

In situ application of non-thermal plasma: preliminary investigations for colorectal and lung tolerance

Marc VANDAMME^{1,2,3}, Eric ROBERT³, Julien SOBILO², Vanessa SARRON³, Delphine RIES³, Sébastien DOZIAS³, Brigitte LEGRAIN⁴, Stéphanie LERONDEL², Alain LE PAPE², Jean-Michel POUVESLE³.

1. GERMITEC, Clichy, France
2. TAAM-CIPA, UPS44 – CNRS, Orleans, France.
3. GREMI, UMR6606 – University of Orleans-CNRS, Orleans, France
4. NOVAXIA, ZA Petit Four, 41220 Saint Laurent Nouan

Contact: marc.vandamme@cnrs-orleans.fr

Abstract: Non-thermal plasma (NTP) has a significant antitumor activity *in vitro* on different cell lines including brain, colorectal and lung tumor. The necessary dose to kill 50% of cells was $\sim 9\text{J}/\text{cm}^2$ for these different cell lines. Reactive Oxygen Species (ROS) generated during treatment appears to be responsible of this effect and induce DNA damages together with a cell cycle arrest leading to an apoptosis induction. These promising results *in vitro* lead us to evaluate NTP *in vivo*. The tolerance study was conducted with NF-kB-luc reported mice. NF-kB is a transcription factor implied in inflammatory process and bioluminescence imaging of this strain of mice allows monitoring of inflammation intensity and time course. Tracheal intubation with Plasma Gun using a small catheter and low gas flow was successfully achieved. As a preliminary result, an induction of the inflammatory process was observed in the treated lung after 6 and 12 min of treatment. Concerning colorectal inflammation following FE-DBD treatment, NF-kB mice failed to reveal any inflammation in preliminary experiments. Further investigations are needed to elaborate a treatment protocol for lung and colorectal tumor treatment.

Keywords: Non-Thermal Plasma, Antitumor activity, Tolerance study

1. Introduction

Numerous studies have described an antitumor activity of NTP on different cell lines with an apoptosis induction using DBD or plasma jet [1-6]. Based on these interesting results, non-thermal plasma appears to be a potential new antitumor strategy for different tumor types. The main goal of this work was to investigate the *in vitro* plasma induced cell death mechanisms and to assess the possibility of *in situ* lung treatment using plasma gun and colon tissue using a FE-DBD.

2. Plasma sources

Two different sources were used in this study: a FE-DBD and a Plasma gun, previously described in [7] and [8].



Figure 1. Left: photography of the FE-DBD source, the discharge operates in air. Right: photography of the Plasma Gun source, the discharge operates in neon.

FE-DBD was applied in ambient air during different periods at a repetition rate of 200 Hz for *in vivo* treatment and at 2 kHz *in vitro*, the voltage pulse amplitude being of 25 kV, the pulse duration of 5 μs (fwhm). Discharge power density of our DBD was 0.52 watts at 2000 Hz, thus a 15s treatment using the 0.78 cm^2 insulated reactor lead to a dose of 10J/cm².

The plasma gun device is one among the very large variety of plasma jets. This plasma gun has two

specificities, a long distance plasma generation (a few tens of centimetres) through flexible capillary of various diameters and the use of very moderate rare gas flow rate down to a few sccm. These parameters appear to be very promising to evaluate potential of plasma gun as a new weapon against *in situ* tumor such as colorectal or lung tumor using endoscopic intervention.

Evaluation of antitumor activity was first performed *in vitro* on different cell lines along with underlying mechanism of NTP induces cell death. Cell lines used in this study was U87MG, a cell line of human glioblastoma, HCT116, a representative cell line of human colorectal carcinoma and H460, a model of lung carcinoma. All these cell lines were purchased transfected by luciferase gene from Caliper® (USA).

3. Antitumor activity of NTP *in vitro*

Antitumor activity of NTP generated by FE-DBD was evaluated *in vitro* by bioluminescence imaging which is an imaging modality dependent on cell proliferation and metabolism. NTP treatment of cells seeded in 24-well plates was performed in open air, 2 mm upper the medium of each well containing adherent cells and 500 μ L of medium.

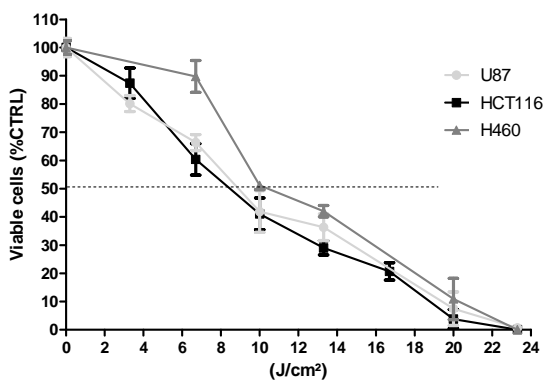


Figure 2. *In vitro* antitumor activity of NTP determined by BLI imaging.

A plasma dose-dependent cells death was observed on different cell lines including colorectal (HCT116), lung (H460) and brain tumor (U87). The DL₅₀ (dose required to kill 50% of cells) was ~9 J/cm² on U87, 8J/cm² on HCT116 and 10J/cm² on H460. Using doses >20J/cm², almost all cells were dead. A very similar DL₅₀ was observed for the 3

cell lines suggesting a common mechanism of action of NTP, whatever cell type considered.

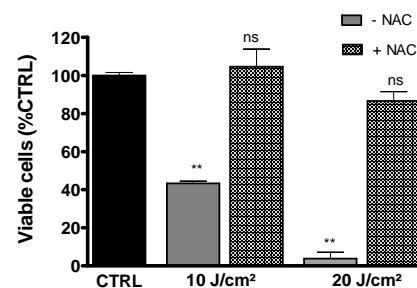


Figure 4. Evidence of ROS implication in the antitumor effect of NTP on HCT116 using NAC as a ROS scavenger.

The antitumor effect was completely lost when a ROS scavenger was used so confirming the implication of ROS in this effect (figure 3). ROS were widely described and these elements are potentially harmful on cell metabolism by affecting cell functions with a direct effect on cell development, growth, survival, as well as tumorigenesis [9].

Radiotherapy is a treatment modality based on interactions between ionizing radiation with atoms or molecules leading to the production of a large amount of free radicals in the vicinity of cells. These free radicals can react and damage protein, DNA and lipids [10], resulting mainly in DNA strand breaks formation. To assess DNA damages following NTP treatment, a specific marker (γ H₂AX) was used. Immunofluorescence of γ H₂AX was monitored by flow cytometer. To avoid visualization of DNA damages occurring during apoptosis process, γ H₂AX phosphorylation was monitored as soon as 1h after the treatment, whereas a minimal 3h delay is required for phosphorylation of γ H₂AX histone triggered by apoptosis associated DNA fragmentation [11].

As observed in figure 5, an induction of DNA damages was observed in the different phases of the cell cycle. A similar result with DNA damages induction was previously observed on a non-tumorigenic cell line. [3].

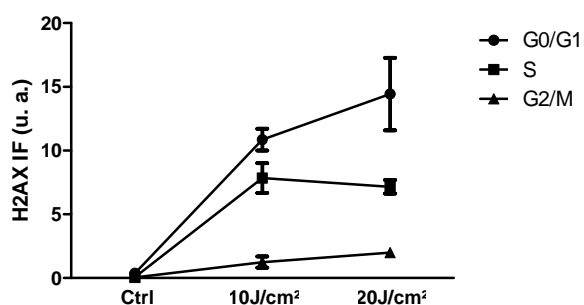


Figure 5. DNA damage induction on HCT116 in the different phase of the cell cycle after NTP treatment.

The evaluation of NTP consequences on cell cycle distribution and apoptosis induction was performed using a propidium iodide staining and annexin-V staining, respectively.

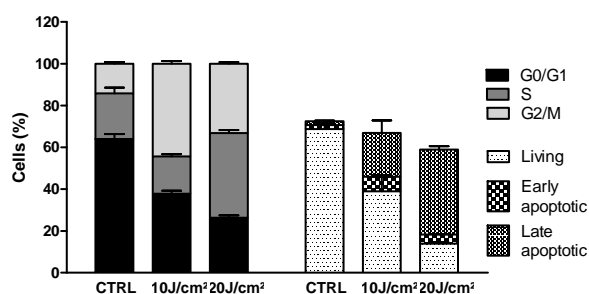


Figure 6. HCT116 cell cycle distribution (left) and apoptosis induction (right) following NTP.

A massive induction of apoptosis was observed together with a multi-phase cell cycle arrest. Yan et al. have also reported a similar result *in vitro* together with a modification of cyclin levels [6]. Finally, main action mechanisms of NTP on tumor cells were closed to consequences of radiation on tumor cells with ROS formation, induction of DNA damages, cell cycle arrest and finally apoptosis induction. DNA damages after NTP treatment need further investigations to determine which type of lesions was involved.

4. Tolerance of NTP *in vivo*

Considering these encouraging results *in vitro*, treatment of lung or colorectal tumor with NTP appears to be very promising. However exploration of tissue inflammation is required to determine the maximum tolerated doses which can be applied on lung or colon mice. To this end, a NF-kB-luc reported mouse (Caliper[®], USA) was used. NF-kB is

a transcription factor implied in the induction of inflammation process and imaging of this reported mouse by bioluminescence imaging (BLI) allows the monitoring of inflammation. In a previous study we defined the maximum tolerated dose on mouse skin at 6 min at 200Hz [7]. Using this dose, a kinetics of inflammation process following NTP treatment was performed on mouse skin using the FE-DBD.

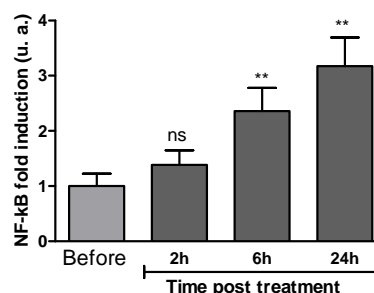


Figure 7. Kinetics of NF-kB activation in the skin following 6 min of NTP.

A dose of 6 min at 200 Hz induced an activation of NF-kB in the treated zone as soon as 2h after the treatment. This induction of NF-kB was higher 24h after treatment reflecting an induction of the inflammatory process despite the fact that no visible damage could be observed. Identification of the molecular mechanisms involved in this inflammation is ongoing [12].

Considering lung tumor treatment using plasma gun, a lung tolerance study was performed. Explorations of inflammatory process were done using NF-kB-luc mice, 24h after treatment according to the kinetics of NF-kB activation in the skin.

To this end, a tracheal intubation was performed on anesthetized mice with a small flexible catheter. The position of the catheter was monitored using high resolution radiological imaging (MX-20, Faxitron X-ray Corporation, USA). Plasma Gun catheter was positioned in the middle of either the right or the left lung. During all this procedure, mice did not exhibit any respiratory disturbance. Once the catheter was positioned, a very low neon gas flow (140 sccm) was used and NTP was generated in the lung. Only a slight modification of mouse breathing was observed during plasma treatment for 6 or 12 min at 100 Hz.



Figure 8. Left: representative picture of mice during NTP treatment with plasma gun. In the right, radiological imaging showing the catheter in the lung.

Using these treatment doses, no visible toxic side effect was observed. 24h after treatment, mice were sacrificed, lungs were excised and bioluminescence imaging was performed. Preliminary results on a small cohort of mice showed an induction of NF-kB in a localized region of the lung suggesting an induction of an inflammatory process resulting from plasma gun treatment. These results on lung tolerance need to be confirmed on a largest mice number.

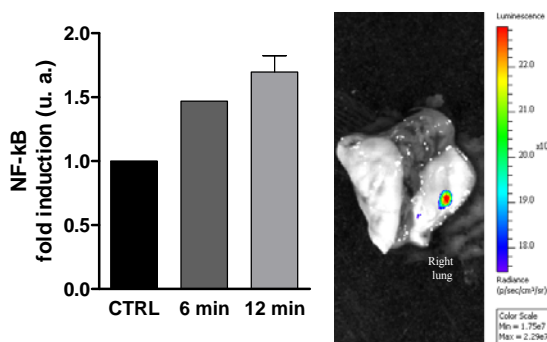


Figure 9. In the left, NF-kB folds induction in the treated lung and in the right a representative pictures showing an inflammation in the right lung after 12 min of treatment.

Concerning colorectal tissue tolerance, mice were anesthetized and a small abdominal incision was performed to extract the caecum. NTP was applied directly on caecum tissue during 6 or 12 min at 200 Hz with the FE-DBD. No visible damage was observed for 6 min of NTP, in contrast with a treatment of 12 min, where a small visible inflammation was noticed 24h after treatment. In a preliminary study, NF-kB mice failed to reveals an inflammatory process in the caecum wall. Studies are ongoing to complete these results. In both cases (lung and colon), a confirmation with histology is needed.

5. Conclusion

Using FE-DBD, we previously described an antitumor effect of NTP on U87 xenografts with an increase of mice lifespan [7, 13]. Treatment was applied during 6 min each day for five consecutive days. With this treatment protocol, a stabilization of tumor volume and an apoptosis induction was observed together with a cell cycle arrest. This major antitumor effect reflects the high cytotoxicity of NTP on tumor. *In vitro* DNA damage, cell cycle arrest and apoptosis induction were observed. Considering the antitumor activity of NTP on lung and colorectal tumor cells *in vitro* together with the ability to performed *in situ* treatment of these pathologies using plasma gun or FE-DBD, NTP appears to be a very promising tool for cancer treatment. Further investigations are required to elaborate a treatment protocol for lung and colorectal tumors.

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