Effect of *Staphylococcus aureus* and *Staphylococcus epidermidis* Debris and Supernatant on Bone Marrow Stromal Cells Growth

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ABSTRAK

Tujuan: *mengetahui pengaruh debris dan supernatant bakteri SA dan SE pada pertumbuhan BMSCs.* ***Metode:*** *bakteri SA dan SE diisolasi dan dikultur dari bahan lesi pasien dengan spondilitis yang ditentukan dengan pewarnaan Gram dan uji biokimia. Kultur BMSCs diinkubasi selama 11 hari lalu dilanjutkan dengan sub kultur dan tripsinasi untuk dihitung jumlah selnya sebelum disemai ke dalam cawan biakan 12-well. Delapan jam setelah penyemaian BMSCs, sejumlah 0,1 mg/mL debris dan supernatant dari bakteri Staphylococcus aureus dan Staphylococcus epidermidis ditambahkan ke cawan biakan 12-well kemudian ditambahkan media kultur. Penghitungan jumlah sel pada setiap cawan dilakukan 2, 5, 7 dan 9 hari setelah penambahan debris dan supernatant untuk mendapatkan profil pertumbuhan BMSCs.* ***Hasil:*** *debris dan supernatant menyebabkan penurunan jumlah BMSCs sejak awal interaksi. Pada hari ke lima interaksi, BMSCs mengalami pertumbuhan. Setelah itu, BMSCs dari kelompok debris mengalami penurunan jumlah sel, sementara BMSCs dari kelompok supernatant mampu mempertahankan jumlah sel. Viabilitas dari semua kelompok lebih dari 80%.* ***Kesimpulan:*** *baik debris maupun supernatant bakteri SA dan SE memiliki pengaruh menghambat pertumbuhan BMSCs sejak awal interaksi. BMSCs tampak seolah-olah berusaha bertahan hidup dari pengaruh debris dan supernatant kedua bakteri pada hari kelima.BMSC dapat memanfaatkan supernatant untuk menggantikan kekurangan nutrisi untuk dapat bertahan hidup.*

Kata kunci: *Staphylococcus aureus, Staphylococcus epidermidis, debris, supernatant, sel stroma sumsum tulang.*

ABSTRACT

Aim: *the goal of this study is to observe the effect of SA' and SE' debris and supernatant on BMSCs growth.* ***Methods:*** *SA and SE were isolated and cultured fromlesion materials of spondylitis patientswhich were determined by gram staining and biochemical tests. BMSCs were cultured and incubated for 11 days to be further sub-cultured and tripsinized for cell counting before seeded. 0.1 mg/ml SA' and SE' debris and supernatant were added into the BMSCs culture media. Cell counting was performed 2, 5, 7, and 9 days after debris and supernatant addition to get the growth profile of BMSCs.* ***Results:*** *debris and supernatant decreases BMSCS number at initial day. At day 5, BMSCs in the group debris were growing down, mean while BMSCs in the group supernatant were able to retain the cell number. Viability of all groups was more than 80%.* ***Conclusion:*** *both debris and supernatant*

from SA and SE have inhibitory effect of the growth BMSCs in the initial day. BMSCs could provide barrier to survive from the debris and supernatant environment of both bacteria in day five. BMSCs can use supernatant to retain the growth to replace the lack of nutrition.

Key words: *Staphylococcus aureus, Staphylococcus epidermidis, debris, supernatant, bone marrow stromal cells.*

INTRODUCTION

The potencies of bone marrow stromal cells (BMSCs) for therapy purposes are well-known. BMSCs were used for various abnormalities in cartilages, bones, adipose tissues and muscles, even in cases of non-union long bone fractures and osteoarthritis of knee.BMSCs have been scientifically proven and applied in humans, although still limited for research purposes.[1-4]

BMSCs are adult multipotent stem cells found in bone marrow and other locations, which have the capacity to develop into many cell types such as osteoblasts, adipocytes, and chondro blasts in-vitro conditions.[1,5-9] Currently, in-vivo studies show that BMSCs are likely to have beneficial effects in the treatment of infections caused by bacteria.[10-14] BMSCs has immune capability to face against bacterial infection.

Infection of the human spine can be caused by various types of bacteria.[8] The three most common bacterias are Mycobacterium tuberculosis, Staphylococcus aureus (SA) and Staphylococcus epidermidis (SE). Staphylococcus genus consists of groups of pathogenic and non-pathogenic organisms, which does not produce endospores but is very resistant to drying, especially when associated with organic matter such as blood, pus, and fluid from other tissues.[2] SA is a commensal bacterium that can be found everywhere such as on human skin, but this bacterium can cause severe infection both acutely and chronically.[2] Similarly, SE has the same characteristics as SA and they both have many resistancies to antibiotics.[3,4,8,9]

In Indonesia, beside spinal infection (spondylitis), post-surgicalinfection is often found too.[9] Based on the report of infection control team at the Department of Surgery RSCM hospital, there are approximately three to four spondylitis surgeries done very month and 35 out of 700 spondylitis cases developed post-surgical pseudo-arthrosis in 10 years. This is occurred due to awide defect of the corpus vertebrae (more than one corpus) which cause difficulty to achieve a stable fusion. This unstable spine will cause pseudo-arthrosis which severely degrade the patient's quality of life.

Therefore, BMSCs become the only best option expected to solve this pseudo-arthrosis problem. The use of BMSCs in cases of vertebral defect caused by the infection becomes a challenge.[10-14]

BMSCs and bacteria are expected to interact one another. There are many possibilities that can happen in BMSCS such as growth rate degradation, death, distortion of differentiation while on the other hand, it could halt the growth of the bacteria. The interactions that occur will greatly influence the decision whether the use of BMSCs to overcome a wide defect of the spine due to the infection process is reasonably acceptable.[1,5,6,16]

An investigation on the effects of SA and SE on BMSCs growth was conducted. The goal of this study is to observe the effect ofSA and SE debris and supernatant on BMSC's growth.

METHODS

SA and SE used in this study were derived from lesion material of spondylitis patients in RSCM hospital that had been isolated at the Laboratory of Clinical Microbiology, Faculty of Medicine University of Indonesia (FMUI) between Septembers to October 2010.

This study was divided into 4 steps: Step 1, Isolation SA and SE from spondylitis lesion material; Step 2, Preparation of supernatant and debris SA and SE; Step 3, Isolation and culture of BMSCs; Step 4, Interaction between Debris and Supernatant of S. aureus and S. epidermidis against BMSCs in-vitro.

Isolation SA and SE from Spondylitis Lesion Material

Two weeks prior sampling, the patient with spondylitis were not allowed to consume any antibotic drugs in order for the bacteria to live in the culture medium.[7] First, the lesion materials

(solid, semi-solid and liquid) were taken from the patients' vertebrae through surgery. Solid material including bone sequester, yellow ligament, lamina; semi-solid material including granulation tissue; and liquid material (pus). They were then separated by the surgeon, put in tube, then immediately sent to the microbiology laboratories to underwent bacterial morphologyexamination by staining and culturing techniques via selective and differential medium.[2,3]

Next, all lesion material put in the thioglycolate liquid medium, were incubated at 37°C for 24 hours. A change in color or turbidity of the medium indicated bacterial growth which then isolated with incised plate method. The isolate can be derived by taking 1-2 osemedium thioglycolate liquid and aseptically streaking them on the medium surface in a 9 cm petri dish, then incubated in an incubator at 37°C for 24 hours. If after 24 hours there was bacterial colony growth, the colony's shape and color werenoted. Then, preparation was made for microscopic bacterial examination (gram staining) along with differential culture medium and biochemical test to determine bacterial species. The biochemical tests conducted were carbohydrate test (glucose, lactose, sucrose and mannitol, starch hydrolysis test), methyl red test, citric test, triple sugar iron agar (TSIA) test, catalase test, niacin and motility test.[7]

Preparation of Supernatant and Debris SA and SE

SA and SE stock bacteria were cultured on blood agar medium and then incubated again at 35°C for 18 hours.[2-4] One colony of the bacteria were then sub-cultured in serum + DMEM medium and incubated in a shaker incubator at 35°C, with rotation speed of 200 rpm for 18 hours. After that, supernatants were separated from bacterial cells by centrifugation using a Beckman centrifuge at 3000 rpm for 10 minutes. Next, a filtration was done to supernatants obtained, to filter out bacteria that were present insupernatant. Supernatant was then filtered andstoredat -20°C.[7]

Pellets (debris) of bacteria obtained from the next centrifugation process were washed three times using sterile H_2O to remove residual of DMEM medium. Pellets were then dissolved in 300 µl of sterile H_2O and were incubated at a temperature of 100°C for 15 minutes. Pellets of bacteria that had become debris were stored at-20°C.[7]

Isolation and Culture of BMSCs

Stem cells were isolated from marrow material of the iliac bone patient with spondylitis. Stem cells isolate was diluted by adding phosphate buffered saline (PBS) solution 1:1. The mixture was centrifuged (2500 rpm, 15 min) at a temperature of 20°C. Pellets were re-suspended in DMEM medium (Dulbecco's Modified Eagles Medium) and moved to a few bottles of culture (Tc-Flask) for being cultured in DMEM medium containing 1000 mg/l D-Glucose, 1000 mg/l L-Glutamine, 110 mg/l sodium pyruvate and 10% Fetal Bovine Serum (FBS), then were incubated for one week at 37°C with a flow of 20% oxygen and 5% CO_2. At the end of the first week, the medium was carefully removed and the culture was washed several times using PBS to remove red blood cells and other cells that were not adhered. Every three days the medium was replaced with fresh culture medium. Culture bottles were examined the nucleated cell adhesion to achieve 75-80% density (confluent). The cell adhesion was released to be harvested by washing them with 20 ml of PBS, then it was released from the flasks surface by adding 4 ml trypsin-ethylene diamine tetra acetic acid (EDTA) and incubated in a CO_2 incubator at temperature of 37°C for five minutes. Ten milliliters DMEM medium containing 10% FBS was added to stop the trypsization reaction and the cell suspension was centrifuged for three minutes at 3000 rpm for then to be stored in liquid nitrogen and re-suspended in DMEM medium upon usage.[11-13]

Interaction Between Debris and Supernatant of S. aureus and S. epidermidis Against BMSCs in-vitro

Cryopreserved BMSCs were thawed and washed in PBS before seeding into Ø10 cm plate of 10.000 cells/cm^2 seeding density. They were culturedfor 11 days and then sub-cultured on day 11. Cells were trypsinized and counted before they were seeded into 12-well plate (seeding density 7.000 cells/cm^2). Eight hours after seeding, 0.1 mg/ml SA and SEexotoxin (supernatant) were added into the culture media. Cell counting was performed 2, 5, 7, and 9 days after toxin addition to get the growth profile. All experiment was done three times.[5-6]

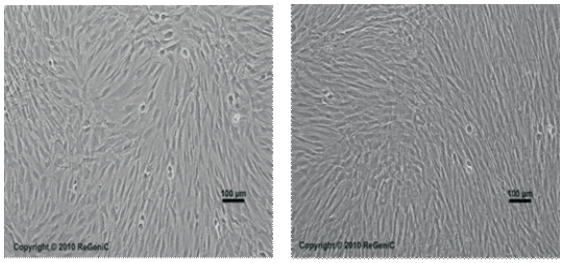
RESULTS

Pellets and Supernatant of Bacteria

Four patients with spondylitis were taken solid (sequester and necrotic tissue) and liquid (pus) materials which were further isolated to derive the bacteria identified as SE and SA. From SA bacteria, 3 samples of supernatant were obtained with concentration of 0.193 mg/mL, 0.187 mg/mL, and 0.230 mg/mL respectively and 3 samples of pellets (debris) were also obtained with concentration of 0.569 mg/mL, 0.543 mg/mL, 0.546 mg/mL respectively. From SE bacteria, 3 samples of supernatant were obtained from with concentrations of 0.534 mg/mL, 0.577 mg/mL, 0.589 mg/mL respectively and 3 samples of pellets were also obtained with concentration of 0.746 mg/mL, 0.756 mg/mL, and 0.760 mg/mL respectively.

Isolation and Culture of BMSCs

At day 17, the confluence reached 85-90% and at day 21 it reached 100%, BMSCs were evenly distributed, the colony was dense. There were less unfilled gaps of BMSCs and the accumulated growth of BMSCs started to appear in some colonies. (**Figure 1**)



A. B.

Figure 1. (A) The confluent of 85-90% was reached at day 17; (B) The confluent of 100% was reachedat day 21.

The Effects of Bacterial Supernatant on the Growth of BMSCs

The results of measurements BMSCs growth in control, SAs and SEs on 2, 5, 7 and 9 day can be seen on **Table 1**. When compared to controls, the effects of each of supernatant on the growth of BMSCs up to day 9 can be seen on **Figure 2**. BMSCs viability affected by SA, SE supernatant or control BMSCs was above 88 percent in average (**Figure 3**).

Table 1. The results of measurements BMSCs growth in control, SAS and SES

Day	Medium	Cell number	SD of cell number	Viability	SD of viability
0	SAS	24500		91.65	4.51
2		19892	2966.09	88.68	4.12
5		33881	4866.72	95.96	1.18
7		32667	2504.16	91.14	2.94
9		33011	2768.27	89.6	7.85
0	SES	24500		91.65	4.51
2		20808	793.86	96.19	1.25
5		28167	2020.73	93.19	4.63
7		31167	3449.03	89.76	2.82
9		29641	2781.9	89.35	6.28
0	SAD	24500		91.65	4.51
2		11825	5520.86	96.98	2.87
5		29167	7324.33	89.56	3.25
7		29000	4205.65	83.72	1.93
9		17652	2599.2	91.62	6.44
0	SED	24500		91.65	4.51
2		10083	2713.09	93.28	7.18
5		27917	1181.45	93.58	3.14
7		27417	946.48	87.16	5.43
9		19088	1270.17	85.86	7.59
0	Control DMEM	24500		91.65	4.51
2		26492	5147.23	89.1	0.98
5		31083	3013.86	95.4	3.67
7		40750	7578.75	93.61	0.65
9		27267	3222.71	93.74	3.13
0	Control DMEM + H2O	24500		91.65	4.51
2		30917	1127.31	93.89	3.39
5		33692	5479.71	93.18	1.3
7		43167	7417.60	94.35	2.9
9		38122	9782.17	80.74	11.14

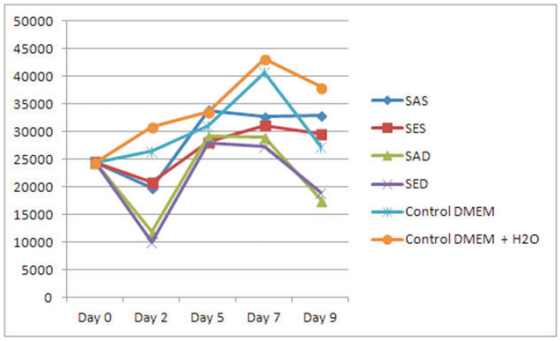


Figure 2. The graphic BMSCs growth in SA and SE Debris (D) and Supernatant (S) compared to control

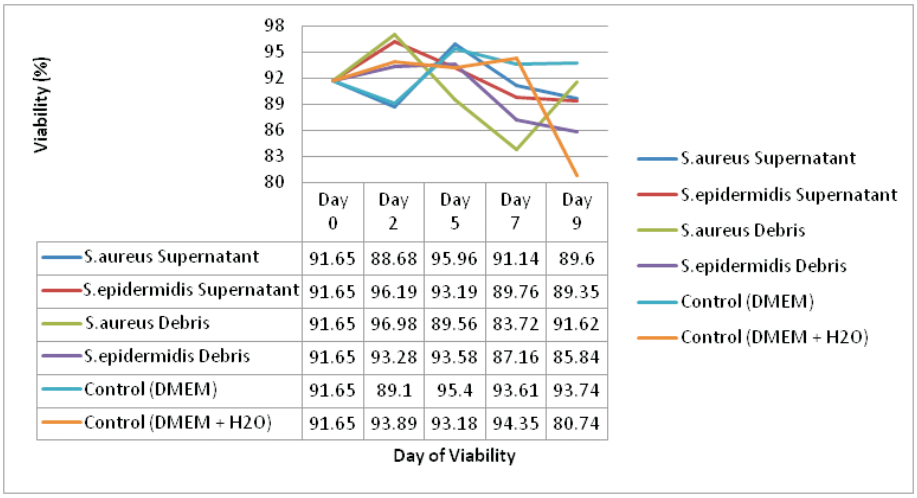


Figure 3. Histogram showed the viabilityis above 80%.

The Effects of Bacterial Debris on the Growth of BMSCs

The results of measurements BMSCs growth in control, SAs and SEs on 2, 5, 7 and 9 day. When compared to controls, the effects of each of debris on the growth of BMSCs up to day 9. BMSCs viability affected by SA, SE debris or control BMSCs was above 80 percent in average.

DISCUSSION

This study is an in-vitro experimental study trying to observe the effects of debris and supernatant of SA and SE on the growth of BMSCs. It is different from the search conducted by Anna Krasnodembskaya et al.[1] which investigated the characteristics of BMSCs immunities to bacteria. Anna suspected and found that the application of stem cells on patients with sepsis resulted in survival improvement and increasing patient's immunity against infection pathogenicity.[1]

Moreover, the bacteria used were SA and SE cultured from the lesion material of spondylitis patients in RSCM hospital. The bacteria were taken directly from patients with the intention that the results obtained could be applied directly on patients in RSCM hospital. This is important, so there will not be any doubt in the application of stem cells in cases of infection in the spine. In addition, the literature does mention that the bacteria caused infections of the spine for the pyogenic infections, SA and SE were top ranked as the cause of the infection.

Cocci bacteria are known to produce exotoxin (supernatant) in defending its life in addition to endotoxin (debris) which already integrated in the cell walls of bacteria and the cell nucleus. In this study, we distinguished the study materials for each bacterial debris and supernatant. These toxins were produced by centrifugation to obtain supernatant containing exotoxin and bacterial debris containing endotoxin.[2-4]

BMSCs were derived from iliac bone marrow of patients with non-infectious problems. The bone marrow was taken and cultured. This step was done to avoid bias on exposure of bacteria on BMSCs.

In general, the effects of debris and supernatant both derived from SA and SE resulted in inhibition of growth of BMSCs at the initial day. From the graph displayed on the results it can be seen that the pattern from debris and supernatant group are relatively different. From the graph it can be seen that endotoxin (of debris) influences in the inhibition of BMSCs growth more strongly compared to exotoxin (supernatant). These result implies that BMSCs applications in case of infection should consider the presence of this debris. Conducting adequate debri dement thus minimizing the amount of bacterial debris, should be a major prerequisite in addressing the use of stem cells in cases of infection.

An increase in the number of BMSCs on day five indicates BMSCs as if they experienced an adaptation to the presence of toxin. However, BMSCs attempted to grow again by (which mechanism has not discovered yet in this study), toxin neutralisation or a spesific immune system pattern that BMSCs strives to resist against the

surroundings (toxin) to be able to proliferate. Therefore, the chart pattern on day five shows as if BMSCs attempt to catch up the normal growth chart.

Debris of SA and SE decreases the number of BMSCs at the initial day. But at day 5, BMSCs adapted to the debris, although it did not succeed, resulting in a growing down of most cells (including control) in day 9.

Supernatant of SA and SE also decreases the number of BMSCs at the initial day. At day 5, BMSCs adapted to supernatant. Hence it can retain the number of cells until day 9. At day 9, most of the cells are growing on relatively the same amount, but are higher than control.

At day 9, BMSCs in the "control" group are growing down because of lack of nutrition. Debris suppressing the BMSCs growth was stronger than the supernatant supressing the BMSCs growth. BMSCs in the group of "supernatant" can retain the cell growth.So that in day 9, the amount of cells are more plenty in the supernatant group than the "control" group.

BMSCs viability of both "debris" and "control" group are more than 80%. While BMSCs viability of both "supernatant" and "control" group are more than 88%. BMSCs viability of all groups therefore is more than 80%. Debris and supernatant does not influence the viability of BMSCs (viability >80%).

CONCLUSION

The characteristics of debris (endotoxin) and supernatant (exotoxin) should be known in related to its effects against the BMSCs growth.Both debris and supernatant from SAand SE have the effects of inhibiting BMSCs growth in the initial day. BMSCs could provide resistance to survive from exotoxin and endotoxin environment of the two bacteria in day five. BMSCs can use supernatant to retain growth as a replacement of nutritional deficits.

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