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Treatment with Dielectric Barrier Discharge (DBD) plasma restricts *Aspergillus niger* growth isolated from wheat grain

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Abstract

Microbiological contamination by fungi impacts the quality and safety of wheat grain storage. This study aimed to evaluate the efficacy of cold plasma in restricting the growth of the fungus, Aspergillus niger, which was isolated from wheat grains. A dielectric barrier discharge (DBD) operating at atmospheric pressure generated cold plasma that was used to treat the fungus, and the impact of this treatment was investigated at various periods 1, 2, 4, 6, and 15 minutes. The results revealed a highly significant decrease in the growth and number of spores of Aspergillus niger compared to the controls. This study revealed an efficient technique for enhancing wheat grain storage that could be a foundation for further large-scale studies.

Keywords: Aspergillus niger, Atmospheric cold plasma, Anti-fungus treatment, Dielectric barrier discharge, Spore deactivation.

Introduction

Food security is a major concern worldwide, and climate change influences both crop quantity and quality [1]. One-third of the global population depends on wheat production [2], and providing the necessary food for growing populations is an international challenge, particularly considering that one-third of food processed during harvesting is discarded [3]. Furthermore, natural factors such as fungus infections also contribute to decreased wheat production [4, 5], and the application of insecticides and fumigants during storage creates a growing risk of chemical accumulation in treated grains [6]. Wheat grains are susceptible to various infections, including those inflicted by fungi during transportation and storage. Fungal infections lead to poor grain quality and inadequate germination of seeds. In addition, such infections create mycotoxins, the harmful constituents that induce allergies and malicious diseases in humans and animals [7].

There are two categories of fungi: field fungi and storage fungi. Seeds of wheat crops located in the field prior to harvesting can be invaded by field fungi such as *Cladosporium, Alternaria, Aspergillus, Fusarium,* and *Penicillium* [8]. Storage fungi invade both wheat seeds and grains, and fungal spores can be found on grains in the harvesting and handling stages, as well as during storage [9]. Fungi change the taste, reduce weight per grain, and decrease germination strength of the wheat, in addition to releasing mycotoxins [10]. In individuals with immune deficiencies, the *Aspergillus* fungus can cause aspergillosis [11].

To combat this challenge, new techniques for disinfecting wheat grain that are safe and acceptable to the environment are required [6]. Numerous disinfectant methods are currently used to eliminate various materials; however, common cleaning techniques such as the use of chemicals and heat have significant disadvantages, as they are

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generally costly and labor-intensive [12]. Chemical residues on wheat seeds may also cause soil contamination [13]. Plasma sterilization can be considered an alternative approach that is cost-effective and rapid, with no negative health or environmental impacts [14].

Recently, atmospheric pressure plasma (APP) systems have replaced low-pressure plasma techniques because they do not require a vacuum and are more practical and affordable. In addition, the performance of APP under non-equilibrium states allows cold neutral gas, ions, and hot electrons to coexist in the plasma. APP operates at a gas temperature of approximately 21 °C, and reactive radicals and ions are generated by energetic electron collisions with the neutral gas. Therefore, APP application does not cause thermal damage to biological systems such as live tissues and cells [6, 12, 15]. Using a high voltage with at least one of the two electrodes covered with a dielectric material, dielectric barrier discharge (DBD), which is a form of cold plasma technology, can be quickly initiated under atmospheric pressure [16]. Furthermore, plasma application could increase the enzyme activity that supports seed germination and the breakdown of seed nutrients, resulting in greater seedling growth [17,18]. This study aimed to identify optimal plasma treatment conditions that inactivate the fungus *Aspergillus niger*, in terms of both their duration and intensity.

Materials and Methods

Plasma apparatus

Dielectric barrier discharge is a system that is powered by an alternating current discharge between two conductors when one or both are covered by a solid dielectric coating (plastic, glass). The barrier prevents the transfer of charges (i.e., current), and the discharge burns due to polarization and electrical induction. Fig 1 depicts a simplified schematic of the DBD system adopted in this study. The system consisted of an electrode connected to a power supply that provided a high voltage (13kV at a frequency of 50 Hz), as described in previous studies (with a modification regarding the output of the high-voltage electrode) [19,20]. The air surrounding the high-voltage electrode was inserted into a quartz tube and used as the working gas. The tube thickness was approximately 1 mm, which represents the dielectric layer. The high-voltage electrode was connected to a copper mesh inserted at the bottom of the tube, which functioned to scatter the discharge over the entire area of the dielectric barrier. The sample was put in a petri dish which was placed on an aluminum stage that served as the ground electrode. Discharge occurred between the bottom surface of the quartz tube and the top surface of the sample, which were separated by a distance of 5 mm. The temperature of the plasma was measured using a digital infrared thermometer (HT-866, Belgium) and values of up to 33 °C were characterized as relatively cool temperatures for direct contact with human skin and viable for use in medical treatments and decontaminants.

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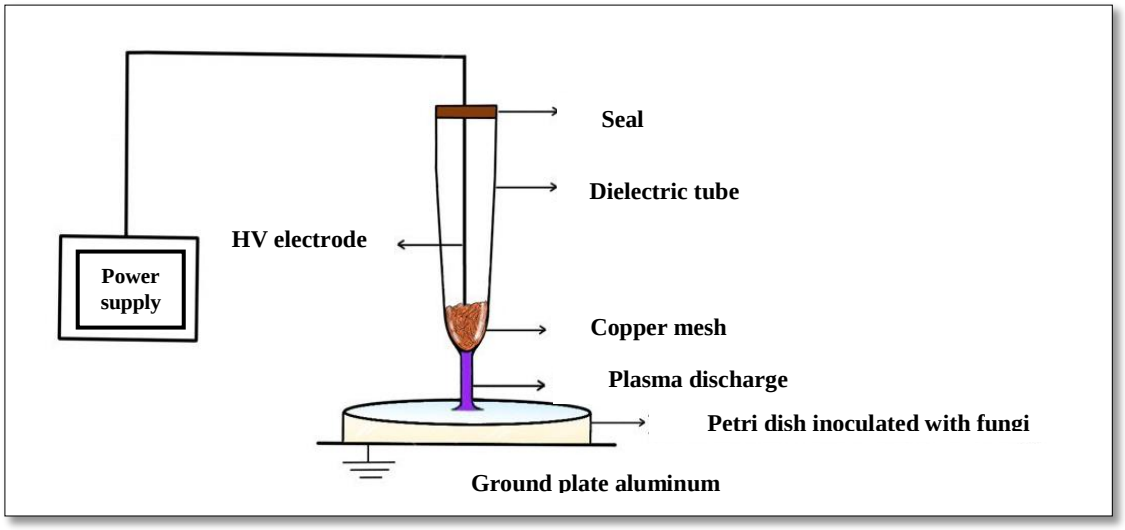

Figure 1. Schematic representation of the experimental set-up of the dielectric barrier discharges (DBD) plasma reactor for plasma treatment of fungi. A high-voltage electrode is connected to a copper mesh to scatter the discharge over the entire area of the dielectric barrier. The sample is placed on an aluminum stage and the discharge occurs between the bottom surface of the quartz tube and the top surface of the sample, which are separated by a distance of 5 mm.

Isolation and identification of microorganisms from wheat grains

To investigate the effects of DBD plasma treatments on grain microbiota and isolate contaminants, organic wheat grains were acquired from a nearby store (Baghdad City, Iraq). The dilution plating technique was used, with a minor modification, to isolate the wheat macroflora [21]. Wheat seeds (1 g) were placed in a test tube with 9 ml of sterilized distilled water and soaked for 15 minutes, after which they were shaken in a mechanical shaker for 10 minutes. The resultant solution was diluted, and aliquots of the various concentrations were evenly placed on potato dextrose agar (PDA, Oxoid, England), along with the antibiotic chloramphenicol at a concentration of 0.05 mg/liter; three replications were performed. The plates were incubated for 72 hours at 28 ± 2 °C. Following incubation, fungal isolates were sub-cultured in PDA and maintained in long-term storage at 4 °C. To tentatively identify the fungal genera, the macroscopic and microscopic morphological features of fungal isolates were compared with those described in the approved classification keys [22, 23]. The fungus was isolated from wheat seeds, and the culture grew overnight. A sterile inoculation needle was used to obtain a sample of the fungus, which was then placed on a glass slide with a drop of Lactophenol. The material was spread out within the drop, covered with a slide, and viewed under a microscope [24]. *Aspergillus niger*, a fungus strain isolated from local wheat grains, was used for the inoculation investigations, as shown Fig 2. The fungus was routinely sub-cultured on potato dextrose agar (PDA) media and maintained at 4 °C until use.

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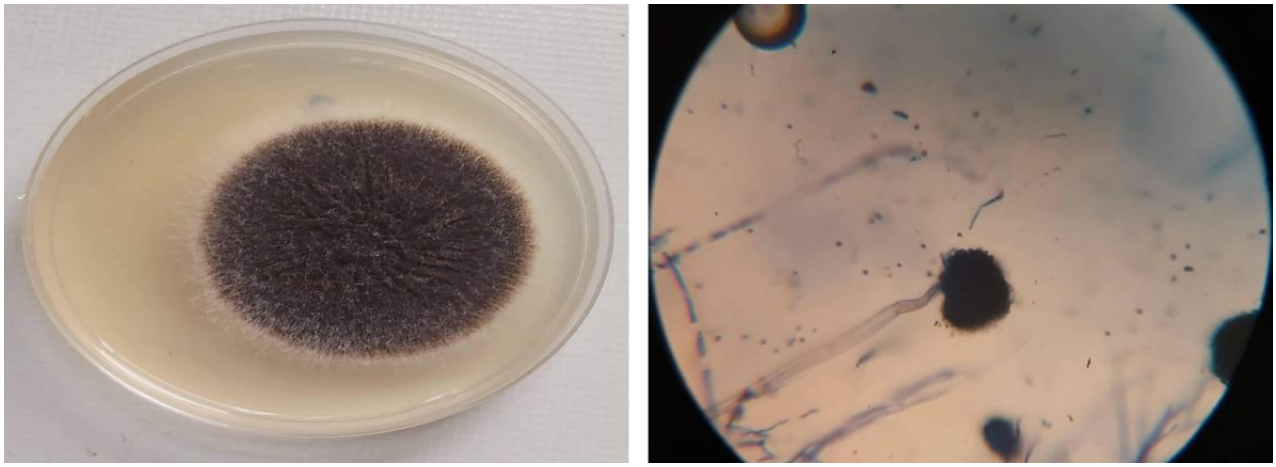

Figure 1. Wheat fungal isolates on PDA after incubation for 72 hours at 28 ± 2 °C. Left panel – colony morphology; Right panel – optical microscopy images (magnification 1000 ×).

Fungi Preparation of treatment

Minor modifications to the single spore technique were employed to prepare the fungus for treatment. The procedure involved mixing a 0.5 cm diameter sample of the fungus with 10 ml of sterile distilled water, diluting 10 µl of the spore suspension to a level that resulted in 1 to 15 spores in the microscopic field (at 10 ×), and dropping the suspension onto a plate containing PDA medium[25,26]. While the number of fungal spores in 1ml of liquid medium can be estimated using a hemocytometer and a microscope counting chamber, the number of spores/ml was instead computed using Eq 1 [27] based on the measured counts.

No. of spores/ml = average spore count in a large square × 10 × 1000 × reverse dilution 1

Plasma treatment

Aspergillus niger-inoculated PDA media were created using a previously described method [24, 25]. The samples were exposed to the plasma at 1, 2, 4, 6and 15 minutes after inoculation. The plates were incubated at 28 ± 2 °C for 48 hours after plasma exposure, as indicated in Fig 3. All treatments were repeated three times at room temperature and atmospheric pressure.

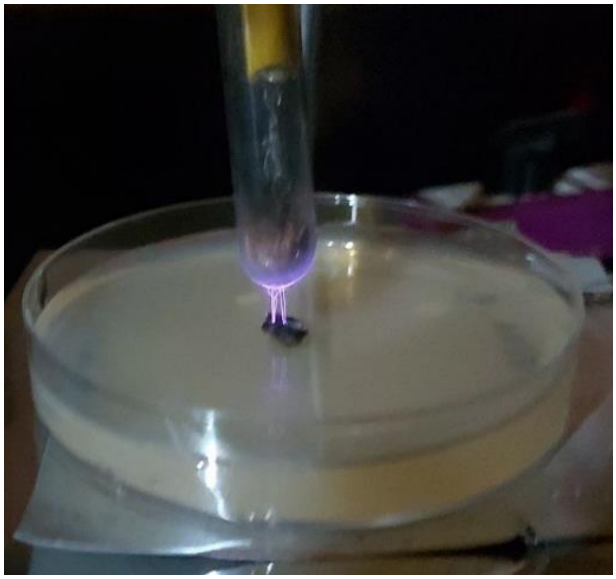

Figure 2. Photograph of a fungal sample in a petri dish during exposure to DBD plasma treatment.

Statistical analysis

One-way ANOVA and Dunnett's multiple comparison test were used to calculate P values using GraphPad Prism software, and P values of <0.05 were considered significant.

Results and Discussion

Wheat grains with fungal infections contain mycotoxins and detrimental substances that cause allergies and malignant diseases in humans. The use of DBD plasma in plant biology and agriculture has been widely studied recently as an efficient, inexpensive, and safe alternative to common methods of seed decontamination. This study illustrated the use of DBD plasma to inhibit

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Aspergillus niger and inactive its spores. The plates inoculated with fungi were treated with DBD plasma at different times 1, 2, 4, 6 and 15 minutes, and the fungal growth rate and number of spores were revealed to be significantly reduced, as shown in Figs 4–6. The plasma treatment exhibited highly significant differences compared with the control (P <0.0001) and (P = 0.0007), as observed in Tables 1 and 2, respectively. DBD plasma may cause fungal and spore inactivation through different mechanisms such as synergetic actions of ionized gas molecules, reactive species, free electrons, reactive oxygen species (ROS), and reactive nitrogen species (RNS), as well as a result of the different wavelengths of UV radiation emitted by the plasma. Plasma reactive particles produce a general mechanical effect on the surface of living organisms. The potential targets in fungal cells include the plasma membrane, cell wall, ribosomes, DNA, RNA, and proteins[28].

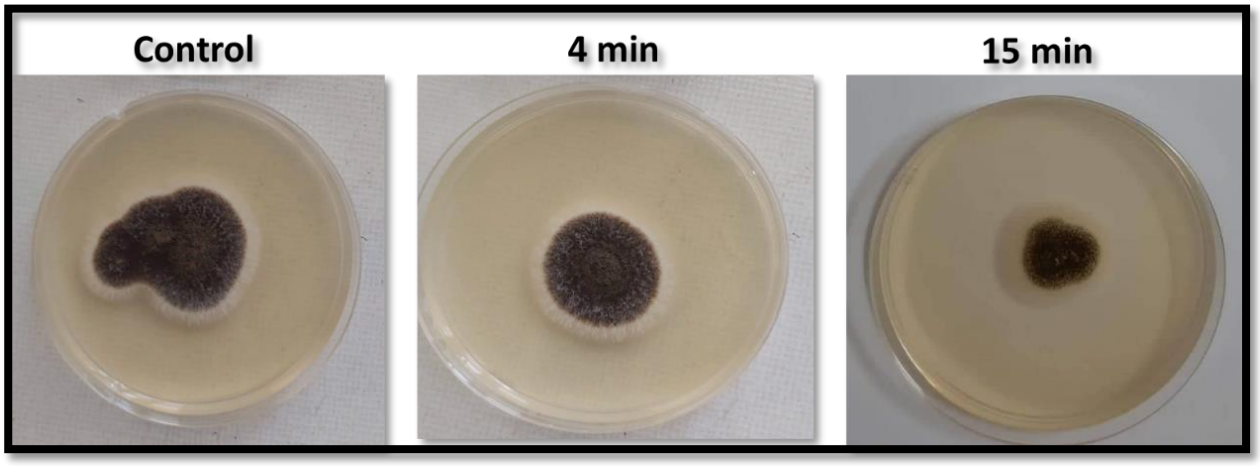

Figure 3. The digital photographs show the samples of the fungus Aspergillus niger in Petri dishes without treatment (control) and after exposure to DBD plasma (4 and 15 min). Aspergillus niger was routinely sub-cultured on potato dextrose agar (PDA) media and maintained at 4 °C until use. The samples were directedly exposed to the plasma at 1, 2, 4, 6 and 15 min. After plasma exposure, the plates were then safely incubated at (28 ± 2 ◦C) for the 48 h.

Table 1. Effects of DBD plasma at different times (1, 2, 4, 6 and 15 minutes) on fungal growth compared with the control, and list of P-values for each treatment.

Dunnett's multiple comparison test	Mean Diff.	Summary	P-value
Control vs. 1 minute	0.88	**	0.0026
Control vs. 2 minutes	0.76	**	0.0099
Control vs. 4 minutes	1.009	***	0.0002
Control vs. 6 minutes	1.063	***	0.0002
Control vs. 15 minutes	1.44	****	<0.0001

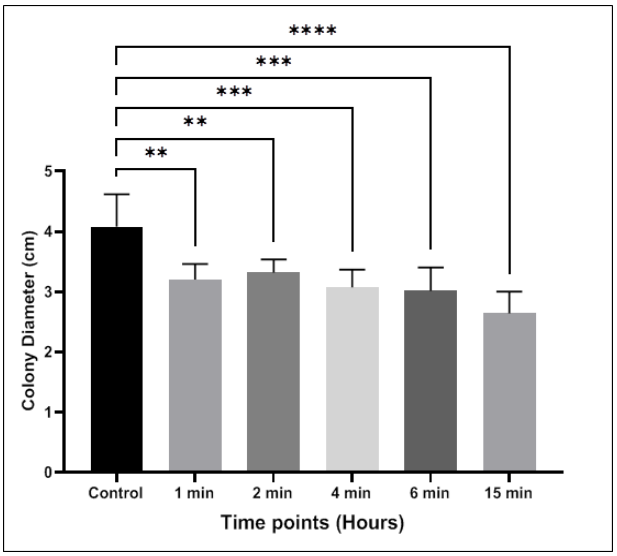

Figure 4. Histograms showing the reduction in *Aspergillus niger* growth after treatment with DBD plasma at different times (1, 2, 4, 6 and 15 minutes) compared with the control. All data are expressed as means ± standard deviations using the LSD test (ANOVA).

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Table 2. Effects of DBD plasma at different times 1, 2, 4, 6 and 15 minutes on number of spores compared with the control, and list of P-values for each treatment.

Dunnett's multiple comparisons test	Mean Diff.	Sum mary	Adjusted P Value
Control vs. 1 min	4.333	*	0.0495
Control vs. 2 min	6.333	**	0.0047
Control vs. 4 min	7	**	0.0022
Control vs. 6 min	8.333	***	0.0005
Control vs. 15 min	9	***	0.0003

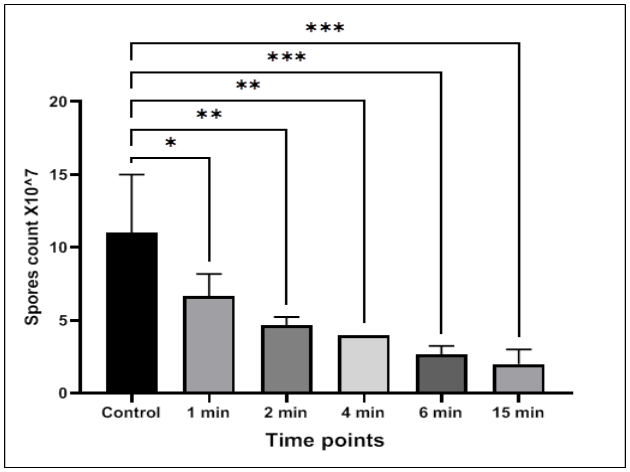

Figure 6. Histograms showing the reduction in the numbers of spores of *Aspergillus niger* after treatment with DBD plasma at different times (1, 2, 4, 6 and 15 minutes) compared with the control. All data are expressed as means ± standard deviations.

This study showed an apparent decrease in filamentous fungi growth and spore number after the first minute of exposure to plasma when compared to the control. A gradual decrease was observed as the exposure time increased, with a minimal growth level observed at minute 15. The data showed significant differences for all treatments, as presented in the Tables 1 and 2. Continuous exposure to the plasma could cause destruction of the outer surface of the fungal cells, as the accumulated charge on the outer membrane created electrostatic forces, which overcame the tensile strength of membrane. These forces can apply pressure that causes alteration and cell surface rupture [29].

Another mechanism of note is the formation of ROS and RNS that oxidize cell components and outer membranes of spores. Oxidization destructs organic components such as amino acids and lipids [30]. Since H, (H·) and (OH·) radicals also exist in the plasma, reactive chemical species such as H and OH radicals may eliminate microbials by oxidizing cellular biomolecules. The cell membrane contains a lipid double layer comprised of unsaturated and saturated fatty acids that support the characteristic of the gel-like structures [31, 32]. Lipid peroxidation is initiated when HO radicals attack unsaturated fatty acids and influence the fluidity of the cell membrane, which results in increased infiltration of plasma-generated ROS and RNS.

Furthermore, structural defects induced by the charged particles enable free radicals to attack, inducing severe cellular damage and subsequent cell death [29, 33, 34]. DNA lesions through the induction of breaks in the DNA could be another important mechanism of cell death, and the DBD plasma was reported to be highly effective at killing the antibiotic-resistant fungi, likely because of changes to cell components induced by the plasma treatment, such as modifications to the proteins and DNA. Furthermore, the engagement of UV photons through cellular molecules led to impairment of proteins, lipids, and DNA due to changes in the oxidative stress status [1, 35, 36].

Further studies are required to clarify the inactivation mechanism of the DBD plasma, including those of DNA and protein damage and cell surface degradation. Thus, our results identified the efficacy of the DBD plasma treatment in inactivating *A. niger* spores, which are frequently reported as contaminants of wheat grain.

Conclusion

The results from the present study highlight that the application of DBD plasma has potential in the decontamination of *Aspergillus niger* spores isolated from wheat grains. In addition, this study demonstrated that the inactivation processes depended on the exposure time. Finally, a process-compatible technology design is required to convert the DBD plasma device into a commercial and practical technology for disinfection in agricultural settings. DBD plasma can prove to be an efficient

and viable approach for wheat seed storage improvement.

Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine. Furthermore, any Figures and images, that are not mine, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in Ministry of Education.

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استخدام بلازما التفريغ الكهربائي عبر حاجز عازل لتقييد نمو فطريات الرشاشيات السوداء المعزول من حبوب القمح

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وزارة التربية، مديرية تربية الكرخ الاولى، بغداد العراق.

الخلاصة

يؤثر التلوث الميكروبيولوجي بالفطر على جودة وسلامة تخزين حبوب القمح. تهدف هذه الدراسة إلى تقييم فاعلية البلازما الباردة في الحد من نمو فطريات الرشاشيات السوداء (Aspergillus Niger)، وهو تلوث فطري معزول من حبوب القمح. أدى التفريغ الكهربائي عبر حاجز عازل (DBD) الذي يعمل تحت الضغط الجوي إلى توليد البلازما الباردة المستخدمة في علاج فطريات الرشاشيات السوداء. تم التحقيق في تأثير بلازما DBD على فطريات الرشاشيات السوداء في فترات مختلفة (1 ، 2 ، 4 ، 6 ، و 15 دقيقة). أظهرت النتائج انخفاض معنوي كبير في نمو فطريات الرشاشيات السوداء ، و ايضا عدد الجراثيم مقارنة بالعينات غير المعاملة . أظهرت هذه الدراسة تقنية فعالة لتعزيز تخزين حبوب القمح ويمكن اعتبارها أساسًا لمزيد من الدراسات واسعة النطاق.

الكلمات المفتاحية : فطريات الرشاشيات السوداء، بلازما الغلاف الجوي الباردة، علاج مضاد للفطريات، التفريغ الكهربائي عبر حاجز عازل، تثبيط الجراثيم.