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The Role of Instrumentation in the Healing Process of Spinal Tuberculosis: An Experimental Study

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Abstract

BACKGROUND: Tuberculosis (TB) is still commonly found in many developing countries. Spinal TB can cause vertebral deformity and neurological disorders. It was discovered 1000 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 using instrumentation to stabilize the deformity of infected vertebral segment and to restore and maintain neurological function. Transforming growth factor-beta (TGF-β) has a major role in angiogenesis in bone healing process. 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 taken by random sampling and divided into five groups, namely, control rabbits, infected rabbits without intervention, infected rabbits treated by instrumentation, infected rabbits given anti-TB drugs, and infected rabbits treated by instrumentation and given drugs. Then, the cytokine levels of TGF-β were evaluated and compared.

RESULTS: The results showed a significant TGF-β level increase in infected rabbits given drugs alone and instrumentation alone compared to infected rabbits without intervention. There was a significant TGF-β increase 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 TB by increasing the body's cytokine levels.

Introduction

Tuberculosis (TB) is a hypersensitive granulomatous infectious disease caused by *Mycobacterium tuberculosis*. TB is a dangerous bacterial infection that is responsible for increasing mortality. TB basil was discovered by Robert Koch in 1882. TB infection occurs due to spread through aerosols, and inhalation of several droplets containing *M. tuberculosis* bacilli. After infection, the pathogenesis of *M. tuberculosis* occurs in two stages. The first stage is an asymptomatic condition that can last for years on the host, which is called latent TB [1].

In 2018, an estimated 10 million people were infected by TB worldwide, as many as 5.7 million men, 3.2 million women, and 1.1 million children and as many as 1.5 million people died because of TB (including 251,000 people with HIV). In worldwide, TB is one of the ten leading causes of death and the main cause of one infectious agent (above HIV/AIDS). TB cases are spread in all countries and age groups. However, TB can be cured and prevented. There are 30 countries with high burden of TB infection which account for 87% of new TB cases. Eight countries accounted for two-thirds of the total cases, with Indonesia are the third after India and China followed by the Philippines, Pakistan, Nigeria, Bangladesh, and South Africa [2].

Spondylitis TB (TB spondylitis) is an infectious disease caused by the *M. tuberculosis* and attacks one or more vertebral bones. Also known as Pott's disease, it is one of the oldest human diseases, which has been documented in spinal fossils from the Iron Age and in ancient mummies from Egypt and Peru [3].

TB Spondylitis treatment in general is divided into two parts that work simultaneously, medical and surgical. Nine-month-opioid agonist therapy (OAT) provided a better remission (nearly 99%) compared

to 6-month OAT therapy. OAT alone can completely cure TB spondylitis only if diagnosis is made earlier, in which bone destruction and deformation are still pretty minimum. Similar to TB therapy, in general, therapy for TB spondylitis infection is multidrug therapy. In general, OAT regiment used in lung TB can also be applied for extra lung TB, but until recently, experts have not provided recommendation for how long OAT for extra lung TB should be applied yet. The World Health Organization suggested chemotherapy is administered for at least 6 months [4], [5], [6]. With the development of effective OAT administration, practitioners have started to leave relative surgical therapy as a major therapy in TB spondylitis. Spinal surgical techniques for spondylitis are varied, but a standardized and empirical surgical technique has not been established; each case has to be evaluated individually. For patients who will undergo surgery, chemotherapy must still be administered at least 10 days before OAT surgery. Administered OAT regimen category should match existing cases and be continued based on each category. Surgery conducted for TB spondylitis involves abscess drainage; radical debridement; bone insertion; arthrodesis or fusion; bone insertion; with or without instrumentation or fixation, both anterior and posterior; and osteotomy [6].

In the management of spinal TB, there are two groups of paradigms. The first paradigm is spinal TB therapy using non-operative/conventional techniques using OAT and external stabilization. In the early days of infection and the absence of severe damage and complications, this technique gives good results [7].

The second paradigm is spinal TB therapy using surgical techniques. The aim of this technique is to clean up the infected segment of vertebra (debridement) then the broken bone is replaced with healthy bone or graft. After debridement and grafting, stabilization of the vertebrae is done externally or internally using metal instrumentation. The purpose of this action is to avoid the sequelae of nerve complications and achieve recovery status with an almost normal spine. Pathological fractures/dislocations of infected vertebral bodies can occur due to mechanical processes. Decompression can add further instability when the infected corpus is cleaned. The insertion of metal implants aims to provide mechanical stability and the use of implants in TB infection is safe [8].

Instrumentation is a new procedure in the treatment of spinal TB to achieve the stable spinal segment, good integrity, increased recovery, which makes cell activity and inflammatory mediators increased, especially the angiogenesis process of the osteogenesis stages of the healing process [9]. There is a very precise statement which says “Rigid and stable fixation will block/negate the infection process” [10]. Oga *et al*. discovered about spinal instrumentation in TB infection that there was no effect of increased number of bacterial colonies and also no new growth colonies around the instrumentation. Since that time, the use of instrumentation caused debates between the pros and cons due to its use in bone infection. The results of the observational evaluation were very good, both in terms of achieving treatment and in eliminating residual deformity. What is interesting to the study here is the benefits of instrumentation and what mechanisms cause instrumentation to play a role in the total healing process of Spinal TB. This mystery has not been studied for its pathophysiology and specifically, but in reality, the use of instrumentation gives and shows very good results [11].

Immune cell, macrophage, is the first cell having interaction with biomaterial. Macrophages are responsible for cleaning wounds, inflammation, and recruiting tissue-generating cells. Macrophage is activated as pro-inflammation (M1) or anti-inflammation (M2); and activation of macrophage will control produced environmental micro-inflammatory response. Activation of macrophage is commonly characterized by pro-inflammatory and anti-inflammatory cytokines and chemokines cells produce. Objective of this study is to describe influence of surface microstructure and energy toward activation of macrophages and cytokine production. M2 anti-inflammatory response is shown by high level of interleukin (IL)-4, IL-10, and Transforming growth factor beta 1 (TGF-βl) [12], [13], [14]. Controlling this activation M2 will suppress response of immune system and facilitate formation of blood vessels and wound-healing [15], [16]. Surface of implant is the only part of biomaterial having interaction with human cells. Based on the data, modifying the surface will change response of immune system and cytokines the cells released. It results in recruitment of cells capable of regenerating tissue instead of chronic inflammation and fibrous encapsulation. Activation of macrophage can be induced or modulated by characteristics of the implant surface and eventually will change healing process and affect long-term stability of the implants. In general, chronic immune response will prevent formation of healthy tissue around the implant [17], [18]. The study aims to investigate TGF-ß expressions in TB spondylitis and their relationship with instrumentation.

The most commonly instrumentation used is metal equipment and whose structure is composed of ions and particles in micro to nano sizes. These particles interact with each other to produce a resonance and transmit energy that will stimulate the immune activity and healing of cells, enhance the healing process and fight TB microbes. The impact of this energy in influencing the healing process of spinal TB can be proven by measuring the cytokines that play a role in the healing process.

Based on the situation, this study investigates TGF-β expression as marker of bone healing process in spondylitis using New Zealand white rabbits as samples. This experimental study used experimental animals allowing control toward the variables. Several studies showed that New Zealand white rabbits can be

infected with *M. 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 3000 g as the samples. The samples were taken by random sampling. All of rabbits were placed in a cage for a week and given same treatment including food, water, and other treatment. The condition of the rabbit will be evaluated. TB spondylitis model was developed by infecting *M. tuberculosis* H37Rv strain on the spinal cord. The samples were divided into five groups. The first group was normal rabbits. The second group (K+) was infected by TB infection (*M. tuberculosis*) strain H37Rv only. The third group (KOAT) consisted of rabbits with TB infection (*M. tuberculosis*) strain H37Rv followed by single oral anti-TB OAT drug treatment, rifampicin. The fourth group (KOAT) consisted of rabbits with TB infection (*M. tuberculosis*) strain H37Rv followed by titanium instrumentation. The fifth group (KI+OAT) was rabbits with TB infection treatment (*M. tuberculosis*) strain H37Rv followed by instrumentation and combined with oral anti-TB drug. The measurement of TGF-β level was done 2 weeks after the instrumentation and drug administration.

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

TGF-β1 and mt38 distribution

Distribution of spinal tissue TGF-β1 was observed using immunohistochemical techniques (IHC) which consisted of the following stages.

Histochemical preparation

For histopathological analysis examination, the tissue was processed for making preparations. Procedures making paraffin preparation block were as follows the tissue was dehydrated using multilevel alcohol (30%, 50%, 70%, 80%, 96%, and absolute) for 60 min each. Clearing with xylol was conducted twice each for 60 min. The following steps were infiltration with soft paraffin for 60 min 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 4–6 um thick with a rotary microtome. From each paraffin block cut, one preparation was stained with hematoxylin-eosin, while one other preparation was used for IHC staining.

Hematoxilen-eosin staining

Slides were washed with phosphate-buffered saline (PBS) pH 7.4 for 5 min and stained with hematoxilen for 10 min. After that, the slides were soaked in tap water for 10 min and rinsed with dH_2O. The following steps were dehydration with 30–50% alcohol for 5 min each, and staining with Eosin solution for 3 min. Then the slides were rinsed with 30% alcohol, washed with dH20 for 5 min and air dried. The last steps were mounting with entanglement, and covering using cover glass.

Immunoperoxidase toward TGF-β1 and mt38

The preparations were deparafinized with xylene for 15 min and rehydrated with 100–70% alcohol for 10 min each. They were washed twice with dH_2O, and incubated with PBS solution for 5 min. The preparations were stored in glass box filled with citrate buffer, and put into autoclave for 15 min to optimize their antigenicity. They are cooled at room temperature for 1 h and after short drying and pap pen were used to draw lines on the tissue separating one from another. The preparations were washed with dH_2O for 5 min and PBS for 5 min before incubation with 0.3% H202 for 15 min, rinsed with PBS pH 7.2 3 times for 5 min each. Incubation with blocking solution was conducted for 30 min. Overnight incubation at the temperature of 4°C was conducted with Mouse monoclonal TGF-β1: SantaCruz cat or Mouse monoclonal mt38. Rinse them with PBS pH 7.4 and incubate on secondary antibodies. Anti-mouse IgG labeled horseradish peroxidase (HRP) for 1 h. Washed them with PBS pH 7.4 and incubated them on the substance for HRP, DAB for 5 min. Washed them With PBS pH 7.4 and then washed with PBS pH 7.4. Counterstain them with Mayer hematoxilen for 10 min rinsed them with dH_2O. Drained and covered them with cover glass.

Results

The study used TB 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 TB Infection.

Figure 1 In the histopathological examination, tissue lesions of corpus vertebrae were identified as tissue reaction toward *M. tuberculosis* bacteria on Figure 1. With 400× magnification, high monocyte distribution was found on the granuloma area. Figure 1 also showed the granuloma area with immunohistochemical staining using anti-mt38. With 1000× magnification, positive result toward 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.

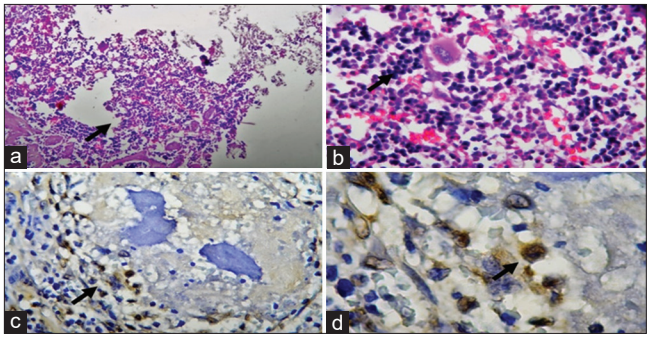

Figure 1: Representative result of histopathological examination using Hematoxilen-Eosin (a and b) staining and immunohistochemistry staining towards mt38 (c and d). (a) Inflammatory cell infiltrate, (b) datia Langhans cells (arrow), (c) immunohistochemical examination of the granuloma area, (d) positive reaction towards anti mt38 on the granuloma area (arrow). (a and c) Photographed at ×200 while (b and d) photographed at ×1000

TGF-β1 expression after M. tuberculosis exposure on the spine with instrumentation

This study observed TGF-β1 expressions of bone tissue after *M. tuberculosis* exposure on the spine with instrumentation using immunohistochemical technique with specific antibody as shown in Figure 2. TGF-βI has pivotal role in maintaining bone mass post-birth combining bone resorption and bone formation. Findings showed significant increase of TGF-βI expression in the spine of TB spondylitis after the administration of OAT drug as shown in Table 1. TGF-β1 also increased 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 TGF-β1 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 toward TGF-βI expressions in the spine of the TB spondylitis model in relation to healing process of the spine.

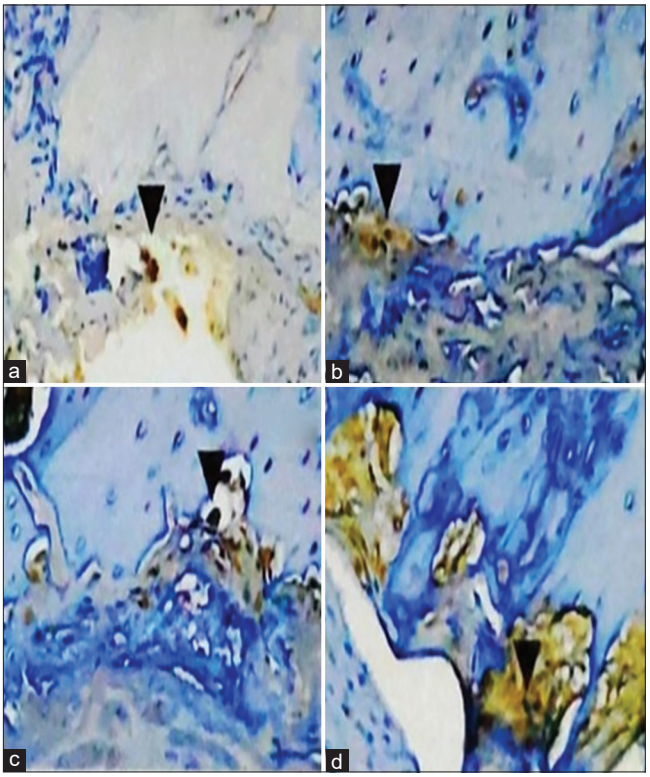

Figure 2: Representative micrographic photograph of the immunohistochemical examination towards transforming growth factor-beta I: (a) KP experimental group, (b). KOAT (group treated with oral antituberculosis drug only) (c) KI (group treated with instrumentation only) experimental group. (d) Opioid agonist therapy +I (group treated with instrumentation and oral anti tuberculosis drug) experimental group. Measured on brown callus tissues (arrow)

Discussion

TB spondylitis in the rabbit

In this study, inoculation of *M. 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 *M. 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.

Table 1: TGF-β1 expressions based on the experimental groups

Group	Average	+SD	p-value
K+	7.80	2.683	0.002*
KOAT	10.00	2.550	
KI	11.80	1.924	
KOAT + I	17.60	5.320	

*One-way ANNOVA test. *Post hoc* Tukey HSD Test: K+ versus KOAT: p = 0.047; K+ versus KI: p = 0.0001; K+ versus KOAT+I: p = 0.0001; KI versus KOAT+I: 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, TGF-β1: Transforming growth factor-beta.

Technical factor is another important element in bacterial inoculation. Inoculation procedures conducted in this study are similar to those in the 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 *M. 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 to obtain a better viewing point before making defect and in other procedures of inoculation. In this study, nerve injury may occur due to *M. 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 *M. 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 *M. tuberculosis* bacteria used in this study is between 10^6 cfu/mL and 10^8 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.

TGF-β1 in osteogenesis in Tb spondylitis

TGF-βI regulates growth, differentiation, migration, adhesion, and apoptosis. TGF-β1 initiates signal transduction through two different serine-threonine kinase receptors, called type I (TBRI) and type II (TbRII). TbRI is activated by TbRII after ligand binding. Activated TBRI phosphorylates the Smads receptor (R-Smad; Smad2; and Smad3), which, in turn, interacts with the general Smad mediator (Smad4) and translocation to the nucleus. The Smad-nucleus complex interacts with various transcription factors and transcription coactivators and will regulate the transcription of target genes. The target genes of TGF-β signaling transcription vary, one of which is the deferensing of osteoblasts. This study examined the expression of TGF-β1 in spinal tissue models or spondylitis TB given instrumentation. Referring to the results of the study, it appears that administration of instrumentation treatment showed a significant increase in expression of spinal tissue TGF-β1. This indicates that the provision of instrumentation provides significant results for the process of differentiating osteoblasts in relation to the process of bone healing (osteogenesis). Bone regeneration ability is based on three concepts, namely: Osteogenesis, osteoinduction, and osteoconduction. Osteogenesis described as the ability to produce new bone and is determined when there are osteoprogenitor cells and osteogenic precursor cells in the affected bone region. Platelet-derived growth factor (PDGF) is found in three to four stages of bone regeneration. Osteoinduction is defined as the ability to stimulate stem cells to differentiate into adult cells through stimulation of local GFs such as PDGF and TGF-β [19], [20]. TGF-β1 has excellent potential for bone regeneration because chondrocytes and osteoblasts have receptors that are compatible with TGF-β1. TGF-β also contributes to bone regeneration at every stage. The combination of PDGF, TGF-β, epidermal growth factor (EGF), insulin-like growth factor (IGF) optimally creates stimulation of differentiation, and proliferation of osteoblasts into osteogenic cells. Osteoblast proliferation is increased due to mitogenic action of PDGF in the differentiation of MSC when TGF-β and EGF are added [21], [22].

Instrumentation provides evidence for a significant increase in TGF-βI expression; this is possible because instrumentation gives effect to the supply of surface free energy. Some proteins (e.g. Fibronectin, vitronectin, laminin, serum albumin, and collagen) facilitate attachment of osteogenic cells to the titanium surface [23], [24]. Therefore, the binding capacity of implant surface proteins is considered an important factor for osseointegration success, due to surface properties, such as micro- and nano-topography [25], physicochemical composition [23] and surface free energy [26], has an influence on the expansion of protein adsorption. It has been documented that osteogenic cells are preferably attached to specific protein sequences, such as the arginine-glycine-aspartate motif. This motif is found in various extracellular matrix proteins, including fibronectin, vitronectin, laminin, and osteopontin [27]. Osteogenic cells attach to this motif by attaching themselves using their membrane receptors, referred to as integrins. Integrin-mediated cell attachment is very important for physiological and pathological mechanisms, such as embryonic development, maintenance of tissue integrity, circulation, migration, and leukocyte phagocytic activity, wound healing, and angiogenesis [28].

Conclusion

Instrumentation using metal/titanium affect directly the immunological process in bone healing

through the increase of TGF-β 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.

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