Effect of mesenchymal stem cells on Mycobacterium tuberculosis growth: in vitro study

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ABSTRACT

Background: TB infection remains one of the major health problems in Indonesia. While stem cells have been developed as new hope and challenge on medical aspect in treatments of various disease. Mesenchymal stem cells applications in treating tuberculosis infections remain controversial.

Objectives: To evaluate interactions between mesenchymal stem cells (MSCs) and Mycobacterium tuberculosis (MTB) in one co-culture growth.

Methods: This study is an experimental in vitro study, where MTB H37Rv strain and BMSCs were co-cultured in the single medium. MTB with 0.5 McFarland and 5000 cells/cm² of MSCs were co-cultured in RPMI medium, MTB 0.5 McFarland in RPMI and 5000 cells/cm² MSCs in RPMI are being our control groups. Acid-fast staining bacilli (AFB), PCR, TB culture were evaluated between the 3 groups on day 3, day 7, and day 9 of observation.

Result: AFB, PCR, and TB culture were found positive (+) in both TB control group and also the co-culture group (+) on day 3, day 7, and day 9. While the AFB, PCR, and TB culture in MSCs control group were found negative (-) on day 3, day 7, and day 9. Cyclus threshold PCR values between co-culture group and control groups were not significantly different (p>0.05). Viable MSCs are hardly found in co-culture group compared with MSCs control group. The difference was significant between co-culture group and MSCs control group (p<0.05).

Conclusion: In this co-culture study, MSCs do not affect MTB growth. Instead, there were suppression of Mesenchymal Stem Cells growth in this co-culture study.

Key Words: immunomodulator, mesenchymal stem cells, Mycobacterium tuberculosis, RPMI (Roswell Park Memorial Institute Medium), co-culture.


INTRODUCTION

Tuberculosis infection remains one of the major health problems in Indonesia. Tuberculosis prevalence in Indonesia reaches 100 per 100,000 population in 2009.¹,² Musculoskeletal tuberculosis accounts for 10% - 15% among all tuberculosis notifications in the non-industrialized world, including Indonesia. Musculoskeletal tuberculosis affects mostly in ostearticular structures, ranging from the spine (50%), hip (12%), knee and tibia (10%), ribs (7%), and multiple sites (3%).³

The primary focus of the disease is visceral (lungs, kidneys, lymph nodes), and musculoskeletal involvement occurs via hematogenous spread. The organisms are focused mostly in the metaphyseal region of the bone, which is the most vascularized area of the bone.⁴,⁵ Once the organisms deposited, they are ingested by mononuclear cells. Mononuclear cells then coalesce into epithelioid cells, and a tubercle is formed when lymphocytes form a ring around a group of epithelioid cells. Caseation then develops within the center of the tubercle. The host inflammatory response intensifies, resulting in exudation and liquefaction, and a cold abscess is formed.⁴

As being explained in the previous studies, tuberculosis infection caused by Mycobacterium tuberculosis (MTB) is due to the interaction of MTB with macrophages, as MTB being engulfed by macrophages. No exotoxins nor endotoxins are produced during tuberculous infections. Once being presented by Major Histocompatibility Complex (MHC) II, MTB induces osteolytic activities through chaperonin 10 (cpn 10) protein. Chaperonin 10 proteins are believed to have an important role as a potent stimulator for bone resorption and inhibit osteoblasts bone forming cells pathway.⁶

Stem cells have been developed as new hope and challenge on medical aspect in treatments of various disease. Mesenchymal stem cells (MSCs) are discovered in 1968 by A.J. Friedenstein, who defines MSCs are a subset of non-haematopoietic pluripotent cells found in adult bone marrow and are capable of differentiating into adipocytes, fibroblasts, and even myoblasts. Mesenchymal stem cells have been broadly known for the ability to self-renewal and differentiation into various mesenchymal elements, such as bone, fat, cartilage, and muscle.⁷

Over these recent years, there is growing understanding among the scientific community that many of infectious disease may be cured or controlled using stem cells. Mesenchymal stem cells (MSCs)
applications in treating tuberculous infections remain controversial. Mesenchymal stem cells are believed to have effective immunomodulator properties in order to antigen eradication.\textsuperscript{8,9,10,11} However, the exact immunomodulatory role of MSCs in MTB infections remains questionable. Previous in vitro study performed by Mensyuknil in 2011 revealed that MTB debris did not affect MSCs growth in Dulbecco’s Modified Eagles Media (DMEM) medium.\textsuperscript{12} In this study, we performed a co-culture study to evaluate the effect of Mesenchymal stem cells on \textit{Mycobacterium tuberculosis} a single medium.

**METHOD**

This study was an experimental in vitro study, performed during May – September 2013 in Laboratory of Microbiology, Faculty of Medicine University of Indonesia, using Roswell Park Memorial Institute (RPMI) medium as the co-culture medium between MSCs and MTB. MTB culture in RPMI and also MSCs in RPMI as our control groups. Minimum sample size was 9 for each group according to Federer’s formula calculation.

Our samples were \textit{Mycobacterium tuberculosis} isolate, ATCC H37RV strain, $10^4$ CFU/mL (= 0.5 McFarland) and Bone Marrow Mesenchymal Stem Cells (BMSCs), 5000 cells/cm$^2$. MSCs samples had been through the ISCT (International Society for Cell Therapy) criteria tests to define human MSCs.\textsuperscript{13} MSCs and MTB samples were cultured (prior the coculture study) separately in RPMI medium, and growth were seen in both MSCs and MTB culture in each RPMI medium.

MTB samples (H37RV) were previously cultured in Lowenstein-Jensen (LJ) medium for 14 days, and the MTB colonies count were $10^4$ CFU/mL. MTB samples then re-cultured and optimized in RPMI medium for 3 days. MTB colonies after optimization were $10^4$ CFU/mL. MSCs samples were cultivated in RPMI medium with Fetal Bovine Serum (FBS) 10% supplementation and antibiotics-antimycotics inside 37°C incubator, CO$_2$ 5%. MSCs cell count were 5000 cells/cm$^2$.

We provided 27 flasks (25 cc Tc Flasks) in this study. Nine flasks were being used in each group (9 flasks for MTB control group; 9 flasks for MSCs control group; and 9 flasks for co-culture group). We put 2.5cc MTB and 2.5 cc RPMI in each flask, as the MTB control group; 2.5 cc MTB and 2.5 cc MSCs in RPMI as the co-culture group; 2.5 cc MSCs and 2.5cc RPMI as the MSCs control group. All flasks were incubated in the 5% CO$_2$ incubator at 37°C. Three flasks from each group were observed on day-3, day-7, and day-9. We performed acid-fast staining bacilli (AFB) using Ziehl Nielsen staining, bacterial culture in LJ (Lowenstein-Jensen) medium, PCR (Polymerase Chain Reaction) real-time assay for MTB identification, and MSCs counting for each group.

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Flasks from 3 groups were observed under inverted microscope, 1 cc of each group was taken for PCR assay, 2cc for AFB and bacterial culture in LJ medium. Each flask was added 2cc trypsin and incubated for 7 minutes, 4cc inactivating medium was added afterward. After centrifugation, debris were taken and transferred to haematocytometer. MSCs counting using haematocytometer were performed under microscope, using cell counting formula (MSC cells: Viable cells x 2 x $10^{14}$ x volume (cc)). Statistical analysis using...
Table 3  Cyclus threshold comparison between co-culture group – MTB culture group – MSCs culture group

<table>
<thead>
<tr>
<th>Cyclus Threshold</th>
<th>MTB</th>
<th>MTB + MSC</th>
<th>MSC</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day-3</td>
<td>22.31 (13.44 – 31.17)</td>
<td>12.79 (12.70 – 13.26)</td>
<td>0.00 (0.00 – 0.00)</td>
<td>0.04</td>
</tr>
<tr>
<td>Day-7</td>
<td>12.72 (12.43 – 13.00)</td>
<td>12.49 (11.12 – 13.00)</td>
<td>0.00 (0.00 – 0.00)</td>
<td>0.07</td>
</tr>
<tr>
<td>Day-9</td>
<td>12.87 (12.34 – 13.39)</td>
<td>13.01 (12.92 – 15.47)</td>
<td>0.00 (0.00 – 0.00)</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Table 4  Cyclus threshold comparison between co-culture group and MTB culture group. The results revealed no significant difference.

<table>
<thead>
<tr>
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<th>MTB</th>
<th>MTB + MSC</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day-3</td>
<td>22.31 (13.44 – 31.17)</td>
<td>12.79 (12.70 – 13.26)</td>
<td>0.08</td>
</tr>
<tr>
<td>Day-7</td>
<td>12.72 (12.43 – 13.00)</td>
<td>12.49 (11.12 – 13.00)</td>
<td>0.77</td>
</tr>
<tr>
<td>Day-9</td>
<td>12.87 (12.34 – 13.39)</td>
<td>13.01 (12.92 – 15.47)</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Table 5  MSCs viable cells comparison between MTB culture group – MSC control group – Co-culture group

<table>
<thead>
<tr>
<th>MSCs Viable Cells</th>
<th>MTB (cells/cm²)</th>
<th>MTB + MSC (cells/cm²)</th>
<th>MSC (cells/cm²)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day-3</td>
<td>0.00 (0.00-0.00)</td>
<td>0.00 (0.00 – 950.00)</td>
<td>135620 (107000-137000)</td>
<td>0.05</td>
</tr>
<tr>
<td>Day-7</td>
<td>0.00 (0.00-0.00)</td>
<td>0.00 (0.00 – 1800.00)</td>
<td>138000 (137000-152000)</td>
<td>0.05</td>
</tr>
<tr>
<td>Day-9</td>
<td>0.00 (0.00-0.00)</td>
<td>0.00 (0.00 – 0.00)</td>
<td>193500 (180000-345000)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 6  MSCs viable cells comparison between MSCs culture group and the Co-culture group

<table>
<thead>
<tr>
<th>MSCs Viable Cells</th>
<th>MTB + MSC (cells/cm²)</th>
<th>MSC (cells/cm²)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day-3</td>
<td>0.00 (0.00 – 950.00)</td>
<td>135620 (107000-137000)</td>
<td>0.046</td>
</tr>
<tr>
<td>Day-7</td>
<td>0.00 (0.00 – 1800.00)</td>
<td>138000 (137000-152000)</td>
<td>0.046</td>
</tr>
<tr>
<td>Day-9</td>
<td>0.00 (0.00 – 0.00)</td>
<td>193500 (180000-345000)</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Picture 1  Tc Flasks Day-3 Observation: Inverted Microscope (a) MSCs control group showed a fibrous pattern. (b)MTB control group were filled with debris and bacteria. (c) Coculture group: no fibrous like pattern were seen, debris were found in the flasks

Picture 2  Tc Flasks Day 7 Observation: Inverted Microscope (a) MSCs control group showed more fibrous pattern cells. (b) MTB control group showed debris and bacteria. (c) In co-culture group, no fibrous pattern were found, debris (+) and bacteria (+)
pattern seemed more apparent over time, as seen respectively on day-3, day-7, and day-9 of the observation. While in the co-culture group, these fibrous patterns were not seen in day-3, day-7, nor in day-9 of the observation under inverted microscope. Instead, we found clumping debris in all over the visual field (microscope). In the MTB control group, flasks were filled with abundant debris and bacteria.

**Acid Fast Staining (AFB) Evaluation**

One cc of each flask was taken for AFB evaluation, using Ziehl-Nielsen staining, and was observed under microscope (1000x). In the MTB culture (MTB control group), AFB results were all positive (+) in all samples within observation on day-3, day-7, and day-9. Positive (+) results were also found in the co-culture group (MTB + MSCs) within observation on day-3, day-7, and day-9. While negative results (-) were found in the MSCs control group within observation on day-3, day-7, and day-9.

**Bacterial Culture**

All flasks in each observation day were taken for bacterial culture in LJ medium. The samples were cultured in LJ medium for 14 days. Positive (+) results of bacterial culture is defined when granular colonies of bacteria (buff, rough, and tough) are seen in the LJ medium.

All bacterial cultures samples of the MSCs culture group were found negative (-) within observation on day-3, day-7, and day-9. Meanwhile, all bacterial cultures of MTB culture group and co-culture group were found positive (+) within observation on day-3, day-7, and day-9.

**Polymerase Chain Reaction Assay**

PCR qualitative assay in observation on day-3, day-7, and day-9 revealed the similar result of proportion as shown in the AFB and bacterial culture in LJ medium. We found all negative (-) results of MTB identification through PCR assay in the MSCs culture group. Positive results (+) were found in both the co-culture group and the MTB culture group.

We also performed PCR Real-Time Assay in each observation for each flask, and to compare the cycling threshold results between co-culture group, MTB culture group, and the MSCs culture group. The median comparison between the 3 groups were analyzed by using Kruskal Wallis test (Day 3: $p = 0.04$; Day 7: $p = 0.07$; Day 9: $p = 0.07$). There were no difference in the cycling threshold value between MTB
culture group and the co-culture group. The median comparison were analyzed using Mann-Whitney test (Day 3: p = 0.08; Day 7: p = 0.77; Day 9: p = 0.56).

**MSCs Counting**
MSCs cell count in each group was evaluated using haematocytometer under microscope. There were no significant viable cells seen in the MTB culture group and the co-culture group, in each observation within day-3, day-7, and day-9. The comparison of viable cells between 3 groups were analyzed using Kruskal Wallis test, while comparison of MSCs viable cells between MSCs group and the co-culture were analyzed using Mann-Whitney test (Day 3: p = 0.046; Day 7: p = 0.046; Day 9: p = 0.037).

**DISCUSSION**
The effect of Mesenchymal Stem Cells on Mycobacterium tuberculosis remains controversial. This study is the first in vitro study in Indonesia to learn the co-culture effect between MSCs and MTB in single medium.

**RPMI as Medium**
It is very difficult to determine the suitable medium to perform co-culture between MSCs and MTB. The medium should have the ingredients that provide growth for both MSCs and MTB. It is universally known that most suitable medium to grow MSCs is in Dulbecco’s Modified Eagles Media (DMEM), and in the other hand, LJ or Mycobacterial Growth Indicator Tube (MGIT) is the most suitable medium for MTB growth.7,12

Roswell Park Memorial Institute (RPMI) medium contains a great amount of phosphate and is formulated for use in 5% CO₂, and has traditionally been used for lymphoid cells culture. Commonly used RPMI is the RPMI 1640.14 RPMI can be used as a medium for culturing MSCs, but not as superior as MSCs being cultured in DMEM.15,16

Some studies revealed that RPMI can be used as a culture medium for MTB, even though RPMI will not be the standard medium for MTB culture.14,17

MSCs and MTB were being cultured in RPMI prior to this co-culture study, and both of MSCs and MTB were successfully grown in the RPMI medium.

**MSCs Effect on MTB Growth**
Using 5000 cells/cm², most effective amount of MSCs that provide efficient cell adhesion, and 10⁴ CFU/ml of MTB in this co-culture study revealed that MTB grows in the co-culture group and in the MTB culture group.18 MTB growth are being identified using AFB, bacterial culture in LJ medium, and by PCR assay.19,20,21 On AFB staining, the co-culture group showed more densed MTB compared to MTB group especially on day-7 and day-9 of observation. And on bacterial culture in LJ medium, both MTB group and co-culture group defines the same positive (+) result. All evaluations for MTB existence/growth in co-culture confirmed that MSCs have no eradication effect on MTB. This result was contradic- tive to the previous study performed by Rahyussalim (2013) that showed there was a significant influence on MTB by MSCs in vivo.22

Based on the evaluation of the MSCs viable cells count, it was shown that MSCs failed to grow or hardly seen in the co-culture group. In this in vitro study, Mycobacterium tuberculosis somehow suppress MSCs growth. The effect of MTB on MSCs growth creates another question, whether there was only a “medium competition” or MTB might produce toxins or virulence factors that affect cells growth.23

This study is an experimental in vitro study, and the samples are quite limited by the small sample size. However, an in vivo study would be required to assert the co-culture between mesenchymal stem cells and co-culture between mesenchymal stem cells and Mycobacterium tuberculosis, and to explain the “phenomenon” resulted in this study.

**CONCLUSION**
Mesenchymal stem cells (MSCs) do not have eradication effect on Mycobacterium tuberculosis. And Mycobacterium tuberculosis somehow suppresses mesenchymal stem cells growth.

**REFERENCES**


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