Infection and Drug Resistance

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ORIGINAL RESEARCH

In vitro Antibacterial Effect Study of Plasma-Activated Saline on *Mycobacterium Tuberculosis*

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Purpose: This study aimed to investigate the antibacterial effects of plasma-activated saline (PAS) on My-cobacterium tuberculosis (Mtb).

Methods: We conducted a growth assay on 3 strains of Mtb and an antibiotic sensitivity test on 4 strains of Mtb. Both tests included groups treated with normal saline (NS), PAS, and hydrochloric acid (HCl). The test of antibiotic sensitivity consisted of parallel tests with two concentrations of bacteria suspension: 10^{-2} and 10^{-4}. The selected antibiotics were rifampicin (RIF), isoniazid (INH), ethambutol (EMB), and streptomycin (SM). The number of bacteria was determined after one month of culture under different conditions. The Kruskal–Wallis test was used to analyze the differences in grouping factors at representative time points.

Results: The growth assay indicated that PAS significantly inhibited the growth of 3 strains of Mtb compared with NS and HCl treatment groups. Furthermore, except for the initial observation time point, the remaining three observation time points consistently demonstrate no significant differences between the NS group and the HCl group. The antibiotic sensitivity test of INH, SM, and RIF indicated that PAS could inhibit the growth of antibiotic-resistant Mtb, and the antibiotic sensitivity test of INH and SM with bacterial suspension concentration of 10^{-2} and SM with bacterial suspension concentration of 10^{-4} showed statistically different results. The antibiotic sensitivity test of EMB indicated that the growth of Mtb in PAS was slower than that in NS and HCl in both antibiotic-resistant and sensitive Mtb, but there was no statistical difference.

Conclusion: The study indicates that PAS contains a significant amount of active substances and exhibits high oxidizability and an acidic pH state. The unique physicochemical properties of PAS significantly delayed the growth of Mtb, compared to the NS and the HCl. PAS not only inhibited the growth of drug-sensitive strains but also significantly enhanced the sensitivity of drug-resistant strains to anti-tuberculosis drugs, which may provide a new therapeutic strategy for the treatment of tuberculosis.

Keywords: plasma-activated saline, mycobacterium tuberculosis, antibacterial effect, antibiotic sensitivity

Introduction

Tuberculosis (TB) remains a significant global health concern, despite recent advancements in its management.[1] Standard TB treatment respond in only 80% of patients, well below the World Health Organisation's target of 90%.[2] Multidrug-resistant TB (MDR-TB) worsens when Mtb develops resistance to anti-TB drugs.[3] As a result, cure rates for MDR-TB and extensively drug-resistant tuberculosis have fallen by 50% and 30%.[4] MDR-TB strains, which resist conventional anti-TB drugs, pose a challenge due to prolonged treatment periods and severe side effects, compounded by patient non-compliance leading to further drug resistance.[5]

Tracheobronchial tuberculosis, affecting a quarter of patients, presents additional complexities. Unlike pulmonary TB, it often requires extended treatment, sometimes necessitating local administration due to fibroplasia and caseous necrosis impeding drug penetration.[6] Even with appropriate medication, tracheobronchial TB can cause irreversible narrowing, often requiring surgical intervention, imposing substantial physical and financial burdens on patients.[7]

Received: 22 December 2023
Accepted: 22 May 2024
Published: 11 June 2024

In this context, exploring alternative treatment modalities is crucial. Plasma, with its potent bactericidal properties driven by reactive oxygen species (ROS), emerges as a promising avenue.[8,9] At present, there are few studies on the inactivation effect of plasma on TB, and most of them focus on the gaseous state of plasma.[10,11] Lee C[12] proved that OH radicals in a Nonthermal Plasma Jet (NTPJ) were used to inactivate mycobacteria in aqueous solution, which can inactivate nonpathogenic Mycobacterium smegmatis and pathogenic Mtb H37Rv. Bar W[10] proved that the low-temperature hydrogen peroxide gas plasma had a bactericidal effect on the bronchoscope infected by Mtb. Daeschlein G'[11] experiments showed that Atmospheric Plasma Jet (APPJ) and Dielectric Barrier Discharge (DBD) Plasma had high inactivating effects on 8 different Mtb strains. It has also been proved that PAS has strong sterilization activity against Shewanella putrefaciae and methicillin-resistant Staphylococcus aureus (MRSA).[13,14] Unlike traditional drug-based approaches, plasma-based therapy offers several advantages, including broader clinical applicability, particularly in irrigating sites affected by TB without contributing to drug resistance.

This study evaluates the in vitro antibacterial activity of PAS against Mtb, presenting a novel therapeutic approach for TB treatment. By harnessing the power of plasma, this method offers a potential solution to the challenges posed by drug-resistant TB strains and the limitations of conventional treatments.

Material and Methods

Plasma Device and Preparation of PAS

PAS is produced by the plasma effluent gas enriched with high valence NOx, which is generated by the plasma discharge configuration, similar to our previous studies.[15] The setup comprises two discharge reactors: a sliding electric arc and a coaxial DBD. The sliding electric arc reactor consists of two stainless steel blades positioned opposite each other (minimum distance = 2 mm, blade length = 35 mm) and an air nozzle with an inner diameter of 1 mm. The blade electrodes are powered by a high-voltage transformer (TH5-NT-1530, Sinolift, Shanghai, China) at approximately 20 W, generating NO_x^-dominated plasma effluent gas. This is because the high temperature of the sliding electric arc completely suppresses the generation of O_3, with NO and NO_2 becoming the primary gaseous reactive species produced. The coaxial DBD reactor involves a quartz tube (outer diameter = 18 mm, thickness = 1 mm) with a ground electrode covering the outer wall (length of 150 mm). A high-voltage electrode is placed at the axis of the quartz tube with a 1mm gap between the high-voltage electrode and the inner wall of the quartz tube. The DBD is powered by a high-voltage power supply (CTP-2000K, Suman, Nanjing, China) with a discharge power of 15 W. In the coaxial DBD reactor, the dissociation energy threshold of O_2 is higher than that of N_2, making O_3 the primary product of the coaxial DBD reactor. Air is introduced separately into the sliding electric arc reactor and coaxial DBD reactor with airflow rates of 1 SLM and 5 SLM, respectively. The two paths of plasma effluent gas are mixed in the gas tube, and the mixed plasma gas is introduced into 100 mL of normal saline for a 15minutes treatment to produce PAS, as shown in Figure 1.

Measurement of Gaseous and Aqueous Plasma Reactive Species

To investigate the gas-phase characteristics of the plasma effluent gas, Fourier-transform infrared (FTIR) spectroscopy is conducted. The mixed plasma effluent gas is introduced into a gas cell with an optical path of 2.4 m, which is integrated with an FTIR spectrometer (Tensor II, Bruker, Billerica, Massachusetts, USA). The absorption cross-section data of each gaseous reactive species is taken from the HITRAN database[16]. For the detection of aqueous reactive species, TEMPONE-H (1-hydroxy-2, 2,6, 6-tetramethyl-4-oxo-piperidine, Enzo, final concentration = 1mM) is used as a spin trap for O_2^- and $ONOO^-$ in PAS, and their concentrations are measured by an electron spin resonance spectrometer (EMXplus, Bruker, Billerica, Massachusetts, USA). The concentration of NO in the aqueous solution is quantized by another spin trap MGD (N-methyl-D-glucaminedithio-carbamate, Dojindo, final concentration = 10 mM). For the long-lived species including H_2O_2, NO_2^-, and NO_3^-, their concentrations are measured by using a microplate reader (Thermo, Varioskan Flash) with the hydrogen peroxide assay kit (Beyotime, Shanghai, China) for H_2O_2 and the nitrate/nitrite colorimetric assay kit (Beyotime, Shanghai, China) for NO_2^- and NO_3^-. The physicochemical parameters of PAS, including pH and oxidation-reduction potential (ORP), were measured using a pH/ORP meter (S210, Mettler Toledo,

Figure 1 The Machine of the production of plasma-activated saline.

Zurich, Switzerland). The measurements of the concentration of aqueous reactive species and the physicochemical properties of PAS have been done three times repeatedly.

Strains of Mtb

We collected 6 strains of Mtb, which were isolated and identified by the Shaanxi Provincial Hospital of Tuberculosis Prevention and Treatment, numbered 5158, 7281, 7454, 9545 and the virulent Mtb strain H37Rv, with the informed consent of the patients. Mtb strains 72819545 and H37Rv were tested for growth assay. Among the 4 strains included in the drug sensitivity test, strain 7281 was found to be resistant to SM, INH, RIF and EMB, while strains 5158, 9545, and 7454 were resistant to SM, INH, and RIF, but sensitive to EMB. This study was approved by the Shaanxi Provincial Hospital of Tuberculosis Prevention and Treatment. The experiments we conducted were under Biosafety Level 3 conditions.

Bacterial Suspension Preparation

To prepare the bacterial suspension, NS was drawn into a sterile tube using a sterile pipette. Fresh colonies from a 2-week-old culture were then collected using an inoculating loop and transferred into the sterile tube. The tube was tightly sealed, and the contents were mixed by shaking at a concentration of 1 McFarland standard (1 mg/mL = 3×10^8 CFU/mL) using an ultrasonic disruptor. The prepared bacterial suspension was then centrifuged at 3000 rpm for 5 minutes, and the supernatant was discarded. The pellet was resuspended in the appropriate diluent to achieve a concentration of 1 McFarland (1 mg/mL) bacterial suspension. Using a micropipette, 50 μL of the 1 McFarland bacterial suspension was aspirated and added to 5000 μL of the corresponding diluent to obtain a concentration of 10^6. Using a pipette to absorb 10^6 CFU/mL bacterial solution 50 μL and add 5000 μL diluent to obtain 10^4 CFU/mL bacterial suspension.

Growth Assay

The suspensions of each strain were divided into three groups: NS control group, PAS-treated group, and HCl-treated group, with three replicates per group. The procedures are as follows:

NS Control Group: Dilute the bacterial suspension to a concentration of 10^4 CFU/mL based on the 1 McFarland standard. Using a pipette, transfer 100 μL of the diluted bacterial suspension and evenly inoculate it onto the surface of the roche culture tube (Baso, Zhuhai, China), ensuring the bacterial suspension is evenly dispersed on the agar slope. Take 500 μL of the diluted bacterial suspension and inoculate it into the prepared BD culture medium (Becton Dickinson, Shanghai, China).

PAS Treatment Group: Take the purified 1 McFarland bacterial suspension and centrifuge it to remove the supernatant. Add 2 mL of PAS to the bacterial pellet. After 30 minutes, follow the same procedure as the NS control group for inoculation.

HCl Treatment Group: Adjust the pH of the HCl with NS to match that of the PAS. Follow the same procedure as the PAS control group for inoculation.

Since Mtb grows slowly, it usually takes 7 days to observe visible colonies on the culture medium, and sufficient colonies are obtained after 14 days. Therefore, the bacterial colony counts will be recorded on day 14, 20, 26, and 31. The selection of these time points is based on ensuring a sufficiently long duration between them to observe significant changes.

Drug Sensitivity Test

NS control group: Adjust the clinical strains 5158, 7281, 7454, and 9545 to a concentration of 1 McFarland. Pipette 20 μL of the adjusted bacterial suspension into 2 mL of NS solution (10^{-2} dilution). Pipette 20 μL of the 10^{-2} bacterial suspension into 2 mL of NS solution (10^{-4} dilution). Inoculate the drug-containing roche culture tube with 10 μL using an inoculation loop.

PAS treatment group: Adjust the clinical strains 5158, 5943, 7281, 7454, and 9545 to a concentration of 1 McFarland. Pipette 20 μL of the adjusted bacterial suspension into 2 mL of PAS (10^{-2}). Pipette 20 μL of the 10^{-2} bacterial suspension into 2 mL of NS solution (10^{-4}). Allow the suspensions to stand for 30 minutes. Inoculate the drug-containing roche culture tube with 10 μL using an inoculation loop.

HCl treatment group: Follow the same procedure as the PAS treatment group, but replace the PAS with HCl.

The bacterial colonies of Mtb will be counted on the 20th, 26th, and 31st days in the drug-containing roche culture tube.

Bacterial Colony Counts

Report of bacterial growth on drug-containing and control roche culture tube: Mtb culture negative (-): No colony growth observed on the slant. Mtb culture positive (+): Colony growth covering 1/4 of the slant area. Mtb culture positive (++): Colony growth covering 1/2 of the slant area. Mtb culture positive (+++): Colony growth covering 3/4 of the slant area. Mtb culture positive (++++): Colony growth covering the entire slant. As shown in the Figure 2, from left to right, different degrees of growth of Mtb are represented.

Statistical Analysis

Statistical analysis was performed using SPSS 26.0 (Chicago, IL, USA). The non-parametric Kruskal–Wallis test was used to compare differences among multiple groups. If the overall test did not detect significant differences between groups, pairwise comparisons were not conducted. Statistical significance was established at a P-value < 0.05.

Results

PAS Worked Due to the Reactive Species Therein

Given that PAS is produced by introducing plasma effluent gas into a saline solution, it is necessary to investigate the gaseous reactive species in the plasma effluent gas. The FTIR absorption spectra of the mixed plasma effluent gas generated by the hybrid plasma discharge configuration are illustrated in Figure 3. The coaxial DBD reactor and sliding electric arc reactor provide mainly O_3 and low valence NO_x (including NO and NO_2), respectively. In the mixed gas, O_3 oxidizes the low valence NO_x to form high valence NO_x (including N_2O_5 and NO_3), where NO is first oxidized by O_3 to NO_2, and then NO_2 is further oxidized by O_3 to NO_3, and finally NO_2 and NO_3 react to produce N_2O_5. N_2O is also present in the mixed plasma effluent gas, but it is an inert gaseous component that is stable and hardly reacts.

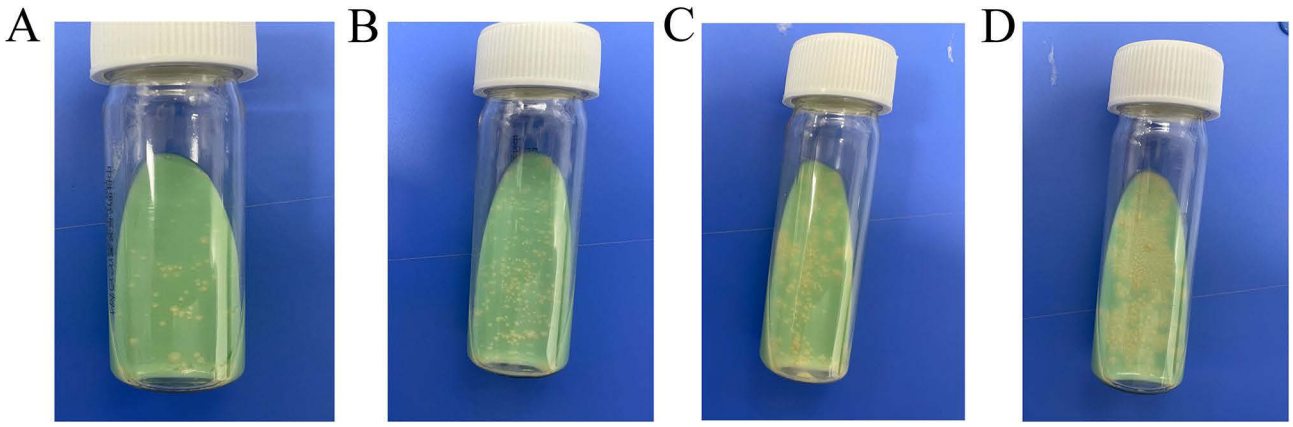

Figure 2 The growth of Mtb in different degrees: (**A**–**D**) respectively represents Mycobacterium tuberculosis culture positive (+), (++), (+++) and (++++).

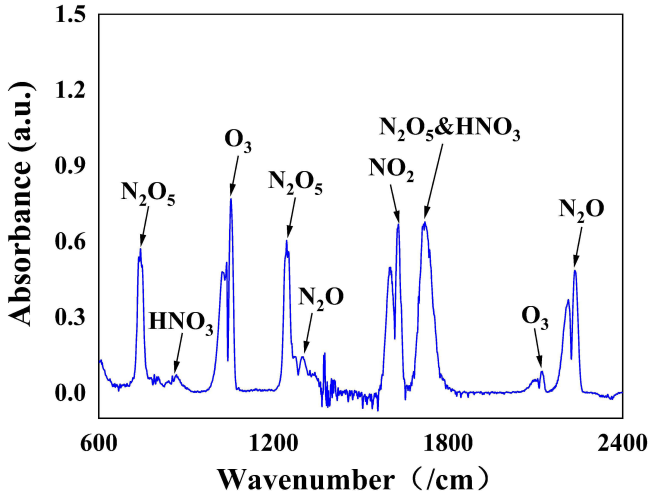

Figure 3 FTIR absorption spectra of the mixed gas from DBD and gliding arc discharge.
Abbreviations: FTIR, Fourier-transform infrared; DBD, Dielectric Barrier Discharge.

Various aqueous reactive species are produced in PAS as a result of a series of chemical reactions between gaseous reactive species and aqueous solutions. As shown in Figure 4, the experimental results indicate that in the 100 mL of PAS after 15 min of plasma treatment, the concentrations of H_2O_2, NO_2^-, NO_3^-, O_2^-andONOO$^-$, and NO radicals are 190.08 ±53.34 μM, 148.08±35.72 μM, 8564.33±523.81 μM, 26.44±6.92 μM, and 9.03±2.37 μM, respectively. In addition, the physicochemical parameters measured by the pH/ORP meter show that the pH and ORP values of the PAS are 2.01±0.11 and 639±43.62 mV, respectively.

Growth Assay

Observation of the growth of Mtb at different time points (Day 14, 20, 26, and 31), revealed the following findings (Table 1): At all observed time points, there was a significant difference in the growth of Mtb between the NS control group and the PAS treated group. As time progressed, significant differences in the growth of Mtb were observed between the PAS-treated group and the HCl-treated group on Day 20, 26, and 31(Table S1). No significant difference in the growth of Mtb was observed between the NS group and the HCl-treated group throughout these time points. It is noteworthy that for the bacterial count in the PAS-treated group was significantly lower than that in the NS group or HCl group at the final observation time point (Day 31). Figure 5 shows the growth of TB strain 7281,9454, H37Rv in different groups on day 31st.

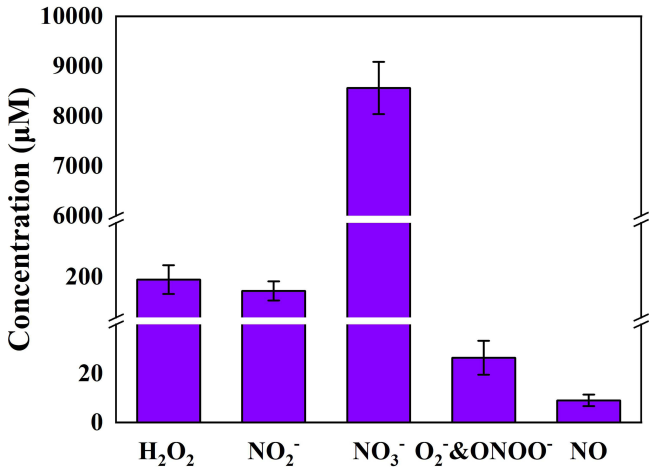

Figure 4 Concentrations of aqueous reactive species in plasma-activated saline.

Drug Sensitivity Test of INH, SM and RIF

Strains 7281, 9545, 7454 and 5158 were resistant to INH, SM, and RIF. Observation of the growth of Mtb at a bacterial concentration of 10^{-2}, revealed the following findings (Table 2): In the INH drug sensitivity test, there was no statistically significant difference in the growth of Mtb between the NS group and the HCl-treated group. The growth of Mtb in the PAS-treated group was slower compared to the other two groups (Table S2). In the SM drug sensitivity test, with the progression of time, the growth of Mtb in the PAS-treated group became progressively slower compared to the other two groups (Table S2). Similar to the INH test, there was no difference in the growth of Mtb between the NS group and the HCl-treated group (Table 2). Although no significant differences were observed in the remaining drug sensitivity test, the overall trend was that the growth of Mtb in the PAS-treated group slower than that of the NS group and the HCl-treated group as time progressed (Table S2), especially on the 31st day, as shown in Figure 6.

Observation of the growth of Mtb at a bacterial concentration of 10^{-4}, revealed the following findings (Table 3): In the SM drug sensitivity test, there was a difference in the growth of Mtb between the NS group and the other two groups at 31st day, but there was no difference between the PAS and HCl groups at the same time. Although no statistically significant differences were observed in the remaining drug sensitivity test, there are still the following expected results (Table S3): In the drug sensitivity test for RIF, strain 5158 exhibited colony formation in both the NS and HCl group,

Table 1 Statistic Analysis of Growth Experiment

Time	Comparison	*P*
14th	NS-PAS	0.005
	NS-HCl	0.025
	PAS-HCl	1.000
20th	NS-PAS	0.000
	NS-HCl	0.333
	PAS-HCl	0.001
26th	NS-PAS	0.000
	NS-HCl	0.231
	PAS-HCl	0.003

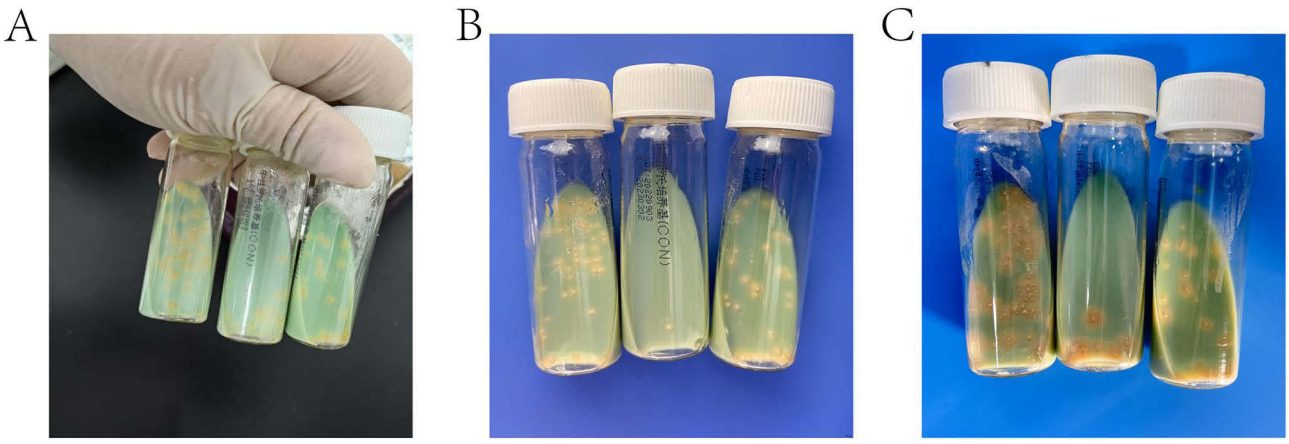

Figure 5 Growth experiment of tuberculosis strains on day 31st: (**A**–**C**) respectively represents the growth of TB strain 72819454 and H37Rv (Each image from left to right represent the NS, PAS and HCl groups).
Abbreviations: NS, normal saline; PAS, plasma-activated saline; HCl, hydrochloric acid.

while no colony formation was observed in the PAS group. In the drug sensitivity test for INH, strain 7454 displayed varying degrees of growth in both the NS and HCl group, but no colony formation was observed in the PAS group. Similarly, in the drug sensitivity test for SM, strain 7281 showed varying degrees of growth in both the NS and HCl groups, but no colony formation was observed in the PAS group.

Table 2 Statistic Analysis of Drug Sensitivity Experiment (Bacterial Suspension Concentration=10^{-2})

Antituberculosis Drugs	Time	Comparison	*P*
RIF	20th	NS	0.253
		PAS	
		HCl	
	26th	NS	0.257
		PAS	
		HCl	
	31st	NS	0.258
		PAS	
		HCl	
INH	20th	NS-PAS	0.005
		NS-HCl	0.398
		PAS-HCl	0.050
	26th	NS-PAS	0.005
		NS-HCl	1.000
		PAS-HCl	0.005
	31st	NS-PAS	0.020

(*Continued*)

Table 2 (Continued).

Antituberculosis Drugs	Time	Comparison	*P*
		NS-HCl	1.000
		PAS-HCl	0.020
SM	20th	NS-PAS	0.054
		NS-HCl	0.477
		PAS-HCl	0.008
	26th	NS-PAS	0.014
		NS-HCl	0.655
		PAS-HCl	0.004
	31st	NS-PAS	0.005
		NS-HCl	1.000
		PAS-HCl	0.005

Abbreviations: RIF, rifampicin; INH, isonicotinic acid hydrazide; SM,streptomycin.

Drug Sensitivity Test of EMB

Strain 7281 is an EMB-resistant strain. The growth of strain 7281 was observed at different bacterial concentrations, and no statistically significant differences were found (Table 4).

It is important to highlight that in the drug sensitivity test conducted with a bacterial concentration of 10^{-2}, the growth of strain 7281 in the PAS group consistently exhibited slower growth compared to the NS and HCl groups, as shown in Figure 6, on day 31, the number of TB bacterial colonies visible in the PAS group was less than in the other two groups. Additionally, in the drug sensitivity test performed with a bacterial concentration of 10^{-4}, no colony formation was observed in any of the three groups.

Strain 5158, 9545, and 7454 were sensitive to EMB. There was no difference in the growth of Mtb in different groups, whether the concentration of bacteria was 10^{-2} or 10^{-4} (Table 5). Although no Mtb growth was observed at 10^{-4} bacterial concentration (Table S3), the growth of Mtb in PAS treatment group was significantly less than that in NS and HCl groups, which could be observed in strains 7454 and 5158 at 10^{-2} bacterial concentration (Table S2, Figure 6).

Discussion

Currently, TB prevention and control in China still face a very severe situation. TB is not only a serious public health problem that poses a threat to the health of the Chinese population but also a significant social issue that hinders national progress and economic development. The current challenges in TB treatment include drug resistance and the lack of effective new drugs. In recent years, the emergence of many innovative treatment methods has provided new therapeutic strategies to end tuberculosis, such as endoscopic injection of anti-tuberculosis drugs, cryotherapy, and bronchial stent implantation.[17] However, these treatments may lead to drug resistance or fail to eliminate the lesions. Therefore, there is an urgent need to search for a treatment method that has fewer toxic side effects and better therapeutic outcomes.

Plasma biomedicine is an emerging interdisciplinary field in recent years, combining plasma science and technology, life sciences, clinical medicine, and other disciplines. The high valence NO_x radicals in the mixed plasma effluent gas reacted with water to generate large amounts of H^+ and NO_3^-, resulting in the acidity of PAS, which helps to ensure high bioactivity.[18] Besides, the aqueous H_2O_2 is likely to originate from the dissolution of gaseous H_2O_2,[15] whereas the aqueous NO radicals and NO_2^- have been reported to be produced from gaseous NO_2.[19] Most importantly, in addition to HNO_3, the gaseous high valence NO_x can generate short-lived aqueous reactive species such as O_2^- and $ONOO^-$,[20] which enables the remarkable chemical and biological reactivity of PAS. The gaseous NO_3 is considered to be the most

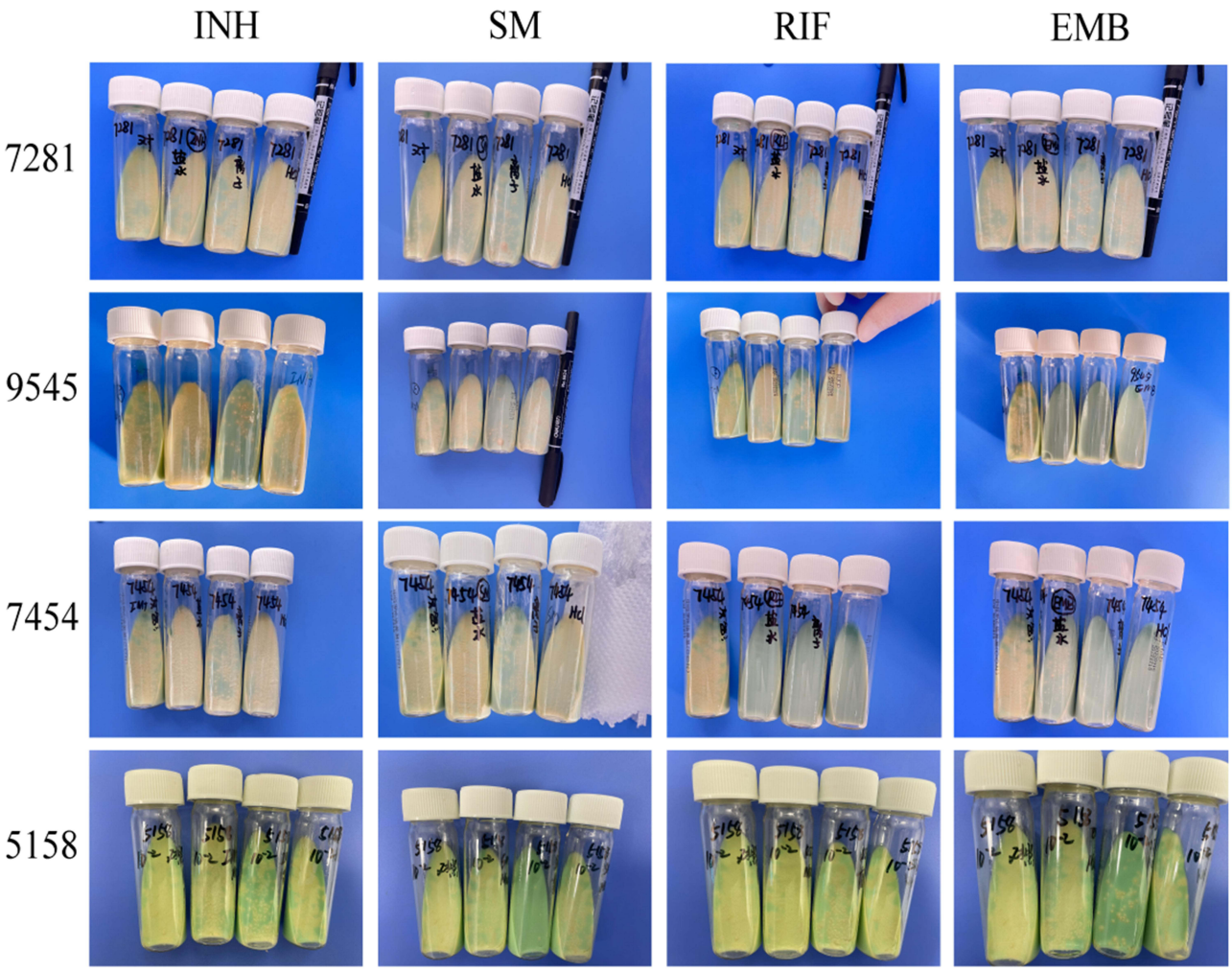

Figure 6 Drug sensitivity experiment of tuberculosis strains (bacterial suspension concentration = 10^{-2}) on day 31st: Each image from left to right represent the Control, NS, PAS and HCl groups.
Abbreviations: NS, normal saline; PAS, plasma-activated saline; HCl, hydrochloric acid; INH, isoniazid; SM, streptomycin; RIF, rifampicin; EMB, ethambutol.

reactive among NO_x and may play the most crucial role in water activation. Currently, plasma has been used for medical device sterilization,[10] treatment of severe infections[14] and cancer.[21] The plasma can promote wound healing by promoting the production of various reaction mediators, such as H_2O_2, hydroxyl radical, O_3, and up-regulating growth factors such as VEGF and FGF.[22] Studies have shown that these mediators have a certain antibacterial effect on

Table 3 Statistic Analysis of Drug Sensitivity Experiment (Bacterial Suspension Concentration=10^{-4})

Antituberculosis Drugs	Time	Comparison	*P*
RIF	20th	NS	0.557
		PAS	
		HCl	
	26th	NS	0.295

(*Continued*)

Table 3 (Continued).

Antituberculosis Drugs	Time	Comparison	*P*
		PAS	
		HCl	
	31st	NS	0.400
		PAS	
		HCl	
INH	20th	NS	0.368
		PAS	
		HCl	
	26th	NS	0.079
		PAS	
		HCl	
	31st	NS	0.304
		PAS	
		HCl	
SM	20th	NS	0.368
		PAS	
		HCl	
	26th	NS	0.111
		PAS	
		HCl	
	31st	NS-PAS	0.006
		NS-HCl	0.039
		PAS-HCl	0.492

Table 4 Drug Sensitivity Experiment of 7281 in EMB

TB Concentration	Time	Comparison	*P*
10^{-2}	20th	NS	0.368
		PAS	
		HCl	
	26th	NS	0.368
		PAS	
		HCl	

(*Continued*)

Table 4 (Continued).

TB Concentration	Time	Comparison	*P*
	31st	NS	0.368
		PAS	
		HCl	
10^{-4}	20th	NS	1.000
		PAS	
		HCl	
	26th	NS	1.000
		PAS	
		HCl	
	31st	NS	1.000
		PAS	
		HCl	

Table 5 Drug Sensitivity Experiment of 5158, 9545, and 7454 in EMB

TB Concentration	Time	Comparison	*P*
10^{-2}	20th	NS	0.360
		PAS	
		HCl	
	26th	NS	0.473
		PAS	
		HCl	
	31st	NS	0.473
		PAS	
		HCl	
10^{-4}	20th	NS	1.000
		PAS	
		HCl	
	26th	NS	1.000
		PAS	
		HCl	
	31st	NS	1.000
		PAS	
		HCl	

Staphylococcus aureus, and can improve the sensitivity of Staphylococcus aureus to antibiotics and enhance the inactivation of antibiotics.[23] ROS produced by plasma can induce the apoptosis of gastric cancer cells.[24]

This study focuses on the liquid form of plasma and successfully produces PAS. It detects activated nitrates, nitrites, H_2O_2, and some activated ions, including O_2^-,·OH, $ONOO^-$ and other active components. The study explores the antibacterial effects of PAS on Mtb through growth assay. By comparing the growth of 3 strains of Mtb in different treatments at 4 observation time points, it was found that the bacterial colony counts of Mtb in the PAS treatment group were less than that in the NS-treated group. Furthermore, at the final observation time point, the bacterial colony counts of Mtb in PAS were less than that in the corresponding NS group and HCl group. This result preliminarily demonstrates the antibacterial effect of PAS on the growth of Mtb.

However, the results indicate no statistically significant difference between PAS and HCl treatment groups on the 14th day, suggesting that pH may affect the growth of Mtb. Pyrazinamide (PZA), a first-line drug for tuberculosis treatment, exerts its bacteriostatic effect on Mtb only in an environment with a pH below 5.5.[25] This leads to a preliminary inference: The low pH of PAS may affect the growth of Mtb. Based on the subsequent test results, statistical differences were observed between PAS and HCl on days 20, 26, and 31. Excluding the influence of pH, PAS can still inhibit the growth of Mtb, possibly because ROS and other long-lived and short-lived reactive species released by PAS affect the growth of Mtb.

Next, we investigated whether PAS affected the sensitivity of drug-resistant Mtb to anti-TB drugs (INH, SM and RIF). PAS significantly enhanced the sensitivity of drug-resistant Mtb to INH and SM compared with NS in the drug-sensitivity test with a bacterial suspension concentration of 10^{-2}. However, there was no statistical difference observed between the HCl group and the NS group. This indicates that PAS can significantly enhance the drug sensitivity of drug-resistant Mtb to INH and SM even if the pH factor is excluded. In experiments with bacterial suspension concentration of 10^{-4}:PAS only affected the sensitivity of Mtb to SM, and did not show significant effects on other drugs. This could be attributed to the low bacterial concentration in the culture.

As the most common drug-resistant form[26], there is a close connection between the mechanism of action of INH and plasma. INH enters the interior of Mtb cells through passive diffusion and is activated by catalase and peroxidase (KatG), which disrupts the growth of Mtb by affecting its internal homeostasis environment.[27] Interestingly, KatG expression in Mtb is peroxide-induced.[28] The activation of INH within the TB produces the same free radicals and superoxide produced by plasma. Timmins showed by Electron spin capture (EPR) that INH activated by KatG produces nitric oxide (NO).[29] Studies have found that endogenous superoxide contributes to the intracellular activation of INH.[30]

It is worth noting that in the INH drug sensitivity test with a bacterial concentration of 10^{-4}, strain 7454 showed varying degrees of growth in both the NS and HCl groups, but no colony formation was observed in the PAS groups. This result indicates that PAS can significantly enhance the sensitivity of INH-resistant Mtb to INH. One possible reason for this is that the reactive radicals and oxidants generated after plasma activation can enhance the expression of KatG and activate INH within the Mtb cells.[27] Additionally, the strong cell-penetrating ability and low pH of plasma can influence the internal environment of Mtb, thereby affecting its normal biological processes and achieving the effect of anti-tuberculosis treatment.[31]

Upon observing the drug sensitivity test of EMB-resistant strain 7281, it is notable that no statistically significant differences were observed between the 10^{-2} and 10^{-4} bacterial concentration groups. In the drug sensitivity test with a bacterial concentration of 10^{-2}, the group treated with PAS consistently displayed slower colony formation compared to the NS and HCl groups. This suggests that PAS may have altered the resistance of strain 7281 to EMB. Combined with the subsequent drug sensitivity test results of EMB-sensitive TB strains, PAS can further enhance the sensitivity of Mtb to EMB and inhibit their growth. We can speculate that PAS may inhibit the growth of both drug-resistant and drug-sensitive TB strains at the same time.

Certainly, there are limitations and shortcomings in this study. Firstly, the number of bacteria included in the experiments was relatively small, and further validation is needed using a larger sample size of Mtb. Secondly, although this study included an HCl control group to eliminate the influence of pH on Mtb, the specific role of pH in inhibiting the growth of Mtb needs further clarification. Additionally, the mechanisms by which ROS and hydroxyl radicals produced in PAS inhibit the growth of Mtb require further investigation.

In summary, the use of PAS for the treatment of tuberculosis is a highly promising strategy. The results of this study demonstrate that PAS has an impact on the growth activity of both drug-sensitive and drug-resistant strains of Mtb. In the next step, we will investigate the specific mechanisms by which PAS inhibits the growth of Mt. Our aim is to apply it in the lavage of bronchial tuberculosis, tuberculous empyema, and joint tuberculosis in the future.

Conclusion

This study demonstrates that PAS, generated by introducing plasma effluent gas into saline, contains a significant amount of active substances, including H_2O_2, NO_2^-, NO_3^-, O_2^-andONOO$^-$. Moreover, PAS exhibits high oxidizability and presents an acidic pH state.

Furthermore, the growth experiments indicate that compared to the NS group and the HCl group, the unique physicochemical properties of PAS can significantly delay the growth of Mtb.

In drug susceptibility testing, we observed that PAS significantly inhibits the growth of Mtb, including both drug-sensitive and drug-resistant strains. It is noteworthy that PAS not only inhibits the growth of drug-sensitive strains but also significantly enhances the sensitivity of drug-resistant strains to anti-tuberculosis drugs, especially INH and SM.

In conclusion, although our study requires further validation and refinement, particularly through larger sample sizes and more comprehensive mechanistic studies, these findings suggest that PAS may become a promising novel therapeutic approach in the treatment of tuberculosis. PAS may have potential clinical applications in treating tuberculosis manifestations such as bronchial tuberculosis, tuberculous pleurisy, and joint tuberculosis.

Ethics Statement

This study was approved by the Shaanxi Provincial Hospital of Tuberculosis Prevention and Treatment. (Ethics approval number: 2023(No.8)). Our study complies with the Declaration of Helsinki.

Funding

This research was funded by Shaanxi Provincial Health Science Research Project (Grant number: 2022D043), the Science and Technology Project of Xi'an City (Grant serial number: 23YXYJ0155), the Nanjing Tianqing Research Fund Project (Grant number: HX202334) and the Institute Fund from First Affiliated Hospital of Xi'an Jiaotong University (Grant number: 2022MS-17), the Science and Technology Project of Shaanxi Province (Grant serial number: 2022SF-423).

Disclosure

The authors declare no conflicts of interest in this work.

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