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journal homepage: <http://ees.elsevier.com/apjtm>Original research <http://dx.doi.org/10.1016/j.apjtm.2016.01.031>Immune formulation-assisted conventional therapy on anti-infective effectiveness of multidrug-resistant *Mycobacterium tuberculosis* infection miceXiu-Li Yuan¹, Qiang Wen¹, Ming-De Ni¹, Li-Kun Wang^{2*}¹Internal Medicine Department No. 3, Eastern Medical District of Linyi People's Hospital, Linyi City, Shandong Province, 276034, People's Republic of China²Infection Department, Eastern Medical District of Linyi People's Hospital, Linyi City, Shandong Province, 276034, People's Republic of China

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ABSTRACT

Objective: To study the effect of immune formulation-assisted conventional therapy on anti-infective ability of multidrug-resistant *Mycobacterium tuberculosis* infection mice.**Methods:** BALB/c mice were used as experimental animals, multidrug-resistant *M. tuberculosis* infection models were built, randomly divided into model group, moxifloxacin group, thymopentin group and combined treatment group and given corresponding drug intervention, and then colony numbers in the spleen and lung, T lymphocyte subset contents and programmed death-1 (PD-1) expression levels in peripheral blood were detected.**Results:** Colony numbers in lung and spleen of moxifloxacin group and thymopentin group were significantly lower than those of model group and colony numbers in lung and spleen of combined treatment group were significantly lower than those of moxifloxacin group and thymopentin group; contents of CD3⁺CD4⁺T cells, Th1 and Th17 in peripheral blood of moxifloxacin group and thymopentin group were higher than those of model group, and contents of CD3⁺CD8⁺T cells, Th2 and Treg were lower than those of model group; contents of CD3⁺CD4⁺T cells, Th1 and Th17 in peripheral blood of combined treatment group were higher than those of moxifloxacin group and thymopentin group, and contents of CD3⁺CD8⁺T cells, Th2 and Treg were lower than those of moxifloxacin group and thymopentin group; PD-1 expression levels on T lymphocyte, B lymphocyte and monocyte surface in peripheral blood of moxifloxacin group and thymopentin group were lower than those of model group, and PD-1 expression levels on T lymphocyte, B lymphocyte and monocyte surface in peripheral blood of combined treatment group were lower than those of moxifloxacin group and thymopentin group.**Conclusions:** Immune formulation thymopentin can enhance the anti-infective ability of multidrug-resistant *M. tuberculosis* infection mice, decrease bacterial load in lung and spleen, and enhance immune function.

1. Introduction

Tuberculosis is a respiratory infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), and affected by bacterial mutation, wide use of chemotherapy drugs and other

factors, the incidence of multidrug-resistant tuberculosis caused by multidrug-resistant *M. tuberculosis* infection is rising [1,2]. The killing effect of conventional chemotherapy drugs on multidrug-resistant *M. tuberculosis* is not ideal, and second-line anti-tubercular drugs have longer course of treatment, more adverse reactions and lower cure rate [3–5]. In recent years, more and more studies have realized that weakened immune function is associated with multidrug-resistant tuberculosis infection, and targeted immune formulation adjuvant therapy has become an important part of anti-tuberculosis comprehensive treatment [6]. Thymopentin is a drug enhancing immune activity and clinical common immune formulation [7]. In the following

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research, the effect of immune formulation-assisted conventional therapy on anti-infective ability of multidrug-resistant *M. tuberculosis* infection mice was analyzed.

2. Materials and methods

2.1. Experimental materials

Experimental animals were 48 SPF level BALB/c mice weighted (18–22) g, were randomly divided into model group, moxifloxacin group, thymopentin group and combined treatment group, each group with 12 cases. Thymopentin was from Beijing Double-Crane Pharmaceuticals Co., Ltd., moxifloxacin was from Bayer Healthcare Co., Ltd., and fluorescent antibodies were from Santa Cruz Company.

2.2. Model establishment and drug intervention methods

7H9 liquid medium containing 1×10^6 /mL multidrug-resistant *M. tuberculosis* was prepared, and multidrug-resistant *M. tuberculosis* infection models of aerosol infection mice were built and given drug intervention from the 21 d after infection. Moxifloxacin group received intragastric administration of 100 mg/kg moxifloxacin, thymopentin group received subcutaneous infection of 1 mg/kg thymopentin, combined treatment group received intragastric administration of 100 mg/kg moxifloxacin and subcutaneous infection of 1 mg/kg thymopentin, and model group received subcutaneous infection and intragastric administration of same doses of saline.

2.3. Detection of colony numbers in visceral organs

Four weeks, eight weeks and sixteen weeks after treatment, mice were killed and anatomized under sterile conditions to get the lung and spleen, appropriate amount of tissue was cut off, homogenized, diluted, then inoculated in 7H11 medium and continuously cultured for 4 weeks, and then colony forming unit was counted.

2.4. Detection of T lymphocyte subset contents in peripheral blood

Sixteen weeks after treatment, mice were taken and killed by decapitation to collect peripheral blood, fluorescent antibodies of CD3, CD4 and CD8 as well as IFN- γ , IL-4, Th17 and CD25 were incubated respectively away from light, hemolysin was added for 15 min of hemolysis, then DPBS 1 000 μ L was added to re-suspend cells, contents of different T cell subsets were detected in flow cytometer, and at the time of detection, excitation light was argon ion laser 488 nm.

Table 1

Comparison of colony numbers in lung and spleen (lg colony forming unit).

	n	4 weeks		8 weeks		16 weeks	
		Lung	Spleen	Lung	Spleen	Lung	Spleen
Model group	12	6.22 \pm 0.62	4.35 \pm 0.49	6.77 \pm 0.82	5.19 \pm 0.62	7.67 \pm 0.83	5.76 \pm 0.59
Moxifloxacin group	12	4.94 \pm 0.53 ^a	3.35 \pm 0.35 ^a	5.32 \pm 0.62 ^a	3.87 \pm 0.46 ^a	5.91 \pm 0.61 ^a	4.47 \pm 0.65 ^a
Thymopentin group	12	5.12 \pm 0.59 ^a	3.29 \pm 0.31 ^a	5.77 \pm 0.49 ^a	4.01 \pm 0.48 ^a	5.24 \pm 0.68 ^a	4.69 \pm 0.50 ^a
Combined treatment group	12	3.15 \pm 0.39 ^{abc}	2.21 \pm 0.27 ^{abc}	3.47 \pm 0.42 ^{abc}	2.85 \pm 0.25 ^{abc}	3.08 \pm 0.19 ^{abc}	2.32 \pm 0.28 ^{abc}

^a: compared with model group, there were differences, $P < 0.05$; ^b: compared with moxifloxacin group, there were differences, $P < 0.05$; ^c: compared with thymopentin group, there were differences, $P < 0.05$.

2.5. Detection of PD-1 expression in lymphocytes in peripheral blood

Sixteen weeks after treatment, mice were taken and killed by decapitation to collect peripheral blood, fluorescent antibodies of CD3, CD19 and CD14 as well as programmed death-1 (PD-1) were incubated away from light respectively, hemolysin was added for 15 min of hemolysis, then DPBS 1 000 μ L was added to re-suspend cells, contents of different T cell subsets were detected in flow cytometer, and at the time of detection, excitation light was argon ion laser 488 nm.

2.6. Statistical process methods

SPSS19.0 software was used to input and process data, comparison among groups was by variance analysis, pair wise comparison was by LSD-*t* method, and $P < 0.05$ was the standard of statistical significance in differences.

3. Results

3.1. Colony numbers in lung and spleen

Four weeks, eight weeks and sixteen weeks after treatment, analysis of colony numbers in lung and spleen was as follows: (1) variance analysis showed that colony numbers in lung and spleen of four groups were different. (2) Pair wise comparison showed that colony numbers in lung and spleen of moxifloxacin group and thymopentin group were significantly lower than those of model group and colony numbers in lung and spleen of combined treatment group were significantly lower than those of moxifloxacin group and thymopentin group (Table 1).

3.2. Contents of T lymphocyte subsets in peripheral blood

Contents of CD3⁺CD4⁺T cells in peripheral blood of moxifloxacin group and thymopentin group were higher than that of

Table 2

Comparison of T lymphocyte subset contents in peripheral blood.

	n	CD3 ⁺ CD4 ⁺ T cells	CD3 ⁺ CD8 ⁺ T cells
Model group	12	37.35 \pm 4.25	39.22 \pm 5.19
Moxifloxacin group	12	41.18 \pm 5.29 ^a	33.61 \pm 3.88 ^a
Thymopentin group	12	46.84 \pm 5.51 ^a	30.17 \pm 3.22 ^a
Combined treatment group	12	57.31 \pm 6.23 ^{abc}	21.36 \pm 3.21 ^{abc}

^a: compared with model group, there were differences, $P < 0.05$;

^b: compared with moxifloxacin group, there were differences, $P < 0.05$;

^c: compared with thymopentin group, there were differences, $P < 0.05$.

Table 3Comparison of the contents of different CD4⁺T lymphocyte subsets in peripheral blood.

	<i>n</i>	Th1	Th2	Th17	Treg
Model group	12	5.82 ± 0.76	6.12 ± 0.87	0.87 ± 0.10	7.23 ± 0.88
Moxifloxacin group	12	7.25 ± 0.83 ^a	5.08 ± 0.38 ^a	1.06 ± 0.10 ^a	5.78 ± 0.84 ^a
Thymopentin group	12	7.71 ± 0.95 ^a	4.77 ± 0.49 ^a	1.14 ± 0.18 ^a	5.12 ± 0.73 ^a
Combined treatment group	12	9.92 ± 1.05 ^{abc}	2.89 ± 0.23 ^{abc}	1.59 ± 0.19 ^{abc}	3.29 ± 0.41 ^{abc}

^a: compared with model group, there were differences, $P < 0.05$; ^b: compared with moxifloxacin group, there were differences, $P < 0.05$; ^c: compared with thymopentin group, there were differences, $P < 0.05$.

Table 4

PD-1 expression levels in lymphocytes and monocytes in peripheral blood.

	<i>n</i>	PD-1 expression in T lymphocytes	PD-1 expression in B lymphocytes	PD-1 expression in monocytes
Model group	12	54.66 ± 6.24	44.44 ± 5.78	33.53 ± 4.23
Moxifloxacin group	12	41.37 ± 5.28 ^a	33.26 ± 4.12 ^a	26.78 ± 2.95 ^a
Thymopentin group	12	39.56 ± 5.47 ^a	30.91 ± 3.79 ^a	25.38 ± 3.17 ^a
Combined treatment group	12	22.78 ± 3.92 ^{abc}	19.32 ± 2.68 ^{abc}	15.30 ± 1.72 ^{abc}

^a: compared with model group, there were differences, $P < 0.05$; ^b: compared with moxifloxacin group, there were differences, $P < 0.05$; ^c: compared with thymopentin group, there were differences, $P < 0.05$.

model group, and contents of CD3⁺CD8⁺T cells were lower than that of model group; content of CD3⁺CD4⁺T cells in peripheral blood of combined treatment group was higher than those of moxifloxacin group and thymopentin group, and content of CD3⁺CD8⁺T cells was lower than those of moxifloxacin group and thymopentin group (Table 2).

3.3. Contents of different CD4⁺T lymphocyte subsets in peripheral blood

Contents of Th1 and Th17 cells in peripheral blood of moxifloxacin group and thymopentin group were higher than those of model group, and contents of Th2 and Treg cells were lower than those of model group; contents of Th1 and Th17 cells in peripheral blood of combined treatment group were higher than those of moxifloxacin group and thymopentin group, and contents of Th2 and Treg cells were lower than those of moxifloxacin group and thymopentin group (Table 3).

3.4. PD-1 expression levels in lymphocytes and monocytes in peripheral blood

PD-1 expression levels on T lymphocyte, B lymphocyte and monocyte surface in peripheral blood of moxifloxacin group and thymopentin group were lower than those of model group, and PD-1 expression levels on T lymphocyte, B lymphocyte and monocyte surface in peripheral blood of combined treatment group were lower than those of moxifloxacin group and thymopentin group (Table 4).

4. Discussion

Multidrug-resistant tuberculosis caused by multidrug-resistant *M. tuberculosis* infection is the difficulty and emphasis of clinical treatment. The main mechanism of *M. tuberculosis* resistance is inhibiting body's cellular immune response [8]. Related study confirms that normal *M. tuberculosis* H₃₇Rv strain doesn't have inhibitory effect on the activation and maturation of Th1 and Th2 cells, and after bacterial infection,

the body can induce Th1 type immune response through autoimmune response and kill *M. tuberculosis* [9]; multidrug-resistant *M. tuberculosis* strains can inhibit the differentiation and maturation of CD4⁺T lymphocytes, inhibit the maturation of Th1 cells, promote the maturation of Th2 cells, and reduce the generation of cytokines such as IL-2, TNF- α and IFN- γ as well as the killing effect of above cytokines on *M. tuberculosis*, and it is difficult for the body to kill *M. tuberculosis* through autoimmune mechanism [10,11]. Multidrug-resistant tuberculosis is mostly insensitive to first-line chemotherapy drugs, second-line chemotherapy drugs are with higher price, more adverse reactions as well as longer drug administration time and course of treatment, and the cure rate is not ideal [12,13].

Broad-spectrum antibacterial drug moxifloxacin is the essential drug of clinical treatment of multidrug-resistant tuberculosis at present [14]. The drug has high affinity to quinolone resistance-determining area of DNA helicase A subunit in multidrug-resistant strains, and it can infect bacteria DNA replication and damage the structure of the bacteria [15,16]. However, the curative effect of moxifloxacin alone is not very ideal. Based on the inhibitory effect of multidrug-resistant *M. tuberculosis* on immune function, more and more scholars advocate use of immune formulation to treat multidrug-resistant tuberculosis [17]. Thymopentin is a kind of bioactive peptide extracted from newborn calf thymus tissue, and it has the function of stimulating T lymphocyte differentiation, maturation and proliferation as well as releasing a variety of cytokines. Related clinical studies prove that thymopentin has promoting effect on cellular immune function, which is specifically manifested as regulating the contents and function of T lymphocyte subsets [18,19].

In the research, based on routine moxifloxacin anti-infection treatment, immune formulation thymopentin was used for adjuvant therapy, aiming to exert the regulating effect of thymopentin on immune function. In order to clarify the effect of immune formulation-assisted conventional therapy on anti-infective ability of multidrug-resistant *M. tuberculosis* infection mice, colony numbers in lung and spleen of different treatment groups were compared. After *M. tuberculosis*

infection, the use of antibacterial drugs and body's autoimmune mechanism can kill pathogenic bacteria or inhibit the growth of pathogenic bacteria, and reduce the number of colonies in the visceral organs. Analysis of colony numbers in lung and spleen showed that colony numbers in lung and spleen of moxifloxacin group and thymopentin group were significantly lower than those of model group and colony numbers in lung and spleen of combined treatment group were significantly lower than those of moxifloxacin group and thymopentin group. This indicated that both moxifloxacin and thymopentin had inhibitory effect on the growth of multidrug-resistant *M. tuberculosis*, and combined use of the two drugs had synergistic effect and enhanced the anti-infective ability of multidrug-resistant *M. tuberculosis* infection mice together.

Cellular immunity is the main immune mechanism of the body to kill *M. tuberculosis*, and T lymphocytes are the main cells to execute cellular immune response [20]. T lymphocyte maturation process experiences positive and negative selection, two types of mature T cell that are generated include CD3⁺CD4⁺T cell and CD3⁺CD8⁺T cell, the former is an important helper cell and the latter is an important suppressor cell [21,22]. In the body's anti-tuberculosis immune response, CD3⁺CD4⁺T cell content and CD4⁺/CD8⁺ ratio increase, and CD3⁺CD8⁺T cell content decreases. In the research, the proportions of T lymphocyte subsets in peripheral blood were compared and analyzed after thymopentin treatment, and results showed that the contents of CD3⁺CD4⁺T cells in peripheral blood of moxifloxacin group and thymopentin group were higher than that of model group, and contents of CD3⁺CD8⁺T cells were lower than that of model group; content of CD3⁺CD4⁺T cells in peripheral blood of combined treatment group was higher than those of moxifloxacin group and thymopentin group, and content of CD3⁺CD8⁺T cells was lower than those of moxifloxacin group and thymopentin group. It indicated that after moxifloxacin and thymopentin monotherapy, the immune function of multidrug-resistant *M. tuberculosis* infection mice was enhanced, and the effect of combined use of the two drugs on enhancing cellular immune function of mice was more significant.

In the process of exerting immune function, CD4⁺CD8⁻T lymphocytes can be activated into different subsets that exert different functions, specifically including Th1, Th2, Th17 and Treg cells [23]. Th1 and Th2 is a pair of cells earliest discovered in CD4⁺T cell subsets, and the former mainly secretes IL-2 and IFN- γ , and can induce cellular immune response and kill pathogenic microorganism; the latter mainly secretes IL-4 and IL-10, and can induce B cells to generate antibodies and inhibit the killing effect of macrophages on pathogenic microorganism to a certain extent [24,25]. Th17 and Treg are newly discovered members of CD4⁺T cell subsets, Th17 can secrete IL-17 and exert pathogen-killing effect similar to that of cytokines such as IL-2 and IFN- γ ; Treg can inhibit the activation of Th17 and the generation of IL-17, and it has immunosuppressive effect [26,27]. In the research, further analysis of CD4⁺CD8⁻T lymphocyte subset contents in peripheral blood of four groups showed that both moxifloxacin and thymopentin monotherapy could increase Th1 and Th17 contents and decrease Th2 and Treg contents, and the modulating effect of combined use of the two drugs on cellular immune function was more significant.

Studies about the body's anti-tuberculosis immune response in recent years believe that PD-1 and its ligand PD-L1, as negative costimulatory molecules, are involved in the regulation of body's immune response and have negative regulatory effect

on lymphocyte activation. There is PD-1 expression on T lymphocyte, B lymphocyte and monocyte surface, PD-1 and its ligand PD-L1 can input suppressor signal through immunoreceptor tyrosine-based inhibitory motif of cytoplasm end sequence, and finally realize the inhibitory effect on immune response. Multidrug-resistant *M. tuberculosis* infection can activate PD-1 and inhibit body's immune response. In the research, analysis of PD-1 expression levels on T lymphocyte, B lymphocyte and monocyte surface showed that PD-1 expression levels on T lymphocyte, B lymphocyte and monocyte surface in peripheral blood of moxifloxacin group and thymopentin group were lower than those of model group, and PD-1 expression levels on T lymphocyte, B lymphocyte and monocyte surface in peripheral blood of combined treatment group were lower than those of moxifloxacin group and thymopentin group.

Based on above discussion, it can be concluded that immune formulation thymopentin can enhance anti-infective ability of multidrug-resistant *M. tuberculosis* infection mice, decrease bacterial load in lung and spleen, and enhance immune function.

Conflict of interest statement

We declare that we have no conflict of interest.

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