BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902019000418547

Article

Influence of isoniazid on T lymphocytes, cytokines, and macrophages in rats

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T lymphocytes, cytokines, and macrophages play important roles in the clearance of *Mycobacterium tuberculosis* (Mtb) by the immune system. This study aimed to investigate the effects of isoniazid on the functions of both innate and adaptive immune cells. Healthy rats were randomly divided into experimental and control groups. Each group was randomly divided into three subgroups and named according to the duration of drug feeding, 1, 3, and 3 months followed by drug withdrawal for 1 month. The experimental groups were fed with isoniazid (12 mg/mL) and the control groups with normal saline. The percentage of $CD4^+$ and $CD8^+$T lymphocytes, level of interleukin (IL)-12 and interferon (IFN)-γ, and function of macrophages were determined at these three time points. Isoniazid significantly increased the percentage of $CD4^+$T lymphocytes and the $CD4^+/CD8^+$T lymphocyte cell ratio ($P < 0.05$). It transiently (<1 month) enhanced the functions of rat macrophages significantly ($P < 0.05$). In summary, isoniazid could increase the percentage of $CD4^+$T lymphocytes, $CD4^+/CD8^+$T lymphocyte cell ratio, and enhance macrophage function in healthy rats.

Keywords: Cytokines. Immune. Isoniazid. Macrophages. T lymphocytes.

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by Mycobacterium tuberculosis (Mtb) bacteria which may damage multiple organ systems and poses a serious harm to human health. According to the World Health Organization's report on the global tuberculosis epidemic, about one-third of the world's population was infected with Mtb in 2015, 10.4 million new TB patients were diagnosed, and more than 1.4 million patients died of the disease (Sisay *et al*., 2016). China has a high incidence of TB, ranking second in the world. At present, due to the increase in human immunodeficiency virus/acquired immunodeficiency syndrome, the use of immunosuppressive and cytotoxic drugs, and the incidence of drug-resistant TB, the severity of TB cases has increased (Das, Satapathy, Murmu, 2016; Ebonyi *et al*., 2016; Lima *et al*., 2017). Therefore, TB has become a serious public health problem in China.

T lymphocytes, cytokines, and macrophages play important roles in the clearance of Mtb-related antigens. Insufficient number or dysfunction of T lymphocytes and macrophages, and the lack of related cytokines may lead to decreased immunity to Mtb. Therefore, immune status is closely related to the occurrence, development, and therapeutic response to TB (Grover *et al*., 2014; Mosquera-Restrepo *et al*., 2017). In recent years, it has been found that some antibacterial drugs not only kill pathogens but also regulate immune function during treatment (Hamilton-Miller, 2001; Schultz, Speelman, van der Poll, 2001; Tauber, Nau, 2008). Some β-lactam antibiotics, such as cefodizime, can improve immune function (Wang *et al*., 2015). Although anti-tuberculosis drugs have been used for many years, studies on

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their immune regulation are lacking. Therefore, it is important to investigate the effects of anti-tuberculosis drugs on immune function, especially those related to the clearance of Mtb.

Isoniazid is one of the most important first-line drugs for treating TB. It has strong antibacterial effects; thus, it is used throughout the period of reinforcement and consolidation. The side effects of isoniazid, such as liver damage, may be related to the high level of immune response (Usui *et al*., 2017; Vinnard *et al*., 2017). At present, it is not clear whether isoniazid has any effect on immune functions related to the clearance of Mtb. Therefore, we explored the effects of isoniazid on the immune functions in healthy rats, and found that isoniazid can serve as a potential immune booster.

MATERIAL AND METHODS

All the experimental animals and procedures were approved by the animal experiment ethics committee of the Southwest Medical University, China (approval number: 201703192).

Animals

Two-month-old male Sprague-Dawley rats of clean grade were purchased from Chongqing Tengxin Bio-Technology Co., Ltd. [Chongqing, China; production license: SCXK (Yu) 2012-0008]. All the rats were fed under the same standard conditions with a temperature of 23 °C ± 3 °C and relative humidity of 50% ± 10%.

Grouping

A total of 60 rats were randomly divided into experimental and control groups (30 rats in each group). Each group was randomly divided into three subgroups (10 rats in each subgroup) and named according to the time points of drug feeding 1, 3, and 3 months, followed by drug withdrawal for 1 month.

Drug delivery method

Experimental group: Isoniazid tablets (Chengdu Jinhua Pharmaceutical Co. Ltd., China) were ground into a powder, and the equivalent dose ratio was calculated according to human and rat body surface area (rats received six times the adult human dosage). Isoniazid powder was dissolved in saline at a concentration of 12 mg/mL (Sichuan Meida Kangjiale Pharmaceutical Co. Ltd., Chengdu, China). Rats were administered isoniazid solution (1 mL/100 g body weight, once every other day) intragastrically.

Control group: The rats were administered normal saline (1 mL/100 g body weight, once every other day) intragastrically. Feeding conditions were the same as those in the experimental group.

Sample collection

The rats were anesthetized with 3% pentobarbital sodium solution (30 mg/kg). Next, 2 mL blood samples were obtained from the hearts. Anticoagulated whole blood (300 μL) was used to determine the percentage of T lymphocytes. The serum of the remaining blood was used to detect the levels of cytokines after letting it stand and centrifugation.

Detection of T lymphocyte percentage by flow cytometry

The anticoagulated whole blood (100 μL) was placed in the test tube for flow cytometry. The samples were mixed with 20 μL of Allophycocyanin (APC) Mouse anti-Rat CD3 (BD, NJ, USA), 20 μL of Fluorescein isothiocyanate (FITC) Mouse anti-Rat CD8a (BD), and 20 μL of Phycoerythrin (PE) Mouse anti-Rat CD4 (BD). The samples (100 μL of anticoagulated whole blood) in the isotype control group were mixed with isotype control. All samples were analyzed to detect the percentage and ratio of $CD4^+$ and $CD8^+$T lymphocytes under a BD fluorescence-activated cell sorter (FACS) Verse II flow cytometer (BD).

Detection of the levels of interleukin-12 and interferon-γ by enzyme-linked immunosorbent assay

The levels of serum interleukin (IL)-12 and interferon (IFN)-γ were detected using a microplate reader (Thermo Fisher Scientific Inc., MA, USA), IL-12 enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, CA, USA), and IFN-γ ELISA kit (eBioscience, CA, USA). All the reagent samples and standards were prepared as instructed. The plate was washed, and the standard and sample were added, followed by antibody. Then the stop solution was added,

and the plate was read. (The detailed instructions on the relevant ELISA kit was followed).

Test of carbon clearance capacity and phagocytic capacity

Diluted ink (0.1 mL/20 g body weight) was injected into the tail vein of the rats. Blood samples (30 μL) were obtained from the hearts after 5 (t1) and 15 (t2) min. The optical density (OD) values of the samples obtained at t1 (OD1) and t2 (OD2) were measured at 650 nm wavelength. The rats were sacrificed by cervical dislocation, and the liver and spleen were weighed. The carbon clearance index K was calculated according to the formula K = (1 g ODl - l g OD2)/(t2 - t1). The phagocytic index a was calculated according to the formula a = body weight/(liver weight + spleen weight) × $K^{1/3}$ (Singh, Yadav, Noolvi, 2012).

Detection of acid phosphatase activity in rat peritoneal macrophages by Gomori assay

Rats were injected intraperitoneally with 0.7 mL of 6% sterile starch broth, and 0.1-0.2 mL of abdominal fluid was extracted after 3 days. The peritoneal fluid was dripped on a precooled slide and fixed with 10% neutral formalin. After adding acid phosphatase solution and 1% ammonium sulfide successively, the cells were stained with Giemsa stain. The slides were examined under ordinary optical microscope to observe brown particles or plaques in macrophages (Anhe, Lima-Oliveira, Azeredo-Oliveira, 2007).

Statistical analysis

The experimental data were analyzed using the SPSS 19.0 software (SPSS Inc., IL, USA). T lymphocytes, cytokines test, carbon clearance capacity and phagocytic capacity test: At the same time point, the experimental and control groups were compared using two independent-sample t test, and t' test was used to assess the heterogeneity of variance. The results were expressed as mean ± standard deviation ($x \pm s$), and a P value < 0.05 was considered statistically significant. Acid phosphatase test: At the same time point, the experimental and control groups were compared using the rank-sum test. The results were expressed as mean rank, and a P value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Most people infected with Mtb develop a latent TB infection without any clinical symptoms that is not cleared by the immune system. Only a small number of patients develop an active TB infection. When various factors cause a decrease in the body's immune function, the latent Mtb can be activated, allowing it to develop into active TB (Petruccioli *et al*., 2016; Riou *et al*., 2016). Therefore, the inhibition and clearance of Mtb are closely related to immune function. This study selected normal healthy rats as subjects to avoid the effects of other factors on the immune system.

Comparison of $CD4^+$T, $CD8^+$T lymphocytes and $CD4^+/CD8^+$T lymphocyte cell ratio

After feeding drug for 1 and 3 months, the percentage of $CD4^+$T lymphocytes and the $CD4^+/CD8^+$T lymphocyte cell ratio was higher in the experimental group than in the control group, and the differences were statistically significant ($P < 0.05$). Moreover, after feeding drug for 3 months followed by drug withdrawal for 1 month, no statistically significant difference was found in the $CD4^+$T lymphocytes or $CD4^+/CD8^+$T lymphocyte cell ratio between the experimental and control groups ($P > 0.05$; Table I). No statistically significant difference was found in the $CD8^+$T lymphocytes between the experimental and control groups at any time point (Figure 1, Table I).

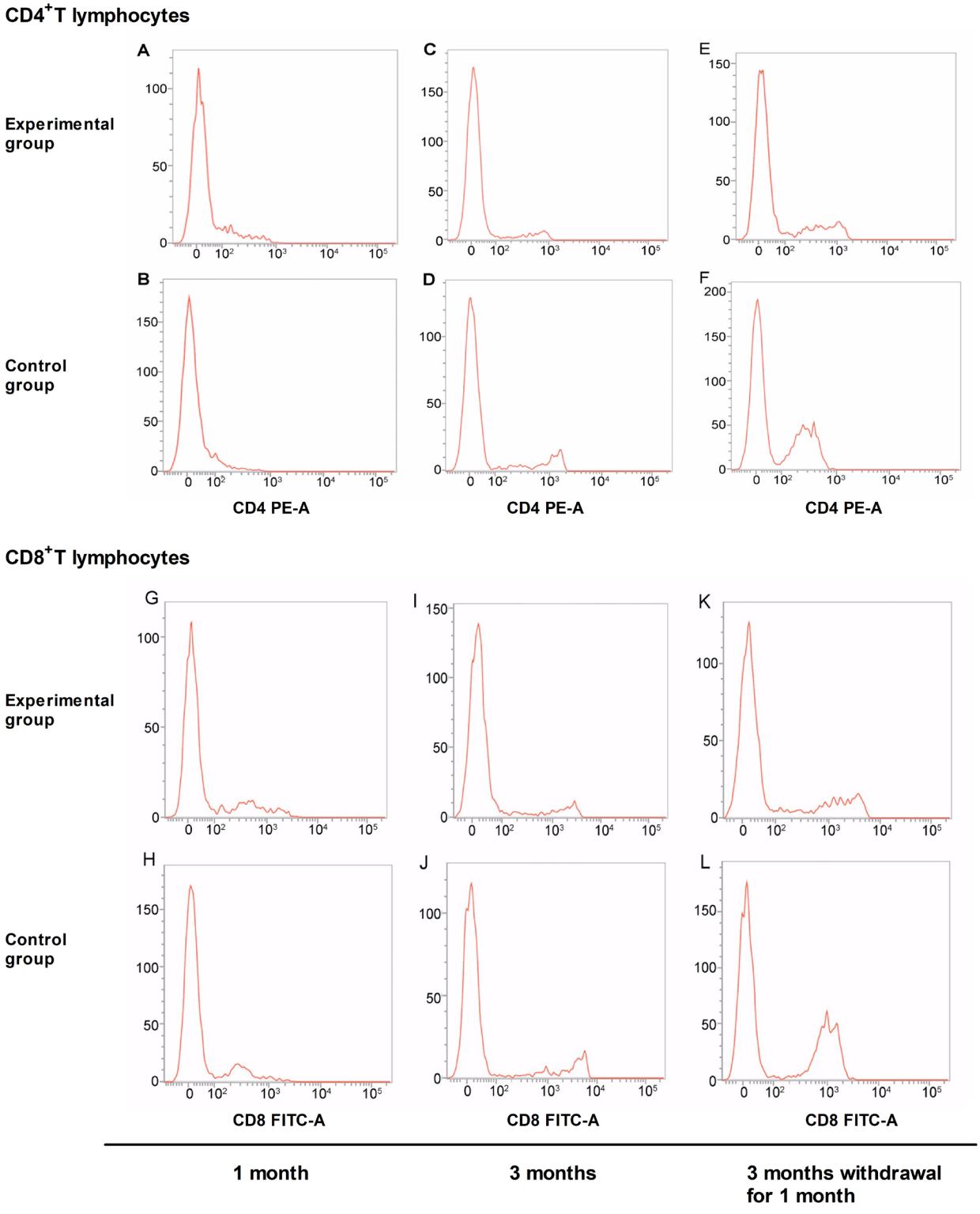

FIGURE 1 - $CD4^+$T lymphocytes in (A) experimental group after drug feeding for 1 month, (B) control group after drug feeding for 1 month, (C) experimental group after drug feeding for 3 months, (D) control group after drug feeding for 3 months, (E) experimental group after drug feeding for 3 months followed by drug withdrawal for 1 month, (F) control group after drug feeding for 3 months followed by drug withdrawal for 1 month. $CD8^+$T lymphocytes in (G) experimental group after drug feeding for 1 month, (H) control group after drug feeding for 1 month, (I) experimental group after drug feeding for 3 months, (J) control group after drug feeding for 3 months, (K) experimental group after drug feeding for 3 months followed by drug withdrawal for 1 month, (L) control group after drug feeding for 3 months followed by drug withdrawal for 1 month.

TABLE I - Comparison of $CD4^+$, $CD8^+$T lymphocytes and $CD4^+/CD8^+$ ratio in the experimental and control groups at the three time points

Time point	Experimental group (*n=10*)	Control group (*n=10*)	*P-value*
$CD4^+$T lymphocytes (%)			
Feeding drug for 1 month	48.332 ± 5.486	37.625 ± 4.192	0.000 [a]
Feeding drug for 3 months	42.169 ± 6.041	35.421 ± 7.882	0.045 [a]
Feeding drug for 3 months followed by drug withdrawal for 1 month	36.656 ± 5.639	32.642 ± 5.325	0.119 [a]
$CD8^+$T lymphocytes (%)			
Feeding drug for 1 month	11.243 ± 1.572	13.642 ± 3.292	0.058 [a]
Feeding drug for 3 months	12.280 ± 4.398	15.611 ± 5.557	0.154 [a]
Feeding drug for 3 months followed by drug withdrawal for 1 month	15.780 ± 6.048	15.963 ± 3.814	0.936 [a]
$CD4^+/CD8^+$ ratio			
Feeding drug for 1 month	4.372 ± 0.780	2.880 ± 0.637	0.000 [a]
Feeding drug for 3 months	3.903 ± 1.579	2.552 ± 1.065	0.038 [a]
Feeding drug for 3 months followed by drug withdrawal for 1 month	2.604 ± 0.869	2.185 ± 0.723	0.256 [a]

a = Two independent-sample *t* test. $P < 0.05$ denoted statistical significant difference

Significance of T lymphocytes in the Mtb clearance

Mtb is an intracellular bacterium; $CD4^+$ and $CD8^+$T lymphocytes play important roles in the immune response against Mtb infection. Previous studies found that for patients with TB, the number of $CD4^+$T lymphocytes decreased and the number of $CD8^+$T lymphocytes increased. The $CD4^+/CD8^+$T lymphocyte cell ratio, which could be used to reflect the severity of TB, decreased or even inverted (Arroyo *et al*., 2016; Reiley *et al*., 2010; Scanga *et al*., 2000).

The effects of isoniazid on T lymphocytes in healthy male rats were investigated in this study. The results showed that isoniazid could increase the percentage of $CD4^+$T lymphocytes and $CD4^+/CD8^+$T lymphocyte cell ratio in the peripheral blood of rats, and the percentages could be restored after isoniazid withdrawal. Moreover, isoniazid had no significant effect on $CD8^+$T lymphocytes in the peripheral blood of rats.

Comparison of IL-12 and IFN-γ level

After feeding drug for 1, 3, and 3 months followed by drug withdrawal for 1 month, no statistically significant difference was found in the IL-12 and IFN-γ level between the experimental and control groups ($P > 0.05$; Table II).

TABLE II - Comparison of IL-12 and IFN-γ level in the experimental and control groups at the three time points

Time point	Experimental group (*n=10*)	Control group (*n=10*)	*P-value*
IL-12 (pg/mL)			
Feeding drug for 1 month	134.409 ± 25.128	131.752 ± 41.084	0.874 [a]
Feeding drug for 3 months	137.034 ± 65.937	96.211 ± 18.836	0.088 [a]
Feeding drug for 3 months followed by drug withdrawal for 1 month	113.241 ± 38.698	96.019 ± 20.463	0.234 [a]
IFN-γ (pg/mL)			
Feeding drug for 1 month	121.552 ± 21.847	107.609 ± 34.765	0.297 [a]
Feeding drug for 3 months	118.444 ± 8.158	127.379 ± 24.003	0.289 [a]
Feeding drug for 3 months followed by drug withdrawal for 1 month	114.280 ± 18.796	105.517 ± 19.753	0.323 [a]

a = Two independent-sample *t* test. $P < 0.05$ denoted statistical significant difference

Significance of cytokines in the treatment of TB

Cytokines, such as IL-12 and IFN-γ, also play important roles in the immune response to Mtb infection (Chen *et al*., 2017; Mortaz *et al*., 2016). IL-12 can promote the proliferation and activation of T lymphocytes (Hamilton *et al*., 2016) and stimulate the production of IFN-γ. IL-12 can also activate macrophages and enhance the ability of anti-Mtb with IFN-γ (Thada *et al*., 2016). Recent studies showed that cytokine imbalance was a key factor in the occurrence and development of TB, and was closely related to TB prognosis (Anuradha *et al*., 2016; Kreins *et al*., 2015).

The effects of isoniazid on the levels of IL-12 and IFN-γ in healthy male rats were investigated in this study. The results showed that the level of IL-12 in the serum of rats decreased as the rat's age increased. However, the change in IL-12 level in the experimental group was not the same as in the control group. It did not decrease as the rat's age increased, indicating that isoniazid might increase the level of IL-12 in the serum of rats, and this effect may become more significant as the administration time is extended. Also, the level of IL-12 could be restored after isoniazid withdrawal. These results were not statistically significant, possibly due to small sample size and short-term administration. Therefore, the effects of isoniazid on the level of IL-12 in the serum of rats may be further examined in future studies by increasing the sample size and extending the administration time. The data presented here suggest that isoniazid may have no significant effect on IFN-γ level in the serum of rats.

Comparison of carbon clearance index, phagocytic index and acid phosphatase level

After 1 month of drug feeding, the carbon clearance index, phagocytic index, and acid phosphatase level in the experimental group was higher than in the control group, and the difference was statistically significant ($P < 0.05$). Moreover, after feeding drug for 3 and 3 months followed by drug withdrawal for 1 month, no statistically significant difference was found in the carbon clearance index, phagocytic index, or acid phosphatase level between the experimental and control groups ($P > 0.05$; Table III).

TABLE III - Comparison of carbon clearance, phagocytic index and acid phosphatase level in the experimental and control groups at the three time points

Time point	Experimental group (*n=10*)	Control group (*n=10*)	*P-value*
Carbon clearance index			
Feeding drug for 1 month	0.017 ± 0.004	0.006 ± 0.001	0.000 [a]
Feeding drug for 3 months	0.011 ± 0.001	0.012 ± 0.002	0.798 [a]
Feeding drug for 3 months followed by drug withdrawal for 1 month	0.024 ± 0.009	0.029 ± 0.009	0.248 [a]
Phagocytic index			
Feeding drug for 1 month	6.383 ± 0.602	4.637 ± 0.374	0.000 [a]
Feeding drug for 3 months	6.366 ± 0.578	6.377 ± 0.491	0.964 [a]
Feeding drug for 3 months followed by drug withdrawal for 1 month	8.391 ± 1.526	8.142 ± 0.989	0.671 [a]
Acid phosphatase level (Mean rank)			
Feeding drug for 1 month	13.40	7.60	0.029 [b]
Feeding drug for 3 months	10.00	11.00	0.739 [b]
Feeding drug for 3 months followed by drug withdrawal for 1 month	11.25	9.75	0.579 [b]

a = Two independent-sample *t* test; b = Rank-sum test. $P < 0.05$ denoted statistical significant difference

Significance of macrophages in anti-tuberculosis

Macrophages play a role in innate immunity, and are critical cells in the immune response to Mtb infection. Activated macrophages can inhibit or even kill Mtb, which plays an important role in the control of Mtb infection (Auricchio *et al*., 2003). Carbon clearance capacity, phagocytic capacity, and acid phosphatase of macrophages can reflect their function.

The effects of isoniazid on macrophage function in healthy male rats were explored in this study. Isoniazid enhanced the carbon clearance capacity, phagocytic capacity, and acid phosphatase activity of macrophages for a short period of time, but the long-term effects were not obvious. A possible explanation is that as innate immune cells, macrophages often respond swiftly to external stimuli then produce high levels of inflammatory mediators. On the other hand, the inflammatory activity of macrophages needs to be tightly regulated in order to prevent the inflammation-induced tissue damage. Many anti-inflammatory cytokines are also produced by activated macrophages to terminate their excessive activation. In contrast, the effects of adaptive immune cells, such as T lymphocytes, are generally persistent over a relatively long time period. Therefore, isoniazid treatment-induced enhancement of macrophage function may be antagonized by negative feed-back mechanisms, thus decreasing the long-term effect of isoniazid.

CONCLUSION

In summary, isoniazid could increase the percentage of $CD4^+$T lymphocytes and $CD4^+/CD8^+$T lymphocyte cell ratio in peripheral blood and enhance macrophage function in healthy male rats.

SOURCES OF SUPPORT

This study was supported by the Sichuan Provincial Health Department (No. 090209).

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Received for publication on 24th July 2018
Accepted for publication on 02nd January 2019