Low-temperature plasma-induced antiproliferative effects on multi-cellular tumor spheroids

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Abstract

Biomedical applications of low-temperature plasmas are of growing interest, especially in the field of plasma-induced anti-tumor effects. The present work is aimed at investigating the regionalized antiproliferative effects of low-temperature plasmas on a multicellular tumor spheroid (MCTS), a model that mimics the 3D organization and regionalization of a microtumor region. We report that a low-temperature plasma jet, using helium flow in open air, inhibits HCT116 colon carcinoma MCTS growth in a dose-dependent manner. This growth inhibition is associated with the loss of Ki67, and the regionalized accumulation of DNA damage detected by histone H2AX phosphorylation. This regionalized genotoxic effect leads to massive cell death and loss of the MCTS proliferative region. The use of reactive oxygen species (ROS), scavenger N-acetyl cysteine (NAC) and plasma-conditioned media demonstrate that the ROS generated in the media after exposure to low-temperature plasma play a major role in these observed effects. These findings strengthen the interest in the use of MCTS for the evaluation of antiproliferative strategies, and open new perspectives for studies dedicated to demonstrate the potential of low-temperature plasma in cancer therapy.
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Keywords: low temperature plasma, multi-cellular tumor spheroid, antiproliferative effect, genotoxic and cytotoxic effects, reactive oxygen species

1. Introduction

In the field of biomedical applications, low-temperature plasmas ejected in open air are an interesting source of active species (charged particles, radicals, long-lived excited species, UV photons, electric field, etc) that can easily be launched, for example, on any prokaryote or eukaryote cells, living tissues, biomaterial surfaces, etc. Such plasma species have been known for many years for their bactericide action useful in decontamination or sterilization [1], and also more recently for wound healing and cell regeneration [2], biomaterial functionalization [3], blood coagulation [4], and gene transfection [5]. The reader can find more exhaustive lists of references on these biomedical applications (for example, in [6–8]) and also on plasma-induced anti-tumor effects that are evocated, hereafter, in the light of a few examples of the literature.

Indeed nowadays, there is a growing interest on research works devoted to the effect of low-temperature plasmas at atmospheric pressure on cancer cells, both in vitro and in vivo. Recent studies have shown, more particularly, a plasma-induced cell cycle arrest and activation of apoptosis in glioma and colorectal carcinoma HCT116 colon cancer cells [9, 10], an activation of p53-dependent cell death in plasma-exposed HCT116 [11], and a mitochondria-mediated apoptosis in the case of human cervical cancer HeLa cells [12]. It has also been suggested that the plasma effect could eradicate lung and/or murine melanoma cancer cells with reduced damaging effects on normal or fibroblast cells [13, 14]. Finally, in vivo reduction of mice xenografted tumors from the bladder [13], glioma cells [10] and pancreatic cancer cells [15] transcutaneously exposed to non-thermal plasma has also been reported.

However, despite the great interest of these works, none provide precise information on the penetration depth, or the species generated by the plasma into the tumor tissue medium, or on the analysis of the effects on cells located, not only on the surface layer of a cancer cell culture, but also inside the tumor tissue volume.

Multicellular tumor spheroid (MCTS) is a 3D culture model that accurately reproduces the organization and the heterogeneity of a microtumor domain with cell–cell and cell–microenvironment interactions [16–20]. The use of this model is currently recognized as an important step in the development of anti-cancer strategies between the studies performed on classical monolayer cultured cells and animal models [21]. The present work is aimed at investigating the regionalized antiproliferative effects of low-temperature plasma on the various layers of MCTS.

2. Materials and methods

2.1. Low-temperature plasma generation

The low-temperature plasma jet is produced by a dielectric barrier discharge configuration already detailed elsewhere [22]. In short, aluminum tape electrodes having a 20 mm width are
wrapped on a quartz tube with a 4 mm inner diameter and a 6 mm outer diameter, and separated by a 10 mm space. Helium gas flows through the quartz tube with a flow rate of 3 liter min$^{-1}$. High-voltage mono-polar square pulses are applied on the powered electrode with specific voltage magnitude, repetition rate and pulse width. A schematic set-up of the plasma jet device is displayed in figure 1(a), while figure 1(b) shows an example of plasma exposure of a spheroid placed inside a well of 96-round bottom well plates. The volume of the media per well = 100 μl. NB: the plasma device displayed horizontally in figure 1(a) is disposed vertically in this picture.

The gas temperature measured from classical thermo-couple and emission spectroscopy based on the OH(A-X) spectrum is around 30 °C due to the very low power consumption required to generate the plasma jet.

The low-temperature plasma jet propagates first inside the tube in a carrier gas flow dominated by the helium concentration. Then, as expected, from the exit of the quartz tube the helium is progressively diluted in the ambient air. For a gas flow of 3 liter min$^{-1}$, the proportion of helium decreases to 90% in the air at a distance of $z=2$ cm from the tube exit. In fact, $z=2$ cm corresponds to the constant distance chosen between the output of the glass tube and the culture medium of spheroids during plasma exposure, as shown in figure 1(b). At this distance ($z=2$ cm), the dilution of helium plasma in the air leads to a rich population in the gaseous phase of more particularly reactive oxygen species (ROS), such as atomic oxygen,
ozone, hydroxyl radical, nitrogen oxide, hydrogen peroxide and several long-lived excited species of molecular and atomic oxygen. They directly interact with the culture medium, immersing the studied spheroid inside each well of the 96-well plates. Such interactions lead, in turn, to the generation of ROS in liquid phase, as, for instance, hydrogen peroxide with hydroxyl, super-oxide and nitrogen oxide radicals.

In the two following sub-sections (2.2) and (2.3), an overview of some physical aspects of the plasma and the liquid medium have been highlighted. An estimation of the plasma dose is given, and the emission spectra of the culture medium exposed to the plasma is analyzed in order to identify some plasma species diffusing in the liquid. The reader can find a detailed description of the present plasma jet in reference [22], and an interesting review on many other physical aspects concerning the plasma surface interactions in the case of various set-ups of low-temperature plasmas in reference [23].

2.2. Power flow for the estimation of the plasma dose arriving on the surface of the culture medium

Usually the power flow (W cm\(^{-2}\)) needed to estimate the plasma doses (J cm\(^{-2}\)) is overestimated in the literature (few hundreds of mW cm\(^{-2}\)). This is due to the power flow determination from the integration of the measured instantaneous current (maximum of a few tenths of A) and voltage (few kV) [22] between the electrodes. In fact, the plasma power flow has to be determined outside the glass tube in open air at the level of the surface of the culture medium. However, due to the lack of experimental information on the specific current and voltage of the plasma jet outside the tube in the downstream flow far from the electrodes, we have chosen to use an alternative validated model of plasma jet dynamics [22] to quantify the plasma power flow.

The estimation of the power flow (in W cm\(^{-2}\)) deposited by the plasma jet on a target surface of, for instance, the culture medium that is positioned at a distance \(z\) from the tube outlet, is performed as follows.

(i) The power density \(p(t, z)\) (expressed in W cm\(^{-3}\)) versus time \(t\) and axial position \(z\) is first calculated from:

\[
p(t, z) = \tilde{j}(t, z) \cdot \tilde{E}(t, z)\left[\text{W cm}^{-3}\right]
\]

\(\tilde{j}(t, z)\) is the total current density with a contribution of positive and negative charged species, and \(\tilde{E}(t, z)\) is the total electric field involved in the plasma jet dynamics. \(\tilde{j}(t, z)\) and \(\tilde{E}(t, z)\) are determined from the model of ionization wave dynamics already described and experimentally validated elsewhere [22].

(ii) For each time \(t\), we calculate, using equation (2), the power inside the plasma ionization wave, i.e. inside a small cylinder volume of \(R = 1\) mm radius and \(\Delta z = 1\) mm length centred on the position of the maximum power density. Indeed, the curves plotted in figure 2(a) show that at a given time \(t\), the maximum power density is mainly confined in a volume of 1 mm extension along the \(z\)-axis, while the radial extension \(R\) is chosen close to the quartz tube radius.
Figure 2. (a) Power density versus axial position from the glass tube outlet at different instants ($\Delta t = 80$ ns) during plasma jet propagation in open air. (b) Schematic representation of the energy flowing through a plane perpendicular to the plasma ionization wave propagation. (c) Power flow versus axial position from the glass tube outlet during the plasma jet propagation in open air.
\[ P(t, z_{\text{max}}) = \pi R^2 \int_{z_{\text{max}}(t) - \Delta z/2}^{z_{\text{max}}(t) + \Delta z/2} p(t, z)\,dz \quad [W] \]

\(z_{\text{max}}(t)\) is the position of the maximum power density at time \(t\). The main assumption is to consider that the power density is constant along the radial direction inside the considered volume.

(iii) If we put a fictive plane perpendicularly to the \(z\)-axis at \(z = z_{\text{max}}(t) + \Delta z/2\), the energy at time \(t\) that is confined inside a plasma bullet just on the left-hand side of the plane may be found fully on the other side of the plane after a delay time \(\Delta t = \frac{v_{\text{bul}}(t)}{\Delta z}\) where \(v_{\text{bul}}(t)\) is the plasma bullet velocity at time \(t\) (see figure 2(b)). \(\nu(t)\) is determined from the model of ionization wave dynamics [22]. Therefore, the energy that crosses the plane through the surface \(S = \pi R^2\) is expressed as follow:

\[ \epsilon(t) = \frac{P(t)\Delta t}{S} \quad [J. \, cm^{-2}] \]  

This energy (per unit surface) crosses the plane one time per voltage pulse. Therefore, if we multiply \(\epsilon(t)\) with the power supply pulse frequency \(f = 1/T = 9.69 \, kHz\), we obtain the power flow for several positions of the plane along the \(z\)-axis. The result is displayed in figure 2(c).

In the case of the position of our interest corresponding to \(z = 2\, cm\) where the culture medium is exposed to the plasma, the plasma power flow is equal to \(0.5\, mW \, cm^{-2}\).

2.3. Spectra of excited species from both plasma jet and culture media exposed to the plasma jet

This sub-section aims to give an insight into the plasma jet excited species diffusing inside the liquid medium, and therefore the ability to directly or indirectly impact the spheroid. For this aim, a specific experiment has been performed to collect spectra of excited species of both plasma jet in open air and liquid medium exposed to the plasma jet.

Lateral emissions of the plasma jet are collected by a 2x magnification optical system offering a 1 mm spatial resolution. It is mounted on a sliding stand to perform a scan along the plasma jet \(z\)-axis (displayed in figure 1(a)). The emitted photons are guided into the entrance slit (slit wide = 200 \(\mu m\)) of the spectrophotometer (0.75 m V MC: Acton Spectra Pro 2750i, in the Czerny–Turner configuration) via a 3 m long optical fiber (OF: UV-Silicon LG-455-020-3). The detecting device is a CCD camera (CCD: PIXIS -100, 256 \times 1024 Imaging array 20 \(\mu m \times 20 \mu m\) pixels) placed at the exit port of the spectrophotometer. The spectral domain lies within 200 nm and 920 nm. The spectra are displayed without any correction for the wavelength dependence of the relevant detection device. For UV emissions (i.e. 200 nm to 400 nm) a grating of 2400 grooves \(mm^{-1}\) is used and air is a natural high-pass filter. For 400 nm to 900 nm spectral ranges, we used a grating of 1800 grooves \(mm^{-1}\), with a high-pass filter being either the Corning 0–53 (cutting wavelength at 340 nm) or the MTO J517a (cutting wavelength at 517 nm).

Excited species produced during the interaction between plasma jet and liquid culture medium are detected by putting the liquid inside a specific well made by synthetic fused silica glass transparent for UV to near infra-red radiations (200 nm to 1000 nm).
The optical fiber collecting the radiations up to the spectrometer slit (200 μm for opening) is placed near the well and the emissions of excited species are collected for two positions. The first position targets the plasma jet in open air just above the liquid medium (a height h ≈ 0 mm above the culture medium). The second position targeted is h = 5 mm (i.e. inside the liquid) where the optical fiber collects the radiations emitted by the culture medium when exposed to the plasma jet. The aim is to identify the spectra of the excited plasma species inside the liquid medium, and also the spectra of the plasma excited species generated in open air before to impact the culture medium. Obviously, the correlation with the presence or absence of the specific ROS underlies such a comparison.

Figures 3(a) and (b) display a selection of collected spectra in UV range (200 nm to 400 nm). The OH(A-X) molecular spectrum is present only in the gaseous plasma stage (h = 0 mm). This means that the OH radical is chemically transformed inside the liquid medium and does not react directly with the spheroid. This is in accordance with literature measurements [24] based on electron spin resonance spectroscopy, confirming that the radical OH, which is unstable (i.e. short live time), is not present in the liquid medium when exposed to low-temperature plasma. Indeed, OH can be detected unless it is blocked by using spin-trapping techniques [24]. Furthermore, the intensities of the molecular band spectra of the second...
positive system of nitrogen N$_2$(SPS) for different band heads (313.6 nm, 315.9 nm, 337.1 nm, etc), and the first negative system N$_2$(FNS) at 391.4 nm, are much higher for plasma jets in the open air (h = 0 mm) than inside the liquid medium, where these nitrogen molecular bands remain present but with a lesser intensity.

Figures 4(a) and (b) display a selection of spectra in the visible range. The presence of a molecular band of He$_2$, with the head at 639.8 nm and the emission lines related to, respectively, H$_\alpha$ (656.2 nm), He (for instance at 667.8 nm or 706.5 nm) and the triplet of atomic oxygen close to 777 nm, is noteworthy. Contrary to the OH(A-X) emission that is completed quenched in the liquid medium, the emission line O(777 nm) shows that the atomic oxygen is present in the liquid medium, but with a lesser intensity than in the gaseous phase, and can therefore directly interact with the spheroids.

2.4. Cell culture and spheroids preparation

HCT116 colorectal cancer cells (ATCC) were cultured in DMEM (Invitrogen) containing 10% FCS with 2 mM l$^{-1}$ glutamine and penicillin/streptomycin in a humidified atmosphere of 5% CO$_2$ at 37 °C. Spheroids were prepared by centrifugation method in low attachment multi-well
plates, as previously described [25]. Briefly, exponentially growing cells were harvested and
were distributed in poly-HEMA-coated 96-round bottom well plates (500 cells/well). Plates
were centrifuged (600 g for 6 min), and then placed in a humidified atmosphere of 5% CO2 at
37 °C. Aggregation of the cells occurred within 24 h, and the diameter of the growing spheroids
was measured over time with a calibrated eyepiece reticule until they reached the expected size
needed to start a given experiment.

2.5. Immunofluorescence on spheroid cryosections

Spheroids were fixed in formalin (Sigma) for 2 to 3 h, then washed with phosphate buffered
saline (PBS) and stored at 4 °C. After fixation, spheroids were incubated in 15% and then 30%
sucrose in PBS at 4 °C for 24 h, embedded in Tissue-Tek (Sakura Finetek) and then processed
for 5 μm cryosections. After blocking in PBS/1% BSA/0.5% Triton, sections were incubated
with antibodies against Ki67 (rabbit polyclonal, Santa Cruz, 1/200 at 4 °C, overnight), cleaved
PARP (rabbit monoclonal, Epitomics, 1/1000 at 37 °C, for 1 h), phosphorylated Histone H2AX
(mouse monoclonal, Millipore, 1/500 overnight at 4 °C). After washes in PBS/0.1% Triton v/v,
the secondary antibody was added for 1 h (anti-mouse or anti-rabbit conjugated with Alexa 488,
Alexa 594 or Alexa 647, Molecular Probes, 1/800, at room temperature). DNA was stained
using 4′,6′-diamidino-2-phenylindole (DAPI). An initial antigen retrieval step (boiling in a
solution containing 2 mM citric acid and 8 mM sodium citrate for 3 × 7 min) was included for the
anti-Ki67 antibodies.

2.6. Image acquisition and analysis

Transmitted light images of spheroids were acquired using a MacroFluo Z16 APO microscope
(Leica) fitted with a CoolSNAP ES2 CCD camera (Roper). Fluorescence images of 5 μm-
spheroid sections were acquired using a DM5000 (Leica) epifluorescence microscope,
fitted with a Roper COOLsnap ES CCD camera. Images were processed using the Metavue and
ImageJ software packages.

2.7. Time-lapse experiments

Transmitted light images of spheroids were acquired using an inverted microscope Axiovert
(Zeiss) under environmentally-controlled conditions (5% CO2, 37 °C) fitted with a CoolSNAP
ES2 CCD camera (Roper) and equipped with a 10X objective.

3. Results

3.1. Exposure of MCTS to low-temperature plasma

Figure 1 displays the low-temperature plasma jet generation procedure, and the application of
the plasma jet on the spheroids grown in 96-wells plates in a 100 μl culture media volume. The
present low-temperature plasma involves pure helium (4.6 purity degree) carrier gas. It is
known that there is a large rate of ROS in the case of plasma generated in pure helium gas flow,
due more particularly to the highest electron energy in comparison to the case of carrier gas
involving additive molecular gas for instance. With this experimental setup, spheroids, one per
well of a plate, could be exposed to different plasma doses by controlling the exposure time of
Figure 5. Plasma exposure inhibits the growth of MCTS. (a) The time-course of the growth increase in HCT116 spheroids after plasma exposure at the indicated doses. The spheroids were cultured in DMEM for three days and then subjected to plasma exposure (day 0). The volume of each spheroid was measured the day of exposure and at the indicated days. The volume increase (ratio between the volume at day n after exposure over the initial volume at the day 0) is plotted as a function of time. The results correspond to the mean of three to four spheroids per condition. (b) Micrographs illustrating the relative variation in the volume of the HCT116 spheroid one, four and seven days after plasma exposure at the indicated doses. Scale bar, 100 μm.
each well. A masking plate (not shown in figure 1(b)) was always used to avoid cross-exposure between neighboring wells during plasma treatment. It is noteworthy that for the chosen position $z = 2\, \text{cm}$ between the 96-wells plates and the plasma jet tube output (see figure 1(b)), the estimated power flow (as shown in section 2.2) is about $0.5\, \text{mWatt cm}^{-2}$ in the case of the considered operating parameters of the power supply ($8.67\, \text{kV}$ for voltage magnitude with $9.69\, \text{kHz}$ and $2\, \mu\text{s}$ for pulse duration and frequency) [22]. Therefore the plasma dose in mJoule cm$^{-2}$ (or mJ cm$^{-2}$) is determined by multiplying this power density ($0.5\, \text{mW cm}^{-2}$) by the exposure time, thus giving, for instance, $30\,\text{mJ cm}^{-2}$ in the case of an exposure time of $60\,\text{sec}$.

### 3.2. Low-temperature plasma inhibits MCTS growth

Colon carcinoma HCT116 multicellular tumor spheroids of $400\,\mu\text{m}$ in diameter were prepared as described in the methods section, and exposed to low-temperature plasma. We first evaluated the impact of low-temperature plasma on spheroid growth. To this aim, we subjected HCT116 spheroids to He plasma exposure at duration times varying between 0 to 150 sec, corresponding to plasma doses ranging from 0 to $75\,\text{mJ cm}^{-2}$. These exposure times and plasma doses were selected on the basis of a prior DL50 (dose inhibiting 50% of growth) determination performed on the same HCT116 cell line grown as 2D monolayer ($40\,\text{mJ cm}^{-2}$ that corresponds to $80\,\text{s}$ of plasma exposure time). Spheroids measuring $400\,\mu\text{m}$ in diameter were subjected to plasma exposure. At that size, HCT116 spheroids already display a slight proliferation gradient with quiescent cells in the most inner cell layers, and do not yet display a necrotic core that will appear at later stages of growth. Variation of HCT116 spheroid volume was monitored during 8 days following exposure, and shown in figure 5(a). During the time of the analysis of the impact of plasma on spheroids growth, control spheroids continued to grow. As the spheroids size increased, cell heterogeneity inside the spheroids also increased with a more pronounced cell proliferation gradient. The progressive size increase was accompanied by the progressive appearance of cell death in the inner cells of the spheroids at the end of the experiment, as already published elsewhere [26]. This growth dynamic exactly corresponds to previously reported observations (see references [16–19] and [20]). Spheroid volume increase was strongly inhibited in a dose-dependent manner. This result was particularly obvious following plasma exposure to a dose above $30\,\text{mJ cm}^{-2}$ that totally inhibited spheroid growth, since a volume reduction was induced on the first day after plasma exposure. The series of micrographs taken at intervals (figure 5(b)) further confirmed these observations, illustrating partial inhibition at low dose and the total absence of growth of spheroids exposed to low-temperature plasma dose above $30\,\text{mJ cm}^{-2}$. In addition, with high doses the overall structure of the spheroids appeared modified with a fuzzy outer-most corona and a dense central region. The calculated DL50 on 3D spheroids growth was in the range of 30 to $35\,\text{mJ cm}^{-2}$, values similar to those observed in the case of 2D monolayer cells. In order to further characterize the growth inhibitory effect of low-temperature helium plasma, the expression of the proliferation marker Ki67 was analyzed by immunofluorescence on spheroid cryosections (figure 6(a)). Ki67 labeling was found to be largely reduced in a dose dependent manner (figure 6(a) and data not shown), and strongly decreased in the MCTS section examined 16 and 24 h after plasma exposure to $60\,\text{mJ cm}^{-2}$. However, surprisingly, no apoptotic cells were detected using PARP-C staining within spheroids exposed to the same plasma regimen (figure 6(b)), indicating that in our experimental
Figure 6. Anti-proliferative, apoptotic and genotoxic effects of exposure to plasma in MCTS. (a) Visualization of the proliferative cells (Ki67, green), on 5 μm cryosections from control HCT116 spheroids or spheroids harvested and fixed 4, 16 or 24 h after exposure to helium plasma at the indicated doses. (b) Immunodetection of the apoptotic marker, cleaved-PARP (C-PARP, green), on 5 μm cryosections from control HCT116 spheroids or spheroids fixed 4, 16 or 24 h after exposure to plasma at the indicated doses. (c) The genotoxic effect due to plasma exposure was analysed by immunodetection of the phosphorylation of the histone variant H2AX (phospho-H2AX, green) on 5 μm cryosections of control HCT116 MCTS or MCTS subjected to 2.5 or 60 mJ cm$^{-2}$ harvested and fixed 4, 8 or 24 h after exposure. The nuclei were stained using DAPI (blue). Representative images from six to eight spheroids from two independent experiments are shown. Scale bar, 100 μm.
conditions, growth inhibition was clearly associated with cell proliferation inhibition but not with detectable apoptosis.

3.3. MCTS growth inhibition is associated with regionalized DNA damage

The DNA damaging effect of the exposure of MCTS to low-temperature plasma was investigated by immunodetection of the phosphorylated form of histone H2AX on spheroid cryosections. As displayed in figure 6(c), phosphorylated histone H2AX was strongly detected in spheroids 4 h after exposure. The staining was extremely intense in the outmost cell layers of the spheroid exposed to the highest plasma dose. Strikingly, this staining was detected at the earliest time-point, but was progressively lost after 16 h and was not detectable after 24 h. On sections from spheroids 16 h after a 60 mJ cm\(^{-2}\) exposure, the outer-most cells appeared to be less organized at the periphery. In the meantime, spheroid diameter appeared reduced, and the fuzzy outmost region previously mentioned (figure 5) was lost. Altogether, these data suggested that the spheroids were peeled off the outmost layers of cells that had DNA damage induced by plasma exposure.

3.4. Time-lapse monitoring of the effect of plasma reveals destruction of the spheroid proliferative region

To obtain more insight into the mechanisms that could result in the observed rapid disappearance of the phospho-H2AXlabeled cells, we performed videomicroscopy monitoring during 24 h of HCT116 spheroids exposed to various doses of low-temperature helium plasma. To this aim, spheroids were exposed to plasma in 96-wells plates and immediately transferred on the stage of a fully motorized videomicroscope under a temperature and CO\(_2\) controlled atmosphere. Figure 7 displays images that were extracted from representative movies recorded with untreated and 60 mJ cm\(^{-2}\) plasma treated spheroids. Movies displaying the observations made with control and gradual plasma doses (2.5, 30 and 60 mJ cm\(^{-2}\)) are provided as online supplementary material (available from stacks.iop.org/NJP/16/043027/mmedia). As shown on these images and on these movies, the outmost cell layers of the spheroids exposed to plasma were progressively disorganized with a corona of cells that disaggregated. In fact, all these dying cells that were loosely associated with the spheroid were lost during the process of fixation and paraffin embedding, and were therefore not detected in the previous experiments. These dying cells therefore correspond to the proliferative layers of the spheroid that becomes phospho-H2AX positive at the earliest time point after DNA damage induced by plasma treatment. However, because these dying cells are lost during the fixation and staining process, they are not detected on cryosections stained for proliferation, DNA damage and apoptosis markers from spheroids eight hours after the plasma treatment.

3.5. Growth inhibition and DNA damage are dependent on ROS

A known feature of low-temperature plasma is the generation of highly reactive species. In order to investigate the role of ROS in the observed growth inhibitory effect and DNA damage induction, we used the ROS scavenger N-Acetyl Cysteine (NAC) in the following experiments. We first examined, over time, the growth of HCT116 spheroids exposed to increasing doses of low-temperature helium plasma in the presence of NAC (10 mM). As shown in figure 8(a), in the presence of NAC plasma exposure did not inhibit spheroid growth even at the
higher doses. This result was confirmed by the examination of Ki67 staining on spheroids frozen sections (figure 8(b)). As displayed, there was no inhibition of Ki67 staining in the proliferative layers, with the exception of the spheroids exposed to a plasma dose of 60 mJ cm$^{-2}$ in which a slight lowering of the percentage of Ki67 cells was detectable.

Strikingly, the DNA damaging effect of low-temperature helium plasma was also fully reversed by NAC as presented in figure 8(c). In all the tested conditions, no increase in phosphorylated-histone H2AX staining was detected on spheroid sections upon treatment. Altogether, these data further confirm that reactive oxygen species are involved in the growth inhibition and in DNA injury observed in HCT116 MCTS exposed to low-temperature plasma.

3.6. Reactive species accumulated in the culture media are responsible for growth inhibition and DNA damage

First, we examined whether growth inhibition and DNA damage were observed upon exposure to conditioned media. To this aim, conditioned media was prepared by exposing the 96-wells plate that did not contain spheroids to the plasma. The media exposed to increasing doses of low-temperature helium plasma was then used to assess its effect on the induction of DNA damage. As illustrated with the representative spheroid sections shown in figure 9(a), treatment of HCT116 spheroids with conditioned media resulted in the induction of DNA damage. Thus, these data indicate that the effect of low-temperature plasma on multicellular spheroid growth is at least in part mediated by reactive species that are accumulated in the culture media.

Secondly, we examined whether the observed spheroid growth inhibition was reversible upon removal of the spheroid from the exposed culture media. To this aim, HCT116 spheroids

![Figure 7. Time-lapse monitoring of the effect of MCTS exposure to low-temperature plasma. Time-lapse video microscopy analysis of spheroids exposed to plasma and grown at 37 °C under CO2 controlled atmosphere. The figure shows representative transmitted light images from video microscopy records (see supplementary movies) performed on a control spheroid (top) or on a spheroid subjected to exposure to plasma at 60 mJ cm$^{-2}$ (bottom). Each column corresponds to the indicated time following exposure.](image-url)
Figure 8. Effect of ROS inhibition on plasma-induced growth inhibition and DNA damage on MCTS. (a) Time-course of the growth increase of HCT116 spheroids after plasma exposure at indicated doses in the presence of 10 mM of NAC in the culture media. The spheroids were cultured in DMEM for three days and then subjected to plasma exposition (day 0). The control corresponds to spheroids grown in the presence of NAC and left untreated. The volume of each spheroid was measured on the day of exposure and at the indicated days. The volume increase (the ratio between the volume at day n after exposure over the initial volume at the day 0) is plotted as a function of time. The results correspond to the mean of three to four spheroids per condition. Data corresponds to the mean of six independent spheroids for each condition. (b) Visualisation of the proliferative cells (Ki67, green) on 5 μm cryosections from control spheroids or spheroids 4 or 24 h after exposure to plasma at 2.5 or 60 mJ cm$^{-2}$ in the presence of NAC. (c) The genotoxic effect of plasma exposure in the presence of NAC in the culture media detected by immunodetection of the phosphorylation of the histone variant H2AX (phospho-H2AX, green) on 5 μm cryosections from control spheroids or spheroids 4 or 24 h after exposure to plasma at 2.5 or 60 mJ cm$^{-2}$. Nuclei were stained using DAPI (blue). Scale bar, 100 μm.
were exposed to increasing doses of low-temperature plasma. Then, after 72 h, the culture medium was replaced by a fresh medium. Growth of the MCTS was subsequently monitored under contrast microscopy (figure 9(b)). Just after culture media replacement (t = 0 h), spheroids exposed to the highest plasma doses displayed the growth inhibitory effect (smaller diameter) and the peeling of the outmost layers already described above (figure 9(b)). Twenty-four hours after media change, no obvious modification was observed, but at four and six days, we observed that spheroid diameter had increased in all exposure conditions. This result shows that spheroids resumed growth when removed from the medium that has been exposed to plasma effect (figure 9(b) and data not shown).

4. Discussion

The effects of low-temperature helium plasmas have been evaluated on HCT116 MCTS, a 3D model representative of the organization and regionalization of microtumors.

We report a strong link between the growth inhibition of MCTS volume and the plasma dose. Indeed, plasma doses ranging from 0 to 75 mJ cm\(^{-2}\) were applied to HCT116 spheroids and resulted in partial growth inhibition for the lowest doses, while the total growth inhibition was from 30 mJ cm\(^{-2}\) plasma exposure. Furthermore, growth inhibition has been correlated with the detection of a regionalized DNA damaging effect detected by immunodetection of the phosphorylated form of histone H2AX. The deleterious consequence of that genotoxic effect was observed by video microscopy, which showed an obvious disorganization of the outmost layers of the MCTS. The latter were gradually disaggregated, leading to a progressive decrease in the MCTS volume. In fact, the spheroid cells of the outmost layers were progressively dying, and released from the spheroid over time. This observation explains why the C-PARP staining presented in figure 6 did not reveal any apoptosis on plasma-exposed MCTS. Thus, our results obtained on 3D spheroids are in agreement with the literature showing that direct exposure to air plasma generated from an anodic nozzle [12] or dielectric barrier discharge (DBD) setup with a floating electrode [11] results in cell apoptosis. However, it is important to note that we used a remote helium plasma jet delivering lower deposited energy in comparison, for instance, to the DBD setup with a floating electrode already used for in vitro cell exposure but with the analysis in a 2D configuration [11, 27]. Furthermore, our results also demonstrate that the DNA damaging effect and the subsequent cell death and growth inhibitory effects are regionalized when helium plasma is applied on a 3D microtumor.

Reactive oxygen species have been reported to be involved in the anti-tumoral effect of low-temperature plasma [27–29]. Here from experiments with and without the NAC scavenger, we show that ROS are responsible for the DNA damaging effect observed in the outmost cell layers up to a certain depth inside the MCTS, depending on the applied plasma dose. It is noteworthy that ROS generated by the plasma jet and diffusing in a liquid medium include those usually emphasized in the literature, and also long-lived excited species and atomic oxygen, as shown from the emission spectroscopy in section 2.3. In fact, in comparison to previous works, the originality of this study is to clearly show in a 3D model the penetration depth inside the MCTS of the effects of the reactive species, such as ROS, generated in the culture media. At 60 mJ cm\(^{-2}\) of plasma exposure, DNA damage was induced in about one-third of the external cell layers of the MCTS. Moreover, the reversion of the spheroid growth inhibitory effect in the presence of a classical NAC, whatever the considered plasma dose,
Figure 9. ROS accumulated in the culture media are responsible for growth inhibition and DNA damage induced in MCTS by exposure to plasma. (a) Spheroids were cultured in DMEM for three days and the media was then replaced by culture media that had just been exposed to 2.5 or 60 mJ cm$^{-2}$. DNA damage was visualized by immunodetection of phospho-H2AX (green) on cryosections from spheroids fixed four hours following media replacement. Nuclei were stained using DAPI. (b) Following exposure to the indicated plasma dose, the culture media was replaced by fresh DMEM media. Micrographs correspond to representative transmitted light images of spheroids just before media replacement, 24 h or 6 days following culture in fresh DMEM. Scale bar, 100 μm.
clearly showed that the growth inhibition of the MCTS is due to the ROS accumulated in the culture media when exposed to the low-temperature plasma. Strikingly, we also show that the conditioned culture media that was first exposed to the low-temperature plasma jet prior to the immersion of MCTS has biological effects similar to the direct exposure of MCTS to low-temperature plasma. Thus, it is clear that ROS involved in the observed antiproliferative effect of plasma on MCTS are first generated in the culture medium (liquid phase) in order to react with MCTS. Obviously, such ROS are generated in the media through the interactions of the gaseous low-temperature plasma with such liquid media. Following partial information in the literature [11, 27], the ROS generated in the media can be, for instance, hydrogen peroxide (H₂O₂), superoxide (O₂⁻), nitrogen oxides with other long-lived excited species and also atomic oxygen, as shown from the emission spectroscopy in section 2.3.

Altogether, our results open various attractive perspectives in the field of low-temperature plasma for antiproliferative purposes. Future studies are needed to identify, from appropriate diagnostic tools (e.g. infra-red and Raman spectroscopy, mass spectrometry), the reactive species involved in the biological effect and the relation with the type of plasma technology. On the other hand, we have shown that the observed effects are regionalized and restricted to the outer layers of the MCTS that are most directly exposed to the plasma effect, which indicate that, from a therapeutic perspective, local application of plasma will have to be appropriately scheduled in a repetitive manner to obtain total elimination of the tumor cells. Furthermore, we have also shown that the MTC5 immersed in a plasma preconditioned media experienced the same anti-proliferative effects as MCTS immersed in culture media exposed to the low-temperature plasma. This finding also opens another potential method for the treatment of micro-tumors using local injections of the preconditioned media.

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References

[1] Laroussi M 2002 Non-thermal decontamination of biological media by atmospheric pressure plasmas: review, analysis, and prospects EEE Trans. Plasma Sci. 30 1409–15
[2] Heinlin J, Morfill G, Landthaler M, Stolz W, Ishary G, Zimmermann J, Shimizu T and Karrer S 2010 Plasma medicine: possible applications in dermatology J. Ger. Soc. Dermatology 8 968–76
[3] Desmet T, Morent R, De Geyter N, Leys C, Schacht E and Dubruel P 2009 Nonthermal plasma technology as a versatile strategy for polymeric biomaterials surface modification: a review Biomacromolecules 10 2351–78
[4] Fridman G, Friedman G, Gutsol A, Shekhter A B, Vasilets V N and Fridman A 2008 Applied plasma medicine Plasma Process. Polym. 5 503–33
[5] Ogawa Y, Morikawa N, Ohkubo-Suzuki A, Miyoshi S, Arakawa H, Kita Y and Nishimura N 2005 An epoch-making application of discharge plasma phenomenon to gene-transfer Biotechnol. Bioeng. 92 865–70
[6] Yousfi M, Merbahi N, Pathak A and Eichwald O 2014 Low-temperature plasmas at atmospheric pressure: toward new pharmaceutical treatments in medicine Fundam.: Clin. Pharmacology 28 123–5

[7] Kong M G, Kroesen G, Morfill G, Nosenko T, Shimizu T, Dijk J V and Zimmermann J L 2009 Plasma medicine: an introductory review New J. Phys. 11 115012

[8] Ishaq M, Evans M M and Ostrikov K K 2014 Effect of atmospheric gas plasmas on cancer cell signaling Int. J. Cancer 134 1517–28

[9] Koritzer J, Boxhammer V, Schafer A, Shimizu T, Klampfl T G and Li Y F 2013 Restoration of sensitivity in chemo-resistant glioma cells by cold atmospheric plasma PLoS One 8 e64498

[10] Vandamme M, Robert E, Lerondel S, Sarron V, Ries D, Dozias S et al 2012 ROS implication in a new antitumor strategy based on non-thermal plasma Int. J. Cancer 130 2185–94

[11] Tuhvatulin A I, Sysolyatina E V, Scheblyakov D V, Logunov D Y, Vasiliev M M, Yurova M A et al 2012 Non-thermal Plasma Causes p53-Dependent Apoptosis in Human Colon Carcinoma Cells Acta Naturae 4 82–7

[12] Ahn H J, Kim K, Kim G, moon E, Yang S S and Lee J S 2011 Atmospheric pressure plasma jet induces apoptosis involving mitochondria via generation of free radicals PLoS One 6 e28154

[13] Keidar M, Walk R, Shashurin A, Srinivasan P, Sandler A, Dasgupta S, Ravi R, Guerrero-Preston R and Trink B 2011 Cold plasma selectivity and the possibility of a paradigm shift in cancer therapy Br J. Cancer 105 1295–301

[14] Kim J Y, Kim S O, Wei Y and Li J 2010 A flexible cold micro-plasma jet using biocompatible dielectric tubes for cancer therapy Appl. Phys. Lett. 96 203701

[15] Brulé L, Vandamme M, Ries D, Martel E, Robert E, Lerondel S, Trichet V, Richard S and Pouveles J-M 2012 Effects of a non thermal plasma treatment alone or in combination with gemcitabine in a MIA PaCa2-luc orthotopic pancreatic carcinoma model PLoS One 7 e52653

[16] Desoize B and Jardillier J 2000 Multicellular resistance: a paradigm for clinical resistance? Crit. Rev. Oncol. Hematol 36 193–207

[17] Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W and Kunz-Schughart L A 2010 Multicellular tumor spheroids: an underestimated tool is catching up again J. Biotechnol. 148 3–15

[18] Sutherland R M 1988 Cell and environment interactions in tumor microregions: the multicell spheroid model Science 240 177–84

[19] Drasdo D and Höhme S 2005 A single-cell-based model of tumor growth in vitro: monolayers and spheroids Phys. Biol. 2 133–47

[20] Drasdo D and Hoehme S 2012 Modeling the impact of granular embedding media, and pulling versus pushing cells on growing cell clones New J. Phys. 14 055025

[21] Pampaloni F, Reynaud E G and Stelzer E H 2007 The third dimension bridges the gap between cell culture and live tissue Nat. Rev. Mol. Cell Biol. 8 839–45

[22] Yousfi M, Eichwald O, Merbahi N and Jomaa N 2012 Analysis of ionization wave dynamics in low-temperature plasma jets from fluid modeling supported by experimental investigations Plasma Sources Sci. Technol. 21 045003

[23] Ostrikov K, Neyts E C and Meyyappan M 2013 Plasma nanoscience: from nano-solids in plasmas to nanoplasmas in solids Adv. Phys. 62 113–224

[24] Tani A, Ono Y, Fukui S, Ikawa S and Kitano K 2012 Free radicals induced in aqueous solution by non-contact atmospheric-pressure cold plasma App. Phy. Lett. 100 254103

[25] Laurent J, Frongia C, Cazales M, Mondésert O, Ducommun B and Lobjois V 2013 Multicellular tumor spheroid models to explore cell cycle checkpoints in 3D BMC Cancer 13 73

[26] Lobjois V, Frongia C, Jozan S, Truchet I and Valette 2009 A Cell cycle and apoptotic effects of SAHA are regulated by the cellular microenvironment in HCT116 multicellular tumour spheroids Eur. J. Cancer 45 2402–11

[27] Kalghatgi S, Kelly C M, Cerchar E, Torabi B, Alekseev O, Fridman A, Friedman G and Azizkhan-Clifford J 2011 Effects of non-thermal plasma on mammalian cells PLoS One 6 e16270
[28] Graves D B 2012 The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology *J. Phys. D: Appl. Phys.* **45** 263001

[29] Zhao S *et al* 2013 Atmospheric pressure room temperature plasma jets facilitate oxidative and nitrative stress and lead to endoplasmic reticulum stress dependent apoptosis in HepG2 cells *PLoS One* **8** e73665