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Research Paper

Effect of non thermal pulsed atmospheric pressure plasma jet on transcription of malignant associated genes

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Abstract

One of challenges is the application of non thermal plasms directly on the surface of human body or on internal organs, tissues and genes, and the ability of therapeutic applications in malignancy. The effect of cold plasma are due to charged particles, reactive nitrogen, UV, photons and intense electric field. For obtaining efficient action on all types of cells generally to reach benefits for cancer cell therapy. Researchers have found that certain types of tumors have proteins on their surface that are specific to the tumors and not found anywhere else in the body. This is key to the type of cancer treatment involved. In our study we used pulsed atmospheric – pressure plasma jet (PAPPJ), The study carried out through four groups of mice, one group is normal mice, and three groups are solid Ehirlich Tumor transplanted mice at thigh region, one of which was non exposed, the second was exposed to 15 pulse of plasma and the third exposed to 30 pulse of plasma. The exposure extended for 4 weeks, twice weekly. Using RNA isolation and cDNA synthesis, the study demonstrated that: firstly, plasma jet exposure reduce the rate of tumor growth without completely treatment, and the reduction of tumor growth rate increase as the result of pulse number increase, secondly, plasma jet induce and affect the amplitude of gene transcription, which may be the genes associated with malignancy (up regulated and down regulated genes are major for treated groups). Non Thermal plasma jet up regulated some types of genes and down regulated another types of genes, these genes may be includes, apoptotic genes, tumor suppressor genes and oncogenes. And because of the detection of the level of transcription was generally and non specific through using arbitrary primers, therefore, we recommend to carry out more specific studies for recognizing the genes which were up regulated and the others which were down regulated as the result of plasma exposure.

Keywords: plasma jet, cancer treatment, Tumor, tissues, genes, RNA isolation.

Introduction

Non-thermal plasmas are frequently called, “on-equilibrium” plasmas because they are characterized by a large difference in the temperature of the electrons relative to the ions and neutrals. Since the electrons are extremely light, they move quickly and have almost no heat capacity. In these plasmas, Te >> Ti » Tn. Ionization is maintained by the impact of electrons (which may have temperatures ranging from 0.1 to more than 20 eV) with neutral species, producing additional electrons and ions. These plasmas are typically maintained by the passage of electrical current through a gas. Atmospheric pressure non-equilibrium plasmas have become powerful experimental tools for many applications in areas such as micro fabrications in microelectronics [1], surface modifications [2], light

sources [3], and environmental processing [4].Atmospheric pressure plasmas have recently received increased attention because several applications[5].

Due to the relative high breakdown voltage of working gases at atmospheric pressure, the discharge gaps are normally from few millimeters to several centimeters, which limit the size of materials to be treated for direct treatment. If indirect treatment (remote exposure) is used, some short life time active species, such as oxygen atom, charge particles may already disappear before reaching the object to be treated [6].

Attempted to characterize the bimolecular effects on atmospheric pressure cold plasma (APCP) system which utilizes helium / oxygen (He / 0 (2)). APCA using He / 0 (2) generates a low level of UV while generating reactive oxygen radicals while probably serve as the primary factor in sterilization, these reactive oxygen radicals have the advantage of being capable to access he interiors of the structures of microbial cells. The damaging effect of plasma explosive of polypeptides, DNA, and enzyme proteins in the cell were assessed [7].

Repetitive cold atmospheric plasma jets which are chemically activated with oxygen, on B16 tumors cells (murine melanoma cell line) and col 0320 DM multidrug resistant cells (human colon cancer cell line). The tests have been performed on human colon cancer cell line COLO 320 DM and murine melanoma cell line B16 – F10. These cells line have been treated with cold helium – oxygen generated plasma jets and the consequent apoptosis has been analyzed by means of flow cytometric method. A treatment time – dependent apoptosis has been observed only in the case of B16 – F10 cells interacting with helium – oxygen plasma and no apoptosis has been identified when the cells were treated only with helium plasma jets. These results indicate the need of oxygen for the chemical activation of plasma. The Colo32DM cells (that over – expressed the MDR efflux pumps) have been exposed to helium – oxygen plasmas only, or in a combination with vegetal extract MCS D161 as MDR efflux pumps inhibitor. For the secondly mentioned case the results have showed an increased apoptosis rate compared to the plasma treatment alone. The obtained data represent a starting point for the study of a possible combined treatment (atmospheric pressure cold plasmas and a MDR efflux pumps inhibitor applied with chemotherapy) [8].

The high reactivity of plasma is a result of different components : electro magnetic radiations (UV/VUV) visible light, IR, high – frequency electromagnetic fields, etc.) on the one hand and ions, electrons and reactive chemical species, primarily radicals, on the other. Besides surgical plasma application like argon plasma coagulation (APC) which is based on high – intensity lethal plasma effects [9].

Materials and Methods

This study carried through four groups of mice which were divided as the following:

1. First group: 20 normal mice as normal control (non treated normal).
2. Second group: 20 thigh transplanted solid Ehirlich tumor mice as cancered exposed.
3. Third group: 20 thigh transplanted solid Ehirlich tumor mice_which exposed to 15 pulse of non thermal plasma jet.
4. Fourth group:_20 thigh transplanted solid Ehirlich tumor mice_which exposed to 30 pulse of non thermal plasma jet.

Group three and group four exposed to plasma twice time a week, and the exposure extend for four weeks.

The transplantation of solid Ehirlich tumor in thigh region of mice, their average weight 17gm (their age 30 days). The exposure started after 8 days of transplantation, the size of the tumors measured twice time weekly, and the size of tumors calculated from the following equation[10]:

$$V = \frac{\pi}{6}(length) \cdot (width) \cdot (height)$$

Character of exposure machine

Cold atmospheric pressure plasma jet devices have recently attracted significant attention [11,12]. The most important devices for generating atmospheric pressure non-thermal plasmas can be considered: atmospheric pressure plasma jet [13,14], plasma needle [15], plasma pencil [16,17], miniature pulsed glow-discharge torch [18], one atmosphere uniform glow-discharge plasma [19], resistive barrier discharge [20] and dielectric barrier discharge [21]. A schematic of the pulsed atmospheric-pressure plasma jet (PAPPJ) discharge and of the experimental set-up is shown in figure (1). The gas is fed through an annular region between the two metal electrodes 15 cm in length. The inner electrode is 5 mm in diameter and is powered with pulsed high voltage power supply, while the grounded outer electrode is separated from the inner electrode by a gap of a few millimeters. A rotating spark gap is made by mounting the ground electrode on a rotating plane.

The APPJ device operates using 10-20kV power supply with a gap between two electrodes is 2-3mm under atmospheric pressure. The electrical discharge inside the reactor of APPJ was induced by a pulsed high voltage power supply. The discharge power was adjusted by the pulse voltage with a pulse frequency of 167 kHz.

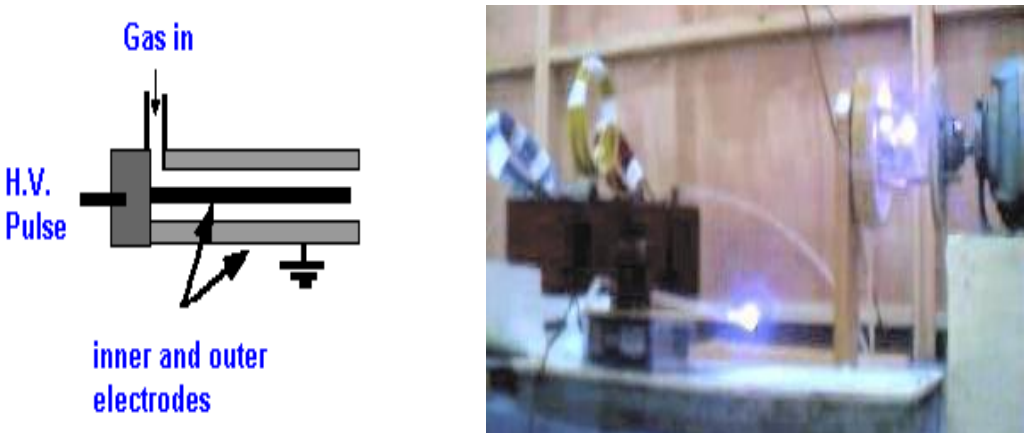

Figure 1: A scheme of the discharge and the experimental set-up

At the end of exposure, the samples of normal and malignant thigh tissue were collected for molecular study. Molecular study deals with detection of transcription of genes through detection of RNA as the following steps:

RNA isolation and cDNA synthesis

Animal tissue samples were subjected to RNA extraction using GStract™ RNA Isolation kit II Guanidium Thiocynate Method. Reverse transcription reaction was performed using oligo (dT) primer. The 25 µl reaction mixture contained 2.5 µl (5x) buffer with MgCl2, 2.5 µl (2.5 mM) dNTPs, 1 µl (10 pmol) primer, 2.5 µl RNA (2mg/ml) and 10 unit reverse transcriptase enzyme. PCR amplification was performed in a thermal cycler programmed at 42 °C for 1 hr, 72 °C for 10 min (enzyme inactivating) and the product was stored at 4 °C until use.

Differential display PCR

Six primers were used in the differential display analysis to examine up-down regulated genes (table 1). The reaction mixture for differential display PCR was carried out in total volume 25µ l containing 2.5 µ l 10x buffer with MgCl, 2 µ l 2.5 mM dNTPS, 1 µ l of 10 pmol primer, 1.5µ l cDNA and 0.2 µ l (5 units/µ l) Taq DNA polymerase. PCR amplification was performed in a thermal cycler (Eppendorf, Germany) programmed for one cycle at 95 °C for 5 min, then 40 cycles as follows: 30 sec at 95°C for denaturation, 1 min at 30-32°C for annealing for 1min and 1min at 72 °C for elongation. Reaction was then incubated at 72 °C for 10 min for final extension. Two µl of loading dye was added prior to loading of 10µ l per gel slot. Electrophoresis was performed at 80 Volt with 0.5 x TBE as running buffer in 1.5 % agarose/0.5x TBE gels and then the gel was stained in 0.5 µ g/ cm3 (w/v) ethidium bromide solution and distained in deionized water. Finally the gel was visualized and photographed using a gel documentation system.

Results and Discussion

The basic understanding of mechanisms of plasma effects on different components of living systems in the early beginning specially for the field of direct therapeutic plasma application, on fundamental knowledge of the mechanisms of plasma interaction with living cells and tissue is essential as a scientific basic. Plasma medicine is a quickly developing area of medical technology that brings recent developments in plasma physics and engineering to bear an important problems such as wound healing, infection control, and also though to treat cancer, by selectively killing cancer cells while leaving normal tissue unharmed.

Low temperature atmospheric pressure plasma is a multi – component system that includes such biologically active agents as charged particles and reactive oxygen species, metastable – state molecules or atoms and UV radiation, the goal of this study is to investigate the effects of this plasma on the transcription of genes associated with malignancy.

Table 1: Primers used in this study for differential display

Primers	Primer sequence 5→3	Annealing (°C)
NS2	GGC TGC TGG CAC CAG ACT TGC	30
NS1	GTA GTC ATA TGC TTG TCTC	*30*
EZ351	AGGAGG TGA TCC AAC CGC	30
18S	CTT CCG TCA ATT CCT TTA AG	32
A2	CAAACGGGTGGTGATCGC	30
A4	GGACTGGAGTGTGATCGA	30

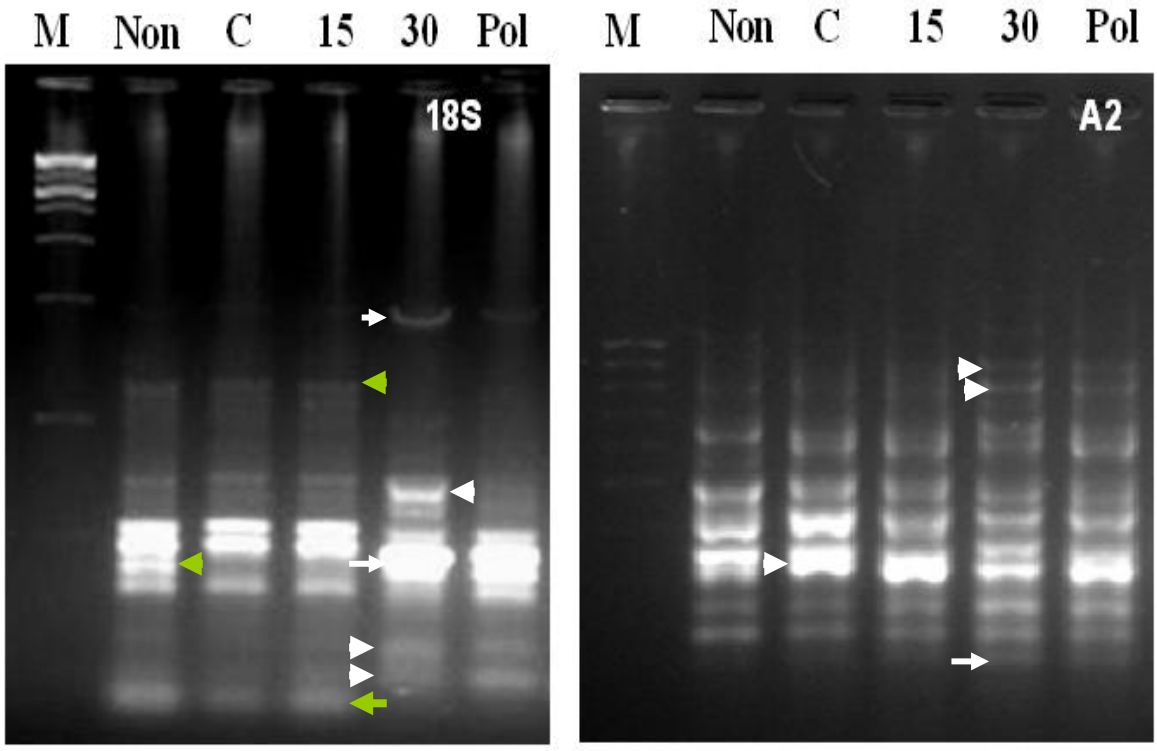

Figure 1: Differential display using 18S and A2 primers as arbitrary primers. M: 1 kbp ladder DNA marker. Lanes, Non: non treated normal animals, C: Cancered animals nonexposed to the plasma. 15: Cancered animals exposed to the plasma rays for 15 pulse. 30: Cancered animals exposed to the plasma rays for 30 pulse. Pol: genetic pool for samples 15 & 30 in combined. *upregulated gene indicated with white arrow. ** The down regulated gene indicated with yellow arrow

When differential display was performed for the RNA extracted from the tissue samples, up and down regulated genes were observed. The observed genes differed in their molecular sizes and the range of molecular sizes of these genes ranged from 1kbp to 50bp. In case of primer 18S five up regulated genes and 3 down regulated genes were recorded. The up regulated genes were found in animals exposed to 30 pulse of plasma and animals exposed to 15 and 30 pulses as well. Whenever, two down regulated genes out of the three were founded in samples exposed to 15 pulses. But only one down regulated gene was shut down in samples control. In case of primer A2 only four upregulated genes were observed and the molecular sizes of these genes ranged from 800bp to 50bp. Three up regulated genes were founded in organisms exposed for 30 pulses and only one gene was observed in the cancred animals nonexposed to plasma.

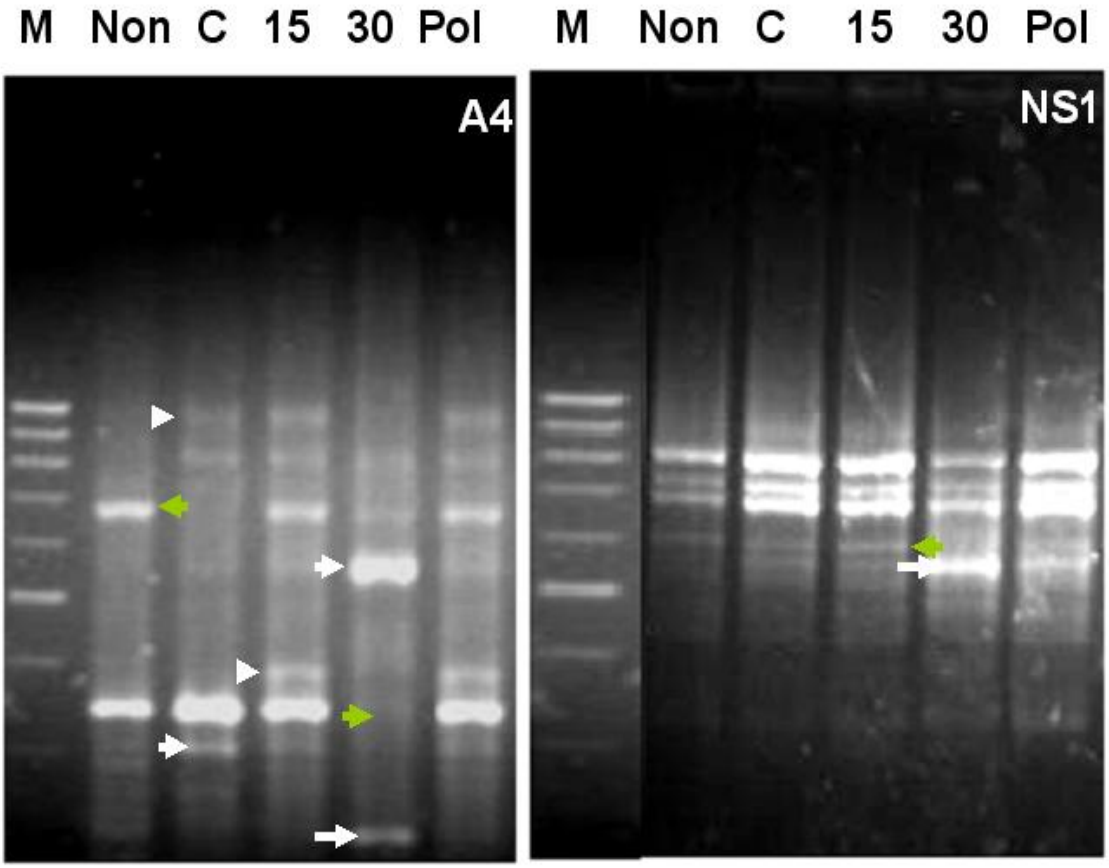

Figure 2: Differential display using A4 and NS1 primers as arbitrary primers. M: 1 kbp ladder DNA marker. Lanes, Non: non treated normal animals, C:Cancered animals non exposed to the plasma . 15: Cancered animals exposed to the plasma rays for 15 pulse. 30: Cancered animals exposed to the plasma rays for 3o pulse. Pol: genetic pool for samples 15 & 30 in combined. *upregulated gene indicated with white arrow. ** the down regulated gene indicated with yellow arrow

Primer A4 succeeded to scan more than 12 mRNA, between them 5 upregulated genes and 2 down regulated genes. The molecular sizes of these genes ranged from 1kbp to 50bp. Here two upregulated genes were observed only with samples exposed to the plasma for 30 pulses, one upregulated gene was demonstrated in animals exposed to 15 pulses and tow upregulated genes were observed with cancered nonexposed animals. Two down regulated genes were observed, one in control animals nonexposed to the plasma and one in animals exposed to 30 pulse of plasma exposure.

In case of the NS1, the primer gave a unique band pattern with all the examined animals except only two genes, one is uprgulated and observed in animals exposed for 30 pulses and the other down regulated was shown gene in same samples.

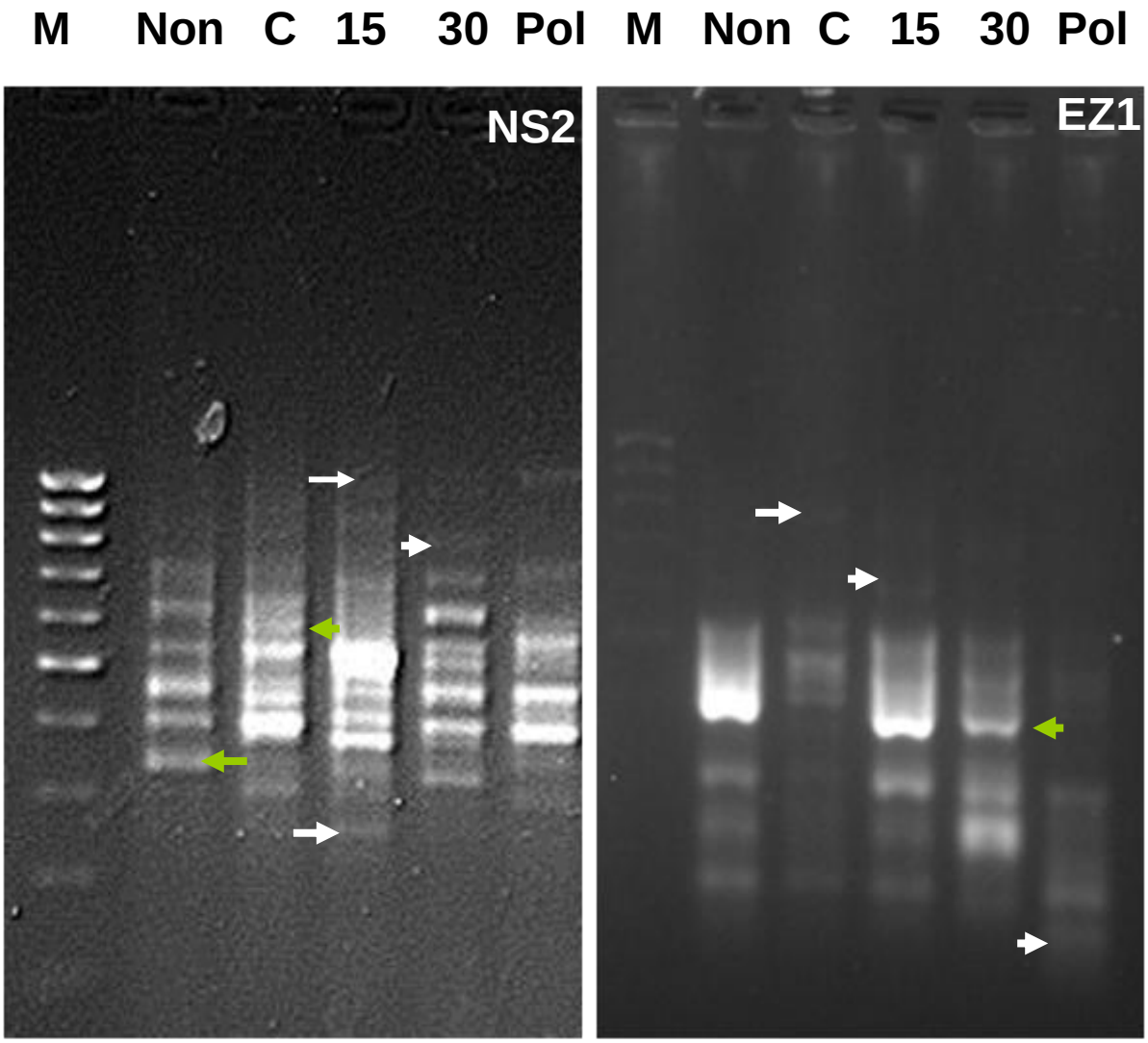

Figure 3: Differential display using NS2 and EZ1 primers as arbitrary primers. M: 1 kbp ladder DNA marker. Lanes, Non: non treated normal animals, C:Cancered animals nonexposed to the plasma . 15: Cancered animals exposed to the plasma rays for 15 pulse. 30: Cancered animals exposed to the plasma rays for 30 pulse. Pol: genetic pool for samples 15 & 30 in combined. *upregulated gene indicated with white arrow. ** the down regulated gene indicated with yellow arrow

Primer NS2 succeeded to scan 11 different m RNA among them 6 is monomorphic genes. The other 5 genes are polymorphic genes and they are grouped into uprgulated (3 genes) and down regulated (2 genes). The molecular weights of the scanned genes ranged between 1kbp to 300bp. The three uprgulated genes were observed in animals exposed for 15 and 30 pulses but the down regulated genes were presented in normal control and cancered nonexposed animals. In case of primer EZ1 three upregulated genes and one down regulated were observed. The molecular sizes of the genes ranged from 700bp to 50 bp. The uprgulated genes were founded with cancered nonexposed animals, animals exposed for 15 pulses and the genetic pool of animals exposed to 15 and 30 pulses respectively. The down regulated gene was observed in control animals and genetic pool of animals exposed for 15 and 30 pulse respectively.

It is clear that exposure of malignancy to non thermal plasma jet leading to change of up regulating and dawn regulating of genes, and because of the detection of transcriped genes of this study was generally (non specific) we think that most of theses genes are associated with malignancy, therefore the study recommends for carrying out more specific detection study to recognize the types of transcriped genes which associated with malignancy.

Table 2: Mean value of tumor growth rate of cancered non exposed © group and each of cancered exposed 15 pulse and cancered exposed 30 pulse animals

Time of exposure per day	1 Day 8	2 Day 11	3 Day 15	4 Day 18	5 Day 22	6 Day 25	7 Day 29	8 Day 32
Cancered non exposed c	3.70	4.20	4.85	5.45	6.10	6.80	7.55	8.30
Cancered non exposed 15 pluse	3.75	4.05	4.45	4.85	5.10	5.60	6.05	6.40
Cancered non exposed 30 pluse	3.65	3.95	4.30	4.60	4.85	5.35	5.70	6.05

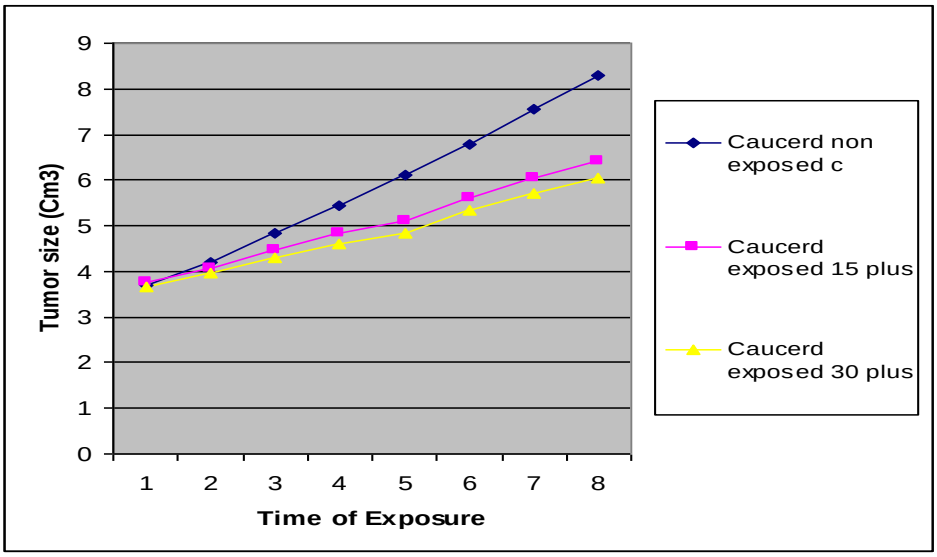

Figure 4: Represents comparison between the tumor growth rate of cancered nonexposed (c) group and each of cancered exposed 15 pulse and cancered exposed 30 pulse animals.

From Figure 4 it was clear that, there was a reduction of tumor growth size rate in comparison between cancer mice and mice exposed to 15 -30 pluse respectively, and reduction of tumor size more increase in case of 30 pluses. The treatment was not completely in the two groups of malignant exposure, and this may related to week penetration of plasma to malignant tissues, and the amount of pulses was not enough to kill all malignant cells, Therefore the range of pulses might be increase to reach more energy enough for completely degradation of malignant cells.

Floating – Electrode Dielectric Barrier Discharge (FE – DBD) plasma treatment was shown to initiate apoptosis in melanoma cancer cell lines – a threshold at which plasma treatment does not cause immediate necrosis but initiates complex cascade of biochemical processes leading to cell death many hours and even days following the treatment .

Melanoma cells treated by plasma at doses significantly below those required for cell destruction survive the plasma treatment but develop apoptosis many hours' post-treatment and die (disintegrate) by themselves gracefully. This could potentially be an intriguing approach for cancer treatment, especially if by manipulation of plasma parameters, the treatment could be made selective to cancerous cells over healthy cells.

Cellular macro molecules during apoptosis are digested into smaller fragments in a controlled fashion, and ultimately the cell collapses without damaging the surrounding cells or causing inflammation with cancer cells, however, a problem arises with apoptosis as the tumor cells frequently learn how to turn off apoptosis as one of the processes they employ in evading the immune system and surviving under un favorable conditions. A way to target apoptosis development only in specific areas of the body is needed, and can be achieved by the non-thermal plasma treatment.

We think that the up regulated genes and down regulated genes may be related to apoptotic genes, tumor suppressor genes and oncogenes therefore, the study have recommended for carring out complementary studies to demonstrate the specific genes up and down regulated.

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