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The Influence of Pyrazinamide Monoresistance on Treatment Outcomes in Tuberculosis Patients from Southern China

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Abstract

This study aimed to explore the influence of pyrazinamide (PZA) monoresistance on the treatment outcome of otherwise drug susceptible tuberculosis (TB). A cohort of 194 TB patients that were infected with strains susceptible to isoniazid (INH), rifampin (RIF) and ethambutol (EMB) were included in a retrospective study at the Guangzhou Chest Hospital. We reported 148 (76.3%) PZA-susceptible TB cases and 46 (23.7%) PZA-monoresistance TB cases identified by the BACTEC MGIT 960 system. All patients were treated with the standard 6 months WHO recommended regimen, which included 2 months of INH + RIF + EMB + PZA in the intensive-phase, and the subsequent 4 months of INH + RIF during continuation-phase. Bacterial burden in the lungs was estimated using sputum smear acid-fast bacillary count while the lung lesions and cavitations were examined by X-ray at the end of first 2 months of chemotherapy. After intensive-phase treatment, there were 164 (84.5%) cases of smear-negative conversion and 151 (77.9%) cases of total or partial lesion elimination. The rates of smear-negative conversion (78.3%) and lesion elimination (39.1%) of the PZA-monoresistant patients were similar with the PZA-sensitive group (P > 0.05). However, lung cavitation was more likely to be resolved in PZA-sensitive patients than in the PZA-patients (X^2 = 9.623, P = 0.002). The smear-negative conversion rates were 95.9% for the PZA-sensitive patients and 87.0% for the PZA-monoresistant patients after 6 months of treatment (X^2 = 3.461, P =

0.063). Together, our data suggest that PZA-monoresistance contributes to the delay of resolution of the lung cavitations in the Southern China population without affecting the sputum conversion and lesion elimination rates.

Keywords

Tuberculosis, Pyrazinamide, Treatment, Monoresistance

1. Introduction

Pyrazinamide (PZA) is a unique first-line anti-TB drug that acts against the tubercle bacilli at the acidic environment (pH < 5.5) of caseous lesions caused by inflammation [1]. Though PZA has no bactericidal activity against the actively growing bacilli, it has sterilizing activity against the semi-dormant tubercle bacilli or persisters [2] [3]. Therefore, PZA is recommended by the World Health Organization (WHO) as an important component of the directly observed treatment short course (DOTS) strategy against TB. Several studies reported bactericidal effect of PZA in the first two months of treatment, which may be related to the acid environment created by active inflammatory lesions [2] [4]. Inclusion of PZA in the first two months of treatment therefore can shorten the overall length of treatment to 6 months despite the fact that the bactericidal effect was not seen when PZA is used alone [5].

Pre-existing drug resistance against the conventional first-line anti-tuberculosis drugs in standardized treatment strategy may increase the risk of acquiring resistance to the new drugs used in combinations [6]. The standardized regimen for initial TB treatment uses combination of isoniazid (INH), rifampin (RIF), PZA and ethambutol (EMB) in the intensive phase for 2 - 4 months, followed by a 4-month continuation phase treatment with INH and RIF. However, whether the PZA-monoresistance can affect the therapeutic outcomes is unclear. In this study we explored the effect of PZA-monoresistance on TB treatment outcome in otherwise drug susceptible TB patients.

2. Materials and Methods

2.1. Study Population

This was a retrospective cohort study at the Guangzhou Chest Hospital between January 2011 and December 2013. Together, 194 patients who met the selection criteria of positive pulmonary tuberculosis and were susceptible to INH, RIF and EMB were enrolled (**Table 1**). There were 124 (63.9%) male and 70 (36.1%) female patients (**Table 2**), gender ratio between male and female was 1.77:1. Patients were aged between 15 to 82 years (mean age 38.1 years), and 45 (23.2%) cases had history of TB.

2.2. Drug Treatment

Patients were treated for 6 months with WHO recommended intensive-phase regimen of INH + RIF + PZA + EMB for the first 2 months and continuation-phase regimen of INH + RIF for additional 4 months. Dosages of INH, RIF, PZA and EMB were in accordance with published guidelines [7]. RIF was administered on empty stomach. All drugs used in this study were purchased from licensed suppliers: INH from Yichang Humanwell Pharmaceutical CO., Ltd (Hubei, China), RIF from Southern China Pharmaceutical CO., Ltd (Guangdong, China), EMB from Chengdu Jinhua Pharmaceutical CO., Ltd (Sichuan, China) and PZA from Guangdong Taicheng Pharmaceutical CO., Ltd. (Guangdong, China).

2.3. Sputum Collection

Early morning sputum specimens were collected for smear testing and liquid culture detection. Baseline sputum specimens were collected from all qualified patients at the date of their clinical enrollment. Another two sputum samples were collected after completion of intensive phase treatment (1 sputum sample on the day of completion and 1 sputum sample prior to the second dose of continuation-phase treatment). Unless otherwise specified,

Table 1. Patient selection criteria.

Inclusion criteria	Exclusion criteria
1) Patients who met the criteria of TB diagnosis and treatment guidelines made by TB Branch of Chinese Medical Association [39];	1) Patients with severe liver and kidney disease history;
2) Mtb strains from them were sensitive to INH + RIF + EMB according to pre-treatment drug susceptibility testing;	2) Mental illness, gout and diabetes history;
3) Alanine aminotransferase (ALT) ≤ 3 times upper limit of normal (ULN);	3) HIV antibody positive, long-term use of immunosuppressive agents, severe malnutrition;
4) Total bilirubin ≤ 2.5 times ULN;	4) Currently enrolled in or was going to start medications of antiretroviral and/or immunosuppressive agents in next 8 weeks.
5) Creatinine ≤ 2 times ULN;	5) Discontinuation of treatment due to serious adverse effects;
6) Hemoglobin ≥ 7.0 g/dL;	6) Failure to complete the six-month treatment program;
7) Platelets ≥ 100,000/mm^3;	7) Pregnancy or breast-feeding;
8) Non-pregnant;	8) Weight < 40 kg;
9) ≤5 days of anti-tuberculosis treatment in last 6 months.	9) Central nervous system tuberculosis;
	10) Pulmonary silicosis.

sputum samples were collected on a monthly basis during the continuation-phase treatment. Sputum samples were digested and de-contaminated using conventional *N*-acetyl-L-cysteine-NaOH (NALC-NaOH) method [8] with final concentrations of 1% - 2% for NaOH and 0.25% for NALC. Sediments were used for smear and culture.

2.4. Acid-Fast Bacilli (AFB) Smear

AFB smear procedures for the sputum samples were adopted from the Chinese Anti-Tuberculosis Association [9]. Briefly, thin smears were prepared from the sputum sediments and were stained using the Nepal luxuriant staining. The smears were examined by hospital technicians under optical microscopes at ×100 oil immersion magnification. Number of AFB was estimated per 100 microscopic fields. Smear was reported negative when no AFB was detected from at least 100 microscopic fields per sample. A score was given to the AFB positive sample according to the WHO guideline [10]: sample with 10 to 99 AFB observed per 100 fields scored as 1+; 1 to 10 AFB per field in at least 50 fields was scored 2+; sample was scored 3+ when ≥10 AFB observed per field in at least 20 fields. Patients were reported as smear-negative when they maintained sputum AFB negative for 3 consecutive months.

2.5. Mycobacterial Culture and Drug Susceptibility Test Using MGIT 960 Liquid Culture System

An antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) was reconstituted with 15.0 ml MGIT growth supplement and 0.8 ml PANTA was added to each MGIT tube prior to sample inoculation. Sputum sediments were diluted 1:5 with PBS and a 0.5 ml inoculums was added to the MGIT tube. Samples were incubated in a BACTEC MGIT 960 Mycobacterium Detection System (Becton Dickinson, Sparks, MD) at 37˚C according to the manufacturer's instructions. Fluorescence was recorded automatically by a 365-nm UV transilluminator. Time of incubation for mycobacterium detection was based on the date of the earliest instrument positivity. The result was confirmed by the AFB smear positivity.

Drug susceptibility of the clinical isolates was performed as described [11] [12] prior to the initiation of drug treatment. The three first-line anti-tuberculosis drugs with final concentration of INH 0.1 μg/ml, RIF 1 μg/ml and EMB 3.5 μg/ml were prepared and added to the MGIT tubes enriched with PANTA respectively according to the manufacturer's instructions. A growth control tube was prepared without the three first-line anti-tuberculosis drugs. The PZA susceptibility test was performed using the BACTEC MGIT 960 PZA Kit (Becton Dick-

inson, Sparks, MD). Isolates were tested against PZA in 100 μg/ml and compared with a growth control at a reduced pH of 5.9. The tubes were incubated with 0.5 ml inoculums at 37˚C in the BACTEC MGIT 960 instrument. Tube inoculated with Mtb strain H37Rv (ATCC 27294) was used as a drug-susceptible (negative) control. The tubes were monitored between the 4th to the 21st day post-incubation. On the day the growth control tube showed positive growth, the sputum from the drug-containing samples were compared to their growth controls and to the Mtb H37Rv. Data of individual anti-tuberculosis drug tested was interpreted and reported automatically by the instrument. Susceptibility profile was determined when the growth unit (GU) of the growth control reached 400, a drug-treated sample with GU ≥ 100 was defined as drug-resistant (R) and sample with GU ≤ 100 was considered susceptible (S). Susceptibility to EMB and PZA was cross-compared with Mtb H37Rv. The patients were categorized into PZA-sensitive and PZA-monoresistant groups with respect to their PZA susceptibility profile before and after treatment.

2.6. Lung Lesion and Cavity Examination

Chest X-ray scanning and computed tomography (CT) images were reviewed by two attending physicians before and after 2-month intensive-phase treatment. Conditions and sizes of the lung lesions and cavities after drug treatment were compared with that of pre-treatment phase.

2.7. Statistics

Data from the study was expressed as percentages and the chi-square (X^2) test was used to compare the rates between PZA-susceptible and PZA-resistant groups. If the theoretical frequency was <5, the continuity correction was used while a P value less than 0.05 was considered statistically significant. Analysis was carried out using SPSS (version 13.0; SPSS; Chicago, IL) statistical software.

2.8. Ethics Approval

This study was approved by the Ethics Committee of Guangzhou Chest Hospital. Written informed consent was given by all participants. Patient information was collected and recorded by attending physicians.

3. Results

Here we report 148 PZA-susceptible TB cases comprising of 100 (67.6%) males and 48 females (gender ratio = 1.48 male:1 female). Mean age was 42.1 years (ranged 15 to 76 years). For the PZA-monoresistance TB group, 24 (52.2%) patients were males and 22 were females (gender ratio = 1.09 male:1 female), mean age was 36.1 years (ranged 16 to 77 years, **Table 2**).

Table 2 shows before the start of drug treatment, there were 69 (35.6%) cases of low sputum smear AFB count (+1), 61 (31.4%) cases of AFB score 2+ and 64 (33.0%) cases were registered as high AFB count (≥3+). About half of the enrolled patients had 5 - 6 pulmonary lesions (98 cases of 194, 50.5%), and 163 of 194 patients (84.0%) had cavity lesions (**Table 2**).

After 2 months of intensive treatment with INH + RIF + PZA + EMB, 164 of 194 (84.5%) patients exhibited sputum smear negative conversion while 30 (15.5%) cases remained non-conversion. Ninety-six of 194 cases (49.5%) had total lesion elimination, 55 of 194 cases (28.4%) showed partial lesion elimination and 43 of 194 (22.1%) failed to resolve lung lesions after treatment (**Table 3**). Overall, 77.9% (151 of 194) of TB patients had total or partial lesion elimination. 78.4% (126 of 148 cases) PZA-sensitive patients had lesion elimination and 75.1% (35 of 46) of the PZA-monoresistance patients had lesion elimination (X^2 = 0.297, P = 0.537) (**Table 3**). The size of cavity lesions shrunk in 113 of 163 (69.3%) patients who had lung cavitations at the time of enrollment. It is of interest to note that 75.4% (95 of 126 cases) PZA-sensitive patients had lung cavitations resolution after 2-month intensive treatment. In contrast, 48.6% (18 of 37) of the PZA-monoresistance patients recovered from lung cavitations (X^2 = 9.623, P = 0.002) (**Table 3**).

On completion of the continuation-phase treatment with INH and RIF, a robust 93.8% (182 of 194 cases) of TB patients reported sputum smear negative conversion while 12 cases (6.2%) did not convert. The conversion rate in PZA-susceptible patients was 95.9% (142 of 148) while 87.0% (40 of 46 cases) of PZA-monoresistant patients had sputum smear negative conversion. Overall, there was no clear difference found between PZA-sensitive and PZA-monoresistant groups in terms of sputum smear negative conversion rate (X^2 = 3.461, P = 0.063).

Table 2. Summary of patient characteristics before treatment.

Characteristic		PZA-sensitive cases (%)	PZA-resistant cases (%)	x^2	P value
Gender	Male	100 (67.6)	24 (52.2)	3.606	0.067
	Female	48 (32.4)	22 (47.8)		
Medical history	Initial treatment[a]	113 (76.4)	36 (78.3)	0.072	0.978
	Retreatment[b]	35 (23.6)	10 (21.7)		
Age (years)	≤25	44 (29.7)	17 (37.0)	3.892	0.273
	25 - 44	53 (35.8)	20 (43.5)		
	45 - 64	35 (23.7)	6 (13.0)		
	≥65	16 (10.8)	3 (6.5)		
Sputum smear	1+	52 (35.1)	17 (37.0)	0.665	0.721
	2+	45 (30.4)	16 (34.8)		
	≥3+	51 (34.5)	13 (28.2)		
Lesions of infection	1 - 2	28 (18.9)	9 (19.6)	3.907	0.142
	3 - 4	40 (27.0)	19 (41.3)		
	5 - 6	80 (54.1)	18 (39.1)		
	Cavitary lesions	126 (85.1)	37 (80.4)	0.577	0.447

[a]A patient was classified as initial-treatment when he or she had no previous anti-tuberculosis treatment; [b]A patient was classified as re-treatment when 1) A TB patient who had completed treatment and was declared cured by a physician in the past, but who reported back to the health facility and found to be sputum smear-positive; 2) A smear-positive TB patient after regular 6 months preliminary treatment and also initial smear negative patient who turned positive again; 3) TB patients who received anti-tuberculosis treatment for one month or more from any source and returns to treatment after having defaulted, *i.e.*, not taken anti-TB drugs consecutively for two months or more, and were found to be sputum smear-positive; 4) Chronic Mtb excreter.

Table 3. Clinical outcomes after 2 months intensive-phase treatment.

Characteristic		PZA-sensitive cases (%)	PZA-resistant cases (%)	x^2	P value
Sputum smear[*]	Conversion	128 (86.5)	36 (78.3)	1.816	0.178
	Non-conversion	20 (13.5)	10 (21.7)		
Lesion elimination[#]	Total lesion elimination	75 (50.7)	21 (45.7)	0.279	0.597
	Partial elimination	41 (27.7)	14 (30.4)		
	Non-elimination	32 (21.6)	11 (23.9)		
	Shrunk cavities	95 (75.4)	18 (48.7)	9.623	0.002
	Non-shrunk cavities	31 (24.6)	19 (51.3)		

[*]On completion of 2 months treatment, if patients had negative results in both sputum AFB smear test and liquid culture, they were considered as sputum negative conversion. Patients with negative sputum AFB smear but were positive for liquid culture after 2 months treatment were considered as sputum negative non-conversion; [#]The terms in lesion and cavity conditions were defined as follows: non-elimination, the lesions did not change obviously; partial elimination, less than one-third of lesions had decreased than in the pre-treatment phase; total lesion elimination, compared with pre-treatment, more than one-third of lesions had decreased; non-shrunk cavities, the sizes of cavities became larger or unchanged compared with pre-treatment; shrunk cavities, cavities decreased in size compared with pre-treatment.

4. Discussion

In this retrospective cohort study evaluating the impact of PZA-monoresistance on treatment outcomes, no significant differences were found in terms of rates of 2- and 6-month sputum smear conversion, while reduced cavity size was more pronounced in PZA-susceptible patients compared to PZA-monoresistance patients.

The rate of smear conversion after two months of treatment is an important indicator for TB cure [13]. Singla *et al.* (2003), showed that a smear conversion rate of at least 75% is a good indicator for non-resistant smear positive TB [14]. In addition, non-conversion of positive smears after the first two months of treatment is one of the reliable predictors for treatment failure [15]. Several factors may delay the time to smear conversion, including lung cavitations, extensive lesions, grade of sputum smear AFB prior to treatment and age. Some studies reported that drug resistance is strongly associated with late (>8 weeks) culture conversion as the failure of TB treatment [16] [17].

PZA is an important first-line drug used in the first two months of treatment to reduce the duration of treatment required [18]. A multicenter clinical study showed that combination of PZA with INH and streptomycin (STR) could reduce TB relapse rate from 29% to 8% after 6 months of treatment [4]. Though it was also suggested that PZA plays no role in combination with RIF and INH to treat *Mycobacterium bovis*, a strain naturally resist to PZA [19].

It has been reported that pre-existing RIF resistance will lower the curative rate and increase the rate of relapse after the 6-month treatment regimen, especially when RIF was given alone during the initial 2-month intensive phase of treatment [20]. However, the pre-existing resistance of INH or STR had little effect on the sterilizing activity of treatment regimen that included RIF or PZA as partner drug, possibly due to the more potent sterilizing activity of PZA [21]. The actual effect of PZA resistance on the treatment outcome was not well studied [22].

This study reports a smear conversion rate of 78.3% (36 of 46 cases) for the PZA-monoresistant patients, while that of PZA-sensitive patients was slightly higher (128/148 cases, 86.5%) after the 2 months intensive-phase treatment. No significant difference ($P > 0.05$) was observed between the PZA-monoresistant and PZA-sensitive patients in total and partial lesion elimination rates after the intensive phase (**Table 3**). This is consistent with our previous study [23] and a recent report in San Francisco, California [24]. Liang *et al.* reported that the multiple drug resistance, but not the single drug-resistance, could alter the sputum conversion after 2 months treatment [25]. As PZA has mainly bacteriostatic activity even when used alone at high dose in mouse [26], the pre-exist PZA-resistance may not affect the culture conversion rate in patients when multiple first-line drugs were used in combination for the first 2-months of treatment [27]. The synergistic effect of the other partner drugs in treatment regimen can compensate the lost of PZA function, though RIF and PZA can contribute to stronger synergy of bactericidal activities for the first two months of treatment [27] [28]. Manca *et al.* reported that PZA could down-regulate the release of pro-inflammatory cytokines and chemokines, including IL-1β, IL-6, TNF and MCP-1. Since the inflammatory cytokines and chemokines can drive the manifestations of cavitations and lung pathology [29], immunosuppression by PZA may be a promising adjunctive treatment to prevent lung pathology or cavitation despite PZA-resistance in Mtb is shown *in vitro* [30].

Cavity formation facilitates bacterial killing by promoting phagosome acidification, production of reactive oxygen and nitrogen species, hydrolytic enzymes, and cationic antimicrobial peptides [31] [32]. After two months of WHO recommended intensive treatment with INH + RIF + PZA + EMB, we found that the proportion of patients with cavitations resolution was significantly lower ($P = 0.002$) in the PZA-monoresistant group (48.7%, 18 of 37 cases) than that of the PZA-susceptible patients (75.4%, 95 of 126 cases). We speculate that the acidification and hypoxic micro-environment in the caseous necrosis and lesions [25] of the PZA-sensitive patients enhances the sterilizing activity of PZA [33] [34]. Therefore it leads to better healing of cavity lesions in the PZA-susceptible group compare to that in the PZA-resistant group.

These findings reveal a slightly higher, but not statistically significant ($P = 0.178$), smear conversion rate in the PZA-sensitive patients (86.5%, 128 of 148 cases) than the PZA-resistant patients (78.3%, 36 of 46 cases) (**Table 3**). Yee *et al.* reported a significantly worse clinical outcome in patients with PZA-monoresistance TB than patients with fully susceptible strains from a study of 218 patients in Canada [35]. The main limitation of this study was the inability to follow the patients for long-term relapses. However, our findings are consistent with previous results that the cavity improvement for PZA-sensitive patients is better than the resistant patients. This may partially explain the differential conclusion of this study that based on the cavity improvement and

smear conversion rate after two months of intensive treatments whereas the other study measured bacteria relapse in long term [36]. It is also in agreement with another report that showed surprisingly high relapse rates in patients with either positive culture after two months of treatment or had cavities prior to treatment [36]. Accurate PZA susceptibility testing is very difficult that some strains may be falsely classified as PZA-resistant by the phenotypic test. However, our recent study indicated that the PZA susceptibility testing using MGIT 960 system was relatively reliable as all the clinical strains with *pncA* mutation were phenotypic PZA-resistant [37]. This is also consistent with the finding that *pncA* encodes the enzyme that activates the pro-drug PZA into its active form pyrazinoic acid for action [38]. Sequencing the *pncA* and *rpsA* genes from the Mtb isolates may help to identify PZA-resistant strain since mutations in these genes could be related to PZA resistance. However, the mechanism of PZA resistance still need to be studied further as mutations in these 2 genes may not be responsible for PZA resistance and many clinical PZA-resistant isolates do not have known mutations in *pncA* and *rpsA* [22].

5. Conclusion

In summary, PZA is an important drug for TB treatment. Although there was no significant difference in smear negative conversion rates between the PZA-monoresistant and PZA-sensitive patients after two months intensive treatment, PZA-sensitive patients achieved better recovery of lung cavitation. Without a reliable PZA susceptibility testing and a definite correlation between the susceptibility of PZA tested *in vitro* and that *in vivo*, we suggest that the doctors should use PZA in patients regardless of the current PZA susceptibility testing results. However, the possibility of Mtb relapse and spreading depends on lung cavities; the delay of cavitations resolution also poses a risk of prolonging Mtb transmission. With technological advances and growing knowledge of the mechanism underlying PZA-resistance, our data suggest that personalized combinations of drugs, other than standard regimen, should be tailored to individual patients in the future with consideration of their initial PZA-susceptibility.

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Conflict of Interest

The authors have no financial conflicts of interests.

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