In-plasma activated PBS solution for the treatment of oral squamous cell carcinoma

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Abstract: In this research, an atmospheric pressure plasma jet is employed to activate phosphate buffered saline (PBS) solutions in their bulk and study their potential in killing oral squamous cancer cells. The effects of the applied voltage and treatment time on several liquid characteristics believed to play a key role in cancer cell death, such as the concentration of H_2O_2 and NO_2^-, pH and temperature are investigated. Results reveal the formation of much higher amounts of H_2O_2 compared to NO_2^-. When exposing the cells to a PBS solution plasma-treated for 5 or 10 min and at 3 kV, a drastic decrease in their viability is noticed thus showing a potential in treating oral squamous cell carcinoma.

Keywords: Plasma jet, oral squamous cell carcinoma, plasma-activated PBS, H_2O_2, NO_2^-.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most malignant epithelial neoplasm and the eighth most common cancer worldwide because of the increase in tobacco use. In spite of the advances in cancer therapeutic approaches, the survival rate of OSCC patients is poor and no decrease in the mortality and morbidity rates was detected along the last 3 decades [1][2]. This suggests the strong need for new therapeutic alternatives replacing/complementing the common chemotherapies, surgeries, and radiotherapies and more efficiently tackling OSCC. In the last decade, atmospheric pressure plasma technology started to be more and more explored as a possible cancer therapy [3]. In fact, plasma produces reactive oxygen and nitrogen species (RONS) such as H_2O_2 and NO_2^- that exhibit cancer killing mechanisms. Such plasma-induced RONS can actually penetrate into cancer cells destabilizing their intracellular anti-oxidant system, and eliciting several signalling pathways causing cell cycle arrest and apoptosis [4]. In contrast to the common cancer therapies, plasma has a selective killing ability towards cancer cells but not healthy cells. One of the suggested causes leading to this selectivity is the expression of more channels transporting RONS on the membrane of cancer cells and less anti-oxidant enzymes inside them [4]. Two basic methods are considered for applying plasma in cancer treatment: direct and indirect treatment. In the direct treatment, cancerous cells and tissues are directly exposed to plasma. Next to impracticability of the direct treatments, the intensity and uniformity of a direct plasma irradiation are affected by the morphology and electrical properties of the cancerous tissue, which elicits variabilities in the anti-cancer ability. In the indirect treatment, a physiological liquid is first exposed to plasma then brought into contact with cancer cells [5]. This approach is very interesting in case of OSCC given the ease of putting plasma-activated liquids (PALs) in the oral cavity for a mouth wash for instance. In most studies involving PALs, an atmospheric pressure plasma jet (APPJ) generated in the gas phase is placed above the liquid to be treated. This study presents a novel approach in which plasma is directly ignited in phosphate-buffered saline (PBS) which is the liquid to be activated using an atmospheric pressure plasma jet (APPJ). The effects of different operational parameters on several liquid characteristics believed to play a key role in cancer cell death, such as the concentration of RONS, pH and temperature are investigated. Thereafter, *in vitro* cell tests using OSCC cells are carried out to evaluate the cancer-killing capacity of the plasma activated PBS.

2. Materials and methods

2.1. Plasma jet

A schematic representation of the liquid submerged APPJ used in this study to activate PBS is shown in Fig. 1. The reactor was fully described in a previous work [6]. To perform the treatment, Ar (purity > 99.99%; Air Liquide - Belgium) was used as carrier gas and was fed into the quartz tube at a flow rate of 2 standard liters per minute (slm) employing a mass flow controller (Model: F-201CV, Bronkhorst, Netherlands). Thereafter, 5 ml of PBS was poured in a tubular glass sample holder that was pierced on its bottom to slide around the end of the quartz capillary. As such, when applying a sufficient voltage, a plasma jet can extend in the PBS bulk. The process parameters that were varied to be further studied were the plasma exposure time (1–10 min) and the applied voltage (2–3.5 kV).

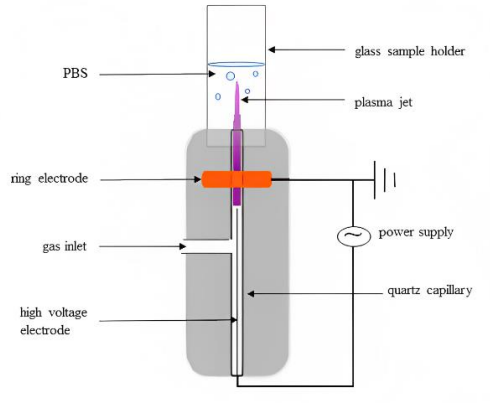

Fig 1: Schematic representation of used APPJ

2.2. Electrical characterization

The discharge mean consumed power of the APPJ was calculated at different applied voltages via the analysis of the voltage-current waveforms (obtained as described in a previous work) [6]. To do so, the following equation was used:

$$P = \frac{1}{T}\int I(t)V(t)dt \quad (1)$$

where T stands for the periodic time and V is the discharge voltage.

2.3. Quantification of hydrogen peroxide in PBS

For the quantification of H_2O_2 in the treated PBS, the titanium oxysulfate assay was performed. The reaction of H_2O_2 with Ti_4^+ leads to the formation of pertitanic acid, which is yellow and has a maximum absorption at around 407 nm. Prior to adding $TiOSO_4$ to the plasma treated samples, sodium azide was added as a nitrite scavenger to avoid the destruction of H_2O_2 by NO_2^-. After mixing 15 µL of NaN_3 with 400 µL of PBS, 200 µLo f reagent was added to the mixture that was incubated in the dark for 20 min to allow the reactions to occur. Thereafter, the absorbance was measured by a Thermo Fisher Genesys 6 spectrophotometer (Thermo Scientific Inc. USA). After constructing a calibration curve, the concentration of H_2O_2 was determined. The reported values are the averages and standard deviations calculated based on 4 measurements per condition.

2.4. Quantification of nitrite in PBS

Nitrite concentration is determined by means of the Griess assay according to the following protocol: 400 µL of plasma-treated PBS was added to 400 µL of the Griess reagent. After incubating the mixture for 10 min in dark, an azo dye was produced which is red-pink and has a maximum absorption at around 540 nm. Thereafter, the absorbance was measured by a Thermo Fisher Genesys 6 spectrophotometer (Thermo Scientific Inc. USA). An untreated sample-Griess mixture was used as baseline to avoid the overestimation of the nitrite concentration. After constructing a calibration curve, the concentration of NO_2^- was determined. The reported values are the averages and standard deviations calculated based on 4 measurements per condition.

2.5. pH values of PBS

The pH values of all PBS solutions was assessed by a FiveEasy Plus pH meter FE20 fitted with a pH combination electrode InLab® VersatilePro with integrated temperature sensor (Mettler Toledo, Switzerland). The reported values are the averages and standard deviations calculated based on 4 different measurements per conditions.

2.6. Evaporation and temperature

The temperature of the PBS solutions was evaluated before and immediately after the treatment by a portable digital thermometer. The difference in temperature between the two states was calculated and reported. For measuring the volume of evaporated PBS after plasma treatment, the sample was weighed with a well-calibrated scale before and after the treatment. Thereafter, from the obtained difference in mass, the volume of the evaporated PBS was calculated considering the density of 1x PBS. The reported values are the averages and standard deviations calculated based on 3 different measurements per condition.

2.7. *In vitro* cell studies

Human oral squamous cancer cell line, purchased from CLS Cell Lines Service (CVCL_7721, Germany), was cultured in a Dulbecco's modified eagle medium F12 HAM (DMEMF12, Gibco, Ireland) enriched with 2 mM L-glutamine, 1% penicillin/streptomycin and 5% heat inactivated foetal calf serum at 37 °C, 5% CO_2 in a humidified area. After 24 h of cell seeding, the cell medium was removed and replaced with 80 µl of fresh medium supplemented with 20 µl of untreated, treated PBS for 1, 5, and 10 min of plasma exposure or one of the control solutions (PBS subjected to the Ar flow without plasma ignition, reconstituted PBS with matching concentrations of exogeneous H_2O_2, NO_2^- and $H_2O_2 + NO_2^-$). To assess the viability of the cells, Alamar blue assay was performed 24 h and 72 h after the treatment. All measurements were performed in triplicate.

3. Results and discussion

3.1. Consumed power

The obtained mean power values as a function of the discharge voltage are shown in Fig. 2 (fixed argon flow rate of 2 slm). The results reveal a linear increase in the mean power with the increase in the voltage which is, with regard to equation (1), rather straightforward.

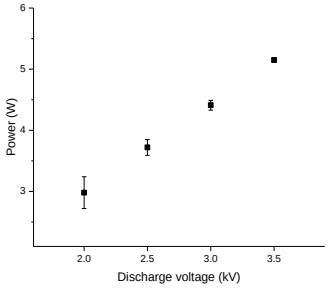

Fig 2: Mean consumed power as a function of the discharge voltage

3.2. Hydrogen peroxide concentration

When Ar plasma is ignited in PBS, a series of electron impact reactions that start with the reaction between Ar atoms and electrons and end up in the formation of OH radicals will occur. Thereafter, a series of liquid phase reactions in which H_2O_2 will be produced from the recombination of 2 OH radicals will befall. All reactions can be found in a previous work [7]. Given the proven anticancer capacity of H_2O_2, its formation in PBS was investigated upon plasma exposure as a function of the treatment time (fixed voltage: 3 kV) and applied voltage (fixed treatment time: 10 min) (Fig. 3). Results reveal that by increasing the voltage and the treatment time, the H_2O_2 concentration increased to reach a value of 1725 µM for a voltage of 3 kV and treatment time of 10 min. In fact, when increasing the voltage, electrons with higher energy will

lead to enhanced dissociation reactions thus forming more reactive species. Moreover, a part of the formed H_2O_2 in plasma-treated liquid is associated with its solubilisation from the gaseous phase [8], thus the longer the treatment time is, the longer the contact period between reactive species in the gas phase and the liquid sample is, which will lead to higher amounts of produced H_2O_2.

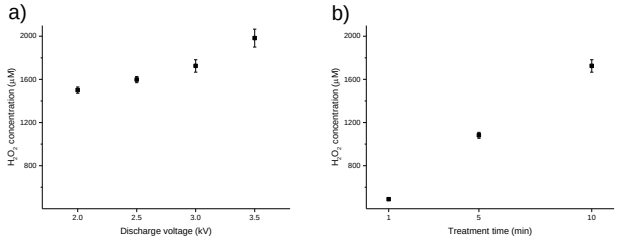

Fig 3: H_2O_2 concentration in PBS upon plasma treatment as a function the (a) discharge voltage and (b) treatment time

3.3. Nitrite concentration

Given the fact that NO_2^- was also shown to have an anticancer capacity, its formation in the plasma-treated PBS was assessed as a function of the treatment time (fixed voltage: 3 kV) and applied voltage (fixed treatment time: 10 min) (Fig. 4). Results show that much lower NO_2^- concentrations (below 20 µM) were obtained compared to H_2O_2 concentrations. Moreover, the nitrite concentration was not changing significantly when increasing the voltage. A slight increment in NO_2^- concentration was only observed by increasing the plasma exposure. The low amount of produced nitrite can be attributed to the lack of nitrogen in the PBS. In fact, pure Ar gas was used as the feed gas, thus the only source of nitrogen that could react with the plasma species to form nitrites is coming from the air above the PBS, thus explaining why for longer treatment time more NO_2^- was formed (longer contact with the surrounding atmosphere).

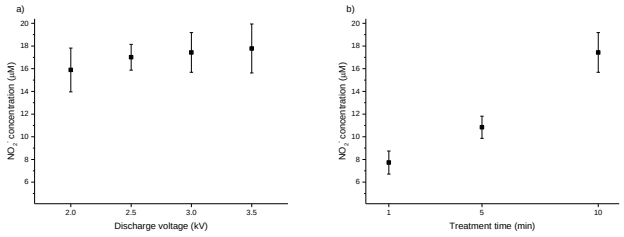

Fig 4: NO_2^- concentration in PBS upon plasma treatment as a function the (a) discharge voltage and (b) treatment time

3.4. pH

The changes in the pH level after exposing PBS to plasma treatments operating in different voltages (fixed treatment time: 10 min) and treatment times (fixed voltage: 3 kV) are illustrated in Fig. 5 a) and b) respectively. The results demonstrate that exposing PBS to plasma did not induce any acidification of the liquid as similar pH values were detected before and after the different treatment conditions. This is probably attributed to the buffer properties of PBS that remained physiological.

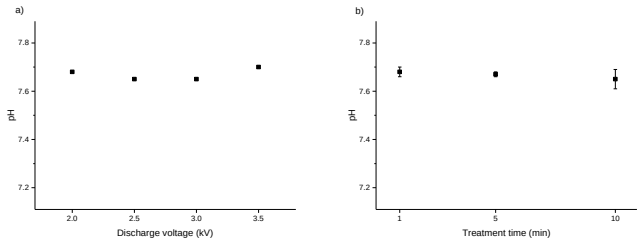

Fig 5: Evolution of the pH of PBS upon plasma treatment as a function the (a) discharge voltage and (b) treatment time

3.5. Temperature and evaporation

During plasma exposure, heat is expected to be transferred from the plasma plume to the PBS solution. Due to this energy transfer, the solution will probably heats up and some amount of water in the sample will evaporate. As such, the difference in temperature before and after plasma treatment and the volume of the evaporated liquid are shown in Fig 6 and 7 respectively, as a function of the treatment time (fixed voltage: 3 kV) and applied voltage (fixed treatment time: 10 min). Results reveal an increase in the temperature difference before and after treatment with the increase in the voltage and the increase in the treatment time (7.8 °C and 9.9 °C for 2 kV and 3.5 kV, respectively and 5.5 °C and 9.6 °C for 1 min and 10 min, respectively). In fact, a higher voltage will lead to a higher energy transfer explaining the increase in the temperature. When increasing the treatment time, temperature accumulation will also occur thus further increasing the PBS temperature.

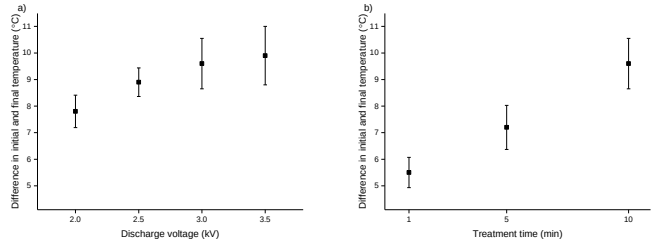

Fig 6: Evolution of the differences in PBS temperature before and after plasma treatment as a function of the (a) discharge voltage and (b) treatment time.

The volumes of the evaporated PBS after plasma treatment are in line with the increased temperature results. In fact, after 10 min of plasma exposure the evaporation reaches 17.6 % and 37.8 % at 2 kV and 3.5 kV respectively. A higher temperature of the sample can actually cause more evaporation. In addition, the gas flow which is applied inside the PBS solution during treatment is also expected to enhance the evaporation. This can be seen by comparing the value of the evaporated liquid after 1 min (0.44 ml) and 10 min (1.39 ml) of treatment. Such observations has been also detected previously [9].

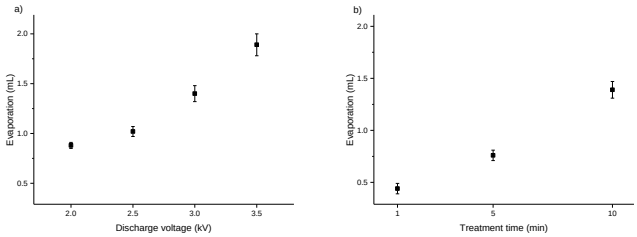

Fig 7: Evolution of the evaporated liquid after plasma treatment as a function of the (a) discharge voltage and (b) treatment time

3.6. *In vitro* cell tests

Fig. 9 shows the OSCC cell viability 24 h and 72 h after adding the treatment (treated PBS and other control solutions) to the cell culture as a function of the plasma exposure time. The controls involve hydrogen peroxide and/or nitrite-rich PBS in which an equal amount of H_2O_2 and/or NO_2^- as in the treated sample was added. Results reveal that when applying the 5 min and 10 min treated PBS to the cells for 24 h, their viability drastically decreased to values below 10 %. When increasing the incubation time to 72 h, the cell viability further decreased to almost 0 %. From the controls, it becomes evident that nitrite alone has no killing effect on the OSCC cells. On the other hand, hydrogen peroxide-rich PBS had a significant impact on the cell viability. This indicates that H_2O_2 has the leading role in cancer cell cytotoxicity. When taking a look at the synergistic effect of H_2O_2 and NO_2^- in PBS, no further killing effect was observed. Previous studies have reported the synergistic effect of H_2O_2 and NO_2^- in the anticancer capacity of plasma-activated liquids [10][11]. However, their plasma reactors produced considerably higher amounts of nitrite compared the APPJ used in this study which can explain the noticed discrepancy.

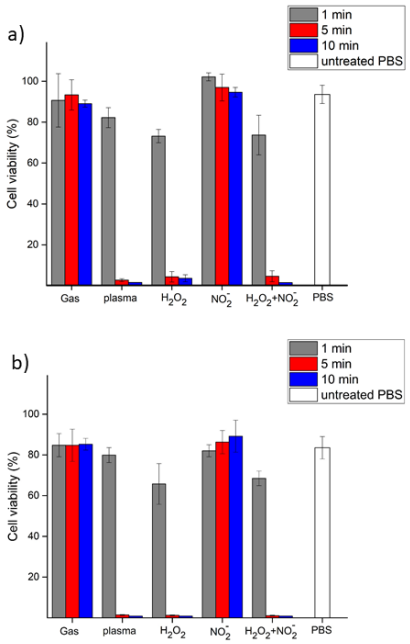

Fig 8: Cell viability after incubation with plasma activated PBS and the different control solutions for (a) 24 h and (b) 72 h.

When taking a closer at the effect of the plasma treatment time on the cell cytotoxicity, one can notice similar cell viability percentages for 5 min (1.10 %) and 10 min (0.88 %) for an incubation time of 72 h. This can be due to the very high concentration of H_2O_2 produced by the APPJ in this study already at 5 min of treatment.

4. Conclusion

The purpose of this study was to assess the potential of plasma-treated PBS solutions to kill human oral squamous cancer cells and on the long term act as treatment for OSCC. To do so, a novel approach in which an Ar APPJ is directly ignited in the PBS solution was adopted. The influence of the voltage and treatment time on different liquid characteristics was studied. Results revealed the formation of much higher amounts of H_2O_2 compared to the formed NO_2^- due to the novel configuration of the APPJ. By increasing the treatment time and voltage, an increase in the concentration of H_2O_2, temperature and evaporated volume of PBS was observed. When analyzing the OSCC cell viability upon exposure to the treated liquids and controls, one could deduce that H_2O_2 plays a major role in the anticancer ability of the plasma-activated PBS, whereas the NO_2^- effect is almost negligible.

Finally, our findings demonstrate that plasma-activated PBS has a potential as a therapeutic method for OSCC but additional studies are necessary.

5. References

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