
</tr>
<tr>
<td>Patient selection</td>
<td>A maximum of 20 patients will be enrolled</td>
</tr>
<tr>
<td>Criteria of inclusion</td>
<td>Patients suffering from locally advanced oral cavity carcinoma with open tumor surfaces, treated with palliative intention and no more curative treatment options</td>
</tr>
<tr>
<td>Criteria of exclusion</td>
<td>No wish for treatment, no compliance and understanding the protocol of the clinical study</td>
</tr>
</tbody>
</table>
| Course of study       | • Election of patients and randomization at the clinics  
• CAP intervention  
• Follow-up and examination of plasma effects                                                                                                                                                    |
| Assessment of efficacy| • Microbiological examination for reduction of microbial burden  
• Documentation of visible changes by photography  
• Pathohistological and biochemical examination of specimen, taken from the tumor area and control areas                                                                                   |
| Effective agent       | Cold atmospheric pressure physical plasma, supplied by kINPen MED®, Neoplas tools GmbH, Germany                                                                                                      |
| Study procedure       | Plasma is applied for 1 minute per cm², producing a spot area of 3 mm diameter with a distance between nozzle and tumor surface of 14 mm. The treatment will be repeated 3 times/week with a break of 1 week followed by a repeated cycle for another week. All kinds of unexpected effects will by documented. |
5. Discussion and outlook

What are the key points for clinical studies and next steps in plasma treatment of cancer on the way to evidence based medicine?

Options of CAP-treatment

First, in palliative medicine, any gain in the relief of patients is a success, and plasma medical devices will complement the standard tools of palliation when no-touch, no-chemical, no-harm treatment of contaminated cancer ulcerations is needed.[79-81] Second, in curative medicine, any reduction of tumor growth counts and possibly means an impact on tumor stem cells. For further studies, if there is a manageable amount of tumor stem cells, like in precancerous lesions of oral mucosa and or in small tumors like early basal cell carcinoma of the skin, plasma could become a clinical treatment option. Plasma treatment does not look like a bulk-removing concept, but in a locally advanced cancer with a tiny tumor stem cell offshoot of the bulk infiltrating an absolutely crucial tissue inaccessible for surgery, radiotherapy, or chemotherapy, it might be very suitable. The advantage is obvious: there is no sudden necrosis of tumor cells, tearing a hole in a blood vessel, but there is apoptosis of tumor cells, the reinforced natural dying of tumor cells and replacement by wound cells, to never let holes arise. At the current level of EBM, treatment with plasma will not be considered when surgery, radiotherapy, or chemotherapy is available and promising. Further clinical pilot studies should be choosing patients for palliation who face no positive effect of the first- to third-line therapy. [68]

Comparing CAP to standard cancer treatment

The fortune of healing and recovery is the sunny side of cancer therapy. The burden of risks, complications, and unavoidable treatment problems is the dark side. In surgery, the scourge of unavoidable side effects means scars from mild to mutilating, loss of functional and aesthetically important tissue, loss of blood, and the need for intensive care post-surgery. In radiotherapy, the most awful burden is the danger of stimulating normal cells to progress wildly to cancer cells themselves after a couple of years. In chemotherapy, the burden is, to some extent, the same as in radiotherapy; moreover, patients are suffering from fatigue caused by loss of red blood cells and low resistance against infections caused by loss of white blood cells. In plasma, there is currently no burden known and no severe and foreseeable
side effects. [4, 5, 88, 89] So, the discussion of plasma as a new option in cancer treatment has the perspective of a new approach at all, not only for the goal of anticancer effectiveness in special circumstances but for better safety, compatibility, and tolerability of cancer treatment in general.

**Outlook**
This is a call for clinical trials in plasma medicine. Due to statistical needs of sample size as well as mutually rewarding international exchange, and to the multiplication of plasma medicine expertise, we are strongly in favor of multicenter participation. Investigators who meet the trial program’s following criteria are cordially invited to participate.

If the results of pilot studies give way to curatively intended randomized clinical trials, it takes 5 years of follow-up of the patients to obtain information about healing. Clinical plasma medicine with support, monitoring, and supervision by scientific plasma research drives the benefit of patients and deceives their trust in medical professionalism. [82-87]

**6. Conclusion**

It makes sense to start clinical trials in plasma medicine with the treatment of head and neck squamous cell carcinoma patients, since (1) favorable ex vivo results were obtained with freshly harvested head and neck cancer specimens, (2) the tumors present with visible, superficial, solid cancer cell masses, that are (3) easily within reach of plasma sources, (4) reactions can be clinically observed directly, (5) well covered medico-legally by the actual license of the devices for treatment of infected wounds and ulcerations, and (6) clinical case studies of CAP on head and neck squamous cell carcinoma have already been published.

Due to a variety of plasma sources in terms of type of discharge, energy yield, working gas or geometric factors, it is recommended to standardize the study protocol by choosing a plasma source with lancet-like precision and easy access to rugged tumor surfaces as demonstrated by the CAP-plasma-jet. The intention of the trial shall be to optimize the plasma jet for tumor site capability and operating room implementation.
CAP is able to reduce contamination of cancer ulcerations and the typical fetid odor that often accompanies head and neck cancer patients. The microbiological diagnostic observed a decrease in the number of bacteria, especially anaerobe species, however no total elimination. The intention of the trial shall be to evaluate the efficiency of decontamination in head and neck cancer ulcerations in terms of pathogenic species, amount of reduction and reliability.

Many patients who received CAP reported a reduction in pain and decrease in the individual request for medication, however, there were some confounders. This effect started early in the treatment period and faded out by the completion of CAP treatment. The extent of pain reduction differed remarkably between different patients receiving CAP. The intention of the trial shall be to differentiate non-responding from responding patients in terms of individualized predictive prescription of drugs.

Tumor surface treatment with CAP does not result in immediate local destruction, but will cause in a certain number of cases visible effects after two weeks of structured exposure, mainly expressed like flattening of a previously augmented tumor surface and stop of granulating tumor proliferation. Some patients showed areas of partial remission in the neighborhood of progressive disease. The intention of the trial shall be to understand the lack of a homogenous cellular response within areas of uniform plasma treatment.

CAP treatment of cancer ulcerations is not free from side effects, in some cases stinging pain was reported when the plasma jet rested more than ten seconds at the same point. Also, a bad taste or collateral edema 24 hours after exposure accompanied intraoral CAP application. The intention of the trial shall be to add to the knowledge of risk factors and look for any other possible complications of CAP-treatment.

CAP is on the edge of widespread clinical application in head and neck cancer palliative medicine. Whether innovation in medical methods and novel technology in general and in detail is of benefit for the people involved is a central research concern [71, 90]: The patient’s personal appraisal of CAP has to be investigated in terms of emotional response to therapy and whether distress and relief from disease are well balanced or even favorable. As current clinical experience shows patients appreciate physical plasma at least in palliation, most notably reduction of odor, reduction of cancer pain and decrease in microbial burden. The
most important intention of the trial from the clinician’s point of view shall be to make CAP-treatment an effective and well-accepted addition to standard cancer therapy based upon EBM at least in palliative medicine.

**Literatur**


38. Ishaq M, Evans MD, Ostrikov KK. Atmospheric pressure gas plasma-induced colorectal cancer cell death is mediated by Nox2-ASK1 apoptosis pathways and oxidative stress is mitigated by Srx-Nrf2 anti-oxidant system. Biochim Biophys Acta 1843(12): 2827e2837, 2014


73. Dröge W. Free Radicals in the physiological control of cell function. Physiol Rev 82: 47-95, 2002


Treating cancer with cold physical plasma: On the way to evidence-based medicine

Hans-Robert Metelmann1 | Christian Seebauer1 | Rico Rutkowski1
Matthias Schuster1 | Sander Bekeschus2 | Philine Metelmann3

1 Department of Oral and Maxillofacial Surgery/Plastic Surgery, Universitatsmedizin Greifswald, Greifswald, Germany
2 Leibniz-Institut für Plasmaphysik und Technologie eV, Greifswald, Germany
3 Department of Orthodontics, Universitatsmedizin Greifswald, Greifswald, Germany

*Correspondence
Hans-Robert Metelmann, Department of Oral and Maxillofacial Surgery/Plastic Surgery, Greifswald University Medicine, Ferdinand-Sauerbruch-Str. DZ 7, 17475 Greifswald, Germany.
Email: metelman@uni-greifswald.de

The application of cold physical plasma in head and neck cancer patients is moving upwards in the pyramid of evidence-based medicine (EBM). The effectiveness of plasma in cancer cell lines, cultivated human tumour cells, human tumour specimens freshly explanted from patients, animal model tumours, and animals with transplanted human tumour stem cells is well documented. There is consent among experts, referred to as EBM-level IV, about the response to plasma in experimental settings and proof of concept by clinical pilot studies, EBM-level III, for plasma treatment of head and neck cancer. If there is a manageable amount of tumour stem cells, like in precancerous lesions of oral mucosa or in small tumours like early basal cell carcinoma of the skin, plasma could become a clinical treatment option soon. Currently, it is already a concept in palliative care of patients with locally advanced head and neck cancer and contaminated ulcerations because of proven effectiveness against microbial pathogens. Patients greatly appreciate that plasma reduces strong fetid odour and pain while not being accompanied by serious side effects.

KEYWORDS
cancer of the oral cavity, face, and neck, clinical cancer research, clinical pilot studies, curative and palliative treatment, evidence-based medicine, physical cold plasma jet

1 | INTRODUCTION

Clinical cancer research features a very specialist expertise within the scientific and technical community of plasma sciences, and its common line of argumentation requires getting used to. Clinicians consider their more-or-less blurred clinical observations data, their figures present patients with cancer, and obtaining better treatment results with cold plasma is a more important objective of a clinical study than gaining better knowledge of the fundamental mechanisms of how plasma works. The only reason to accept this exceptional conduct is the sense-giving instance of medicine, demonstrating a particularly useful and beneficial opportunity to apply cold plasma medical devices based on the fine knowledge of plasma sciences and technology, if you are interested in any application at all.

The treatment of a patient suffering from cancer is, of course, a personal and individual mission; however, mercifully, considering the doctor’s responsibility and by coincidental agreement with health authorities, medical care and research may sensibly follow the rules of evidence-based medicine (EBM), as defined by the U.S. Agency for Health Care Policy and Research 1992, whenever possible. There is a rigid hierarchy in this system (Figure 1): introducing a new concept of cancer therapy that requires full recognition has to start with (a) simulation studies and investigations in cell cultures and animals, leading in summary to a (b) consent of experts (EBM-level IV) that gives way to (c) clinical pilot studies (EBM-level III) and further proceeds to (d) non-randomized studies (EBM-level II). EBM-level I of an innovative cancer treatment concept is reached with successfully evaluated (e) randomized clinical studies (EBM-level Ib), concluded by the positive outcome of a (f) structured review of several randomized clinical trials as EBM-level Ia.
1.1 What are the objectives of EBM-targeted and appropriate investigations, studies, and trials?

The first intention of cancer treatment is the healing of a patient, which will be achieved by the total extinction of the tumour and no evidence of disease for at least for 5 years, and is referred to as curative treatment. Logically, the first objective of EBM-targeted clinical cancer studies is the removal or killing all tumour cells that are able to generate new tumour cells, termed tumour stem cells. In case of predictable or eventuated failure of healing, the first intention of treatment, the second intention is relief from the health problems of the patient caused by cancer, referred to as palliative treatment. Typical health problems of cancer of the oral cavity, face, or neck are pain, fatigue, mental burden, loss of weight, shortness of breath, swallowing difficulties, and infection of tumour ulcerations. Consequently, the second objective in clinical cancer research is alleviation and easing of these severe complaints.

With regards to curatively intended treatment of head and neck cancer, and especially squamous cell carcinoma of the oral cavity, the total removal of tumour bulk and inclusion of potent stem cells by radical surgery is currently the first-line therapy and EBM-level Ia. Second-line treatment and EBM-level Ia, likewise, includes radiotherapy—attacking tumour stem cells locally in the area under radiation. Third-line treatment as well as EBM-level Ia is chemotherapy—attacking tumour stem cells wherever they occur and whenever they build up distant metastasis. To select one of these established therapy options or combine them complies with the specific individual conditions of the tumour and the state of health of the patient.

1.2 Where is cancer treatment by plasma found right now within the pyramid of EBM?

We see a tremendous amount of cell studies and animal investigations, as listed at first, by Schlegel and coworkers,[5] reporting the effectiveness of plasma in cancer cell lines, cultivated human tumour cells, human tumour specimen freshly explanted from patients, animal model tumours, or animals with transplanted human tumour stem cells. There is consent among experts, referred to as EBM-level IV, that in experimental settings, plasma kills various types of cancer, including melanoma, brain tumours, lung cancer, colon cancer, pancreatic cancer, breast cancer, leukaemia, thyroid cancer and head and neck cancer. Plasma treatment of head and neck cancer reaches the next stage, proof of concept by clinical pilot studies, EBM-level III.[6–8]

Admittedly, the abovementioned clinical benefit of plasma was discovered by chance in observations of patients receiving palliative therapy.

The regulatory approval of the first plasma sources for medical treatment purposes in 2013 was based on their well-documented effectiveness against microbial pathogens. Therefore, decontamination of infected ulcerations complies with the on-label use of these plasma devices. Many patients with very advanced squamous cell carcinoma of the oral cavity suffer severely from tumour ulcerations, with strong microbial contamination and infection causing pain, risk of infection, and the stench of rotten tissue. We have recently started treating these patients routinely for decontamination and palliation by an Argon-driven cold atmospheric pressure plasma jet kINPen® MED (neoplas tools GmbH, Greifswald, Germany)[2] every 2–3 days for 1 min/cm² in a meandering manner at 10 mm. This kind of medical care is usually very much appreciated by these patients because it reduces the strong fetid odour and pain[6] and is not accompanied by serious side effects. Moreover, the application of CAP has been observed several times to cause visible changes of the tumour surface, similar to small and local regression.[7] The following case presents the first real clinical benefit of a patient’s plasma treatment, initially intended for palliative antimicrobial control only, which goes beyond just visible changes of the tumour surface:

A 51-year-old Caucasian male presented with a well-differentiated squamous cell carcinoma of the oral cavity. The patient’s medical history was significant for long-term tobacco addiction of more than 25 years. The patient underwent curatively intended tumour surgery. Four months later, a CT scan indicated a large, contrast-enhancing mass at the neck, a tumour relapse that was not removable by surgery. For the next 2 months, the patient underwent palliative radiotherapy and two cycles of cisplatin...
administration as chemotherapy. After this, the tumour still in progress was characterized by an extended bacterially contaminated ulceration (Figure 2a). Due to the vulnerability of the nearby carotid artery, wound care, and especially mechanical or chemical removal of the bacterial layer, was difficult, and palliative plasma treatment was started.

To report the efficacy against pathogens: Within the plasma-treated zones, the infected necrotic tumour appears to be cleaned of cell detritus and bacteria (Figure 2c). Microbiological examination reveals a reduction of bacterial colonization of significantly anaerobic bacteria, such as *bacteroides* spp., which leads to decrease of bacterial decomposition products and wound odour as well. Thus, due to the decrease of local and perifocal inflammation, vulnerability and wound algesia have been reduced significantly.

To report the tumour reaction: A significant response is obvious (Figure 2a–c) with the ulcerated tumour area decreasing to one-quarter of its original size. Margins and the centre of the wound are sclerosed and calloused. The wound bed is covered by a physiological fibrin coating. Despite infiltration of the vascular wall of the underlying external carotid, the carotid artery is still intact, and sonographic investigation reveals regular blood flow without thrombosis.

To report the biopsy results taken at the beginning and after 4 months of plasma treatment: Incisional biopsies were performed to verify changes at the cellular level. Similar to healthy skin, immunofluorescence staining of CD11b+ cells (*cluster of differentiation 11b*, investigation of cell surface molecules providing targets for immunophenotyping of cells) in the plasma-treated tumour reveals only a minor presence of myeloid cells, whereas high numbers of CD11b+ cells were found in tissue sections of other patients who did not receive physical plasma. TUNEL analyses (*terminal deoxynucleotidyl transferase dUTP nick end labelling, a method for detecting DNA fragmentation*) demonstrates a moderate amount of apoptotic tumour cells. Furthermore, a desmoplastic reaction of the conjunctive tissue through an increased production of extracellular matrix is histologically visible, whereas proliferating cells remain very sparse.

In summary, there is considerable improvement of the patient not only by tumour decontamination but also by plasma-induced tumour mass reduction (Figure 2a–c). The main plasma effect seems to be a combination of a superficial cancer cell interaction, apoptosis, and a deep desmoplastic kind of a scar formation. The tumour regression by plasma appears to be promising; however, the tumour is still persistent, with the potential of relapse. No plasma related side effects have been observed.

1.3 What are the next steps in plasma treatment on the way to EBM?

First, in palliative medicine, any gain in the relief of patients is a success, and plasma medical devices will complement the standard tools of palliation when no-touch, no-chemical, no-harm treatment of contaminated cancer ulcerations is needed.[10–12]

Second, in curative medicine, any reduction of tumour growth counts and possibly means an impact on tumour stem cells. If there is a manageable amount of tumour stem cells, like in precancerous lesions of oral mucosa and or in small tumours like early basal cell carcinoma of the skin, plasma could become a clinical treatment option. Plasma treatment does not look like a bulk-removing concept, but in a locally advanced cancer with a tiny tumour stem cell offshoot of the bulk infiltrating an absolutely crucial tissue inaccessible for surgery, radiotherapy, or chemotherapy, it might be very suitable. The advantage is obvious: there is no sudden necrosis of tumour cells, tearing a hole in a blood vessel, but there is apoptosis of tumour cells, the reinforced natural dying of tumour cells and replacement by wound cells, to never let holes arise. At the current level of EBM, treatment with plasma will not be considered when surgery, radiotherapy, or chemotherapy is available and promising. Further clinical pilot studies should be choosing patients for palliation who face no positive effect of the first- to third-line therapy.[13]
If the results of these pilot studies give way to curatively intended randomized clinical trials, it takes 5 years of follow-up of the patients to obtain information about healing.

Third, clinical plasma medicine without support, monitoring, and supervision by scientific plasma research jeopardizes the benefit of patients and deceives their trust in medical professionalism. The role of clinicians on the way to EBM in plasma medicine is to ask the right questions to stimulate basic research,\textsuperscript{[14-19]}

Fourth, the fortune of healing and recovery is the sunny side of cancer therapy. The burden of risks, complications, and unavoidable treatment problems is the dark side. In surgery, the scourge of unavoidable side effects means scars from mild to mutilating, loss of functional and aesthetically important tissue, loss of blood, and the need for intensive care post-surgery. In radiotherapy, the most awful burden is the danger of stimulating normal cells to progress wildly to cancer cells themselves after a couple of years. In chemotherapy, the burden is, to some extent, the same as in radiotherapy; moreover, patients are suffering from fatigue caused by loss of red blood cells and low resistance against infections caused by loss of white blood cells. In plasma, there is currently no burden known and no severe and foreseeable side effects.\textsuperscript{[2,6,7,20]} So, the discussion of plasma as a new option in cancer treatment has the perspective of a new approach at all, not only for the goal of anticancer effectiveness in special circumstances but for better safety, compatibility, and tolerability of cancer treatment in general.

2 CONCLUSION

There are many possible methods to cut a wooden plank. At best, we take a saw. There is no need to replace the saw with something else until we realize that there is a hidden nail in the wood with adverse consequences or sawdust becomes a problem. At this point, we have to start thinking about alternative ways to cut the plank. Cancer treatment by plasma is such an alternative. The concept is promising but has to provide level-I evidence that this kind of treatment is more effective as the standard procedures of EBM in special circumstances, has substantially fewer side effects, and currently offers a new tool in palliative care of patients with locally advanced head and neck cancer (Figure 3).

REFERENCES


Clinical experience with cold plasma in the treatment of locally advanced head and neck cancer

Hans-Robert Metelmanna,m,⁎, Christian Seebeaua,h,m, Vandana Millerb, Alexander Fridmanb, Georg Bauer, David B. Gravesd, Jean-Michel Pouvesle, Rico Rutkowskia, Matthias Schuster, Sander Bekeschusf,h,m, Kristian Wendef,h,m, Kai Masurf,h,m, Sybille Hasseh,m, Torsten Gerlingf,h, Masaru Horig, Hiromasa Tanakag, Eun Ha Choii, Klaus-Dieter Weltmannh,m, Philine Henriette Metelmannaj,m, Daniel D. Von Ho, Thomas von Woedtkeh,l,m

a Oral & Maxillofacial Surgery/Plastic Surgery, University Medicine Greifswald, Germany
b AJ Drexel Plasma Institute, Drexel University, Philadelphia, USA
c Institute of Virology, Medical Center-University of Freiburg, Germany
d Chemical and Biomolecular Engineering, University of California, Berkeley, USA
e GREMI, Université d’Orléans, Orleans, France
f ZIK plasmatis, Leibniz Institute for Plasma Science and Technology, Greifswald, Germany
g Institute of Innovation for Future Society, Nagoya University, Japan
h Leibniz Institute for Plasma Science and Technology, Greifswald, Germany
i Plasma Bioscience Research Center, Kwangwoon University, Seoul, Republic of Korea
j Department of Orthodontics, University Medicine Greifswald, Germany
k Translation Genomics Research Institute, Phoenix, USA
l Hygiene and Environmental Medicine, University Medicine Greifswald, Germany
m National Center for Plasma Medicine e.V., Berlin, Germany

ARTICLE INFO

Keywords:
CAP
Oropharynx cancer
Immunotherapy
Apoptosis
ROS/RNS

ABSTRACT

Purpose: Cold atmospheric pressure plasma (CAP) is well known for inactivating microbial pathogens and stimulation of tissue regeneration in chronic wounds. Several authors have reported the effectiveness against cancer in different cell lines and animal models. This is the first report of patients with real clinical benefit following application of CAP, not just visible change of the tumor surface but lasting partial remission. The authors discuss the CAP treatment approach and the efficacy for inoperable head and neck cancer patients.

Methods: The trial enrolled six patients with locally advanced (pT4) squamous cell carcinoma of the oropharynx suffering from open infected ulcerations. Patients were treated with a jet plasma source (kINPen MED, neoPlas tools GmbH, Greifswald, Germany) in cycles of 3 single applications (1 min/cm² from a distance of 8 mm) within 1 week, each followed by an intermittence of 1 week.

Results: CAP treatment resulted in a reduction in odor and pain medication requirements, in improvement in social function and a positive emotional effect. Further observance revealed partial remission in two patients for at least nine month. Incisional biopsies at remission demonstrate a moderate amount of apoptotic tumor cells and a desmoplastic reaction of the connective tissue.

Conclusion: The trial demonstrates the clinical relevance of CAP in cancer treatment. There are three approaches for discussion of tumor remission: (i) the role of myeloid cells, (ii) the ROS/RNS model of cellular impact and (iii) the immunogenic cell death model of cancer treatment, and there is a reflection on non-sustainable tumor response due to adapted tumor microenvironment.

1. Introduction

The development of sources providing physical plasma at body temperature and under atmospheric pressure has opened the use of plasma components for medical purposes. From the clinical point of view the approval of the first plasma sources for medical treatment purposes in 2013 has been based upon their well-known effectiveness not only to stimulate tissue regeneration but also against microbial

H.-R. Metelmann et al.

pathogens. Cold atmospheric pressure plasma (CAP) has shown to be effective in cancer as well. Schlegel and coworkers in 2013 were the first to list the antitumor activity of CAP. The antitumor of effect of CAP has also been demonstrated in various types of cancer cell lines in vitro, such as melanoma, glioblastoma, lung cancer, colon cancer, pancreatic cancer, breast cancer, leukemia, thyroid cancer, and head and neck cancer. Several studies in experimental animal models have underlined this notion using e.g. melanoma, colon cancer, pancreatic cancer, endometrial adenocarcinoma, and ovarian cancer cells [1].

We have observed from our clinical experience that many patients with locally advanced squamous cell carcinoma of the oropharynx suffer severely from microbial contamination and odorous infection of their tumor ulcerations. After approval of the CAP device we began local application of CAP for decontamination. Palliation therapy by CAP was effective in the reduction of the strong fetid odor and pain [2]. In passant, application of CAP has been observed to cause visible changes of the tumor surface similar to small and local regression [3] that was not accompanied by serious side effects [4].

This paper is documenting and discussing six patients’ clinical CAP treatment results in terms of survival time, course of disease, tumor remission and safety. The approved indication was antimicrobial control as part of a standard treatment protocol for palliation.

2. Material and methods

The protocol was designed as a prospective treatment observation (protocol H) including 6 patients (H1 - H6).

2.1. Compliance with ethical standards

All procedures including assessing data from the patients were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments. All patients gave written informed consent before inclusion in the study.

2.2. Patient recruitment

Patients suffering from locally advanced cancer of the oropharynx (pT4) with contaminated tumor ulcerations and with no lasting remission following curative intended tumor treatment were offered CAP treatment (Table 1). Recruitment, treatment and follow-up of all patients were performed at the department of oral and maxillofacial surgery, Greifswald University Medicine, between January 2015 and March 2017.

2.3. Inclusion, exclusion and dropout criteria

Appropriate indications for CAP treatment were infected cancer ulcerations, easily and dependably accessible for CAP hand piece and jet stream. The linguistic, physical and mental ability to understand the nature of CAP treatment and to participate in the study was necessary. No special exclusion criteria despite the manufactures instructions were applied. Dropout criteria were the occurrence of undesired effects and aggravation of the microbial contamination.

2.4. Therapy

CAP was made applicable by a jet plasma source (kINPen MED, neoplas tools GmbH, Greifswald, Germany), a medical device previously described in basic technical detail [5]. The device consists of a hand-held unit that discharges plasma under atmospheric conditions, requiring a DC power unit and Argon gas reservoir. In the center of a ceramic capillary (inner diameter 1.6 mm) a pin-type electrode (1 mm diameter) is mounted. The needle is powered by a miniaturized RF generator producing a sinusoidal voltage waveform ranges from 2 kV to 3 kV amplitude peak at a frequency of 1 MHz and modulated with 2.5 kHz and a plasma duty cycle of 1:1.

The plasma is generated at the tip of the central electrode and expands into the surrounding air outside the nozzle. The system works with argon gas and flow rate of 5 slm. During operation, the length of the plasma jet (effluent) extends 10 mm from the ceramic capillary. Under these conditions, the maximum temperature of the plasma jet contacting the skin surface is 38 C. The physical effects of plasma generated by this device are well within a safe range for medical applications. The device is portable, allowing treatments to take place in a dental chair.

With regard to the study protocol CAP was delivered in cycles of 3 single treatments within 1 week, followed by an intermittence of 1 week without CAP exposure. CAP treatment proceeded by repeatedly scanning the area of the tumor with the visible plasma effluent for 1 min/cm² from a distance of 8 mm vertically from naturally moist tissue. Technical compliance with the study protocol was difficult in rugged and fissured ulcerations. Total treatment time increase to more than 30 min for patients with large ulcerations exceeding 30 square centimeters.

Tumor development was evaluated by calculating the total area of the tumor ulceration under treatment monthly and comparing the actual size with the starting size. Progressive disease means obviously increasing area of tumor surface. No response means there was no change of size of the cancer ulceration following CAP. Partial remission means reduction of tumor area.

2.5. Outcome parameters

As parameters of clinical outcome were documented (i) the survival time of the patients from the beginning of CAP treatment, (ii) the patients’ reports concerning effects on contamination, tumor growth, need of pain medication, side effects and benefit of palliation in terms of quality of life, (iii) shrinking or growing of the tumor area under treatment, calculated and documented as difference (+/- %) to the baseline (central 0-line).

Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>Tumor Type</th>
<th>Ulceration Status</th>
<th>Treatment Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>76 y, f,</td>
<td>locally advanced oro-pharyngeal cancer (pT4) with contaminated tumor ulceration, no lasting remission by surgery, radiation and chemotherapy, no curative treatment available, CAP-treatment for palliation (microbial control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2</td>
<td>55 y, f,</td>
<td>locally advanced oro-pharyngeal cancer (pT4) with contaminated tumor ulceration, no lasting remission by surgery, radiation and chemotherapy, no curative treatment available, CAP-treatment for palliation (microbial control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td>78 y, m,</td>
<td>locally advanced oro-pharyngeal cancer (pT4) with contaminated tumor ulceration, no lasting remission by surgery, radiation and chemotherapy, no curative treatment available, CAP-treatment for palliation (microbial control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td>56 y, f,</td>
<td>locally advanced oro-pharyngeal cancer (pT4) with contaminated tumor ulceration, no lasting remission by surgery, radiation and chemotherapy, no curative treatment available, CAP-treatment for palliation (microbial control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H5</td>
<td>53 y, m,</td>
<td>locally advanced oro-pharyngeal cancer (pT4) with contaminated tumor ulceration, no lasting remission by surgery, radiation and chemotherapy, no curative treatment available, CAP-treatment for palliation (microbial control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H6</td>
<td>56 y, m,</td>
<td>locally lasti y advanced oro-pharyngeal cancer (pT4) with contaminated tumor ulceration, no lasting remission by surgery, radiation and chemotherapy, no curative treatment available, CAP-treatment for palliation (microbial control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
For laboratory investigations, tissue biopsies of the tumor site were taken and embedded in OCT™, and 5 µm thin cryo-sections (Leica, Germany) were generated. To detect myeloid cells, fluorescently labeled anti-CD11b monoclonal antibodies (BioLegend, UK) were incubated on the tissue for 1 h at room temperature, and images were acquired using confocal laser scanning microscopy (Leica). Apoptotic cells were detected by TUNEL assay employing in situ death detection kit (Roche, Germany). Proliferating cells were visualized staining with a primary antibody to Ki67 (DAKO, Germany) followed by an Alexa 594 labeled secondary antibody incubation (Life Technologies, USA). Nuclei counterstaining was achieved using DAPI (4′,6-Diamidin-2-phenylindol). Images were captured on an AxioObserver Z.1 microscope (Zeiss, Germany).

3. Results

3.1. Survival time

5 of the patients passed away between 1 month and 12 months of CAP-treatment. One patient is still under control and receiving treatment by now for more than 9 months (Fig. 1). This result corresponds to expectations: With regard to the very limited literature available, the median survival time of patients with advanced cancer of oropharynx just under palliative treatment amounts to 7.5 months [6].

3.2. Clinical results

Table 2 is demonstrating the individual benefit of CAP treatment from the clinical point of view. Five of six patients were enjoying a reduction of odor as obvious effect of decontamination. Four of six patients presented with less demand of pain medication under CAP treatment. There were no deaths related to the application of CAP. There were no side effects in two patients, four patients complained of fatigue and dry mouth like symptoms. We attributed these symptoms resulted from the long lasting CAP application in the treatment of large-area tumor and the airflow of the plasma jet. Four of the patients considered CAP treatment as a noticeable palliation in terms of quality of life, in particular fatigue, nausea, vomiting, social function, emotionality and cognitive functions. The individual appreciation corresponds with the individual outcome concerning tumor growth. Viewed from the other side, the two patients with no obvious palliation were suffering from no effect on their demand of pain medication as well, some at least mild side effects, in one case no reduction of odor and, causing a lot of distress, no noticeable effect on tumor growth. In one patient CAP treatment failed in every category and the patient died after one month.

3.3. Tumor development

Six curves are following the individual courses of tumor surface size development of the six patients (Fig. 2). Patient H3 was suffering from a fast growing carcinoma that increased its size in 1 month by 50%, and...
he passed away after just one cycle of CAP treatment very soon. The tumor of patient H2 was responding to CAP treatment in terms of slower growth at the beginning, however between month 3 and 4 and independent from steady treatment the speed of tumor growth became the same as in patient H3. Patient H4 was suffering from a tumor that was not as fast growing. However, there was never a partial remission to be documented. At the time of exitus letalis following 12 months of increasingly unsteady CAP treatment the tumor had expanded by 80%. Patient H1 is presenting a tumor staying at the same size under CAP treatment for many months. She considered this as an encouraging course of cancer. However, when the tumor started to slowly grow after 8 month, the patient gave up and died.

Patient H5 experienced a strong response to the treatment for about 7 month and he witnessed 80% reduction of tumor surface (Fig. 3). However, tumor shrinkage did not reach total remission of 100% and the tumor started growing immediately with the same velocity as in patients H3 and H2, cutting the base line after 10 month and ending with exitus letalis shortly after. Patient H5 non-complaint with the rigid treatment protocol. Patient H6 is enjoying almost the same speed of tumor reduction as patient H5 for many months, and the shrinkage is continuous. He is still under CAP treatment aiming for 100% reduction that means total remission.

From a clinical point of view, it is important to pick the winners when considering CAP for tumor treatment more than palliation. From a scientific point of view patient H5 is most interesting because he started as a winner and then for no reason known became failing. The question as to why tumor remission for 7 month turned suddenly to tumor progression while still under constant unmodified CAP treatment has to be further investigated.

3.4. The case of patient H5

In February 2015, a 51-year-old Caucasian male patient presented with a well-differentiated squamous cell carcinoma of the oropharynx. The patient’s medical history was significant for long-term tobacco addiction of more than 25 years. The patient underwent curatively intended tumor resection and neck dissection on both sides. The tumor was found to be AJCC stage II (pT2 pN0 pM0 pL0 pV0 pR0 G1).

In June 2015, CT-scan indicated a large contrast enhancing mass in contact with the external left carotid artery and operative findings revealed tumor tissue infiltrating the vascular wall of the external carotid, which prevented R0 (full resection). The tumor recurrence was classified as large lymph node metastasis of a well to moderately differentiated squamous cell carcinoma at AJCC stage IVb (pT2 pN2c pM0 pL1 pV1 pR2 G2). Between July and August 2015 the patient underwent palliative radiotherapy with a daily dosage of 2 Gy up to a total radiation dosage of 66.0 Gy and 2 cycles of cisplatin chemotherapy.

In October 2015, the tumor was characterized by an extended bacterially contaminated ulceration (Fig. 3a). Due to the vulnerability of the underlying carotid artery, wound care was difficult. In January 2016 patient H5 was enrolled into the study and a supportively intended palliative cancer treatment started, applying CAP according to the trial protocol.

3.5. Clinical response

Efficacy against pathogens: Within the plasma treated zones infected necrotic tumor appears to be cleaned of cell detritus and bacteria (Fig. 3c). Microbiological examination reveals a reduction of bacterial
colonization, significantly anaerobic bacteria like bacteroides spp., which leads to decrease of bacterial decomposition products and wound odor, too. Decrease of local and perifocal inflammation resulted in significant reduction of vulnerability and wound algesia.

Tumor response: A significant reduction of wound area is obvious (Fig. 3a-c) with the ulcerated tumor area being reduced to one-quarter of its original size. Margins and center of the wound are sclerosed and calloused. A physiological fibrin coating coats the wound bed. Despite infiltrating of the vascular wall of the underlying external carotid, carotid artery is still intact and sonographic investigation reveals a regular blood flow without thrombosis.

3.6. Biopsy results

Incisional biopsies were performed to verify changes at the cellular level. Similar to healthy skin, immunofluorescence staining of CD11b+ cells in the plasma-treated tumor reveals an only minor presence of myeloid cells whereas high numbers of CD11b+ cells were found in tissue sections of other patients that did not receive physical plasma (Fig. 4a). TUNEL analysis demonstrated a moderate amount of apoptotic tumor cells (Fig. 4b). Furthermore, a desmoplastic reaction of the conjunctive tissue by an increased production of extracellular matrix is histologically visible (Fig. 4c), whereas proliferating cells remain very sparse (Fig. 4d).

In summary, CAP induced tumor reduction and significant improvement in tumor decontamination and tumor mass (Fig. 3). The main plasma effect seems to be a combination of a superficial cancer cell interaction, apoptosis and a deep desmoplastic kind of a scar formation. The tumor remission by CAP appears to be promising, however it turned out, that this remission has not been lasting.

4. Discussion

The number of clinical reports concerning CAP treatment of cancer areas is small, and so is the understanding of what might explain the clinical anticancer effect in this case and maybe the later loss in contrast. There are especially three specific approaches as food for discussion to understand at least the mechanism of action in cancer.

4.1. The role of myeloid cells

In healthy skin, myeloid cells such as tissue-resident macrophages usually reside around epidermal ridges in the papillary layer of the dermis [7], and accordingly CD11b+ cells have been detected in this region. Like in other cancer entities such as breast cancer [8], prostate cancer [9], or endometrial cancer [10], large numbers of tumor-associated macrophages are associated with a poor prognosis also in head and neck cancer [11]. Interestingly, tumor tissue of the patients that had received frequent plasma treated was almost devoid of myeloid cells, which is generally associated with a good prognosis in progressive head and neck cancer [12]. It can only be speculated whether this was either a direct consequence of the plasma treatment or a secondary effect due to a plasma-assisted decrease in bacterial burden of the infected cancer tissue.

4.2. The ROS/RNS model of understanding the clinical effect

It is intriguing to see that CAP had a profound effect both on bacterial and on tumor growth. Assuming that ROS/RNS (reactive oxygen species/reactive nitrogen species) contained in CAP were the most likely responsible effector molecules during therapy, the question may be raised whether the species that caused the antibacterial effect were the same as those that caused the antitumor effect. In a recent study, Wu and coworkers have demonstrated that the dominating
antibacterial effect of CAP was due to singlet oxygen (\( ^1O_2 \)) and that hydroxyl radicals contributed to a minor degree [13]. Singlet oxygen, an excited state of molecular oxygen, is not only contained in CAP, but in addition, it can be generated from certain CAP components [14].

Importantly, model experiments with a defined source of singlet oxygen showed that extracellular application of singlet oxygen had a strong and selective apoptosis-inducing effect on tumor cells, but did not affect nonmalignant cells. In contrast, intracellular generation of singlet oxygen caused nonselective cell death in both cell types. The detailed analysis of this process showed that extracellular singlet oxygen inactivated membrane-associated catalase, which is characteristic of tumor cells [15]. In addition, tumor cells show sustained activity of membrane-associated NADPH oxidase-1 that generates extracellular superoxide anions. These are required for maintaining proliferation and the malignant state of tumor cells, but also drive two intercellular ROS/RNS-dependent apoptosis-inducing signaling pathways. Membrane-associated catalase of tumor cells interferes with intercellular apoptosis-inducing signaling. Local inactivation of membrane-associated catalase prevents decomposition of \( H_2O_2 \) and peroxynitrite. As a result, these species interact in a complex reaction chain and generate secondary singlet oxygen that inactivates additional catalase molecules. This autoamplification of singlet oxygen generation, originally triggered by exogenously applied singlet oxygen, finally depletes the tumor cells of their protective membrane-associated catalase and causes their selective death through ROS/RNS signaling-mediated apoptosis induction.

In a recent analysis, these data obtained from model experiments were compared to data related to the potential effects of other CAP-derived ROS/RNS. This allowed to conclude that singlet oxygen from CAP seemed to be the most likely primary candidate to explain selective apoptosis induction in tumor cells in vitro and tumors in vivo. Therefore, if the data related to the connection between NOX1 and catalase in tumors can be translated to human tumors in a clinical situation, it seems reasonable to consider that the beneficial effects of CAP for the patient presented in this manuscript might be due to the same biochemical and cell biological scenario, dependent on an initial singlet oxygen-dependent step.

A rigid analysis is required that is suitable to determine whether application of CAP on tumor cells can be fully explained by singlet oxygen, in analogy to the model experiments described earlier. This analysis will not be trivial, as it will require to differentiate between the effects of CAP-derived singlet oxygen, singlet oxygen generated by CAP components, singlet oxygen potentially generated in plasma activated medium [16] and secondary singlet oxygen generated by targeted tumor cells.

If the anticipated scenario can be proven to be true, these findings should open ways to optimize CAP treatment of tumors through modulation of the CAP components, e.g. favoring a relatively high content of singlet oxygen. Based on the high standard of the physics of CAP, such modulations seem to be feasible in due course [17].

However, the experimental elucidation of the primary CAP-derived species that triggers the onset of the selective cascade of death inducing effects in tumor cells should not prevent us from reflecting on co-application of other species during treatment. For example, though singlet oxygen is discussed as primary antitumor agent in CAP in a recent analysis [14], and NO at moderate concentrations was found not to have the potential for effective initial interaction with the protective system of tumor cells against ROS/RNS signaling, the application of NO in parallel to singlet oxygen might enhance the steps that follow catalase inactivation by singlet oxygen. These steps are generation of secondary singlet oxygen through the interaction between \( H_2O_2 \) and peroxynitrite as well as apoptosis induction through formation of peroxynitrous acid. More examples of such useful and enhancing interactions have already been experimentally established.

If singlet oxygen inactivation of tumor cell protected catalase can be shown to be indeed the driving force for selective tumor therapy in vivo, the established knowledge on the optimization of singlet oxygen-dependent apoptosis induction in tumor cells should allow us to define regimens that utilize mechanism-based synergy effects. In this way, the required doses for CAP might be lowered. This mechanism then might open the way for effective treatment of tumors through micro-invasive CAP generators.

A concerted scientific action, based on work in vitro and animal models in vivo, focusing on future therapeutic applications to patients, is therefore required. The intellectual and technical basis required to achieve this goal is available and should be further activated and encouraged.

4.3. The immunogenic cell death model of cancer treatment

Tumors employ several immunosuppressive strategies to escape the body’s normal immune surveillance and elimination. Recent advances in our understanding of mechanisms involved in tumor immunity have led to increased efforts in development of immunotherapeutics for cancer [17]. One such modality relies on exposure of new antigens on tumor cells via the immunogenic cell death (ICD) pathway [18,19]. Since several steps in this pathway are ROS dependent, plasma is a viable candidate for this type of oncoimmunotherapy [20].

As such, plasma effects are largely mediated by ROS, but it is the concentration of ROS that influences the kind of biological effects achieved [21]. Rapid delivery of large amounts of toxic species causes cells to die. In context of cancer treatment, this would result in a quick, ablative debulking of tumor mass, as is seen in the patient case study presented here. The smaller tumor may then become more manageable by the compromised immune system. In addition, plasma decontamination of open ulcers and removal of immunosuppressive nature cannot be reversed. Hence, their elimination may be the only option and plasma at these ablative regimes can do that, as shown in this case study. Together, the debulking of tumors, decontamination of open ulcers and removal of immunosuppressive cells provides symptomatic relief to the patient and perhaps increased life expectancy.

Another method plasma may be employed for active immunotherapy of cancer is treatment in the ICD-causing regime. Softer than the ablative plasma regime, it operates by inducing endoplasmic stress pathways that result in generation of several molecules called damage associated molecular patterns (DAMP) that include ATP, calreticulin (CRT), HMGB1, HSP70, HSP90 etc. [18,22,23], cells follow a canonical pathway where they release ATP as a “find me” signal for antigen presenting cells (APC) immediately after plasma exposure [24,25]. This signal brings about the recruitment of APCs like DCs and macrophages to the local area [26]. Soon after, CRT is externalized on the membrane of plasma-stressed cells [24] to act as the “eat me” signal for the recruited APCs to phagocytose and destroy these cells [23]. The tumor cell laden APCs travel to draining lymph nodes to present the neoantigens exposed during this process to cognate T-cells. The tumor specific T-cells thus generated travel to the tumors (plasma treated or untreated, metastatic masses) and destroy them through production of cytotoxic molecules like TNFα [27]. Hence, tumors are destroyed by the highly selective natural defense pathways of the body. Another advantage of this ICD-inducing plasma regime is that it actively recruits and stimulates APCs instead of destroying them [24,28]. Therefore, we achieve tumor debulking through concomitant stimulation of immune responses.

Cancer is a complex disease and it is unlikely that a single treatment modality will successfully cure patients. Perhaps, a combination therapy with plasma - first plasma hit in ablative regimes followed by ICD-inducing regime - would be the ideal way to treat tumors with plasma. Alternatively, better optimization of plasma systems may
achieve both outcomes. In a recently completed small clinical trial, we show that actinic keratosis lesions resolved with a single nanosecond pulsed dielectric barrier discharge (DBD) plasma treatment. This system is adaptable for treatment of different surface areas by altering electrode geometry. A few of these patients reported overall clearing of skin and reduced incidence of new lesions at the 4-month follow-up. None of the patients reported immediate or late adverse effects.

While these small studies highlight the promise that plasma has in the field of oncoimmunotherapy, further exploration of mechanisms, both plasma and biological, are required to achieve consistently reproducible results [29–31].

4.4. Reflections on non-sustainable tumor response

In patient H5, the tumor remission by CAP appeared to be promising for several months. What might be the reason why it turned out that this remission has not been sustainable?

The loss of effectiveness under long-term plasma treatment of cancer tissue opens questions about plasma application and protocol. At first, it must be considered that the treated area is morphologically and chemically changing over the time, from activated surrounding to more normal tissues which are less humid and bacteriologically cleaner. This implied that over the time there is a modification of the tumor microenvironment and of the tissue conductivity that can greatly affect the plasma composition itself and the plasma induced processes mentioned in the discussion. It is known from literature [32–34] that for any type of plasma jet, the nature of the target, and specially its conductivity, influences the production (not only concentrations but also production zones in the jet) of reactive species, such as NO, OH, H2O2 and others, which play a direct role in the observed results. Concentration can increase by orders of magnitude and zone of production can change from plasma column volume to near target surface when the target characteristics change from near insulator to highly conductive material. Therefore, it can be expected that changes will occur in the ROS/RNS production between the start of the treatment and the treatment after few months due to tissue modifications, in the present case most probably leading to a decrease in the reactive species production. This will also affect the potential diffusion of species through the tissue upper layers, then change the cell signaling pathways and thus the triggering of the immune system that has been evoked earlier in this paper. This may also affect the potential role of the plasma jet generated electric field since penetration is also clearly related to the tissue characteristiques and can play on local cell permeabilization important for the exchanges through the cell membranes. Other possible changes occurring during the overall treatment are the tissue oxygenation and local blood flow that have been shown to vary under plasma action [35–37]. This last mechanism can consequently affect all other processes previously mentioned in this paragraph in a way that cannot be determined yet. It clearly appears that tissue modifications all along the treatment may play a deleterious role that reduces plasma efficacy and then partly explains the non-sustainable remission. All of this would imply to pay extreme attention to possible modifications of the plasma characteristics during treatment over months, which is not easy to realize in real conditions. In a first step, it would be nice to add simple spectroscopic measurements (realized with the use of a fiber optic connected to a compact spectrometer) in the plasma zone near the treated surface at different stages of the patient treatment to follow probable plasma emission modifications leading to a qualitative evaluation of changes in the excited radical species production. This will not lead to a direct evaluation of the treatment efficacy, but will potentially help to modify the treatment protocol by varying single fraction treatment time accordingly. In the future, in a specific study, it will be helpful to follow the treated tissue modifications to go deeper into the comprehension of the involved mechanisms in the observed results.

5. Conclusion

Clinical observation indicates a relevance of CAP in cancer treatment. Cancer patients under palliation with CAP resulted in a reduction of odor as obvious effect of decontamination, and less demand of pain medication, and very mild side effects. Four of the six patients enrolled in this observation considered CAP treatment as a noticeable palliation in terms of quality of life, in particular fatigue, social function and emotionality. Following their individual courses of tumor development, two patients were suffering from fast growing carcinoma, another two from slow or during longer periods not growing tumors and two patients enjoyed a strong response to CAP, one still persistent, the other having experienced a sudden relapse. Incisional biopsies of this tumor at the time of remission revealed minor presence of myeloid cells, a moderate amount of apoptotic tumor cells, a desmoplastic reaction of the conjunctive tissue by an increased production of extracellular matrix, whereas proliferating cells remained very sparse. To explain the clinical anticancer effect, there are three approaches for understanding: (i) the role of myeloid cells, (ii) the ROS/RNS model of cellular impact and (iii) the immunogenic cell death model of cancer treatment. Reflections about why some tumors are responding very well to CAP and why some do not, and why some change their reaction under constant treatment are of outstanding clinical importance to pick the winners when considering CAP in the future for tumor treatment more than palliation.

Acknowledgements

This work could not have been completed without the support of Wolfram Kaduk and Axel Schriewer as surgeons, Anke Friedrich, Martina Büttcher and Runa Tschersche-Mondry as wound nurses and Kerstin Böttger as system consultant in the Department of Oral and Maxillofacial Surgery, Greifswald University.

Conflict of interest

The authors declare no potential conflicts of interest. The CAP medical device is in clinical just due to approval and supplied by the manufacture without financial obligations.

References


Visible tumor surface response to physical plasma and apoptotic cell kill in head and neck cancer

Matthias Schustera,*, Christian Seebauera, Christian Seebauerc, Rico Rutkowskia, Anna Hauschilda, Fred Podmellea, Camilla Metelmannb, Bibiana Metelmannb, Thomas von Woedtkec,d, Sybille Hassec, Klaus-Dieter Weltmannec,d, Hans-Robert Metelmanna,d

a Department of Oral and Maxillofacial Surgery/Plastic Surgery (Head: Prof. Dr. Dr. Hans-Robert Metelmann), Greifswald University Medicine, Ferdinand-Sauerbruch-Str. DZ 7, 17475 Greifswald, Germany
b Greifswald University Medicine, Department of Anesthesiology, Anesthesia, Intensive Care-, Emergency- and Pain Medicine, Ferdinand-Sauerbruch-Str., 17475 Greifswald, Germany
c Leibniz Institute for Plasma Science and Technology (INP), Felix-Hausdorff-Str. 2, 17489 Greifswald, Germany
d National Centre for Plasma Medicine (ZPM), Charitéplatz 1, 10117 Berlin, Germany

ARTICLE INFO

Article history:
Paper received 11 December 2015
Accepted 1 July 2016
Available online 18 July 2016

Keywords:
Cold atmospheric pressure plasma (CAP)
Head and neck cancer
Tumor growth
Palliative medicine
Decontamination
Apoptotic cell kill

ABSTRACT

The aim of the study was to learn, whether clinical application of cold atmospheric pressure plasma (CAP) is able to cause (i) visible tumor surface effects and (ii) apoptotic cell kill in squamous cell carcinoma and (iii) whether CAP-induced visible tumor surface response occurs as often as CAP-induced apoptotic cell kill.

Twelve patients with advanced head and neck cancer and infected ulcerations received locally CAP followed by palliative treatment. Four of them revealed tumor surface response appearing 2 weeks after intervention. The tumor surface response expressed as a flattened area with vascular stimulation (type 1) or a contraction of tumor ulceration rims forming recesses covered with scabs, in each case surrounded by tumor tissue in visible progress (type 2).

In parallel, 9 patients with the same kind of cancer received CAP before radical tumor resection. Tissue specimens were analyzed for apoptotic cells. Apoptotic cells were detectable and occurred more frequently in tissue areas previously treated with CAP than in untreated areas.

Bringing together both findings and placing side by side the frequency of clinical tumor surface response and the frequency of analytically proven apoptotic cell kill, detection of apoptotic cells is as common as clinical tumor surface response.

There was no patient showing signs of an enhanced or stimulated tumor growth under influence of CAP.

CAP was made applicable by a plasma jet, kINPen® MED (neoplas tools GmbH, Greifswald, Germany). © 2016 Published by Elsevier Ltd on behalf of European Association for Cranio-Maxillo-Facial Surgery.

1. Introduction

Clinical case reports and select trials have demonstrated that cold atmospheric pressure plasma (CAP) is a useful tool for decontaminating severely infected wounds and ulcerations (Isbary et al., 2010, 2012; Brehmer et al., 2015). For this purpose, it has been applied in our unit as part of the palliative medicine program for patients within the final stages of advanced head and neck carcinoma and grossly contaminated tumor ulcerations. Indeed, head and neck cancers present difficult clinical problems as cancer proximity to significant anatomic structures calls for better local therapy.

1.1. CAP for decontamination

The reduction of microbiological contamination is a result of one of the, actually in fact the most, promising characteristics of CAP, its ability to very effectively inactivate multi-resistant strains of microorganisms (Daeschlein et al., 2014). Whether therapeutic application of CAP might inactivate cancer cells as well is not a
matter of clinical concern for patients with advanced stages of head and neck cancer, since the effect of CAP is limited to very superficial tissue structures. However, they receive CAP for decontamination and en passant the question of cancer cell response has been attracting researchers around the world since Fridman et al. (2007) published their landmark paper entitled “Applied plasma medicine”. Keidar et al. (2007) introduced the idea of CAP selectivity and the possibility of a paradigm shift in cancer therapy. Their idea has been recently supported by clinical observations and findings of immunological interactions (Metelmann et al., 2015a, 2015b; Miller et al., 2015).

CAP is physical plasma generated by adding energy to a gas resulting in ionization and excitation of gas molecules. Biological tissue is primarily affected by two components of physical plasma: 1) electromagnetic radiation (UV, VIS, IR, high-frequency electro-magnetic fields, etc.) and 2) ions, electrons and reactive chemical species. The technical possibility of generating physical plasma at low temperatures in an atmospheric environment opens up new chances to use CAP for medical therapies (Isbary et al., 2013).

According to the current state of knowledge, plasma effects on biological systems are mainly caused by reactive oxygen and nitrogen species (ROS and RNS) which influence cellular processes via impacts on the redox balance of cells (von Woedtke et al., 2014) that might be applicable for cancer stem cells, too.

1.2. CAP for inducing apoptosis

The single cancer stem cell is the therapeutic target, when treating malignant tumors with curative intention, as this is the source of clonal tumor growth, metastasis, recurrent cancer and development of cancer disease (VonHoff et al., 1982). The evidence to date suggests that CAP has a significant apoptotic effect on cancer cells as demonstrated in several tumor lines, tumor models in in vitro and in vivo studies using nude mice (Vandamme et al., 2010; Schlegel et al., 2013; Utsumi et al., 2013). Triggering reactive oxygen and nitrogen species to derange the redox balance and redox signaling of cancer stem cells is considered as a key pathway for understanding CAP-induced apoptosis, since the survival and proliferation signaling network is one of the most essential signaling networks. Two major signaling pathways within this network are the Phosphoinositide 3-kinase (PI3K)-AKT signaling pathway and the Rause-associated sarcoma (RAS)-Mitogen-activated Protein Kinase (MAPK) signaling pathway (Downward, 2003; Martelli et al., 2010). Activation of this signaling network often seen in cancer patients leads to induction of cell growth and inhibition of apoptosis. CAP treatment hinders both pathways (Tanaka et al., 2012) and induces apoptosis of tumor cells due to down-regulation of the survival and proliferation signaling network (Chalhoub and Baker, 2009; Laplante and Sabatini, 2012).

1.3. Tumor cells sensible to CAP

Head and neck cancer cells are of confirmed sensibility to CAP (Guerrero-Preston et al., 2014; Kang et al., 2014; Han et al., 2013; Chang et al., 2014a), and also bladder cancer (Keidar et al., 2011), brain tumor (Tanaka et al., 2011, 2012; Vandamme et al., 2010; Koritzer et al., 2013; Kaushik et al., 2012, 2013), breast cancer (Kim et al., 2010a; Wang et al., 2013), cervical cancer (Leduc et al., 2009; Ahn et al., 2011; Sato et al., 2011; Huang et al., 2013), colorectal cancer (Lupu et al., 2009; Vandamme et al., 2012; Kim et al., 2010b, 2010c; Ishaq et al., 2014), gastric cancer (Tori et al., 2014), leukemia (Thiagarajan et al., 2012; Barekzi and Laroussi, 2012), liver cancer (Gweon et al., 2010), lung cancer (Huang et al., 2011; Kim et al., 2011; Adachi et al., 2014; Panngom et al., 2013), malignant melanoma (Fridman et al., 2007; Lee et al., 2009; Zirnheld et al., 2010; Daeschlein et al., 2013; Sensenig et al., 2011; Yajima et al., 2014; Iida et al., 2014), ovarian cancer (Iseki et al., 2012; Utsumi et al., 2013, 2014), pancreatic cancer (Brullé et al., 2012; Partecke et al., 2012), prostate cancer (Hirst et al., 2014) and thyroid cancer (Kaushik et al., 2014; Chang et al., 2014b).

The aim of this study is to learn whether clinical application of cold atmospheric pressure plasma (CAP) in head and neck cancer patients is able to cause (i) visible tumor surface effects, (ii) apoptotic cell kill and (iii) whether CAP-induced visible tumor surface response occurs as often as CAP-induced apoptotic cell kill.

2. Material and methods

2.1. Study design

The study is designed as a descriptive evaluation of the clinically visible influence of the intervention in one group of patients (EudraCT number 2014-000416-34) together with a histological analysis of tissue effects in a comparable second group of patients. Patients suffering from advanced squamous cell carcinoma (n = 21) were assigned to one of the both groups due to their individual different clinical treatment plan.

2.1.1. Group I

Group I (n = 12) was treated with CAP as part of their palliative program, not primarily intended to influence tumor growth but to reduce microbiological contamination of their infected ulcerations. The descriptive clinical evaluation was based upon an intra-individually comparative, prospective, blindly evaluated study protocol. The objectives of the analysis were to prospectively look for and assess changes of the tumor surface as intra-individual differences between a spot treated with CAP and the surrounding area of the same tumor lesion untreated. The assessment of CAP effects was based on photo evaluation by a remote panel of three blinded experts, since blinded, remote photographic analysis is feasible for clinical studies and correlates well with direct clinical assessments (Rennekampff et al., 2015). Additional clinical assessment was performed by the patients themselves and their clinical treatment team evaluating the effects of CAP unblinded. Statistical analysis was not performed, due to the small sample size and cancer-related multi-morbidity of patients causing a very inhomogeneous sample.

The evaluation was conducted in compliance with International Conference on Harmonisation guidelines for Good Clinical Practice and the principles in the Declaration of Helsinki. All blinded viewers and treatment team members received training in the protocol and in the standardized acquisition of photographs. Informed consent was obtained from all patients before inclusion in the analysis.

2.1.2. Group II

Group II (n = 9) was treated by curatively intended surgery and received CAP before total tumor resection. Tissue specimens were analyzed for apoptotic cells.

2.2. Patients

The study was conducted between October 2013 and November 2015 and involved 9 female and 12 male Caucasian patients, age of 40–77 years.

2.2.1. Group I

Group I patients were scheduled for palliative care due to the advanced stage of carcinoma disease beyond curative standard cancer therapies. All patients selected were in Karnofsky
performance status of 60–80 and presented superficial intraoral or extraoral ulcerations. CAP treatment was part of their palliative program to reduce risk of infection, pain medication and typical strong fetid odor causing social and even family isolation. All patients consented to a CAP study protocol for possible tumor response prior to their standard CAP treatment for microbial decontamination. No patient consented in offering tumor specimen to confirm clinical findings by histology.

2.2.2. Group II
Group II patients were scheduled for surgical removal of cancer due to curative standards. All patients consented to receive CAP intra-operatively for study purposes before resection.

2.3. Interventions
CAP was made applicable for medical use by a plasma source, kINPen® MED (neoplas tools GmbH, Greifswald, Germany), a plasma jet tool previously described in basic technical detail (Weltmann et al., 2009), licensed for treatment of infected wounds and infective skin diseases since 2013. The medical device consists of a hand-held unit that discharges plasma under atmospheric conditions requiring a DC power unit and Argon gas reservoir (Fig. 1). In the center of a ceramic capillary (inner diameter 1.6 mm), a pin-type electrode (1 mm diameter) is mounted. The needle is powered by a miniaturized RF generator producing a sinusoidal voltage waveform that ranges from 2 kV to 3 kV amplitude peak at a frequency of 1 MHz and modulated with 2.5 kHz and a plasma duty cycle of 1:1.

2.3.1. Group I
CAP was delivered to the superficial tumor tissue in a cycle of 3 single treatments within 1 week, followed by an intermittence of 1 week without CAP exposure. Each single treatment occurred as a spot exposure of the ulceration to CAP for 1 min from a distance of 8 mm, vertically to naturally moist tissue surface (Fig. 2).

2.3.2. Group II
CAP was delivered to the superficial tumor tissue in a one-time application followed by total resection of the tumor. Technically like in group I application, this proceeded as a spot exposure of the ulceration to CAP from a distance of 8 mm, vertically to naturally moist tissue surface, however for 3 min (Fig. 2).

2.4. Evaluation and analysis
According to the different issues, both groups’ data were used in different procedures, group I for evaluation of visible tumor surface changes, group II for histological analysis of apoptotic cell kill within the tumor tissue.

2.4.1. Group I
For the blinded evaluation of tumor surface changes, photos were taken with a Canon EOS 70D camera with fixed settings. Photos were checked by medical experts knowing about the area of CAP exposure but not involved in the treatment of the patients or safety and appraisal assessments. Only photos confirmed to be free of treatment related markings were considered for evaluation. For each patient, photos were presented in chronological order. The assessment was counted for records only when the viewers formed their opinion unanimously.

For the unblinded evaluation of tumor surface changes and documentation of adverse effects and personal benefit, patients and treatment team estimated the effects of CAP application directly and clinically with concern for adverse effects, personal benefit and appraisal of novel plasma technology. Adverse events had to be documented in accordance with International Committee for Harmonisation guidelines for Good Clinical Practice as unrelated or as unknown, unlikely, possibly, or probably related to the treatment. For assessing personal benefit and appraisal patients and treatment team answered questionnaires about emotional estimation of the treatment on a Likert scale (Likert, 1932; Metelmann, 2016).

2.4.2. Group II
For the histological detection of apoptotic cells, specimens from the freshly removed total tumor bulk, formerly with or without local CAP application, were embedded and cryo-sectioned. In order to detect DNA fragmentation as a hallmark of apoptotic cells, tissue samples were subjected to terminal dUTP nick-end labeling (TUNEL). Counterstaining was realized by DAPI staining where nuclei appear as blue fluorescent. Microscopic pictures were taken by fluorescence microscopy. Image software was employed for quantitative image processing (Hasse et al., 2016).

The ratio of apoptotic cells was compared in tissue with and without plasma treatment.
3. Results

The outcome of CAP application in 15 patients with a total of 20 interventions (group I) was documented in 60 photographs, sampled in stakes 0, 1 and 2.

Stake 0 of 20 photographs of tumor surface areas was taken before CAP application, stake 1 of 20 photographs of identical pattern was taken 1 week after start of CAP application and full cycle of treatment, stake 2 of 20 photographs of identical pattern was taken 2 weeks after start of CAP application at the end of the post-treatment intermittence.

The viewers were asked to decide whether there was any different appearance in clinical aspect of the tumor surfaces intra-individually after 1 week or 2 weeks of CAP application, comparing the CAP-exposed spot and the surrounding tissue.

Viewing stake 0 the experts confirmed, that in all 20 photographs the area to be exposed with CAP was clearly recognizable. Viewing stake 1 all reviewers agreed in their assessment, that there was no remarkable change in appearance after 1 week in the aspect of the area under observation.

Viewing stake 2, in 3 photographs viewers disagreed in their assessment. These photographs and in consequence these patients were taken out of further analysis.

3.1. Time of appearance and kind of visible tumor surface response

Among the 12 patients in group I left for further consideration, photographs of 4 interventions were presenting a consented intra-individual difference between the spot treated and the surrounding tumor surface, that appeared in stake 2, that means 2 weeks after CAP application (Figs. 3–6).

The intra-individual differences of development between CAP-exposed spot and untreated neighboring tissue surface expressed as (type 1) a flat area with vascular stimulation (Figs. 3 and 4) or (type 2) a contraction of tumor ulceration rims forming recesses covered with scabs (Figs. 5 and 6), in each case surrounded by tumor tissue in visible progress.

There was no photograph showing the aspect of an enhanced or stimulated tumor growth under influence of CAP.

Patients and treatment team came to similar assessments in general, concerning time and kind of tumor surface change. Additionally they decided for positive influence of CAP in the 4 patients formerly excluded by the blinded viewers from further analysis.

3.2. Safety

There were no severe adverse effects or adverse effects to be reported in group I. CAP was safe and well tolerated, both treatment team and patients never considered effects of CAP to be a reason for discontinuation of procedure. However patients complained about a stinging, but moderate pain starting when duration of CAP exposure reached the one-minute application time continuously at the same spot due to the study protocol.

3.3. Benefit and personal appraisal

The most common discomfort mentioned in group I was related to bad taste and exhaustion. Bad taste is obviously following intraoral CAP application, and some of the patients with larger ulcerations report extreme fatigue when it comes to CAP palliative treatment due to the greater duration of decontamination procedure required. As a most highly evaluated benefit of CAP application in general, patients report the reduction of odor, the reduced need for pain medication and in few cases even gain of weight as a result of improved appetite and general condition. Patients in general appreciate physical plasma and the balance of emotional distress and relief in palliation very much.
3.4. Apoptotic cell kill

Staining for detection of cancer cells in the state of apoptotic cell death (group II) visualizes cells as green fluorescent (Fig. 7). The ratio of apoptotic cells was compared in tissue with and without plasma treatment. As a result apoptotic cell kill is more frequently detectable in tissue areas previously treated with CAP (Table 1).

Bringing together both findings and placing side by side the frequency of clinical tumor surface response and the frequency of analytically proven apoptotic cell kill, detection of apoptotic cells is as common as clinical tumor surface response.

4. Discussion

This study yields visible information, that CAP can affect cells in clinical head and neck cancer as known for cell lines (Guerrero-Preston et al., 2014; Kang et al., 2014; Han et al., 2013; Chang et al., 2014a) and is causing apoptotic cell kill in head and neck cancer tissue as well. In case of cancer response visible effects at the tumor surface became obvious 2 weeks after CAP application and appeared in 2 different types. There was no clinical observation of stimulated tumor growth in all of the patients.

From the clinical point of therapeutical applicability these findings are limited by study protocol restraints, however the small number of case reports so far published or presented is in line with the first impression of these results (Metelmann et al., 2015a; Seebauer et al., 2015; Schuster and Metelmann, 2015).

Concerning the different efficacy of CAP in different tumor patients, it is not surprising and maybe simply due to the biological idiotypes and clinical presentations unique to each individual patient. VonHoff and his group have mentioned clonal evolution and therapeutic resistance in solid tumors (Barrett et al., 2013): Tumors frequently arise as a result of an acquired genomic instability and the subsequent evolution of neoplastic populations with variable genomes. That might be due to response or no-response to CAP, too.

Another limitation of the analysis is the unknown cellular and histological background of tumor surface changes. Apoptotic cell kill is obvious, however, some of the adverse effects of CAP treatment published recently (Metelmann et al., 2015a) have to be interpreted as stroma reactions, i.e. bleeding and erythema, not directly to be related to cancer stem cell behavior (Whattcott et al., 2013). There is no evidence for CAP influence on cancer stem cells exclusively, but a particular thought, that apoptotic cell kill might be a necessity for tumor regression starting with tumor surface...
change, seems to be not a sufficient premise. From a clinical point, this is a matter of minor concern, however, as long as tumor growth in general might be reduced by CAP.

5. Conclusion

Twelve patients with advanced head and neck cancer received superficial CAP followed by palliative treatment. Four of them revealed tumor surface response appearing 2 weeks after intervention. The tumor surface response expressed as a flat area with vascular stimulation (type 1) or a contraction of tumor ulceration rims forming recesses covered with scabs, in each case surrounded by tumor tissue in visible progress (type 2).

In parallel, 9 patients with the same kind of cancer received CAP before radical tumor resection. Tissue specimens were analyzed for apoptotic cells. Apoptotic cells were detectable and occurred more frequently in tissue areas previously treated with CAP than in untreated areas.

Bringing together both findings and placing side by side, the frequency of clinical tumor surface response and the frequency of analytically proven apoptotic cell kill, detection of apoptotic cells is as common as clinical tumor surface response.

As a result of, and according to the discussions of the 3rd International Workshop on Plasma for Cancer Treatment, Washington DC, April 2016, further data concerning clinical response and apoptotic cell kill induced by CAP need further clinical research.

Acknowledgments

The authors thank Daniel D. VonHoff for his continuous stimulation to look for new horizons in cancer treatment and for his consultations. The patients’ study participation is gratefully acknowledged. This paper is dedicated to the patients that have supported the development of plasma medicine studies tremendously.
References


Metelmann HR: Personal approach to Metelmann B, Metelmann (eds), Telemed- icine at the emergency site – evaluated by team members in simulated sce- narios. Greifswald University, 2016 [Inaugural dissertation]


Hyperspectral imaging for in vivo monitoring of cold atmospheric plasma effects on microcirculation in treatment of head and neck cancer and wound healing

R. Rutkowski\textsuperscript{a,*}, M. Schuster\textsuperscript{a,1}, J. Unger\textsuperscript{b,1}, C. Seebauer\textsuperscript{a,d,e}, H.R. Metelmann\textsuperscript{a,e}, Th.v. Woedtke\textsuperscript{d,e}, K.D. Weltmann\textsuperscript{a,e}, G. Daeschlein\textsuperscript{c}

\textsuperscript{a} Department of Oral and Maxillofacial Surgery/Plastic Surgery, University Medicine Greifswald, Greifswald, Germany
\textsuperscript{b} Department of Obstetrics and Gynecology, University Medicine Greifswald, Greifswald, Germany
\textsuperscript{c} Department of Dermatology, University Medicine Greifswald, Greifswald, Germany
\textsuperscript{d} Leibniz-Institute for Plasma Science and Technology (INP), Greifswald, Germany
\textsuperscript{e} National Center for Plasma Medicine e.V., Berlin, Germany

ARTICLE INFO

Keywords:
Cold atmospheric plasma
Head and neck cancer
Hyperspectral imaging
Maxillofacial surgery
Microcirculation
Wound healing

ABSTRACT

Beside a proven antimicrobial and inflammatory-modulating spectrum of plasma therapy, recent studies point to impressive molecular and cellular effects against tumor cells. However, underlying mechanisms of anticancer effects and improved wound healing, in particular plasma associated influence on microcirculation, have not been sufficiently clarified yet. To date, there is no convincing method for monitoring therapy with cold atmospheric plasma (CAP) in vivo. TIVITA™ Tissue System is an innovative hybrid technology that combines imaging and spectroscopy for assessment and documentation of tissue. By collecting spectral information, HSI generates a three-dimensional (3D) data cube that enables calculation of different microcirculation parameters. In this research two patients were treated with CAP in context of head and neck tumor therapy. HSI was able to demonstrate CAP associated effects on microcirculation showing a relevant increase of superficial and deeper cutaneous oxygen saturation, hemoglobin concentration and distribution. This effect may contribute to healing support by CAP in wounds as well as tumor disease. Still in scientific development, HSI appears to be suitable for analyzing and monitoring CAP effects in clinical setting. With regard to the limited amount of data, these findings must be verified in a larger study population.

1. Introduction

While the origins of plasma technology goes back to the 19th century, biomedical applications are increasingly becoming the focus of academic and clinical interest [1]. In addition to different types of disinfecting, sterilizing and surface conditioning treatments, increasing understanding of both individual components and complex interactions with biological tissues also extends current indications as well as theoretical horizon of medical-therapeutic treatment [2]. Several cold plasma sources have been developed for use on biological tissue [3,4], for which a broad antibacterial and tissue-promoting potential has been demonstrated in vitro and in vivo [5–8]. Cold atmospheric plasma consists of various components that combine specific biological effects. In this so-called plasma cocktail, reactive oxygen and nitrogen species (ROS, RNS) play a dominant role for plasma-induced biological reactions. According to the actual state of scientific research main cellular and subcellular effects of CAP are based on plasma induced changes of the liquid environment of cells [9]. There are three important aspects of RONS. First, reactive species are an intrinsic part of physiological cellular homeostasis and involved in several intracellular and extracellular signaling networks [10,11]. Second, pathophysiological effects of reactive oxygen and nitrogen species are documented in different contexts including ageing process [12], cardiovascular [13] as well as metabolic [14] disease and carcinogenesis [15]. Third, reactive species are already part of evidence based therapies ranging from the involvement in antibiotic infections [16] to radiation therapy [17] and redox cancer chemotherapy [18]. Using CAP with the intention to control and modify the cellular redox balance in order to affect respectively induce lethal or non-lethal mechanisms is the central subject of biomedical plasma application. Against this background physical
plasma in low-temperature range is also becoming increasingly important in cancer therapy. In this context successful treatment was reported within palliative treatment of head and neck cancer [19]. While essential plasma effects seem to be caused by redox biological processes the role of plasma-modified microcirculation remains partly unclear. Another clinical difficulty is the fact that there is no convincing technique for monitoring hemodynamic CAP effects in vivo, neither in wound healing nor in tumor therapy. While invasive procedures such as angiography are routinely used for the imaging of macrocirculation, the use of invasive methods for the spatial visualization of the microcirculation is not justified. Particularly in view of the fact that the biomedical use of cold atmospheric plasma is a non-invasive technique, there is an urgent need for an adequate, non-invasive monitoring technique as well. In recent years, significant advances in development of spectral imaging have been made, leading to Hyperspectral Imaging (HSI) technology [20,21]. Hyperspectral imaging is a hybrid technology composed of digital photography and spectroscopy which allows analyzing the microcirculation of a large area tissue area in nearly real time. Based on the spectral information different microcirculation parameters such as superficial and deeper tissue oxygen saturation as well as hemoglobin and water distribution in tissue are calculated. This technique has already been used successfully in wound diagnostics, in particular for postoperative transplant monitoring [22,23]. In the present study we have used HSI to analyze possible CAP effects on microcirculation.

2. Material and methods

2.1. Plasma device and treatment

Plasma therapy was performed with kINPen® MED (neoplas tools GmbH, Greifswald, Germany). Operation details and parameters were previously described [5]. Generated plasma effluent length is 9–12 mm and with 1 mm in diameter. After five minutes of atmospheric acclimatization CAP application was performed for 60 s per 1 cm² in constant sinusoidal movement (moving velocity between approximately 8 mm/s and 10 mm/s). To ensure that during treatment the distance of the jet was not lower than 8 mm, an autoclavable spacer was used.

2.2. Hyperspectral imaging

For hyperspectral imaging TIVITA™ Tissue System (DiaSpersive Vision, Pepelow, Germany) was used. Examination lasted about three minutes per patient and was carried out by suitably trained staff after an acclimatization phase of approximately five minutes in an examination room (temperature-controlled at about 22 °C and 50% humidity) in complete absence of other light sources. TIVITA™ Tissue System allows the acquisition of hyperspectral data between 500 and 1,000 nm and so in both, visible (up to 750 nm) and near-infrared range. For each image pixel, an optical spectrum with a resolution of up to 5 nm can be recorded, which corresponds to a total of about one hundred spectral channels. Based on the spectral information, a three-dimensional (3D) data cube consisting of two spatial dimensions and
one spectral dimension is generated which constitutes the basis for calculation of oxygen saturation at tissue surface (\% StO2) as well as in deeper layers (NIR, 8 mm depth, calculated index (0–100)) and hemoglobin content (THI, calculated index (0–100)). Monitoring of microcirculation was performed before, immediately after and ten minutes after plasma application.

2.3. Study population

Two Patients of the Department for Oral and Maxillofacial Surgery/Plastic Surgery of the University Medicine Greifswald who, due to different oncological indications, received a therapy with CAP were examined with TIVITA™ Tissue System. The investigation was performed in accordance with the standards set by the Declaration of Helsinki. Written informed consent was obtained from all participants prior to treatment and examination.

First patient was a 66-year-old man with T4 squamous cell carcinoma of oral cavity. After initially successful surgical removal a highly aggressive tumor recurrence developed which showed progressive growth trend even under adjuvant radiation and cytotoxic therapy (Fig. 1). The skin-breaking tumor showed an ulcerating infected surface with complex bacterial load (e. g. Staphylococcus, Streptococcus, Klebsiella, Pseudomonas and Enterococcus species) including partial resistances. CAP application was performed as part of palliative concept and was mainly intended for microbial decontamination of infected tumor surfaces.

Second patient was a 55-year-old man with T3 squamous cell carcinoma of oral cavity, primary treated by surgical resection including neck dissection (Fig. 3). During surgery there was a temporary connection between oral cavity, tumor mass and neck wound. Due to the high risk for spreading of microbiological colonization (intra- and postoperatively) we used CAP with the intention of synergistically using the plasma-specific combination of chemical and physical active components for reduction of microbial load and stimulation of cell proliferation to optimize wound healing.

3. Results

All patients could be examined easily and reproducibly with HSI. Clinically relevant areas could be visualized and analyzed over time.

3.1. CAP as part of palliative tumor treatment

HSI allows specific wavelength analysis of each pixel in generated image and moreover image presentation at exactly one wavelength (Fig. 1). A distinct impact on superficially (StO2) and deeply (NIR) oxygen saturation as well as hemoglobin distribution (THI) could be detected after CAP treatment of tumor tissue. In detail, immediately after plasma application an increase of 7% in superficial tissue oxygen saturation (StO2) and 5 index points in oxygen saturation in deeper lied tissue layers (NIR) was observed. In addition, a rapid plasma-mediated increase in local hemoglobin concentration (THI) by 14 index points was observed (Fig. 2A,B). The measurements ten minutes after completion of the plasma treatment not only confirmed these observations, but also showed a further increase in the microcirculation parameters examined. In contrast, there were no relevant changes in the control area not treated with CAP. Corresponding HSI measurement data are shown in Table 1.

3.2. Early stage of wound healing after surgical removal of cervical lymph nodes in context of head and neck tumor surgery

HSI showed deficient oxygenation of superficial as well as deeper tissue layers before CAP application in wound area in comparison to neighboring skin area (Figs. 3B, 4A). A distinct impact on superficially (StO2) and deeply (NIR) oxygen saturation as well as hemoglobin distribution (THI) could be detected after CAP treatment in wound area. In more detail, immediately after plasma application an increase in superficial tissue oxygen saturation (StO2) ranging between 26% and 30% (depending on the POI considered) was observed (Table 2). With regard to the oxygenation of the deeper tissue layers (NIR), an immediate increase between 9 and 12 index points in the plasma-treated area could be determined (Table 2). In contrast to oxygen saturation, the HSI measurements prior to plasma application showed a localized increased hemoglobin distribution in the wound area (POI 01-03) compared to the adjacent tissue (POI 04) (Table 2). However, even for THI an immediately increase after CAP application could be shown. Measurements ten minutes after completion of the plasma treatment showed a further increase in the microcirculation parameters examined (Table 2 and Figs. 3C, 4B). However, there were no relevant changes in the control area (POI 04) that was not treated with CAP. Corresponding HSI measurement data before, immediately after and ten minutes after plasma therapy are shown in Table 2.

4. Discussion

In this study, we demonstrated a three-dimensional CAP-mediated effect on microcirculation in different indications of two patients with head and neck cancer for the first time. Using the TIVITA™ Tissue System (Diasp ective Vision, Pepelow, Germany), it was possible to identify a less perfused tissue in the early stage of wound healing after neck dissection compared to surrounding skin area. In detail, a discrepancy between the distribution of hemoglobin in tissue and the actual oxygen saturation could be analyzed. The completely non-invasive HSI measurements could also be performed on tumor tissue in situ and the measured values could be evaluated in relation to those of a control region. Furthermore, it was shown that cold atmospheric plasma (CAP) has a distinct influence on microcirculation both in early postoperative wound healing and in the area of a locally advanced tumor. Based on the three-dimensional analysis, it can be stated that the microcirculatory effect (increase of superficial and deeper lying oxygen saturation as well as increase of local distribution of hemoglobin) clearly exceeds over the area of the actual plasma application. In addition, our study results show a plasma-mediated influence of the local microcirculation beyond the actual duration of application. The microcirculatory effects of plasma treatment seem to differ from one another depending on the treated tissue (e. g. tumor tissue, tumor surrounding tissue and tissue in different stages of wound healing). Dysfunction of microperfusion is present in both wound healing disorders and tumor disease. However, transport of oxygen, cells and nutrients into/from tumor and wound area is an indispensable prerequisite for complex healing and regeneration processes.

Plasma medicine represents a new highly interdisciplinary research area at the interface between physics, life sciences and medicine. Numerous in vitro and in vivo studies have demonstrated a comprehensive antibacterial and healing-promoting potential [24–27]. In addition to this extensively investigated treatment indication, recent studies indicate several impressive molecular and cellular effects on tumor cells of different entities [28–30]. From a clinical point of view, however, there is no practicable monitoring technique for CAP effects in treated tissue. Furthermore, there is little knowledge of the detailed effects of CAP on perfusion and oxygenation. Metelmann et al. [19] showed a partial size reduction of tumor volume, a significant reduction in microbial wound contamination as well as reduced infection induced pain and fetid wound odor in palliative concept of head and neck cancer. Several studies indicate that the oxygenation status of malignant tumors is an important factor in the response to radiation therapy, thermoradiotherapy and a number of chemotherapeutic agents [31,32]. Other research projects also dealt intensively with oxygen saturation as a supposed regulatory key factor for tumor growth and metastasis [33]. Martinez et al. [34] described a rapid tumor progression of a head and neck squamous carcinoma under hyperbaric oxygen therapy. Based
on our research, we conclude that HSI is a helpful technique to monitor the oxidation status of tumor tissue. In addition to the cellular and subcellular mechanisms against cancer cells that are currently the subject of intensive scientific research, based on our research it seems possible to modify the oxidation status in tumor tissue by means of CAP and thus possibly generate a higher sensitivity to other therapies. These results represent an innovation approach in clinical plasma research and could form the starting point of an alternative understanding tumor therapy.

The decisive role of oxygen supply in physiological and disturbed wound healing as well as in wound treatment is well studied [35,36]. Chang et al. [37] showed hypoxia in wound and surrounding tissue after mastectomy using an invasive technique. They also demonstrated that hypoxia was most pronounced immediately after surgery. This illustrates an important starting point for plasma therapy already in the early wound healing stage, not only for chronic, poorly healing wounds. Pellini et al. [38] classified a deficient tissue oxygen saturation as an essential factor for the development of wound complications after neck dissection. Coskun et al. [39] found that the wound infection rate was higher after neck dissection than other surgical procedures. This is consistent with our experience. Kisch et al. [40] showed that CAP (applied by DBD plasma device) increases cutaneous tissue oxygen saturation and capillary blood flow at the radial forearm of healthy volunteers. In addition, same research group was able to show that the repetitive use of cold atmospheric plasma boosts and prolongs cutaneous microcirculation [41]. To our knowledge, there are no comparable studies in the area of acute postoperative wound healing or in vital tumor tissue.

While for macrocirculation beside clinical tests different procedures such as angiography and duplex sonography are routinely used, comparable procedures for spatial visualization of microcirculation are missing. With HSI an easy-to-use and non-invasive tool for visualization of complex blood perfusion parameters of larger tissue zones in real-time is available. In general, only a limited number of suitable devices are available for investigation of tissue perfusion including Laser Doppler Flowmetry (LDF), Laser Doppler Imaging (LDI) and transcutaneous oxygen-tension probing (tcpO2) [42–44]. LDF and tcpO2 are disadvantageous since significant changes may appear from point to point, which cannot be overcome by simply adding more measuring.

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before CAP</th>
<th>Immediately after CAP</th>
<th>10 min after CAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>POI01 (tumor area)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>StO2 (%)</td>
<td>75</td>
<td>82</td>
<td>87</td>
</tr>
<tr>
<td>NIR (Index)</td>
<td>70</td>
<td>75</td>
<td>77</td>
</tr>
<tr>
<td>THI (Index)</td>
<td>36</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>POI02 (internal control, non-plasma treated tissue)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>StO2 (%)</td>
<td>67</td>
<td>66</td>
<td>67</td>
</tr>
<tr>
<td>NIR (Index)</td>
<td>63</td>
<td>64</td>
<td>63</td>
</tr>
<tr>
<td>THI (Index)</td>
<td>39</td>
<td>39</td>
<td>40</td>
</tr>
</tbody>
</table>

Chang et al. [37] showed hypoxia in wound and surrounding tissue after mastectomy using an invasive technique. They also demonstrated that hypoxia was most pronounced immediately after surgery. This illustrates an important starting point for plasma therapy already in the early wound healing stage, not only for chronic, poorly healing wounds. Pellini et al. [38] classified a deficient tissue oxygen saturation as an essential factor for the development of wound complications after neck dissection. Coskun et al. [39] found that the wound infection rate was higher after neck dissection than other surgical procedures. This is consistent with our experience. Kisch et al. [40] showed that CAP (applied by DBD plasma device) increases cutaneous tissue oxygen saturation and capillary blood flow at the radial forearm of healthy volunteers. In addition, same research group was able to show that the repetitive use of cold atmospheric plasma boosts and prolongs cutaneous microcirculation [41]. To our knowledge, there are no comparable studies in the area of acute postoperative wound healing or in vital tumor tissue.

While for macrocirculation beside clinical tests different procedures such as angiography and duplex sonography are routinely used, comparable procedures for spatial visualization of microcirculation are missing. With HSI an easy-to-use and non-invasive tool for visualization of complex blood perfusion parameters of larger tissue zones in real-time is available. In general, only a limited number of suitable devices are available for investigation of tissue perfusion including Laser Doppler Flowmetry (LDF), Laser Doppler Imaging (LDI) and transcutaneous oxygen-tension probing (tcpO2) [42–44]. LDF and tcpO2 are disadvantageous since significant changes may appear from point to point, which cannot be overcome by simply adding more measuring.

### Fig. 2. A, B: Tissue Hemoglobin Index (THI) before (A) and ten minutes after (B) CAP treatment. THI shows distribution of hemoglobin in image area. White circle marks tumor tissue. Pink circle points on healthy and non-plasma treated tissue. Color ranges from blue (= low THI) to red (= high THI). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

### Fig. 3. A, B, C: A shows a partial screenshot of analysis tool for better orientation in HSI images. Deficient oxygen saturation (StO2%) in superficial tissue before (B) and highly increased ten minutes after CAP application (C) is shown. Three measuring points (POI) are located in the area of neck dissection wound (white, pink, purple). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).
points. Although LSI allows spatial investigations, it does not enable a clear evaluation of surface and deep oxygen saturation, since it measures the kinetics of particles that are not necessarily erythrocytes. In addition, LSI takes considerably more time what excludes quick serial measurements.

Regarding to the limited set of data our findings need to be proved in a larger study population. Another limit is the currently not available optical reference measurements.

HSI data of the second patient who received CAP therapy during early wound healing after neck dissection. Superficial oxygenation saturation (StO2 in %), Near-infrared perfusion Index (NIR) and Tissue Hemoglobin Index (THI) were measured before, immediately after and ten minutes after CAP treatment. POIs 01–03 were located in the area of neck dissection wound while POI 04 was set in cheek region as internal control.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before CAP</th>
<th>Immediately after CAP</th>
<th>10 min after CAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>POI_01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>StO2 (%)</td>
<td>40</td>
<td>70</td>
<td>74</td>
</tr>
<tr>
<td>NIR (Index)</td>
<td>52</td>
<td>64</td>
<td>69</td>
</tr>
<tr>
<td>THI (Index)</td>
<td>43</td>
<td>49</td>
<td>51</td>
</tr>
<tr>
<td>POI_02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>StO2 (%)</td>
<td>42</td>
<td>69</td>
<td>72</td>
</tr>
<tr>
<td>NIR (Index)</td>
<td>55</td>
<td>64</td>
<td>69</td>
</tr>
<tr>
<td>THI (Index)</td>
<td>40</td>
<td>49</td>
<td>52</td>
</tr>
<tr>
<td>POI_03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>StO2 (%)</td>
<td>42</td>
<td>68</td>
<td>73</td>
</tr>
<tr>
<td>NIR (Index)</td>
<td>50</td>
<td>60</td>
<td>68</td>
</tr>
<tr>
<td>THI (Index)</td>
<td>41</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>POI_04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>StO2 (%)</td>
<td>56</td>
<td>57</td>
<td>56</td>
</tr>
<tr>
<td>NIR (Index)</td>
<td>61</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>THI (Index)</td>
<td>22</td>
<td>22</td>
<td>23</td>
</tr>
</tbody>
</table>

5. Conclusions

HSI is the first technique that enables detection of ongoing changes in oxygen saturation and hemoglobin distribution caused by CAP treatment in this entirety. TIVITA® Tissue System was shown as a useful clinical diagnostic tool for the analysis of oxygen supply and hemoglobin distribution in CAP therapy. In this way, HSI provides further information for a better understanding of the microcirculatory CAP effects in cancer therapy and wound healing processes. First results are promising with regard to the implementation of hyperspectral imaging as useful tool in clinical practice for monitoring and further optimization of plasma therapy. However, a causative biophysical correlation between our findings and improved wound healing on the one hand and effective tumor therapy on the other hand has to be evaluated in clinical trials. Considering the limitations of this project, there is still need for further evidence-based, prospective and comparative studies to enhance understanding the role of microcirculation in CAP treatment.

Conflict of interest

None.

References


[17] P. Riley, Free radicals in biology: oxidative stress and the effects of ionizing radia-


D. Weltmann, S. Kindler, P.H. Metelmann, S.E. Finkelstein, D.D. Von Hoff, Head and

Oxygenation and perfusion monitoring with a hyperspectral camera system for
2064.

(2014) 010901-010903.

Randeberg, Hyper spectral imaging as a diagnostic tool for chronic skin ulcers,

imaging for wound healing evaluation in the context of a comparative preclinical

K.D. Weltmann, Physical and microbiological characterisation of Staphylococcus
epidermidis inactivation by dielectric barrier discharge plasma, Plasma Process.

Woedtke, K.D. Weltmann, M. Jünger, In vitro susceptibility of important skin and
wound pathogens against low temperature atmospheric pressure plasma jet (APPJ)
and dielectric barrier discharge plasma (DBD), Plasma Process. Polym. 9 (4) (2012)
380–389.

[26] G. Daeschlein, T. von Woedtke, E. Kindel, R. Brandenburg, K.D. Weltmann,
M. Jünger, Antibacterial activity of an atmospheric pressure plasma jet against
relevant wound pathogens in vitro on a simulated wound environment, Plasma

[27] G. Isbary, G. Mortill, H. Schmidt, M. Georgi, K. Ramrath, J. Heinlin, S. Karrer,
M. Landthaler, T. Shimizu, B. Steiner, A first prospective randomized controlled trial
to decrease bacterial load using cold atmospheric argon plasma on chronic wounds

[28] N. Barczi, M. Laroussi, Dose-dependent killing of leukemia cells by low-tempera-

D. Gaset, C. Kieda, B. Legrain, ROS implication in a new antitumor strategy based

Plasma induced-death of HepG2 cancer cells: intracellular effects of reactive spe-

[31] M. Höckel, C. Koop, K. Schleger, B. Vorndran, E. Baßmann, M. Mitze,
P.G. Knapstein, P. Vaspel, Intratumoral pO2 predicts survival in advanced cancer of

G.J. Broder, Oxygen distribution in squamous cell carcinoma metastases and its


[34] S.A. Martinez, J.J. Bradfield, J.B. Kanella, J.T. Mader, E.W. Bridges, K.H. Calhoun,
Rapid progression of head and neck squamous carcinoma after hyperbaric oxyge-


[36] A.A. Tandara, T.A. Mustoe, Oxygen in wound healing—more than a nutrient, World

[37] N. Chang, W. Goodson 3rd, F. Gottrop, T.K. Hunt, Direct measurement of wound

[38] R. Pellini, G. Mercante, C. Marchese, V. Tenerzi, I. Sperduti, V. Manciocco,
P. Ruscito, G. Cristalli, P. Marchesi, B. Pichi, Predictive factors for postoperative
wound complications after neck dissection, Acta Otorhinolaryngol. Ital. 33 (1)
(2013) 16.

[39] H. Coskun, L. Erissen, O. Basut, Factors affecting wound infection rates in head

Kraemer, Improvement of cutaneous microcirculation by cold atmospheric

Kraemer, The repetitive use of non-thermal dielectric barrier discharge plasma

[42] J.D. Briers, Laser Doppler, Speckle and related techniques for blood perfusion


laser-based methods for determination of burn scar perfusion: laser Doppler versus
Original research article

Side effects in cold plasma treatment of advanced oral cancer—Clinical data and biological interpretation

Christian Seebauer*

*Department of Oral and Maxillofacial Surgery/Plastic Surgery, University Medicine Greifswald, Ferdinand-Sauerbruch-Str., DZ 17475 Greifswald, Germany
**Dental Clinic, Tehran, Iran

ARTICLE INFO

Keywords:
Clinical plasma medicine
Physical cold atmospheric pressure plasma
Side effects
Radical oxygen and nitrogen species
Switch-on mechanism

ABSTRACT

Purpose: Treating oral cancer with cold atmospheric plasma (CAP) is an evidence-based-medicine level III concept in ongoing cancer research. There is a discussion concerning the potential risk in medical application of CAP due to the formation of free radicals, which may have an adverse impact. After in recent studies the risk of dramatic effects like genotoxic impact was basically excluded, the focus of this discussion is mainly on acute unwanted clinical effects.

Methods: A retrospective analysis is including 20 patients suffering from locally advanced head and neck cancer and contaminated ulcerations, who underwent palliative treatment with CAP for decontamination. The focus lies on documented side effects related to CAP.

Results: There are no, mild or moderate unwanted effects related to the application of CAP, especially never life threatening.

Conclusion: Understanding the lack of severe side effects in plasma medicine, the role of radical oxygen species (ROS) and radical nitrogen species (RNS) is discussed, proposing a model in which CAP is not a direct effector of antitumor action but rather triggers a singlet oxygen-mediated switch-on effect on the specific target, leading to reactivation of intercellular ROS/RNS-dependent apoptosis signaling in tumor cells. As these processes are strictly restricted to the specific targets and as normal tissue is devoid of the required target, it is neither harmed nor affected.

List of abbreviations

CAP cold atmospheric pressure plasma
ROS reactive oxygen species
RNS reactive nitrogen species
CE Conformité Européene (CE mark)
UV ultraviolet
DC direct current
RF radio frequency
ATP adenosine triphosphates
SOD superoxide dismutase
NADPH nicotinamide adenine dinucleotide phosphate
NOX nitrogen oxide
HOCl hypochlorous acid
NO nitrogen oxide

1. Introduction

Patients with infected ulcerations of locally advanced oral cavity carcinoma have poor prognosis and few treatment options. The standard one is cytostatic drug therapy including cisplatin, methotrexate, bleomycin, fluorouracil, cetuximab or docetaxel. This kind of
Clinical Plasma Medicine 10 (2018) 9–15

M. Schuster et al.

Literature reporting side effects.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Indication for treatment</th>
<th>Region of treatment</th>
<th>Plasma source</th>
<th>Treatment time</th>
<th>Number of cases</th>
<th>Number of side effects</th>
<th>Kind of side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulrich et al. 2015 [4]</td>
<td>Support of wound healing</td>
<td>Chronic leg ulcers</td>
<td>Plasma jet</td>
<td>3 times/week for 2 weeks</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Safe, no side effects</td>
</tr>
<tr>
<td>Emmert et al. 2013 [5]</td>
<td>Not reported</td>
<td>Safe, no side effects</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
<tr>
<td>Isbary et al. 2014 [8]</td>
<td>Support of wound healing</td>
<td>Chronic wounds</td>
<td>Indirect plasma</td>
<td>5 min/5 days</td>
<td>37</td>
<td>12</td>
<td>Bad taste, pain, collateral edema, bleeding, skin blisters brain</td>
</tr>
<tr>
<td>Metelmann et al. 2013 [10]</td>
<td>Infection eczema, Skin</td>
<td>Dielectric barrier discharge</td>
<td>Plasma jet</td>
<td>3 times-week between 1 and 9 weeks</td>
<td>2 patients, single occasion: pain (before and after treatment)</td>
<td>6</td>
<td>Red burns, pain, collateral ulcers, bleeding, skin blisters</td>
</tr>
<tr>
<td>Metelmann et al. 2015 [2]</td>
<td>2 patients, well tolerated in all cases</td>
<td>Plasma jet</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Not reported</td>
<td>No reported</td>
<td>Safe, painless</td>
</tr>
<tr>
<td>Metelmann et al. (ViperGroup) 2013 [11]</td>
<td>Palliative decontamination</td>
<td>Head and neck</td>
<td>Plasma jet</td>
<td>3 times-week</td>
<td>5 patients with 3 laser sessions each</td>
<td>0.15</td>
<td>No precancerous skin features after 12 months</td>
</tr>
<tr>
<td>Metelmann et al. 2014 [7]</td>
<td>Skin lesions following laser ablation</td>
<td>Lower arm</td>
<td>Plasma jet</td>
<td>10 s to 3 times 10 s</td>
<td>5 patients</td>
<td>Not reported</td>
<td>No side effects</td>
</tr>
</tbody>
</table>

2. Material and methods

2.1. Patients

10 female and 10 male Caucasian patients (aged between 49 and 84 years) suffering from locally advanced squamous cell carcinoma of the head and neck area and presenting intraoral or extraoral ulcerations, had been treated with CAP for palliative decontamination [2,3]. The clinical data were including especially side effects or discomfort under or after CAP treatment. All the patients selected were in Karnofsky performance status of 60–80 and beyond reach of standard cancer therapies, however, all were able to undergo clinical CAP treatment and all have signed a consent form.

2.2. Treatment indication

The aim of CAP treatment was to decontaminate infected cancer ulcerations as part of a palliative treatment program. None of the ulcerations had received special treatment before due to individual medical reasons.

2.3. Treatment site

Appropriate areas for CAP treatment were infected cancer ulcerations, in general easily and dependably accessible for a CAP jet. Infected lesions with clear borders were selected so the surface area of CAP application could be accurately observed.

2.4. CAP medical device

CAP had been made applicable for medical use by a plasma source, kINPen® MED, (neoplasm tools GmbH, Greifswald, Germany) a medical device previously described in basic technical details [5], licensed for...
treatment of contaminated wounds and infective skin diseases since 2013. The device consists of a hand-held unit that discharges plasma under atmospheric conditions, requiring a DC power unit and an Argon gas reservoir. In the center of a ceramic capillary (inner diameter 1.6 mm) a pin-type electrode (1 mm diameter) is mounted. The needle is powered by a miniaturized RF generator producing a sinusoidal voltage waveform from 2 kV to 3 kV amplitude peak at a frequency of 1 MHz and modulated with 2.5 kHz and a plasma duty cycle of 1:1.

2.5. Treatment technique

Prior to the CAP intervention, the biofilm covering the ulcerations was gently removed with gauze. The CAP treatment proceeded by repeatedly scanning the hand-held visible plasma jet over the area of the ulceration.

2.6. Application of CAP

CAP was delivered in cycles of 3 single treatments within 1 week, followed by an intermittence of 1 week without CAP exposure, exceptionally due to the patient’s individual circumstances of 2–3 weeks. Each single treatment occurred in such manner: exposure of every accessible square centimeter of ulceration to CAP for 1 min from a distance of 8 mm vertically from naturally moist tissue. Clinical compliance with the study rules was difficult in rugged and fissured ulcerations. The total treatment time went up to more than 30 min, for patients with large ulcerations exceeding 30 cm².

2.7. Outcome evaluation

The reaction to CAP was analyzed for any clinically obvious side effects, and related to general condition, history of cancer treatment, state of disease at present, palliation procedure and outcome at all of the patients.

3. Results

What do we find when looking for risks of CAP-treatment in cancer patients?

3.1. Kind and frequency of side effects

Fig. 1 presents unwanted observations and summarizes their clinical frequency in terms of how many times they happened among 20 patients.

Most of the patients met discomfort, uneasiness or several side effects while under CAP-treatment, and just 6 of 20 did not report anything like this. However, all of the unwanted effects were mild to moderate and never life threatening.

3.2. Bad taste

Table 2 presents clinical details and the outcome of patients experiencing bad taste under treatment, in comparison with patients not suffering from bad taste.

Bad taste is more a discomfort than a side effect and has been usually described as ozone odor or bitter taste. Bad taste is only happening when CAP-treatment takes place intraorally. Whilst comparing the 2 groups of patients with or without this discomfort, the complaint about bad taste had nothing to do with the outcome of plasma palliation, which was usually better. Males and patients in rather good general condition seem to suffer more from bad taste.

3.3. Exhaustion

Table 3 presents the clinical aspects of patients reacting to plasma medicine with exhaustion, in comparison to the one without this uneasiness.

Patients suffering from exhaustion happen to meet frequently no palliation effect in general, having no tumor pain relief or a noticeable decontamination of ulcerations. Remarkably, the survival time was not too bad in comparison with patients without exhaustion.

3.4. Bleeding

Table 4 is concerned with the most relevant side effect in this study, bleeding.

Bleeding is mainly occurring in patients with no palliation effect under treatment, however the bleeding itself might have caused the feeling of there is no palliation. Nevertheless, these patients enjoyed some relief from the contamination, including the odor, too. Bleeding was more frequent in male than in female patients and in patients with better general condition in comparison with the patients not complaining about bleeding. It took mainly only 1 to 2 cycles to meet bleeding, and in contrast to patients in an overall better general condition, the patients suffering from this side effect mostly did not enjoy the survival time of those patients without bleeding.

3.5. Lack of side effects

Table 5 compares patients reacting to plasma medicine without any side effect or discomfort, with all the other patients.

Patients not suffering from any side effect or even discomfort were female, in the age of 50–60 years and in relatively good general
condition. They all had a history of full standard cancer treatment, smaller ulcerations in comparison with the other patients and they re-
ceived plasma treatment for a long time. Even having no remarkable
relief of tumor growth or bacterial contamination by CAP and without a
remarkable di-

diff

erence in the survival time in comparison with the other
patients, they always enjoyed the feeling of palliation provided by CAP-
treatments.

4. Discussion

What might be the biochemical basis for the lack of severe side
effects in clinical plasma medicine?
Successful decontamination of infected cancer ulcerations and
wounds by cold atmospheric plasma, without harming nonmalignant
tissue indicates that the effect of plasma on bacteria cannot be due to a
reaction that is analogous to the direct antimicrobial effect of chemical
disinfectants or radiation, otherwise a certain degree of damage of the
tissue would be unavoidable. Rather, plasma is suspected to exert a
specific triggering function that is low in its own chemical reactivity
and thus free of side effects. This triggering signal seems to turn on a
subsequent, target-defined reaction that is powerful, but restricted to
the target. Thus it does not harm healthy surrounding tissue.

Wu et al. [19] have reported that singlet oxygen is responsible for
the antibacterial effect of cold atmospheric plasma. This finding is in
perfect agreement with established data on the very efficient anti-
bacterial potential of singlet oxygen. Singlet oxygen-dependent bac-
terial killing was about 3–4 orders of magnitude more effective as
bacterial killing by \( \text{H}_2\text{O}_2 \). Even extremely radiation-resistant bacteria
like Deinococcus radiodurans were highly sensitive to singlet oxygen-
dependent inactivation. An important clue for the understanding of the
remarkable inactivation of bacteria by singlet oxygen is derived from
the report on the inactivation of bacterial respiratory chain enzymes by
singlet oxygen. It seems likely that the damaging effect of singlet
oxygen on bacteria is not based on the general destruction of bacterial
components by singlet oxygen (which would require a large number of
hits), but rather, that the site-directed inactivation of central elements
of the ATP generating system by a relatively low dose of singlet oxygen
causes a strong subsequent effect on bacterial survival through the

Table 2
Clinical aspects of patients’ reaction to plasma medicine with bad taste versus no bad taste.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cancer Treatment</th>
<th>State of Disease</th>
<th>Plasma Palliation</th>
<th>Outcome</th>
</tr>
</thead>
</table>
| Bad taste          | more male than female, age between 50 and 77, Karnofsky index between 60 and 90 | combined therapy of surgery, chemotherapy and radiotherapy | intracranial ulcerations, 10 to 15 cm


t, G2 and G3 | CAP plasma-jet, 1 min/cm


t, 3 times/week, mainly 2 to 4 cycles | always feeling of palliation, good effect on pain, tumor growth and contamination, survival up from 12 month |
| No bad taste       | male and female equal, age between 50 and 80, Karnofsky index between 60 and 70 | mainly combined therapy, single-treatment too | all size of cancer between 5 and 25 cm


t, extracranal and intraoral, even distant metastasis, G2 and G3 | CAP plasma-jet, 1 min/cm


t, 3 times/week, between 1 and 16 cycles | some cases of no palliation, no effect on pain medication, mainly no response on tumor growth, between good and poor effect on contamination, survival between 3 weeks and 25 month |

4. Discussion

What might be the biochemical basis for the lack of severe side
effects in clinical plasma medicine?
Successful decontamination of infected cancer ulcerations and
wounds by cold atmospheric plasma, without harming nonmalignant
tissue indicates that the effect of plasma on bacteria cannot be due to a
reaction that is analogous to the direct antimicrobial effect of chemical
disinfectants or radiation, otherwise a certain degree of damage of the
tissue would be unavoidable. Rather, plasma is suspected to exert a
specific triggering function that is low in its own chemical reactivity
and thus free of side effects. This triggering signal seems to turn on a
subsequent, target-defined reaction that is powerful, but restricted to
the target. Thus it does not harm healthy surrounding tissue.

Wu et al. [19] have reported that singlet oxygen is responsible for
the antibacterial effect of cold atmospheric plasma. This finding is in
perfect agreement with established data on the very efficient anti-
bacterial potential of singlet oxygen. Singlet oxygen-dependent bac-
terial killing was about 3–4 orders of magnitude more effective as
bacterial killing by \( \text{H}_2\text{O}_2 \). Even extremely radiation-resistant bacteria
like Deinococcus radiodurans were highly sensitive to singlet oxygen-
dependent inactivation. An important clue for the understanding of the
remarkable inactivation of bacteria by singlet oxygen is derived from
the report on the inactivation of bacterial respiratory chain enzymes by
singlet oxygen. It seems likely that the damaging effect of singlet
oxygen on bacteria is not based on the general destruction of bacterial
components by singlet oxygen (which would require a large number of
hits), but rather, that the site-directed inactivation of central elements
of the ATP generating system by a relatively low dose of singlet oxygen
causes a strong subsequent effect on bacterial survival through the

Table 3
Clinical aspects of patients’ reaction to plasma medicine with exhaustion versus no exhaustion.

<table>
<thead>
<tr>
<th>Condition</th>
<th>General Condition</th>
<th>Cancer Treatment</th>
<th>State of Disease</th>
<th>Plasma Palliation</th>
<th>Outcome</th>
</tr>
</thead>
</table>
| Exhausted | more female than male, age between 50 and 60, Karnofsky index mainly 70 | mainly single therapy like surgery or radiotherapy | mainly intracranial cancer ulceration between 5 and 25 cm


t, no distance metastasis, grading G2 | CAP plasma-jet, 1 min/cm


t, 3 times/week, mainly for cycles, | no feeling of palliation, effect of pain medication uncertain, mainly no effect on tumor growth, mainly poor effect of decontamination, survival time between 6 and 12 month |
| Exhausted | almost always combined therapy of surgery, chemotherapy and radiotherapy | all sizes of cancer ulceration, some cases G3 | CAP plasma-jet, 1 min/cm


t, 3 times/week, mainly 2 to 3 cycles | almost always feeling of palliation, positive effect on pain medication, some cases of partial response of tumor growth, mainly good effect on contamination, survival time mainly more than 15 month, but 3 weeks to. |
depletion of their ATP-generating system. Based upon direct measurements of singlet oxygen action directed towards mammalian cells [21], it may be concluded that the singlet oxygen doses required for the decontamination of bacteria are not sufficient to harm nonmalignant cells. This conclusion, that fits into the actual findings for CAP-based contamination, is also substantiated by the fact that singlet oxygen generated by photosensitizers is effective towards bacteria-induced dental caries without affecting the tissue in the mouth [22]. This is just another striking example of singlet oxygen-dependent decontamination without unwanted side effects at the location of treatment.

Certain pathogenic bacteria express SOD and catalase close to their surface and thus attenuate ROS/RNS-dependent attack by professional phagocytes. As both enzymes can be inactivated by singlet oxygen, CAP-derived singlet oxygen may also be effective in the sensitization of these bacteria for ROS/RNS-based attack by cells of the innate immune system.

The impressive effects of CAP on healing of chronic wounds seem to be based on the concerted action of antimicrobial action, stimulation of tissue proliferation and recruitment of immune cells [23]. Assuming that the antimicrobial effect related to induction of wound healing is explained by the action of a low dose of singlet oxygen on the bacterial respiratory chain (as outlined in the preceding paragraph), this beneficial effect would be combined with stimulation of tissue proliferation and recruitment and action of immune cells. Both of these signaling processes are unlikely to inherit the potential for any unwanted side effects due to their selectivity and biological specificity. Thus, in total, the absence of unwanted side effects during CAP-mediated wound healing can be predicted, provided the dose of CAP is well adjusted.

The selective effects of CAP directed towards tumor cells in vitro [24] and in vivo [6] are impressive and encouraging. The underlying mechanisms are, however, just beginning to be understood. There seems to be consensus in the field that reactive oxygen species and reactive nitrogen species (ROS/RNS) are central elements that control CAP-dependent antitumor mechanisms [25]. Initial attempts to explain selective CAP action through direct apoptosis-inducing effects of CAP-derived ROS/RNS were not satisfactory, as the proposed models could not convincingly present a candidate of the ROS/RNS family that would induce apoptosis directly as well as selectively in tumor cells.

In a recent manuscript, Bauer and Graves [26], analyzed the potential interactions between CAP-derived ROS/RNS and cells from different stages of tumor development. This analysis not only evaluated potential interactions between ROS/RNS and tumor cells, based on

<table>
<thead>
<tr>
<th>Table 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical aspects of patients’ reaction to plasma medicine with bleeding versus no bleeding.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>general condition</th>
<th>cancer treatment</th>
<th>state of disease</th>
<th>plasma palliation</th>
<th>outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bleeding</strong></td>
<td>more male than female, age 50 to 75, Kamofsky index 70 to 80</td>
<td>more combined therapy than single surgery</td>
<td>more intra- than extracranial ulcerations, 10 to 25 cm², mainly grading G3</td>
<td>CAP plasma-jet, 1 min/cm², 3 times/week, mainly 1 and 2 cycles</td>
</tr>
<tr>
<td><strong>No bleeding</strong></td>
<td>more female than male, mainly about age 50 to 60, Kamofsky index mainly 60 to 70</td>
<td>some single therapy cases, mainly combined therapy</td>
<td>almost only intracranial ulcerations, 5 to 15 cm², some distant metastasis, mostly grading G2</td>
<td>CAP plasma-jet, 1 min/cm², 3 times/week, mainly 3 to 4 cycles</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical aspects of patients’ reaction to plasma medicine with side effects versus without side effects and discomfort.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>general condition</th>
<th>cancer treatment</th>
<th>state of disease</th>
<th>plasma palliation</th>
<th>clinical follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No side effects and discomfort</strong></td>
<td>female, age 50 to 60, mainly Kamofsky index 60</td>
<td>always surgery, radiotherapy and chemotherapy</td>
<td>mainly intracranial ulcerations, 5 to 15 cm², no distant metastasis, grading G2</td>
<td>CAP plasma-jet, 1 min/cm², 3 times/week, between 3 and 12 cycles</td>
</tr>
<tr>
<td><strong>Side effects and discomfort</strong></td>
<td>more male than female, age 52 to 80, Kamofsky index 60 to 80</td>
<td>some cases of single radiotherapy, mainly combined chemotherapy</td>
<td>some extracranial ulcerations, some distant metastasis, 5 to 25 cm², some grading G3</td>
<td>CAP plasma-jet, 1 min/cm², 3 times/week, mainly 3 cycles</td>
</tr>
</tbody>
</table>
established data, but also took into consideration that tumor cells might actively contribute to their own destruction after they had been triggered by CAP-derived ROS/RNS. The unique ROS/RNS-related phenotype of tumor cells with its high expression of NADPH oxidase-1 (NOX-1) and the expression of membrane-associated catalase and SOD seemed to be the key for the understanding of selective CAP action towards tumor cells [27]. The high expression of NOX-1 leads to abundant generation of extracellular superoxide anions, whereas membrane-associated catalase and SOD interfere with apoptosis-inducing signaling pathways that are driven by superoxide anions. These are the HOCl and the NO/peroxynitrite signaling pathway as reviewed. Based on model experiments performed with a pure source of singlet oxygen [28], it was concluded that CAP-derived singlet oxygen initially caused local inactivation of a few catalase molecules. As a consequence, the extracellular ROS/RNS chemical biology of tumor cells allowed for the generation of secondary singlet oxygen in an autoamplificationary loop. This then caused inactivation of further catalase molecules and the onset of intercellular ROS/RNS-dependent apoptosis induction selectively in the tumor cells, as it was controlled by active NOX1. Therefore, the CAP-related ROS/RNS effect is based on an initial triggering effect of a low dose of CAP-derived singlet oxygen. Triggering requires few hits on the specific target catalase of tumor cells. Nonmalignant cells, that lack membrane-associated catalase (and also sustained NOX1 expression) seem not to be damaged by the potential interaction of these few singlet oxygen molecules with undefined constituents of the membrane. In the case of tumor cells, however, the initial hit with its low own reaction potential causes a strong biological impact, as it switches on a cascade that leads to massive singlet oxygen generation, catalase inactivation and reactivation of intercellular ROS/ RNS-dependent apoptosis-inducing signaling. As the resultant damaging effect is restricted to the site of superoxide anion generation (due to limited free diffusion path lengths of the key players in this system), tumor cells are selectively killed, whereas nonmalignant tissue remains unaffected.

These ROS/RNS-related signaling effects that are under the control of an initial singlet oxygen-dependent “switch-on effect” might be instrumental for the induction of an additional cooperative immunological process. Tumor cells, damaged through specific ROS/RNS effects might attract and stimulate immune cells that enhance the antitumor effect of ROS/RNS signaling [29,30]. This conceivable immunological process may be crucial for the final therapeutic outcome, as it can be predicted to attack individual tumor cells. It thus can remove tumor cells that have survived ROS/RNS signaling. ROS/RNS signaling is a cell density process and therefore might lose its efficiency after destruction of the majority of tumor cells. This overall scenario is in good agreement with the finding of selective CAP-mediated destruction of tumor cells and tumors without severe side effects on normal tissue.

5. Conclusion

Clinical application of plasma in the treatment of contaminated cancer ulcerations is carrying the potential risks of formation of free radicals. However, there are no severe side effects to be observed, mainly mild reactions, uneasiness and discomfort in some of the cases. To understand the lack of side effects, this paper proposes a model in which CAP is not a direct effector of antimicrobial or antitumor action, but rather triggers a singlet oxygen-mediated switch-on effect on the specific target, leading to energy depletion in bacteria and to reactivation of intercellular ROS/RNS-dependent apoptosis signaling in tumor cells. As these final processes are strictly restricted to the specific targets (due to the biochemistry of the switch-on mechanism and its consequent reactions) and as normal tissue is devoid of the required target structure to allow switch on by singlet oxygen, it is neither harmed nor affected. This explains the lack of severe side effects at least in CAP palliative cancer treatment. Basic research is required to experimentally verify or falsify these conclusions and to work out more biochemical details that may be instrumental for further optimization of CAP-dependent medical applications. At present and from a clinical point of view, there is no risk of severe side effects obvious when applying CAP in cancer patients for palliation.

Acknowledgments

This study could not have been completed without the support of Wolfram Kaduk and Axel Schriewer as surgeons, Anke Friedrich, Martina Böttcher and Runa Tscherishes-Mondry as wound nurses and Kerstin Böttger as system consultant in the Department of Oral and Maxillofacial Surgery, Greifswald University.

Conflict of interest

The authors have no conflict of interest to declare.

Financial disclosure

On behalf of all authors I certify that we have no financial interest to declare.

Ethical tatement

All procedures including assessing data from the patients were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments. All patients gave written informed consent before inclusion in the study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cpm.2018.04.001.

References


**Eidesstattliche Erklärung**

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät, keiner anderen wissenschaftlichen Einrichtung vorgelegt worden.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

07. August 2018

Matthias Schuster