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Original Article

Molecular Effects of Atmospheric Pressure Plasma Jet on the Double-Stranded DNA

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Abstract

Introduction
The aim of this study was to investigate the sterilization potential of atmospheric pressure plasma jet (APPJ) and interactions of this technology with double-stranded DNA using the polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) techniques.
Materials and Methods
The plasma jet was produced through a high voltage sinusoidal power supply using a mixture of argon and oxygen gases with the flow rate of 1 L/min. *Escherichia coli* cells and double-stranded DNA (dsDNA) fragments were amplified by *T7* universal primer through the PCR technique and treated with argon/oxygen APPJ at different exposure times. The data were analyzed by the agarose and polyacrylamide gel electrophoresis, SSCP and renewed PCR techniques.
Results
According to the results of the study, the APPJ could serve as an effective instrument for sterilization at > 30 sec discharge. The destruction of DNA was detectable by different techniques after 120 sec from APPJ discharge.
Conclusion
Our findings revealed that the active species of plasma can lead to cell death. These species may break or nick the dsDNA, exchange DNA nucleotides, and lead to transition and transversion mutations. These mutagenesis effects of APPJ might be the reason of microorganism cell death after the treatment in addition to other destructive effects of APPJ on macromolecules.

Keywords: APPJ, Single Strand Break, Double Strand Break, Atmospheric Pressure, DNA

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1. Introduction

In the recent years, much of the literature has been devoted to the employment of atmospheric pressure plasma jet (APPJ) in the fields of medicine and biology [1-4]. Seminal studies have been carried out on the sterilization property of the APPJ [5-7]. Recently, the researchers have proposed the use of APPJ in dermatology and wound healing [8,9], cancer therapy [10,11], dentistry [12], and tissue culture [13,14]. However, to achieve this aim and use plasma as a safe therapy, the identification of molecular effects of APPJ on cells and biomacromolecules is essential.

The previous studies have analyzed the effects of APPJ on such biological macromolecules as proteins [15], lipid [16], and deoxyribonucleic acid (DNA) [17]. DNA molecules are the most important biological macromolecules, which can be a good indicator for probing the molecular effects of APPJ on cells. The researchers commented that some of the damages and degradations may occur in the single- or double-stranded DNA (ssDNA or dsDNA) after the treatment with APPJ [18,19]. However, the tiny but steady variations such as transition and transversion mutation in DNA can cause large effects in cells.

For instance, the tiny but steady changes in the genes that have critical biological roles, such as housekeeping genes, can cause cell death. Therefore, further studies are needed with more details on DNA in order to understand the effects of APPJ on DNA as the most important biomacromolecules. Polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) are two sensitive molecular methods that can help to achieve this purpose.

The PCR allows the amplification of a predetermined DNA region via the use of two small and specifically designed fragments of DNA, namely primers or oligonucleotides. The primers start DNA amplification from 3'-OH. In other words, the PCR technique provides a simulation replicated *in vitro* condition. The SSCP method is based on the conformational differences of single strand DNA (ssDNA) segments that can be detected as mobility variations in non-denaturing polyacrylamide gel electrophoresis (PAGE) [20]. This technique is capable of showing only one nucleotide change (transition or transversion) in DNA [21]. In the present study, the PCR and SSCP methods, which are considered as sensitive and reliable techniques, were used to detect the mutations in double strand (dsDNA).

2. Materials and Methods

2.1. APPJ device

A general scheme of the experimental setup is presented in Figure 1. Our reactor consists of a 42 mm Pyrex glass tube. Powered electrode was set inside the Pyrex tube and the grounded ring electrode was attached to the surface of a Pyrex nozzle. The APPJ was driven by a sinusoidal alternating voltage with frequency of 18.56 kHz. The APPJ was generated at the gas gap between two copper electrodes and expanded into the surrounding air outside the nozzle (Figure 1).

The working gases were a mixture of argon (Ar) (99%) and oxygen (1%) with a flow rate of 1 L/min. In addition, the APPJ outside the nozzle had a length of about 27 mm. The distance between the nozzle and the sample was approximately 17 mm. And the temperature and humidity of the room were 25˚C and 70%, respectively. For the purpose of optical emission spectroscopy of plasma, we employed the Ocean Optic spectrometer (HR2000+CG). Furthermore, an ozone (O_3) sensor (A-21ZX, Eco Sensors, United States Inc) was used to measure the O_3 concentration above the sample.

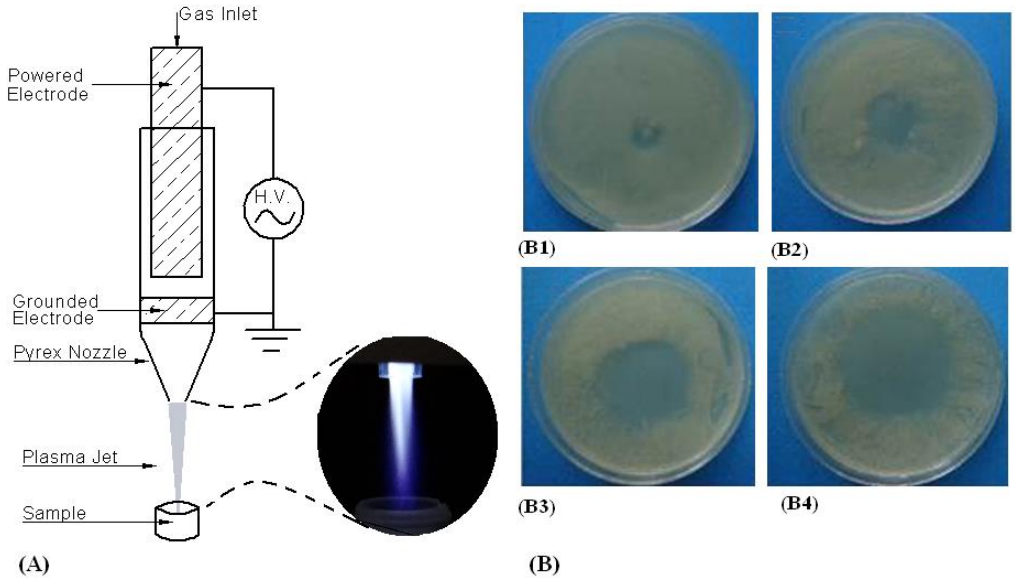

Figure 1. Schematic drawing of APPJ device and inactivation of E. coli: (A) Schematic drawing and a picture of the plasma jet/flame; (B) Inhibition zones of growth inactivation of E. coli following the exposure to argon/oxygen APPJ at different exposure times: 30 sec (B1), 60 sec (B2), 120 sec (B3), 180 sec (B4)

2.2. Plasma Treatment and Growth Conditions

The bacterial strain of *Escherichia coli* ATCC 35218 was obtained from the Pasteur institute of Iran and prepared for plasma treatments according to the method explained by Hosseinzade Colagar *et al*. (2010) on the Luria-Bertani (LB) broth and solid (LB-agar) media [3]. The LB media contained the following components per liter of distilled water: 10 g Bacto Tryptone, 5 g yeast extract, and 10 g NaCl for the broth; in addition, 15 g agar were added for the solid media. One milliliter of bacterial culture liquid media (with OD_{600nm}=0.25) was added to 9 ml of fresh LB broth.

Additionally, 100 µl of this solution was spread on the LB-agar media surface with four treatments (i.e., for 30, 60, 120, and 180 sec). Furthermore, one control Petri dish of solid LB was prepared for this experiment. Following the plasma treatment, all the Petri dishes were incubated at 37°C overnight.

2.3. PCR Technique

The amplification of the multiple cloning sites of pET26b with 309 bp fragment was performed in 50 µl final volume reaction. The mixture contained 20 ng of template DNA, 5 µl of 10X PCR buffer (i.e., 100 mM KCl, 20 mM Tris-HCl, pH of 8.8 at 25°C, 0.1 mM DTT, 50% Glycerol, 0.5% Tween 20, and 0.5% NP-40), 1.5 µM $MgCl_2$, 0.3 µM dNTP, 0.5 µM of each of the T7 universal primers [forward: 5'-TAATACGACTCACTATAGG, reverse: 5'CCGCTGAGCAATAACTAGC], and 0.25 U *Taq* polymerase. As illustrated in Table 1, the PCR was carried out for 32 cycles on a DNA thermal cycler (Eppendorf Master Cycler Gradient, Germany). After DNA polymerization, the PCR products were quantified. Subsequently, the concentration of DNA products was measured by a $T80^+$ UV/VIS spectrophotometer (PG Instruments, Beijing, China) at 260 nm using the following general formula: [dsDNA]µg/ml= OD260 * [number dilution] * 50, which has been described in a study conducted by Green and Sambrook [22].

2.4. DNA Treatment and Renew-PCR

About 1800 ng of DNA segments that is up to 24 µl with double distilled H_2O (ddH_2O) were treated with the APPJ for 0, 30, 60, 90, 120, 150, 180, 210, and 240 seconds. Then, the final volumes were aliquoted into two distinct parts to be analyzed with agarose gel electrophoresis and renew-PCR.

The PCR products were treated with APPJ as template DNA instead of *pET26b*. All other stages were performed like the way previously described in the PCR subsetion, which is known as a renew-PCR.

Table 1. Highlighted spectral lines and inactivation zones of APPJ treatments

A) Highlighted spectral lines of the APPJ				
Species	Wavelength (nm)			
OH ($A^2\Sigma \rightarrow X^2\Pi$)	308			
N_2 ($C^3\Pi \rightarrow B^3\Pi$)	337			
N_2 ($C^3\Pi \rightarrow B^3\Pi$)	357			
N_2^+ ($B^2\Sigma \rightarrow X^2\Sigma$)	391			
Ar	696			
Ar	763			
Ar*	794			
Ar*	811			
O ($3^5P \rightarrow 3^5S$)	777			
O ($3^3P \rightarrow 3^3S$)	844			
B) Areas and percentages of the inactivation zones				
Treatment time (s)	30	60	120	180
Area of inactivation(mm^2)	170.5	364.2	660.2	1,133.5
Inactivation zones (%)	4	9	17.2	29.5

2.5. Agarose and Polyacrylamide Gel Electrophoresis

The standard electrophoresis method was performed on 1% (w/v) agarose gels in 1X Tris-Borat-EDTA (TBE) buffer in the Paya Pajoohesh submarine system (HU95-Model, Paya Pajoohesh Laboratories Inc., Iran) at 70 V for 2.30 h. Subsequently, these gels were stained with ethidium bromide (1 µg/ml). The gels were captured under UV-illumination by a CCD camera. Agarose gel electrophoresis and gel staining were performed according to the method explained by Green and Sambrook (2012) [22].

Additionally, the general PAGE and gel staining with $AgNO_3$ were also performed following Green and Sambrook (2012) [22]. To this aim, after plasma treatment, 1µl renew-PCR products were run in 12% polyacrylamide gel [10 ml polyacrylamide (30% acryamide/bisacrylamide ratio of 29:1), 2.5 ml 10X TBE buffer, 350 µl 10% ammonium persulfate, 150 µl 10% tetra-methyl-ethylen-diamin, and 12 ml ddH_2O] with 1X TBE buffer on a vertical electrophoresis system (VEU7305-Model, Paya Pajoohesh Laboratories Inc., Iran) at a constant power of 90 V for 4 h at 4-12°C.

2.6. SSCP Analysis

One microliter of the renew-PCR products were mixed with 9 µl SSCP dye [9.5 ml formamide, 400 µl 20 mM $EDTA_{Na2}$, pH of 8.0, and 100 µl 1% bromophenol blue dissolved in absolute ethanol]. The mixture was subsequently placed in a 95°C water bath for 5 min. Afterwards the samples were rapidly chilled on ice before loading to the SSCP gel. Six percent polyacrylamide gel [5 ml polyacrylamide 30%, 1.25 ml 10X TBE, 350 µl 10% ammonium persulfate, 150 µl 10% N,N,N',N'-tetramethylethylenediamine, and 18.25 ml ddH_2O] was used with 0.5X TBE buffer on a vertical electrophoresis system (VEU7305-Model, Paya Pajoohesh Laboratories Inc., Iran) at constant power of 200 V for 10 h at 4-12°C.

2.7. Statistical Analysis

All data shown demonstrate the results of at least three independent experiments. The differences between the control group and experimental groups were verified using the t-test.

3. Results

3.1. Species Present In the Plasma

The optical emission spectroscopy at a range of 200-1000 nm revealed the presence of various excited species, including reactive hydroxyl (OH), metastable and excited state Ar, and reactive atomic oxygen (OI) in the plasma jet (Table 2). Highly reactive oxygen species (ROS) such as $\bullet O^-_2$ and •OH, excited Ar, and O_3 played a crucial role in eroding the key elements of DNA. In addition, O_3 is one of the most important products of plasma jet at ambient air. The O_3 concentration was found to be 0.2 ppm in our experiment [15]. Some reactions that Hosseinzadeh Colagar et al. (2013) described previously could be responsible for the generation of OH**,** Ar, OI, $\bullet O^-_2$, O^-, and O3 [23].

Table 2. The PCR program for the amplification of multiple cloning sites of pET26b

Stages	Temperature	Time	Cycle number
Initial denaturation	94°C	5 min.	
Denaturation	94°C	30 sec	32
Annealing	56°C	30 sec	
Elongation	72°C	45 sec	
Final Elongation	72°C	3 min	

3.2. Sterilization

After the treatment and incubation of the samples, the extent of inhibition area was studied. The inhibition zones increased by the enhancement of the exposure time as following the sterilization zone was clearly obvious (Figure 1). To quantify the sterilization efficiency, the areas and percentage of inactivation were shown in Table 2. The inactivation zone was increased by 2% in 60 sec, compared with that in 30 sec. According to the results, the inactivation zone at 180 sec was approximately seven times wider than that at 30 sec.

3.3. Agarose Gel Electrophoresis Analysis

Electrophoresis of the treated DNA samples in the 1% agarose gel demonstrated that the DNA band intensity was reduced by increasing the time of exposure in the lines 1-9, and after 240 sec of plasma bombardment, the dsDNA band disappeared. In addition, lines 1, 10, and 11 had equal intensity (Figure 2a). In addition, more than 50 nucleotide deletions could be detected by the agarose gel. On the other hand, the band intensity reflects the amount of healthy DNA. Based on the incidence of destruction in some or all of the DNA molecules, the intensity of the DNA band will be reduced or disappear, respectively.

Therefore, in lines 1-9, the DNA destruction increased with the elevation of the exposure time (Figure 2a). After 240 sec, the amount of healthy DNA reduced to the extent that the agarose gel could not detect it. Likewise, based on the agarose gel, after 240 sec of treatment, all healthy DNA molecules were destroyed. The equal intensity of lines 1, 10, and 11 in figure 2a indicted that the working gas did not have any effect on DNA, and the effective factor was APPJ.

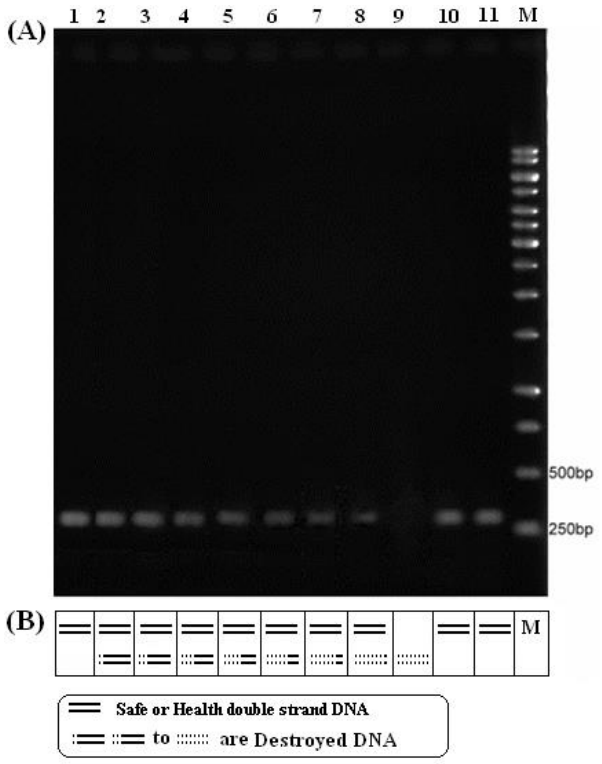

Figure2. Agarose gel electrophoresis pattern of the PCR products after plasma treatment in different exposure times: (A) Double-stranded DNA after treatment with plasma in 1% agarose gel electrophoresis; line M: Marker (#SM0311, Fermentas Inc., USA), lines 1-9: plasma treatment for 0, 30, 60, 90, 120, 150, 180, 210, and 240 sec, respectively, lines 10 and 11: control groups of minimum (30 sec) and maximum (240 sec) exposure times treated with the working gas at the same flow rate with plasma off

The agarose gel, which was used by previous researchers [15, 16] had low sensitivity to show DNA deletion and only demonstrated whether DNA destruction existed or not. Moreover, it could not accurately indicated the amount of destruction, especially in the short-time exposures. The other researchers reported just big damages in DNA like DNA single-strand and double-strand breaks after the treatment with APPJ, and did not investigate the mutational effects of APPJ on DNA [23-25].

3.4. Renew-PCR Analysis

To quantify the amount of plasma destruction and examine the mutation effects of APPJ including deletion, transition, and transversion, we used the renew-PCR and analyzed these PCR products with the PAGE and SSCP techniques. Since the destroyed DNA cannot act as a

template DNA in a PCR reaction, we can accurately quantify the amount of plasma destruction effects through measuring the concentration of the PCR products. Figure 3a displays the concentration of the renew-PCR products, and figure 3b shows the destructive effect of APPJ in percentage. The results illustrated that the concentration of these PCR products reduced and the percentage of degradation enhanced with increasing the exposure time. It was also observed that even in a short time (30 sec treatment), the APPJ had destructive effects on DNA (~12%).

The electrophoresis of the PCR products in 12% polyacrylamide gel is shown in the figure 4a. As can be seen in this gel, after replication with renew-PCR, only one band from each samples was observed, which were expected, but intensity of bands have been reduced (lines 1-9). The reason of the reduction in the intensity of bands was that the concentration of the PCR products reduced with increasing the treatment time. The existence of single bands indicates that there is no deletion in this segment, because if there were even one nucleotide deletion, we would not have single band.

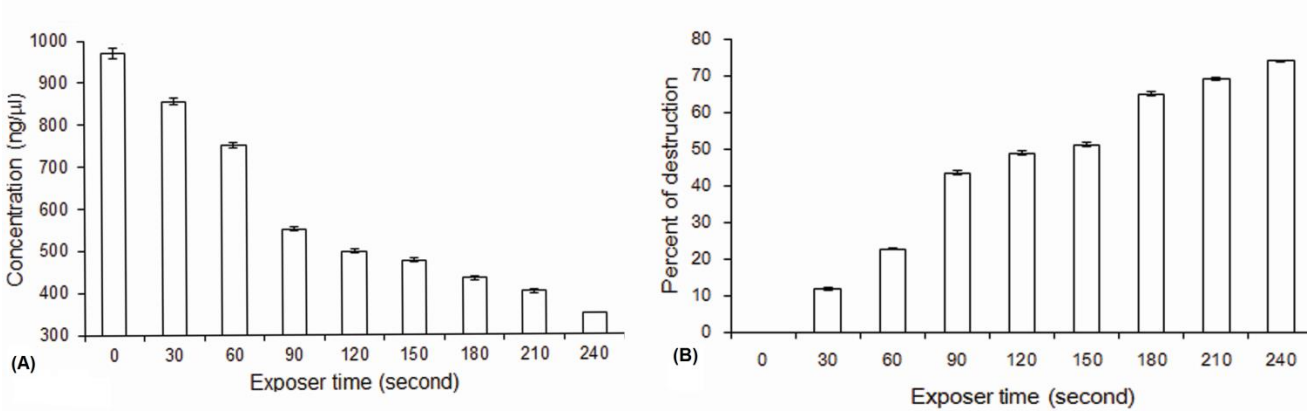

Figure 3. Concentration of renew-PCR products and percentage of destructive effect: (A) Concentration diagram of renew-PCR products, (B) Destructive effect percentage diagram

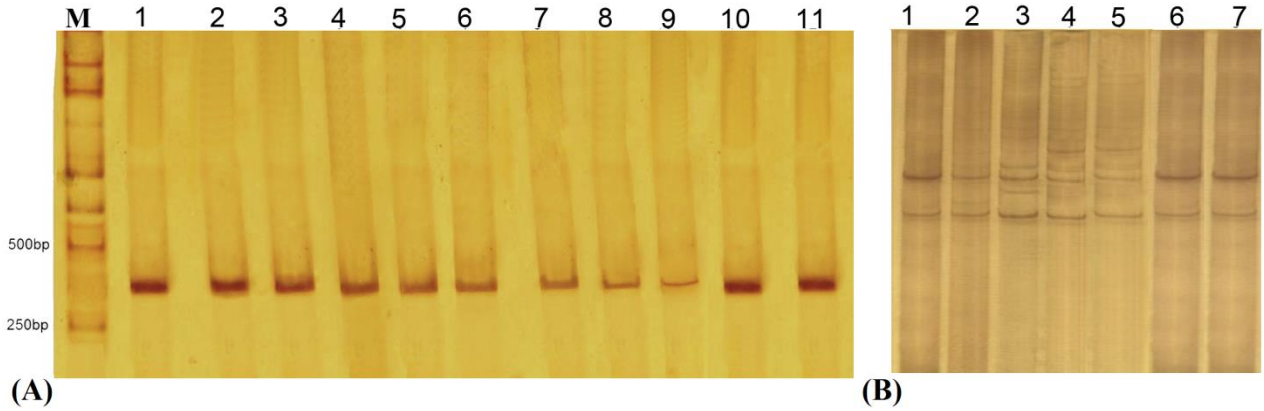

Figure 4. Renew-PCR products electrophoresis in polyacrylamide gel and SSCP: (A) Renew- PCR products of the samples analyzed on 12% polyacrylamide gel electrophoresis; line M: Marker (#SM0311, Fermentas Inc., USA), lines 1-9: renew-PCR products of the samples treated with plasma for 0, 30, 60, 90, 120, 150, 180, 210, and 240 sec, respectively, lines 10 and 11: control groups of minimum (30 sec) and maximum (240 sec) exposure times treated with the working gas at the same flow rate with plasma off; (B) Renew-PCR products analyzed by SSCP method, lines 1-9: renew-PCR products of the samples treated with plasma for 0, 30, 60, 120, and 180 sec, respectively, lines 6 and 7: control groups of minimum (30 sec) and maximum (180 sec) exposure times treated with the working gas at the same flow rate with plasma off

In other words, single bands indicate that plasma does not have any effect on the DNA, which would result in deletion mutation, like inducing thymine dimer or other similar changes in DNA,

which results in deletion after replication. Since the PAGE cannot show transition and transversion mutations, the SSCP method was used to check these mutations.

The results of the SSCP revealed that the renew-PCR products of the DNA fragment, which was treated for 30 sec or more had a polymorph bands in addition to a normal band (lines 2-5) (Figure 4b). These polymorph bands in the SSCP show that the nucleotides of DNA (i.e., adenine, cytosine, thymine, and guanine) are replaced with each other. These changes are called transition and transversion mutations. Therefore, this illustrates the effects of the APPJ on DNA through causing transition and transversion mutations following the replication.

4. Discussion

The argon/oxygen plasma, which was used in this study, produced reactive species that could affect the bacterial cells and their components. We found that these species inactivate *E.coli* in a short time (under 30 sec). The excited components of APPJ presumably play an important role in the inactivation of *E.coli*. The argon/oxygen plasma treatment exposes the cell surface to active neutral atoms and molecules, such as O_3, NO, OH radicals, and singlet oxygen ($O_2{}^1\Delta g$) as well as a flux of charged particles, including both electrons and ions such as super oxide radicals. These components can break the covalent bonds and initiate various chemical reactions [11]. The biological targets for the reactive species are DNA, RNA, proteins, and lipids [12].

It should be accentuated that the inactivation zone is not circumscribed to the dimeter of the plasma jet. Noticeably, it was shown that an increase in the exposure time led to more efficient inactivation (Figure 1). Laroussi *et al.* used the atmospheric cold plasma for the sterilization of the equipment and heat-sensitive materials [26]. Moreover, Ramona *et al.* stated that non-thermal helium plasma inactivated *staphylococcus aureus* [27].

However, it should be noted that the synthetic media acts better than the environmental conditions in terms of the bacterial growth. For example, the LB medium has sufficient nutrients and optimum temperature for bacteria, which lead to bacterial growth in logarithmic phase. Furthermore, the damaged bacteria can repair their failure. But this condition does not exist in the natural environment.

Regarding this, following the APPJ, it is expected to have fewer surviving bacteria in the natural environment than those in the bacterial growth medium. The sterilization experiments suggest that the APPJ is an effective method to disinfect *E.coli* from certain environments. These environments could include restaurants, hospitals, operating rooms, and food factories [28]. Furthermore, the non-thermal argon/oxygen plasmas can be used for the sterilization of heat-labile instruments and medical devices, such as the dental tools [14].

The previous studies have demonstrated the destructive effects of plasma on DNA, proteins, and lipids causing the inactivation of the micro-organisms. The APPJ causes an etching effect on the cell surface, and cell material is eroded by producing ions and electrons. As a result, the cell membrane ruptures and the contents effuse, causing death of bacilli [5]. Changes in the integrity of the membrane can directly affect the DNA, especially in bacteria where DNA is anchored to the membrane [15]. Moreover, the ROS affects the bacterial membrane lipids by causing the formation of unsaturated fatty acid peroxides.

In line with the findings reported in the literature, this study demonstrated that DNA can be affected by the APPJ, and the ROS of plasma leads to DNA degradation. For example, ROS and O_3 result in the induction of breaks in the ssDNA or dsDNA [15, 16, 23]. Moreover, our results revealed that in parallel DNA destruction after treatment with the APPJ, changes would occur in the DNA even in a short time. These changes can cause transition and transversion mutations in DNA after several PCR-simulated replications.

In the biological membrane systems, lipid peroxidation produces products that often react with DNA and proteins leading to oxidative modification [11, 15]. The lipid peroxidation chain reaction is initiated by the attack of unsaturated fatty acid by ROS, which abstracts the hydrogen atom from a methylene group (-CH2). This reaction leads to the formation of a fatty acid radical, which can readily react with an oxygen molecule to give lipid peroxide radical. These radicals can abstract the hydrogen atoms from other lipid molecules. Lipid peroxyl radical is unstable and breaks down to form various products such as aldehydes [29].

Malondialdehyde is a potentially important contributor to DNA damage and mutation [30]. These mutation effects can deactivate the repair system of the micro-organism and cause cell death in the short time treatment. In addition, previous studies suggested the plasma UV radiation may be causes to creating of thymine dimers in the DNA structure [15]. Our results demonstrated that the APPJ does not cause thymine dimer or similar changes in DNA, which cause deletion mutation.

5. Conclusion

As the findings of the present study indicated, the argon/oxygen APPJ could serve as an effective instrument for both sterilizing the micro-organism and decomposing DNA from the surface of the objects being sterilized. Therefore, it can have medical, healthcare, food processing, and air purification applications. Moreover, understanding the biological pathways of bacterial inactivation by APPJ can help us to take more advantage of this technology in the future, as a valuable alternative to the established approaches. To this aim, further studies should be conducted investigating the mutagenic effects of APPJ.

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