Delayed Effects of Cold Atmospheric Plasma on Vascular Cells

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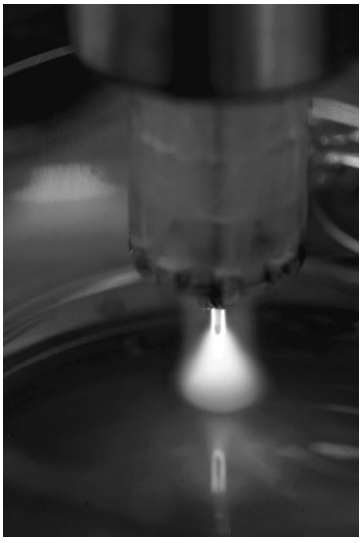
We investigated the long-term behaviour of vascular cells (endothelial and smooth muscle) after exposure to a cold atmospheric plasma source. The cells were treated through a gas-permeable membrane, in order to simulate intravenous treatment with a gas plasma-filled catheter. Such indirect treatment resulted in (dose-dependent) apoptosis and necrosis in smooth muscle cells; these effects were visible 6–10 h after exposure. We ascribed cell damage to poisoning by active nitrogen species. A not yet resolved phenomenon of total (irreversible) cell inactivation without necrosis was observed in endothelial cells. Conditions for non-inflammatory plasma treatment (no cell necrosis) can be realized. Furthermore, the current experiments have brought new insights in the mechanism of cell detachment, which was observed in many previous studies.



Introduction

Cold atmospheric plasma (CAP) treatment may become an extremely powerful and versatile medical therapy.[1–5] Local and precise treatment with CAP does not involve complex chemicals with their inevitable (harmful) side effects, and does not heat or drastically damage the tissue. Plasma releases controllable amounts of short-lived reactive oxygen and nitrogen species (ROS/RNS)[6] that address only the target area in the tissue. ROS and RNS have different physiological functions: antibacterial, pro-apoptotic and pro-inflammatory (ROS), or anti-inflammatory and pro-apoptotic (RNS, in particular nitric oxide NO).[7] External administration of ROS or RNS by the plasma will locally reinforce the natural physiological processes. The hitherto performed *in vitro* tests provide us with faith in the great potential of CAP therapy. Promising applications include:

a) Disinfection, blood coagulation:[1] applicable to wound and skin care, and treatment of dental caries;[5]
b) Non-inflammatory tissue treatment: applicable to cancer treatment,[2] removal of (vascular) stenoses, post-trauma scar treatment and skin rejuvenation; and
c) Various cell manipulation techniques: mobilisation, intra-cellular drug administration, non-viral transfection, etc.

The applicability of CAP in medicine and dentistry is still being treated with caution and reserve in the medical community. Several issues regarding efficacy and side effects (e.g. toxicity and DNA damage) must be resolved,

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and even though excellent *in vitro* models are available, eventually *in vivo* verification will be necessary. However, there are already a few examples of non-equilibrium plasmas that are established in medical practice, such as Argon Plasma Coagulation and coblation.[8,9] Non-inflammatory removal or growth suppression of tissues can be achieved by controlled induction of programmed cell death (apoptosis), or by suppressing cell proliferation in other way, but without inflicting accidental cell death (necrosis). Especially induction of apoptosis is a desired effect. Apoptosis is a programmed cell self-destruction pathway that is executed in old, damaged or not functional cells in complex organisms. It involves a series of biochemical events leading to changes in cell morphology and behaviour, such as membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. In the later stages, the cell is phagocytised by other cells. The whole process removes the cell, but does not induce inflammatory reactions.

Our previous works showed that treatment with a micro-plasma usually does not induce necrosis, but triggers sub-lethal cell reactions (detachment, apoptosis) in various cell types.[10,11] Apoptosis induction was also investigated by various researchers. Fridman et al.[2] used a large area air discharge, and diagnosed the cell condition with a flow cytometric TUNEL assay. We chose simple DNA staining with propidium iodide and microscopic observation, since it is much easier this way to observe local effects induced by our micro-plasma. We chose to investigate apoptosis in cells constituting the artery tissue (endothelial and smooth muscle cells). This is because of a high mortality and morbidity of cardiovascular diseases (e.g. atherosclerosis), and thus a high societal urgency for introducing new remedies. The pathology of atherosclerosis is very complex.[12] Short, chronic inflammatory processes cause overgrowth of the arterial muscle layer (media) that leads to artery constriction. A frequently applied remedy is based on mechanical recanalisation, followed by placing arterial supports (stents). However, the inflammation is not removed, and the damaged artery wall is prone to secondary tissue overgrowth (restenosis). We envision that cold plasma treatment may reduce both the primary and the secondary growth, while helping to suppress the existing inflammation. Nitrogen-rich plasmas would be the first choice, because of anti-inflammatory properties of RNS. Of course, a micro-plasma device would have to be modified to enable intravenous catheterisation. The latter is feasible, yet technically complex. Before developing such devices, we intend to use simple in vitro models to collect general information on local apoptosis induction by CAP. To simulate catheterisation, we treated the cells not directly, but through a porous membrane. The membrane provides an efficient gas-fluid barrier (a solution that will be used in CAP-catheters), but is permeable to small molecules (oxygen, nitrogen and plasma species). In this indirect manner of treatment, certain plasma species (e.g. charged particles and UV photons) were unable to reach the cells. The current experiment provided new insights in the mechanism of cell detachment.

Experimental Methodology

Plasma Source

Our CAP source was the standard plasma needle, operated by a 13.56 MHz generator in combination with a matching network (both the 10 W generator and the matching network are homemade).[13] The complete equipment including the CAP probe is shown in Figure 1. The probe consisted of a 0.3 mm metal alloy pin, confined in a 5 mm Perspex tube, which was flushed with helium at a rate of 2 $l \cdot min^{-1}$. Under these conditions the air content in the plasma was 0.5% and the ROS density about 10^{19} m^{-3}.[14] Gas temperature measurements were performed earlier.[11] Here, the temperature was controlled by simple skin exposure; at the chosen power settings (100 and 190 mW) there was no warmth sensation. Thus, thermal effects on treated samples could be excluded.

Cell Culture

Two cell types were used in the experiments which are prevalent in arterial walls: endothelial cells and smooth muscle cells. The specific cell types are Bovine aortic endothelial cells (BAEC) (Cell Applications Inc., San Diego, CA) and rat aortic smooth muscle cells (A7r5, ATCC, Manassas, VA). The cells were transferred every three or four days into a new flask with fresh culture medium and incubated at 37 °C with 5% CO_2. The culture medium for both cell types was composed of: 500 ml Dulbecco's

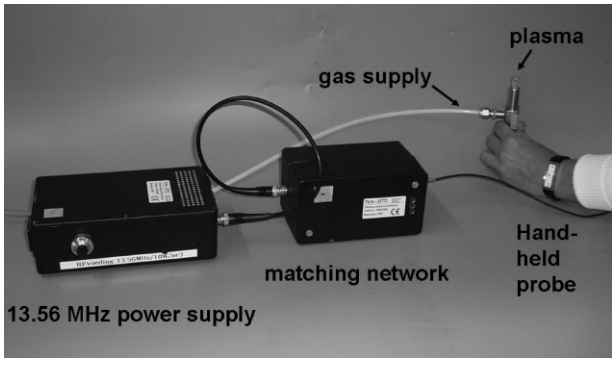

Figure 1. A complete cold plasma setup for cell treatment.

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modified eagle medium (DMEM) with 4.5 $g \cdot l^{-1}$ glucose and with L-glutamine (Biowhittaker, Cambrex, Verviers, Belgium), 50 ml foetal bovine serum (FBS) (Biochrom AG, Berlin), 5 ml nonessential amino acids (100×) (Biochrom AG), 2.5 ml gentamycin (10 $mg \cdot ml^{-1}$) (Biochrom AG); and 10 ml HEPES-buffer (1M) (Biochrom AG). The cells were transferred into an OptiCell™ for treatment, which was a square 2 mm thick chamber enclosed with two microporous 75 μm thick membranes. The membrane was permeable for gases (thus also for plasma species), but not for larger molecules (e.g. bacteria). Each membrane provided 50 cm^2 of surface for cell attachment. Cells were grown on one membrane, until nearly confluent. Cell density was estimated by counting (about $3.7 \cdot 10^5$ $cells \cdot cm^{-2}$).

Treatment and Diagnostics

Cells were exposed to the plasma at 100 or 190 mW from outside the chamber (through the membrane); the distance between the probe and the membrane was fixed (1 mm). The arrangement is schematically depicted in Figure 2. Dosage was adjusted by varying the treatment time (2–50 s). The influence of the antioxidant addition was studied. Medium was enriched with L-ascorbic acid (Merck) at a total concentration of 3×10^{-3} M. Cells were incubated with enriched medium for about 15 min to allow intracellular uptake of the antioxidant. Immediately after the plasma treatment, cell samples were observed under a phase contrast microscope; some samples were stained with propidium iodide (PI, 10 $mg \cdot ml^{-1}$, Molecular Probes) and examined under a confocal laser microscope Zeiss LSM410 to check for immediate cell necrosis. Cells were incubated for several hours and examined periodically for morphological changes. Each time, some samples were stained with PI and observed under the laser microscope to check for (delayed) necrosis. Moreover, some samples were fixed with 3% formaldehyde prior to staining with PI. This way, it was possible to detect apoptotic (non-necrotic) cells, because they would have an altered staining pattern with PI. After 24 h, cells on the samples were permeabilized with 3% formaldehyde, rinsed with phosphate buffered saline (PBS) and stained with 2% eosin. Damaged cells were removed by rinsing. The whole cell sheet was thus stained red, except the circular empty areas from where the plasma-damaged cells had been removed. The diameter of these voids was measured. Each measurement point (for different treatment times and powers, with and without antioxidant) was obtained in triplicate. Additionally, several samples were left in the incubator and periodically observed during several days after treatment.

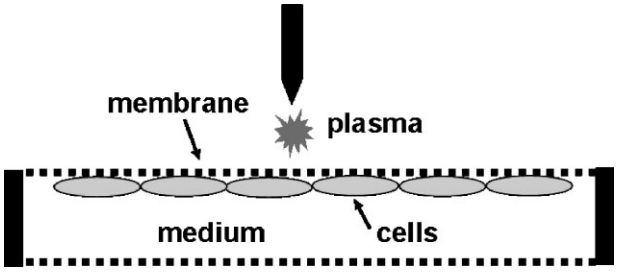

Figure 2. A scheme of the membrane-bound chamber in which cells are treated.

Results and Discussion

Immediately after the treatment, there were no visual changes to the samples. Phase contrast microscopy showed that the cells retained their shape and remained attached to the membrane; there was no PI uptake as seen under a fluorescence microscope. The occurrence of immediate necrosis was found to be negligible. The effects started to manifest themselves several hours after exposure to CAP; the onset was about 8 h for endothelial (BAEC) and 6 h for smooth muscle (A7r5) cells. A circular area concentric with the point of incidence of the microplasma probe (needle) was visible; cells within the area changed their shape to rounded (BAEC) or unorganized with typical blebbing (A7r5). 10 h after treatment, the area did not increase in size anymore. Typical micrographs are shown in Figure 3. Affected cells were loosely attached to the membrane and could be removed by rinsing with PBS. The cells surrounding the affected area appeared unharmed. The shape of A7r5 was indicative of membrane blebbing and formation of apoptotic bodies. For short plasma treatment times (<10 s), there was only sporadic uptake of PI by unfixed BAEC and A7r5. Thus, no delayed necrosis in both cell types was diagnosed. For plasma exposures longer than 10 s at 190 mW, the A7r5 cells in the middle of the treated area (coincident with the needle electrode) were necrotic (Figure 4). This necrotic zone was

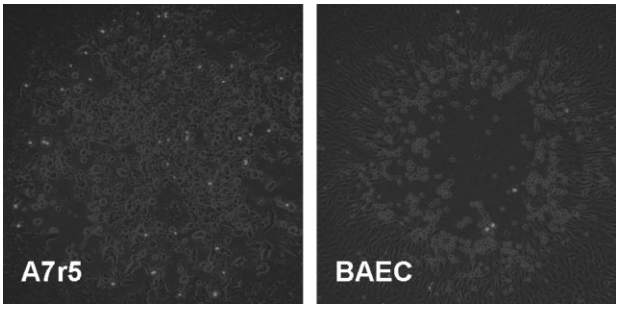

Figure 3. Micrographs of A7r5 and BAEC cells 10 h after plasma treatment at 100 mW (for 5 s). The cells were stained with PI (but not permeabilised). Morphological changes of affected cells and a few cases of necrosis (PI positive cells) are visible. The diameter of the affected spot is about 0.5 mm.

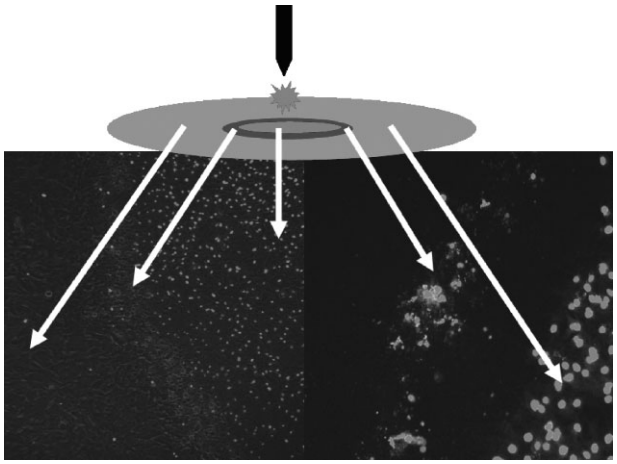

Figure 4. Micrographs of the A7r5 cells treated for 20 s at 190 mW, stained with PI and observed 10 h after treatment. Top: a schematic structure of the total cell sample and the affected area, showing the position of the plasma needle, a necrotic spot at the incidence of the needle, and a surrounding ring of apoptotic cells. Left: a not permeabilised sample, showing the edge of the affected area. Right: a permeabilised sample, showing DNA fragmentation in apoptotic cells. The arrows indicate approximate locations of imaged area on the total sample.

surrounded by a ring of apoptotic cells (with typical morphological changes). To examine the condition of affected cells, samples were permeabilised with formaldehyde and stained with PI. The A7r5 cells showed the characteristic DNA fragmentation (karyorrhexis) that occurs before the cell fragmentises into apoptotic bodies (Figure 4). The necrotic cells did not display karyorrhexis. The response of BAEC was different. Firstly, no morphological changes typical for apoptosis were seen; 8 h after treatment the cells assumed a rounded shape and retained it during the whole observation period. There were no necrotic cells left in the middle of the treated area: few necrotic cells were scattered randomly within the affected spot. However, cell density in the affected area was lower, and there was no cell proliferation in the whole spot. Dose-response curves were determined for each cell type, as a function of the plasma exposure time and the plasma power. Twenty four hours after treatment cells were fixed, rinsed and stained with eosin; the loosely attached cells were removed and characteristic circular voids became visible. The voids reached maximally 1.6 mm, and their size was about the same in BAEC as in A7r5. For A7r5, the total void diameter (corresponding to all influenced cells, apoptotic or necrotic) was measured, and the diameter of the necrotic spot was estimated from the corresponding micrographs (in analogy to Figure 4). From the void diameter and the cell density the total amount of removed cells was calculated. Figure 5 shows the total void diameter, the necrotic zone diameter for A7r5, and the corresponding numbers of cells that were influenced by plasma treatment (total affected and necrotic cells). The

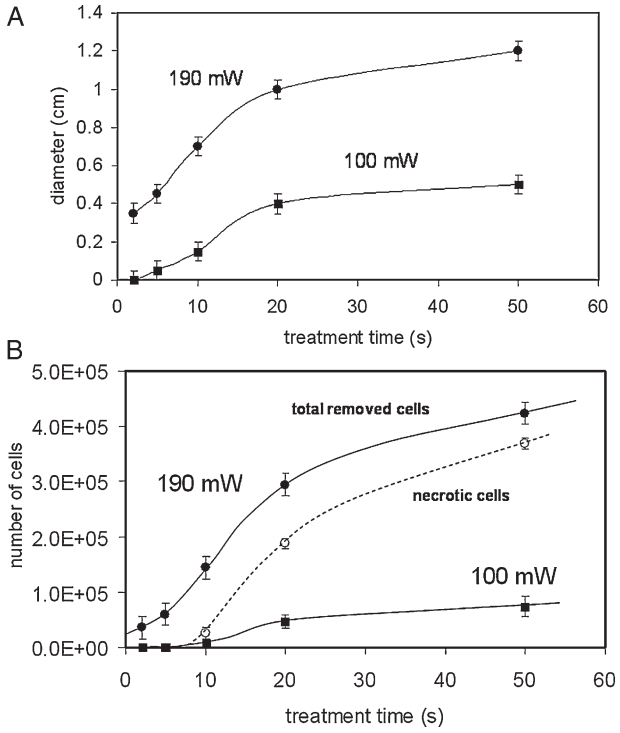

Figure 5. The A7r5 cells (24 h after treatment). The damaged cells were removed from areas affected by plasma treatment; the diameter of the affected areas (voids) was measured. A: The diameter of the void in function of plasma power and treatment time. B: The total number of removed (dead) cells and the number of necrotic cells as a function of the plasma power and the plasma treatment time (note that necrotic cells were found only for 190 mW and not for 100 mW).

corresponding data set for BAEC (without necrotic zones) is shown in Figure 6. The behaviour and void sizes in samples incubated with an antioxidant (L-ascorbic acid) prior to treatment were not significantly different from the samples treated without antioxidant.

Behaviour of Treated Cells

The current results are in line with our previous studies on apoptosis induction in fibroblasts,[11] and with the results on human melanoma cells.[2] A remarkable observation in all studies was that the onset of cell death was seen many hours after irradiation. In fibroblasts and melanoma cells (cancerous melanocytes) the responses to plasma treatment manifested themselves as apoptosis, 12–72 h after exposure.[2,11] In vascular cells, reactions begun about 6–8 h after treatment. At low plasma dose (low power and short irradiation times), smooth muscle cells displayed typical patterns for apoptosis (morphological changes and nuclear DNA condensation). Thus, we presume that apoptosis was the predominant mechanism of cell death under these conditions. Apoptosis was also seen at higher plasma dose, but necrosis was prevalent in the middle of the affected

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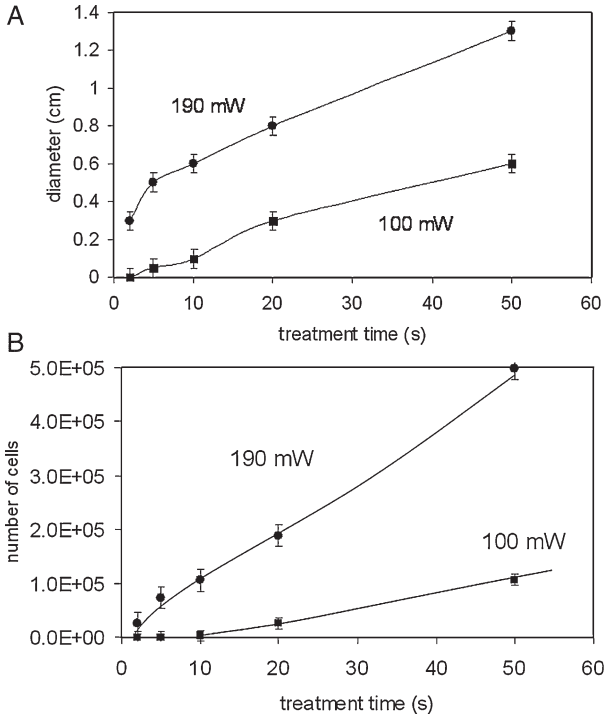

Figure 6. The BAEC cells (24 h after treatment). The damaged cells were removed from areas affected by the plasma treatment; the diameter of the affected areas (voids) was measured. A: The diameter of the void as a function of the plasma power and the treatment time. B: The total number of removed cells in function of plasma power and plasma treatment time.

area, coinciding with the point of highest irradiation. This was not the secondary necrosis (permeabilisation of apoptotic cells) because the nuclear DNA of dead cells was not fragmented. Thus, higher dosages of plasma treatment must have led to (delayed) cell death that was different from apoptosis. Since plasma treatment was indirect (membrane-mediated), no heat effects were observed, and necrosis was not instantaneous, we can exclude cell death due to desiccation and heat. A possible mechanism may be related to depletion of cellular energy resources (ATP), as described by Villena et al.[15] This depletion can lead to apoptosis or necrosis, dependent on the severity of the effect. The time scale on which cell death occurs is comparable to our situation. Depletion of cellular ATP can result from ROS-induced damage,[15] oxygen deficiency and many other types of cell damage and poisoning. Therefore, it is plausible that cell poisoning by plasma species is responsible for apoptosis at lower doses and for necrosis at higher. We have to stress that necrosis occurs only after prolonged treatment at higher powers. Cold atmospheric plasma does not induce necrosis at low dosages, and as such, it does not contribute to inflammation in treatment of arterial walls. The nature of plasma-induced effect in case of endothelial cells (BAEC) is not yet resolved. We can only state that the cell condition is totally different from apoptosis and necrosis. Cells appear to be impermeant (intact membranes), but they do not display any activities such as movement or proliferation. Their morphology is also changed (rounded shape) and they are loosely attached to the membrane. This feature is reminiscent of the first stages of apoptosis, but apparently the BAEC cells remain in this stage without proceeding into later phases. We observed similar effects in cancerous epithelial cells, which did not undergo apoptosis but were inactivated in a comparable way to BAEC.[16] A phenomenon called "cell retraction" in research on X-ray radiography may provide a clue to explain plasma effects on endothelial cells. In the study of cell retraction by Onoda et al.,[17] mouse pulmonary microvascular (endothelial) cells were found to display morphological changes, loss of cell-to-cell contact, and severe cytoskeletal damage after receiving high dosages of X-ray irradiation. Although in this particular work[17] the role of oxidants was not mentioned explicitly, oxidative stress is a known effect of X-ray irradiation,[18] and a known damage factor. Damage induced by plasma species may be of similar nature to the X-ray induced cytoskeletal damage. However, at this stage we cannot decide whether cell retraction due to X-ray has the same mechanism as the observed plasma inactivation of BAEC cells. Independent of the precise pathway of injury, plasma effect on BAEC is different from necrosis. In this respect, plasma is suitable for non-inflammatory treatment of this type of cells. It is striking that the addition of large amounts of antioxidant (L-ascorbic acid) had no significant effect on cell damage. In our previous studies[14] we established that the level of ROS induced by plasma treatment is of the order of several μM, and we used 0.1×10^{-3} M concentrations of L-ascorbic acid to protect the samples from oxidation. In the current experiment, the concentration of the antioxidant was 30 times higher (3×10^{-3} M); yet it did not prevent cell damage. In this view, we have to revise the working hypothesis of cell damage due to plasma-released ROS. Although oxidants are the prime candidates for damage factors that lead to apoptosis and necrosis,[19] in this case, the plasma must release another cell injury agent. In our previous mass spectrometric studies, we found that our plasma contained RNS (especially atomic nitrogen and nitric oxide), and their densities were 5–100 times higher than ROS densities.[6] It is plausible that RNS like NO are responsible for apoptosis in A7r5 and damage in BAEC, while the role of ROS and oxidative stress is of lower importance.

Cell Detachment

There is one significant difference between these results and our previous findings.[10,11] The immediate cell detachment that was ubiquitous in our previous experiments did not occur here. The latter fact brings a new

insight into the mechanism of cell detachment after CAP treatment. In our earlier observations, cells detached readily only when exposed directly to the plasma glow. The effect was induced even by very short irradiation (1–2 s), and occurred during or immediately after treatment. Similar effects were also observed by other authors.[20] The presence of supernatant greatly reduced this effect, e.g. when the thickness of supernatant was more than 0.1–0.2 mm, no detachment occurred.[10] This was tentatively ascribed to limited penetration of active plasma species in the fluid, but the responsible species and the nature of their interactions with cells were not specified. Here, in our membrane-bound models instantaneous detachment did not occur at all. On the other hand, plasma effects on cells (other than detachment) were evident, which means that certain active species from the plasma were transmitted through the membrane. This means that agents responsible for detachment can be totally blocked by the membrane or by a layer of supernatant. Furthermore, we observed previously that instantaneous detachment was not cell-dependent; it occurred in all investigated cell types, and when the exposure time to CAP was short (<5s) the cells were unharmed and could reattach. This indicates that i) detachment is a physical rather than biological process, and ii) it is caused by certain short-living plasma species that are not particularly toxic. We postulate that cell charging with plasma electrons induces instantaneous cell detachment observed in our previous experiments (without membrane and without fluid). Negative electric charging of objects in direct contact with the plasma glow is a commonly known phenomenon that causes e.g. electric confinement of dust particles in complex plasmas.[21] In our case, a living cell can be seen as a non-spherical conducting dust particle. When it is exposed directly to the glow, it becomes negatively charged by the incoming plasma species. This superficial charging cannot be disposed of immediately, because the cell is placed on an insulating surface (Petri dish). When a conducting object is charged, the charge will spread on the surface. In contrast to solid dust particles, the cell is a deformable object, which can assume another shape if it results in a lower energy. To minimize the electrostatic energy (due to Coulomb repulsion of the charge), the cell should become spherical. However, change of shape can occur only if the electrostatic energy gain is higher than the energy needed to detach from the surface and to deform the cytoskeleton. Below we shall roughly estimate the energy gain for the charged cell when it changes its shape from attached (roughly ellipsoidal) to detached (spherical). We shall use a very simple electrostatic approach, and assume that the cell does not change its volume during deformation (no swelling etc.). We shall approximate the shape of an attached cell by an ellipsoid with typical dimensions of about 40 μm length, 10 μm width and 5 μm thickness.

The electrostatic energy of a chargeable object with a capacitance C at the potential V is given by:

$$E = \frac{1}{2}CV^2 \tag{1}$$

Thus, when the cell is exposed to the field in the plasma sheath, it will try to minimize its capacitance. For the potential V, we shall take a typical value for the potential drop in the plasma sheath (V_{plasma}) at the grounded object, which is at least 5 V according to simulations by Sakiyama and Graves.[22] Now we shall evaluate the reduction of the electrostatic energy after reshaping:

$$\begin{aligned}\Delta E &= E_{\text{sphere}} - E_{\text{ellipsoid}} \\ &= \frac{1}{2}(C_{\text{sphere}} - C_{\text{ellipsoid}})V_{\text{plasma}}^2\end{aligned} \tag{2}$$

The capacitance of a sphere with a radius R is obviously $4\pi\varepsilon\varepsilon_0 R$, and the one of an ellipsoid can be calculated from:

$$C_{\text{ellipsoid}} = \frac{8\pi\varepsilon\varepsilon_0}{\int_0^\infty ((t+a^2)(t+b^2)(t+c^2))^{-1/2}\mathrm{d}t} \tag{3}$$

where a, b, c are the three principal radii. This integral can be solved numerically, but it is easier to estimate the capacitance using the equivalent radii approach, as proposed by Shumpert and Galloway,[23] i.e. $C_{\text{ellipsoid}} = 4\pi\varepsilon\varepsilon_0 R_{\text{eq}}(a,b,c)$ where the equivalent radius is a function of the ellipsoid dimensions. In our case $a \approx 5\cdot10^{-6}$, $b \approx 2.5\cdot10^{-6}$ and $c \approx 2\cdot10^{-5}$ m; thus $R_{\text{eq}} \approx 8\cdot10^{-6}$ m.[23] Assuming a constant volume during reshaping, the radius of the spherical cell will become $R = (abc)^{1/3} \approx 6.3\cdot10^{-6}$ m. For ε we shall take the vacuum/air dielectric constant ($\varepsilon = 1$) because we assume that the cell is directly exposed to plasma (thus, in contact with gas phase). Finally:

$$\Delta E = 2\pi\varepsilon_0 V_{\text{plasma}}^2 (R - R_{\text{eq}}) \approx -2.4\cdot10^{-15} J \tag{4}$$

Similarly, we can evaluate the charge deposited on the (spherical) cell:

$$Q = C_{\text{sphere}} V_{\text{plasma}} \approx 3.5\cdot10^{-15} C \approx 2.2\cdot10^{4} e \tag{5}$$

Note that this is a typical number of elementary charges on a few μm large dust particle in a dusty plasma.[24] The energy gain of the cell after changing its shape from attached to spherical is respectably high. Now we shall compare it with typical energies needed to detach the cell and to deform its cytoskeleton. Cell adhesion to the substrate and to other cells is mediated by cell adhesion molecules. Detachment energies vary dependent on the

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cell type and binding type. Typical integrin-mediated adhesion energy is about $1.4 \cdot 10^{-6}$ J $\cdot$ m^{-2}.[25] A single cell occupies a surface of about $3 \cdot 10^{-10}$ m^2, as can be obtained from the experimental cell density of $3.7 \cdot 10^9$ cells $\cdot$ m^{-2}, or from the projection surface of an ellipsoid with the above-mentioned dimensions. The resulting adhesion energy would be (maximally) $4 \cdot 10^{-16}$ J per cell. The mechanics of cytoskeletal deformation was extensively studied in relation to erythrocyte transport through capillary vessels.[26] We shall use the average values on deformation energy for this type of cell. Typical values for the compression moduli (spring constants K) associated with cytoskeletal stretching or compression are of the order of 10^{-6} J $\cdot$ m^{-2}. The corresponding deformation energy for a displacement $\Delta R = R - R_{eq}$ is about $(1/2)K\Delta R^2$, in our case maximally $2 \cdot 10^{-17}$ J per cell. Other methods yield similar or lower estimates (10^{-18}–10^{-17} J).[27] Both the cell adhesion energy and the deformation energy are lower than the electrostatic energy (10^{-15} J) that can be gained by cell reshaping. Therefore, it is plausible that the cell will detach when exposed to the plasma and charged by plasma electrons. Of course, cell deformation after charging is much more complex than presented here: one could take into account membrane stretching, cell swelling, intracellular charge transport and relaxation, and many other effects. The above calculation serves merely as an order-of-magnitude estimation, while the whole phenomenon should be modelled using more sophisticated (numerical) techniques.

Conclusion

We observed delayed effects caused by cold plasma treatments on vascular cells. Cell treatments with micro-plasmas were performed indirectly, using a porous cell culture membrane as an interface. The effects manifested themselves 6 to 8 h after the plasma treatment, and were strictly localized in the area addressed with the micro-plasma. We distinguished between apoptosis, necrosis and cell inactivation that was reminiscent of "retraction" reported elsewhere. The experiment brought insight in the mechanism of cell detachment observed in our previous works. Detachment was ascribed to electrostatic interactions of plasma with cells. This results obtained from this simple in vitro model indicate that cold atmospheric plasma is capable of non-inflammatory treatment of arterial walls.

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Keywords: apoptosis; cold atmospheric plasma; vascular cells

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