Use of non-thermal plasma for decontamination of titanium implants

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Abstract. Non-thermal plasma (NTP) has a non-specific bactericidal effect as a result of the synergistic activity of biologically active components contained in the plasma torch. As part of this work, we investigated the potential of using NTP for the antibacterial treatment of titanium implants. Applying samples made of the VT6 titanium alloy used to create implants and a strain of antibiotic-resistant staphylococcus MRSA, we showed that 1) plasma pretreatment of the sample surface led to the formation of a film containing titanium oxides which have a weak bactericidal effect reducing the colonization of the surface with staphylococcus; 2) direct plasma treatment of a polished titanium disk for 120 seconds reduced the contamination of S.aureus by 563 times; 3) the relief of the titanium surface itself plays an important role in the effectiveness of decontamination: the bacteria on the rough surface were less susceptible to the effects of NTP than the bacteria on the polished surface. In total, the data obtained indicate the need to continue research aimed at increasing the effectiveness of the bactericidal activity of NTP against bacteria on the surface of titanium implants.

1. Introduction
Surgical site infections (SSIs) are defined as infections that occur within 30 days after surgery or up to one year after surgery in patients receiving implants and involve either the incision or deep tissues of the surgical site [1]. Despite advances in prevention, SSIs remain a serious clinical problem, as they are associated with significant mortality and morbidity, and place considerable demands on health care resources. The incidence of SSI can be as high as 20%, depending on the surgical procedure, the follow-up criteria used, and the quality of the data collection [2], [3]. The most frequently SSI-associated microorganisms are Staphylococcus aureus, coagulase-negative staphylococci, Pseudomonas aeruginosa, Escherichia coli and some others [4], [5]. The risk of SSI is influenced by numerous patient- and procedure-related factors, and therefore prevention requires a comprehensive approach with systematic attention to multiple risk factors to reduce the possibility of bacterial contamination. A particular risk for the development of SSI is infection of the implant surface. The adhesion of bacterial cells to titanium implants followed by the formation of biofilms on them is still the main cause of unsuccessful osseointegration, despite the use of various coatings that prevent the colonization of the
surface of medical devices by microorganisms and improve the adhesion of osteoblasts [6], [7]. Thus, there are good reasons for the introduction of new technologies that ensure sterility into everyday clinical practice.

Non-thermal gas plasma (NTP) is one of the bactericidal methods based on the action of physical and physical-chemical active factors. The biologically active components of non-thermal plasma include charged and metastable particles, reactive oxygen and nitrogen species (ROS and RNS), and ultraviolet light [8], [9]. NTP has a nonspecific bactericidal effect on a wide range of bacteria, including those resistant to antibiotics [10]. The targets of NTP are, first of all, the surface structures of the bacterial cell. The destruction of the lipid bilayer and the formation of pores in the membrane as a result of lipid peroxidation ultimately lead to the penetration of the active components of the plasma torch into the cell and damage to internal structures. The purpose of this work was to evaluate the potential of using low-temperature plasma for antibacterial treatment of titanium implants and to select the optimal modes of antimicrobial plasma exposure.

2. Materials and methods

2.1. Source of non-thermal plasma jet
The used NTP source was previously described in paper [11]. A non-thermal plasma jet was generated by a coaxial barrier discharge created in a narrow dielectric tube blown by argon at atmospheric pressure.

2.2. Bacterial strains and growth conditions
The Staphylococcus aureus strain SA180-F (MRSA, antibiotic resistance profile PG-OXC-E-CLI-CIP-VAN-TPM-LEV) from the collection of the Gamaleya Centre was used in the work. Bacteria were grown on BHI medium (BD, USA) at 37°C.

2.3. Samples of titanium implants
In the experiments, samples of implants made by 3D printing from titanium alloy of the VT-6 brand were used. The appearance of the samples is shown in Figure 1. As one can see, these are discs with a diameter of 15 mm and a thickness of 2.5 mm. The discs were cut by the spark method in distilled water from the rod of a long length. During the cutting process, the surface is modified by hot spark plasma. As a result, the surface of the discs after cutting by the spark method became rough. The experiments were carried out with discs whose surface was mechanically polished after their cutting off, and with discs with a rough surface, i.e. without polishing them.

Figure 1. The appearance of samples made of titanium alloy VT6. a) disc with a mechanically polished surface; b) disc with a rough surface after its cutting off by spark method from a long rod.
2.4. Surface modification using NTP.
VT6 titanium samples with a polished and rough surface were treated with a jet of argon plasma at atmospheric pressure. The plasma jet was created by a coaxial barrier discharge with a frequency of 100 kHz and a power of 8 Watts. The jet velocity at the plasma source outlet was 30 m/s. The distance from the source outlet to the sample to be treated was 15 mm. The exposure time was varied.

2.5. Methods of examination of the implant surface before and after plasma treatment.
The study of the topography of the disk surface after it was cut off by the spark method and after mechanical polishing, as well as after prolonged treatment of the polished disk with plasma jet in the ambient air was carried out with an ADF I350 optical microscope (inverted metallographic microscope) with a maximum 1000x magnification and equipped with an ADF SPD16 digital camera with a volume of 16 megapixels (matrix size 8.7 x 6.6 mm²).

Four methods of observation and registration of the surface image of the samples were used: 1) light field mode, when light falls on the sample normally from the eyepiece of a microscope; 2) dark field mode, when light falls on the sample from the side of an annular source at an angle of 45 degrees; 3) light field mode using reflected polarized light; 4) differential contrast mode, used to create contrast in unpainted transparent samples. In this case, the polarized beam from the light source is divided into two beams that pass through the sample by different optical paths. The length of these optical paths (that is, the product of the refractive index and the geometric path length) is different. Farther, these rays interfere at their intersection. This allows one to create a three-dimensional relief image corresponding to the change in the optical density of the sample, accentuating the lines and borders. However, this picture is not an accurate topographic picture.

The obtained surface topography of the samples is shown in Figures 2 and 3. It can be seen that the rough surface has numerous cavities with a characteristic depth and width of about 20-30 microns, in which bacteria can "hide" from plasma exposure. The polished surface is practically free of such cavities.

![Figure 2](image_url). Topography of the mechanically polished surface of VT6 titanium alloy samples at 500x magnification. The vertical and horizontal size of each frame is equal to 150 microns.

a) light field; b) differential contrast; c) polarization in reflected light: an image of a translucent speck of small dust in a form of a butterfly casually deposited on the surface of the disk is given.
Figure 3. Topography of the rough surface of a sample made of titanium alloy VT6 modified by hot spark plasma during cutting off the disc. a) dark field, magnification 200, vertical frame height 375 microns; b) magnification 500, vertical frame height 150 microns; polarized light, one can see an outsider orange inclusion in the center of the frame. Note the low depth of the lens focus does not allow one to see sharply the entire structure of cavities in the vertical direction. This circumstance leads to the appearance of cloudy images which correspond to the surface areas above the plane of the lens focus.

The elemental composition and topography of the initial and plasma-modified implant surfaces were studied using scanning electron microscopy (Zeiss EVO MA10 electron microscope with Bruker XFlash 5030 energy dispersive X-ray detector). This technique allows one to study the composition of the surface at a point, by area, or the distribution of elements along a line on the surface, while the depth of the information collection layer does not exceed 1 micron. The phase composition of the modified surface was studied by X-ray phase analysis using an X-ray diffractometer DRON-8 on Cu Kα radiation with a nickel filter. This method makes it possible to detect the presence of crystalline phases on an area of several square millimeters and at a depth of up to several tens of microns. Phase identification based on the results of diffractogram processing was carried out using an ICDD PDF2 X-ray file.

2.6. Titanium disc infection
5-6 bacterial colonies were seeded in BHI nutrient broth and grown for 18 hours (overnight culture). The overnight culture of bacteria was diluted with saline. Solution up to a concentration of 10⁹ CFU / ml according to the McFarland standard. The resulting suspension was further diluted 10 times. Bacteria in an amount of 10⁶ CFU taken in a volume of 10 μl were applied to the surface of a titanium disc and incubated at a temperature of 37°C for 1 h until complete drying. To obtain biofilms, titanium samples were immersed in the overnight culture and incubated at 37°C for 24 hours.

2.7. Antibacterial titanium disc treatment with NTP
Bacterial suspension was applied to the sample, and the discs were incubated until completely dry as described above. The infected discs were treated with NTP for 60, 120, or 300 s at a distance of 15 mm. An untreated disc was used as a control.

2.8. Bacterial load counting
Two methods were used to determine bacterial loads on the titanium disc surface: qualitative and quantitative. Fluorescent dyes included in the Live/Dead Cell Viability Assay kit (Invitrogen) were used according to the manufacturer's instructions for qualitative assessment and to determine the ratio of live and dead bacterial cells. Surface swabs were made for quantification using 100 μl of
phosphate-buffered saline (PBS). The washed bacterial suspension was diluted in PBS, making a series of consecutive decimal dilutions. From each dilution, bacteria were plated on BHI agar. Colonies were counted after 24 h incubation at 37°C.

2.9. The effect of the titanium surface pre-treatment.
To evaluate the property of the titanium surface modified with NTP to affect bacterial viability, 10⁶ CFU of methicillin-resistant *S. aureus* (MRSA) were applied to a disk treated with NTP 3 times for 120 s each time. The infected disk was incubated for 1 h. Alternatively, the disc was immersed in a bacterial suspension for 24 h to form biofilms. After incubation, samples were stained with Live/Dead vital dye and micrographs and washes were taken from the disc surface. An untreated disc was used as a control.

2.10. Statistics
All experiments were done in duplicate and repeated 3 times. The one-way ANOVA method was used to calculate statistical significance.

3. Results

3.1. Characteristics of the plasma jet and the barrier discharge forming the NTP jet
The dielectric barrier discharge (DBD) was created in a quartz tube with an inner and outer diameter of 2.5 and 4.5 mm, which was blown with argon at atmospheric pressure at a speed of 20-30 m/s. Inside the tube along its axis, there was a thin rod, to which a sinusoidal voltage with a frequency of 100 kHz and an amplitude of 4.5 kV was applied. A grounded electrode in the form of a thin metal foil was wrapped around the outer surface of the tube. The electric characteristics of a coaxial dielectric barrier discharge forming a non-thermal plasma jet at the outlet of the tube are shown in Figures 4 and 5. Figure 4a shows a visible image of a glowing plasma jet that blows out freely into the surrounding air. Figures 4b and 4b show shadow images of the argon jet itself both in the absence of DBD (Fig.4b) and in the presence of DBD (Fig.4b). These figures show that, firstly, the plasma jet has a smaller diameter compared to that of the gaseous jet (i.e., the plasma jet is inside the gas one) and, secondly, the argon jet exits the tube in the laminar regime. However, a comparison of Figures 4b and 4b shows that the inclusion of DBD leads to a decrease in the distance from the tube outlet, after that the laminar jet goes into turbulent mode. The occurrence of turbulent pulsations in the gas jet leads to a faster mixing of the plasma jet with the surrounding air containing oxygen and water vapor. This process is accompanied, on the one hand, by the appearance of useful OH-type radicals in the plasma jet, but, on the other hand, leads to the rapid disappearance of the primary reactive particles created by the dielectric barrier discharge.

The number and sort of reactive particles created by the dielectric barrier discharge depend not only on the composition of the plasma-forming gas but also on the electrical power inserted in the discharge. The oscillograms of current I(t) and voltage U(t) characterizing DBD are shown in Figure 5a. Sharp current splashes on the I(t) oscillogram are caused by an electrical breakdown of the gas-discharge gap happening in each half-cycle of the applied sinusoidal voltage. These breakdowns provide the formation of non-thermal plasma inside the plasma source. After every breakdown, energy is released into an argon plasma. The residence time of the gas inside the discharge zone of the plasma source is about 10⁻³ s. Thus, at the frequency of the applied voltage of 10⁵ kHz, the activated gas is energized by more than 200 breakdowns, saturating the gas with numerous active particles. The growing dependence of the period-averaged electrical power W of the barrier discharge on the amplitude U of the applied voltage is shown in Fig. 5b.
Figure 4. Visible (a) and shadow (b, c) image of an argon plasma jet flowing into free space (ambient air); the speed of the plasma jet at the discharge tube outlet is 30 m/s; shadow image of a gas jet in the absence of a barrier discharge (b) and in the presence of a barrier discharge (c). The frequency of the sinusoidal voltage exciting the barrier discharge is 100 kHz, the voltage amplitude is 4.5 kV.

Figure 5. a) Oscillograms of the current and voltage DBD generating the plasma jet. b) The discharge electrical power dependence on the amplitude U of the applied voltage. In both cases, the velocity of the argon jet at the outlet is 30 m/s.

3.2. The results of the materials science study of the implant surface before and after plasma treatment.

The implants were made of titanium alloy of the VT6 brand, which should not contain oxygen in its composition. However, the implant surface after the spark-cutting method looked rough and gray in color, corresponding to the presence of an oxide film on its surface. Our studies of the composition of the implant surface layer with the diagnostics described above really showed a high concentration of oxygen (up to 30 or more atomic percent) due to the formation of titanium oxides during the spark-cutting. Cleaning of the implant from the gray oxide film by its mechanical removal to a state of gloss with subsequent analysis of the surface showed that the oxygen concentration on the cleaned and smooth surface of the titanium alloy sharply decreased. At the same time, the long treatment of the cleaned and smooth surface of the disc with a plasma jet in the ambient air again led to an increase in oxygen in the
surface layer. The essential advantage of the oxide thin layer formation by the NTP is that the surface keeps the smoothness without the formation of any cavities. The results of the analysis are shown in Figure 6. It is clear that the presence of titanium oxides can lead to unusual results of plasma exposure to bacteria. It is important to note that the oxygen concentration on the implant surface layer depends on the exposure time of the surface by the plasma jet. This circumstance makes it possible to vary the oxygen concentration on the implant surface to the desired level if it is necessary.

![Figure 6](image)

**Figure 6.** Radial distribution of the content of elements, including oxygen (in Atom%) on the treated and untreated implant surface by argon plasma jet. The distance of 0-0.2 mm corresponds to the untreated surface, 0.2-2.9 mm - the treated surface.

3.3. Results of NTP bactericidal activity.

3.3.1. The effect of the titanium surface pre-treatment. The rough titanium disc surface was pre-treated with NTP as described in Materials and Methods, and MRSA bacteria were applied to the surface. After 1 h incubation bacterial loads on the treated surface were lower if compared with loads on the disc that was not pre-treated with NTP. These data suggested that the titanium surface, pre-modified with NTP, had weak antibacterial properties against MRSA when the bacteria were completely dried on the sample (Fig. 7). On average, plasma pre-treatment led to a decrease in the contamination of the disc surface contaminated with S. aureus MRSA by 44.3±5%. At the same time, the plasma pretreatment of the titanium surface did not prevent the formation of biofilms during 24 h disc incubation in the bacterial liquid culture (data not shown).

![Figure 7](image)

**Figure 7.** The effect of plasma pre-treatment of the titanium surface on the viability of *S. aureus* MRSA cells. Bacteria were visualized with the Live/Dead staining after 1 h incubation on the surface of the pre-treated or control titanium disc. a) - control, b) – disc was pre-treated with NTP. Green for live bacteria, red for dead bacteria.
3.3.2. Direct bactericidal effects of titanium treatment with NTP. Next, we applied NTP to decontaminate discs infected with MRSA bacteria. Two types of surfaces were studied, rough and polished. The efficiency of direct plasma treatment depended on the geometric properties of the surface. The Live/Dead staining showed that damaged bacteria appeared after 60 s NTP treatment when bacteria were placed on the polished surface. 300 s NTP treatment of polished discs was enough for dead bacteria to prevail in the field of view with only a few alive green bacteria (Fig. 8). On the rough surfaces treated for 300 s, Live/Dead staining revealed conglomerates formed by damaged and living bacteria in relatively similar proportions (Fig. 8).

![Control, Polished disc 300s, Rough disc 300s](image)

**Figure 8.** Bactericidal effects of infected polished and rough disc treatment with NTP. Disc surface was infected with 10^6 CFU MRSA. After drying, surfaces were treated with NTP for 300 s, stained with the Live/Dead marker kit, and analyzed with fluorescence microscopy.

The quantification of survived bacteria confirmed the data obtained using fluorescence microscopy. Plasma treatment of a smooth titanium disk led to a decrease in bacterial loads by 154 ± 83, 187 ± 91, and 563 ± 237 times after 60, 120, and 300 s of treatment, respectively. At the same time, the rough surface protected bacteria much better. 60 s treatment led to a decrease in the number of living bacteria by 6.7 ± 3.1 times, and an increase in exposure time to 120 and 300 s reduced the number of surviving bacteria by only 14.4 ± 6.6 and 25.1 ± 13.3 times, respectively (Fig. 9).

![Figure 9](image)

**Figure 9.** Bactericidal effects effect of NTP applied to rough and polished titanium surfaces contaminated with MRSA.

4. Discussion

In the framework of this work, the potential of non-thermal plasma to fight against contamination of the surface of titanium implants was studied. Investigating the effects of plasma surface pre-treatment with NTP as a means to prevent bacterial adhesion to the surface of titanium implants, and the effects of NTP treatment of titanium surfaces contaminated with MRSA, we found that 1) plasma pre-treatment of the sample leads to the formation of a film of titanium oxide, which has a weak bactericidal action; 2) direct
plasma treatment of a contaminated polished titanium disk for 300 s reduced the bacterial loads by 563 times; 3) the relief of the titanium surface played a primary role in decontamination: bacteria on a rough surface are better protected from direct plasma exposure than on a polished one.

Titanium oxide and dioxide have a nonspecific bactericidal effect due to the development of free radical reactions, although the mechanisms of bacterial death have not been fully elucidated [12], [13]. Our results indicate that the pretreatment of the implant surface with NTP is able to form sufficient concentrations of surface titanium oxides for these mechanisms to come into play. It is possible that these mechanisms operate not only during surface pretreatment, but also during the action of NTP on the contaminated surface of a titanium implant. However, under the direct action of NTP on bacteria located on the surface, the bactericidal effect is much higher, which indicates a relatively minor contribution from the formed titanium oxides, and suggests the main contribution to the death of bacteria from the active components of the plasma torch. Indeed, the plasma torch contains a whole complex of antibacterial components, the effect of which is synergistically enhanced during the action of the plasma torch on the target. The main antibacterial agents of NTP include charged particles, reactive oxygen and nitrogen species formed during the interaction of a plasma torch with air, and ultraviolet radiation [8], [9].

At the same time, the obtained results indicate that the process of bacteria inactivation on the surface of implants is less efficient than, say, on the surface of an agar medium, i.e. under conditions commonly used to assess the bactericidal activity of NTP sources [10]. For the MRSA strain used in this work, a similar treatment for 300 s led to the complete destruction of living bacteria (a drop in the concentration of living cells by more than 10^6 times, data not shown) compared with a drop of 563 times on the surface of titanium. The decrease in the effectiveness of the antibacterial properties of NTP in the treatment of the surface of titanium implants can be associated with various reasons. One of these reasons is the rough surface of titanium implants used to enhance the adhesion of human cells. Roughnesses in the micrometer range, comparable to the size of the bacterial cell, protect bacteria from the impact of NTP, which was proved in this work by comparing the results of processing bacteria placed on a rough and polished surface. Other mechanisms may include the low surface moisture of the titanium compared to the same nutrient agar, which is actually a gel whose surface is wet. Additional studies on the contribution of air humidity are needed to evaluate this parameter.

5. Conclusions
Taken together, obtained data indicate that NTP has a bactericidal effect on bacteria that contaminate the surface of titanium implants, which is the sum of their total effect of the active components of the plasma torch and the activity of titanium oxides formed on the surface during processing. The relief of the titanium surface has a great impact on the outcome of the treatment. Further research is needed to increase the effectiveness of NTP impact

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