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The interactions between non-thermal atmospheric pressure plasma and ex-vivo dermal fibroblasts

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Abstract

The cold atmospheric plasma (CAPs) is the fourth state of matter, which means that it is an ionized gas, obtained at room temperature and atmospheric pressure when energy is supplied to a gas. Its composition is complex, involving chemical (gas’s molecules, free radicals, ions and electrons) and physical (Ultra-violet radiation and electric field) components. It has been successfully used in the industry for the processing of materials and the recent discovery of its efficacy in sterilization of microorganisms has trigged a large quantity of research in the biomedical field. The expectations of good that the CAPs may bring to medical treatments are high and therefore this study analyzes the effects caused by cold atmospheric pressure plasma in ex-vivo dermal fibroblasts. For that, fibroblast cell culture was obtained from ex-vivo human skin and treated with different conditions of CAPs, such as treatment type (direct or indirect). This work aimed to determine if ex-vivo human fibroblasts remain viable after these treatments. For that, we counted the number of cells right after the experiments and compared it with the control, which did not receive any kind of plasma. For further results we also counted the number of viable cells 3 and 5 days after the application. The fibroblasts were treated with 85W plasma, from 30 seconds up to 60 seconds. Our findings show that CAPs obtained from Argon gas can be applied to in-house human fibroblasts culture directly and indirectly without altering the cells’ viability. It does not cause apoptosis in all of the cells treated nor does it exacerbate its proliferation. Further studies need to be done in order to analyze if it alters the cells’ functionality.

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1. Introduction

Plasma is known as the fourth state of matter or as an ionized gas. In other words, when energy is sufficiently offered to a gas, collisions between neutral particles start to role. These collisions have enough energy to separate electrons, generating a mixture of components of a highly ionized gas. Being a mixture it is similar to the body’s fluid and therefore was named plasma [1].

By the 1950s, low collision and high temperature plasma had been considerably researched. In the 1990s, the headlines belonged to the non-thermal plasma, which was obtained in low pressure and temperature. After the year 2000 the plasma was obtained at room temperature and pressure, allowing its use in the biomedical field [2].

Nowadays, the thermal plasma is used in medical procedures such as tissue removal, cauterization, cuts and sterilization of materials. It is delivered at temperatures higher than 100 Degrees Celsius, which has several effects on tissues. The non-thermal plasma is delivered at room temperature, which causes less damage to living tissues, allowing it to be used in several medical therapies. Studies show that it is able to kill gram-positive bacteria, gram-negative bacteria, fungi and others parasites, without harming cellular tissues. Parasites such as Escherichia coli, Streptococcus species, Enterococcus faecium, Pseudomonas aeruginosa, Burkholderia cepacia, Bacillus cereus and Candida albicans were sterilized with treatments of 5 to 30 seconds. Heinlin et at (2011) showed a reduction of 1x10⁶ bacteria after CAPs treatment. The sterilization that plasma causes may be an excellent contribution to the treatment of ulcers. However it could harm the healthy tissue. In this matter, it is necessary to understand the interactions between the non-thermal atmospheric pressure plasma and the human cells in order to use it for ulcers treatment [3-11].

Studies proved that skin does not get burned by CAPs because it is delivered at a temperature of 33 Degrees Celsius. Burns happen when skin is heated to 42 Degrees Celsius. It was also shown that the ozone delivered did not cause harm to the cells and that the Ultra-violet radiation was not enough to cause important mutations [12, 13]. CAPs treatment can, therefore, be used in medical therapies, such as wound healing, blood vessels’ cauterization. It can also be used in the treatment of purulent inflammation of the appendages of the womb, pleural empyema, chronic gingivitis, psoriasis, eczemas, scleroderma, melanoma skin cancer and others [14].

This greater human cell resistance to plasma may exist because of its disposition. A mammalian cell has an envelope that protects its nucleus, while the bacteria does not. Therefore it is possible that plasma can cause direct damage to the nucleus of the bacteria, since it is more vulnerable to Ultra-violet radiation and plasma delivers this kind of radiation [4, 15].

Considering all these aspects, the evolution of human fibroblast culture after different kinds of CAPs treatments was analyzed. In order to use this technology in medical treatments, this work aimed to determine a minimum base treatment that allows these cells to remain viable. For that, a capacitive device was used to generate plasma, which was formed inside a quartz tube between powered electrodes and was ejected due to Argon gas flow, acquiring a torch form. Being a non-equilibrium system the plasma torch runs at room temperature (around 22 Degrees Celsius). The torch is delivered in a micro atmosphere, which mainly has Nitrogen and Oxygen. These gases, when mixed with the plasma’s compounds (positive and negative particles) trigger several chemical reactions that culminate with the formation of free radicals [16-18].
2. Materials and Methods

2.1. Ethical aspects

This study was approved by the Ethics committee of the University of Campinas’ Faculty of Medical Sciences, protocol number 92.993/2012, Campinas, São Paulo, Brazil.

2.2. Skin collection

Skin fragments were obtained from eyelid plastic surgery at the University of Campinas’ (UNICAMP) Clinical Hospital, Campinas, São Paulo, Brazil.

2.3. Cells culture

In accordance with Rehder et al (2004), the eyelid skin fragments were placed on Petri dishes with 2.5% trypsin and 0.1% Ethylenediaminetetraacetic acid (EDTA) (Trypsin-EDTA GIBCO cat. 25200-056). The mixture was kept in the incubator at 37 Degrees Celsius with 5% of CO₂ for 3 hours. The Trypsin-EDTA induces the dermal-epidermal separation. The supernatant was then neutralized with fetal bovine serum (FBS) centrifuged at 1.200 rpm for 10 minutes [19].

After that, the cells were distributed into culture bottles. Then, they were incubated at 37 Degrees Celsius with 5% CO₂ tension, immerse at culture medium (Medium 199 – GIBCO) supplemented with 10% of FBS. The culture was changed three times a week.

2.4. Plasma treatment

The equipment used to treat the samples was Surface® plasma tool model: SAP – Lab applications (Figure 1). Argon (Praxair 4.8) was used as the working gas with 1.0 liter per minute output (lpm).

![Fig. 1. (a) Surface plasma tool model – SAP – lab applications used as plasma source; (b) A plasma torch of 15mm long was obtained with Argon as the working gas at 1 lpm.](image)

The plasma treatments were distinguished in two types of plasma exposure: directly and indirectly (Figure 2).

- **A) Directly**: The active plasma zone (lighting zone) is in direct contact with the cell culture. In this arrangement the short-living species have the highest probability to reach the surface of treatment. The transferred energy and the species fluxes are very high. It allows the positive ions bombardment and consequently a physical current transfer. If the system is not correctly tuned, necrosis and cell devitalization may occur [20, 21].

- **B) Indirectly**: It is after the plasma zone (post discharge zone), thus there are low chances of short-living species reaching the surface although there is highest chance of free radicals (mainly nitric oxide and hydroxyl radicals) reaching it. If correctly tuned, it has a low chance of physical current transfer but can
be chemically aggressive if a high micro molar concentration of peroxides reaches the cells (it causes pH reduction, which may harm the cells) [22, 23].

Fig. 2. (a) Direct plasma exposure; (b) Indirect plasma exposure.

A previous study was made to gauge the time of treatment. Plasma exposure of 2, 3, 4, 6, 8 and 10 minutes were performed. After a 4 minute-treatment neither the control group nor the cells treated survived. This probably happened because long periods without the culture media (FBS) lead cells to death. Therefore, successful answers to plasma treatments in cell cultures are obtained with short treatments (from 30 up to 60 seconds). Considering this previous study the treatments were adjusted at 30 and 60 seconds.

2.5. Cell count

The fibroblasts culture was obtained from eyelid skin from three different patients (A, B and C) that were treated with the direct and the indirect plasma. For each type of treatment there was a control group (CG), that did not receive any kind of plasma treatment and remained with the FBS while the other two groups – group one (G1) and group two (G2) received a treatment of 30 seconds and 60 seconds, respectively (Figure 3).

Cells were counted right after the experiments, 3 and 5 days after the treatment. For that, the cells were dyed with Trypan blue, which turns the viable cells blue. Through optic microscope the number of viable cells present in one quadrant of the Neubauer chamber was counted.

3. Results

3.1. Cell count

All groups had their cells counted immediately, 3 and 5 days after plasma application. There were three groups: control group (without treatment), group one (30 seconds of plasma treatment) and group two (60 seconds) for each type of plasma application (direct and indirect).

3.1.1. Patient A

The fibroblasts culture obtained from patient A’s eyelid skin treated with direct plasma showed the following count: on day 0, the CG, G1 and G2 presented 50.000, 70.000, 55.000 cells/mL, respectively. On day 3, the CG,
G1 and G2 presented 170,000, 110,000 and 90,000 cells/mL, respectively. On day 5, the CG, G1 and G2 presented 260,000, 195,000 and 160,000 cells/mL, respectively (Figure 4).

For the indirect mode, the results were: On day 0 the CG, G1 and G2 presented 200,000, 135,000 and 90,000 cells/mL, respectively. On day 3, the CG, G1 and G2 presented 305,000, 245,000 and 240,000 cells/mL, respectively. On day 5, the CG, G1 and G2 presented 375,000, 340,000 and 320,000 cells/mL, respectively (Figure 5).

3.1.2. Patient B

The fibroblasts culture obtained from patient B’s eyelid skin treated with direct plasma showed the following count: On day 0, the CG, G1 and G2 presented 300,000, 97,500, 105,000 cells/mL, respectively. On day 3, the CG, G1 and G2 presented 210,000, 135,000 and 65,000 cells/mL, respectively. On day 5, the CG, G1 and G2 presented 280,000, 125,000 and 65,000 cells/mL, respectively.
For the indirect mode, the results were: On day 0 the CG, G1 and G2 presented 375,000, 165,000 and 90,000 cells/mL, respectively. On day 3, the CG, G1 and G2 presented 170,000, 115,000 and 45,000 cells/mL, respectively. On day 5, the CG, G1 and G2 presented 210,000, 120,000 and 65,000 cells/mL, respectively (Figure 7).

3.1.3. Patient C

The fibroblasts culture obtained from the patient C’s eyelid skin treated with direct plasma showed the following count: On day 0, the CG, G1 and G2 presented 450,000, 300,000, 180,000 cells/mL, respectively. On day 3, the CG, G1 and G2 presented 345,000, 292,500 and 217,500 cells/mL, respectively. On day 5, the CG, G1 and G2 presented 470,000, 305,000 and 210,000 cells/mL, respectively (Figure 8).
For the indirect mode, the results were: On day 0 the CG, G1 and G2 presented 300,000, 360,000 and 270,000 cells/mL, respectively. On day 3, the CG, G1 and G2 presented 382,500, 285,000 and 108,750 cells/mL, respectively. On day 5, the CG, G1 and G2 presented 405,000, 295,000 and 175,000 cells/mL, respectively (Figure 9).

4. Discussion

The results observed show that not only the direct plasma treatment, but also the indirect one do not alter ex-vivo human viability of fibroblasts. Even though the cells did not present a similar number increase, they all proliferate. In order to accomplish mitosis the cell cycle must be controlled by specific intracellular proteins. When significantly damaging mutation happens the cell can go through apoptosis, proliferate excessively, overexpress a gene or even suppress a gene.

Apoptosis takes a few hours to happen, so it could have happened with some of the cells that were treated, but not with all of them, otherwise there would be no cells in the second and in the third cell count (after 3 and 5 days,
respectively). As the exacerbation of proliferation, our findings suggest that it is not present, since the fibroblasts treated did not present a higher growth than the corresponding control group.

It can also be noticed that in all treatments the control group had more cells than the other groups. This could have happened because of the plasma treatment, but it is more likely to be due to the method of treatment. While G1 (30 seconds treatment) and G2 (60 seconds treatment) remained without culture medium (CM) during the treatments, the CG was kept immerse in CM. The lack of culture medium cause a great cell stress, frequently culminating with its death. In a preliminary study, cell culture samples were treated by several minutes and neither the CG nor the treated cells survived. So, it was decided to treat one sample many times but with short periods of plasma exposure in order to avoid lack of culture medium. Between the treatments the cell culture was supplied with CM. The results were as expected: the cells kept their viability. This can also explain the reason why the 60 second-treated cells showed lower counts in all experiments. Besides that, the reactive oxygenated species (ROS) created in the environment by the plasma torch could be responsible for micro-molar pH reduction, leading the ex-vivo fibroblasts to cell death. Also, plasma generates reactive nitrogenated species (RNS) as well, which could have triggered apoptosis in some cells [24-26].

As for the proliferation pattern, it did not present a common characteristic when patients were compared. Our findings show that each patient had a specific treatment response. It is an unusual finding when compared with other plasmas biomedical research. In latter, it is common to observe a certain pattern after treatments since the cells used come from cell line [27–33]. Our study was made with in-house cells. Therefore, the samples are expected to be different. Another important aspect is that the patients did not have the same age (old people on average) and the eyelid skins obtained from the plastic surgery had different sizes. Finally, it was observed that the same patient’s CG cell culture presented cell count differences between the direct and indirect treatment mode. This could be justified by the sample’s heterogeneity, even though the methodology was followed in all samples.

In vitro studies with human in-house cells are closer to the possibility of an in vivo experiment, since it can analyze how an actual body’s cell evolves after stimulation [34].

5. Conclusion

Non-thermal atmospheric pressure plasma obtained from Argon gas can be applied to ex-vivo human fibroblasts directly and indirectly without altering the cells’ viability. It does not cause apoptosis in all cells treated nor does it exacerbates its proliferation.

References


