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Effects of Atmospheric Pressure Plasma Jet Treatment Duration to the Viability of HeLa Cells and Microtissues

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ABSTRACT

Non-thermal atmospheric plasma jet (NTAPPJ) is believed to be effective in treating monolayer cells cultured in-vitro due to the oxidative stresses induced. However, it is controversial whether studying the impact of plasma to cells in monolayer can be representative to a model close to the in-vivo tissues. This study revealed and compared the effects of NTAPPJ treatment directly to both monolayer of HeLa cells and microspheroids in the culture media, at different exposure time from 15 to 60 seconds at a fixed power of 80 W. Optical emission spectroscopy (OES) revealed that the plasma applied to the cells is rich in hydroxyl, nitrogen, oxygen and argon species. The experimental results revealed that the treatment time from 15 seconds onwards was detrimental to the monolayer of cells, but not to the spheroids compared with the negative controls. After long NTAPPJ exposure, the HeLa cells in the spheroids were found viable, regardless of the plasma exposure time applied. The multicellular structure of the spheroids has prevented the penetration of free radicals into the cells encapsulated in the spheroids.

Keywords: Atmospheric Pressure Plasma Jet, Microspheroids, Microtissues, Non-Thermal, Cell Viability.

1. INTRODUCTION

Non-thermal atmospheric pressure plasma (NTAPP) is an ionized gas consisting of positive ions, protons and free electrons generated under the atmospheric pressure and low temperature conditions when high electrical energy is applied. In NTAPP, the ions and free radicals are in thermodynamically non-equilibrium state. There are different setups of NTAPP, such as plasma jet, dielectric barrier discharge, corona discharge, radio frequency plasma and gliding arc discharge [1]. High electrical energy *via* a pair of electrodes is required to be discharged in different gases such as helium, argon, oxygen, hydrogen, nitrogen or a mixture of these gases. NTAPP has wide applications in disinfection [1], cancer treatment [2], drug delivery [3], wound healing [4] and treatment on wound dressing [5]. In plasma medicine applications, the atmospheric plasma generated interacts with air and the media where the plasma plume is discharged to. The media is usually of liquid or solid surfaces. Due to the interaction of the discharged plasma with the air, various active agents that include ultraviolet (UV), reactive oxygen species (ROS), reactive nitrogen species (RNS), free radicals, free electrons, positive and

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negative ions can be produced. If the media is humidified, O+ ions, OH− ions, OH• radicals or hydrogen peroxide (H_2O_2) can also be generated [1]. These compositions vary by the type of gas component, intensity and pulse duration of the electric field applied [1]. The applications of NTAPP are expanding due to its applicability in air and non-damaging thermal conditions [6].

Many studies [7-10] investigated the effects of non-thermal atmospheric pressure plasma jet (NTAPPJ) in inhibiting the proliferation of cancer cells or inducing apoptosis of cancer cells. NTAPPJ based on helium gas could induce cell death to numerous cancer cell lines, including p53 mutated cancer cell lines [10, 11]. In cancer research, NTAPPJ was applied both directly and indirectly for cancer cell treatment [6, 12]. In the direct exposure of NTAPPJ, the plasma plume is directly exposed to the cells or tissues. Increasing evidences [8, 11, 13, 14] indicate that oxidative stress, induced by ROS, is the major factor in inducing apoptosis of cells *in-vitro*. It is believed that cancer cells usually contain elevated levels of reactive oxygen [15]. By treating the cancer cells with plasma, the reactive oxygen level can go over the threshold of free radicals that is beyond the adaptable level of the cancer cells, leading to cell apoptosis.

The Indirect plasma method involves exposing culture media [13] or phosphate buffered saline [16] to NTAPP jet in minutes, to produce a plasma activated medium (PAM). Then, the species in the medium interacts with the cells, inducing different biological responses [6]. This method was innovated to circumvent the deep tissue tumors that are not reachable by the plasma treatment. A previous report indicated that indirect method was found not comparable to direct method in reducing the growth of tumor [11]. Contrarily, another study indicated that indirect NTAPP could effectively reduce the tumor size [17]. These contradicting findings are subjecting the effectiveness in suppressing cell growth of NTAPP to the plasma dose and the plasma exposure time [18]. The effects of plasma treatment are also influenced by different sources of gases, cell types and treatment to adherent or non-adherent cells. Despite of plasma-inducing lethal effects, plasma can also provide stimulating effects on cells, leading to wound healing. In addition, general acceptance for plasma treatment is that short plasma treatment and low plasma doses could stimulate the proliferation and migration of cells [4]. *Vice versa*, long plasma treatment or high plasma doses will reduce cell adhesion, induce cell apoptosis and inhibit cell proliferation [4]. These findings mainly derived from research on plasma treatment to monolayer of cells. The studies on the effect of plasma treatment to multi-layered cells or tissues are still limited.

This paper presents the investigation on the effects of argon gas based NTAPP treatment to monolayer of cancer cells in comparison to microtissues. Under the exposure to similar dose of plasma, plasma treatment duration to the cell samples were varied, and the physical changes and cell viability were examined. The objective is to examine whether the microtissues composed of multilayer or monolayer cancer cells have stronger resistance towards direct cold plasma treatments, under different duration of NTAPPJ exposure.

2. MATERIALS AND METHODS

2.1 The setup of NTAPPJ and OES Measurement

The setup of the NTAPPJ with varying power input is as shown in Figure 1. The power source to the plasma torch was supplied from a neon power transformer, in which the power of the transformer was controlled by an alternative current (AC) dimmer which supplies different input power to the neon-transformer. The AC dimmer was adjusted between 60 – 240V. The power from the neon-transformer was connected to a pair of anode and cathode of the plasma torch. The anode of the plasma torch was a copper needle electrode while, the cathode was a copper tape wrapped around the terminal end of the plasma torch (quartz tube). The quartz tube of the plasma torch has an inner and outer diameter of 3 and 5 mm, respectively. Argon gas

was purged through the plasma torch at a flow rate of 40 L/min to discharge plasma plume when the torch was energized with high electrical power. The neon power transformer can deliver an output power of 60 W at 8.6 kVp-p and 35 kHz. The power output was measured by using a digital oscilloscope (Agilent DSOX2022A, USA) *via* a high voltage probe (Tektronix P6015A, USA) and current probe (Tektronix A622, USA).

To characterize the optical emission output of the plasma plume, an Optical Emission Spectroscopy (OES) system was used. The system consisted of an optical fibre sensor connected to a monochromator (Ocean Optic HR 4000) or a focus lens located 15 mm from the plume emission. To align the point-of-view of the optical fibre sensor, a laser source was used to ensure the optical emission is in line-of-sight with the focus lens at 1:1 reflective ratio and the optical fibre sensor. The optical sensor was used to measure the plasma emission spectrum from 200 to 1100 nm of wavelength.

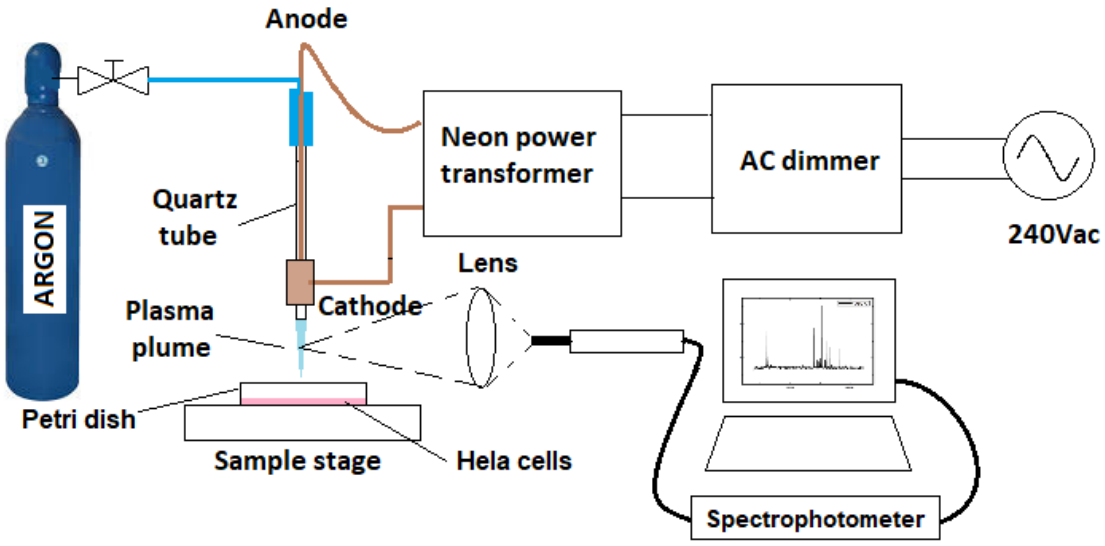

Figure 1. Setup of an OES measurement system for an NTAPPJ system.

2.2 Cell Culture and Preparation

The human cervical carcinoma cell lines (HeLa) were purchased from ATCC (CLL-2, Virginia, USA). The HeLa cells were maintained in a 25 cm^2 culture flask with Dulbecco's Modified Eagle's Medium (DMEM) added with 10 % Fetal Calf Serum (Gibco®, Life Technologies, US), Penicillin (100 units/mL, Sigma-Aldrich, UK), L-Glutamine (Gibco®, Life Technologies, US), Streptomycin (100 mg/mL, Sigma-Aldrich, UK) and Fungizone (2.5 mg/L, Sigma-Aldrich, UK). The cells were incubated at 37°C in a 5 % CO2 perfused incubator. When the cells reached 80 % confluency, the culture media was discarded from the culture flask and washed three times with Hank's Balanced Salt Solution (HBSS). The HBSS was discarded from the culture flask and the cells were enzymatically dissociated with 1 mL of 0.25 % crude EDTA-trypsin. Then, the culture flask was incubated at 37°C for 5 min. After incubation, 5 mL of supplemented DMEM was deposited into the culture flask and the cells were transferred to a 15 mL polypropylene tube. The tube was centrifuged at 1000 rpm for 5 minutes and the supernatant was removed. The cells were re-suspended with DMEM media. Approximately 1 mL of cells at a cell density of 5.2 x 10^5 cells/mL was added into a petri dish and allowed to grow to 80% confluency. Then, the monolayer of HeLa cells was ready for the plasma treatment experiment.

2.3 Microtissues Preparation

Microcapsules ladened with cells at a density of 6.2 x 10^7 cells/mL were produced using an in-house flicker microencapsulation machine [19]. The microcapsules of alginate containing cells were polymerized in a calcium chloride ionic bath for 30 minutes. The microcapsules of cells were washed with HBSS, deposited in DMEM culture media and incubated in a CO_2 incubator maintained at 37°C. The cells in the alginate encapsulation were continuously being monitored for 24 hours. The microencapsulated HeLa cells were found growing into microtissues after 15 days of culture. During early stage of the encapsulation, individual cells were scattered at high density in the microcapsules of calcium alginate. From day 3 onwards, aggregates of cells were observed, and more clumps of microtissues were formed after 5 days of culture. The cells proliferated, microtissues had grown and filled the entire volume of the microcapsules up to 15 days of culture. In a three-dimensional (3D) culture environment, the 3D matrix of alginate has supported the growth of the cells. The size of microcapsules produced by the flicking technique was small enough (200 – 350 µm) [19] to serve as a condition for exchange of catabolites and nutrients into the cells. Approximately 10 pieces of microtissues were deposited in 3 petri dishes containing 1 mL of complete culture media. These microtissues samples were readied for the subsequent plasma treatment experiment.

2.4 NTAPPJ Treatments of Cells and Microtissues

In the cell treatment experiment, cells grown in a petri dish were positioned approximately 15 mm under the tip of the plasma torch. Before plasma exposure, the cells or microtissues were washed in HBSS and the petri dish was filled with 1 mL of media before plasma treatment. Then, the plasma torch was discharged with pure argon gas at a flow rate of 40 L/min, and the plasma plum was focused to a small group of cells or microtissues. For NTAPPJ application at different exposure times, the dimmer was set at 60 W for energizing the plasma torch. Under similar output power, the cells were treated with NTAPPJ plasma for 15, 30, 45 and 60 seconds. A control experiment containing cells and culture media were treated without NTAPPJ. After the plasma treatment, the images of the plasma treated monolayer cells and microtissues were captured using a phase contrast microscope (Nikon Eclipse, TS-100, Japan).

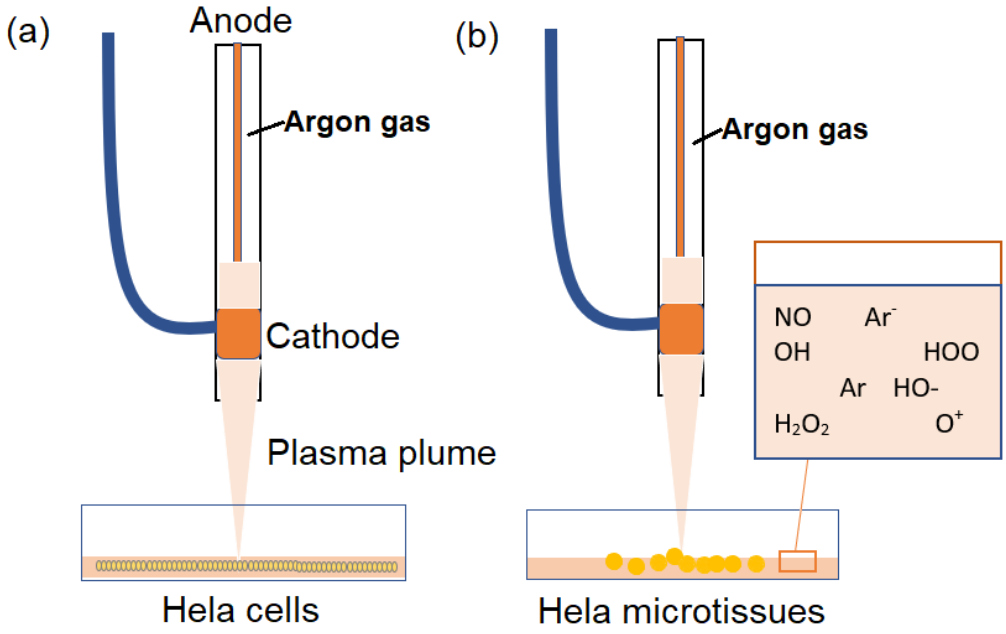

Figure 2. Non-thermal atmospheric pressure plasma jet discharge to HeLa (a) cells and (b) microtissues.

2.6 Live and Dead Assay of Cells and Microtissues

The viability of the cells in the microtissues cultured using the alginate microencapsulation was investigated using a live/dead viability kit for mammalian cells (Invitrogen, Paisley, UK). The live/dead viability kit containing calcein-AM (20 µL, 2 mM in 10 mL HBSS) and ethidium homodimer-1 (EthD-1, 5 µL, 4 mM in 10 mL HBSS) reagents were prepared. The live/dead cell viability kit can differentiate live cells from the dead cells by double staining the cells or microtissues with green-fluorescent calcein-AM (Invitrogen, Paisley, UK), which indicates intracellular esterase activity, while, red-fluorescent ethidium homodimer-1 (EthD-1) (Invitrogen, Paisley, UK) indicates loss of plasma membrane integrity. The cells or microtissues were stained in the live/dead cells staining assay for 10 minutes. The stained cells and microtissues were captured using a BX53 fluorescence microscope (Olympus, Tokyo, Japan) mounted with a DP73 microscope (Olympus, Tokyo, Japan) CCD camera. The stained live and dead cells in the plasma treatment region of 1 mm^2 were counted under the microscope and percentage of live cells was calculated and computed. The live and dead cells staining experiments for the cells and microtissues were repeated for three times.

2.7 Statistical Analysis

The data for cell viability from three repeats of experiments were expressed as means ± standard deviations (SD). For comparison of means, Student's t-test was applied to determine the significance of differences of the means. The differences were considered statistically significant for $p \leq 0.05$ and marked with an * in the graph.

3. RESULTS AND DISCUSSION

Figure 3 shows a typical OES characteristic curve of the NTAPPJ jet generated and energized by a neon-transformer. There were two distinct groups of spectra lines found; one close to 300 nm, the other is between 600 and 1000 nm. These two groups are associated with the spectra lines of hydroxyl group and argon, whilst, the spectra at 334 and 362 nm were attributed to N_2. The atomic oxygen emission appeared at 777 nm were identified for the spectra produced at AC voltages of 240 V. However, the intensities of these spectra were influenced by the dimmer voltage or power output of the neon-transformer [20]. These lines were associated with the excitation and dissociation of the argon plasma jet and the ambient air, in which the ambient air contains oxygen, water and nitrogen. There were little changes of N_2 regardless of the increased AC voltages. This might be due to the power applied was not enough to induce excitation of nitrogen gas. Figure 3 indicates that the main plasma output is composed of argon, oxygen, nitrogen and hydroxyl species.

The duration of the argon NTAPPJ treatment is a critical factor in determining the survival of the monolayer cells, as indicated in Figure 4. Cells in monolayers were observed with reduced adhesion at 15 seconds of treatment time, and further increment of plasma exposure time led to shattered cell body. The effects of plasma treatment on monolayer of cells seemed to be in good agreement with previous reports [21, 22] which claimed the anticancer effects of NTAPPJ to cells in monolayer. In plasma treatment to monolayer cells grown in culture flask (Figure 2a), fifty percent of the cell surfaces were exposed to the plasma plume. The impact of the treatment is severe because the positive ions, negative ions, electrons, photons and free radicals contribute to the lethal action of interacting directly with monolayer of cells that are less resilient to external cues [13]. The degree of damage to monolayer HeLa cells increased with the argon plasma treatment time as observed in Figure 4b-f. This is due to the increase in the intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) quantities that was proportional to the NTAPPJ treatment time [12]. Usually, RNS works collectively with ROS

to damage cells [13] . Under short exposure of NTAPPJ (< 15 seconds), the surfaces of the cells were exposed to bombardment of the radicals that induced surface lesions, leading to irreversible cellular impairment. Under longer plasma exposure (> 15 seconds), the oxidative agents bombarded on the exterior lipid membrane were strong enough to disintegrate the cell body as observed in Figure 4e and 4f. This is due to the active species of plasma discharges initiating lipid peroxidation in which, the free radicals removed electrons from the lipids in cell membranes or oxidatively degrade lipids, leading to significant cell damage [23]. However, overdosing argon gas can also damage cells because argon gas is inert and its asphyxiant character can induce deprivation of oxygen to the cells.

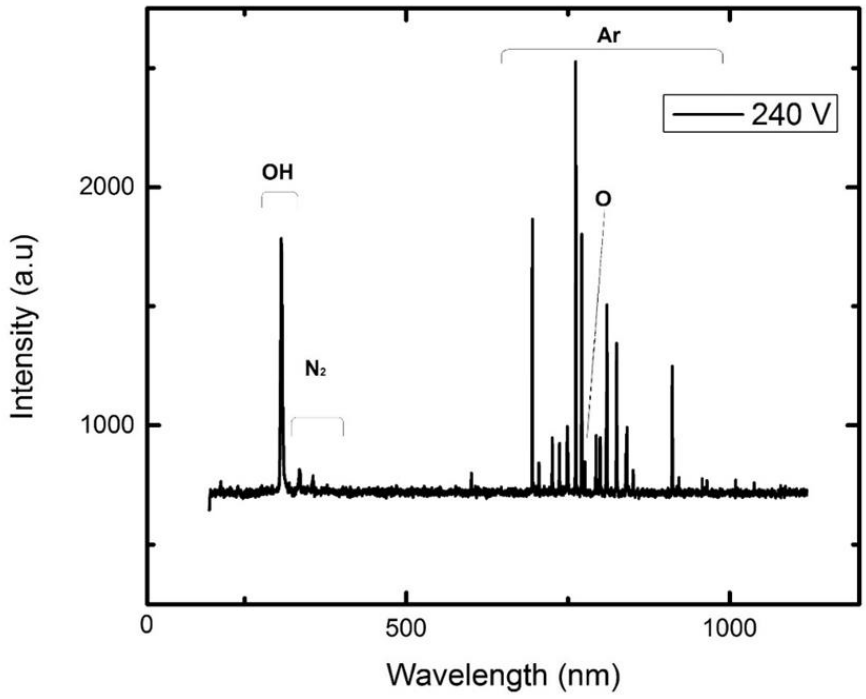

Figure 3. OES characteristics spectra generated with plasma jet of argon gas at 240V, 80W power.

For plasma treatment on the HeLa spheroids, there were no significant changes of morphology to the cells in the spheroids released from the alginate capsules. Under phase contrast microscope, the cells remained in spheroid structures with good cell-cell integrity, despite of short or long plasma exposure up to 60 seconds (Figure 4h-l). There were no apparent physical changes or shape distortion to the HeLa spheroids after plasma treatment. The effects of the plasma treatment on the microspheroids were not easily observed *via* the phase contrast microscopy. Further investigations on cell viability were required to confirm the survival of the cells.

Live/dead cells staining micrographs reveal that no cells in monolayer culture survived when the plasma treatment time was 15 seconds and beyond (Figure 5c-f and 6c-f). This result is contrary to the plasma treatment of the HeLa spheroids in which, the cells in the HeLa spheroids showed high green fluorescence stains of calcein-AM, indicating high cell viability regardless of treatment time, up to 60 seconds. Comparatively, similar plasma exposure time has incurred lethal effects to the cells in monolayer, but it was not lethal to the cells in multilayer structures (Figure 5). Negative controls with air treatment alone have no significant effects on the HeLa cells and spheroids that have similar cell viability found in the control (Figure 6). This revealed that cells in the form of microtissues are structurally resilient to the attack of free radicals released directly from the plasma plume.

From the experimental results revealed that the effectiveness of plasma treatment in anti-cancer in monolayer of cells [21] needs to be interpreted with care because it may not be effective in treating multilayer tissues. In this case, the spheroids represented a closer model to actual implementation of plasma medicine to multilayer tissues. However, it is important to highlight that tissue can absorb plasma reactive species from a few tens of micrometers to 1.5 mm, which depends on the duration of exposure [24].

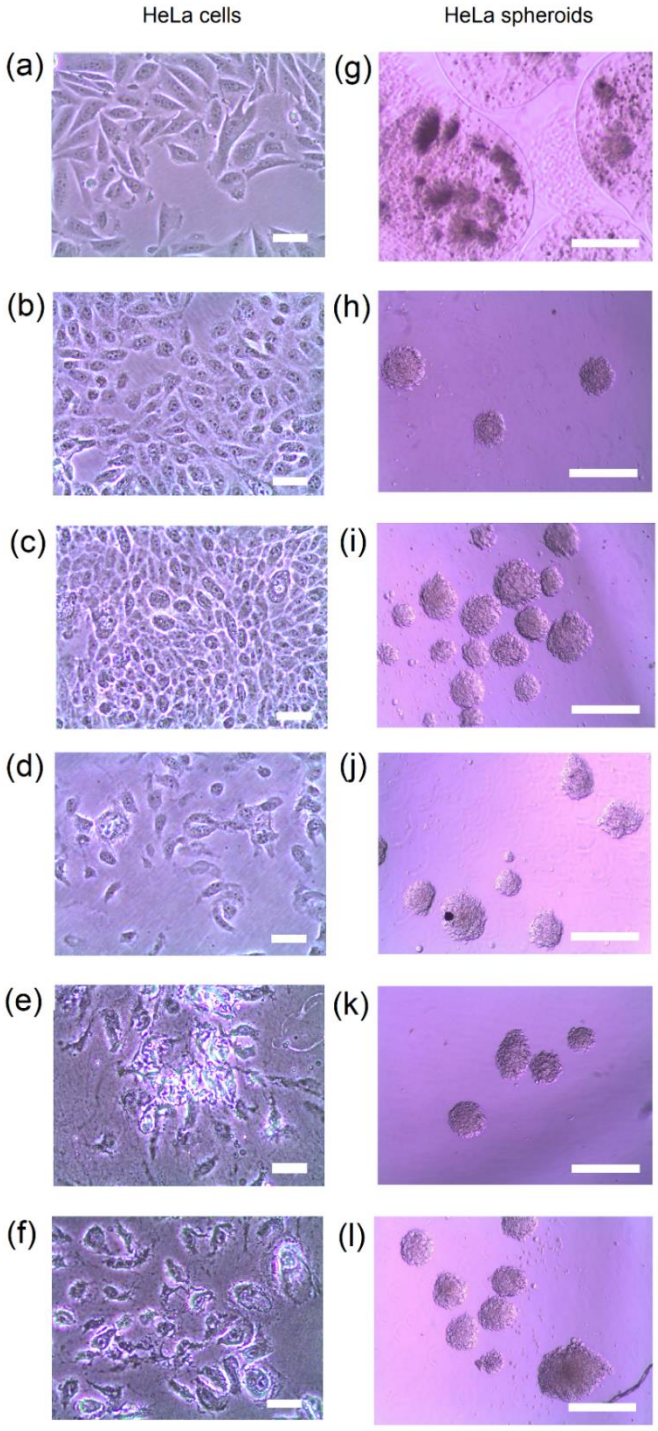

Figure 4. Phase contrast micrographs of Hela cells and microtissues in (a, g) controls, (b, h) negative controls and after argon NTAPPJ exposure time of (c-f, h-l) 15s, 30s, 45s and 60s, respectively. (Scale bar: 100 µm).

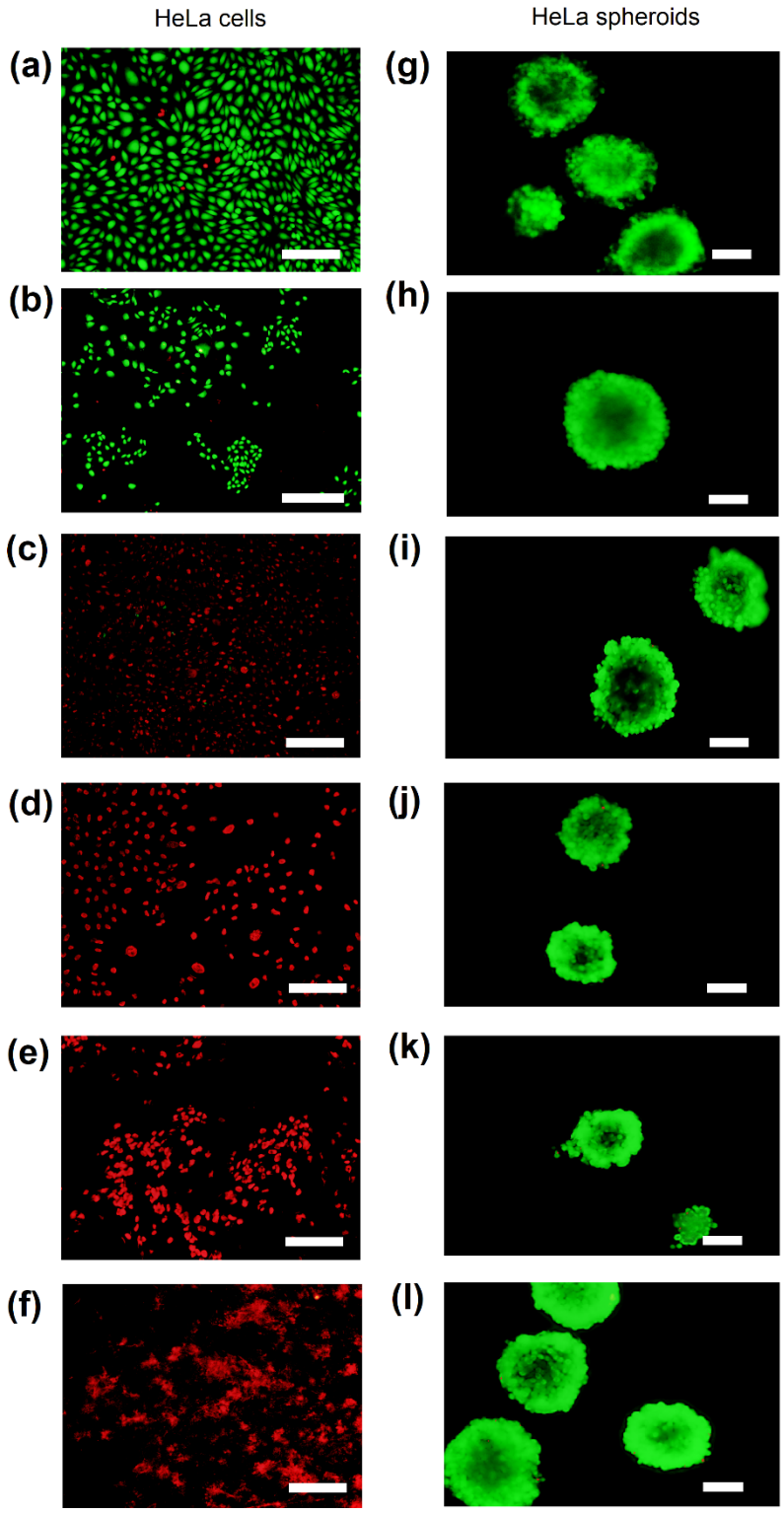

Figure 5. Fluorescence micrographs for calcein-AM and EthD-1 stainings of HeLa cells and microtissues in (a, g) controls, (b, h) negative controls (air) and after argon plasma treatments for (c-f, h-l) 15s, 30s, 45s and 60s, respectively. (Scale bars: 100 μm).

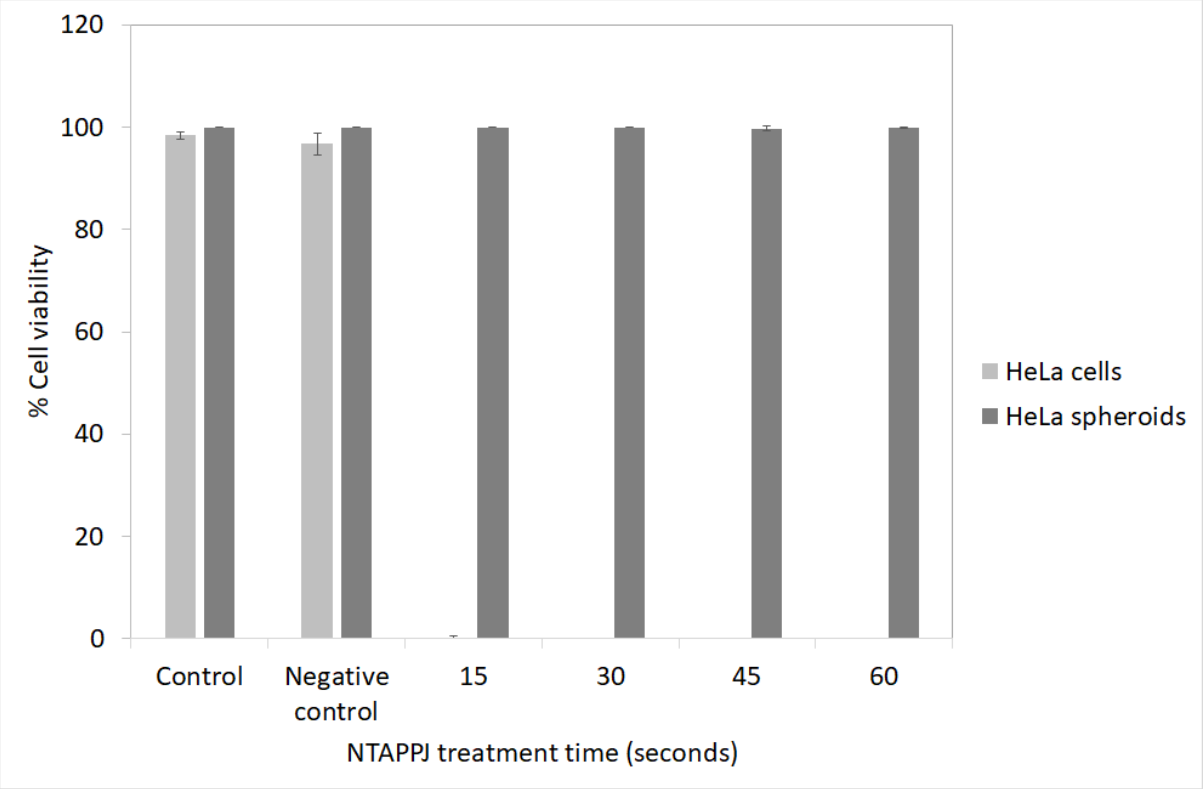

Figure 6. Effects of NTAPPJ to the viability of cells and microtissues.

4. CONCLUSION

The NTAPPJ produced in this experiment was indicated with the production of argon, oxygen, nitrogen and hydroxyl species in the atmospheric air. These species were soluble in the culture media and had shown fatal effects to the monolayer of cells for treatment time > 15 seconds. Short exposure of NTAPPJ to monolayer of cells, less than 15 seconds, reduced the adhesion of cells due to ROS and RON. Bombardment of oxidative agents and UV in prolonged period > 15 seconds seemed to be the main factor in inducing cell apoptosis. However, similar plasma treatment time under similar power output applied to HeLa spheroids did not cause any detrimental effect to the cells in multicellular structure. The protective layers of multicellular structures have prevented the direct attacks of ROS and RNS. Hence, plasma treatment application to monolayer of cells needs to be interpreted with care. These findings provide new insights into adopting 3D cell models for future plasma medicine study.

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