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Effects of cold atmospheric plasma on mucosal tissue culture

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Abstract

Thermal plasmas have been commonly used in medical applications such as plasma ablation and blood coagulation. Newer developments show that plasmas can be generated with ion temperatures close to room temperature: these non-thermal or so-called cold atmospheric plasmas (CAPs) therefore open up a wide range of further biomedical applications. Based on the understanding of the bactericidal, virucidal and fungicidal properties of CAPs, information about the effects of CAP on mucosal cells and tissue is still lacking. Therefore this study focuses on the interaction of CAP with healthy head and neck mucosal cells on a molecular level.

To analyse this interaction in detail, fresh tissue samples from healthy nasal and pharyngeal mucosa were harvested during surgery, assembled to a three-dimensional tissue culture model (mini organ cultures) and treated with CAP for different treatment times. Effects on the viability, necrosis induction and mutagenic activity were evaluated with the trypan blue exclusion test, Annexin-V/PI staining and alkaline microgel electrophoresis (comet assay). Trypan blue exclusion test revealed that the CAP treatment significantly decreases the cell viability for all tested treatment times (5, 10, 30, 60 and 120 s; $p < 0.05$), but only a treatment time of 120 s showed a cytotoxic effect as the viability dropped below 90%. Annexin-V/PI staining revealed a significant increase in necrosis in CAP treated pharyngeal tissue cultures for treatment times of 60 and 120 s ($p < 0.05$). For nasal tissue this effect was already detected for a 30 s treatment ($p < 0.05$). Comet assay analysis showed no mutagenic effects after exposure to CAP.

(Some figures may appear in colour only in the online journal)

1. Introduction

In the past few years different cold atmospheric plasma (CAP) devices—ranging from dielectric barrier discharge (DBD) [1], surface microdischarge (SMD) [2], plasma jet/bullets [3, 4] to microwave driven plasma sources [5]—have proved their effectiveness for applications in health care and medicine. It is well known that all these CAP devices produce a so-called

‘chemical cocktail’ composed of electrons, charged particles, reactive species (mainly reactive oxygen and nitrogen species), UV light and heat, which may vary in concentration—depending on the used device. CAPs are able to inactivate bacteria (even multi-resistant strains such as EHEC and MRSA), fungi, viruses and spores very efficiently in a dose-dependent manner [2, 6].

Concerning the effects of CAP on eukaryotic cells and tissue, first results have also been obtained. Maisch *et al* for example showed that CAP treated porcine skin (up to several minutes) histologically did not possess any differences compared with the untreated control [7]. Newer



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experiments with lung fibroblast cells from Chinese hamster showed no toxicity or induction of mutagenicity for different dosages, including those which could easily inactivate different microorganisms [8]. In addition to these results other *in vitro* studies on benign and malignant epithelial cells were able to demonstrate that CAP influences the cell proliferation, cell cycle and apoptosis [9].

Applications in medicine—such as clinical studies on chronic wounds—showed the vast potential of CAPs: highly significant higher germ reduction compared with the untreated control wounds was obtained from studies on more than 260 patients using the CAP technology as an add-on to the standard topical treatment therapy [10]. Furthermore, newest results show that the CAP treatment accelerates wound healing in chronic [11] and in skin graft wounds [12]. Concerning skin diseases, a report in 2010 was published which showed the successful treatment of the Hailey–Hailey disease [11].

To date only wounds (chronic or skin graft) of the skin or different skin diseases were addressed in clinical studies and no investigations of the effects of CAP on mucosa were studied. The mucosal membranes line all cavities that are exposed to the environment and to the internal organs. Acute bacterial infections of mucosal lined organs are very prevalent [13]; particularly pharyngotonsillitis and rhinosinusitis which are the most frequent diseases in humans. Because of the complex physiological and anatomical conditions of head and neck organs and the resulting difficulty of access, topical therapies of head and neck infections have commonly only a symptomatic and not a curative aspect.

In order to find a new approach for the treatment of mucosal diseases, the CAP influence on mucosal tissue cultures was evaluated in this study. To predict the CAP dosage, i.e. treatment time dependent toxicity potential and to elucidate the capability of DNA damage of CAP, fresh tissue samples from healthy nasal and pharyngeal mucosa were assembled to a three-dimensional tissue culture model and treated with a CAP device using the SMD technology for plasma production in air [2].

2. Materials and methods

Biopsies. Fresh tissue samples of oropharyngeal and nasal mucosa were obtained during surgery of tonsillectomy ($n = 10$, 7 male, 3 female, average age 34.7 years) and conchotomy ($n = 10$, 6 male, 4 female, average age 41.5 years). To avoid any additional risks for the concerned patients, specimens were gathered after the surgery from the resectates. Therefore no disadvantages for the patients occurred and the intended therapy remained unaffected. The study design was approved by the local ethics committee. All donors were informed about procedures and aims of the study, and provided a written informed consent. After excision, all samples were immediately covered with 0.9% NaCl solution.

Mini organ cultures (MOCs). After proximate transport (<2h after excision) to the laboratory, the mucosal samples were assembled to tissue cultures (mini organ cultures, MOCs)—a three-dimensional *in vitro* model with

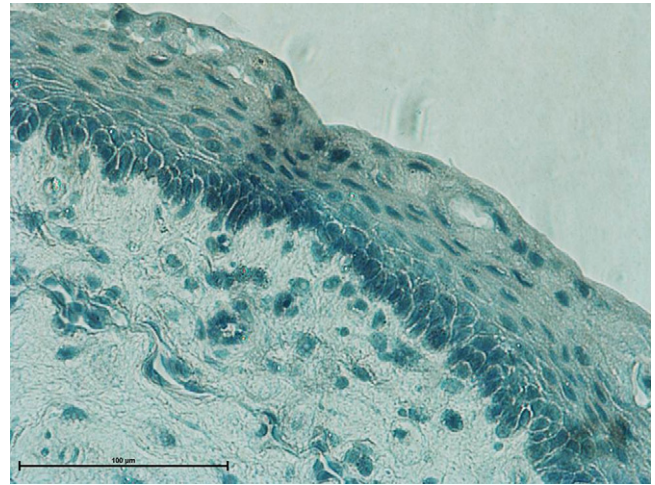


Figure 1. Histological slide of a representative oral MOC (Haematoxylin/Eosin staining, $\times 40$). The outside MOC multilayer is built up of 3–5 layers of epithelial cells. Each layer contains $0.3\text{--}1.0 \times 10^6$ cells. The inside of the MOC is composed of (almost exclusively) connective tissue with a small number of embedded epithelial cells (5–15% of the total cell amount). In contrast a partly ciliated surface can be found in nasal MOCs under higher magnification.

biotransformative activity [14] (figure 1). For this, the samples had to be dissected into mucosal cubes of 1 mm^3 excluding deeper layers, vessels and lymphatic parts and washed three times in bronchial epithelial cell basal medium (BEGM; Promocell, Heidelberg, Germany). The prepared cubes were placed in 24-well plates. Each well was coated with 0.75% Agar Noble (Difco; Detroit, MI, USA) dissolved in Dulbecco's Modified Eagle Medium (Gibco; Eggenstein, Germany), 10% fetal calf serum (FCS) (Gibco), non-essential amino acids (Gibco), and amphotericin B (Gibco). After a mean cultivation time of 20 days in $250\ \mu\text{l}$ bronchial epithelial cell growth medium (BEGM supplemented with bovine pituitary extract, insulin, hydrocortisone, epinephrine, triiodothyronine, transferrin and retinoic acid; Promocell, Heidelberg, Germany) and incubation at 37°C , 5% CO_2 , and 100% relative humidity, the MOCs were completely recoated with interacting endogenous epithelium, no additional cells for recoating were added. Growth medium was renewed every second day, and the MOCs were transferred to a new multi-well plate every seventh day. For all methods and treatment times 3 MOCs from each donor were cultured.

Plasma treatment. This study was carried out by employing a CAP device which uses the SMD technology for plasma production in air [2]. Details of the so-called MiniFlatPlaSter device were published in Maisch *et al* [7] and others [8, 14]. Briefly, the handheld CAP device is portable and the high voltage power supply for generation of the plasma is therefore incorporated inside. The used SMD electrode consists of a glass Epoxy board, which is sandwiched by a copper foil layer and a stainless-steel mesh grid. By applying a high pulse-like voltage of 7 kV (peak-to-peak) with a repetition frequency of 6.75 kHz the plasma is produced homogeneously on the mesh grid side in the ambient air. The power consumption was

Table 1. Characteristics of the MiniFlatPlaSter. The concentration of reactive species was measured as a function of time; the above values are averages over 1 min [30].

Charged particles	Electrons, ions	At the surface of the SMD electrode $\sim 10^{11} \text{ cm}^{-3}$
Reactive species	O ₃	Mean value for a treatment time of 1 min ~ 25 ppm
	NO	Mean value for a treatment time of 1 min < 1 ppm
	NO ₂	Mean value for a treatment time of 1 min < 15 ppm
Heat		At maximum 5°C increase (referring to the ambient temperature for a treatment time of 1 min)
Photons	UV, visible	Power density $< 0.66 \mu\text{W cm}^{-2}$
Static electric field		$\sim 10^7 \text{ V m}^{-1}$ on the mesh electrode
Electrical current through the samples		$< 50 \text{ nA cm}^{-2}$

measured to approximately 0.5 W cm^{-2} . The applied potential across the dielectric is transformed from an approximately 4 V dc rechargeable battery unit to a 7 kV pulsed voltage using suitable electronics and a mini-transformer.

The main constituents produced by the MiniFlatPlaSter are listed in table 1. Concerning the UV emission the main components are in the wavelength range between 280 and 400 nm. The UV power density was measured to be less than $0.6 \mu\text{W cm}^{-2}$. For a plasma treatment time of 60 s the UV dose of the MiniFlatPlaSter therefore is less than $36 \mu\text{J cm}^{-2}$, which is a factor of 100 below the ICNIRP (International Commission Non-Ionizing Radiation Protection) safety limits for intact human skin. Looking at the production of toxic gases, mean values of 25 ppm for O₃, < 1 ppm for NO and < 35 ppm for NO₂ for a treatment time of 2 min were measured close to the plasma electrode. So far, no safety limit values were published for the application of O₃, NO or NO₂ on healthy skin or mucosa. Nevertheless, the measured values for O₃ and NO₂ are above the safety limits published by NIOSH (National Institute for Occupational Safety and Health) for 8 h continuous inhaling. As the MiniFlatPlaSter produces the plasma indirectly—i.e. without using the sample (in our case the MOC) as a counter electrode—the current through the sample is far below the safety limits published by ICNIRP and even below the value of $100 \mu\text{A}$ given by the Canadian Health Authority for healthy intact skin.

For the CAP treatment of MOCs, cultivated MOCs were transferred to dry and uncoated 6-well plates. The CAP source/electrode was built and designed in such a way, that its dimension exactly fits the rim of one well of a 6-well plate (distance between the electrode and the tissue equalled 17.5 ± 0.5 mm). Therefore a closed volume was generated throughout the CAP treatment to confine the produced species (including O₃ and NO₂) inside. CAP treatment times of 5, 10, 30, 60 and 120 s were used. Unexposed MOCs (control groups) were maintained under the same conditions according to the respective CAP treatment time. To avoid dehydration effects caused by the experimental conditions MOCs were removed from the media for plasma treatment only (max. 180 s). The temperature at the bottom of the well did not increase by more than one degree Celsius after 120 s of CAP treatment so that dehydration effects on the specimen are considered to be unlikely.

Cell separation. For all further experiments single cells had to be extracted out of the united cell structure of the MOCs.

For this, after the CAP treatment, all MOCs first underwent enzymatic digestions for 1 h, after being covered with a solution of 50 mg protease (Biochrom, Heidelberg, Germany), 10 mg hyaluronidase (Roche, Mannheim, Germany) and 10 mg collagenase P (Roche) dissolved in 10 ml BEGM. After digestion, connective tissue and extracellular matrix components were separated by vortexing and removed mechanically under a dissection microscope. Histolytic enzymes were neutralized with FCS (Gibco, Germany) and the cell suspension was washed twice in phosphate buffered saline (PBS) (Gibco, Germany). As a precaution against collateral DNA damage induced by ultraviolet light caused by daylight and/or fluorescent tubes [31], all following steps were made under red light conditions.

Cell viability/trypan blue exclusion test. Trypan blue is one of the main recommended dye techniques for exclusion staining and for counting cellular populations, therefore evaluating cellular viability and acute cellular toxicity [16]. The method is based on the principle that living cells exclude the dye, because of their intact cell membrane. However, non-viable cells take up the dye due to their damaged membrane and are stained blue. To monitor the cell viability changes after different CAP treatment times as described above, the trypan blue exclusion test was performed using the following protocol. Supernatant PBS from cell separation steps was discarded after centrifugation at 800 rpm for 5 min at 4°C. Pellets of treated cells were resuspended in 1 ml of $1 \times$ PBS. $50 \mu\text{l}$ of cell suspension was mixed with an equal volume of trypan blue 0.4% (Merck), transferred to a hemocytometer slide and afterwards counted using light microscopy. A minimum of 200 cells were metered for each data point in a total of sixteen microscopic fields. The defined percentage of viability equals non-stained cells/(stained + non-stained cells) $\times 100$ [17].

Necrosis detection Annexin-V/Propidium iodid IHC staining. Acute necrosis was measured 2 h after the CAP treatment with fluorescence microscopy using an Annexin V-FITC detection kit (Merck for Biosciences, Germany). CAP treatment and MOC preparation to single cells were carried out as described above. Cells were stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions. Structural changes according to the plasma membrane of the cell are early steps in the apoptotic pathway. One of these changes is the translocation of phosphatidylserin (PS) from the internal to the external face of the plasma membrane. Annexin-V is a calcium-dependent phospholipid

binding protein with a high affinity to PS. Therefore, this protein can be used as a sensitive marker for the exposition of PS on the outer surface of the cell membrane of apoptotic cells. If there is a loss of membrane integrity necrotic cells bind annexin, too. For this reason the Annexin-V labelling is combined with a PI staining. PI is excluded from live, intact and early apoptotic cells, but permeates late stage apoptotic and necrotic cells. Accordingly this method allows us to distinguish between viable, early apoptotic, late stage apoptotic and necrotic cells. 100 cells per CAP treatment were counted and necrotic cells were identified and indexes were calculated.

DNA damage/comet assay. To detect DNA damage after the CAP treatment, assessment of the alkaline microgel electrophoresis (comet assay) was performed subsequently to the cell separation steps. The used protocol allows the detection of DNA-strand breaks in single cells, alkali-labile sites (ALSS) and incomplete excision repair sites [18].

Cell pellets were resuspended in 75 μ l of 0.7% low-melting agarose (Biozym, Hameln, Germany) and applied to slides (Langenbrinck, Emmendingen, Germany), frosted at the long edges and covered with 0.5% normal melting agarose (Biozym, Hameln, Germany) to provide stability to the agarose layers.

For cell lysis the slides were placed in alkali solution for 1 h (10% DMSO, 1% Triton-X, 2.5 M NaCl, 10 mM Trizma-Base, 100 mM Na₂EDTA and 1% *N*-lauroylsarcosine sodium salt). Prior to electrophoresis, the slides were placed in a horizontal gel electrophoresis chamber (Renner, Dannstadt, Germany), positioned close to the anode and incubated with alkaline buffer solution containing 300 mM NaOH and 1 mM Na₂EDTA at pH 13.2. After a 20 min DNA unwinding period, electrophoresis was started at 0.8 V cm⁻¹ and 300 mA and continued for 20 min. Following neutralization (Trisma base, 400 mM, pH 7.5; Merck Germany) fluorescent DNA staining was performed with 75 μ l ethidium bromide (Sigma; [51 μ l]). After staining, the slides were analysed with a DMLB microscope (Leica, Bensheim, Germany). 80 cell nuclei per slide (2 slides per CAP treatment time) were selected with random pattern and digitized with the attached monochrome CCD camera (Cohu Inc., San Diego, CA, USA).

A high DNA damage/fragmentation leads to a faster and further migration in the electric field. Undamaged DNA does not migrate, which results in a figure shaped like a comet with undamaged DNA in the head and damaged DNA in the tail. Migration was measured by the image analysis software Komet++ (Kinetic Imaging, Liverpool, UK) using the % of DNA in tail and the Olive tail moments (OTM). The OTM represents the multiplication of the relative amount of DNA in the tail with the median migration distance. The % tail DNA is a measure of the relative fluorescent intensity in the head and tail. The % tail DNA further enables a comparison of data over studies whereas the OTM, although consistent within a study, may not be comparable across different studies [19].

Statistical analysis. Unless elsewhere noted data were reported as the arithmetic mean \pm SEM. The SPSS 16.0

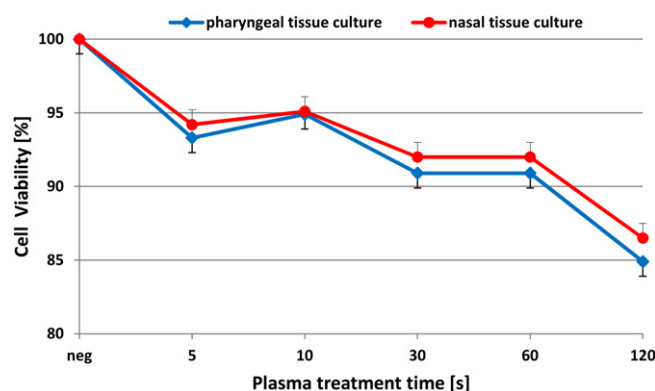


Figure 2. Measured cell viability for different CAP treatment times. For both tissues pharyngeal (blue) and nasal (red) the viability decreases for increasing CAP treatment times. Standard errors of the mean, depicted by whisker blots, ranged from 0.78 to 2.17 (pharyngeal tissue experiments), respectively, from 0.27 to 0.77 (nasal tissue experiments). (As a matter of lucidity the errors were only depicted in one direction).

software (SPSS GmbH, Munich, Germany) was used for statistical analyses and imaging. For calculating the statistical significance of the results the Mann–Whitney test was used. Differences were considered significantly at p values < 0.05 , prior the statistical analysis. Bonferroni correction was used if necessary [20].

3. Results

Cell viability. Figure 2 shows the viability of oropharyngeal and nasal tissue culture cells after CAP exposure for treatment times of 5, 10, 30, 60 and 120 s compared with the untreated controls. Cell viability was measured 2 h after the CAP treatment using the trypan blue exclusion test as described in section 2. This time point was chosen to investigate the acute cytotoxic effects on healthy cells and to further eliminate influences of other cell mechanisms like apoptosis or cell repair which usually occur later. Viabilities of the controls were set to 100% so that the results—expressed in per cent of the control—allow the comparison between the different tissues. For the pharyngeal tissue culture the viability was significantly reduced to 93.3% ($p < 0.05$) for a treatment time of 5 s, respectively, to 94.9% for 10 s ($p < 0.05$) as compared with the controls. Longer treatment times (30 and 60 s) induced a further reduction to 90.9%. Compared with the shorter treatment times (5 and 10 s) this was no further significant decrease in the viability. After a duration of 120 s the viability significantly dropped to 84.9% ($p < 0.01$). For the nasal tissue a similar significant reduction of the viability was observed. Compared with the controls the viability was reduced to 94.2% for a treatment time of 5 s ($p < 0.05$) and to 95.1% ($p < 0.05$) for 10 30 and 60 s led to a further decreased viability of 92% ($p < 0.05$). A treatment time of 120 s showed a viability of 86.5% ($p < 0.01$). Compared with the untreated cells and compared with all other treatment times a significant decrease in the cell viability after 120 s of CAP treatment in pharyngeal and nasal tissue ($p < 0.05$) was observed (table 2).

Table 2. p values (Wilcoxon test) for cell viability tests of pharyngeal and nasal tissue cultures level of significance $p \leq 0.05$.

p values for Cell Viability tests (Pharyngeal tissue culture)						p values for Cell Viability tests (nasal tissue culture)					
	5 s	10 s	30 s	60 s	120 s		5 s	10 s	30 s	60 s	120 s
0 s	0.012	0.018	0.005	0.008	0.005	0 s	0.028	0.030	0.011	0.012	0.012
5 s		0.232	0.234	0.905	0.028	5 s		1.00	0.547	0.203	0.025
10 s			0.056	0.172	0.012	10 s			0.260	0.149	0.017
30 s				0.497	0.07	30 s				0.734	0.027
60 s					0.015	60 s					0.043

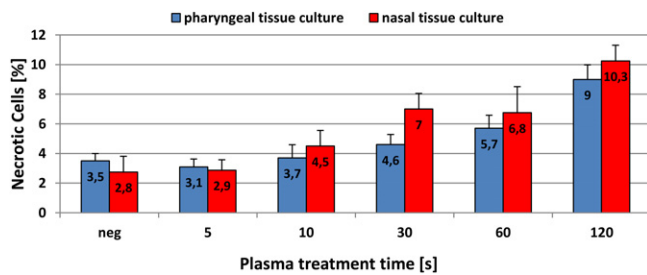


Figure 3. Percentage of necrotic cells in pharyngeal (blue) and nasal (red) tissue cultures after different CAP treatment times. Whisker blots depict standard errors of the mean (SEM). These ranged from 0.5 and 0.99 (pharyngeal tissue experiments), respectively, from 0.7 and 1.77 (nasal tissue experiments).

Necrosis detection. The trypan blue exclusion test gives a good indication for the cell viability in short term assays. Assessing the toxicity with the trypan blue exclusion test alone, may underestimate the toxicity since it is based on the loss of membrane integrity which is a late step in cell death. Furthermore, this test does not allow the discrimination between apoptotic and necrotic cells, and moreover false positive blue stained cells, with a mechanical damaged cell membrane but a viable cell function can occur. To confirm our suggestions of a treatment time dependent acute toxic effect on mucosal cells, Annexin-V/PI staining 2 h after the CAP treatment was measured (see section 2).

The untreated controls showed an average of 3.5% (pharyngeal tissue), respectively, 2.8% (nasal tissue) of necrotic cells (figure 3). For the pharyngeal tissue, a slight increase of necrosis was detected for CAP treatment times of up to 30 s, but these data are not significant. A CAP treatment time of 60 s showed an average of 5.7% necrotic cells which is a significant increase compared with the control ($p < 0.05$) and the 5 s treatment ($p < 0.05$). The 120 s treatment increased the rate of necrotic cells to 9% which was significant compared with the control and all other treatment times especially with the 60 s treatment ($p < 0.05$). For the nasal tissue a treatment time of 30 and 60 s led to a similar average of 6.8%, respectively, 7% of necrotic cells. Compared with the controls and the 5 s treatment these results are statistically significant ($p < 0.05$). 120 s of CAP treatment increased the average of necrotic cells to 10.3% which was significant compared with the control and to treatment times of up to 30 s ($p < 0.05$) (table 3).

DNA-damage measurement. To clarify if the time dependent reduction in cell viability and the increasing cell death

(necrosis) is induced through DNA damage and to further investigate the mutagenic potential of increasing CAP treatment times the alkaline microgel electrophoresis (comet assay; as described in section 2) was used for the detection of DNA damage in human mucosal cells. DNA damage was quantified by analysing the percentage of DNA in the comet tail (% tail DNA) and the OTM (median DNA migration distance \times relative amount of DNA in the tail of the comet). Figure 4 shows the % tail DNA in detail.

Analysis of all experiments using pharyngeal tissue revealed that there was no significant increase of DNA damage compared with the controls ($p > 0.05$) for all CAP treatment times (table 4). For the nasal tissue a significant increase of % tail DNA after 60 s (6.7%) compared with the untreated controls (5.0%; $p < 0.05$) and the treatment time of 5 s (5.1%; $p < 0.05$) was observed. Nevertheless, all measured OTM were under 2 (data not shown). As only OTMs >2 are considered to reflect relevant DNA damage [21], we can conclude that no significant genotoxic effect was observed for all CAP treatment times of up to 120 s. Surprisingly, there was a significant reduction of DNA fragmentation after 120 s treatment time for both tissues (pharyngeal 4.1%; nasal 5.0%) compared with the 60 (5.8%; $p < 0.05$) and 30 s (5.6%; $p < 0.05$) treatment in pharyngeal and to the 60 s (6.7%; $p < 0.05$) in nasal tissue. This issue was analysed in detail using a theoretical model, which is described below. This model has as a major assumption a space/time dependent CAP effect on the cells.

Theoretical model. As described above MOCs are three-dimensional *in vitro* models, built up of a few layers of ‘target cells’ (either mucosal cells from pharyngeal or nasal tissue) around an organic substrate (figure 1). Some of the ‘target cells’ may also become embedded inside the substrate.

The following model is partly based on known facts (e.g. the structure of MOCs, the possibilities for cell changes etc), and partly on assumptions. These assumptions are stated in the mathematical development of the model at the appropriate steps.

The basic aim is to define an ‘untreated’ DNA marker $\langle D_u \rangle$, based on the natural evolution of the MOC system, and a ‘plasma treated’ DNA marker $\langle D_p \rangle$ based on additional plasma effects on the cells that are in principle possible. The ratio of these markers can be related to the measurements (figure 4).

The main assumptions are CAP treatment affects the surface layer of cells; we can define ‘DNA markers’ as an average over the cells in parametric form; the plasma treated

Table 3. p values (Wilcoxon test) for necrosis detection tests of pharyngeal and nasal tissue cultures. Level of significance $p \leq 0.05$.

p values for necrosis detection tests (Pharyngeal tissue culture)						p values for necrosis detection tests (nasal tissue culture)					
	5 s	10 s	30 s	60 s	120 s		5 s	10 s	30 s	60 s	120 s
0 s	0.605	1.00	0.206	0.029	0.007	0 s	0.792	0.068	0.018	0.012	0.018
5 s		0.552	0.072	0.031	0.005	5 s		0.121	0.012	0.018	0.012
10 s			0.341	0.102	0.007	10 s			0.017	0.075	0.017
30 s				0.203	0.008	30 s				0.672	0.050
60 s					0.024	60 s					0.068

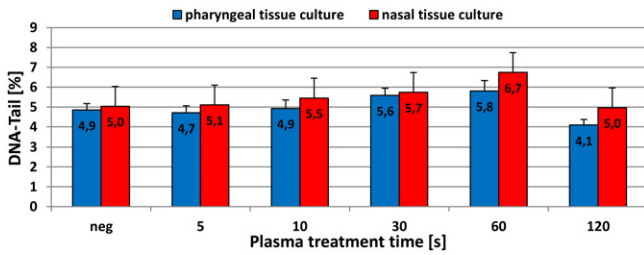


Figure 4. Detection of DNA damage (using the comet assay) in pharyngeal (blue) and nasal (red) tissue culture after different CAP treatment times. Whisker blots show the standard errors of the mean, which ranged from 0.28 to 0.53 (pharyngeal tissue experiments), respectively, from 0.27% to 0.77% (nasal tissue experiments).

‘DNA marker’—if it leads to additional cell effects—therefore is additive to the untreated case.

With these three basic assumptions one can generate an expression how the ‘DNA marker’ would evolve, depending on the plasma dose, without specifying this dose directly. This is done by looking at different limits for the derived expression (equations (9)–(12)) and by comparing this with the measurements.

For the following calculation we divide the ‘target cells’ into two groups:

- (1) Surface cells—total number N_s ,
- (2) Interior layer cells—total number N_i .

Furthermore, we assume that the CAP treatment only affects the surface cells directly. There are at least four possibilities due to this:

- (1) some cells remain normal, i.e. totally unaffected— N_{SN} ,
- (2) some cells are turned into dormant forms (so-called ‘hedgehogs’)— N_{SI} ,
- (3) the plasma inflicts DNA damage— N_{SD} ,
- (4) some cells become necrotic— N_{ST}

and also we have for the sum of all cell types $\sum_i N_{Si} = N_s$.

For DNA damage testing, the dormant forms (hedgehogs) are usually not considered. Let us call the DNA marker ‘ D ’.

The value for D , as determined in the DNA damage measurements, is the cumulative sum of all the individual markers summed over all the target cells, except for the dormant forms.

For the untreated case, we assume that there are no dormant cells:

$$\langle D \rangle_u = \{N_s D_s + N_i D_i\}. \quad (1)$$

where the subscript S stands for ‘surface’ and i for interior. We may safely assume that $D_s = D_i$, so that

$$\langle D \rangle_u = D(N_s + N_i). \quad (2)$$

However, there is always a natural level of DNA damage that occurs in such samples, dependent on the life time of the MOC, so that we may write

$$\langle D \rangle_u = [fD + (1 - f)D'](N_s + N_i), \quad (3)$$

where $f(t)$ is the fraction of normal target cells after time t , and D' refers to the natural DNA damaged marker (average). If some cells naturally become dormant or necrotic then $N_s + N_i$ have to be reduced accordingly, and we get

$$\langle D \rangle_u = [fD + (1 - f)D'](N_s + N_i) f_i f_T. \quad (4)$$

where $(1 - f_i)$ and $(1 - f_T)$ are the fraction of cells which naturally (after time t) have become dormant or necrotic. We put the factor

$$\left[f + (1 - f) \frac{D'}{D} \right] f_i f_T \stackrel{\text{def}}{=} F \quad (5)$$

so that (4) becomes simply

$$\langle D_u \rangle = FD(N_s + N_i). \quad (6)$$

F may be regarded as the natural evolutionary factor of the target cell sample. Anything that happens as a result of CAP application is additional.

For the CAP treated sample we can in principle have an additional effect for dormancy, DNA damage and necrotic action.

This effect is assumed to be simply additive to the natural ones described above. Then we may write

$$\langle D_p \rangle = F(N_s + N_i - N_{SI} - N_{SD} - N_{ST})D + FN_{SD}D', \quad (7)$$

where the level of N_{SI} , N_{SD} and N_{ST} depends on the plasma dose p . Compared with the untreated case, the DNA marker becomes

$$\frac{\langle D_p \rangle}{\langle D_u \rangle} = 1 - \frac{N_{SI} + N_{SD} + N_{ST}}{N_s + N_i} + \frac{N_{SD}}{N_s + N_i} \frac{D'}{D} \quad (8)$$

we see that there is a term that increases the (relative) DNA marker, and another one that decreases this marker.

Table 4. p values (Wilcoxon test) for DNA damage detection of Pharyngeal and Nasal Tissue Cultures. Level of significance $p \leq 0.05$.

p values for DNA- damage detection tests (pharyngeal tissue culture)						p values for DNA- damage detection tests (nasal tissue culture)					
	5 s	10 s	30 s	60 s	120 s		5 s	10 s	30 s	60 s	120 s
0 s	0.799	0.878	0.203	0.203	0.093	0 s	1.00	0.484	0.093	0.036	0.484
5 s		0.415	0.17	0.241	0.139	5 s		0.401	0.327	0.012	0.674
10 s			0.169	0.203	0.047	10 s			0.674	0.263	0.575
30 s				0.208	0.022	30 s				0.208	0.069
60 s					0.022	60 s					0.017

Special cases:

- (1) At low plasma doses we may assume that there are no additional modifications over the natural ones. Then

$$\langle D_p \rangle / \langle D_u \rangle = 1. \quad (9)$$

- (2) At moderate plasma doses the sum of modified cells = $N_S - N_{SN}$ will begin to increase. If this is only due to DNA damage, then equation (8) reduces to

$$\langle D_p \rangle / \langle D_u \rangle = 1 + \frac{N_{SD}}{N_S + N_i} \left(\frac{D'}{D} - 1 \right). \quad (10)$$

- (3) At high plasma doses we need to consider the full equation (8). Rewriting gives

$$\frac{\langle D_p \rangle}{\langle D_u \rangle} = 1 + \frac{N_{SD}}{N_S + N_i} \left(\frac{D'}{D} - 1 \right) - \frac{N_{SI} + N_{ST}}{N_S + N_i}. \quad (11)$$

The cross-over point, A, occurs when

$$N_{SD} \left(\frac{D'}{D} - 1 \right) = N_{SI} + N_{ST} \quad \text{or}$$

$$N_{SD} \frac{D'}{D} = N_S - N_{SN}. \quad (12)$$

- (4) At very high plasma doses, in the extreme limit we may argue that the whole surface cell population has become necrotic—i.e. $N_{ST} = N_S$ and correspondingly $N_{SI} = N_{SD} = 0$. Then we get

$$\langle D_p \rangle / \langle D_u \rangle = 1 - \frac{N_S}{N_S + N_i}. \quad (13)$$

This feature of our model is in qualitative agreement with the observations concerning DNA damage shown in figure 4. Quantitatively, we can determine this effect, for instance, by assuming the MOC to consist of n equal layers of cells. Then we have simply

$$\langle D_p \rangle / \langle D_u \rangle = 1 - 1/n. \quad (14)$$

Interpretation of the measurements according to this model.

The model results and the measurements (figures 3 and 4) together with the MOC structure (figure 1) suggest the following:

- (1) The MOCs consist of a few layers, n , of cells were $2 \leq n \leq 10$. We take an average $n = 5$.
- (2) The DNA marker does not vary significantly with plasma dose (up to 120 s), so that in first approximation the ratio $\langle D_p \rangle / \langle D_u \rangle = 1$. This corresponds to equation (9), i.e. ‘low’ plasma dose, and implies that practically all cells are unaffected by the plasma, i.e. $N_S \approx N_{SN}$.

- (3) There is a hint of a systematic effect in the data, suggesting that $\langle D_p \rangle / \langle D_u \rangle$ increases slightly up to 60 s of plasma treatment. This would imply using the ‘moderate plasma’ dose solution in a second approximation. If the DNA marker changes only due to DNA damage (and not because some cells may become dormant or necrotic) the model reduces to equation (10). This is an extreme assumption, but worth investigating further. Using this interpretation on figure 4 yields $1 + \frac{N_{SD}}{N_S + N_i} \left(\frac{D'}{D} - 1 \right) = 1.34$ (nasal time) and $= 1.18$ (pharyngeal time), respectively (and by implication D' is larger than D). From our model assumptions, $N_{SD} \leq N_S$ and $N_S / N_S + N_i = 1/n$ with n the typical number of cell layers. Then $D'/D \geq 2.7$ (nasal time) and ≥ 1.9 (pharyngeal time), respectively, taking n to be 5 on average.

- (4) As the plasma dose is doubled to 120 s, the amount of DNA in the tail (of the comet) (according to figure 4) decreases again. This result is statistically borderline and we may overinterpret its significance. Nevertheless, the model is capable of explaining this seemingly curious result, too. The data suggest that $\langle D_p \rangle / \langle D_u \rangle \approx 1$ again (within the statistically accuracy). This corresponds to the situation described in equation (12). Rearranging this and substituting for D'/D we obtain the result that at this stage $N_{SD} \approx N_{SI} + N_{ST}$, i.e. the number of DNA damaged cells in the surface layer of the MOCs is similar to the number of dormant and necrotic cells.

- (5) In the extreme limit, where the plasma dose is so high that the whole surface layer of cells might become necrotic, we obtain the situation given in equation (14). Then the amount of DNA in the tail of the comet can become smaller than the control at most (for $n = 5$) by 20%. This result is also broadly consistent with the measurements of increase of necrotic cells (up to 9%), shown in figure 3, hence there is some internal consistency in such model assumptions, which encourages further investigations in this direction.

4. Discussion

CAPs are known to have excellent bactericidal properties. Furthermore, *ex vivo* studies have shown that they are benign with respect to human skin [15]. There is however practically no information about the effects on mucosal cells. Because of the high prevalence of bacterial infections of the head and neck sided organs, this study addresses the interaction of CAP with healthy mucosal tissue for the first time. The results for pharyngeal and nasal tissue demonstrate that CAP leads

to a treatment time dependent reduction in viability without significant detectable DNA damage. For up to 60 s of CAP treatment only weak cytotoxic and no significant mutagenic effects were observed. The *in vitro* model of MOCs used in this study was chosen because, in contrast to single cell experiments in culture, MOCs have a connective cell structure with a biotransformative activity which simulates *in vivo* conditions and therefore may reflect the effect of CAPs better than common two-dimensional single cells experiments [22]. Furthermore, we were able to carry out the CAP treatment in a more realistic setting, without cell medium covering the cells (only a slight film remained on the MOCs).

Up to now, many studies on several benign and malignant cell lines have been published, reporting a power- and/or treatment time dependent cell death suggesting CAP induced apoptosis and/or cytotoxic CAP effects [9, 23]. Whereas in almost all of these studies the median lethal dose, LD₅₀ (LD₅₀ = dose that is required to kill half the members of a tested 'population') was reached easily also for short treatment times, i.e. plasma dosages, our experiments only showed a maximum of viability reduction of 14% after 120 s of CAP application. A concomitant significant increase of necrotic cells after a treatment of 120 s confirmed these results. Shorter treatment times induced significant reduction of viable cells too, but the viability was still larger than 90% compared with the controls. These findings of a lower decrease in viability (than that reported by the other authors) may be explained by the fact that we used tissue culture in our experiments, whereas other groups used single cell cultures. As already mentioned above it is well known that tissue culture models have a biotransformative activity and are therefore more resistant to exogenous influences [24]. Furthermore, the studies performed in other groups were all carried out with different CAP devices producing different concentrations of reactive species, ions, etc. The viability measurements in our experiments were carried out shortly (~2 h) after the CAP treatment, so that only the acute effect on the viability was observed and effects on the viability that take more time, such as apoptosis induction, were not investigated yet. Overall, further investigations regarding long term survival of healthy mucosal cells still have to be made, but a strong cytotoxic effect for CAP treatment times of up to 120 s is considered to be unlikely.

To evaluate the mutagenic potential of CAP on healthy human mucosal single cells, the microgel technique involving electrophoresis (comet assay) was used. The version used for our experiments is very efficient for detecting frank DNA single-strand breaks (SSBs), double strand breaks (DSBs) and ALSs. Because almost all genotoxins induce more SSB and/or ALS than DSB and other DNA alterations such as DNA crosslinks or intercalations, the comet assay is highly sensitive for detecting low levels of DNA damage and requires only a small number of cells per sample, which makes it superior to other genotoxicity tests [18]. Compared with the untreated controls no significant treatment time, respectively, dose-dependent increase, of DNA damage was observed in our tests. The results therefore show that exposure of mucosal tissue cultures to CAP does not have a significant mutagenic effect for

treatment times of up to 120 s. Similar results were obtained by Boxhammer *et al*, who carried out the HPRT assay to analyse the induction of mutagenicity in V 79 lung fibroblast hamster cells: in this study, which was carried out with the same plasma device used here (MiniFlatPlaSter), treatment times of up to 240 s did not induce any mutations beyond those which occur naturally [8]. In contrast to these findings Arndt *et al* showed that 120 s of CAP treatment—using the MiniFlatPlaSter—induced phosphorylation of H2AX, a histone variant that is phosphorylated in response to DNA-DSBs in two melanoma cell lines. Nevertheless, a lower CAP treatment time of 60 s did not show any DNA-DSBs in the same setup [25]. This finding of a CAP dosage dependent increase in DNA damages was also reported by other authors using different CAP devices and cell lines [9, 26].

At this point we want to mention that a comparison of diverse experiments carried out with different CAP devices is difficult due to the differences in the production of their respective components. Nevertheless, experiments also carried out with one plasma device—in this case the MiniFlatPlaSter—give different results when different cell lines (malignant/non-malignant) or cell tissue cultures (MOCs) are targeted (and/or different detection assays are used).

From the experiments Kalghatgi *et al* performed, it was suggested that the dose-dependent DNA damage, measured with the γ -H2AX detection method, is mediated by stable organic components in the cell medium, such as organic peroxides (i.e. peroxidized amino acids) induced by intracellular reactive oxygen species (ROS) [26]. This means that DNA interacting, respectively, damaging agents are reactive ingredients of the medium and that the shown effects are probably indirect effects of the CAP treated medium and no direct CAP effects on the cells. These indirect effects can be ruled out in our study—as well as in the studies performed by Boxhammer *et al* and Arndt *et al*—as the MOCs were treated under almost 'dry conditions', i.e. no medium covered the cells during the CAP treatment.

Nevertheless, our results surprisingly show a highly significant time-/dose-dependent decrease of DNA fragmentation after 120 s (see figure 4), which can be interpreted in two ways. On the one hand, this phenomenon may be explained by the formation of DNA crosslinks mediated by plasma induced intracellular ROS. Several studies have shown the formation of intracellular ROS induced by CAP [9, 27]. ROS are potentially harmful for the cellular metabolism. At low doses they are known to have a positive effect on cell proliferation but induce mutagenesis, whereas high levels of ROS inhibit cell proliferation, induce cytotoxic effects and apoptosis [28]. ROS including superoxide, hydrogen peroxide and free hydroxyls are also able to generate peroxidized proteins that react with the DNA by forming strong intermolecular protein-DNA-crosslinks (PDC) or to promote DNA-DNA interstrand crosslinks [29]. The comet assay used in this study is very sensitive for the detection of DNA-single strand breaks, ALSs and DNA-DSBs. The presence of DNA-DNA or DNA-protein crosslinks reduces the ability of DNA to migrate in the agarose gel. Assuming a dose-dependent increase of intracellular ROS and the proportional formation of DNA crosslinks this mechanism may explain our results with a decrease in DNA migration

after 120 s of CAP treatment. Nevertheless, further investigations concerning this topic have to be carried out to prove this explanation.

On the other hand, a model calculation was set up in order to explain both the observed cell viability and the DNA measurements. The basic model assumption is that CAP influences the surface layers of cells, causing necrosis above a certain dose. This model is in good qualitative and quantitative agreement with the measurements. The two interpretations address different aspects of the observations (the DNA issue and the necrosis) and are therefore complementary.

Against the medical background this is the first study which ever evaluated the effects of CAP on mucosal cells. In conclusion no mutagenic effects for treatment times of up to 120 s were detectable. Nevertheless, a slight reduction in cell viability was observable. Regarding the undeniable bactericidal effect of the MiniFlatPlaSter within even lower treatment times our results could contribute to future applications such as the gentle treatment of mucosal bacterial infections.

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