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Effect of Rifampicin on the Adhesion Ability and Biofilm of *Mycobacterium tuberculosis* in diabetic patients Suffering from Tuberculosis

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XF and BL conducted the experiments in this study. GZ and XF contributed to the design and interpretation of the current study and wrote the article. All authors read, revised, and approved the final manuscript.

Key words
Rifampicin, Diabetes, *Mycobacterium tuberculosis* (MT), Adhesion ability, Biofilm

ABSTRACT

The objective of this study was to explore the effect of Rifampicin on the Adhesion Ability and Biofilm of *Mycobacterium tuberculosis* (MT) in diabetic patients with tuberculosis complicated. Seventy patients with type II diabetes and tuberculosis who were admitted to Shangrao Municipal Hospital from June 2019 to June 2020 were randomly divided into a control group (CG, n=35) and an observation group (OG, n=35). The CG was treated with rifampicin capsules, and the OG was treated with rifampicin sterilized powder. The adhesion ability and biofilm formation of MT were analyzed by electron microscope. After eight months of the treatment, the total effective rate of the OG was much higher than that of the CG. Compared with the CG, the probability of gastrointestinal reaction and liver function damage in patients of the OG was significantly lower, and the difference was statistically significant ($P<0.05$). After the rifampicin intervention, the number of adherent colonies on the interface of the three implants decreased significantly ($P<0.05$). The biofilm is destroyed in varying degrees under 400 times field of vision. The use of rifampicin for injection in patients with diabetes and tuberculosis can improve the condition of tuberculosis, which has good safety and medical propaganda significance. MT adheres to the surface of implant and forms tuberculosis biofilm, which has certain selectivity and specificity. After the rifampicin intervention, the number of colonies attached to the interface of titanium alloy, CoCrMo, and polyethylene decreased significantly, and the biofilm formed on the surface of polyethylene is damaged with ladder style. It was concluded that the local application of anti-tuberculosis drugs has a great effect.

INTRODUCTION

Tuberculosis is one of the major infectious diseases affecting the public health safety in the world, especially in developing countries (Bloom *et al*., 2017; Ahmad *et al*., 2018). However, because continuous research on the biological characteristics of MT and a series of anti-tuberculosis drugs such as rifampicin was discovered, the spread of tuberculosis was controlled due to the emergence of these drugs (Bourai *et al*., 2012). With the continuous development of modern transportation industry, the gap between the rich and the poor is too large, wars often erupt, the flow of population is accelerated, the nutritional conditions are poor, the natural environment is deteriorated, medical resources are spread unfairly, the spread of AIDS and other immune problems, and various dissemination factors make the incidence of tuberculosis increase year by year (Al-Sayyad and Abumunaser, 2011). According to the survey, about 33% of the global population may be infected with MT, of which more than 20 million patients need to receive anti tuberculosis drug treatment (Sun *et al*., 2020). In recent years, the spread and prevalence of multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis have been caused by irregular use of anti-tuberculosis drugs, poor patient compliance, and the increase in the number of people living with HIV. The prevention and treatment of tuberculosis is an unprecedented problem (Oztürkmen *et al*., 2010). Drug

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resistance of MT was very serious in China (Golden and Vikram, 2005).

In recent years, some scholars proposed that the drug resistance of MT seems to be related to the existence of biofilm. Biofilm is a way of existence of bacteria in the world, which is related to many aspects of people's life. After bacteria attach to the implant interface, they can start or turn off the expression of specific genes with extremely accurate and coordinated procedures, and secrete many polysaccharide-protein complexes, wrap it up, and become a biofilm structure with a lot of bacteria (Costerton *et al*., 1999). Biofilm is an important defense of bacteria against hostile environments. General biofilm has a very powerful immune escape technology, which can prevent bacteria from being attacked by host defense changes and chemicals, making biomaterial related infection (Veerachamy *et al*., 2014). Adhesion is a technology for bacteria to adhere to the host surface and is the first step for bacteria to form biofilms. Bacterial adhesion is mainly the result of recognition of receptors on host surface by specific adhesion proteins on bacterial surface, so biofilm has selectivity and specificity (Ha *et al*., 2005). Some studies indicated that bacterial biofilm is closely related to the occurrence and development of implant infection (Chocholáč *et al*., 2013). Diabetic tuberculosis and diabetes are very common in clinic. Studies showed that diabetes is an independent cause of tuberculosis. The incidence of tuberculosis in diabetic patients is 3.9 times higher than that in the general population. The clinical treatment of diabetes complicated with tuberculosis requires effective anti-tuberculosis treatment, while reducing blood glucose (Arnold *et al*., 2014). It is the purpose of this study to explore the effect of Rifampicin on the adhesion ability and biofilm of MT in diabetic patients with tuberculosis complicated.

MATERIALS AND METHODS

Subjects

Seventy patients with type II diabetes complicated with tuberculosis admitted to Shangrao Municipal Hospital from June 2019 to June 2020 were enrolled. All patients were treated for tuberculosis, which met the relevant diagnosis and treatment requirements of tuberculosis and diabetes. The patients in this study also knew and agreed, and this study was also agreed by the hospital ethics committee. The patients were randomly divided into a control group (CG, n=35) and an observation group (OG, n=35). The only definitive criterion for exclusion from the study is pregnant people with diabetes, for which it is not possible to perform X-ray due to possible damage to the fetus.

Preparation of materials

In this study, titanium alloy, co-cr-mo alloy, and super polymer polyethylene were used as prosthesis samples for MT biofilm experiment. The three implanted material samples were processed into disks with a diameter of 0.7cm and a thickness of 0.3cm. All samples were polished. It is usually difficult to avoid injury implantation due to surgical instruments in surgery and other reasons, but it is not clear whether this will increase the possibility of MT adhesion, and whether it will affect the recurrence risk of bone tuberculosis after surgery. Therefore, in this study, vascular clamp was used to scrape the other half of the smooth surface of the same material block ten times to form a rough surface and form self-observation. All implants were divided into titanium alloy group, co-cr-mo group, and polyethylene group according to their properties and interface roughness. A total of 50 implants were cleaned with saline and ultrasonic vibration alternately. Four same implants were placed in 40mL centrifuge tube with tweezers, a total of 25 implants. The power and the switch were turned on. When the external pressure gauge of high temperature and high-pressure sterilization pot showed that the pressure in the pot reached 1.1 kg/cm^2, and the temperature in the pot rose to 120°C, the timing switch was opened and the timing was started for 10 min. A small amount of frozen MT standard strains were carefully inoculated in Geynrovich medium with inoculation ring. After three weeks of incubation at 36°C, the colonies were obtained. Single colony was inoculated again in modified Rovich medium, and pure bacteria were obtained after three weeks of continuous culture at 36°C. The colonies with good maturation conditions were selected for subculture, and the bacterial suspension with a concentration of 1,000 CFU/mL was prepared by inverted plate method and sterile saline.

Treatment methods

On the basis of reasonable diet control, oral hypoglycemic agents were given with fasting blood glucose lower than 12.0mmol/L, and insulin was given with fasting blood glucose ≥ 12.0mmol/L. Anti-tuberculosis treatment: Both groups were given isoniazid tablets 0.5g, orally, once a day. Ethambutol 0.9g, orally, once a day. Pyrazinamide tablets 1.3g, orally, three times a day. Patients in the OG were given rifampin sterilization powder 0.5g, intravenous drip, once a day. Patients in the CG were given 0.5g rifampicin capsules, orally before bedtime every day. Both groups were treated for eight months.

Experimental methods

Before the culture experiment, the prepared bacterial suspension was examined by anti-acid staining in

laboratory, and it was confirmed to be MT. No other mixed bacteria grew (Fig. 1). Four pieces of titanium alloy, co-cr-mo alloy, and ultra-high molecular weight polyethylene were randomly put into two 60mL centrifuge tubes, and 7mL Middlebrook 6H8 liquid medium was added. Then, 3mL prepared MT suspension was inoculated, and shaken to mix. After incubation at 36℃ for five weeks, one piece was randomly taken from each centrifuge tube, and 3mL of rifampicin solution with a concentration of 1.2 mg/L was added to each centrifuge tube. After three weeks of culture under the same conditions, the other two implants were removed. The interface of all material blocks was observed under scanning electron microscope. When the material block was taken out: the cap of the centrifuge tube was screwed, the cap of the centrifuge tube was opened, and carefully the implant block was clamped with pre-sterilization tweezers. Be careful to clamp the height of both sides of the implant specimen with tweezers in the process of clamping the implant block, so as to avoid contacting the effective interface of the implant block during the sampling process. In order to avoid unnecessary damage to MT colony and biofilm formed by implant interface adhesion, which can affect the accuracy of colony count and MT biofilm formation observation during the experiment. Whenever the material block was taken, the bacteria in the centrifuge tube were subjected to anti-acid staining to eliminate the contamination of the experimental group by miscellaneous bacteria.

Observation indicators

According to tuberculosis clinical treatment manual standards to evaluate the therapeutic effect: Full absorption the lesions were completely absorbed. Important absorption, after treatment, the lesion was reduced to less than 1/2. Focal absorption, part of the lesion but less absorbed 1/2 of the primary lesion. No change, no change after treatment. Deterioration, the lesions expanded or diffused after treatment. The study took absorption, significant absorption, and complete absorption as curative effect. The status of patients before and after treatment between groups was compared.

Scanning electron microscope observation

After all samples were processed, the sample was dehumidified by CO_2 critical point dryer for 20h to ensure that the sample was dry. The coating was treated by gold ion sputtering. The colony and biofilm formation of MT at the interface of four implant materials were observed by scanning electron microscopy. In the case of amplification, the visual field of 9μm × 9μm was randomly selected on the different interfaces of each implant material, and the number of colonies per unit area was carefully examined. Then, the number of colonies attached to the smooth interface and rough interface was calculated on the unit area of each implant material, and the colony adhesion point line diagram was drawn.

Statistical analysis

SPSS software was used to analyze the overall data. Measurement data and enumeration data were expressed as $\bar{x}\pm s$ and percentage (%). When $P<0.05$, the difference was statistically significant. t test or x^2 test was used for comparison between groups. Before rifampin interference, variance analysis was designed with material properties and interface types as grouping variables and the number of colonies adhered to the material per unit area as dependent variables. Paired data t test was used to compare the number of adherent colonies per unit area of implant interface before and after rifampicin intervention, and the detection level α value on both sides was set to 0.05.

RESULTS

In the OG, there were 16 females and 19 males, aged from 16 to 67 years old, with an average age of 45.3±2.3 years old. The duration of illness ranged from 7 months to 8 years. There were 14 cases of pulmonary cavity. In the CG**,** there were 15 females and 21 males, aged from 18 to 67 years old, with an average of 46.5±2.3 years old. The disease time was 9 months to 11 years, and 15 cases of pulmonary cavity. In terms of basic data, there was no significant difference between the two groups of diabetic patients with tuberculosis, and there was no statistical significance ($P>0.05$).

The results for change of lesions and comparison of adverse reactions are shown in Table I. As the table shows, the total effective rate of the OG was significantly much higher than that of the CG after 8 months of treatment ($P<0.05$). When the patients were treated, compared with the CG, the gastrointestinal response and liver function damage of the OG were significantly lower ($P<0.05$).

Before and after rifampicin treatment, no MT colonies were observed on the smooth and rough surfaces of titanium alloy and co-cr-mo alloy. After rifampicin intervention, the number of colonies on the polyethylene surface decreased (Fig. 1).

Before rifampin intervention, MT adhesion at the polyethylene material interface was observed under 400 times of viewing, showing snowflakes and clouds, and some colonies were fused into a piece. After amplification, many MT collection were showed, forming a three-dimensional complex membrane structure. At the periphery of mycobacterium tuberculosis, “membrane” mucus can be seen linking and wrapping bacteria, forming a biofilm

Table I. Change of lesions and comparison of adverse reactions between groups.

Indicator	Control group (n=35)	Observational group (n=35)	x^2	p
Change of lesions (%)				
Complete absorption	6	28		
Absorption	45	17		
Significant absorption	34	51		
No change	8.4	6.1		
Change	8.4	0		
Total effective rate	82	95	5.9	<0.05
Adverse reactions (%)				
Gastrointestinal response	26	12	7.1	<0.05
Liver function damage	29	12	9.1	<0.05
Skin rash	12	7.9	0.6	<0.05
White blood cell reducing	7	3	1	<0.05

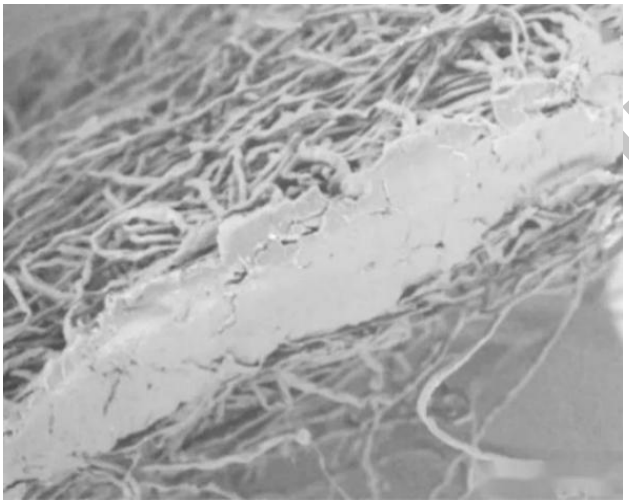

Fig. 1. The number of colonies on the polyethylene surface.

structure that had a protective effect on bacteria "under the membrane" (Fig. 2A). After the rifampin intervention, the number of adhered colonies on the ethylene material interface decreased at 400 times field of vision. After continuous amplification, some MT were cracked and the biofilm was destroyed to varying degrees. Before and after the rifampin intervention, only a few MT colonies were observed at the interface of titanium alloy materials in each field of vision. Occasionally, the adhesion of single MT was observed, and the bacteria were either dried up or cracked. No classical biofilm structure was formed (Fig. 2A, C).

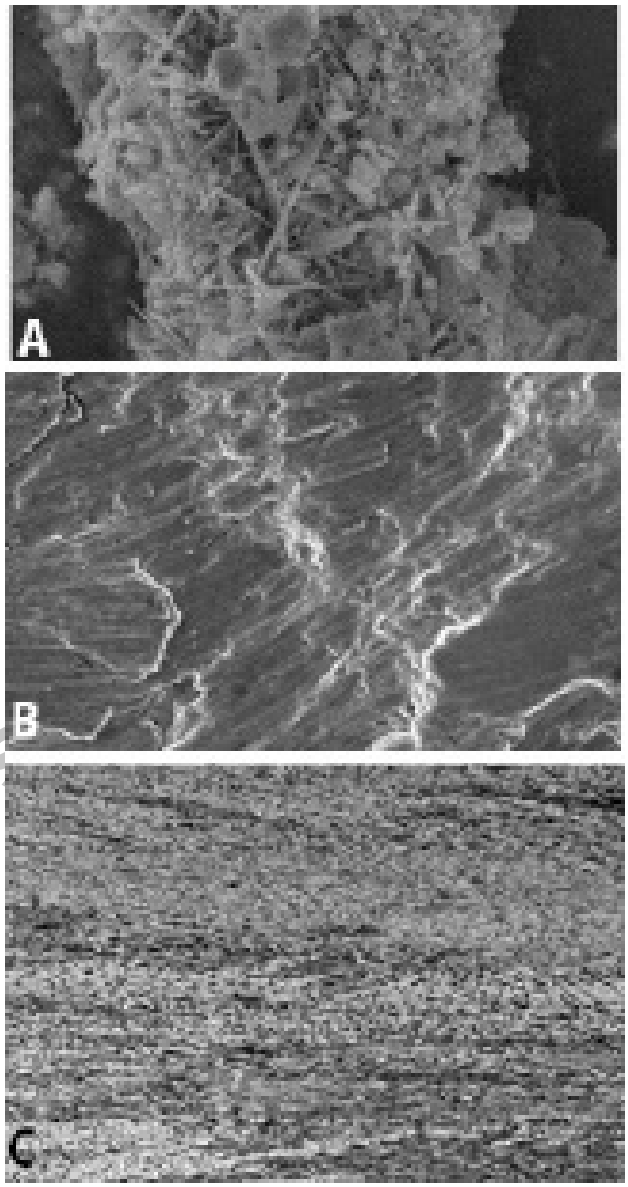

Fig. 2. Results of scanning electron microscopy. (A), polyethylene surface after rifampicin intervention; (B), Surface of titanium alloy before rifampicin intervention; (C), Surface of titanium alloy after rifampicin intervention.

Before the rifampicin intervention, the number of adhesive colonies per unit area on polyethylene surface was higher than that on co-cr-mo surface, and the difference was statistically significant ($P<0.05$). The number of adhesive colonies per unit area of co-cr-mo interface was higher than that of titanium alloy interface, and the difference was statistically significant ($P<0.05$). The adhesion ability of implant to MT varied with the roughness of implant interface, and the difference was

statistically significant ($P<0.05$). MT was more likely to adhere to the rough implant interface, and there was a first-order interaction between the implant interface state and the implant characteristics ($P<0.05$). The results showed that the interface state and properties of implants would interfere with the adhesion ability of MT to a large extent. After the rifampicin interference, the number of adherent colonies per unit area of the smooth interface of the three implants decreased slightly, and the differences were statistically significant ($P<0.05$). Explanation: Since the number of colonies adhered to the material interface after rifampicin intervention was the same, only the number of colonies adhered to the smooth interface of the material was calculated.

Table II. Comparison of colonies attached and colonies adhered.

Prosthesis samples	Colonies to different surfaces		Colonies to smooth interface of implants	
	Rough	Smooth	Before	After
Polyethylene	10	6	6±1.2	2.8±1.1
co-cr-mo	4	2	2.2±1.3	1.9±0.8
Titanium alloy	3	1.8	1.73±0.71	0.48±0.5

Before, before rifampicin intervention; After, after rifampicin intervention; co-cr-mo, cobalt chromium molybdenum.

DISCUSSION

Diabetes and tuberculosis often coexist and interact, and the clinical treatment of the two diseases should be carried out together (Tzeng *et al*., 2015). First, diabetes should be controlled. Insulin should be the first choice when blood glucose is higher than 11.1mmol/L, and oral hypoglycemic drugs should be used when blood glucose is lower than 11.1mmol/L (Sauer *et al*., 2002). In anti-tuberculosis drugs, rifampicin is the first choice (Alteri *et al*., 2007). Studies indicated that the injection of rifampicin capsules works faster and more secure, because of the injection of rifampicin into the blood to treat and high bioavailability. Absorption of oral preparation is significantly related to gastric PH, food, and gastrointestinal pathology, such as isoniazid in acidic gastric juice, which can largely affect the absorption of rifampicin (Ramsugit *et al*., 2013). Some patients may have gastrointestinal discomfort after taking rifampicin. If the condition is serious, it is necessary to stop taking rifampicin and discontinue treatment. However, no gastrointestinal changes were observed after injection of rifampicin, which did not affect the appetite of patients. According to the results of this study, it was believed that the occurrence and changes of type II diabetes were closely related to inflammation. Injection of rifampicin made tuberculosis poisoning quickly controlled, and appropriately reduced inflammatory factors, thereby alleviating hyperglycemia and regulating blood glucose. In addition, the lesion absorption efficiency in the OG was significantly higher than that in the CG, and the difference was statistically significant ($P<0.05$). Compared with oral rifampicin preparation, injection of rifampicin can significantly improve tuberculosis. In terms of adverse reactions, the gastrointestinal reaction rate and liver damage rate of the OG were lower than those of the CG, and the differences were statistically significant ($P<0.05$), indicating that rifampicin for injection was safer.

At present, there are few studies on the biofilm formation process of MT attached to the prosthesis surface (Judd and Noiseux, 2011). There are many studies on the adhesion of MT to certain specific living cells. Moreover, current studies only show that MT can form biofilm at the liquid-gas interface, and it is still controversial whether biofilm can be formed on the surface of the implant (Hosman *et al*., 2010). The surface roughness, properties, and composition of the implanted material can affect the adhesion of bacteria, thereby changing the formation of biofilm. Researchers believe that MT does not form flagella, fimbriae, and other cilia-like structures, but some studies showed that MT can form fimbriae under certain conditions. It is also concluded that the MT gene can encode and express the MT hair on the bacterial surface, which promotes the adhesion of MT and the formation of biofilm (Siddiqi *et al*., 2021). Many genes related to biofilm formation and regulation of MT were introduced at home and abroad. Each gene has its unique structure and function to inhibit or promote biofilm formation (Dunstan *et al*., 2005), but its main regulatory mechanism still needs further study. Studies revealed that polyketide synthase can catalyze the formation of a variety of lipids and plays an extremely important role in the immune regulation and virulence of mycobacterium tuberculosis. The synthesis of MT polyketide synthase protein gene is positive, which accelerates the presence of MT biofilm in some specified form (Milosev *et al*., 2005). In addition, like common bacteria, the formation of MT biofilm is also related to the density sensing system.

When the number of MT adhesion increases, in order to adapt to the surrounding environment, it can also produce and release a signal molecule of autologous inducer expanded due to the expansion of bacterial density. When the concentration of autologous inducer accumulates to the change of specific gene expression of bacteria, bacteria will regulate their common behavior through sensory signals and regulate the existence of bacterial biofilm. Studies suggested that there is a plateau

phase in the adhesion process between MT and prosthesis, and the adhesion quantity varies greatly and dynamically. This study found that the bacterial density sensing system began to play a role in regulating the formation of bacterial biofilm.

CONCLUSION

After the rifampicin interference, the number of colonies attached to the interface of titanium alloy, co-cr-mo, and polyethylene decreased significantly, and the biofilm formed at the polyethylene interface was damaged to varying degrees. It shows that the local application of anti-tuberculosis drugs has a very important impact. The limitation of this study is that the sample size is slightly small. It is of great significance to further understand the molecular mechanism and related genes of MT adhesion and biofilm formation. It is expected that in the near future, with the application of two-dimensional chip, genetic engineering, nuclear magnetic resonance, and laser technology in the field of microstructure, the genetic level and molecular level of MT biofilm will be beneficial to reduce the synthesis of related factors and inhibit the regulation of gene expression, control the adhesion of MT to cells and repair materials in vivo and the emergence of biofilms. Then, the incidence of tuberculosis infection and drug resistance are reduced, and there is an opportunity to provide accurate drug targets for the prevention and treatment of tuberculosis biofilm formation in the future.

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IRB approval

This study was approved by the Advanced Studies Research Board of Shangrao Municipal Hospital, Jiangxi Province, China.

Ethical approval

The study was carried out in compliance with guidelines issued by ethical review board committee of Shangrao Municipal Hospital, China. The official letter would be available on fair request to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

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