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The effect of cold plasma on the treatment of external otitis: an experimental study in rats

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Abstract
In this paper we investigate the influence of cold plasma as novel method on the external otitis treatment which is a frequent cause of earache. 24 infected external auditory canals in 24 rats were categorized in four experimental groups including control, plasma exposed, ciprofloxacin drug and mixed of plasma-ciprofloxacin groups. In plasma group, dielectric barrier discharge was employed as the source of cold plasma in 5 days. All rats were observed with otoscope daily and a scoring system was used to evaluate swelling and effusion of the ear canal. Number of colonies in microbiological culture were counted in each group during the first 5 days after treatment. For the multiple group comparisons of swelling and effusion measured in the external auditory canal, Kruskal–Wallis analysis was applied and one-way anova and Kruskal–Wallis analysis was used for the statistical analysis of the results of the cultures in different days. Also, Tukey and Mann–Whitney tests was applied for multiple comparisons. Our findings show that swelling and effusion were obviously reduced in plasma group compared to control group ($P<0.01$). Number of colonies in control group was statistically different from those in drug, plasma, and mixed group on the second to fifth day ($p<0.001$). According to the results cold plasma can be introduced as an impressive method for external otitis treatment. Moreover, when cold plasma joined to antibiotic method, it leads to a superior performance respecting plasma or antibiotic method alone.

Keywords Cold plasma, External otitis, Rat, Effusion

Introduction

Acute otitis external (AOE) is the most common infection of the external auditory canal which is known as swimmer's ear (Demirel et al. 2018). High temperature, humidity, absence of cerumen, trauma, alkaline pH, excessive sweating and the use of hearing aid are some of significant risk factors that can cause acute external otitis (Kantas et al. 2007; Demirel et al. 2018).

AOE starts with the rapid onset of ear canal inflammation, leads to redness and swelling of EAC, feeling of fullness in the ear and discharge from the ear canal (Schaefer and Baugh 2012). Sensitivity by touching the tragus or pina complaints of hearing loss may also be reported if the ear canal is excessively swollen (Kaushik et al. 2010; Schaefer and Baugh 2012).

Inflammation of ear canal in acute otitis external generally results from the growth of bacteria and, in certain cases, fungi. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are some of the most common bacterial pathogens in AOE. Although these microorganisms can also be cultured in low numbers from the healthy EAC, increment of those number leads to severe complications.

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Around 25 to 64% of external otitis cases contain *P. aeruginosa* which is a type of gram-negative bacteria and grows easily on a moist environment. *Pseudomonas aeruginosa* is an opportunistic pathogen with a particularly high rate of antibiotic resistance, in part due to its potent ability to form biofilms. It is responsible for many hospital-acquired infections, in particular of chronic wounds, and represents a major problem for patients with otitis externa (Mai-Prochnow et al. 2015; Yau et al. 2018; Maybin et al. 2023). Moreover as reported by Emgard, *Candida albicans* as a fungal-pathogen occurs in less than 10% of external otitis cases (Emgård et al. 2005; Demirel et al. 2018).

Nowadays various popular treatment strategies have been utilized for external otitis. Topical glucocorticoids with or without antibiotics, an acid with or without antibiotics, an ethanol mixture, antimycotics and oils are some of these typical methods (Emgård 2005).

The degree of patient satisfaction is always essential to choose the treatment strategies. According to literature overall patient satisfaction depends on four factors: relief of signs, rate of return to normal conditions, ease of administration and lack of medication side effects. Surely a treatment that reduces itching, relieves symptoms, and treats quickly with fewer side effects will lead to greater patient satisfaction (Emgård 2005).

In topical treatment, ear drops containing anti-inflammatory steroid and antibiotic components is routinely used (Emgård et al. 2005; Demirel et al. 2018). However, studies have shown that up to 40% of patients are prescribed oral and/or injectable medications in addition to topical treatment, many of which are not active against *P. aeruginosa* or *S. aureus*. This type of treatment come with a higher cost and risk of side effects due to use of oral antibiotics in addition to the topical treatment (Kaushik et al. 2010). Therefore, when using drug therapy for otitis externa, both the potential toxicity of the ear and the risk of bacterial drug resistance should be considered (Emgård 2005).

New approaches to minimize or avoid the use of antibiotics are required to diminish the development of antibiotic resistance and increased improvement in symptoms such as pain, canal edema, and erythema. In this regard, cold atmospheric plasma (CAP) can be proposed as an alternative non-pharmacological method.

In physical science, plasma is the fourth state of matter, along with solid, liquid and gas phases and should not be confused with the more familiar blood plasma. Generally, plasma is an ionized gas consists of neutral and excited spices like oxygen, nitrogen and hydrogen radicals, ions, electrons, photons and ultraviolet radiation (Isbary et al. 2010, 2012).

Plasma technology offer a new and innovative solution for many biomedical application (Fathollah et al. 2016). High-temperature plasmas are already in medical use for the sterilization of equipment, tissue destruction, cutting and cauterizing. In recent years, atmospheric pressure plasma has demonstrated many potential applications in skin wound healing, blood coagulation, bacteria disinfection and cancer therapy (Fathollah et al. 2016). Cold atmospheric plasma have the same advantages as the high-temperature plasmas but without the enormous heat production (Isbary et al. 2012). It is therefore applicable to a broad range of new medical applications, such as the in vivo treatment of normal skin or infected wounds (Isbary et al. 2010).

Various laboratory studies have shown the antimicrobial effect of CAP against various bacterial and fungal pathogens, regardless of their resistance. In addition, there are lots of clinical studies and case reports on utilization of plasma for animals and humans, particularly in dermatology and otorhinolaryngology (Isbary et al. 2010, 2012, 2013; Herbst et al. 2015).

Despite large number of studies on the use of plasma in the field of medicine and biology, there is little published research on the use of plasma to control ear infections. As the first experience, Isbary et al. proposed that CAP can be utilized in a patient with chronic post-operative ear infection. Their results show that applying CAP lead to a significant reduction in pain and clearance of bacteria (Isbary et al. 2013).

In the current study we apply dielectric barrier discharge (DBD) cold plasma for the treatment of acute otitis external in a rat model. We evaluate the efficacy of cold atmospheric plasma procedure to control of ear infection. Furthermore, we compared the results of plasma methodology with the conventional drug therapy to demonstrate the advantages of this method.

Materials and methods

Experimental material and animal care

All animal maintenance and procedures were in accordance with recommendations established by the Animal Ethics Committee of Shahid Beheshti University of Medical Sciences as well as the United States NIH guidelines. The experimental protocol was approved by the Ethical Committee of Shahid Beheshti University of Medical Sciences with the Document No. 1398.818.

The study included a total of 24 male rats, aged 12–16 weeks, weighing 180–200 g, with a healthy external auditory canal, tympanic membrane, and middle ear bilaterally accepted by an otoscope examination.

The rats were kept in applicable cages under standard environmental conditions with the standard guidelines (room temperature between 22 and 24 C, 50% relative humidity and 12-h light and dark cycles). The animals had free access to water and were fed with conventional laboratory diet until they were euthanized.

Experimental design and animal groups

To create the external otitis model, primarily, the rats were intraperitoneal administered anesthesia of 0.1 (90 mg/kg) ketamine hydrochloride and 0.2 mL (10 mg/kg) xylazine. Then both EACs were traumatized with a sandpaper for 4 min. Approximately 1 min after the trauma, 0.1 mL *P. aeruginosa* (1.5×10^7colony-forming units (CFU)/mL) drops were administered. The standard strain of *P. aeruginosa* ATCC 27853 was used. The external otitis model was applied to right ear of rats in the study. After 24 h, result of culture confirmed the development of external otitis in the right ear of 24 rats. Exclusion criteria for the external otitis groups of rats were determined as the death of an animal during the experiment or no visualization of symptoms of otitis under the otoscope view. The rats were randomly divided into 4 groups each with six rats. Group I was the control group with no treatment applied. In Group II, 0.2 mg/kg ciprofloxacin drops were applied once daily to right ear. In Group III, cold plasma was applied once daily about 5min. In Group IV, both 0.2 mg/kg ciprofloxacin drops and cold plasma were applied once daily.

Plasma generation

The schematic diagram of the experimental set up for plasma treatment of external otitis of rat is presented in Fig. 1A. Power supply (Model: PS-100A), from Kavosh Yaran Fann-e Pouya Company (Iran) was used to create a uniform DBD plasma. It generates high voltage pulses with the amplitude of 13 kV at the frequency of 16 kHz with adjustable power setting in the range of 0-10W. In this study a specific design of DBD plasma probe based on a mold prepared from rat's ear canal was used to allow the researchers to access to the rat's ear canal. As it is shown in Fig. 1B, it consists of two basic parts. The main part is the probe head which composed of an end-closed quartz tube with the diameter of 2 mm and thickness of 0.5 mm filled by cupper filing. The second part of probe is a conical Teflon which surrounded the quartz tube except for 2 mm at the end of the quartz tube. The quartz end of the probe is located about 1 mm from the tympanic membrane when the probe is inserted into the ear canal. Applying the voltage to the probe produce cold plasma inside the ear space. Optical emission spectroscopy (OES) measurement was performed to know the chemical properties of the plasma. It was carried out using an Ocean Optics HR2000+spectrometer (USA) with the spectral range of 190–1100 nm.

Plasma treatment and follow-up

The treatment was administered regularly for 5 days in the total 24 rats after external otitis creation. No rat from any group died during the study period.

In all groups, the status of the EAC was observed under anesthesia once daily from days 1 to 5. It was calculated according to a scoring scale, which included grading swelling and effusion. Swelling was graded using funnels of standardized size in four categories of no swelling (the width of the EAC measuring at least 4 mm), 3 mm, 2 mm and 1 mm EAC size. The occurrence of effusion was classified as 0=dry, 1=moist, 2=fluid in the EAC, 3=otorrhea. On days 1, 2, 3, 4 and 5, samples from EACs were collected from all animals for culturing of bacteria. To know the bacterial levels in rat's ear canal, they were examined by microbiological culture over all days of study. Samples was taken by ear-swabs from the ear's discharges. The swabs were then quickly transported to the microbiology laboratory of Qom Azad University, and they were promptly cultured onto freshly nutrient agar and were incubated aerobically at 37 °C for 18–24 h. Then number of colonies in each plate were counted.

The treatment was performed daily after recording observations and taking swabs from the ear canals for culture.

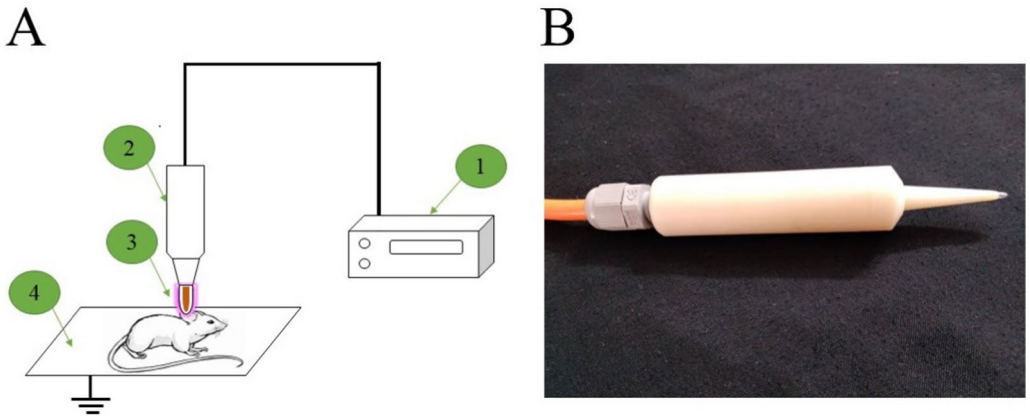

Fig. 1 **A** The schematic diagram of the experimental set up including (1) high voltage generator (2) DBD plasma probe (3) rat and (4) ground electrode **B** an image of DBD plasma probe

Table 1 Data of swelling during the experiment days

Group name	Canal size (mm)																			
	Day 1			Day 2				Day3					Day 4					Day 5				
	3	2	Mean rank	3	2	1	Mean rank	4<	3	2	1	Mean rank	4<	3	2	1	Mean rank	4<	3	2	1	Mean rank
Control	3	3	14	0	0	6	21.5	0	0	0	6	21.5	0	0	0	6	21.5	0	0	0	6	21.5
Plasma	3	3	14	0	6	0	9.8	2	1	3	0	9.8	2	3	1	0	11.8	4	1	1	0	11.5
Drug	4	2	12	6	0	0	10.67	0	4	2	0	10.67	3	3	0	0	9.75	6	0	0	0	8.5
Mixed	5	1	10	5	1	0	8	0	6	0	0	8	5	1	0	0	6.9	6	0	0	0	8.5
P-Value	0.59*			0.000*				0.002*					0.001*					0.000*				

*Based on the Kruskal–Wallis test

Table 2 Data of effusion during the experiment days. Effusion type (0 = dry, 1 = moist, 2 = fluid and 3 = otorrhoea)

Group name	Effusion type																				
	Day 1			Day 2				Day3				Day 4					Day5				
	2	3	Mean rank	1	2	3	Mean rank	1	2	3	Mean rank	0	1	2	3	Mean rank	0	1	2	3	Mean rank
Control	0	6	13	0	0	6	14.5	0	0	6	21.5	0	0	0	6	21.5	0	0	0	6	21.5
Plasma	0	6	13	0	0	6	14.5	2	4	0	9	0	6	0	0	11	0	6	0	0	12.5
Drug	0	6	13	0	0	6	14.5	0	6	0	12	3	3	0	0	6.5	4	2	0	0	6.5
Mixed	1	5	11	1	3	2	6.5	3	3	0	7.5	0	6	0	0	11	2	4	0	0	9.5
P-Value	0.39*			0.003*				0.001*				0.000*					0.000*				

*Based on the Kruskal–Wallis test

Statistical analysis

The Statistical Package for Social Sciences version 26.0 (IBM Corp.; Armonk, NY, USA) software was used for statistical analyses. The defined numerical values were compared statistically. The log transformation was used to make the data of colony counts conform to normality. For the multiple group comparisons of swelling and effusion measured in the EAC, Kruskal–Wallis analysis was applied, and in the multiple comparisons, the post-hoc Mann–Whitney test was applied. The statistical analysis of the results of the cultures taken on days 1 and 2 of the study were analyzed using one-way anova analysis and on days 3,4 and 5 of the study were analyzed using Kruskal–Wallis analysis. Multiple comparisons using Tukey and Mann–Whitney test were made for significant parameters. A P value of <0.05 was accepted as statistically significant for all the statistical data.

Results

Swelling

Number of samples in each swelling category and statistical mean rank of swelling in each experimental group were shown in Table 1. On the first day of the study, the P value of swelling between the groups was 0.59. This result implies that before the intervention, there was no significant difference between the sizes of the ear canal in all samples of experimental groups. On the second to fifth day, in control group, the EAC size or swelling of rats was statistically different from those in drug, plasma and combination group with the significant level of $p=0.000$, $p=0.002$, $p=0.001$, $p=0.000$ respectively. In fact, no change was observed in the control group until the last day of treatment, while all treatment groups start to improve from the second day. Multiple comparison revealed that swelling was significantly higher in the plasma group than the drug and combination groups just on the second day ($p=0.002$, $p=0.01$ respectively).

Statistical analysis showed that on the second day, with one therapeutic intervention, the amount of swelling in the plasma group increased and this difference was significant compared to the drug and combination groups and from the third day, the recovery process began in the plasma group. However, in the following days, the results of this group came closer to the other groups so that there was no significant difference between all treatment groups on the third to fifth days. In the drug and combination groups, amount of swelling decreased from the second day. It is worthy to point that on the fourth day of follow up, swelling in the combination group was less than the other treatment groups so that 5 rats completely recovered while this number was 3 in the drug group and 2 in the plasma group.

Effusion

Number of samples in each effusion category (0 = dry, 1 = moist, 2 = fluid and 3 = otorrhoea) and statistical mean rank of effusion in each experimental group were shown in Table 2. Comparison of effusion in the EAC on the first day of study shows no statistically significant difference between the groups ($p=0.39$). This result means that before the intervention, all samples in the experimental groups had similar effusion. Rate of effusion in the control group was statistically different from those in the drug, plasma and combination groups on the second to

fifth day ($p=0.003$, $p=0.001$, $p=0.000$, $p=0.000$, respectively). In the other words, in the control group, there was otorrhea until the last day while effusion in the EAC was controlled in the other groups. Post hoc analysis revealed that effusion was significantly lower in the combination group than the plasma and drug groups on the second day ($p=0.021$, $p=0.021$).

Statistical analysis showed that on the second day there was a decrease in effusion in the combination group, while in the plasma and drug groups, an improvement was observed from the third day. This indicates that combining the two methods of drug and plasma can lead to an early improvement.

Colony count

Data of colony counts in the groups and days were shown in Fig. 2. Data from colony counts of the samples showed no significant difference between the groups on the first day ($p=0.553$). This means that all samples were almost equally infected initially. However, number of colonies in the control group was statistically different from those in the drug, plasma, and combination groups on the second to fifth days of follow up ($p=0.000$, $p=0.000$, $p=0.000$, $p=0.000$ respectively). Although number of bacterial colonies in the control group decreased slightly during the different days of follow up, it is still significantly different from the other groups.

Post hoc analysis indicated that number of colonies was significantly higher in plasma group than the drug and combination groups on 2,3 and 4 days ($p<0.05$). Moreover, statistical analysis showed that in the drug and combination groups, rapid improvement began on the second day of the study, and on the third day no bacteria were observed on the plate. In the plasma group, the recovery started from the second day of follow up at a slower rate,

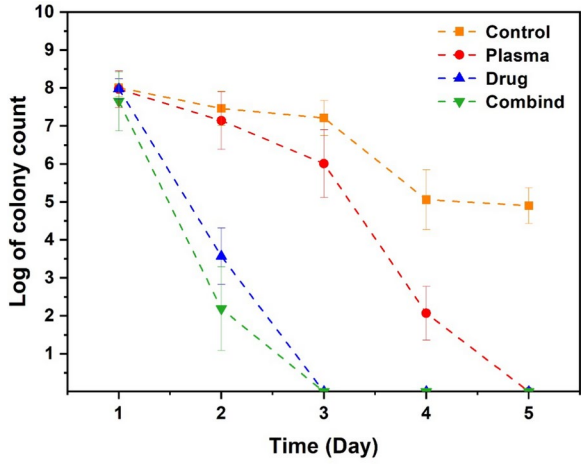

Fig. 2 Data of colony count during the experimental days. Mean and standard deviation of log of colony counts in experimental groups in each day was shown

so that it was significantly different from the other treatment groups, and however, on the fifth day, no bacteria were seen on the plate.

Discussion

The issue of appropriate, reliable with a fewer side effects treatment of acute otitis external is still contentious. Therapeutic approaches may include a steroid alone, an antibiotic or a combination of the above (Kantas et al. 2007). Although employing of chemical drugs as was also observed in our results, is a reliable method for AOE treatment, it suffers from some common side effects. In this study aims to figure out if plasma could be used as an alternative method for AOE treatment. Therefore, the effect of CAP on acute otitis external in rat was investigated and acquired results were compared with the effect of the drug.

The data obtained in this report showed that swelling and effusion of ear canal could be resolved completely when the ear canal exposed with the CAP. Our results demonstrated that exposure to cold plasma without the need for medication can relieve inflammation in the skin of the ear canal. In addition, culturing of bacteria revealed that CAP exposure for 5 days can result in the elimination of bacteria from the external ear canal compared to the control group whose infection increases over all 5 days of follow up. These results show cold plasma could be a new approach for a more efficient disinfection of external ear. Although the accurate mechanisms of CAP for microorganism's inactivation are still unclear, some research groups proposed different chemical and physical mechanisms involving cells' inactivation. Plasma generates reactive ions, molecules and free radicals such as atomic oxygen(O), hydroxyl(OH), nitrogen molecule(N_2), super oxide anion(O_2^-) and nitric oxide(NO) (Fathollah et al. 2016). It is extensively believed that these reactive agents play an important role in the interaction between the plasma and cell (Fathollah et al. 2016). Moreover, serious damage to the bacteria cell wall and leakage of bacteria cytoplasm from cracks (Navabsafa et al. 2013), destruction of DNA molecules (Dobrynin et al. 2009)and UV radiation from plasma excited species (Lackmann and Bandow 2014) are some probable mechanisms of plasma interaction with microorganisms. It is worthy to note that an important benefit of plasma application is the high antibacterial activity without the increase of resistance or adverse effects on surrounding tissues (Dobrynin, Fridman, Friedman and Fredman 2009, Fathollah, Mirpour, Mansouri, Dehpour, Ghoranneviss, Rahimi, Naraghi, Safaie, Chalangari and Chalangari 2016).

According to our results, in the drug group treated with antibiotics and in the combination treatment group (drug and plasma), the recovery process begins on the second

day and the external ear infection is completely cured on the third day. However, in the group treated with cold plasma, the recovery process started from the third day and complete treatment of the external ear infection occurs on the fifth day. Obviously accompanying drug chemicals with plasma spices promotes inhibitory activity of the treatment method against the bacteria.

Results of our statistical animal model is in confirmation of what is reported by Isbary et al. for a patient with chronic post-operative ear infection (Isbary et al. 2013). They showed that only the application of CAP leads to a significant reduction in pain and clearance of bacterial carriage which is well in line with our animal model results.

Although treatment of AOE by both plasma and drug methods has been done in a short time, there are no ototoxicity and body resistance to the drug in the plasma method. It can be considered as a big advantage of the plasma method over the drug. However, the results showed that by combining two methods of drug and plasma, the best results can be achieved in the treatment of AOE.

Along with all were discussed in comparing the treatment methods, it should be noted that these results can be valid for other kinds of bacteria as well. However, the most common type of bacteria in AOE disease was tested in this article, the other kinds of bacteria will also be affected by plasma. According to many articles and references, CAP is bactericidal and fungicidal against a broad spectrum of microorganisms, regardless of resistance patterns (Isbary et al. 2010, 2013; Navabsafa et al. 2013). *P. aeruginosa* is one of highly resistant bacterium due to its properties which was chosen as the test bacteria in our work.

In conclusion, this report indicates the effect of cold atmospheric plasma as a new treatment method on acute otitis external. The findings demonstrated that the exposure of cold plasma can suppress the infection of AOE. Moreover, combining the two methods of antibiotic and plasma will lead to an early improvement. The outcome of this study proposes cold atmospheric plasma as an available treatment method for resolving the infection in otitis media in the future. The results of this article are promising for its possibility to be used in clinical trials and it is suggested that its human model be tested for further studies.

Abbreviations

AOE	Acute otitis external
CAP	Cold atmospheric plasma
CFU	Colony-forming units
DBD	Dielectric barrier discharge
EAC	External auditory canal
OES	Optical emission spectroscopy

Author contributions

T.T. and N.N.S. designed research. T.T. performed research, analyzed data, and wrote the manuscript; A.A.B helped for the statistical analysis. T.T. and N.N.S. edited the manuscript.

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Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate

This article used infected rat as an animal model. All procedures were carried out in accordance with the international, national, and institutional ethical standards. The experimental protocol was approved by the Ethical Committee of Shahid Beheshti University of Medical Sciences with the document no. 1398.818. Moreover, all the authors have checked and agreed to submit this manuscript into AMB Express.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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