To ground or not to ground?
That is a key question… during plasma medical treatment

A Stancampiano[1], T-H Chung[2], S Dozias[1], J-M Pouvesle[1], L M Mir[2], E Robert[1]

[1] *GREMI, UMR7344 CNRS Université d'Orléans, 45067 Orléans, France*
[2] *VTA, CNRS, Univ. Paris-Sud, Gustave Roussy, Université Paris-Saclay, Villejuif, France*

Abstract:
Target electrical parameters (conductivity, potential…) play a major role in determining plasma treatment conditions. This effect is of particular relevance in plasma medicine where we move from treating small *in vitro* samples to humans, passing by animal models. We demonstrate how by means of a small and affordable electrical circuit it is possible to "*compensate*" the electrical differences between *in vitro* models, mice and human body and in such a way to reach closer treatment conditions between *in vitro* and *in vivo* targets.

Keywords: plasma medicine, target, potential, compensation, in vitro, in vivo, models.

1. Introduction

The antitumor effect of non-thermal plasma (NTP) is now well established. The list of studies demonstrating plasma action on tumor cells *in vitro* is too long to be reported here but we can estimate that more than 50 cell lines of most types of cancers have nowadays been tested [1]. Similarly, the number of studies demonstrating other plasma medical applications, such as wound healing and decontamination, *in vitro* is nowadays significant and constantly increasing. Nevertheless, the number of studies involving patients is still limited and the exact plasma action mode is so far not fully understood [2, 3]. In general, the translation of *in vitro* plasma effects to *in vivo* effects remains far from being intuitive. This task is even more challenging due to the physical and chemical complexity of NTP interactions with liquids/cells/tissues which depends on a number of variables such as applied voltage, pulse duration, pulse repetition frequency, gas mixture, distance to the target, time of exposure, protocols of application, evolution of the target physical and chemical characteristics, etc…

Among these parameters, the electrical condition of the target is one of the most neglected and goes often unmentioned, especially in papers which were more focused on the biological effects. Nevertheless, as already reported in the literature, the target can strongly influence the plasma characteristics [4–7]. Physically, during a direct NTP treatment, the target (e.g. cell culture or living tissue) is part of a '*transient electrical circuit*' that is formed between the pulse generator and the ground. This means that target electrical parameters (conductivity, potential, total impedance…) play a major role in determining treatment conditions. Even with all the other parameters fixed, different targets can lead to very different plasmas characteristics and therefore very different effects. In this study we demonstrate that even the same target (culture medium in a multiwell plate) but in different electrical conditions (grounded vs floating potential) can lead to very different treatment results.

The variation of NTP treatment conditions due to target characteristics is of extreme relevance especially in the plasma medicine field where we move from treating small *in vitro* samples to humans passing by various animal models. The standard approach for medical validation that foresees the sequence *in vitro* model → *in vivo* model → patients may not be the best option if applied straightforward to plasma treatments. The plasma generated on a small *in vitro* sample, characterized by a high conductivity and a low capacitance, can hardly be compared with the one produced on a human body, with typically a lower conductivity and a much higher capacitance. This in turn ends up potentially affecting the reliability of the results achieved on *in vitro* and even *in vivo* models and their possibility to be translated to patients.

In this study we propose and successfully test a simple and versatile solution to reduce the degree of variation encountered in NTP studies moving from *in vitro*/animal models to human body. The main idea is to "*compensate*" the electrical differences between the models and the human body by means of a small and affordable electrical circuit that can be applied both on animal and *in vitro* models (Fig. 1).

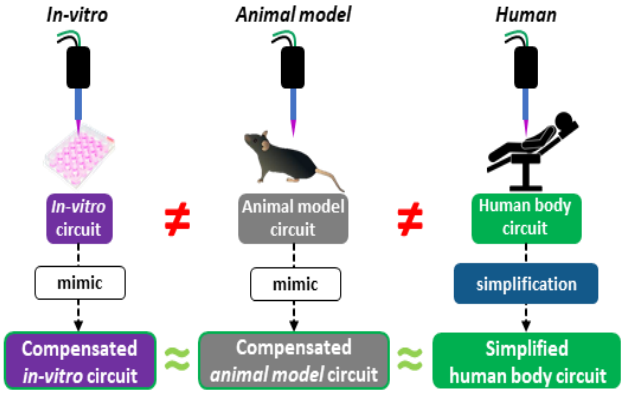

Figure 1: Logical scheme of the proposed method

2. Materials and methods

Plasma source

The atmospheric plasma jet used in this work is a Plasma Gun (PG) already described in detail in [4]. Briefly, the PG is a coaxial DBD reactor with a quartz capillary flushed with helium and powered by a micropulsed high voltage generator. The 12 cm long capillary was tapered at the outlet ($Ø_{in}$=1.5mm, $Ø_{ext}$=3mm). The plasma source was operated with a helium flow of 0.5 slm and powered by µs duration voltage pulses of +10kV peak with a 1 kHz repetition rate. For all the experiments the distance from the treated surface was kept constantly at 15 mm.

In vitro targets

To represent *in vitro* models we used a common 24-multiwell plate (Nunclon® Delta Surface, Thermo Fisher Scientific, DK) and a custom made rectangular well (2x5x0.9 cm, PVC). The liquid adopted for the test was high purity water (distilled, conductivity < 1µS, by Chem-lab) with dissolved NaCl (Fisher Scientific, UK) to adjust its conductivity to the desired values of 10 and 20 mS/cm. The conductivity of the solution was measured by means of a liquid conductivity probe (InLab Conductivity Probes 51344030, Mettler Toledo). A volume of 1-3 ml and 9 ml were introduced respectively in the multiwell plate and the custom well before each experiment.

The multiwell plate and the custom well were either positioned on a conductive plane (aluminium, 3 mm thick) electrically connected to the ground or on a dielectric plane (low density polyethylene LDPE, 15 cm thick) that was itself positioned on the conductive plane.

Floating potential (FP): the liquid at floating potential was simply not in contact with any conductive material.

Grounded (GR): the liquid in the multiwell plate was connected to ground by means of a stainless-steel plate (3.6x7x0.5mm; Fig.2a) while in the custom well by a stainless-steel plate of bigger dimensions (19x8.1x1mm). Both connections were positioned close to the wall, so to be as far as possible from the plasma impinging point at the centre of the well.

In vivo targets

To represent *in vivo* models we adopted mice (BALB/cJ) with or without tumour (CT26, murine colon carcinoma sub-cutaneously at 500.000 cells/100 µL on each flank).

Treated flank of the mice has been shaved and depilated the day before the treatment. Mice were anesthetised and positioned on a metal heating plate connected to the ground to maintain a constant body temperature of the mice during the treatment (Fig. 2b). To represent the human body, the Authors volunteered as targets. The chosen treated area was the fingertips of the hand (Fig. 2c). Ground connection was realized by means of a metal cylinder that was hold in the hand. No other grounded surfaces were in contact with the person during tests.

Compensation circuit

The compensation circuit adopted to reduce the electrical differences between the models and the human body was realized according to the International Standard IEC 60601-1 for medical electrical equipment [8].

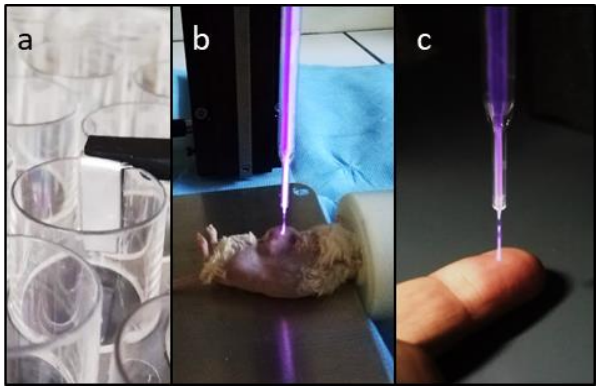

Figure 2: a) Ground connection for the 24-multiwell plate; b) NTP treatment on animal model on a grounded plate; c) NTP treatment on a human fingertip

This standard has been chosen as internationally recognized and widely adopted. The circuit was realized with a variable resistor in such a way to take into account the resistance already introduced by the compensated model and adjust the circuit in such a way that the total equivalent circuit was that of the reference standard. The circuit was connected in series between the model and the ground. The total cost of the circuit resulted very low and the dimensions can be reduced down to that of a sugar cube.

Electrical measurements

Voltage measurements were performed by means of a high voltage passive probe (Tektronix P6015A) on the high voltage cable connecting the PG and the generator. Current measurements were made by a current passive probe (Pearson 6585). The probe was positioned around the plasma source capillary (5 cm away from the outlet) or on the cable connecting the target to the ground.

Reactive species in liquid evaluation

A semi-quantitative chemical analysis of peroxide, nitrate and nitrite concentrations produced in plasma treated liquid in *in vitro* models was performed to support the understanding of the influence of the target electrical conditions. For these measurements Quantofix® test strips (Macherey-Nagel GmbH & Co. KG, DE) were adopted.

3. Results and Discussion

To ground or not to ground

As already mentioned, the electrical conditions of *in vitro* targets are often neglected or considered as marginal. In this work we demonstrate how even by positioning the same liquid (1 ml salt water 10mS/cm), in the same 24-multiwell plate, but on a conductive grounded plane (e.g. biosafety cabinet) or on a dielectric plane (e.g. standard lab table) we can greatly influence the treatment conditions. Considering the cases with the liquid at floating potential (FP), we see that moving the plate from a dielectric support to a conductive one significantly modify the current going through the plasma in the capillary (Fig. 3). Changing the support, we changed the capacitance associated to the *in vitro* target and therefore the characteristics of the transient electrical circuit created during the treatment.

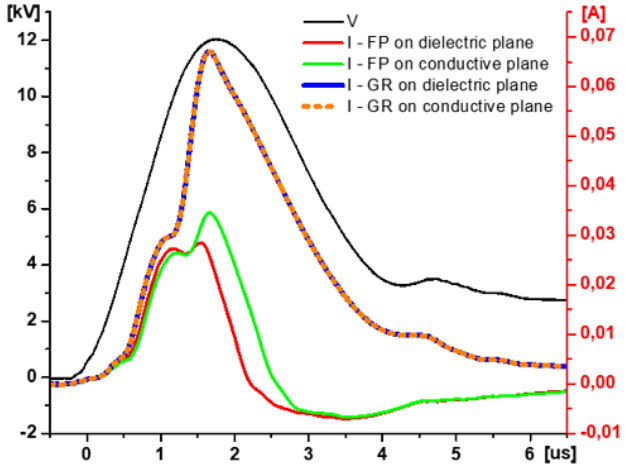

Figure 3: Voltage (V) and current (I) waveforms during direct treatment of water in 24-multiwell plates at different electrical potentials (floating FP or grounded GR) and on different supports (dielectric or conductive)

This affects especially the conduction current (peak between 1.5 and 2 µs) that takes place after the impact of the primary plasma front and the formation of a conductive channel between the high voltage electrode and the target [4]. A variation of the treatment conditions moving from one support to another is also testified by the treated liquid analysis (Tab. 1) where we observe that a significantly greater amount of peroxides and nitrates are produced when the support is conductive. Two biological assays on cells, performed inside a biosafety cabinet and on a dielectric table would supposedly produce different results.

The same test was repeated with the liquid grounded (GR). In this case the recorded currents resulted virtually identical between the two supporting planes (Fig. 3). Grounding the liquid is like connecting a resistor in parallel to the capacitance we already discussed above. Since the conductivity of the tested liquid (as well as that of most common culture media) is relatively high, the equivalent resistance results small. This in turn favours the passage of a greater conduction current compared to the cases at floating potential (Fig. 3) and greatly reduces the impact of capacitance and therefore of the support. This is also confirmed by the liquid analysis that recorded that the shift from floating to grounded potential induced the production of higher concentrations of reactive species (Tab. 1) and hindered the influence of the support.

Table 1: Concentrations of H_2O_2, NO_3- and NO_2- generated in 1 ml of water in 24-multiwell plates for different target electrical conditions. Treatment time 5 min.

Target potential	**Support**	**H_2O_2 [mg/l]**	**NO_3- [mg/l]**	**NO_2- [mg/l]**
Floating	Dielectric	2 - 5	10	<1
	Conductive	10	10-25	<1
Grounded	Dielectric	25	100	20
	Conductive	25	100	20

This first part of results suggests that the grounding may beneficially affect the treatment making it more stable and independent of the support nature while increasing the amount of reactive species produced.

Electrical compensation of models

In the second part of the study, we tested the proposed compensation circuit. As mentioned, the goal of the circuit is to adjust the total impedance of the model to match that of a human body. As a first step we recorded the characteristic current on the ground connection of a human body treated by the PG (Fig. 4). This measurement was adopted as a reference for future tests.

Then, the current going through 3 ml of salt water (10mS/cm) in a 24-multiwell plate and connected to the ground was measured. The same was repeated for the same setup but connecting the compensation circuit between the liquid and the ground. The equivalent resistance of the liquid was estimated (conductivity and geometry of the setup are known) and the circuit was matched accordingly.

As visible in Figure 4 the current peak going through a normal liquid sample (not compensated) is significantly higher than that one through a human body. The introduction of the compensation circuit greatly reduces these differences and approaches the current found on the *in vitro* targets to that on a human body. Since the recorded current depends on the characteristics of the plasma, we can assume that when with the compensation circuit the current on the *in vitro* sample results more similar to the one generated on a patient also the plasma would be more similar.

The same test was repeated with similar results (data not shown) changing the geometry (custom well) or the liquid conductivity (up to 20 mS/cm). Interestingly, the circuit, originally designed to make *in vitro* samples look more similar to the human body, may also be used to homogenize the behaviour of different *in vitro* samples (e.g. different well or liquid) from an electrical point of view.

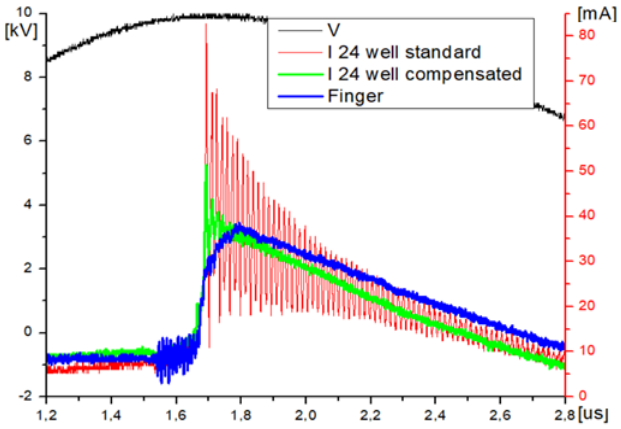

Figure 4: Voltage (V) and current (I) waveforms during direct treatment on grounded human body and on grounded water in 24-multiwell plate with (compensated) and without (standard) compensation circuit.

Table 2: Concentrations of H_2O_2, NO_3- and NO_2- generated in water in 24-multiwell plate (3ml) and in the custom well (9ml) with and without the compensation circuit. Treatment time: 5 min for each 3 ml of water.

Compen.	Well	H_2O_2[mg/l]	NO_3-[mg/l]	NO_2-[mg/l]
no	24-multiwell	2	10-25	1
	Custom	5	25	1 - 5
yes	24-multiwell	2	10	1
	Custom	2	10	1

This is confirmed in Tab. 2 where the compensation leads to the production of similar amounts of reactive species inside the treated liquid.

As a further validation of the compensation circuit method the same approach was repeated on animal models. As expected, the electrical characteristics of an animal model are already closer to those of a human body than those of an *in vitro* sample. Nevertheless, even in this case the addition of the compensation circuit contributed to yield higher similarity (Fig. 5). The same experiment was repeated on mice with grafted tumours (data not shown). For this model, the compensation resulted less satisfactory probably in virtue of the different resistivity of the tissues exposed to the plasma (healthy vs tumour). In fact, tumours presents a conductivity that is usually higher than the healthy tissue [9] meaning that the equivalent circuit will as well be different.

4. Conclusion

In the first part of this study we highlighted the importance of taking into account, and control, the electrical conditions of usual *in vitro* samples employed in plasma medicine studies. When the target is at floating potential, even the nature of the plane supporting the sample can influence the treatment. For example, different concentrations of reactive species in the treated medium can be the result of treating the sample on a dielectric table rather than inside a biosafety metallic cabinet. For this reason, it may be advisable to impose the potential of the target to ground level in order to reduce the influence of the surroundings and increase the reproducibility of the experiments.

In the second part of the study we propose a new method aimed to reduce the degree of variation encountered in NTP studies moving from *in vitro*/animal models to human body. Keeping in mind that the final target of plasma medicine studies are human patients it is proposed to adopt a small and affordable electrical circuit to "compensate" the electrical differences between models and human body and, as result, have the same NTP effect on very different targets.

Compensation circuit setting was proven to lead to both closer current waveforms through different targets but also much closer reactive species delivery in liquid solutions.

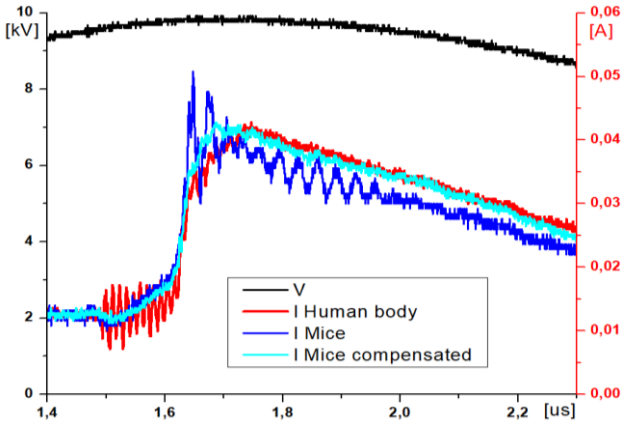

Figure 5: Voltage (V) and current (I) waveforms during direct treatment of human body and healthy mice with and without compensation circuit.

Results confirm that the circuit helps maintaining the same treatment consequences from one target to another.

Moreover, the mimicking circuit could also help to keep the same treatment conditions when there is a change from one *in vitro* support to another (e.g. from a well plate type to another).

In conclusion, we hope that this solution will help to ease the transition from *in vitro* to *in vivo* studies in the plasma medicine field.

5. Acknowledgment

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6. References

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