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Study on the killing effect of cold atmospheric pressure plasma on MRSA *Staphylococcus aureus in vitro* and *in vivo* infection model

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ABSTRACT

Aims: Non-thermal atmospheric-pressure plasma (cold plasma), is described as a partly ionized gas. Cold plasma is a new method of medicine for killing the bacteria, treatment of cancer diseases, accelerates the healing of infectious ulcers, especially in infection caused by Meticillin-resistant *Staphylococcus aureus (*MRSA). This study aimed to investigate the impact of *Non-thermal atmospheric-pressure plasma* on MRSA *S. aureus* organism isolated from burn wound infection *in vitro* and *in vivo*.
Methodology and results: Five *MRSA S. aureus* strains were recovered in burn patients from *Shahid Motahari* Burns *Hospital*, Tehran, Iran. They confirmed by microbiology and biochemical tests. Antibiotic susceptibility testing was performed using Kirby Bauer Disk Diffusion Method with selected antibiotics. Then, the antibacterial impact of atmospheric non-thermal plasma on MRSA *in vitro* and *in vivo* at different times was assessed. After that, the tissue was randomly separated from control and treated mice with plasma and transferred to the Histopathology Laboratory for further evaluation. Results of the inactivation of MRSA by non-thermal atmospheric plasma showed no bacterial growth. Also, results of the impact of non-thermal helium plasma *in vivo* environment revealed that, in addition to healing in the animal wound, the burn wounds infection was healed and treated according to the histological results.
Conclusion, significance and impact of study: Our results confirmed the inactivation of MRSA *S. aureus*, healing of animal burn wound and complete treatment by non-thermal atmospheric plasma. It recommended that cold plasma can be used for the treatment of burn wounds infection due to the gentle on the human skin.

Keywords: Argon plasma, *Staphylococcus aureus,* burn, MRSA, cold plasma, mouse model, histology

INTRODUCTION

Staphylococcus aureus is a Gram-positive coccus in the nose (20-50% of people carrying the *S. aureus* in their nose) and the skin of healthy people in different proportions, and can be pathogenic in people who their body resistance due to the reasons has been reduced, can virulent and caused acute infection (Selton-Suty *et al.*, 2012). Some *Staphylococcus* strains are resistant to methicillin, which is referred to as MRSA (Waness, 2010). MRSA strains have become an increasing concern for health systems in worldwide. Multidrug-resistance accompanying with an extensive range of extracellular enterotoxin genes, virulence factors, can confer life-threatening characteristics on MRSA and makes them highly pathogenic and be challenging to treat *(Johnson et al.*, 2012). MRSA is considered as one of the most important hospital-acquired agents which are resistant to different antibiotics which have faced the treatment of infections caused by this bacterium with serious problems (Hiramatsu *et al*., 2014). The number of people with chronic ulcers, burn colonization, mainly due to methicillin-resistant *S. aureus*, is growing. Treatments

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with routine methods are time-consuming, and thus very expensive (Werdin *et al*., 2009).

For these reasons, new concepts and strategies regarding the control of wound infection and thus improving chronic wound care are powerfully recommended, which one of those is the use of non-thermal atmospheric-pressure plasma (Haertel *et al*., 2014).

Plasma is a gas or a mixture of several gases that have been converted into ionized molecules and free electrons under the influence of an electric field, which is referred to as the fourth state of matter. One type of plasma is cold plasma at temperatures of 30-40 °C under atmospheric pressure (Haertel *et al*., 2014). Plasma Medicine", combining plasma physics with life science and medicine advanced quickly (Von Woedtke *et al*., 2014). Plasma has many uses such as; food sterilization, decreasing the microbial load of drinking water, killing the bacteria in dental plaques, puncturing canals the teeth, instead of the common painful methods and the removal of contamination from heat sensitive materials and equipment, treatment of cancer diseases, also accelerates the healing of infectious ulcers, especially in cases where the microorganisms causing infection are MDR organisms (Weltmann *et al.*, 2010). In this study, we try to investigate the impact of *Non-thermal atmospheric-pressure plasma* (cold plasma*)* on MRSA *S. aureus* organism isolated from burn wound *in vitro* and *in vivo* environments. The study is completely new in our country, and so far there is no report of the use of *Non-thermal atmospheric-pressure plasma* in this regard.

MATERIALS AND METHODS

Power supply for plasma jet production

The DC power was used to generate jet plasma. The power supply used in this research produces voltages from 0 to 10 kV. The operating frequency of the power supply was 20 kHz and the pressure was from 6 to 8.

Source of strains

Five MRSA *S. aureus* strains were recovered in burn patients from *Shahid Motahari* Burns *Hospital*, Tehran, Iran. They were cultured in agar nutrient medium. For gaining of single colony and confirmation of diagnosis, they were grown in Muller Hinton Agar medium. Then, the plates were incubated for 24 h at 37 °C. Then for confirmation, Gram staining, colonial morphology, lysostaphin sensitivity, coagulase, catalase, mannitol fermentation was conducted.

Antibiotic susceptibility testing

For isolated strains, antibiotic susceptibility testing was performed using Kirby Bauer Disk Diffusion Method with polymyxin B, amikacin, gentamicin, tobramycin and ciprofloxacin antibiotics (*Merck* KGaA, Darmstadt, *Germany*).

Antibacterial impact of atmospheric non-thermal plasma on Meticillin-Resistant *S. aureus* (MRSA) *in vitro*

To measure the anti-bacterial effect of the non-thermal atmospheric plasma, at first of desired strains a *standard* Turbidity of. 0.5 McFarland was prepared according to CLSI standards and plated on the Muller Hinton Agar medium and agar Plates containing cultured bacteria were subjected to non-thermal atmospheric radiation with argon and helium gases at 5 sec, 15 sec, 30 sec, 60 sec, 90 sec and 120 sec. Nasal was placed at a distance of 2 cm from the plate, then the plates were incubated in an incubator 37 °C for 24 h, finally, the results were analyzed.

Antimicrobial impact of atmospheric non-thermal plasma on meticillin-resistant *S. aureus* (MRSA) *in vivo*

At this step, 10 female rats with weight 30±2 g were placed in separate groups and the mice back were shaved in size of 3 cm. And then the rats were anesthetized by injecting 10 mL of ketamine and xylosin substances (depending on the weight of the mice). Then was formed a burn wound with a diameter of 2 cm. Then was taken with an insulin syringe from a bacterial suspension of 0.5 McFarland and inoculated on the wound, the procedure 5 times repeated for 5 days until the infection was visible on the skin. After creating the infection, 4 mice were kept as controls and 6 mice treated with plasma with the same conditions. All six mice were exposed to helium plasma 5 days for 90 sec. After that, the tissue was randomly separated from control and treated mice with plasma equally and transferred to the Histopathology Laboratory of Baqiyatallah Medical Sciences Hospital for evaluation.

Histopathology

To evaluate the impact of atmospheric non-thermal plasma, after staining and preparing the pathology sections, they were stained with Haematoxylin-Eosin dyes and considered histologically.

Statistical analysis

Analysis was performed using SPSS software through Chi-square test. The P value less than 0.05 was considered statistically significant.

RESULTS

According to the CLSI standards 2016, a strain of *S. aureus is* MRSA that is resistant to oxacillin in addition to methicillin. The results of susceptibility testing showed that all these strains are resistant to oxacillin and confirmed that they are MRSA (Table 1).

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Table 1: Results of antibiotic susceptibility testing for isolated strains.

Antibiotic Strains	Ceftazidime (30 µg)	Gentamicin (10 µg)	Amikacin (30 µg)	Piperacillin (100 µg)
Number 1	R	R	R	R
Number 2	R	R	R	R
Number 3	R	R	R	R
Number 4	R	R	R	R
Number 5	R	R	R	R

Results of the impact of non-thermal atmospheric pressure argon plasma jet on methicillin-resistant *S. aureus* (MRSA) *in vitro*

The confirmation of antibiotic properties of non-thermal atmospheric argon plasma jet was performed by observing the inhibition zone diameter on MRSA. The diameter of the inhibition zones was mentioned in the Table 2 and Figure 1.

Table 2: The diameter of the inhibition zone of non-thermal atmospheric argon plasma jet on MRSA at different times.

Time Strains	5 sec	15 sec	30 sec	60 sec	90 sec	120 sec
No: 1	5 mm	7 mm	10 mm	12 mm	15 mm	18 mm
No: 2	5 mm	7 mm	10 mm	12 mm	15 mm	17 mm
No: 3	6 mm	9 mm	10 mm	13 mm	15 mm	18 mm
No: 4	5 mm	8 mm	10 mm	11 mm	14 mm	19 mm
No: 5	6 mm	7 mm	9 mm	10 mm	15 mm	18 mm

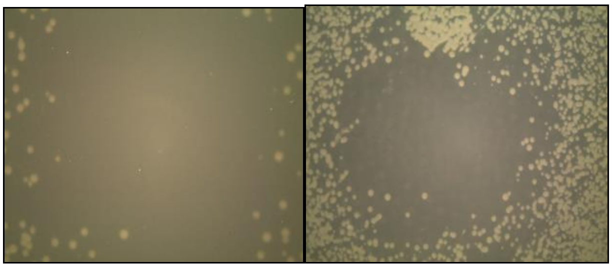

Figure 1: The diameter of the inhibition zone of non-thermal atmospheric argon plasma jet on MRSA at different times of plasma radiation, Left image; after 30 sec, Right image; after 2 min.

Results of the impact of Non-Thermal Atmospheric Helium on Methicillin-Resistant *S. aureus* (MRSA) *in vitro*

The confirmation of antibiotic properties of non-thermal atmospheric helium plasma jet was performed by observing the diameter of the inhibition zone on MRSA. The diameter of the inhibition zone was stated in the Table 3 and Figure 2.

Table 3: The Diameter of the inhibition zone of non-thermal atmospheric helium plasma on MRSA.

Time Strains	5 sec	15 sec	30 sec	60 sec	90 sec	120 sec
No: 1	0 mm	3 mm	5 mm	6 mm	6 mm	10 mm
No: 2	0 mm	3 mm	5 mm	6 mm	7 mm	9 mm
No: 3	0 mm	2 mm	5 mm	6 mm	7 mm	9 mm
No: 4	0 mm	3 mm	5 mm	6 mm	6 mm	8 mm
No: 5	0 mm	2 mm	4 mm	5 mm	7 mm	8 mm

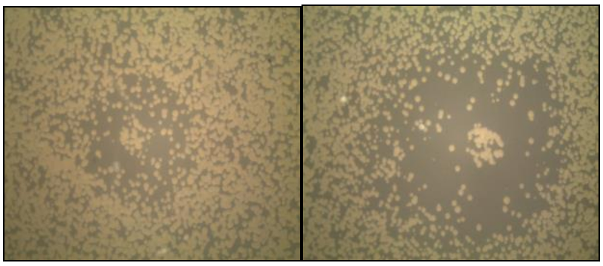

Figure2: Diameter of the inhibition zone of non-thermal atmospheric helium plasma on MRSA at different times of plasma radiation, Left image; after 30 sec, Right image; after 120 sec.

Results of confirmation of the inactivation of MRSA by non-thermal atmospheric plasma

It was prepared a subculture of the diameter of the inhibition zone by a loop and after 24 h incubating the culture medium, the result was surveyed, but no bacterial growth was observed. This indicates that the atmospheric non-thermal plasma was completely effective in inactivating the MRSA strains.

Results of the impact of non-thermal hemisphere helium plasma *in vivo*

After 5 days of plasma treatment on mice, the results were evaluated. Our results revealed that, in addition to healing in the animal wound, the burn wounds infection was healed and completely treated according to the histological results (Figure 3).

Histopathology results

Histology classifying was performed according to the Sydney protocol and scales of 0 to 3 were considered for the activity of atrophy and inflammation. In sample 1 (control), chemotaxis and number of macrophages and neutrophils in the tissue were lower and the wound healing was also slower, and the numbers of bacteria were increased.

In sample 2 (treated), chemotaxis and abundance of macrophages and neutrophils were observed that indicating recovery and wound healing. By increasing the number of defensive cells and inflammation, the number of bacteria was reduced.

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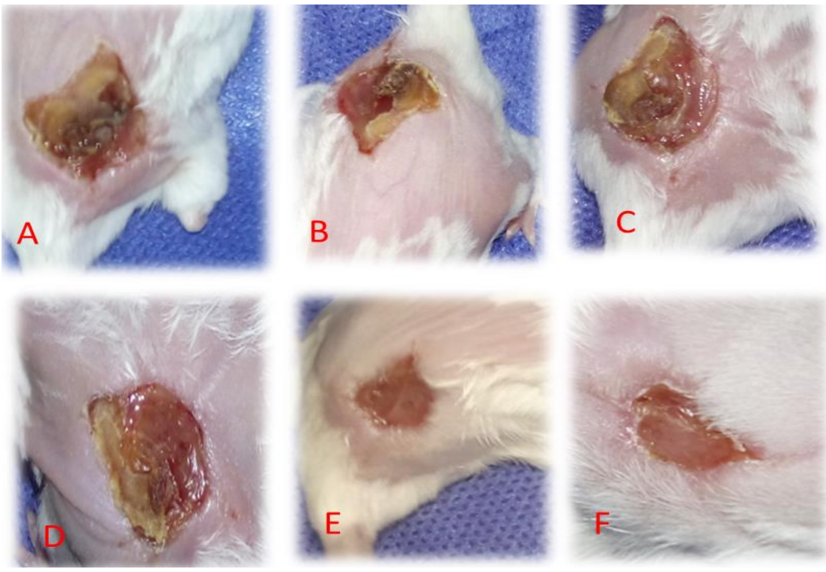

Figure 3: Mice treated with non-thermal atmospheric plasma, A to F, respectively, the first days of treatment until the 6th day. Result showed burn diameter decrease with cold plasma (A=2mm, B=1.65mm, C=1.5mm, D=1.35mm, E=1mm, F=0.7 mm).

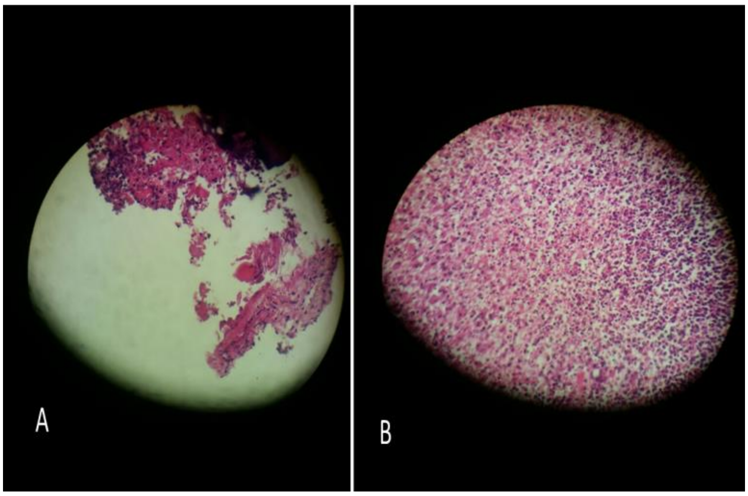

Figure 4: Effect of atmospheric non-thermal plasma after staining with Haematoxylin-Eosin and preparing the pathology sections with magnification power 1000×. A, Pathology image of the control sample; B, Pathology image of the plasma-treated sample.

DISCUSSION

Because burn in addition to severe tissue damage, it creates a good environment for the growth of bacteria such as MRSA and *P. aeruginosa* strains consequently the onset of infection caused by them (Ge *et al.*, 2011).

Staphylococcus aureus is clearly known to be a potential pathogen that causes many infections(Hosseini *et al*., 2017). These infections are classified into three types of skin infections and various toxicities such as food poisoning, septic shock, toxic shock syndrome and scaled skin syndrome (Freeman-Cook *et al*., 2006). Amongst *S. aureus* strains, Methicillin-resistant *S. aureus* (MRSA) strains are one of the main causes of burn, hospital/community-acquired infections, which nowadays have multiple resistance to a wide range of antibiotics including beta-lactams, aminoglycosides, tetracyclines, fluoroquinolones and macrolides (Khademi *et al*., 2016;

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Skov and Jensen, 2009), therefore, currently a limited number of antibiotics are available as anti-staphylococcal drugs in burn patients (Francis *et al.*, 2005).

As previously described there are several strategies for control of wound infection, including cold plasma, for that we study the impact of *Non-thermal atmospheric-pressure plasma* (cold plasma*)* on MRSA *S. aureus* organism recovered from burn wound *in vitro* and *in vivo*. Results of the inactivation of MRSA by non-thermal atmospheric plasma showed no bacterial growth. This indicated that atmospheric non-thermal plasma is completely effective in inactivating MRSA strains and also, results of the impact of non-thermal hemisphere helium plasma *in vivo* environment revealed that, in addition to healing in the animal wound, the burn wounds infection was healed and treated according to the histological results. One of the disadvantages of our study is that here helium plasma was used which due to the high cost of helium is not economical and if it can be designed in such a way that argon plasma or helium is produced with a percentage of oxygen that minimizes damage to the skin is much more suitable for the patient to tolerate it. For this reason, Isbary *et al*. (2010) afflicted 36 chronic ulcers in 36 patients with moderate daily exposure to argon plasma at 5 °C, and the results of 291 tests in 36 wounds (regardless of the type of bacteria) were investigated, which resulted in a significant reduction in the microbial load in the treated wounds, and no complications were observed and treatment was successful and painless (Isbary *et al*., 2010). In another study carried out by Brun *et al*. (2012), they studied the effect of disabling the pathogens of eye infections without causing specific damage to the conjunctiva tissue or horny cells of the skin with the use of a cold, atmospheric plasma atomic device producing ionized helium gas flow with treatment of the cornea of the human eye with the plasma for 2 minutes, their results were very successful (Brun *et al*., 2012).

In our study we used of the atmospheric non-thermal plasma owing to sterilization with the help of low temperature plasmas, which are partially ionized gases, are the most appropriate and modern method. Low-temperature plasmas that can easily be produced at low pressures (eg less than 10 volts) with the application of an electric field, including highly active species such as atoms, ions, high energy electrons, methane particles and UV radiation). Due to the presence of these active species in the plasma, it has been proved that the use of plasma with low temperature is preferable than many common methods due to its high impact (Yu *et al*., 2006).

As mentioned in the result section, inconsistent with confirmation other results, Pathological studies showed that cold atmospheric pressure plasma was able to degrade the macrophage, increase the activity of phagocytosis and chemotaxis, which ultimately led to bacterial clearance.

Finally, this work similar to other studies showed the inactivation of MRSA by non-thermal atmospheric plasma and healing in the animal wound, but it should be investigated with different times on the various tissues of humans and animals.

CONCLUSION

Our results confirmed the inactivation of MRSA *S. aureus* and no bacterial growth of it by non-thermal atmospheric plasma, healing in the animal wound, and the burn wounds infection was healed and treated, too. It recommended that helium plasma is used for the treatment of burn wounds infection of human after the investigation at different times on the various tissues of humans and animals.

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