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Abdelmonsef A. Elhadary
Biological Applications Department, EAEA (Egyptian Atomic Energy Authority), Cairo, Egypt, eng.amira85@yahoo.com

A. El-Zein
Electrical Power &Machines Department Faculty of Engineering, Zagazig University, Zagazig, P.O. 44519, Egypt, eng.amira85@yahoo.com

M. Talaat
Electrical Power &Machines Department Faculty of Engineering, Zagazig University, Zagazig, P.O. 44519, Egypt\\ Electrical Department, College of Engineering, Shaqra University, Dawadmi, Al Riyadh, Saudi Arabia, eng.amira85@yahoo.com

G. El-Aragi
Plasma Physics and Nuclear Fusion Department, EAEA, Cairo, Egypt, eng.amira85@yahoo.com

A. El-Amawy
Electrical Power &Machines Department Faculty of Engineering, Zagazig University, Zagazig, P.O. 44519, Egypt\\ Plasma Physics and Nuclear Fusion Department, EAEA, Cairo, Egypt, eng.amira85@yahoo.com

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Studying The Effect of The Dielectric Barrier Discharge Non-thermal Plasma on Colon Cancer Cell line

Abdelmonsef A. Elhadary[1], *A. El-Zein*[2], *M. Talaat*[2,3], *G. El-Aragi*[4], *and A. El-Amawy*[2,4,*]

[1]Biological Applications Department, EAEA (Egyptian Atomic Energy Authority), Cairo, Egypt
[2]Electrical Power &Machines Department Faculty of Engineering, Zagazig University, Zagazig, P.O. 44519, Egypt
[3]Electrical Department, College of Engineering, Shaqra University, Dawadmi, Al Riyadh, Saudi Arabia
[4]Plasma Physics and Nuclear Fusion Department, EAEA, Cairo, Egypt

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Abstract: Non-thermal plasma is a future tool for the treatment of cancer, some diseases, and for many medical applications. Dielectric Barrier Discharge Plasma (DBDP) is a type of plasma that has been used for the treatment of cancers in different researches. In this work (DBDP) was used to evaluate its effect on Colon Cancer Cell-line (CaCo Cell-line), through three doses depending on the time of exposure (40, 60, and 80 sec.). After exposure, the cell-line were cultured for 72 hours and followed by two types of analysis. The first was MTT assay for the determination of cell viability. The second was molecular analysis for two genes associated with cancers; Caspase 9 gene (apoptotic gene) and Bax gene (pro-apoptotic gene) through detection of their proteins. The results of the study proved that the DBD Plasma has a strong killing effect on the cancer cell-line (CaCo), and this effect increased with the time of exposure increased. The study proved also that; the effect of DBDP coming from its effect on the transcription of genes associated with cancer, where the study found that the degree of transcription of Caspase 9 and Bax genes was significantly increased as the result of the exposure. Therefore, the results of this study make us consider that DBD Plasma Device is a promised tool for the treatment of cancer, where the plasma promotes the process of apoptosis in cancer cells.

Keywords: DBD; plasma; CaCo cancer cells; non-thermal plasma, MTT assay, Molecular assay.

1 Introduction

The plasma concept is created by shedding high voltage energy to the gaseous state that atoms strike together and diminish their electrons [1]. Plasma has a complicated structure, It contains a group of electrons, ions, excited and neutralization atoms, ultraviolet (UV), free radicals, thermal, infrared radiation, electric fields, and molecules [2].

Several investigations have proved the antitumor effectiveness of plasma on various kinds of cancer cells [3-4].

Over the past decade, cold atmospheric plasma (CAP), ionized gas near room temperature has a promising application in cancer therapy. Two devices, dielectric barrier discharge and plasma jet show a significant anti-cancer effect on many different cancer cell-lines (in vitro) and several subcutaneous xenograft tumors (in vivo). In contrast to conventional anti-cancer approaches and drugs, cold atmospheric plasma is a selective anti-cancer modality [5].

In vitro plasma-exposed eukaryotic cells demonstrate several effects such as cell migration alteration and apoptosis or necrosis according to cell type and exposure parameters (power, time of exposure) [6].

Different studies suggested that cancer cells are more sensitive to cold atmospheric plasma treatment than normal cells concluding a selective decrease in cancer cell viability with less cytotoxic effect on nonmalignant cells [7].

Cold atmospheric plasma includes physical effects (production of ultraviolet, and electromagnetic fields) and beside chemical components (production of RONS). Whereas the physical effects seem to have a negligible cellular impact [8], but RONS may induce cell membrane alterations, where the increase of the intracellular ROS,

*Corresponding author E-mail: eng.amira85@yahoo.com

leads to the decrease of the antioxidant potential and DNA double-strand brakes and subsequently apoptosis [9].Many basic cellular responses following cold atmospheric plasma are(apoptosis, growth inhibition, selective cancer cell death, cell cycle arrest, DNA and mitochondrial damage, selective increase of ROS or even immunogenic cell death) [10].

The Bax gene translates a protein which upon activation changes the mitochondria in the cell to motivate cell death [11].

Upon activation, the Bax gene leads to depolarize the membrane and to leak the mitochondria to its contents like cytochrome C [12]. And these lead to changes in the form or, the structure of the apoptotic cell which is called morphological differences such as shrinkage and nuclear condensation, i.e. the shrinkage of the cell and the bleeding of the membrane, and these changes lead to the release of vesicles consists of the cellular ingredients which will be swallow by living cells [13].

The Caspase-9 gene is the fundamental initiator Caspase that is responsible for the programmed cell death or mitochondrial apoptosis path [14]. Under exposure to the apoptosis exciters inside the cell, the cytochrome C is released by the mitochondria. The activated Caspase-9 can stimulate the executioner Caspases (Caspase-3, Caspase-6,

and Caspase-7) and release a cascade of steps causing apoptosis in the cell [15].

2 Materials and Methods

2.1 Experimental Setup

The current study was performed in the Biological applications Department, Plasma and Nuclear Fusion Department in the Egyptian Atomic Energy Authority.

The electrical circuit of the DBD plasma device consists of a High Voltage (HV) power supply of voltage up to 15 kV Alternating Current (AC), a frequency of 50 Hz is connected to a variable resistance controller to adjust the input voltage and a charging resistor of 10 kΩ. The input voltage is connected to the HV electrode (anode) via the HV probe of attenuation ratio 1000:1 linked to a digital oscilloscope [16].The biomedical device of the DBD plasma mainly contains the HVAC power supply, two electrodes, and a glass dielectric as shown in Fig. 1(a). The upper electrode is made of iron and the lower electrode facing the sample is made of iron mesh. They are separated by 2 mm thick glass.

Fig. 1(b) illustrates the construction of the DBD plasma apparatus where the diameter of the HV electrode is a 6 cm iron plate. Plasma is transferred from a source located at a constant distance of 5 cm up the treated sample; the plasma is generated as an electrical discharge results from the electrical breakdown of argon gas in the gap between the HV electrode and the treated sample.

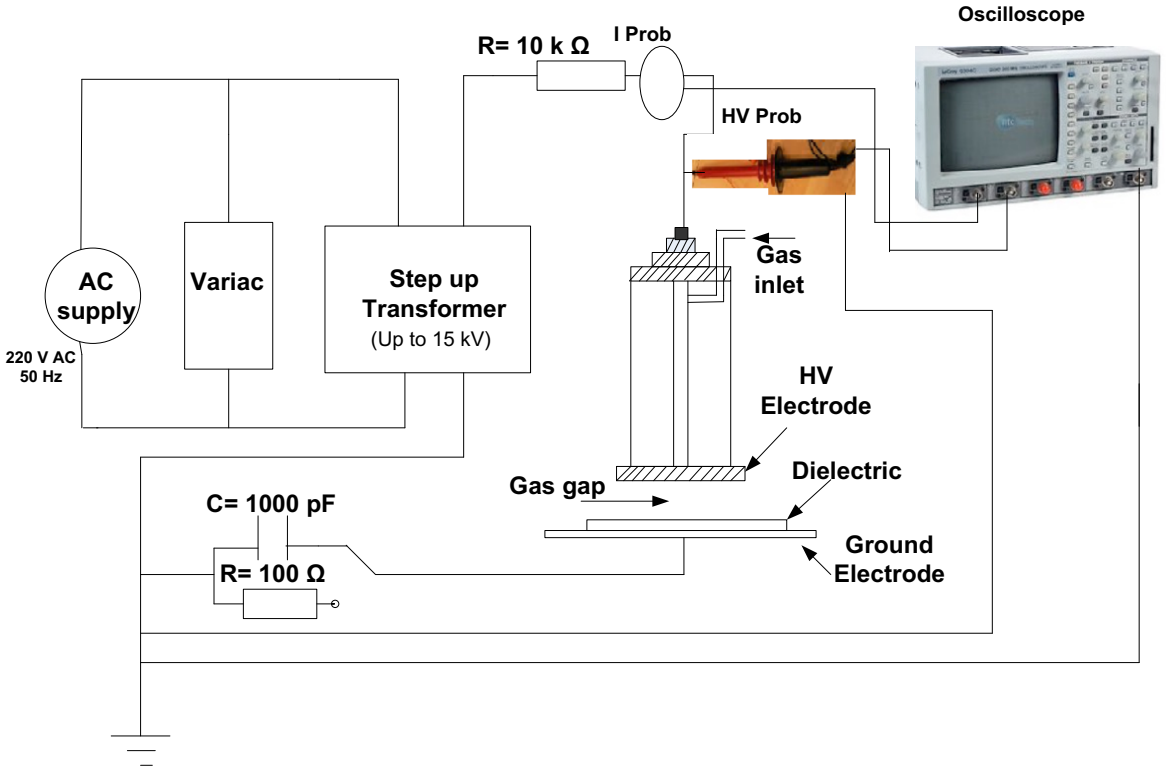

Fig.1(a): the schematic diagram of the biomedical device of the DBD plasma [16].

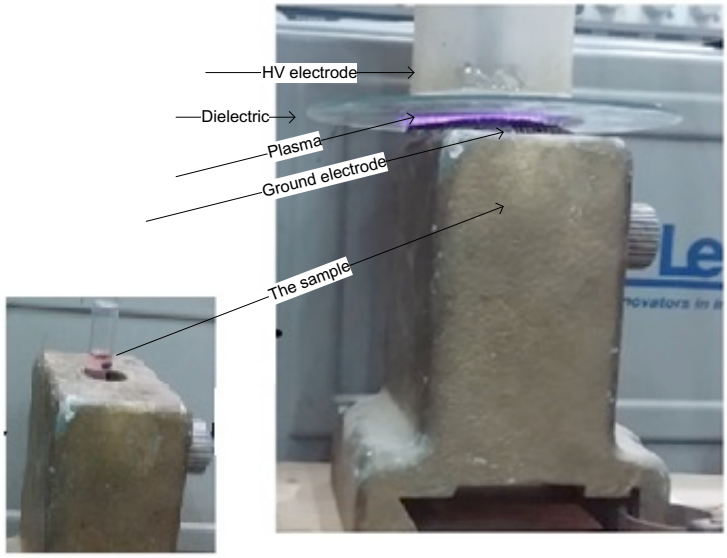

Fig.1(b): The DBD plasma photo for biomedical application.

The distance between the sample (cell-line) and the ground electrode was 5 cm. the gas of plasma was argon, the atmospheric temperature was 38 ºC. The exposure time of the samples was as the following:

First sample: Control sample, non-exposed.

Second sample: Cell-line exposed for 40 sec.

Third sample: Cell-line exposed for 60 sec.

Fourth sample: Cell-line exposed for 80 sec.

After treatment, each Cell-line sample was divided into 7 portions, and they were cultured for 3 days, then the determination of the viability of the cell-lines groups for all samples were obtained by MTT assay.

I. MTT Assay:

Cytotoxicity activity was evaluated for CaCo cells via MTT 3-(4,5-Dimethylthiazol-2yL)-2,5 Diphenyl tetrazolium bromide) assay (MTT assay kit(cell proliferation) Abcam, (ab 211391) to Horiuchi et al.,(1988) [17,18] as follow:-

1. Cells ($1x10^5$ /well) were plated in 0.2 mL of medium /well in 96-well plates.
2. Removing medium from the wells carefully after incubation.
3. Washing each well with MEM (W/O) FCS 2-3 times and 200µL of MTT (5 mg/ml) was added.
4. Incubating plates for 6-7 hrs. in 5% CO_2 incubator for cytotoxicity.
5. Adding 1 mL of DMSO (Solubilizing reagent) was to each well and mixed well by micro-pipette and left for 45 sec.
6. The presence of viable cells was visualized by the development of purple color due to the formation of formazan crystals.
7. Transferring suspension to the cuvette of a spectrophotometer and the OD (Optical Density) values were read at 595 nm by using DMSO as a blank.
8. Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. The standard graph was plotted by taking the dose of treatment on the x-axis and relative cell viability on the y-axis.

The viability of the cell (%) = x100.

After determination of viability, the cells were going for another analysis (molecular analysis).
The molecular analysis included proteomics analysis of caspase-9 and Bax genes.

II. Molecular assay (proteomics analysis):

The proteomics analysis included two genes the caspase-9 gene and the Bax gene

as the following:

Blotting technique:

1- Total soluble proteins for all samples were purified through tri fast(peq lap VWR company)[isolation of RNA, DNA, and protein simultaneously]
2- Blotting Solutions:
   a- Blotting buffers:

25 mM Tris, pH 7.4, 0.15 M NaCl and 0.1% Tween 20

b- Blocking solution:

2-5% nonfat dry milk in Blotting Buffer Adjust pH to 7.4

c- Antibody solution:

1-5% nonfat dry milk in Blotting Buffer Adjust pH to 7.4

3. Blotting protocol:

Electrophoresed proteins on SDS-PAGE were transferred to a *Hybond*™ nylon membrane (GE Healthcare) via TE62 Standard Transfer Tank with Cooling Chamber (Hoefer Inc. and incubate for 1 hour at room temperature in Blocking Solution. Additionally, β-actin was applied as a housekeeping protein**.**

4. The membrane was incubated overnight at 4°C in Antibody Solution containing Anti- Caspase 9 primary antibody (Abcam, USA, ab25758), and Anti-Bax (ab232479). For normalizing data, Anti- β-actin primary antibody (Abcam, ab228001) was used.

5. The membrane was washed at room temperature for 30-60 minutes with 5 changes of Blotting Buffer.

6. The membrane was incubated for 1 hour at room temperature in Antibody Solution containing appropriate dilution of HRP-conjugated secondary antibody (Antibody concentration. 0.1-0.5 microgram/mL. Adjust antibody concentration from 0.05 to 2.0 microgram/mL to obtain desired signal strength and low background.

7. The membrane was washed for 30-60 minutes with 5 changes of Blotting Buffer.

The technique is called the **Western Blot Protocol References [19, 20, and 21].**

Data analysis:

Gel documentation system (Geldocit, UVP, England) was applied for data analysis using totallab analysis software, www.tatallab.com (ver. 1.0.1) [22].

3 Results

3.1 Statistical Analysis

The obtained data were presented as mean ±SE. One-way analysis of variance (ANOVA) was carried out using a statical package program (COSTAT). A probability level of $p < 0.05$ was considered.

The results of viability were as the following:

Table 1 The mean value of viability (%) for all groups. The values of G_1, G_2, G_3, and G_4 represent the percent of changes regarding normal control group 1.

Groups	Control	40 sec	60 sec	80 sec
	G_1 ±SE	G_2 ±SE	G_3 ±SE	G_4 ±SE
Viability	95±1.3(a)	81±2.6(b)	57±2.4(c)	55±2.4(c)

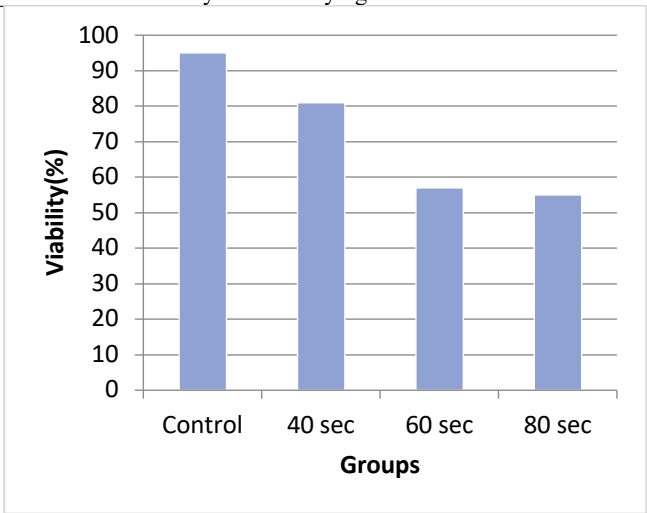

Fig. 2 Mean value of viability (%) for all groups.

Table 1 and **Fig. 2** represent the percent of changes related to the normal control group. Comparing to the normal control group, the levels of viability showed a significant decrease in all treated groups (G_2, G_3, and G_4). At the same time, the levels of changes were highly significant in G_3 and G_4 (60 seconds and 80 seconds of exposure time) respectively.

i.e. increasing the time of exposure leads to good treatment.

1. Caspase-9 gene

Fig. 3 The indication level of caspase-9 for CaCo cell line protein of four treatments.

Table 2: data parameters for the indication level of caspase-9 of CaCo cell line protein of the four treatments.

Groups	**G_1 (control)** ±SE	**G_2** ±SE	**G_3** ±SE	**G_4** ±SE
Caspase 9	82.5±1.3(c)	89.91±2.4(b)	89.91±2.1(b)	92.72±2.4(a)

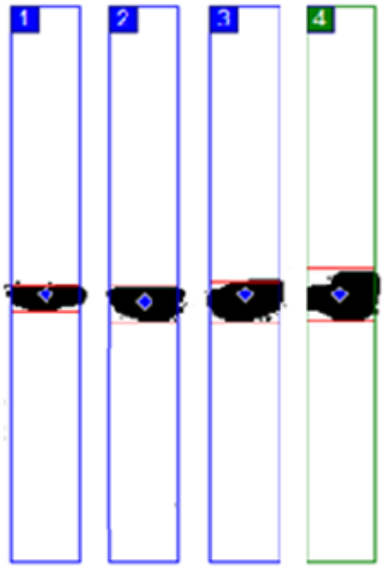

Fig. 4 Computerized detection of the indication level of caspase-9 for CaCo cell line protein of four treatments.

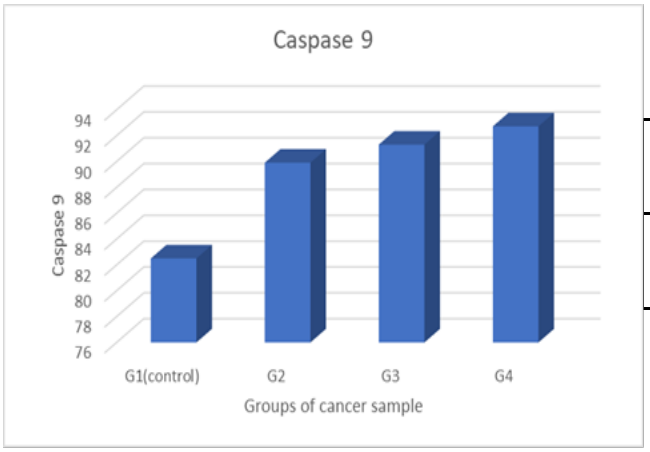

Fig. 5 the indication level of caspase-9 of CaCo cell line for four treatments.

1. Bax gene:

Fig. 6 Bax protein expression level for CaCo cell line protein of four treatments.

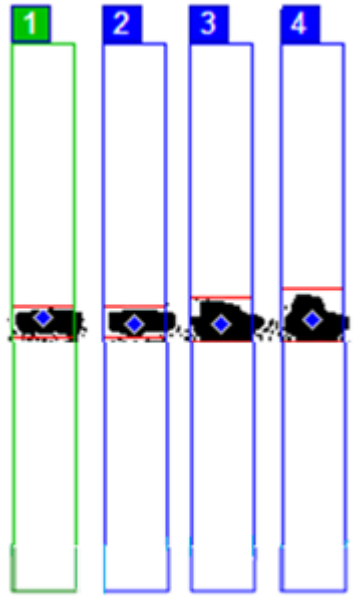

Fig. 7 Computerized detection of the indication level of Bax protein for CaCo cell line protein of four treatments.

Table 3: data parameters of the indication level of Bax protein of CaCo cell line protein for the four treatments.

Groups	**G_1 (control)** ±SE	G_2 ±SE	G_3 ±SE	G_4 ±SE
Bax	7.69±0.22 (d)	8.61±0.21 (c)	11.25±0.30 (b)	12.75±0.41 (a)

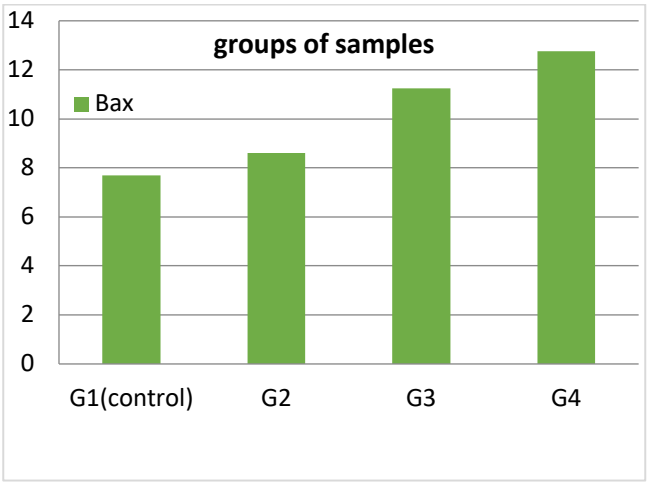

Fig. 8 The indication level of Bax protein of CaCo cell line for four treatments.

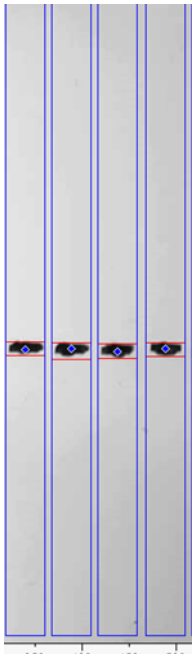

Fig. 9 β-actin protein expression level for CaCo cell line protein of four treatments.

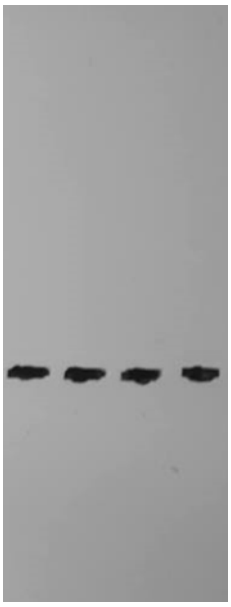

Fig. 10 Computerized detection of β-actin protein expression level for CaCo cell line protein of four treatments.

Table 4 data parameters of β-actin protein expression level for CaCo cell line protein with four treatments.

Groups	G1(control)	G_2	G_3	G_4
β-actin	10.25	10.37	10.77	10.12

4 Discussions

Plasma is an ionizing gas that contains charged particles such as electrons, ions, electronically excited molecules, and atoms, free radicals, and UV photons. Plasma treating exposes tissue surface or cells to active long and short-lived neutral atoms and molecules, including singlet oxygen (O_2), ozone (O_3), OH radicals, and NO, and a remarkable flowing of charged particles, including both positive ions, negative ions, and electrons [23].
Our physical cells are generated by the mitosis process and nearly all will stop living or die by the apoptosis process that is a physiological process of cell to commits suicide. Cancer happens when this equilibrium is disturbed, either by the reduction of cell death or the rising in cell reproduction. The purpose of cancer treatment is to increase the death of cancer cells without further destruction of the living cells. [24].

The results of this study proved that Dielectric barrier discharge plasma has a strong killing effect on malignant cells (Table 1 and Fig. 2) where the viability of colon cancer cells (CaCo) was significantly decreased as the result of exposure, and increasing the time of exposure leads to good treatment.

For supporting this result with another analysis (molecular protein analysis of two genes associated with cancer), and to know the mechanism of action for DBD plasma on cancer cells the study proved that; the DBD plasma exposure induces protein transcription of the Bax gene (pro-apoptotic gene) as shown in Table 3 and Fig.6 and the level of transcription increased with increasing time of exposure.

The same result was found in the case of Caspase 9 gene transcription (apoptotic gene), where the level of protein densities was significantly increased as the result of exposure, and increasing the dose of exposure increase the level of protein transcription (Table 2 and Fig. 5). This means that; the plasma induces apoptosis and cell programmed death of the malignant cells, giving finally more degradation of cancer cells.

The result of this study is in agreement with many studies where a significant portion of human breast cancer cells was observed to exhibit apoptosis fragmentation with the application of a pulsed atmospheric pressure plasma jet.

The apoptotic effect depends mainly on the components of the plasma plume [25].

In another study, a comparison of cancerous cells with their non-cancerous counterparts was performed to determine the selectivity of treatment. Through analysis of seven human

cell lines (cancerous; A549, U87, A375, and Malme-3M; non-cancerous: BEAS-2B, HA, and HEMA) and five different cell culture media (DMEM, PRMI 1640, AM, BEGM, and DCBM) revealed that the tested parameters influence indirect cold atmospheric plasma treatment, while direct treatment was less affected. The study demonstrated that cell type, cancer type, and culturing medium must be taking into account [26].

The conclusion of this study stated that Dielectric Barrier Discharge Plasma has a selectivity degradative effect on cancer cells through inducing the apoptosis process and able to destroy cancer cells without any damage to healthy cells.

In comparison between the densities of resulted protein (transcription) of the Caspase -nine gene between different doses of treatment and control group (see Fig. 7 and Table. 2, it was found that group 3 and group 4 (60 seconds and 80 seconds of exposure time) significantly increased which means that, the DBD plasma increase the transcription level of Caspase-9 (Apoptotic gene).

Increasing the level of this gene leads to an increase in the level of degradation of cancer cells.

Regarding the Bax gene, the densities of G_3 and G_4 (60 seconds and 80 seconds of exposure time) were highly significant increase in comparison with the control group (see Fig. 12, Table 3).

This result indicates the same result of the Caspase-nine gene, and the result of the two genes illustrates that the exposure time of the cell-line to DBD plasma induces the transcription of Apoptotic and pro-apoptotic genes which resulted in a killing effect on malignancy with different degrees according to the dose of exposure.

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