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Atmospheric Pressure Plasma Jet Application on Human Oral Mucosa Modulates Tissue Regeneration

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ABSTRACT: With the development of plasma sources generating nonthermal plasmas at atmospheric pressure the applications broaden enormously, especially in the medical field.

While earlier applications of physical plasma on biological matter focused on inactivation of microorganisms, the latest studies have revealed a modulation of eukaryotic cells by the same type of plasma. The aim of this study was to elucidate molecular mechanisms triggered by non-thermal plasma in human oral mucosa. *Ex vivo* biopsies were treated directly with the cold atmospheric pressure plasma jet kinpenMED and analyzed for distinctive markers of proliferation, apoptosis, DNA damage, and differentiation. Moreover, secreted cytokines and growth factors were measured in the supernatant. These results revealed stimulating effects indicated by proliferation consolidated by secretion of VEGF. We also proved that these effects were mediated into deeper layers of the mucosa, without harming the cells on top of the treated samples. DNA damage detected as H2A.X-immunoreactivity was nearly constant between all samples.

Our study revealed for the first time that human oral mucosa can be modulated by plasma treatment. Molecular markers for both cell damage and proliferation helped to identify the mechanisms triggered by plasma treatment. Moreover, our results strengthen a safe application of the kinpenMED on human oral tissue. Further studies on diseased skin are being performed in order to proceed towards a safe introduction of plasma medicine into the clinics.

KEY WORDS: plasma jet; proliferation; apoptosis; oral mucosa; cytokines

I. INTRODUCTION

The application of cold atmospheric plasma in the oral cavity is an interesting new field of plasma medicine. Cold atmospheric plasma is used for teeth bleaching,[1] for functionalization of surfaces of dental implants, for disinfection, and to improve dental implant incorporation.[2,3] The newly emerging field of plasma medicine raises hopes in new therapeutic options.[4] However, serious medical side effects occurring after using cold atmospheric plasma are discussed.

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Besides bactericidal effects plasma can also exert diverse effects on eukaryotic cells. More recently plasma has been discovered to have subtle modulatory effects on skin cells[5] as well as immune cells.[6] While short exposure times stimulated predominantly pro-proliferative signals, longer plasma treatment times resulted in cell death by apoptosis.

Based on *in vitro* experiments researchers together with clinicians conducted successful efforts to apply plasma in dermatology, namely for wound healing, atopic dermatitis, or skin cancer.[7–9]

The oral cavity is lined by a stratified epithelium similar to the skin. The majority of the cells are keratinocytes at different stages of differentiation.[10] Although similar to human skin, oral mucosa differs in dimension and differentiation. Human oral mucosa spans 100–500 µm and is divided into several layers as indicated in Fig. 1. Depending on localization within the mouth it either holds a cornified layer (stratum corneum) or is devoid of it. This also determines the absorption of chemical characteristics. Oral mucosa lines the entire oral cavity. On the cellular level it consists predominantly of keratinocytes and in total as much as 10% other types, including melanocytes, Langerhans cells, Merkel cells, and inflammatory cells (e.g., lymphocytes). The turnover is about 14 days as opposed to 28 days in skin.[11]

For introduction in the clinic, plasma devices should be easy to use, feasible, and working at low temperatures and at the same time well characterized regarding electrical

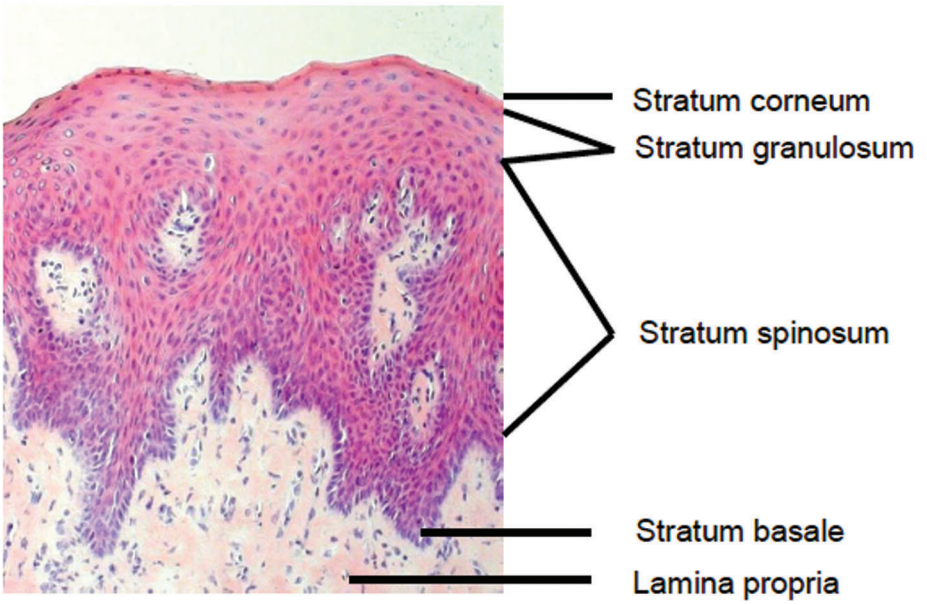

FIG. 1: Histological structure of oral epithelium. A parakeratinized section of gingiva indicates the different layers of the epithelium that were taken as a basis for further analysis. H&E staining shows nuclei in blue-purple while cytoplasm shows in red-pink

current, electric field, and UV emission. Considering these requirements the cold atmospheric pressure plasma jet kinpenMED was introduced as a medical device in 2013.[12] In contrast to dermal skin the surface of oral mucosa is covered with saliva. Furthermore the challenges are to understand the interactions of the highly reactive plasma with the cells within their physiological environment—humid or dry—that influences the plasma properties as well.

Oral mucosa is an appropriate model for analyzing possible side effects of cold atmospheric plasma in the oral cavity. However, literature on application of plasma on the oral mucosa is very limited. In 2013 Welz et al. presented data on a plasma-treated mucosa of nasal and pharyngeal origin.[13] In order to use plasma as a treatment option it needs to be safe for surrounding cells or even beneficial with respect to regeneration of retracted gingiva on the neck of the tooth.

Herein we present a suitable model for the testing of plasma application on oral mucosa. This compartment is easily reachable and feasibility is provided. To the best of our knowledge this is the first study that investigates epithelial cells of oral cavities after plasma treatment. In this investigation *ex vivo* explants of healthy oral mucosa were exposed to plasma and processed for histological investigations. Moreover, secreted cytokines and growth factors were quantified.

II. MATERIALS AND METHODS

A. Clinical Samples

Samples of oral mucosa (masticatory and lining mucosa) were collected from the clinic of maxillofacial surgery/plastic surgery at the University Medicine Greifswald, Ernst Moritz Arndt University Greifswald, after patients' consent during routine ambulant surgery. A total of 10 patients were included with a mean age of 53 years (ranging from 29 to 81 years). There were 5 males and 5 females. Sample size circumscribed about 2–3 mm.

Immediately after excision samples were placed in serum-free Williams' E medium (Lonza, Verviers, Belgium) containing 0.25 µg/ml amphotericin B (PromoCell GmbH, Heidelberg, Germany), 100 IU/ml penicillin/10 µg/ml streptomycin, 10 µg/ml insulin, 10 ng/ml hydrocortisone, and 2 mmol/l L-glutamine (all from Lonza, Verviers, Belgium) and transported to the laboratory. This medium functions also as a wash step.

B. Plasma Source

The medical device kinpenMED was employed as plasma source (neoplas tools GmbH, Greifswald, Germany).[12,14] It consists of a handheld part that contains the pin-type inner electrode. This is surrounded by a ceramic capillary (1.6 mm in diameter) and a grounded ring electrode, as well as a power supply station. It generates a RF signal with a frequency of about 1 MHz and a voltage amplitude of 2–3 kV. With a frequency of 2.5 kHz and a duty

cycle of 50% the discharge is switched on and off. Argon flow rate was set to 5 standard liters per minute. Plasma is generated at the tip of the pin-type electrode and expands to the surrounding air outside the capillary. Samples were directly treated on a spot at a distance of about 7 mm from the capillary outlet to the surface. In order to avoid drying the culture medium was added dropwise every 30 s during treatment. Plasma exposure took place for 1, 3, or 5 min. The gas temperature at the working distance was determined at 35–39°C. Samples of mucosa without any treatment served as a negative control.

C. Tissue Culture

After plasma treatment tissue samples were transferred to 500 µl fresh serum-free Williams' E culture medium supplemented with 100 IU/ml penicillin/10 µg/ml streptomycin, 10 µg/ml insulin, 10 ng/ml hydrocortisone, and 2 mmol/l L-glutamine (all from Lonza, Verviers, Belgium) and cultured in 24-well plates (Costar®, Corning, NY) for 24 h. Samples were maintained at 37°C and 5% CO_2. Subsequently tissue samples were embedded in optimal cutting temperature compound (OCT) (VWR International GmbH, Darmstadt, Germany) and snap frozen in liquid nitrogen. Prior to use 5-µm sections were cut using a cryotome (Leica CM 199, Leica Microsystems, Nussloch, Germany).

D. Histology and Immunofluorescence

Routine hematoxylin and eosin stain (H&E stain) was performed by incubating slides in hemalum for 5 min at room temperature followed by washing in tap water. Afterwards eosin was applied for 5 min and again washed off in tap water. Slides were dehydrated and covered in Eukitt (Sigma-Aldrich Co, St. Louis, MO).

In addition specific antibodies were employed for DNA damage (detected by γH2A.X, Cell signaling Technology, Frankfurt, Germany), apoptosis (detected by TUNEL; *in situ* cell death detection kit, Roche diagnostics GmbH, Basel, Switzerland), proliferation[15] (detected by Ki67; Dako, Deutschland GmbH, Hamburg, Germany), and differentiation (detected as keratin 14; Covance Hiss diagnostics GmbH, Freiburg, Germany). Tissue sections were first blocked using DAKO block (Dako), incubated with the primary antibody (as above), labeled with the secondary antibody Alexa Fluor 594, goat anti-mouse IgG and goat anti-rabbit IgG Alexa Fluor 546, respectively (Biolegend Inc., San Diego, CA).

E. Luminex and ELISA

Supernatant of tissue culture was subjected to an initial screening for released proteins. The first screening was performed by a multi-analyte bead based Luminex assay. Analytes were chosen based on *in vitro* data using HaCaT keratinocytes. Because IL-6 and

IL-8 concentrations were much higher than the other analytes an additional ELISA was employed (ELISA MAX™ Deluxe Set Human, BioLegend Inc., San Diego, CA).

III. RESULTS

This *ex vivo* tissue model was employed for analyzing the impact of atmospheric pressure plasma directly on the cellular compartment and on the modulation of secreted mediators, i.e., cytokines and growth factors.

Mucosal integrity. Hematoxylin and eosin stain was applied to assess the histological integrity of mucosa after plasma application. Nuclei stain blue-purple while the cytoplasm shows a red-pink color. In Fig. 2 one representative example of the microscopic pictures after each treatment time is shown. In all treated samples no thermal damage, formation of blisters, or other skin damage could be detected. These samples were directly plasma treated at one single stationary treatment area of 5 mm² for 1, 3, and 5 min, respectively. Even for the longest plasma exposure the integrity of the mucosa remains. No difference with regard to tissue impairment was detected when compared to the untreated skin (Fig. 2a). It has to be mentioned that though all four samples originated from the same patient, the control, 1 min, and 5 min samples were derived of multilayered epithelium with parakeratinization while the 3 min sample displays a multilayered squamous epithelium without keratinization. Hence the histology appears different (Fig. 2c).

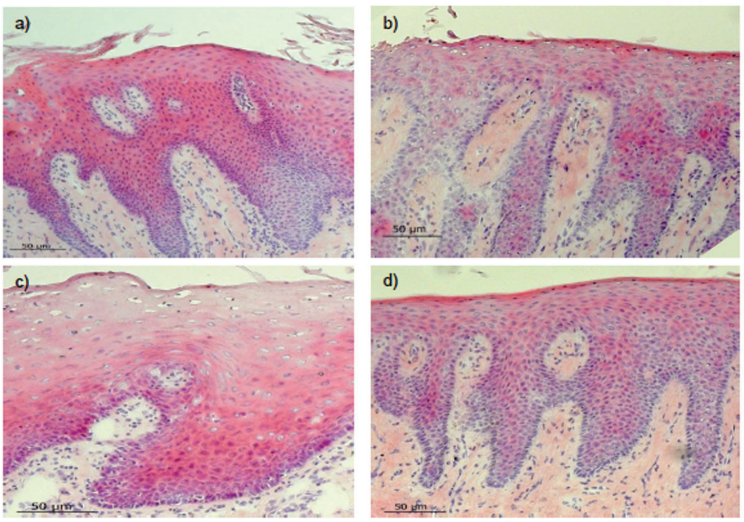

FIG. 2: Representative histological photographs of human oral mucosa without plasma treatment (a), after 1 min (b), 3 min (c), and 5 min (d). No plasma induced damage of epithelial layers and underlying lamina propria was detected. Magnification 100×. Scale bar represents 50 µm.

DNA damage (γH2A.X) and apoptosis (TUNEL). Immunofluorescence staining for γH2A.X was employed to detect possible DNA damage after plasma treatment. Positive cells occurred predominantly in the basal layer and fewer cells were detected in the stratum spinosum. This was consistent between all tested specimens as presented in Fig. 3. Compared to the total number of cells as visualized by DAPI their number increased only slightly with prolonged plasma treatment time. Remarkably, in untreated mucosa about 44% of keratinocytes stained positive for γH2A.X increasing up to 58% after 5 min plasma exposure (Fig. 3). This result suggests that keratinocytes of the oral mucosa are relatively robust towards the components of plasma for up to 5 min. γH2A.X, however, indicates rather a phosphorylation at a checkpoint in the cell cycle. Skin cells hold a repair mechanism and therefore are able to repair themselves.

In order to check whether the increasing number of γH2A.X-positive cells results in more apoptotic cells we applied TUNEL staining. The number of positive cells was normalized to the individual control. Due to the method and based on the differentiation process also cells stain positive during the transition from the stratum granulosum to the stratum superficiale/corneum. Between the specimens great variations were observed (Fig. 4a). For all three treatment time points an elevated mean value was detected, however, with a reverse tendency to the plasma treatment duration. Looking at the individual data revealed that this was not the case for each sample indicating a variation of susceptibility.

In this context it is noteworthy that the absolute numbers are very small being 0-6 TUNEL positive cells per analyzed area (50 cm²).

Proliferation (Ki67). Earlier results of *in vitro* experiments revealed a plasma treatment time-dependent modulation of pro-proliferative signaling molecules, i.e., ERK1/2.[6,16] Therefore it was of interest whether plasma application stimulates proliferation of basal keratinocytes within the oral mucosa. Ki67 immunofluorescence revealed a high number of proliferating cells in the basal layer (Fig. 4b) as expected. After plasma exposure the number of proliferating cells could be further increased on average by 61% after 1 min and 64% after 3 min as is indicated by the line within the bars (Fig. 4b). The heights of the bars highlight large variation between probands (Fig. 4b). Only 4 out of 6 specimens showed this increase after 1 min and 4 out of 5 after 3 min. After 5 min treatment only 3 out of 5 probands showed an increased number of Ki67-positive cells. These results underline once more the individual susceptibility of oral mucosa epithelial cells towards plasma stimulation. In this respect it is noteworthy that the human oral mucosa represents a tissue with a relatively high turnover of 14 days which is reflected in the absolute number of Ki67-positive cells in this compartment. With our method we counted between 25 and 188 Ki67-positive cells per 50 cm²—outnumbering the number of TUNEL-positive cells detected for the same treatment time.

Differentiation (keratin 14). All basal cells of oral stratified epithelia contain keratin 14 and keratin 5 while differences in keratin expression patterns emerge in suprabasal layers with differentiated cells.[10] In this study the focus laid on keratin 14

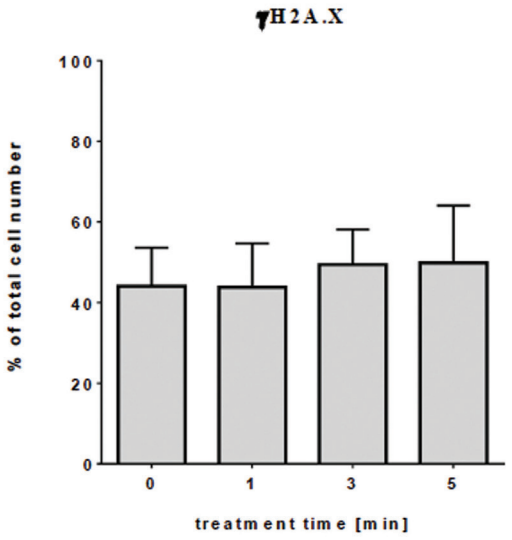

FIG. 3: Results of the quantitative analyses of the γH2A.X-staining in human oral mucosa. All γH2A.X-positive cells were counted and correlated to the total cell number as detected by DAPI-staining (equals 100%). The bars represent mean and standard deviation of 10 specimens

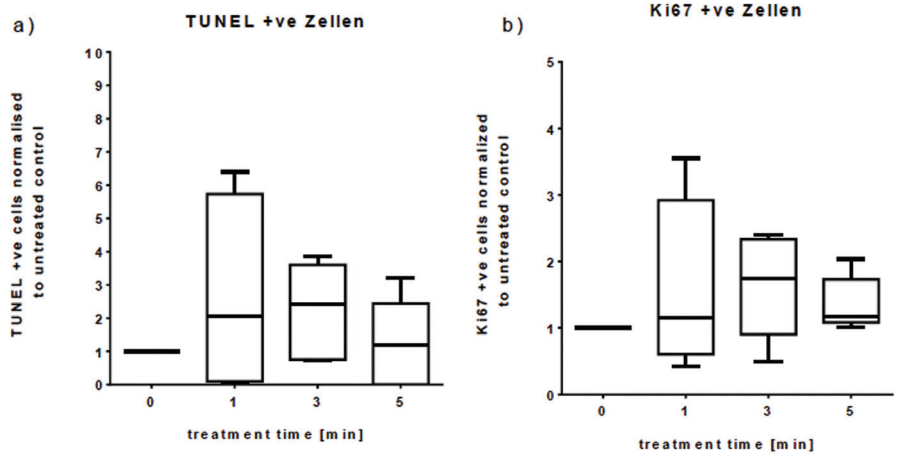

FIG. 4: Results of the quantitative analyses of the TUNEL-staining (a) and Ki67-staining (b). Apoptotic and proliferating cells were counted and normalized to their individual respective untreated control. Therefore the relative percentage of positive cells is represented as a floating bar with a mean for all tested treatment times (control: *n*=10; 1 min: *n*=6; 3 min: *n*=5; 5 min: *n*=5). The bar height highlights the large variation between probands.

expression. The left-hand panel of Fig. 5 displays representative microscopic pictures of the localization within the oral epithelium after 1, 3, and 5 min (b,c,d) and the untreated control (a). Keratin 14 expression is strongest in the basal layer and the stratum spinosum as expected. Green fluorescence occurred also in suprabasal layers although to a lesser extent. In the right-hand panel of Fig. 5 semiquantitative, relative analyses are presented where fluorescence intensities per mucosal layer were

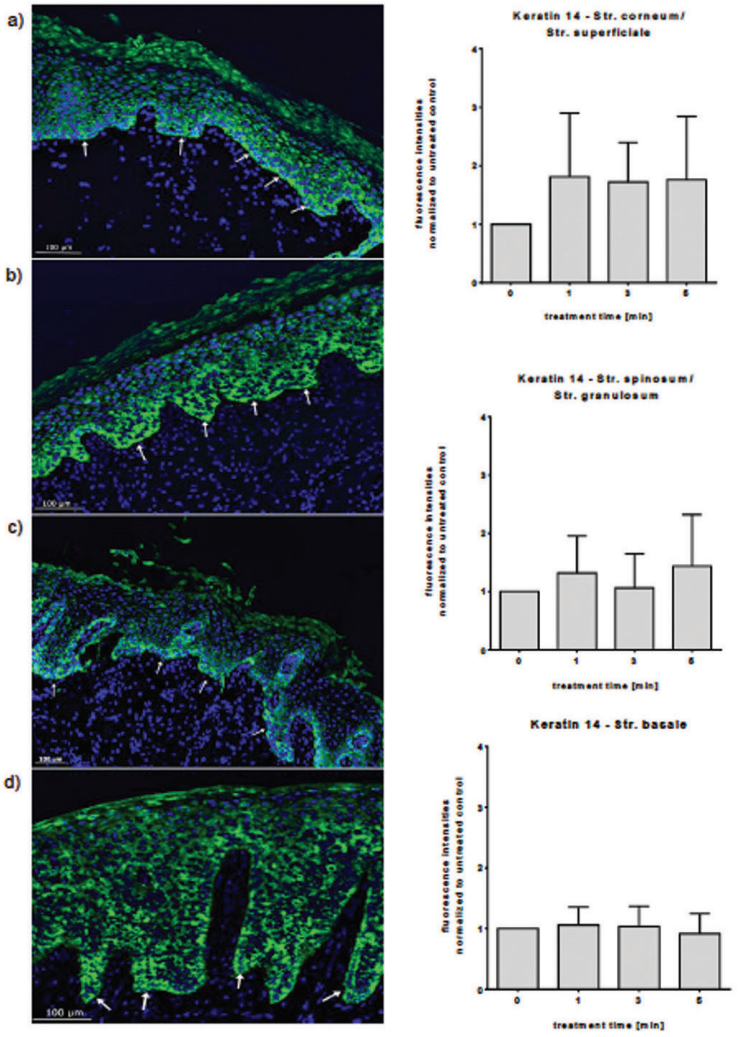

FIG. 5: Keratin 14 expression in human oral mucosa. Representative microscopic photographs of keratin 14 (green fluorescence) detected in oral mucosa after plasma treatment for 1 min (b), 3 min (c), and 5 min (d) as well as in untreated control (a). Nuclei are stained by DAPI (blue fluorescence). Arrows indicate the strong expression in the basal layer. Scale bar: 100 µm. The right-hand panel displays the quantitative analyses of the fluorescence intensities in different layers of the oral epithelium (control: n=10; 1 min: n=6; 3 min: n=5; 5 min: n=5)

analyzed (stratum basale, stratum spinosum/granulosum, stratum corneum). Each specimen is normalized to its untreated control. This expression pattern and the fluorescence intensity were not grossly impaired by the plasma application for all tested time points. Even after long plasma exposure of 5 min the keratin 14 intensity was at the same level compared to the control (Fig. 5). However, this was true only for 7 out of 10 probands.

Mediators. A large number of biologically active substances such as cytokines and growth factors play a major role in the control of proliferation processes. Using a multiplex assay we screened the plasma-treated biopsies for secreted cytokines and growth factors. Tissue samples were incubated and hence are given time to produce proteins that function as communicators between the cells. In order to test for the stimulation of secretory proteins after plasma treatment several cytokines (IL-1β, IL-2, IL-6, IL-8, IL-10, TNFα, IFNγ) and growth factors (basicFGF, GM-CSF, VEGF) were screened for, within which the pro-inflammatory cytokine IL-6, the anti-inflammatory cytokines IL-8 and IL-10, and the growth factors VEGF showed evaluable results. The highest concentrations occurred for IL-6 and IL-8 which may be attributed to the stress situation during surgery. Therefore an ELISA was performed. The results are presented in Fig. 6. After plasma treatment the concentrations of both cytokines were reduced. However, IL-6 concentration rose to the level of control after 5 min plasma treatment. IL-10 secretion was slightly reduced after 1 and 3 min plasma treatment but reached above the control level after 5 min. Interestingly for VEGF a slightly different pattern emerged. After 1 min and 3 min plasma treatment the concentrations were as high as the control. Similar to the other growth factor 5 min treatment resulted in an increased secretion. However, the changes were not significant as tested by one-way ANOVA with a 95% confidence interval. Therefore, and due to the small sample size, they can only be considered as hints for plasma mediators in the tissue.

IV. DISCUSSION

In this approach we treated biopsies from human oral mucosa of healthy volunteers with a cold atmospheric pressure plasma jet for different lengths of time and analyzed afterwards for molecular modulations in the tissue. To our knowledge this is the first time that epithelial cells of the oral cavity were investigated after plasma treatment. Besides its antimicrobial properties plasma was also able to stimulate eukaryotic cells *in vitro*, which opens a wider field of utilizations.[17,18] This could be proven by other researchers using a treatment time-dependent increase of IL-6 as well as VEGF secretion *in vitro*.[19] Both factors are known to be stimuli of cell proliferation which could be exploited in dental care and after surgery of the oral cavity. Our tissue samples were excised during oral surgery and therefore revealed a very high level of IL-6 even in untreated tissue.

Applications in dentistry seem to be very promising.[20–23] In 2013 the kinpenMED was certified as a medical device and consequently can be applied in clinical depart-

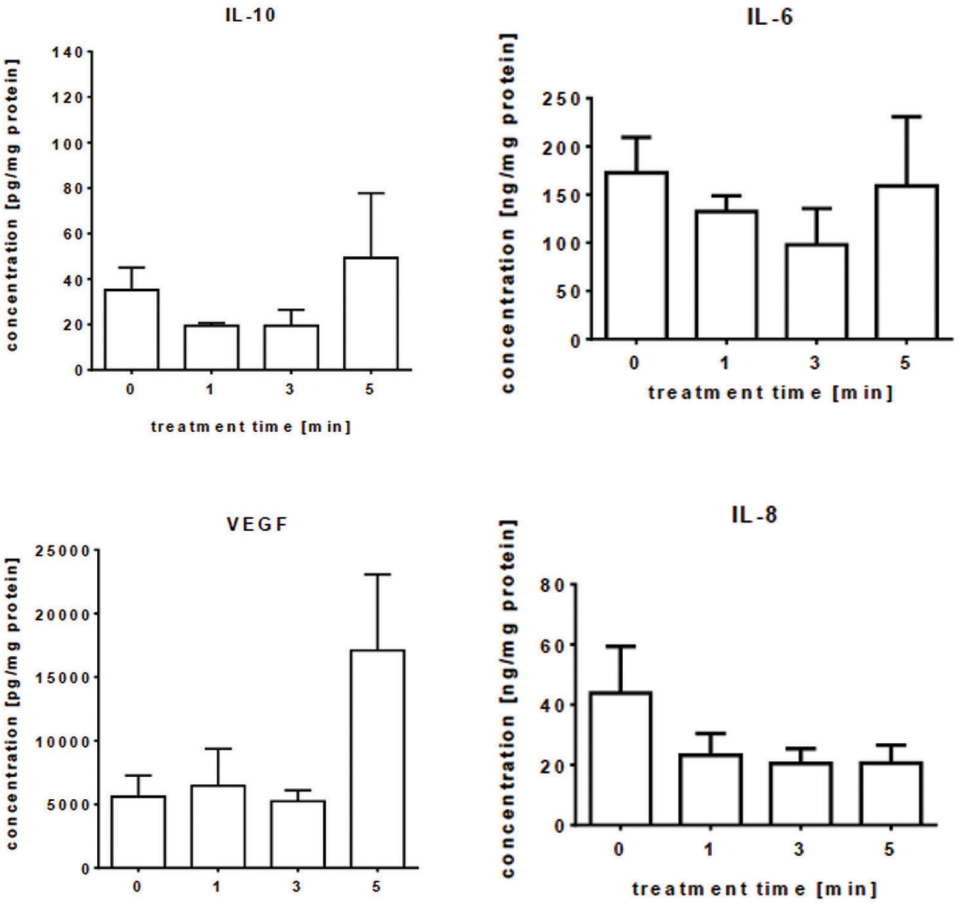

FIG. 6: Concentration of IL-10, VEGF, IL-6, and IL-8 in the supernatant of plasma-treated oral mucosa tissue as determined by Luminex-assay (IL-10 and VEGF) or ELISA (IL-6 and IL-8)

ments. Therefore we aimed to investigate the impact of plasma on cells of the oral cavity. Many patients face problems with periodontal diseases or inflammation of gingiva. As a consequence teeth may be impaired as well. Plasma application holds great hopes in dentistry and maxillofacial surgery due to its very locally and timely limited mode of application, its good tolerability, and the feasibility on surfaces. The literature on plasma interaction with mucosal tissue, however, is very limited.

The oral cavity is lined by a stratified epithelium similar to the skin. The majority of the cells are keratinocytes at different stages of differentiation. *In vitro* experiments using HaCaT keratinocytes revealed a stimulation of the pro-proliferative (ERK1/2) as well as the pro-apoptotic (JNK) cell signaling cascade after plasma exposure that depended on treatment time and incubation time.[6,16] Hence the question remains, what im-

pact has plasma on tissue comprised of keratinocytes. Consequently future applications could evolve based on these results.

Although this study included very few samples we think it gives some important hints towards the effects of plasma treatment on human oral mucosa. Certainly a higher sample number would improve statistics. The herein presented data used healthy human mucosa that derived from the surroundings of different lesions including hyperplasia and hyperkeratosis.

The age of the included probands varies over a large range. To date it is not entirely clear whether mucous epithelium ages and how.[10] Functional changes remain to be elucidated since they are less obvious than in skin.

Individual susceptibility became very obvious in this study and should be considered in future clinical applications of plasma. The variety within this group is reflected clearly in the number of proliferating and apoptotic cells.

Moreover the highest percentage in γH2A.X-positive cells occurred after 5 min treatment but is not concomitant with the number of apoptotic cells. Most TUNEL-positive cells were detected after a short plasma exposure of 1 min. It has been shown that near γH2A.X foci the concentration of repair proteins was elevated.[24] In some specimen we found even a decreased number of γH2A.X-positive cells with prolonged plasma exposure.

DNA damage as detected by γH2A.X was relatively high in all tested specimens and only increased slightly—but not significantly—with plasma treatment time. Predominantly basal keratinocytes stained strongly positive for this marker while apical the number of positive cells decreased. DNA strand breaks occur during cell division as a physiological process and can be repaired normally. In our hands there was no correlation between the number of γH2A.X-positive cells and smoking behavior, age, and sex, respectively. Isbary et al. revealed similar findings on *ex vivo* plasma-treated skin.[25]

The rate of proliferation in oral mucosa is already relatively high compared to skin. A turnover of 14 days has been documented for buccal mucosa while in skin the turnover is 28 days.[11] This may be considered when interpreting the results of Ki67-positive cells. Moreover the thickness of the epithelium above proliferating basal cells is far larger than in skin and hence the impact of reactive species from the plasma may be diminished. The epithelial ridges in oral mucosa are more pronounced than in skin which may explain the small effect on proliferating cells. Skin revealed a stronger elevation of proliferating cells after 1 and 3 min in a comparable experimental setup (Hasse et al., submitted).[26]

To date it is not well understood what triggers proliferation in keratinocytes after plasma exposure. Direct impact of ROS and RNS needs to be considered. Therefore it is of great interest how deep certain species can penetrate into the tissue. In addition indirect mediators are good candidates to exert growth stimulation. *In vitro* experiments revealed enhanced release of IL-6 and GM-CSF, both being able to influence cell proliferation.[19]

Moreover the applied method of tissue culture is useful for preclinical studies of plasma applications. It represents a simple tool to investigate molecular modulations by plasma *in situ*.

Unfortunately most of the detected growth factors showed no correlation with the disposed plasma treatment time. Therefore the mediators exerting the pro-proliferative effect in some specimens is still not clear and needs further investigation.

With the method applied herein the tolerability of plasma was provided for all tested treatment times. Although not tested before on oral mucosa this is in good agreement with other dermatological applications.[7,27] In contrast the findings by Welz et al.[13] using nasal and pharyngeal mucosa tissue and the MiniFlatPlaSter device[13] detected decreased viability and a significant increase in necrosis/apoptosis after 60 s treatment. One has to keep in mind that comparability between plasma devices and experimental setup is limited due to the lack of standardization to date. In an *ex vivo* skin model plasma could be applied for up to 20 min without any histological and electron-microscopic changes.[25]

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