

THE ROLE OF DOXYCYCLINE AS AN MMP-9 INHIBITOR IN TUBERCULOUS SPONDYLITIS RABBIT MODEL WITH AN IMMUNOHISTOCHEMISTRY EVALUATION

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Abstract

Background

Tuberculous spondylitis is a disease that occurs throughout the world. The diagnosis can usually only be made at an advanced stage with severe spinal deformity and significant neurological deficits such as paraplegia. Due to chronic inflammation in bone, there will be an increase in cellular immune response and the release of inflammatory cytokines, which will eventually trigger an increase in the expression of matrix metalloproteinases (MMP). In vitro studies, doxycycline inhibited MMP secretion induced by TB infection. This study assessed the effect of doxycycline on MMP-9 expression in rabbits exposed to tuberculous spondylitis by immunohistochemical examination.

Methods

This randomized controlled trial study was carried out from January to June 2020. Adult New Zealand rabbits (*Oryctolagus cuniculus*) were used in this study. A total of 40 rabbits were included in this study. The inoculation of *Mycobacterium tuberculosis* procedure was carried out in groups. Doxycycline was administered to the control group, the group with a dose of 1 mg/kgBW/day, and the group with a dose of 5 mg/kgBW/day for each group based on time. . The outcome of this study was the examination of the IHC expression of MMP-9 from blood samples after doxycycline administration.

Result

Forty rabbits inoculated with *Mycobacterium tuberculosis* and treated with doxycycline for 4 weeks were incubated in individual cages in one large room. The rabbit's appetite does not decrease; the rabbits can defecate and urinate normally, remain active, and respond to the environment well. Four rabbits died during the experiment. There was no significant differences in MMP-9 expression between the control group and intervention group at 2 and 4 weeks. Meanwhile, MMP-9 expression was found greater at 6 and 8 weeks during doxycycline administration between the control and intervention groups.

Conclusion

Based on the results of this study, there is a positive relationship and effect between doxycycline administration and MMP-9 expression based on immunohistochemical examination of rabbits inoculated for 6 and 8 weeks.

Keyword: Tuberculous Spondylitis, Doxycycline, MMP-9

Introduction

Tuberculous spondylitis, also known as Pott's disease of the spine, is a disease that occurs throughout the world. This disease affects the spine caused by the bacteria *Mycobacterium tuberculosis* (Mtb). Approximately 3 million deaths occur each year due to this disease.[1]

In the United States, from 2002 to 2011, 75,858 patients were diagnosed with Mtb, of which 2,789 were diagnosed with Mtb spondylitis. The average Mtb spondylitis patient per year is 278.9 cases/year. Over 10 years, the incidence of tuberculous spondylitis decreased sharply from 0.07 cases in 100,000 people in 2002 to 0.05 cases in 100,000 people in 2011.[2]

Tuberculous spondylitis can cause severe morbidity, including neurological deficits and permanent spinal deformities. The diagnosis of tuberculous spondylitis is challenging to establish and is often mistaken for spinal neoplasms or other pyogenic spondylitis. The diagnosis can usually only be made at an advanced stage with severe spinal deformity and significant neurological deficits such as paraplegia. Due to chronic inflammation in bone, there will be an increase in cellular immune response and the release of inflammatory cytokines, which will eventually trigger an increase in the expression of matrix metalloproteinases (MMP).[3]

A previous study showed that variations in the cellular system in the production of MMP and Tissue Inhibitors of Metalloproteinase (TIMP) inhibitors contribute to the pathophysiology of bone erosion. The balance between MMP and TIMP is essential in determining the integrity of the extracellular matrix.[4] Another study showed an increase in MMP-9 levels in pulmonary tuberculosis infection. However, until now, no literature specifically discusses MMP-9 expression in tuberculous spondylitis in Indonesia.[5]

Doxycycline, approved tetracycline by Food and Drug Administration (FDA), was used in an in vivo tuberculosis model for the temporal control of mycobacterial gene expression. In vitro studies, DOX inhibited MMP secretion induced by TB infection at 5 mg/liter and higher.[5]

This study assessed the effect of doxycycline on MMP-9 expression in rabbits exposed to tuberculous spondylitis by immunohistochemical examination.

Methods

This randomized controlled trial (RCT) study was carried out from January to June 2020 at the Integrated Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara. Adult New Zealand rabbits (*Oryctolagus cuniculus*) weighing 1.8-2 kg were used in this study. A total of 40 rabbits were included in this study. The rabbits were divided into 12 groups, where each group consisted of 3 rabbits with different research periods. Groups 1-3 are groups of rabbits with a study period of 2 weeks, groups 4-6 with a study period of 4 weeks, groups 3 with a study period of 7-9 weeks, and groups 10-12 with a study period 8 weeks.

Inoculation processes

The inoculation procedure was only carried out in groups 2, 3, 4, and 5. Prior to the inoculation procedure, clinical and radiological examinations were carried out on rabbits. Under general anesthesia, using ketamine anesthetic at a dose of 44 mg/kg, the back of the rabbit at the T13-L1 level was shaved, and aseptic and antiseptic procedures were performed using 70% alcohol

and betadine, and then a sterile cloth was applied. The rabbit was prepared in a side-facing position with the left back facing the surgeon; the 12th thoracic was identified by palpating the 12th rib and then tracing to the transverses process. A transverse incision was made to reach the spine at the T12 level, starting from the spinous process 3-5 cm wide to the left lateral through the cutis and subcutis. The paraspinal muscles were separated up to the 12th rib, transverse process, and 12th thoracic plate. Then a drill was used to make a hole at the midpoint of the 12th thoracic body (+ 5 mm from the transverse process) 6 to 10 mm deep using a 1.5 mm drill bit. 0.2 mL of *Mycobacterium tuberculosis* bacterial suspension was inoculated with a quantity of 1×10^8 CFU/mL aseptically into the hole made in the body, then exposed for 5 minutes to open air, and then closed by sewing the fascia, muscle, and subcutis. The success rate of inoculation was assessed using AFB examination, culture, and immunohistochemical examination (IHK).[6]

Doxycycline administration

Doxycycline was given to the control group, the group with a dose of 1 mg/kgBW/day, and the group with a dose of 5 mg/kgBW/day for each group based on time.

The outcome of the study

In this study, the rabbit group was divided into a control group, a group that was given doxycycline 1 mg/kg/day, and doxycycline 5 mg/kg/day for 4 weeks based on the group for the 2nd, 4th, 6th, and 8th week of inoculation. The outcome of this study was the examination of the IHC expression of MMP-9 from blood samples after doxycycline administration.

Data analysis

In the bivariate analysis, with a categoric-categoric measuring scale through doxycycline administration and interpretation of IHC, the Chi-Square or Fisher's exact test was used. The R value can be done with the Pearson test with the alternative Spearman test. To see the effect of doxycycline with MMP-9 based on immunohistochemistry in rabbits, the researchers used an ordinal correlation model adjusted to a variable measuring scale. This correlation test value is interpreted by the $Y = \alpha + \beta_1X_1 + \beta_2X_2$ model by looking at the positive or negative β value. The SPSS version 15 was used to perform statistical analysis.

Results

Forty treated and control rabbits inoculated with *Mycobacterium tuberculosis* and treated with doxycycline for 4 weeks were incubated in individual cages in one large room [Table 1]. The rabbit's appetite does not decrease; the rabbits can defecate and urinate normally, remain active, and respond to the environment well. Four rabbits died during the experiment.

Table 1. Distribution of treatment for rabbit groups

No	Group	Treatment I	Treatment II
1	Group A	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 2 weeks	Without doxycycline for 4 weeks
2	Group B	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 2 weeks	Doxycycline 1 mg/kg/day for 4 weeks

3	Group C	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 2 weeks	Doxycycline 5 mg/kg/day for 4 weeks
4	Group D	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 4 weeks	Without doxycycline for 4 weeks
5	Group E	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 4 weeks	Doxycycline 1 mg/kg/day for 4 weeks
6	Group F	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 4 weeks	Doxycycline 5 mg/kg/day for 4 weeks
7	Group G	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 6 weeks	Without doxycycline for 4 weeks
8	Group H	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 6 weeks	Doxycycline 1 mg/kg/day for 4 weeks
9	Group I	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 6 weeks	Doxycycline 5 mg/kg/day for 4 weeks
10	Group J	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 8 weeks	Without doxycycline for 4 weeks
11	Group K	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 8 weeks	Doxycycline 1 mg/kg/day for 4 weeks
12	Group L	Bacteria inoculation <i>Mycobacterium tuberculosis</i> , Incubated for 8 weeks	Doxycycline 5 mg/kg/day for 4 weeks

Examination of healing with the duration of inoculation in this study is shown in Figure 1.

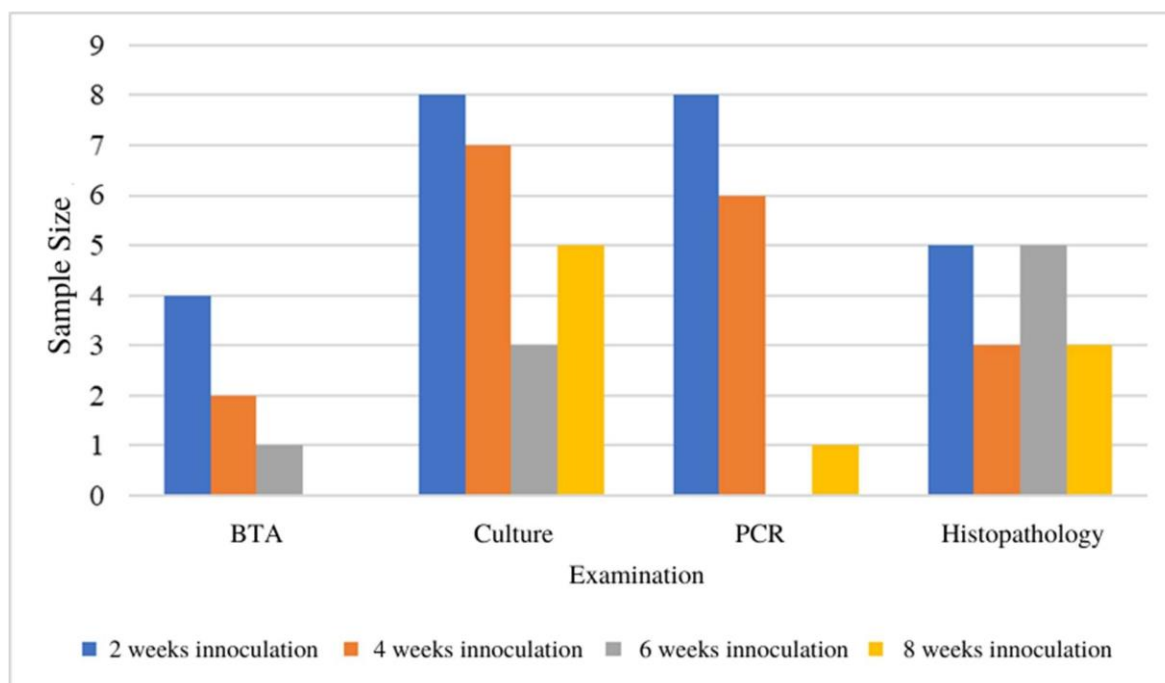


Figure 1. Graph of Healing Check with Inoculation Time

The effect of doxycycline administration on MMP-9 with IHC was shown in Table 2.

Table 2. Administration of doxycycline to MMP-9 with CPI

Inoculation	Group	MMP-9 IHK		p value	β value
		Negative	Positive		
2 weeks	Without	2	1	0.238	β1=0.106 β2=0.078
	doxycycline	(66.7%)	(33.3%)		
	Doxycycline 1 mg/kg/day	2	1		
	Doxycycline 5 mg/kg/day	1	2		
4 weeks	Without	1	2	0.357	
	doxycycline	(33.3%)	(66.7%)		
	Doxycycline 1 mg/kg/day	1	2		
	Doxycycline 5 mg/kg/day	2	1		
6 weeks	Without	2	1	0.023*	
	doxycycline	(66.7%)	(33.3%)		
	Doxycycline 1 mg/kg/day	1	2		
	Doxycycline 5 mg/kg/day	2	1		
8 weeks	Without	2	1	0.038*	
	doxycycline	(66.7%)	(33.3%)		
	Doxycycline 1 mg/kg/day	0 (0%)	3		
			(100%)		

Doxycycline 5 mg/kg/day	2 (66.7%)	1 (33.3%)
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Discussion

Statistically, developing countries account for 90% of tuberculosis cases worldwide. After Mtb infection, the innate immune response is activated, and macrophages are an essential part of the innate immune system. Importantly, they play a critical role in recognizing, responding to, and reacting to Mtb infections. Phagocytosis of Mtb by macrophages can be triggered by non-specific pinocytosis or by activation of specific receptors. In addition, Mtb can also be recognized through pattern recognition receptors (PRR) such as Toll-like receptors (TLR) and Nod-like receptors (NLR). This interaction of Mtb and macrophages ultimately activates the inflammatory response.[7]

For decades, various animal models have been used to predict the body's immune response to tuberculosis infection for vaccine and diagnostic research purposes. Rabbits have the ability to form cavities and can help study the factors that cause this disease.[8]

This study used New Zealand White rabbits as the sample (model). This was similar to the theory that currently, many animal species have been used as Mtb models such as rats, guinea pigs, monkeys, and one of them is the rabbit. The spinal tuberculosis model in rabbits is the best model to evaluate the effectiveness of drugs/regimens in bone TB. The most widely used strains in rabbits are *Mycobacterium tuberculosis* H37RV and Erdman.[9]

The highest success rate for inoculation of *Mycobacterium tuberculosis* bacteria in the vertebral bodies of rabbits was in group 3, specifically 7 of 8 samples (87.5%) with an incubation period of 6 weeks, followed by group 2 (75%, with an incubation period of 4 weeks), group 1 (37.5%, with an incubation period of 2 weeks), and group 4 (12.5%, with an incubation period of 8 weeks). These findings were similar to Rahyussalim et al.[6] study, showing the potential for the spread of *Mycobacterium tuberculosis* into the environment with the rabbit spinal Mtb model, that based on PCR examination, the inoculation was successful in the first week and based on the histopathological examination the inoculation was successful in the seventh week. 6.

Histopathology has a much higher diagnostic success rate than culture, with 100% confirmation. Cytology shows characteristic epithelioid cell granulomas, granular necrosis, lymphocytes, and Langerhans giant cells.[10]

This is also found in this study. In this study, the positive histopathological examination results (HE view) were tubercles, macrophages, and epithelioid cells. Two studies that observed a rabbit spinal TB model's establishment found suitable histopathological results.[11,12]

Effect of Doxycycline on MMP-9 Serum Levels of Tuberculosis Spondylitis Rabbit Blood

Mtb is a lung disease. Lung extracellular matrix biochemistry predicts that matrix metalloproteinases (MMPs) will be the dominant proteases that promote lung matrix destruction in TB. MMP-9 regulates monocyte recruitment to granulomas in a zebrafish model, suggesting that MMP modulates the immune response against *Mycobacterium tuberculosis* (Mtb) and triggers pathological conditions. Many tetracyclines, including DOX, have anti-inflammatory properties mediated by suppressing tumor necrosis factor (TNF- α) and matrix

metalloprotease (MMP). In vitro, DOX inhibited MMP secretion induced by Mtb infection at 5 mg/liter and higher.[13]

Like other body tissues, inflammation of the central nervous system (CNS) also increases MMP secretion and affects the permeability of the blood-brain barrier (BBB). Recent studies have shown that upregulation of MMP-9 has been observed in brain biopsies of patients with tuberculous meningitis. This increased activity of MMP-9 in brain tissue may be involved in the breakdown of the blood-brain barrier (BBB), edema, and exudation of inflammatory cells. Li et al.[14] analyzed the expression of MMP-9 in the pathophysiological process of tuberculous meningitis in a mouse model. Several studies have shown that serum MMP-9 levels have significant differences in the group with tuberculosis infection compared to the control group.[5,15,16]

Doxycycline can inhibit MMP activity in cellular tuberculosis models, and the decrease in MMP level was associated with reduced tissue damage in tuberculosis infection.[17] MMP activity was implicated in various lung disorders characterized by the destruction of the extracellular matrix. MMP-9 was widely released by macrophages and had high concentrations in the lung and pleural fluid in TB patients depending on the severity of the infection.[10]

Hence, in this study, it was seen that at 6 and 8 weeks of inoculation, doxycycline administration with MMP-9 had p-values of 0.023 and 0.038, respectively ($p < 0.05$). These results indicated a relationship between doxycycline administration and MMP-9 based on immunohistochemistry at 6 and 8 weeks of inoculation. Miow et al.[17] demonstrated that doxycycline significantly reduced MMP-1, -8, -9, -12, and -13 sputum, suppressed the breakdown of type I collagen and elastin, reduced lung cavity volume without changing the mycobacterial load of sputum, and was safe. The beta value in the ordinal correlation test showed a 1 value of 0.106 2 of 0.078 with a value of 1.444. These results indicate a positive effect of doxycycline administration with MMP-9 based on immunohistochemistry in rabbits inoculated for 6 and 8 weeks. This may be because the immunohistochemical examination did not show any significant changes with doxycycline administration for 6 and 8 weeks or the lack of samples. In comparison to the study of Miow et al.[17], they conducted a study with 30 patients that randomly assigned to receive 100 mg of doxycycline or placebo two times a day for 14 days. They found that administration of doxycycline significantly reduced sputum MMP-1, -8, -9, -12 and -13 in pulmonary TB patients. Naomi FW et al.[18] study, observed Doxycycline Reducing Mtb Growth in a TB Guinea Pig Model, found that doxycycline can suppress the colony forming unit (CFU) of TB lung and was positively correlated with the percentage of guinea pig granulomatous infiltrates. However, there was no independent effect of doxycycline could be identified. This indicated that doxycycline acted directly to limit mycobacterial proliferation in a guinea pig model rather than on MMP activity to alter immunopathology. Therefore, further research is needed on this matter because there are no other studies that specifically examine TB spondylitis cases.

Conclusion

Based on the results of this study, there is a positive relationship and effect between doxycycline administration and MMP-9 expression based on immunohistochemical examination of rabbits inoculated for 6 and 8 weeks.

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