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Molecular Studies for Myelogenous Leukemia Exposed to Gliding Arc Plasma

A. Elsayed[1,*], *A. A. Elhadary*[2], *G. El-Aragi*[3] *and F. Fouda*[1]

[1]Zoology Department, Faculty of Women Arts, Science and Education, Ain Sham University, Cairo, Egypt
[2]Biological Application Department, EAEA (Egyptian Atomic Energy Authority), Cairo, Egypt
[3]Plasma Physics and Nuclear Fusion Department, EAEA, Cairo, Egypt

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Abstract: Gliding Arc Discharge (GAD) non thermal plasma is one type of important new technology in the field of biomedical applications, such as skin disease, wound healing and cancer treatment. This low temperature atmospheric-pressure plasma is a multi-component system that contains such biologically active agents, charged particles, reactive nitrogen and oxygen species, metastable-state molecules or atoms, and UV radiation among other things. The main purpose of this study is to investigate the effect of Gliding Arc Discharge on Acute Myelogenous leukemia cell line (AML). Different doses depend on the time of exposure (40, 60 and 80 sec) applied for one time, the cells going for culture 72 hours. Two types of analysis were carried out. The first one is MTT assay where the viability of cells showed significant decrease in all treated samples, also it's realized that increasing the time of exposure leads to good treatment. The second analysis was a molecular proteomics assay for two genes which are caspase-3 and cox-1gene. It was found that exposure to non-thermal plasma gliding arc increases the level of gene transcription (protein product) of caspase-3 gene (Apoptotic gene) which led to more degradation of malignant cells, where the transcription of cox-1 gene (inflammatory gene) reduced.

Keywords: Gliding Arc Discharge (GAD), Acute Myelogenous leukemia cell line (AML).

1 Introduction

Cancer is a genetic disorder caused by changes (mutations) in DNA in which cancerous cells continue to grow, divide and re-divide instead of dying and resulting in the formation of new abnormal cells [1, 2]. Acute myeloid leukemia (AML) is a heterogeneous disease distinguished by clonal expansion of myeloid progenitors (blasts) in the bone marrow and peripheral blood [3]. AML is mainly treated with chemotherapy, radiation therapy, or stem cell transplantation [4, 5]. Non thermal plasma is an ionized gas generated by electrical discharges in the atmospheric pressure at room temperature [6].

Cold pulsed atmospheric pressure plasma is a multicomponent system that contains biological active agents such as charged particles, reactive nitrogen and oxygen species, and electromagnetic radiation (ultraviolet, visible, infrared light). Therapeutic application of plasma at or in the human body is a challenge both for medicine and plasma physics [7].

Non-thermal atmospheric pressure plasma has drawn more and more attention worldwide in the biomedical sector over the last two decades. Plasma effects on mammalian cells are of basic interest in vitro experiments to describe plasma-cell interactions should include the basic cellular parameters such as morphology, viability or proliferation as well as the cellular responses like influence on DNA or cellular proteins [8]. Furthermore, the plasma acted at the cellular level to eliminate diseased tissue without causing inflammation and damage, to inhibit infectious and to modulate the viability (apoptosis/necrosis) of tumoral cells [9].

Gliding arc discharge (GAD) plasma is divided into thermal and non-thermal categories, characterized by their ionization which enables them to deliver high temperature. Non thermal plasma including low pressure glow radiofrequency and corona discharges, which give high selectivity and energy efficiency. These types are able to operate effectively at low temperatures without any quenching [10].

Apoptosis is phenomenon, (programmed cell death) carried out by two pathways [intrinsic and extrinsic] that includes the

*Corresponding author E-mail: amalelarabi264@gmail.com

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activation of a set of cysteine proteases known as “caspase”. The process of cell death by means of apoptosis is accompanied by a number of distinctive morphologic and metabolic changes [11].

Apoptosis is distinguished by different morphological characteristics and energy-dependent biochemical mechanisms. In addition to DNA fragmentation, apoptosis is morphologically characterized by the cytoplasmic condensation, nuclear pyknosis, chromatin condensation, cell rounding, membrane blebbing, cytoskeletal collapse and the development of membrane-bound apoptotic bodies that are rapidly phagocytosed and digested by macrophages or neighboring cells without activating immune response [12].

Caspase family members are critical regulatory networks controlling cell death and inflammation [13]. Caspase divided into 3 groups:

Initiator caspase [caspase-8 and caspase -9], executioner caspases [caspase-3, -6, and -7] and Inflammatory Caspases [Caspase-1, -4, -5, and -12]. Caspase-3 is one of the major executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of several important proteins, such as the nuclear enzyme poly (ADPribose) polymerase (PARP), which are cleaved in many different systems during apoptosis [14]. Cyclo-oxygenese -2 (cox-2) is causally linked to cancer. Identification of an enzyme catalyzing fatty acid oxidation as a rate limiting step in the progress from normal cell growth then new approach is far from the usual approaches based on nucleic acid metabolism, chemoprevention based on cox-2 has been achieved in a restricted group of patients [15]. Description of cox-2 enzyme concentrating on those of its characteristics particularly relevant to its participation in the processes of carcinogenesis, and this can be found in a number of reviews [16, 17].

2 Material and Methods:

2. Characteristics of the Plasma Machine

2.1 Gliding Arc Discharge (GAD) Experiment

The gliding arc (GA) is an intermediate system among thermal and non-thermal discharges, and is able to extend simultaneously high plasma density, power and operating pressure with high level of non-equilibrium, high electron temperature, low gas temperature and possibility of stimulation selective chemical processes without any quenching. The main advantages of GA are the “memory effect” and essential influence of convective heat and mass transfer on plasma properties and the space/time arc evolution.

Gliding arc gas discharge (GA) is an auto-oscillating phenomenon occur when at least two electrodes are immersed in a laminar or turbulent gas flow, and results in significantly non-equilibrium plasma area at elevated power level [18-19].

The reactor used for this study derives from the gliding arc device as illustrated in Fig. 1.

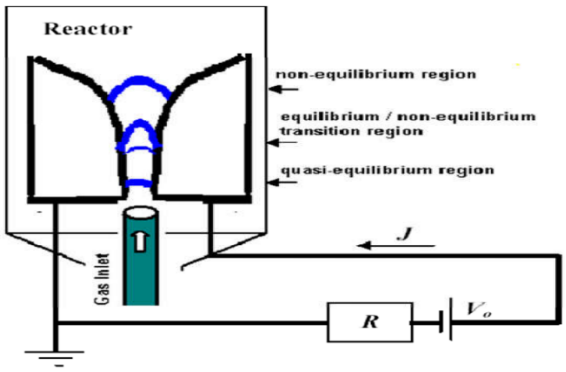

Fig. 1: Simplified scheme of the gliding arc

Fig. 2 Shows photographs of gliding arcs for various gas flow rates at constant discharge power. When the gas flow rate increased, emission intensity increased on the upstream side. On the other hand, the electric discharge area increased with increasing gas flow rates in the downstream side. The positive column of the main arc discharge which is controlled by discharge power, and downstream domain is a plasma jet plume which exists across between electrodes that depend on the gas flow.

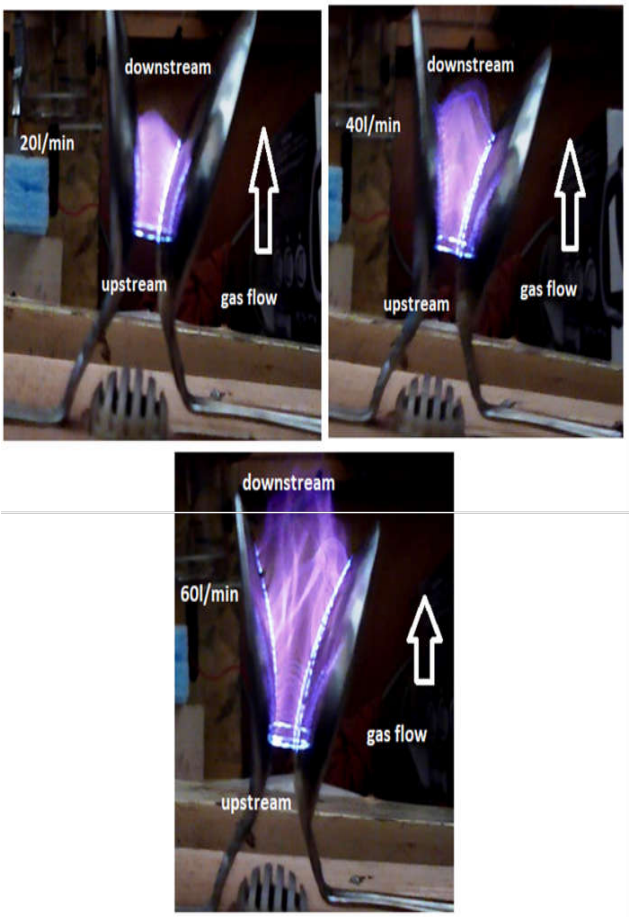

Fig. 2: The photographs of gliding arcs for different gas flow rate at constant discharge voltage

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The gliding arc discharge for various discharges of power at 60 l/min in air gas flow rate shows that, in the upstream side, emission intensity was strong, and it increased with increasing discharge voltage. Furthermore, electric discharge area is small and it was increased with increasing the discharge voltage. On the other hand, emission intensity is low, and the electric discharge area is high in the downstream side. Although emission intensity is almost constantly independent for the discharge voltage, discharge area decreased with increasing discharge voltage in the downstream.

The species formed in air discharge plasma region were detected with spectroscopic emission, and the result has been presented in Fig.3.

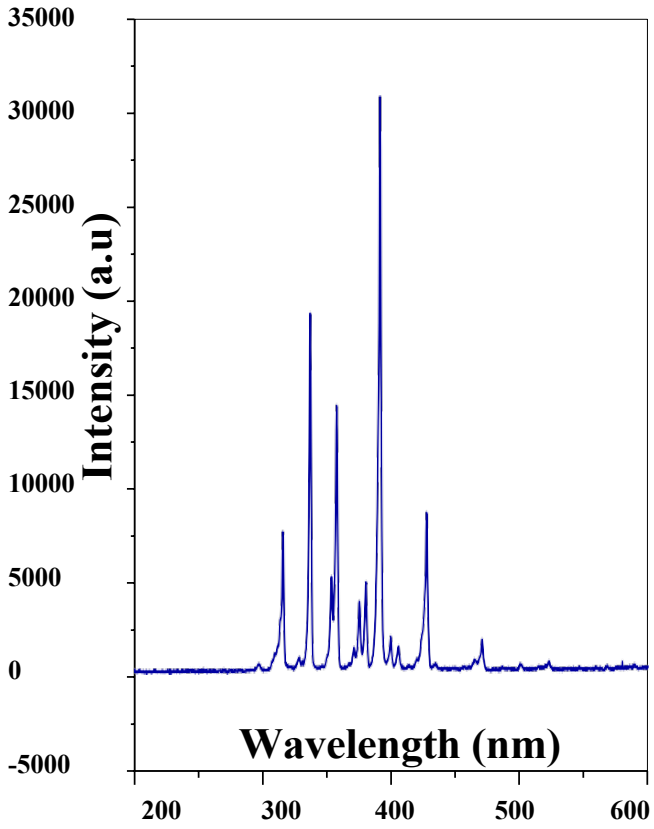

Fig. 3: Spectroscopic emission in air discharge plasma region

The Gas source is Hydrogen, Pressure 30 Bar, Atmosphere temperature 38, Device temperature 44 and The Distance between the device and the sample is 3 cm.

2.2 Cell-line and Treatment:

Cell-line of acute myeloid leukemia obtained from The Genetic Engineering unit, National Research Center of Egypt.

The Cell-line was cultured in EMEM medium (ATCC) supplemented with 10% fetal bovine serum (FBS, thermo Fisher Scientific) as Recommended by the supplier. Culture kept in a humidified 5% CO_2 Atmosphere at 37ċ and the medium was changed every week.

Tissue sampling was approved by the clinical and experimental medicine and the Atomic Energy Authority of Egypt Ethics committee.

The study carried out on a cell-line (Acutemyeloid leukemia model) where the cell-line was exposed to three different doses of physics non thermal plasma called (Gliding Arc Discharge) device.

The samples of cell-line divided into four groups and exposure includes three groups only as the following:

1- **First group:** control sample non exposeds

2- **Second group**: sample exposed to plasma for 40 seconds

3- **Third group**: sample exposed to plasma for 60 seconds

4- **Fourth group**: samples exposed to plasma for 80 seconds

After treatment the cell-line cultured for 3 days. viability of the cell-line determined by MTT assay.

2.3 MTT Assay:

Cytotoxicity activity was evaluated on cell-line (Acute myeloid leukemia) via MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (MTT assay kit(cell proliferation) Abcam, (ab 211391) according to Horiuchetal ; 1988 [20,21] as following :

1- cells ($1x10^5$ | well) were ploted in 0.2 ml of medium /well in 96 – well plates

2- Elimination medium from the wells carefully after incubation.

3- Washing each well with MEM (minimum essential medium), w/o(zero hormone) and FCS(fetal calf serum) for 2-3 times and 200 ML of MTT (5 mg/ml) was added

4- Incubation plates for 6-7 hrs –in 5%CO_2incubator for cytotoxicity.

5- To each well, add 1ml of DMSO (Diethyl sulpho oxide) solubilizing reagent, mix well with micropipette and left for 45 sec.

6- Presence of viable cells was visualized by the production of purple color due to formation of formazone 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 standard graph was plotted by

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taking concentration of the drug in X axis and relative cell viability in Y axis.

Cell viability (%) = mean OD| control ODx100

After determination of viability, there were a follow up of another analysis, these analyses were molecular analysis. The molecular analysis included proteomics analysis of caspase -3 and cox-2genes.

The data obtained were presented asmean SE. One way analysis of variance [ANOVA] was carried out using a statically package program [COSTAT]. Approvability of **P<0.05**was considered.

Antitumor activity of non-thermal plasma on cell-line (Acute myeloid leukemia) determined through the following analysis:

Determination of viability conducted through MTT assay and molecular proteomics assay for two genes, caspase-3 and cox-2gene.

* Caspase-3 gene represents apoptotic gene.

*Cox- 2 gene which increased in case of malignancy [cox-2 gene which associated with carcinogenesis of malignancy].

Molecular assay (proteomicsanalysis):

The proteomics analysis included two genes, caspase-3 gene and cox-2 gene. These analyses were as follows:

Protein extraction and purification:

Total soluble proteins for all cell line samples were purified through Tri fast (peq lab VWR company) isolation of RNA, DNA and protein simultaneously.

Data analysis:

Gel documentation system (Geldoc –it , UVP, England) was applied for data analysis using Totallab analysis software , ww, totallab .com (ver.1.0.1) .[22].

Blotting technique:

1. Blotting Solutions:

1-a- Blotting buffers:

25 mMTris, pH 7.4, 0.15 M NaCl and 0.1% Tween 20.

1-b- Blocking solution:

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

1- c- Antibody solution:

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

2. Blotting protocol:

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

3. Membrane was incubated the overnight at 4°C in Antibody Solution containing Anti- caspase -3primary antibody (abcam,USA,ab13847) and Cyclooxygenase-2 (cox-2) (abcam,USA, ab15191). For normalizing data, Anti- β-actinprimary antibody (abcam ,ab 228001) was used.

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

5. 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.

Western Blot Protocol References:

Vedpaletal ; 1994 [23] .

John D Pound ; 1998 [24] .

Harlow and David Lane, 1999 [25] .

The results of viability were as following:

Table 1: mean values of viability (%) of all groups .

Groups	G1 (Control) $X_1^- \pm$ SE	G2 (40 sec) $X_2^- \pm$ SE	G3 (60 sec) $X_3^- \pm$ SE	G4 (80 sec) $X_4^- \pm$ SE
Values	100 ± 0.0^a	75 ± 2.6^b	62 ± 2.3^c	45 ± 2.1^d

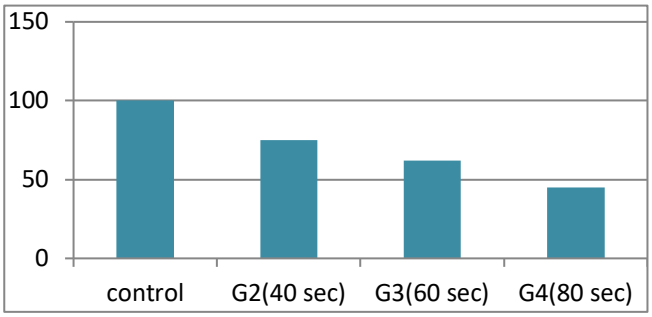

Fig. 4: mean values of viability (%) of all groups.

Table (1) and Figure (1) represent the percent of changes relative to normal control group, comparing to normal control group, the levels of viability showed significant decrease in all treated groups (G2: G4) .

The values of G1, G2, G3, G4, represent the percent of changes relation to normal control group1 .

The changes were highly significant in G3 and G4 (60 sec and 80 sec exposure) respectively. Increasing the time of exposure leads to good treatment.

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3 Results of genes analysis

1-caspace-3 gene

2-cox-2 gene

Fig. 5: Cyclooxygenase-2 (COX-2) protein expression level for Acute myeloid leukemia cell line protein with four treatments.

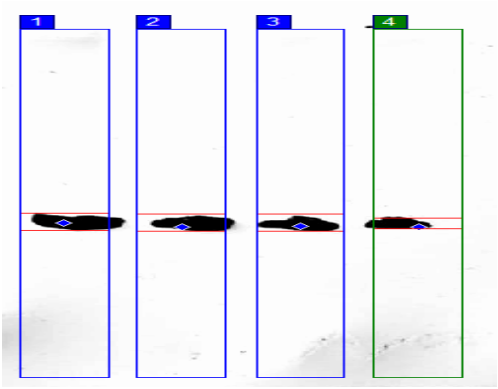

Fig. 6: Computerized detection of Cyclooxygenase-2 (COX-2) protein expression level for Acute myeloid leukemia cell line protein with four treatments.

Table 2: Data parameters of Cyclooxygenase-2 (COX-2) proteinexpression level for AcutemyeloidLeukemiacell line protein with four treatments.

Groups	Lane 1 % Control $X_1^- \pm$ SE	Lane 2 % 40 sec $X_2^- \pm$ SE	Lane 3 % 60 sec $X_3^- \pm$ SE	Lane 4% 80 sec $X_4^- \pm$ SE
Values	72.63±2.2[a]	72.13±2.1[a]	70.82±2.1[a]	44.31±1.6[b]

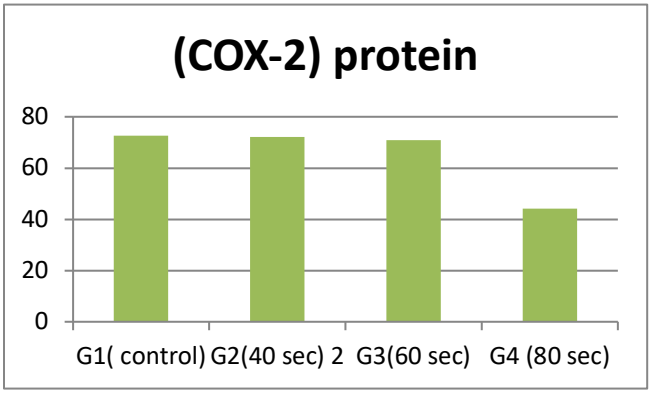

Fig. 7: Cyclooxygenase-2 (COX-2) expression level of Acute myeloid leukemia cell line for four treatments.

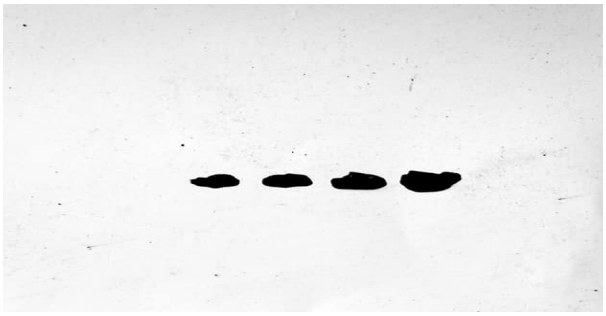

Fig. 8: Caspase- 3 protein expression level for Acute myeloid leukemia cell line protein with four treatments

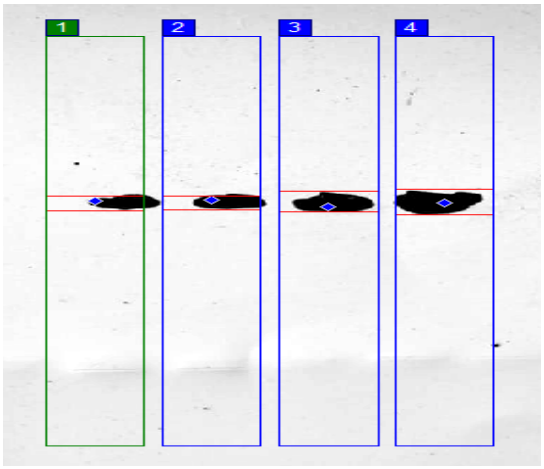

Fig. 9: Computerized detection of Caspase 3 protein expression level for Acute myeloid leukemia cell line protein with four treatments.

Table 3: Data parameters of Caspase 3 protein expression level for Acute myeloid leukemia cell line protein with four treatments

Groups	Lane 1% Control $X_1^- \pm$ SE	Lane 2% 40 sec $X_2^- \pm$ SE	Lane 3% 60 sec $X_3^- \pm$ SE	Lane 4% 80 sec $X_4^- \pm$ SE
Values	24.79±0.7[d]	27.47±0.9[c]	37.29±1.1[b]	42.13±1.7[a]

ANOVA DUNCAN`S test at < 0.05

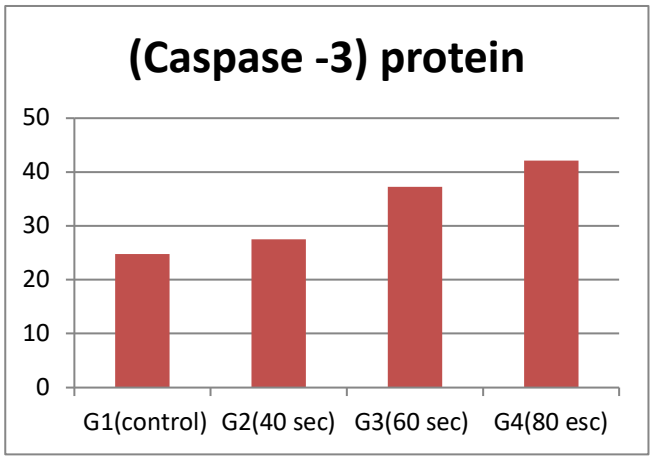

Fig. 10: Caspase 3 expression level of cell Acute myeloid leukemia line for four treatments.

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4 Discussion:

Many researchers are studying cold atmospheric plasma (an ionized gas) as a possible therapy in dentistry and oncology. Helium, Argon, Nitrogen, Heliox and Air are some of the gases that can be used to produce cold atmospheric plasma. There are many methods of production by which cold atmospheric plasma is created. It has been demonstrated that reactive oxidative species, charged particles and UV photons play the main role of treatment. It has been shown that cold plasma induces apoptosis, necrosis, cell detachment and senescence by disrupting the S phase of cell replication in tumor cells. This finding opens up its potential therapy in oncology [26].

Cold Atmospheric Plasma has been overall studied in the treatment of cancer, aiming to increasing tumor cell death and decreasing the therapy `s effect to healthy tissue [27].

The reactive ionized species such as OH ., H_2O2, N_2, NO, and O_2 are the major component of the cold plasma jet that supplies for therapeutic effects, not only with cancer, but also with biological disinfection [28].

This study proved that; exposure to Gliding Arc Plasma produce significant degradative effect on the cell of leukemia (table1), and the level of the death increased with increasing the time of exposure.

This result is in agreement with the study of (Neha, etal 2014), which demonstrated that; A plasma treatment significantly decreases cell viability, causing more severe damage to DNA and mitochondria, as determined by means of apoptosis markers and related genes expression. Taking together these findings indicate a significant level of apoptosis in plasma treated lymphoma cells [29].

Also, the study proved that; treatment using Gliding Arc Plasma leads to increase the level of (caspase -3 Apoptotic gene) transcription, this appeared from the significant increase of its protein product, and this product was more increased with increasing time of exposure.

At the same time the level of cox-2 gene transcription was significantly decreased, and the level was more decreased with the time of exposure increased.

These results mean that; the treatment by using Gliding Arc Plasma may induce the transcription of genes associated with cancer (Acute myelogenous leukemia), where this effect coming from up regulation of genes responsible of apoptosis and down regulation of genes associated with tumorgenesis [30].

Proved that transferred cells are subject to elimination through intercellular reactive oxygen / nitrogen species (RONS) – dependent apoptosis – inducing signaling. Tumor progression requires expression of membrane – bound catalase, thus inducing apoptosis selectively in tumor cells, therefore Cold Atmospheric Plasma (CAP) derived o2 induces the mechanism through which CAP acts selectively against cancer cells in vitro and tumors in vivo [30].

5 Conclusion

This study proved that; the gliding Arc non thermal plasma have a highly toxic effect on leukemia (blood cancer), and mechanism of action coming from, the chemical and physical component of the plasma on transcription of apoptotic genes and tumorgenesis genes.

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