The Study of TNFα Regulation in the Management of Spinal Tuberculosis Using Instrumentation

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Abstract

**Introduction:** Tuberculosis is still commonly found in many developing countries. Spinal tuberculosis can cause vertebral deformity and neurological disorders. It was discovered thousands years ago and its management was aimed to eradicate infection and maintain the integrity of the vertebrae. Previously, the management of spinal TB was using drugs and external stabilization. Surgical techniques were developed afterwards to clean the infected vertebral segment. Because of the vertebral deformity remained inevitable and had impacts on neurological disorders, new paradigm had been developed by using instrumentation to stabilize the deformity of infected vertebral segment and to restore and maintain neurological function. TNF-a has a major role in immune process of spinal TB. Spinal TB instrumentation uses metal devices composed of ions and particles that can interact each other so it could produce physical and chemical energy that is transmitted to the vertebrae. The energy is expected to enhance the biomolecular and biocellular activity of the body's immune cells so the healing process could be better. **Methods:** An experimental study was carried out on New Zealand Rabbits which were given TB H37Rv strain infection in the vertebral body. Samples were divided into five groups namely control rabbits, infected rabbits without intervention, infected rabbits treated by instrumentation, infected rabbits given anti-tuberculosis drugs and infected rabbits treated by instrumentation and given drugs. Then the cytokine levels of TNFα were evaluated and compared. **Results:** The results showed a significant TNF-a level increase in infected rabbits given drugs alone and instrumentation alone compared to infected rabbits without intervention. There was a significant TNFα decrease in infected rabbits given drugs and treated by instrumentation compared to control rabbits and rabbits who received drugs only. **Conclusions:** Instrumentation can improve the healing process in spinal tuberculosis by increasing the body's cytokine levels.

**Keywords:** TNF-a, Spinal TB infection, immune response, Instrumentation, Cytokine Expression, Healing Process.

**INTRODUCTION**

Mycobacterium tuberculosis infection occurs when several popular airborne tubercle bacilli from a patient with pulmonary TB reach the alveoli of the host. Here, Mtb is rapidly phagocytosed by alveolar macrophages which can normally kill bacteria that enter by innate immune response (Innate Immune Response). (Urdahl et al., 2011). If the bacillus can survive this first line of defense, it begins to actively replicate in macrophages, spreads to cells in the lobby including epithelial and endothelial cells, and within a few weeks through exponential growth will reach a bacterial load that is high. (Wolf et al., 2008). During the early stages of this infection, Mtb can diffuse to other organs via lymphatics and through hematogenous spread where it can infect other cells including the spine. (Wolf et al., 2008). After that, once the adaptive immune response appears, neutrophils, lymphocytes, and other immune cells to the primary infection site form cellular infiltrates which then form a typical granuloma structure. (Ottenhoff et al., 2012). The fibrosis component covers the granuloma which becomes calcified so that the bacilli remains confined and protected by the host immune response. These primary lesions, classically referred to as the Ghon complex, are referred to as "sanctuaries" of Mtb during latent infection, with the bacilli remaining dormant, metabolically inactive, for years, decades, or even alive. In this scenario, when, during latent infection, for
unknown reasons, bacilli will replicate within these primary lesions, and disease will result. (Bishai, 2000; Delogu et al., 2013).

The clinical manifestations of Mycobacterium tuberculosis infection describe the complex interactions between the causative bacteria and the host immune response. The immune response to TB infection includes innate and adaptive immune responses. The innate immune response is played by macrophage cells, while the adaptive immune response is played by T lymphocytes, both CD4+ and CD8+. Granuloma formation is the most prominent picture of the immune response to TB germs, while the role of antibodies is still being debated (Bhatt et al., 2007; Potian et al., 2007). TNF-α is referred to as multifunctional cytokines, proinflammatory cytokines and is produced by dendrites, macrofages, and T cells. These cytokines have a role in the recruitment of phagocytes by stimulating or activating other macrophage / dendrite cells. Besides, it stimulates and activates Th1 cells by stimulating IFN-γ. IL-6 is a pro-inflammatory and inhibitory cytokine produced by macrophages. Has activities in collaboration with TNF-α to continue advanced stage inflammation. IFN-γ, is a cytokine needed by adaptive immunity cells, namely CD4-Th1 cells, in regulating NK cells - as a direct killer of Mycobacterium. IL-17 is a cytokine produced by Th-17 cells and has the ability to reactivate the adaptive immunity response to Mycobacterium and is useful for delivering the healing process of spinal TB (Orme and Juarrero, 2007).

Immune cells, macrophages, are the first cells to interact with biomaterials. Macrophages are responsible for cleaning wounds, inflammation, and recruiting tissue-producing cells. Macrophages are activated as pro-inflammatory (M1) or anti-inflammatory (M2); and macrophage activation will control the resulting environmental micro-inflammatory response. Macrophage activation is generally characterized by pro-inflammatory and anti-inflammatory cytokines and chemokines. The purpose of this study was to describe the effect of microstructure and surface energy on macrophage activation and cytokine production. M2 anti-inflammatory response is indicated by high levels of IL-4, IL-10, and TGFβ1 (Gordon & Taylor 2005; Brancato & Albina 2011; Novak & Koh 2013). Controlling M2 activation will suppress the immune system response and facilitate the formation of blood vessels and wound healing (Arranz et al. 2012; Gordon & Martinez 2010). The surface of the implant is the only part of the biomaterial that interacts with human cells. Based on the data, modifying the surface will change the response of the immune system and the cytokines released by cells. This results in cell recruitment capable of regenerating tissue instead of chronic inflammation and fibrous encapsulation. Macrophage activation can be induced or modulated by the surface characteristics of the implant and will ultimately alter the healing process and affect the long-term stability of the implant. In general, a chronic immune response will prevent the formation of healthy tissue around the implant (Mavrogenis et al., 2009; Han et al., 2015). This study aimed to investigate the expression of TNFα in Tuberculous Spondylitis (TB) and its relationship with instrumentation.

Based on the situation, this study investigates TNFα expression as marker of bone healing process in Spondylitis using New Zealand white rabbits as samples. This experimental study used experimental animals allowing control towards the variables. Several studies showed that New Zealand white rabbits can be infected with Mycobacterium tuberculosis bacterium using aerosol infection system after 6-33 weeks of exposure using the laboratory strain M. tuberculosis namely H37Rv to create a spinal infection.

METHODS

This study used pure experimental design in vivo with a randomized post-test only controlled group design and male New Zealand rabbits (Oryctolagus cuniculus) aged 4-5 months and weighed 3,000 grams as the samples. Tb spondylitis model was developed by Infecting Mycobacterium tuberculosis H37Rv strain on the spinal cord. The samples were divided into 5 groups. The first group was normal rabbits. The second group (K+), was infected by TB infection (Mycobacterium tuberculosis) strain H37Rv only. The third group (KOAT) consisted of rabbits with TB infection (Mycobacterium tuberculosis) strain H37Rv followed by oral anti tuberculosis (OAT) drug treatment. The fourth group (KOAT) consisted of rabbits with TB infection (Mycobacterium tuberculosis) strain H37Rv followed by oral anti-TB drug. The measurement of TNF-α level were done 2 weeks after the instrumentation and drug administration.

The rabbits anesthetized with intraperitoneal xyla (25mg/kg BW) and ketamine (50mg/kg BW). Spinal tissue biopsy was conducted by cutting across a 50 mm spinal transverse. Result of the biopsy was stored into a 2mL tube containing 10% formalin solution for histology and immunohistochemistry preparation.

Histochemical Preparation

For histopathological analysis examination, the tissue was processed for making preparations. Procedures m making paraffin preparation block were as follows the tissue was dehydrated using multilevel alcohol (30%, 50%, 70%, 80%, 96% and absolute) for 60 minutes each. Clearing with xylol was conducted twice each for 60 minutes. The following steps were

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infiltration with soft paraffin for 60 minutes at the temperature of 48°C, and block where hard paraffin was pour into mold and left for a day. The next day, the paraffin block was attached to a holder and cut into 4-6um thick with a rotary microtome. From each paraffin block cut, one preparation was stained with hematoxylin-eosin, while one other preparation was used for immunohistochemical staining.

Hematoxilen-Eosin Staining

Slides were washed with PBS pH 7.4 for 5 minutes and stained with hematoxilen for 10 minutes. After that, the slides were soaked in tap water for 10 minutes and rinsed with dH2O. The following steps were dehydration with 30% and 50% alcohol for 5 minutes each, and staining with Eosin solution for 3 minutes. Then the slides were rinsed with 30% alcohol, washed with dH2O for 5 minutes and air dried. The last steps were mounting with entanglement, and covering using cover glass.

Immunoperosidase towards TNFα and mt38

The preparations were deparafinized with xylene for 15 minutes and rehydrated with 100% and 70% alcohol for 10 minutes each. They were washed twice with dH2O, and incubated with PBS solution for 5 minutes. The preparations were stored in glass box filled with citrate buffer, and put into autoclave for 15 minutes to optimize their antigenicity. They are cooled at room temperature for one hour and after short drying, pap pen were used to draw lines on the tissue separating one from another. The preparations were washed with dH2O for 5 minutes and PBS for 5 minutes prior to incubation with 0.3% H2O2 for 15 minutes, rinsed with PBS pH 7.2 3 times for 5 minutes each. Incubation with blocking solution was conducted for 30 minutes. Overnight incubation at the temperature of 4°C was conducted with Mouse monoclonal TNFα: SantaCruz cat or Mouse monoclonal mt38. Rinse them with PBS pH 7.4 and incubate on secondary antibodies, anti-mouse IgG labeled HRP for 1 hour. Washed them with PBS pH 7.4 and incubated them on the substance for HRP, DAB for 5 minutes. Washed them With PBS pH 7.4 and then washed with PBS pH 7.4. Counterstain them with Mayer hematoxilen for 10 minutes. Rinsed them with dH2O. Drained and covered them with cover glass.

RESULTS

The study used tuberculosis strain H37Rv infection technique, by injecting 100 cfu (colony forming units) to the rabbits’ spine (L4). Rabbits became the samples because of the size of their spinal tissues and its relationship to instrumentation as well as their response and resistance to tuberculosis infection.

In the histopathological examination, tissue lesions of corpus vertebrae were identified as tissue reaction towards Mycobacterium tuberculosis bacteria on Figure 1. With 400x magnification, high monocyte distribution was found on the granuloma area. Figure 1 also showed the granuloma area with immunohistochemical staining using anti mt38. With 1000x magnification, positive result towards mt38, indicated by browning of monocyte cells (black arrow). Both histopathological and immunohistochemical examination showed good results of infection evaluation, proved by significant difference between all of the experimental groups compared to the control.

TNF-α Expression after Mycobacterium tuberculosis exposure on the Spine with Instrumentation

This study observed TNF-α expressions of bone tissue after Mycobacterium tuberculosis exposure on the spine with instrumentation using immunohistochemical technique with specific antibody. TNF-α has pivotal role in maintaining bone mass post-
birth combining bone resorption and bone formation. Findings showed significant expression of TNF-α in the spine of TB spondylitis after the administration of OAT drug as shown in Table 1. TNF-α also decreased significantly after the Instrumentation. Between the OAT drug and instrumentation, there was no significant difference, but combination between the two resulted in significant increase of TNF-α expressions compared to the control or the OAT drug only, and was not significant towards the instrumentation. Therefore, it can be concluded that instrumentation had major contribution towards TNF-α expressions in the spine of the TB spondylitis model in relation to healing process of the spine.

Table-1: TNFa Expressions based on the Experimental Groups

<table>
<thead>
<tr>
<th>GROUP</th>
<th>AVERAGE (pg/ml)</th>
<th>+ SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>708.4167</td>
<td>242.8874</td>
<td></td>
</tr>
<tr>
<td>K+</td>
<td>2938.0667</td>
<td>593.70993</td>
<td>0.000*</td>
</tr>
<tr>
<td>KOAT</td>
<td>1055.0833</td>
<td>162.63261</td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>742.5000</td>
<td>279.82472</td>
<td></td>
</tr>
<tr>
<td>KOAT + I</td>
<td>357.4167</td>
<td>278.76974</td>
<td></td>
</tr>
</tbody>
</table>

*One-way Annova test, post hoc Tukey HSD test; K- vs K+: p=0.000; K+ vs KOAT: p=0.000; K+ vs KI: p=0.000; K+ vs KOAT-I: p=0.000; KOAT vs KI: p=0.040; KI vs KOAT+I: p=0.038, KOAT vs KOAT: p=0.000

K+: infected group without treatment; KOAT: infected group treated with oral anti tuberculosis drugs only; KI: infected group treated with instrumentation only; KOAT+I: infected group treated with instrumentation and oral anti tuberculosis drug.

DISCUSSION

Tb Spondylitis in the Rabbit

In this study, inoculation of Mycobacterium tuberculosis bacteria into corpus vertebrae was conducted by making defect or drilling. Histopathological examination showed that the inoculation succeeded since the result of each examination was positive. Successful bacterial inoculation depends on several factors, namely host (rabbits), bacteria, and the environment. Host (rabbit) plays pivotal role in administration of this procedure (bacterial inoculation). The host should be kept in an optimum cage and environment so that Mycobacterium tuberculosis bacteria, injected to the rabbit, can live and grow well. It is important to select healthy sample so that the sample can survive until the end of this study. Technical factor is another important element in bacterial inoculation. Inoculation procedures conducted in this study are similar to those in previous study, which is osteomyelitis model on the bone of the host. An issue the researchers encountered is related to the type of bacteria used during the inoculation. Inoculation of Mycobacterium tuberculosis has never been conducted previously.

Technical factor can also contribute to nerve injury in the host, which is indicated by paralysis after the inoculation. Paralysis was immediately detected after the host was free from the influence of anesthetic drugs, and as the result, it is predicted that paralysis or decline in motor strength occurs due to direct injury to the nerve during the inoculation. In this case, paralysis occurred due to some errors in making defect on corpus vertebrae and as the drill bit hit the spinal cord. This technical error can be prevented by improving the exposure technique used during inoculation in order to obtain a better viewing point prior to making defect and in other procedures of inoculation. In this study, nerve injury may occur due to Mycobacterium tuberculosis bacteria that can cause infection to the spinal cord or compression of the nerves due to formation of puss, necrotic tissue in the spinal canal. This process can be identified several weeks after the inoculation.

Another aspect that influences the inoculation is type and preparation of Mycobacterium tuberculosis bacteria since the bacteria were obtained from the laboratory and therefore, it is predicted that its virulence is different from bacterial strain obtained directly from the environment. Concentration of the bacteria is another important element and is investigated in this study.

Concentration of Mycobacterium tuberculosis bacteria used in this study is between $10^8$ cfu/mL and $10^9$ cfu/mL. In general, $10^4$ - $10^6$ organisms/ ml are the requirement for detecting TB, the basis to determine the lowest concentration used in this study.

Inflammatory Cytokines in Tb Spondylitis

Mycobacterium tuberculosis is a unique bacterium in terms of its source of infection, route of infection, and characteristics of infection as well as: infection in the bone at first in the lung microbes then follow the bloodstream and filtered by regional lymph nodes and become a primary effect / primary complex that will heal or dormant.

At present, applications that focus on the immunological aspects are the best solution to provide better healing outcomes of spinal TB. The healing process is in line with the biomolecular and biosellular activities of the immunological process against TB microbes which are needed by the human body’s immune system to stop or kill spinal TB microbes.

There are many unique processes and actions regarding host – agent interactions, especially regarding the immunological process against TB which is controlled by the human immune system known as Mediated Immune Response (cell defense and cell products such as cytokines and growth factors). The archetorial of the inflammatory process and followed by the healing process is a complex and comprehensive route.

The literature states that TNF-α, IFN-γ, IL-6 and IL-7 are the cytokines that most often contribute significantly to TB infection. The activity of these
cytokines starts from the invasion and incubation of TB microbes to systemic spread / bacteremia. The microbial killing process will be followed by the regulation and coordination of cytokines, especially TNF-α, IFN-γ, IL-6 and IL-7. There are many types of processes to kill bacteria such as: a / direct killing by NK cells, b / apoptosis with granuloma formation, c / intra-cellular phagocytosis. All these processes are regulated by immunological defense processes and carried out by a mediated immune response, that is, cell defense with molecular products.

In conclusion, instrumentation using metal / titanium affects directly the immunological process in bone healing through the decrease of TNF-α caused by energy emissions produced by instrumentation. Further research is needed that provides clearer information about the process of resonance of Ti energy into the diseased tissue that activates the immunological process to eliminate the bacteria and enhance healing process.

REFERENCES


