Plasma-Induced Death of HepG2 Cancer Cells: Intracellular Effects of Reactive Species

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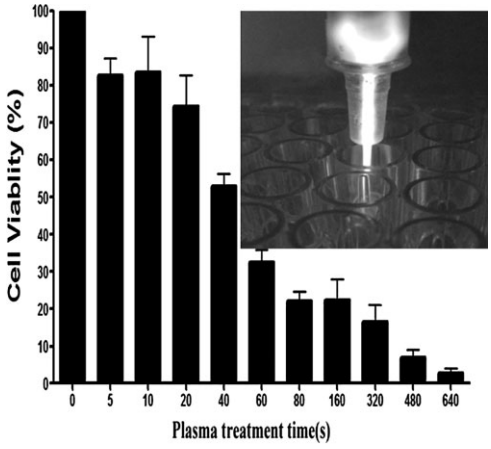
Reports show that cold atmospheric-pressure plasmas can induce death of cancer cells in several minutes. However, very little is presently known about the mechanism of the plasma-induced death of cancer cells. In this paper, an atmospheric-pressure plasma plume is used to treat HepG2 cells. The experimental results show that the plasma can effectively control the intracellular concentrations of ROS, NO and lipid peroxide. It is shown that these concentrations are directly related to the mechanism of the HepG2 death, which involves several stages. First, the plasma generates NO species, which increases the NO concentration in the extracellular medium. Second, the intracellular NO concentration is increased due to the NO diffusion from the medium. Third, an increase in the intracellular NO concentration leads to the increase of the intracellular ROS concentration. Fourth, the increased oxidative stress results in more effective lipid peroxidation and consequently, cell injury. The combined action of NO, ROS and lipid peroxide species eventually results in the HepG2 cell death.



1. Introduction

Non-equilibrium plasmas have been used for a long time for sterilization of medical instruments, bacterial inactivation, blood coagulation, wound healing, oral hygiene, etc.[1–13] Recently, with the rapid advances in the multidisciplinary research areas of cold atmospheric-pressure plasmas and plasma health care/medicine, interactions of such low-temperature, non-equilibrium plasmas with a large number of biologic objects, have attracted a major attention.[14–24] These objects include but are not limited to eukaryotic (mammalian) and prokaryotic (bacterial) cells, viruses, spores, fungi, DNA, lipids, proteins, cell membranes, as well as living human, animal, and plant tissues and organs. Of particular interest are the plasma interactions with cancerous cells. It has been shown by several groups that the plasma is able to induce death (the programmed death, apoptosis or the necrotic cell rupture) in a number of cancer cell types.[25–31] This offers exciting prospects for clinical applications of cold atmospheric plasmas for aggressive treatment of malignant cells and

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ultimately as a viable alternative to the present-day interventional oncology that is capable of cancer resolution without surgery. However, very little is presently known about specific mechanisms of the plasma-induced death of cancer cells.

The apoptosis, a self-regulated cell death, can be triggered via various extrinsic and intrinsic signaling pathways. In contrast to necrosis, which is caused by profound cellular injury accompanied by rapid cell swelling and lysis, the apoptosis is morphologically characterized by the cytoskeleton disruption, cell shrinkage, rapid outgrowth of a plasma or nuclear membrane, as well as nuclei fragmentation.[32] The apoptotic cellular response is executed by the caspase family of cysteine proteases via the activation cascade as well as by nucleases. The apoptotic responses are effectively controlled by a number of pro- and anti-apoptotic molecules.[33]

It is well known that atmospheric-pressure plasmas can generate nitric oxide (NO), reactive oxygen species (ROS), and some other species. On the other hand, cells can also produce NO, a short-lived radical gas, which acts as a messenger in most mammalian organs. The NO radicals participate in vascular homeostasis, neurotransmission, antimicrobial defense,[34] as well as immune system regulation and host defense processes.[35] This reactive gas can cause apoptosis, necrosis or, alternatively, protect the cells from death, depending on the cell type, radical concentration, as well as the duration and specific areas of the exposure.[36]

In addition, cells can also produce ROS, which play a critical role in cancer cell apoptosis, can induce damage to lipids, proteins, DNA molecules, and eventually cause cell death.[37] Intracellular ROS are generated by highly respiring mitochondria and peroxides.[38,39] Excessive production of ROS, such as superoxide anion, hydroxyl radical, and hydrogen peroxide, may either directly damage the cellular structure to cause cell necrosis or indirectly affect normal cellular signaling pathways and gene regulation to induce apoptosis.[40] Various stimuli such as anticancer drugs and chemopreventive agents are used to stimulate cells to produce excessive amounts of ROS and subsequently induce apoptotic responses.

Importantly, NO and ROS radicals can be produced either by the cells themselves or by the plasmas. Therefore, the NO and ROS generated by the cold atmospheric-pressure plasmas may affect the concentration of NO and ROS inside the cells during the plasma-cell interactions.

An increased intracellular concentration of ROS (larger oxidative stress) in turn causes lipid peroxidation (LPO) leading to profound cellular injuries. The ROS attack the side chains of unsaturated fatty acids of the membrane lipids, which results in the formation of lipid hydroperoxides.[41–43] Accumulation of the lipid hydroperoxides in cell membranes disrupts their normal functions and can lead to the membrane collapse. This in turn results in profound leaks and a major loss of selective permeability,[44] eventually triggering cellular apoptosis or necrosis.

Our previous studies show that the plasma treatment can increase the percentage of apoptotic cells, which is associated with the cell cycle arrest at the G2/M phase by modulating the genes related to the cell cycle and apoptosis.[31] In this paper, investigations into the temporal dynamics of the intracellular concentrations of the ROS, NO and lipid peroxide species after the non-equilibrium plasma treatment are carried out to further understand the mechanisms of the plasma-induced cell death. The results indicate that the plasma-enhanced production of NO, ROS and lipid peroxide species in the intracellular space is intimately related to the effective HepG2 cell death.

2. Experimental Section

2.1. Cell Line and Cell Culture

Human hepatocellular carcinoma cells (HepG2) were purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China). The HepG2 cells were maintained in a high-glucose Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY), supplemented with 10% (v/v) fetal calf serum (FCS; SiJi Qing, Hangzhou, China) at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2.

2.2. In Vitro Plasma Treatment

A single-electrode plasma jet device is used to generate the non-equilibrium plasma plume. The high voltage (HV) wire electrode, which is made of a copper wire with a diameter of 2 mm, is inserted into a 4 cm long quartz tube with one end closed. The inner and outer diameters of the quartz tube are 2 and 4 mm, respectively. The quartz tube along with the HV electrode is inserted into a hollow barrel of a syringe. The diameter of the hollow barrel is about 6 mm and the diameter of the syringe nozzle is about 1.2 mm. The distance between the tip of the HV electrode and the nozzle is 1 cm. When helium with a flow rate of $2\,L \cdot min^{-1}$ is injected into the hollow barrel and the HV pulsed DC voltage is applied to the HV electrodes, the room-temperature plasma plume is generated in the surrounding air along the nozzle axis. The length of the plasma plume can be adjusted by the gas flow rate and the amplitude, frequency, and pulse duration of the applied voltage. A detailed description of the experimental setup can be found elsewhere.[16] Figure 1(a) and (b) are the schematic and the photograph of the plasma jet device. In the experiments reported in this paper, the pulse frequency, pulse width t_{pw}, and the rms amplitude of the applied voltage were fixed at 8 kHz, 1.6 μs, and 8 kV, respectively. The current generated by the plasma source has two distinct current pulses per applied voltage at both the rising edge and the falling edge of the voltage pulse with a peak value of approximately 300 mA.

Before the plasma treatment, the medium was changed to phosphate-buffered saline (PBS, calcium-free, 7.9 g of NaCl, 1.8 g

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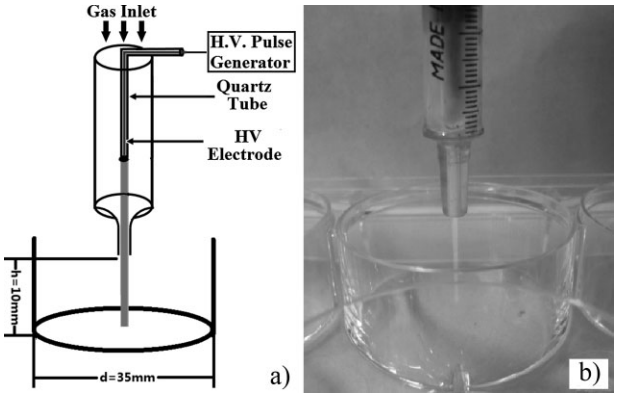

Figure 1. Schematic of the atmospheric-pressure non-equilibrium plasma jet device used in the HepG2 carcinoma cell treatment. (a) The schematic of the plasma jet device and the 6 well; (b) the photograph of the plasma jet device and the 6 well.

of K_2HPO_4, 0.24 g of KH_2PO_4, and 0.2 g of KCl per 1 L of distilled water). The thickness of the liquid layer in this series of experiments was ≈1 mm. Each well of 6-well cluster dishes was placed right under the nozzle during the treatment. The distance between the nozzle and the surface of the PBS was fixed to 10 mm. The working gas He/O_2 (1%) with a flow rate of 1 $L \cdot min^{-1}$ was used. Addition of a small amount of oxygen was motivated by the previous success of atmospheric plasmas of helium–oxygen gas mixtures in inactivation of bacterial cells.[6] The experiment was carried out at room temperature. Immediately after the treatment, the PBS was removed, and then fresh culture medium was added to the dishes and returned to the CO_2 incubator. The control cells were subjected to the identical procedure without turning on the plasma. After being cultured for additional 24 h (unless stated otherwise), the cells were used in the following experiments. The thickness (≈1 mm) of the liquids (PBS/medium) in each well was carefully selected to avoid the undesirable drying effects even after 640 s (and even longer) of the plasma treatment. To show that gas blow does not have any significant effect on the cell viability, the untreated control samples and the gas blow control samples were compared, with no significant difference found between them. This procedure has been used in our previous works.[30,31]

2.3. Cell Viability Assay

The HepG2 cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) method. The assay depends on the reduction of the MTT (Sigma, Inc.) to a blue-black formazan product by living rather than dead cells. Dimethylsulfoxide (DMSO) is the best solvent for dissolving the formazan product. A solution of the MTT formazan in DMSO (Sigma, Inc.) changes the intensity of the absorbance spectrum of the solution. The optical absorption of the solution with dissolved formazan correlates with the number of living cells. If the cell growth is inhibited, the reduction of MTT to formazan will decrease, thus the optical density will reduce accordingly.

The HepG2 cells in 96-well plates at a density of $\approx 10^4$ cells per 96 well were treated by the plasma in processes of different durations. Three wells of a 96-well plate were treated per each plasma treatment time. After the plasma treatment, the cells were continuously cultured for 24 h (unless stated otherwise). Then 20 μl of MTT was dissolved in a PBS solution at a concentration of 5 $mg \cdot ml^{-1}$. The resulting solution was added to each well and subsequently incubated in a CO_2 incubator for 4 h. Finally, the medium was aspirated from each well and 100 μl of DMSO was added to dissolve formazan crystals. The optical density of each well was obtained using a Microplate Reader (Sunrise, Tecan) operated at a wavelength of 492 nm.

2.4. Intracellular NO Measurements

HepG2 cells were grown at a concentration of $\approx 2.5 \times 10^5$ cells per 6 well and allowed to attach for 8–12 h. After the plasma treatment, the cells were continuously cultured for 24 h (unless stated otherwise). The cells were washed twice with ice-cold PBS after removing the media, and then were lysed using cell lysis buffer (Beyotime, Jiangsu, China). The lysates were collected and centrifuged at 10 000 rpm at 4 °C for 10 min. The production of NO was measured by assaying NO_2^- in the supernatants using a calorimetric Griess reaction. The Griess Reagent System is based on a diazotization reaction which detects the presence of organic nitrite compounds. Nitrite is detected and analyzed through the formation of a red pink color upon treatment of a NO_2^--containing sample with the Griess reagent. The optical density of the red pink color solution is in turn related to the concentration of the NO_2^-. The nitrite detection kit was used according to the guidelines provided by the manufacturer (Beyotime). Fifty microliters of samples or standard $NaNO_2$ was incubated in a 96-well plate for 10 min using the equal volume of the Griess reagent at room temperature. The optical density was measured using spectrophotometry at 560 nm (Sunrise, Tecan). The optical absorbance measurements were then converted to moles of NO_2^- per well using a standard curve of $NaNO_2$. PBS was used as a buffer in this case as well, similar to other analyses in this series of experiments.

2.5. Detection of Intracellular ROS

The determination of concentrations of the ROS was based on the oxidation of 2,7-dichlorodihydrofluorescin (DCFH, Beyotime) and performed following the manufacturer's protocols. The 2,7-dichlorofluorescein diacetate (DCFH-DA) is a well-established compound to detect the presence and quantify the concentration of intracellular ROS. DCFH-DA can be transported across the cell membrane and deacetylated by esterases to form the non-fluorescent DCFH. This compound is trapped inside the cells. Then, DCFH is converted to the highly fluorescent compound DCF through the action of ROS, which can be detected and quantified by the fluorescence intensity. In brief, following the incubation for predetermined times after the plasma treatment, the cells were collected and washed with DMEM without FBS, and then incubated with DCFH-DA at 37 °C for 30 min. Dichlorofluorescein (DCF) fluorescence distribution was detected by fluorospectrophotometry analysis at an excitation wavelength of 488 nm and an emission wavelength of 525 nm (PerkinElmer LS55).

2.6. LPO Assay

The level of the LPO was determined by the amount of malondialdehyde (MDA) formed, which is the final product of the LPO process. The determination of the intracellular MDA level was based on the thiobarbituric acid (TBA) method. The MDA can react with TBA, and form stable thiobarbituric acid-reactive substances (TBARS), which absorb light at 532 nm. The concentration of MDA was assessed using a LPO assay kit (Beyotime) according to the manufacturer's guidelines. The lipid peroxide content was expressed as nmol of MDA per ml using a standard curve.

3. Results and Discussion

3.1. Reduction of HepG2 Cell Viability by the Plasma Treatment

The effect of the plasma treatment on the viability of HepG2 cells is shown in Figure 2. The data suggest that the percentage of the number of viable cells rapidly decreased (compared to the non-treated control sample) as the plasma exposure time increased from 5 to 640 s. The plasma treatment time required to reduce the cell viability by 50% (EC_{50}) was measured to be 34.75 s.

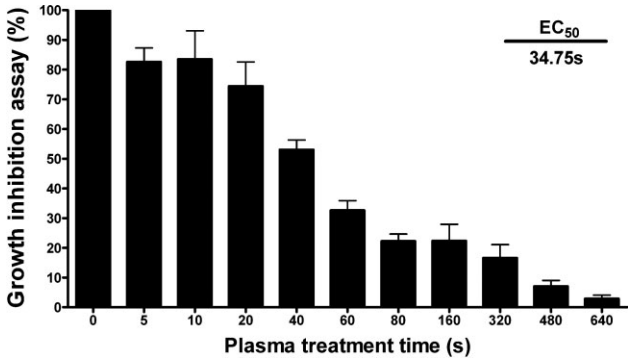

Figure 2. Effect of the plasma on the viability of HepG2 cells. Cell viability was determined by the MTT assay and was expressed as a mean value ± standard deviation (SD) of three separate experiments.

3.2. Intracellular NO Concentration 24 h After the Plasma Treatment

Figure 3(a) shows the intracellular NO concentration in the HepG2 cells after 24 h of the plasma treatment. When the time of the plasma treatment was varied from 120 to 960 s, the concentration of intracellular NO greatly increased. It is interesting to point out that the concentration of

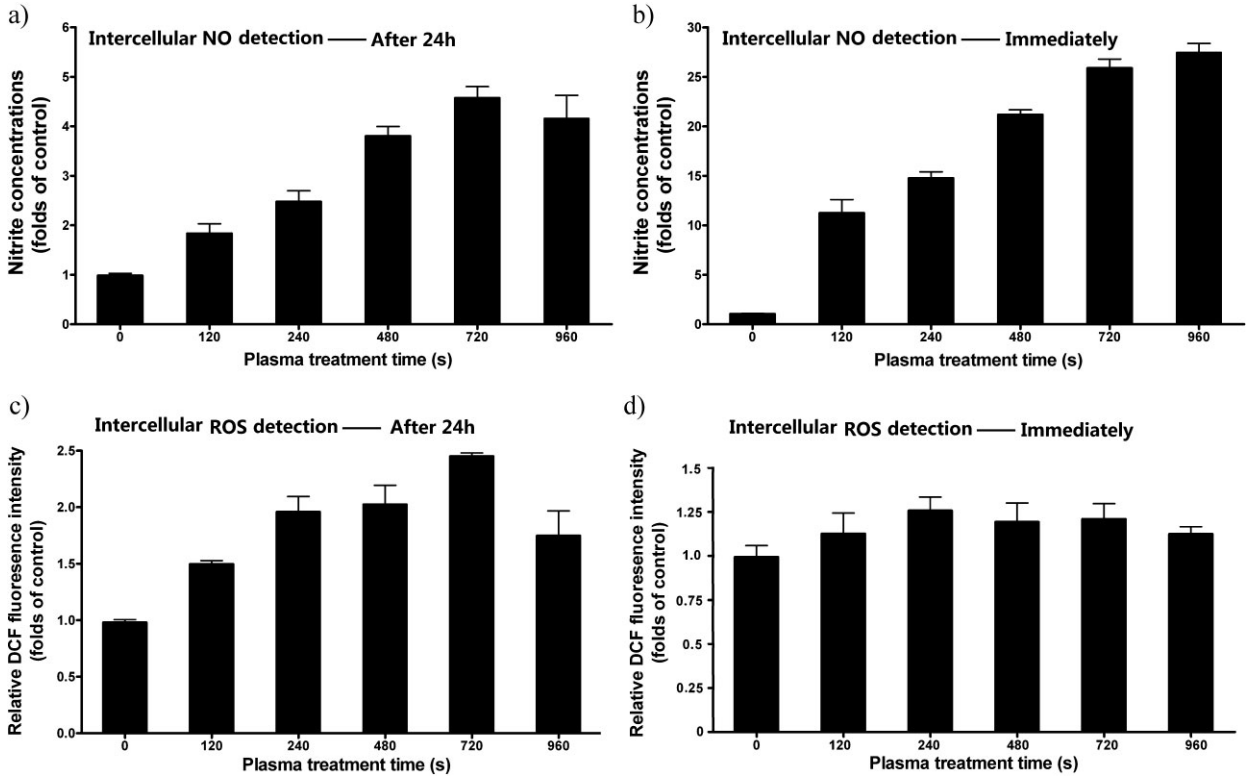

Figure 3. Variation of the intracellular NO and ROS concentration in HepG2 cells after 24 h and immediately by plasma treatment. (a) Effect of the plasma treatment on the intracellular NO concentration in HepG2 cells after 24 h culture; (b) same as in (a) for ROS; (c) same as in (a) but immediately after the plasma treatment; (d) same as in (b) but immediately after the plasma treatment. All the results are expressed as a mean value ± SD of three separate experiments.

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the intracellular NO species peaked at approximately 720 s into the plasma treatment. At this time, the percentage of the viable cells has decreased by more than one order of magnitude (Figure 2). Therefore, a high intracellular concentration of NO species appears well after effective inactivation of the overwhelming majority of the cells. Since dead cells cannot produce NO species, this remarkable increase can be attributed to the plasma production and transport of the NO radicals. A further decrease of the intracellular concentration of NO after 720 s of the plasma treatment suggests that some internal mechanisms (e.g., in the remaining viable cells or in the extracellular medium) lead to the conversion of a fraction of the NO radicals into some other radicals, possibly ROS.

3.3. Elevated Intracellular ROS Concentrations Induced by the Plasma

The cells were stained with DCFH-DA to examine the concentration of intracellular ROS in HepG2 cells. Fluorospectrophotometry was used to measure the DCF fluorescence. As shown in Figure 3(b), the level of ROS in the cells exposed to the plasma increased with the treatment time. Interestingly, the concentration of intracellular ROS also peaks at 720 s, similar to the results in Figure 3 for NO species. Thus, the trends of the concentrations of the NO and ROS radicals are very similar after 24 h culturing — a larger content on NO corresponds to the larger content of ROS and vice versa. This is consistent with the results of other authors suggesting that ROS can stimulate the intracellular production of NO and vice versa and several pathways (e.g., via mitochondrial channels) are considered viable.[45,46] Therefore, there is a possibility that a higher content of ROS after 24 h culturing is closely related to the effect of NO radicals; the data of the following sections will substantiate this. Nonetheless, at this point it is clear that the plasma treatment has significantly increased the intracellular presence of ROS in the HepG2 cells, which in turn may promote mitochondrial dysfunction and trigger mitochondria-mediated apoptosis. However, the question whether the larger intracellular concentrations of ROS were produced during the plasma exposure or during the culturing process is still open and will be addressed in the following sections.

3.4. Variation of Intracellular NO and ROS Concentrations Right after the Plasma Treatment

As pointed out above, the NO and ROS radicals can be produced either by the cells themselves or by the plasmas. The plasma plume that was used to treat the HepG2 cell cultures in open air using the $He + O_2$ (1%) working gas can effectively generate these species.

During the cold plasma treatment, the NO and ROS generated by the plasma can affect the NO and ROS concentrations both in the cell culture medium and within the cells. Therefore, the concentration of NO radicals should also be measured in the extracellular medium. However, the ROS concentration in the extracellular medium could not be measured directly by using the same method as for the intracellular ROS concentration, because of the cell presence. Therefore, in order to conclude whether the NO and ROS were generated by the plasma or by the cells themselves, we have measured the NO concentrations within the cells (immediately after the plasma-exposed medium is replaced) and in the as-replaced plasma-exposed medium immediately after the plasma treatment. All the procedures were the same except for the avoided cell culture for 24 h.

Figure 3(c) shows the intracellular concentration of NO species immediately after the plasma treatment. This measurement reveals that the intracellular NO concentration immediately after the plasma treatment is several times higher than that after culturing for 24 h. Since it takes time for the cells to produce NO, these results suggest that the NO species are more likely to be produced by the plasma rather than by the cells. It was previously reported that in water saline without hemoglobin, NO concentration fell slowly over 20 min with a half-life of 445 s.[47] This indicates that both the intercellular NO concentration and extracellular concentration generated by the plasma may in fact be higher than what is measured. Figure 3(c) also shows that the intracellular NO concentration reaches the highest value at 960 s rather than at 720 s. This trend is quite different compared to Figure 3 and indicates on the significant role of the plasma in the continued production and delivery of the reactive species.

Second, the intracellular concentration of the ROS was also carried out. Figure 3(d) shows the effects of the plasma exposure on the intracellular concentration of the ROS in the HepG2 cells immediately after the plasma treatment. As can be seen from Figure 3(d), there is no significant change of the DCF fluorescence intensity after the plasma treatment of 120–960 s. This trend is very different from the results in Figure 3(b). More importantly, these results suggest that the intracellular ROS are more likely generated by the cells during the 24 h cell culturing process rather than during the plasma treatment.

3.5. Effect on MDA Content

We have also studied the effect of the plasma treatment on the LPO in the cells. The measurements of the MDA content, which is widely accepted as a reliable indicator of the LPO level, have also been performed.[48,49] As shown in Figure 4, the level of MDA in the untreated control group was about 2.3 μM. In contrast, the plasma treatment led to a

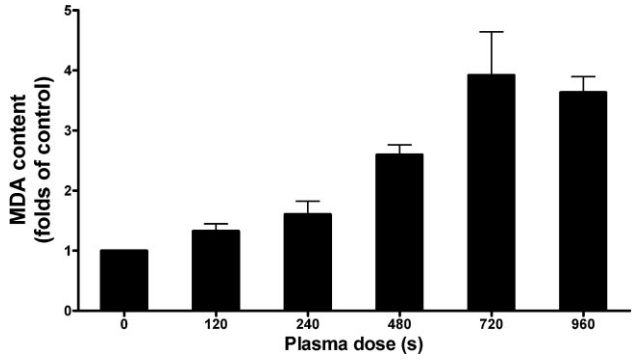

Figure 4. Malondialdehyde content in HepG2 cells treated with the plasma. The results are expressed as a mean value ± SD of three separate experiments.

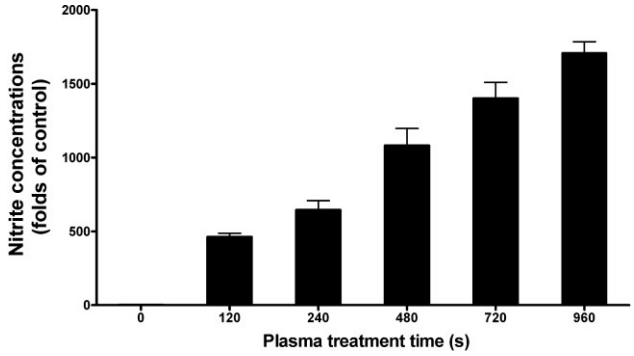

Figure 5. Effects of the plasma treatment on NO concentration in the plasma-processed cell culture medium immediately after the plasma treatment.

significant dose-dependent increase of the MDA. The MDA content increased in approximately four times when the HepG2 cells were treated by the plasma for 720 s. This is consistent with the increased intracellular concentrations of the ROS species [Figure 3(b)] and the oxidative stress they cause.

3.6. Plasma Exposure

It is important to point out that despite using 6 and 96 well configurations for different analyses, care was taken to ensure that cells were subjected to the same plasma exposure in all experiments. Indeed, according to the Paracelsus' rule (''only the dose makes a thing not a poison''), the dose of reactive species determines the desired biological or medical effect. Here we recall that for the cell viability assay experiments, 96 cell wells are used. For all other measurements, 6 cell wells are used.

However, the cell density ($\approx$30 000 cm^{-2}) and liquid thickness ($\approx$1 mm) for both 96 wells and 6 wells cases were the same. The exposed surface areas for the 96 and 6 wells were approximately 0.33 and 9.6 cm^{-2}, respectively. Therefore, in order to have the cells exposed by the same plasma dose, the treatment time in the 6 wells configuration was about 30 times longer than in the 96 wells case. In addition, during the treatment, the plasma plume was moved around the surface of the liquid to ensure uniform exposure in different areas. However, there is a possibility that the cell culture was a bit more susceptible to direct plasma exposure factors than in a 6 well configuration. This effect can be minimized by using the cell death and apoptosis (e.g., Annexin V/PI staining) assays under the same geometrical/volume conditions.

3.7. Variation of Extracellular NO Concentration after the Plasma Treatment

To verify the effect of the extracellular medium on the transport of NO and ROS radicals, in a separate set of experiments, we have measured the NO concentration in the plasma-exposed medium immediately after its replacement by a new medium and followed the similar procedures described above. Figure 5 shows that the NO concentration in the plasma-exposed medium is about five time higher than the intracellular NO concentration immediately after the plasma treatment. This indicates on the possibility of diffusion of NO radicals from the medium into the intracellular space.

Reactive oxygen species, such as $-$OH, O_2^-, H_2O_2, O_2^*, O, O^*, and several others are important signal mediators that regulate cell death.[50] They are broadly defined as oxygen-containing chemical species with reactive chemical properties.[51] ROS produced by a cell when stimulated by environmental stress or other factors are considered as potential signaling molecules.[52] The increase of the ROS concentration in the cells may cause DNA damage, genetic instability, cellular injury and eventually trigger cell death. Our results show that HepG2 treatment by the plasma indeed leads to the increase of the intracellular ROS concentration.

It is also well known that the presence of nitrogen-containing radicals is closely related to the oxidative stress produced by ROS.[52] NO could be biosynthesized endogenously from L-arginine, oxygen and NADPH by various nitric oxide synthase (NOS) enzymes.[53,54] The intracellular NO is a pleiotropic mediator and a signaling molecule involved in a large number of cell functions.[55] In some situations, NO activates the transduction pathways causing the cells to undergo apoptosis, whereas in other cases NO was found to protect cells against spontaneous or induced apoptosis.[56] In this study, we have found that the intracellular NO concentration increased significantly immediately after the plasma treatment. A striking observation [Figure 3(d)] was made that this is not the case for the intracellular ROS concentration.

This leads to the following conclusions. First, the plasma increases the NO concentration in the extracellular medium. Second, a very large difference between

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the NO concentrations in the medium (Figure 5) and within the cells [Figure 3(c)] leads to the strong diffusion of NO species from the medium into the cells. Thus, the intracellular NO concentration increases due to the diffusion. Third, the intracellular ROS concentration also increases since the nitrate tolerance of the cells is closely related to the oxidative stress caused by the ROS, as mentioned above. Finally, the increase of the intracellular ROS and NO concentrations eventually leads to the cell death.

There is considerable evidence that oxidative stress caused by the excessive ROS concentrations results in LPO, which may contribute to cell/tissue damage.[57] Apoptosis caused by LPO has been related to the activity of p53, which is best known as a tumor suppressor capable of triggering the cell-cycle arrest and apoptosis.[58] As pointed above, the MDA content was considered as a general indicator of the effectiveness of the LPO. In the present study, a significant increase in the MDA levels in HepG2 cells treated by the plasma was observed (Figure 4). This indicates that the cell treatment by the plasma leads to the LPO. According to the discussion above, this can be attributed to the increase of the intracellular concentrations of NO and ROS species. In order to conclusively confirm this possibility, further studies are required.

3.8. Cell Death: Apoptosis versus Necrosis

This study has reported on enabling effective cell death by atmospheric-pressure plasma treatment. However, specific mechanisms of the cell death require more detailed research. Indeed, a mere use of an MTT assay does not allow one to specify the predominant cell death pathway, for example, through the induction of apoptosis or necrosis. A clear differentiation between the cell death types is important, particularly because necrosis is often associated with unwarranted cell loss and can lead to local inflammation. The reported decrease in cell viability with longer plasma treatment time indicates that both mechanisms are in principle possible. On one hand, the intracellular NO and ROS-induced mechanisms can induce apoptosis; however these reactive species can also induce cell necrosis as it is shown that ROS can also kill cells by lysosomal membrane permeabilization.[59] Precisely tailored assays should be used to differentiate between the apoptotic and necrotic cell death pathways.[60]

4. Conclusion

In conclusion, we have studied the effect of the atmospheric-pressure plasmas on HepG2 cells. This study is the first to evaluate the duration of the plasma exposure (only 34.75 s) required to reduce the number of the viable HepG2 cells by 50%. This characteristic is similar to the drug/toxin potency in pharmacology and is of a significant interest for the clinical applications of non-equilibrium atmospheric-pressure plasmas.

It was shown that the plasma exposure was effective to control the concentrations of reactive NO and ROS species both in the extracellular medium and within the intracellular space. The increased concentrations of NO, ROS and lipid peroxide during the plasma exposure correlated with the decreasing numbers of viable cells. The measurements of the intracellular concentrations of NO and ROS radicals immediately after the plasma exposure and after 24 h culturing in a fresh medium, as well as the NO concentration in the plasma-exposed medium made it possible to elucidate the plausible death mechanism of the HepG2 cells.

The very large difference between the NO concentrations within the cells and in the extracellular medium was found immediately after the plasma treatment. Moreover, the ROS concentration within the cells did not change significantly even after 960 s of the plasma treatment. However, a major increase in the ROS concentration was measured after the 24 h culture. This indicates that the plasma delivers a significant amount of NO radicals to the extracellular medium, form where they diffuse into the cells. Excessive NO concentrations presumably lead to more effective ROS generation within the cells. This in turn increases the oxidative stress and the lipid peroxide levels. The enhanced LPO processes are the likely cause of the observed very strong (possibly apoptotic) cellular response. The specific mechanism of the so significantly increased intracellular ROS concentration still remains unclear and further studies are warranted.

Nevertheless, this study has proposed the mechanisms of the HepG2 death under exposure to cold atmospheric-pressure non-equilibrium plasmas. Our results contribute to the understanding of the mechanisms of cancer cell inactivation and as such are highly relevant to the development of the next-generation therapies and biomedical devices for the interventional oncology of the future.

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