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Cytogenetic and Immunological Effects on Human Blood Cultures Resulting from Cold Pulsed Atmospheric Pressure Plasma Jet Exposure

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ABSTRACT: Plasma medicine is an emerging field of research that aims to increase understanding and utilization of the interaction of plasmas with living tissues and cells. Low-temperature atmospheric-pressure plasma is a multi-component system that includes such biologically active agents as charged particles, reactive nitrogen and oxygen species, metastable-state molecules or atoms, and UV radiation. The main objective of this study was to investigate the effects on human blood cultures of exposure to the plasma components for different time periods (20 s, 40 s, 60 s, and 80 s). The present study recorded the scoring of micronuclei in both mono- and bi-nucleated lymphocytes as well as the apoptosis and necrosis of cells for each time period of exposure. Three blood samples for each experimental dose were compared with a non-exposed (control) group. In addition, the levels of interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) were analyzed for each experimental dose and for the control samples. The results showed that the exposure of blood samples to the plasma jet yielded significant incremental differences in micronuclei, IL-1β, and TNF-α, except in the first dosage group. Based on these results, we conclude that plasma can be used to repair tissues, cure diseases, and treat tumors.

KEY WORDS: plasma jet, micronucleus test, interleukin-1β, blood culture, tumor necrosis factor alpha

I. INTRODUCTION

Advances in life science increasingly utilize unrelated technologies and knowledge. Microelectronics, optics, material sciences, and nanotechnologies have become key technologies in modern medicine. Now, a similar trend is expected concerning plasma technology. Plasma medicine is emerging worldwide as an independent medical field comparable to the launch of laser technology into medicine years ago.[1]

During recent years, the application of low-pressure, atmospheric-pressure plasma has been well established in several industrial processes. New applications of plasma in medicine will exceed these current uses because their main focus is the direct application of physical plasma to the human or animal body.[2]

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One of the most important advances under development is the plasma treatment of chronic wounds to enable selective antimicrobial activity without damaging the surrounding tissue, combined with a controlled stimulation of tissue regeneration. Other promising fields in plasma medicine include tissue engineering, treatment of skin disease, and tumor treatment based on specific induction of apoptic processes.

The main characteristic of plasma sources for use in medical therapy is its direct application to the human body. Therefore, plasma effects on mammalian cells are of primary interest. *In vitro* tests to characterize plasma–cell interaction should include characterization of basic cellular parameters like morphology, viability, or proliferation of special cellular responses, such as influences on DNA or cellular proteins.[3–6]

The most important aspect of field of the atmospheric-pressure plasma jet (APPJ) is its interaction with DNA in terms of safety and the estimation of potential risks. To address these questions, several research studies have used comet assay and electrophoresis to investigate the APPJ treatment of suspended human cells (cell cultures) in terms of DNA fragmentation. Preliminary results have shown that APPJ significantly influences DNA, but a tendency of repair within 4 to 24 hours after plasma treatment has also been revealed. Because of the importance of this new technology, current investigations are using additional techniques, such as micronucleus assay and flow cytometry, to obtain more detailed information about basic plasma influences on the genetic code of cells.[2,6,7]

Apoptosis, one of the major types of cell death, can be modulated by programmed control mechanisms. In contrast to acute traumatic cell death, apoptosis is important not only in the turnover of cells for regeneration in all types of tissues but also during the normal development, differentiation, and senescence of an organism.[8] In contrast to its significant roles in physiological processes, defective apoptotic processes have been implicated in a variety of diseases, such as atrophy, autoimmunity, neurodegenerative disorders, acquired immune deficiency syndrome, and uncontrolled cell proliferation (as found in cancer).[9]

Several investigation methods allow cytokines to be used as radioprotective or radiosensitizing agents. They can also be used to minimize the effects of irradiation indirectly by neutralizing other harmful cytokines. Several studies recorded the radioprotective effects of IL-1β and TNF-α. Natural levels of IL-1β and TNF-α confer radio-resistance by promoting repair and restoring the host defenses.[10,11] IL-1β has been hypothesized to induce hematopoietic growth factors and endogenous antioxidant mechanisms such as metallothionein, cerruloplasmin, and MnSOD.[12]

II. MATERIALS AND METHODS

A. Characteristics of the Plasma Machine

The most important devices for generating non-thermal atmospheric plasmas are the atmospheric pressure plasma jet (APPJ),[13,14] the plasma needle,[15] the plasma pencil,[16,17] the miniature pulsed glow-discharge torch,[18] the one-atmosphere uniform glow-discharge plasma,[19] resistive barrier discharge,[20] and dielectric barrier discharge.[21]

The plasma generator used in the present experiments consists of a negative direct current (dc) source, a Blumlein-type pulse-forming network (E-PFN), and a dynamic spark gap switch. A triggered spark gap switch is used as a closing switch for the E-PFN. The E-PFN has four stages of the LC ladder, composed of 5 nF of capacitor and 3 μH of inductor. The characteristic impedance ($2\sqrt{L/C}$) and the pulse width ($2N\sqrt{LC}$) of E-PFN, calculated from capacitance (C) and inductance (L) of the LC ladder and number (N) of LC ladder stages, are approximately 49 Ω and 1.0 μs, respectively.

The configuration of this apparatus is as follows: The charging resistance is 50 kΩ; this value corresponds to a charging RC time constant of 1 ms, which is 40 times faster than the repetition rate of the pulse.

A schematic of the pulsed atmospheric-pressure plasma jet (PAPPJ) device for generating high-voltage (HV) pulsed, cold atmospheric plasma jets is shown in Figure 1. The HV wire electrode, which is made of a copper wire, is inserted into a hollow barrel of a syringe. The distance between the tip of the HV electrode and the nozzle is 0.5 cm.

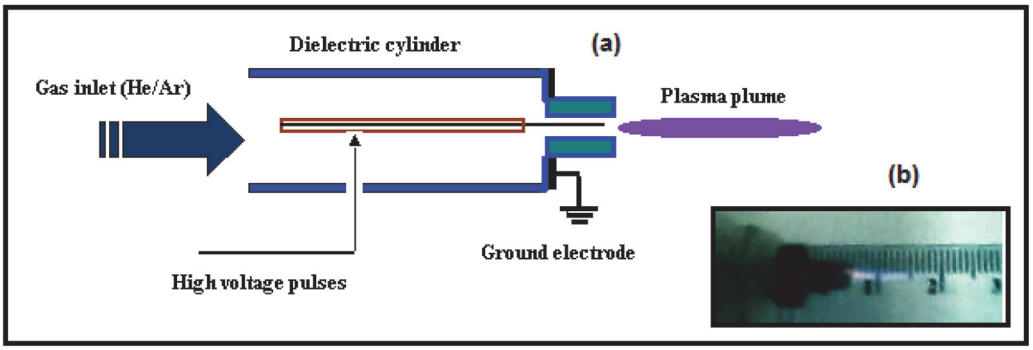


FIG. 1: (a) Schematic diagram of the plasma jet generator. (b) Image of the plasma jet with argon gas.

HV pulsed dc voltage (amplitudes up to 25 kV, repetition rate up to 25 Hz) is applied to the HV electrode and helium gas is injected into the hollow barrel. This device uses a medical syringe (made out of an insulating material cylinder). The gas is fed into the system via a flow meter. The applied voltage to and the discharge current through the discharge chamber are measured using a voltage divider (homemade) connected between the two electrodes, and a current monitor, which is located on the return to the ground. The signals from the voltage divider and the current monitor are recorded using a digitizing oscilloscope (Lecroy, USA) with a 200-MHz bandwidth.

The HV pulses are applied between the needle electrode positioned inside a dielectric cylinder (a simple medical syringe) and a metal ring is placed on the exterior of this cylinder. To obtain electric discharges at atmospheric pressure, high-voltage pulses (tens of kV), which have limited duration (hundreds of nanoseconds) and are repeated (tens of pulses per second), in addition to an inert gas (argon), are introduced into the cylinder. The gas flows are in the range of 0.5–10 L min^{-1}. The discharge takes place

between the metallic needle top and a metallic ring fit on the outer surface of the syringe. Under optimal conditions, plasma is emitted as centimeter-long jets, just millimeters in diameter or even smaller.

The working gases are supplied by high-pressure cylinders. Gas pressure regulators are used to reduce the pressure of gases to a workable level. Then, gas flow controllers deliver the gases with the desired flow. For voltage amplitudes of 15–18 kV, the plasma jet is very weak. The plasma jet disappears at voltage amplitudes lower than 15 kV.

When argon is injected from the gas inlet and a high-voltage pulse (26 kV voltage) is applied to the electrode, the plasma jet is generated, and a plasma plume reaching a length of 21 mm is launched through the end of the tube and into the surrounding air. The length of the plasma plume is adjusted using the gas flow rate and the applied voltage.

A Lecroy 200 MS/s 4-channel digital storage oscilloscope model (9304c) records voltage and current waveforms via a high-voltage probe and a pulse-current transformer, respectively, and to calculate the discharge power. The measured peak value of the discharge current is approximately 10.5 A during the pulse. Figure 2 shows the current and voltage waveforms measured as a function of time at an input energy of 6.76 J (maximum applied voltage 26 kV). Figure 3 shows the power input as a function of time; the maximum power is approximately 150 kW at 167 ns.

The high reactivity of plasma result from different components such as electromagnetic radiation (UV/VUV) visible light, IR, high-frequency electromagnetic fields, etc. on the one hand, and as a result of ions, electrons, and reactive chemical species,

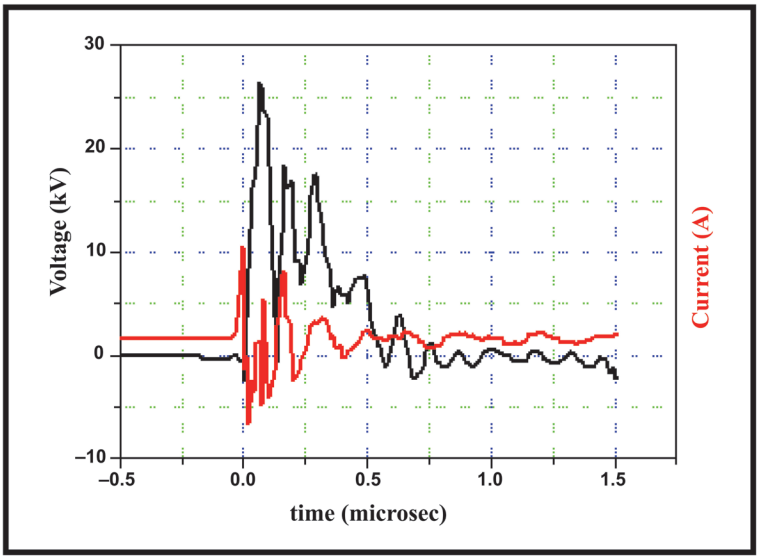


FIG. 2: Typical discharge current and the voltage waveforms.

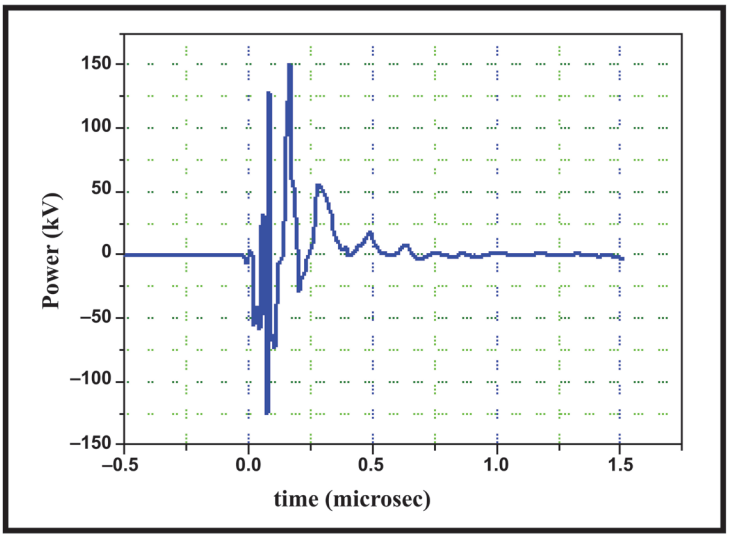


FIG. 3: The power waveform of the device.

primarily radicals, on the other. In addition, argon gas is fed through the annular region between the two electrodes (high-intensity lethal plasma effects).[22]

B. Chemicals

The chemicals for the blood culture were purchased from Gibco (Carlsbad, CA, USA); heat-inactivated fetal calf serum (FCS) was purchased from Sigma-Aldrich (St. Louis, MO, USA), a human IL-1β ELISA kit was purchased from AviBion (Finland); and a human TNF-α ELISA kit was purchased from Anogen Co. (Ontario, Canada).

C. Blood Sampling

To overcome possible inter-individual variability in response to treatments, a blood sample was obtained from one healthy female donor (age 36 years, non-smoker) who provided informed consent for participation in the study. The donor was selected according to the current International Program on Chemical Safety (IPCS) guideline for the monitoring of genotoxic effects of carcinogens in human.[23] Venous blood was collected under sterile conditions in a heparinized vacutainer tube (v = 5 ml; Becton Dickinson, USA) containing lithium heparin as an anticoagulant.

D. Experimental Design

The heparinized blood was divided into five groups; three samples were processed (n = 3) for each culture. The APPJ was applied directly to the heparinized blood samples

(four groups) at a distance of 3 cm from the blood surface in each tube at the four exposure periods selected for study (20 s, 40 s, 60 s, and 80 s). One non-exposed sample was reserved to represent the control group. The blood groups were incubated at 37°C for 4 h before the initiation of cultures and biochemical investigations. Group C was the control group, and the exposure groups were D_1 (20 s exposure), D_2 (40 s exposure), D_3 (60 s exposure), and D_4 (80 s exposure).

E. Blood culture

Blood cultures were set up for 72 h according to the protocol described by Evans and O'Riordan (1975).[24]

1. Micronucleus Assay

The micronucleus assay was performed as described by Fench (2000)[25] and Kirschvolders et al., (2001)[26] with some modifications. We recorded mononucleated and binucleated cells for the presence or absence of micronuclei.

Moreover, apoptic and necrotic cells were detected in 1000 scored cells for each sample.

2. Determination of IL-1β

IL-1β was determined according to the method described by Orgenium laboratories (IL-1β ELISA kit) from AviBion Human IL-1β ELISA.

3. Determination of TNF-α

TNF-α was determined according to the method described by Anogen laboratories (TNF-α ELISA kit).[27]

F. Statistical Analysis

Data are presented as distribution analyses, percentages, means ± SE. Data were analyzed using two-way analysis of variance and an "F" test according to Abramowitz and Stegum.[28] The level for statistical significance was $p < 0.05$.

III. RESULTS

The results show non-significant differences between the control group and D1 for all data presented in Tables 1–3. The percentage and the probability values between means of mononucleated, binucleated, apoptic, and necrotic cells in cultured human blood are recorded in Table 1.

APPJ exposure for the D2, D3, and D4 groups resulted in significantly increased frequencies of mononucleated cells with one and two micronuclei, binucleated cells

TABLE 1. The incidence of mononucleated and binucleated cells with and/or without micronuclei and of apoptic and necrotic cells in exposed and control blood groups (count in 1000 cells).

Groups	Control	D1	D2	D3	D4
Mononucleated cells	92.2% 922 ± 4.36	90.8% 907.7±3.94	72.1% 721±1.53[a,b]	75.9% 759.3±2.91[a,b,c]	54.7% 547±3.61[a,b,c,d]
Mono+ 1 Mn	0.83% 8.33±0.34	1.1% 11±2.00	4.47% 44.67±2.03[a,b]	4.77% 47.70±2.97[a,b]	4.8% 48±1.53[a,b]
Mono+ 2 Mn	0.07% 0.67±0.66	0.07% 0.67±0.34	0.6% 6±1.03[a,b]	0.97% 9.67±0.66[a,b,c]	0.5% 5±1.53[b,d]
Binucleated cells	6.47% 64.70±3.85	7.3% 73±7.24	15.97% 159.7±1.20[a,b]	9.2% 92.3±1.34[a,b,c]	6.97% 69.7±0.88[c,d]
Bi+ 1 Mn	0.17% 1.67±0.34	0.27% 2.67±0.34	1.73% 17.33±0.88[a,b]	3.01% 30.67±0.88[a,b,c]	1.63% 16.33±1.34[a,d]
Bi+ 2 Mn	0.00% 0.00±0.00	0.03% 0.33±0.34	0.2% 2±2.00	0.2% 2±1.16	0.13% 1.33±0.88
Apoptic and necrotic cells	0.27% 2.67±0.66	0.47% 4.67±1.46	4.93% 49.33±0.66[a,b]	5.83% 58.33±0.33[a,b,c]	31.27% 312.7±0.44[a,b,c,d]

Mononucleated cell= Mono
Binucleated cell= Bi
Micronuclei= Mn

a: Significant difference from group (control).
b: Significant difference from group (D1).
c: Significant difference from group (D2).
d: Significant difference from group (D3).

with one micronucleus, or apoptic and necrotic cells compared with the control and D1 groups. However, the incidences of mononucleated cells were significantly decreased for the same groups compared with the control and D1 groups. The percentage values of binucleated cells significantly increased in groups D2 and D3 compared with the control, D1, and D4 groups.

Table (2) shows the percentage values of normal, aberrant, apoptic, and necrotic cells. In addition, the total number of micronuclei that expressed in mononucleated and binucleated cells were represented.

The frequencies of the normal cells showed gradually decreasing counts from the D1 group to the D4 group, whereas the number of aberrant cells increased up to the D3 group. In the D4 group, the lowest number of aberrant cells was recorded and the highest number of apoptotic and necrotic cells, compared with D2 and D3 groups. The total number of micronuclei significantly increased in the D2, D3, and D4 groups compared with control and D1 groups, while in the D4 group the lowest percentage of micronuclei was recorded, compared with the D2 and D3 groups.

Table 3 represents the IL-1β and TNF-α data in exposed and control blood cultures. The levels of IL-1β non-significantly increased in the D1 and D2 groups but significantly increased in D3 and D4 groups compared with the control group.

TABLE 2. The frequencies of the total numbers of normal cells, aberrant cells, apoptic and necrotic cells and micronuclei in exposed and control blood groups.

Groups	Control	D1	D2	D3	D4
Normal cells (Mono+Bi)	98.7% 986.7±1.46	98.07% 980.7±3.53	88.07% 880.7±2.73[a,b]	85.2% 851.7±3.34[a,b,c]	61.67% 616.7±3.34[a,b,c,d]
Aberrant cells (Mono + Bi)	1.07% 10.67±0.88	1.47% 14.67±2.19	7% 70±2.09[a,b]	9% 90±3.51[a,b,c]	7.07% 70.70±2.67[a,b,d]
Mono+ 2 Mn	0.07% 0.67±0.66	0.07% 0.67±0.34	0.6% 6±1.03[a,b]	0.97% 9.67±0.66[a,b,c]	0.5% 5±1.53[b,d]
Total apoptic and necrotic cells	0.27% 2.67±0.66	0.47% 4.67±1.46	4.93% 49.33±0.66[a,b]	5.83% 58.33±0.33[a,b,c]	31.27% 312.7±0.44[a,b,c,d]
Total number of Mn	1.13% 11.33±1.46	1.57% 15.67±2.19	7.8% 78±4.36[a,b]	10.17% 101.7±4.26[a,b,c]	7.7% 77±5.01[a,b,d]

Mononucleated cell= Mono
Binucleated cell= Bi
Micronuclei= Mn

a: Significant difference from group (control).
b: Significant difference from group (D1).
c: Significant difference from group (D2).
d: Significant difference from group (D3).

TABLE 3. The levels of IL-1β and TNF-α in human blood cultures exposed to APPJ.

Groups	IL-1β (pg/ml)	TNF - α (pg/ml)
Control	3.10±0.04	28.59±0.19
D1	3.28±0.11	29.06±0.58
D2	3.41±0.13	30.33±0.09[a]
D3	3.94±0.03[a,b,c]	30.73±0.11[a,b,c]
D4	4.97±0.17[a,b,c,d]	31.55±0.29[a,b,c]

Mononucleated cell= Mono
Binucleated cell= Bi
Micronuclei= Mn

a: Significant difference from group (control).
b: Significant difference from group (D1).
c: Significant difference from group (D2).
d: Significant difference from group (D3).

In contrast, the mean values of TNF-α data showed increases incrementally in the D1 to D4 groups. The D4 group showed significant increases in IL-1β and TNF-α compared with the D2 and D3 groups.

The previous levels of IL-1β and TNF-α were represented in Figs. 4 and 5.

IV. DISCUSSION

The plasma jet has been proposed as a novel therapeutic method for anticancer treatment. However, its biological effects and mechanisms of action remain elusive.[29] In the future, sophisticated evaluation of biological plasma effects will be facilitated by a com-

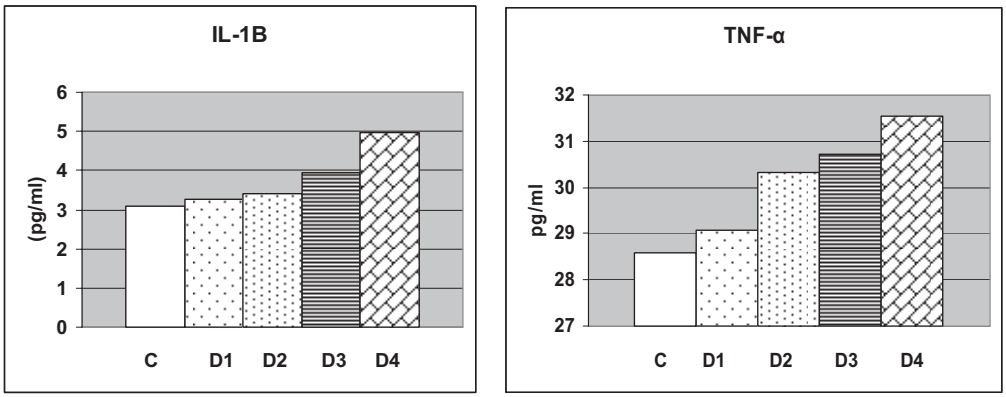


FIG. 4: represent the levels of IL-1 β and TNF-α in human blood cultures exposed to APPJ.

bination of plasma technology and plasma diagnostics with cell biological, biochemical, and analytical techniques based on *in vitro* models using microorganisms as well as cell and tissue cultures.[30]

To achieve the sustained success of plasma medicine, for any potential application, optimal plasma composition (i.e., radicals, irradiation, temperature, etc.), useful application rate (time periods for exposure), and acceptable relations between desired therapeutic effects and adverse reactions must be determined.[2] In the present study, we investigated the relation between the dose rate and the cellular responses (division, genetic damage, and apoptosis) and immunological induction and responses. We showed that micronuclear assay is suitable tool for these investigations, and that IL-1β and TNF-α act as the protypic multifunctional cytokines, which are potent immune-modulators, mediating a wide range of immune responses.

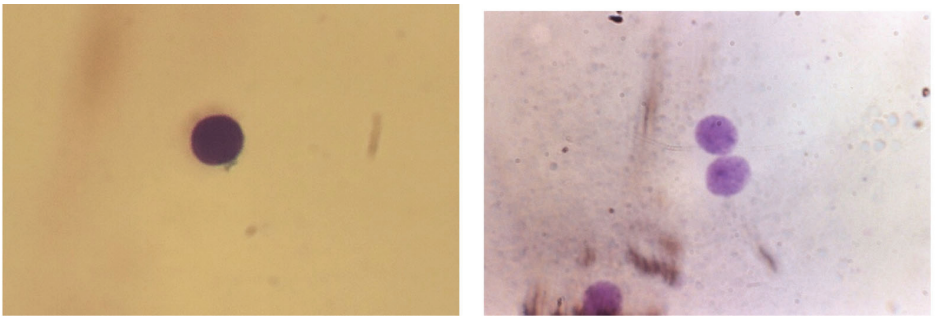
A micronucleus is formed during the metaphase/anaphase transition of mitosis (cell division). Scoring of micronuclei can be performed relatively easily and on different cell types relevant for human biomonitoring.

An *in vivo*/ *in vitro* analysis of lymphocytes in the presence of cytochalasin-B (added 68 h after the start of cultivation) allows the researcher to distinguish easily between mononucleated cells and binucleated cells that have completed nuclear division during *in vitro* culture. Moreover, apoptic cells, necrotic cells, the nucleoplasmic bridge, and nuclear buds can be scored and detected according to new criteria with the cytokinesis block micronucleus assay.[26]

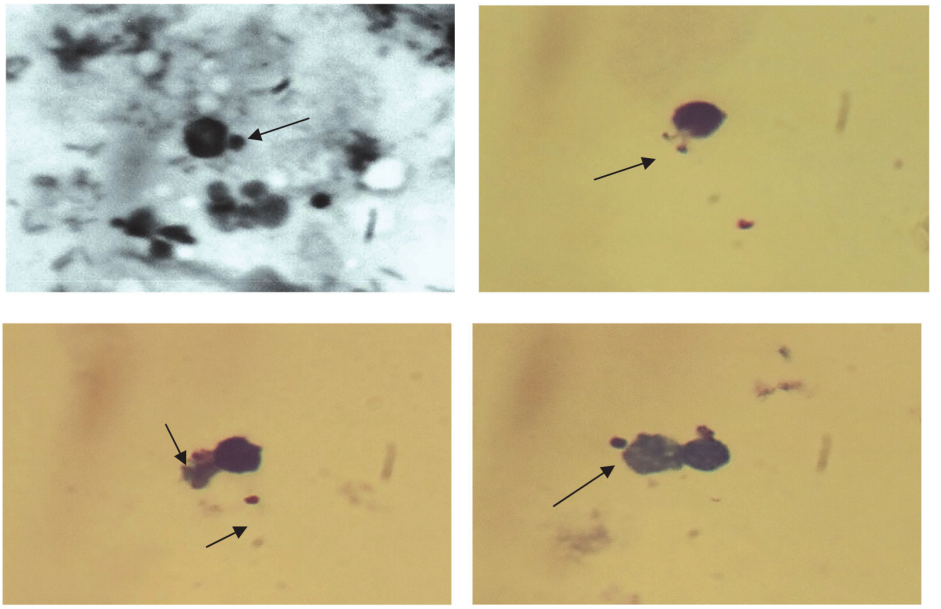
Recently, several studies have reported that APPJ and nitrogen jets induce apoptosis of cancer cells by generating DNA damage. However, the molecular mechanism by which plasma induces apoptosis and which signal(s) stimulate plasma-induced apoptosis remain unclear.[31]

APPJ can generate reactive oxygen species (ROS) such as ozone, atomic oxygen, superoxide, peroxide, and hydroxyl radicals. There is growing evidence that the redox environment of a cell is able to control apoptosis by dissipation of mitochondrial transmembrane

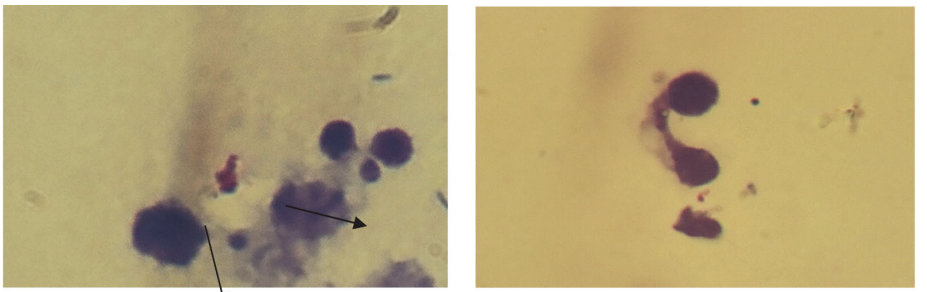
Micrograph of peripheral blood lymphocytes showing



(A) Normal cells (mononucleated and binucleated cells).

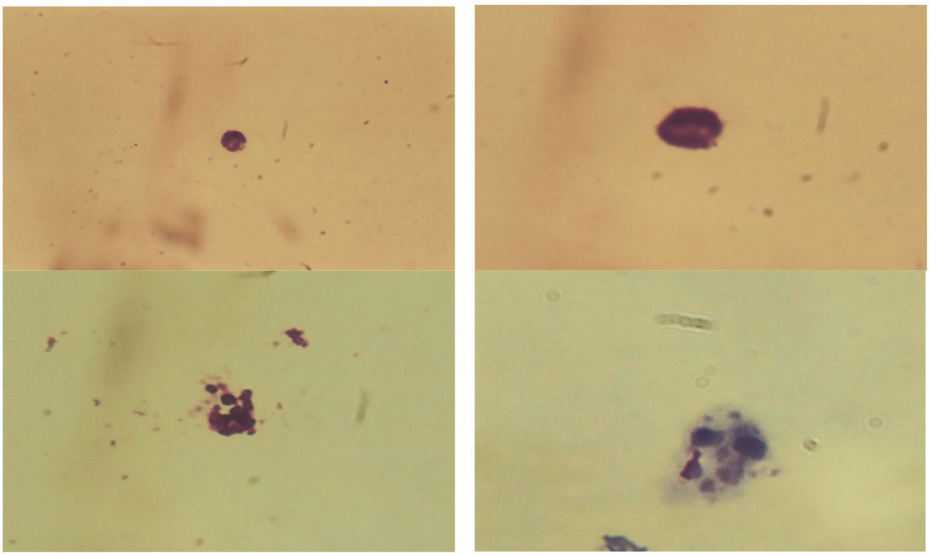


(B) Mononucleated cells with one and two micronuclei.

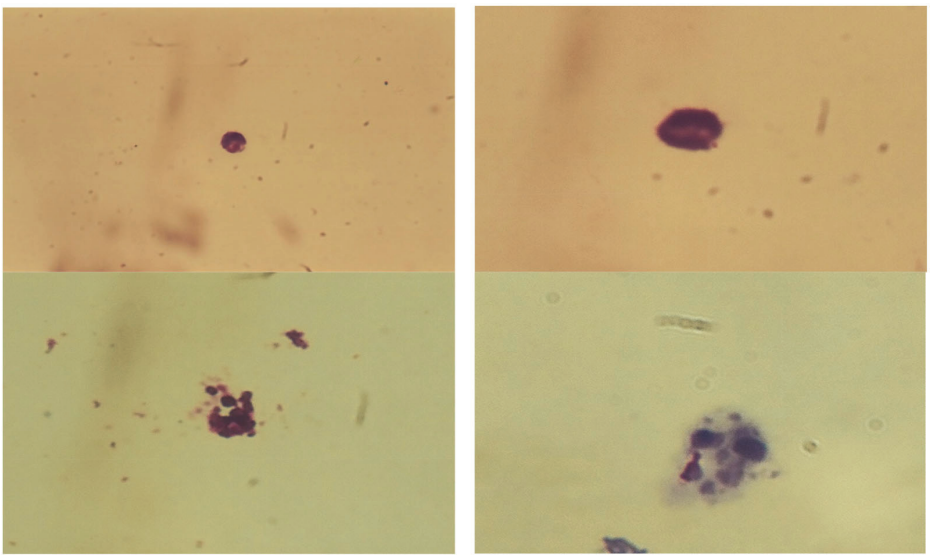


(C) Binucleated cells with one micronucleus and nucleoplasmic bridge.

FIG. 5: represent the levels of IL-1 β and TNF-α in human blood cultures exposed to APPJ.



(D) Different stages of apoptic cells.



(E) Different stages of necrotic cells.

FIG. 5: Continued

potential.[32] The major free radicals such as ROS and nitric oxide (NO) play an important role in a number of biological processes, such as the intercellular killing of bacteria by phagocytic cells. They have also been implicated in cellular redox signaling. However, because of their reactivity, excessive amounts of free radicals can lead to cell damage and death.[33]

In the present study, the total number of normal and aberrant cells in D1 (20 s) group were not significantly different compared with control group. In addition, an increase in the number of micronuclei was recorded in the mononucleated cells, but its value was within normal levels.

While the percentages of the normal cells gradually decreased in the D2 (40 s) group and in the D3 (60 s) group, the aberrant cells and the micronuclei gradually increased for these groups.

The D4 (80 s) group showed the lowest level of normal cells and the highest levels of apoptic and necrotic cells.

These results are presented in view of the importance of cellular responses and the rate of exposure to the APPJ. Cellular responses occur according to the type, amount, and time of exposure to any stressor. Blood culture cells exposed to APPJ were responding to a diversity of stressors such as UV irradiation, visible light, IR, high-frequency electromagnetic fields, ions, electrons, reactive chemical species, and primarily radicals. ROS are generated and can assault intracellular organelles and membranes, proteins, DNA, and lipids. Cells are equipped with a variety of defense mechanisms, including scavenging enzymes such as catalyses and superoxides (SOD), repair machinery (micronuclei formation), and regeneration pathways. However, cellular damage can be too severe for cells to adapt or survive, leading to cell death.[29] According to the previous APPJ stressors and the rate of exposure, the results pointed to three trends of responses, as recorded in D1 group, D2 and D3 groups, and D4 group as compared with control group.

The primary source of TNF-α is thought to be the monocytes/macrophage, but various cell types are known to express this cytokine: lymphocytes, basophiles, eosinophils, mast cells, NK cells, T cells, B cells, astrocytes, and some types of tumors. TNF-α is produced upon stimulation with cytokines such as IL-1, IL-2, or TNF-α itself and with bacterial lipopolysaccharide (LPS), which is a potent inducer.[27] It has been reported that TNF-α is responsible for mediating (LPS) toxicity, cell toxicity, and that it is involved in cellular proliferation.[34] Various pathological conditions are associated with the production of high levels of TNF-α.

On the other hand, IL-1β is a cytokine with a broad spectrum in terms of its impact on immune response processes and on inflammatory cells. It is well known that the response of eukaryotic cells to different types of radiation includes activation of DNA repair mechanism, cell cycle arrest, mutagenesis, and lethality. All of these processes are mediated by the induction of several cytokines such as TNF-α, platelet-derived growth factor, fibroblast growth factor, and IL-1β.[35]

Therefore, therapies may be developed by blocking harmful effects of IL-1 and TNF-α and enhancing beneficial effects of IL-1 and TNF-α.

The data presented in Table 3 as well as Figs. 4 and 5 show that IL-1β and TNF-α in the D1 group increased but within the normal range compared with the control group.

In contrast, the values of IL-1β and TNF-α in groups D2, D3, and D4 increased significantly more than in the control and D1 groups. These increments were related to the time periods of APPJ exposure, especially for the 80-s dosage rate.

The present results confirm the assertion that APPJ is the most developed and effective plasma tool. Because of the contracted plasma resulting from this type of plasma generation, APPJ is especially suitable for focused, small-area treatments.[36]

Moreover, we conclude that medical plasma applications may be used as curative tools (i.e., enhancing the immunological responses) and as treatment tools (i.e., inducing apoptosis) through different rates of exposure.

Next, complementary studies must be conducted to determine the dose-response curve for APPJ exposure to normal and cancer cells. Thereafter, a set of basic plasma physical as well as biological performance parameters must be formulated and translated into legal rules and standards for use in different fields and for different applications.

In previous studies, only the chemical effect of free radicals produced by plasma exposure on the component of the cells was recorded; the signal causing apoptosis as the result of plasma exposure has not been determined. We hypothesize that the signal by which plasma exposure causes apoptosis and stimulates the immune system originates from metabolic biomagnetic resonance of the electromagnetic field and electromagnetic waves with certain genes and biochemical molecules.

The plasma jet produces many bands and many frequencies of electromagnetic waves, and some of these frequencies resonate with some genes and some biochemical effector molecules, leading to the up-regulation and down-regulation of these genes and enhancing of biochemicals. Some of these genes are *FAS*, *TNF-RI* Signaling, *CTL*, *AICD EGL-1*, *BCL2*, *CED-3*, *CED-4*, *APOF-1*, *TRAIL*, *FADD*, *TRADD*, Caspases genes family, *SMAC*, *IAPS*, *BCL-XL*, *BAX* , *BAK*, *DH3* and *P53*.

It is well known that processes of inflammation and apoptosis take place as the result of chemical responses to foreign microorganisms, to the internal workings of the cells itself, or to neighboring cells, all of which are known as internal chemical effects. With plasma exposure, the same effects and results can be obtained but as a result of external physical effects. Therefore, more and more studies must be conducted to investigate gene transcription associated with the processes of apoptosis and immune response as they are related to doses of plasma jet exposure.

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