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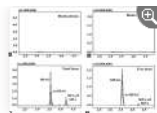
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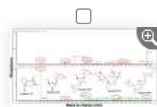


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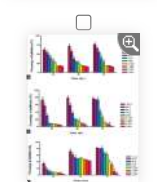


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The enhancement apoptosis of osteosarcoma mesenchymal stem cells co-cultivation with peripheral blood mononuclear cells sensitized by secretome and granulocyte macrophage colony-stimulating factor

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ABSTRACT

The advanced, metastasis, and recurrent of osteosarcoma (OS) patients have a poor prognosis postaggressive surgery and chemotherapy. Peripheral blood mononuclear cells (PBMCs) as cell-based immunotherapy may successful in the OS treatment. To investigate the enhancement apoptosis of OS-mesenchymal stem cells (OS-MSCs) co-cultivated with PBMCs sensitized using the secretome and granulocyte macrophage colony-stimulating factor (GM-CSF). This true experimental study with posttest only control group design and *in vitro* study. The sample was cultured OS-MSCs which confirmed by Cluster of Differentiation-133 using immunocytochemistry (ICC) and histopathology analysis. The sample divided into six groups accordingly: OS-MSC, OS-MSC + PMBC, OS-MSC + PMBC + Secretome, OS-MSC + PMBC + GM-CSF, OS-MSC + PMBC + Secretome + GM-CSF ($n = 5/N = 30$). The enhancement of OS-MSCs apoptosis was analyzed through Interleukin-2 (IL-2) level through the Enzyme-Linked Immunosorbent Assay examination, expression of Signal Transducers and Activators of Transcription (STAT)-3 and caspase-3 by ICC. One-way analysis of variance test and Tukey Honestly Significant Difference to analyze the difference between the groups ($P < 0.05$). The highest of IL-2 level was found in the PMBC + Secretome + GM-CSF group. The highest expression of caspase-3 was found in OS-MSC + PMBC + Secretome + GM-CSF group with significant different between groups ($P < 0.05$). There was insignificant difference of STAT-3 expression and IL-2 level between groups ($P > 0.05$). The co-cultivation of OS-MSCs and PBMCs activated using secretome and GM-CSF has a great ability to enhance OS-MSCs apoptosis.

Key words: Granulocyte macrophage colony stimulating factor, mesenchymal stem cells, osteosarcoma, peripheral blood mononuclear cells, secretome

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INTRODUCTION

Osteosarcoma (OS) is a malignant tumor with neoplastic cell producing bone that the most common primary sarcoma

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arises from the bone tissues.^[1] Treatment for the newly diagnosed OS including three main modalities: presurgical chemotherapy, surgical resection, and postsurgical chemotherapy that increased the outcome of localized OS cases. However, on the advanced, metastatic, and recurrent OS cases remain to have a poor prognosis. After treated aggressively by resection and chemotherapy, the 5-year survival rate on the localized OS about 65% and <20% for metastatic cases.^[2]

Recent treatment failure is related to the inability to target cancer stem cells due to capability of self-renewal, increase tumorigenicity, and chemotherapy resistance.^[3,4] Cell death resistance and immune screening avoidance are the two main mechanisms of tumor growth. Reinforcing and intensifying the cancer recognition by the immune system can improve the treatment efficacy. Formerly, necrosis was considered as the only cell death mechanism that affects the immune system and stimulates inflammatory response. Otherwise, apoptosis was considered as physiologic cell death pathway without immune stimulation. It is discovered that apoptotic cells were rapidly and specifically recognized by a phagocyte. Compared to classic apoptosis or immunogenic cell death can stimulate and intensify the immune system and response.^[5,6]

Apoptosis occurs through caspase-dependent and caspase-independent pathways. The caspase-dependent pathway implicates the extrinsic and intrinsic factors. Activated cytokine receptors on the membrane cancer cells will induce Janus Kinase (JAK) and Signal Transducers and Activators of Transcription (STAT-3 and STAT-5). Those activations detain Bcl-xL and activate the Cytochrome-C. Subsequently, apoptotic protease activating factor-1 (APAF-1) will be increased then activate caspase-9 and stimulate caspase-3 that lead to apoptosis of OS cells.^[7,8]

Nowadays, cell and antibody-based immunotherapy have gained success in childhood cancer management. Peripheral blood mononuclear cells (PBMCs) that considered superior to other cell-based treatment sources due to isolation process is less invasive compared to bone marrow. Moreover, autologous PBMCs administration does not need long-term immunosuppressive treatment and can be performed without ethical problem.^[9,10]

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a potent cytokine on stimulating the differentiation of myeloid cells. Furthermore, GM-CSF has a role as an additional immune stimulus to generate anti-tumoral immunity. GM-CSF also plays an important task in dendritic cells differentiation.^[11,12]

The other new treatment modality is stem cell-based application, such as mesenchymal stem cells (MSCs) secretome.^[13] Secretome application is also considered safer

due to containing no cellular element that makes it is less possibility of mutation and transforms into cancer-associated fibroblast within cancer microenvironment.^[14,15,16]

GM-CSF, as well as secretome, will increase PBMCs immunogenicity by inducing it's containing immune cells. Thus, The aim of this study is to investigate the enhancement apoptosis of OS-MSCs cultivated with PBMCs activated by secretome and GM-CSF sensitization *in vitro*.

SUBJECT AND METHODS

Isolation, expansion, and culture of osteosarcoma mesenchymal stem cells

OS tissues were obtained from an open biopsy of patient at Orthopaedic Department Dr. Soetomo General Hospital Surabaya. Patient was confirmed and filled the written informed consent. The tissues firstly were confirmed as OS by histopathology. The tissues were washed by phosphate-buffered saline (PBS) (Sigma Aldrich, US) containing 5% penicillin/streptomycin (Gibco, US). Tissue samples were sliced using a scissor then put into Erlenmeyer tube with 5 ml Type I collagenase 0.075% within PBS containing 2% of penicillin/streptomycin. Samples were stirred with a magnetic stirrer for 30 min on 37°C temperature. Then 5 ml of α -minimal essential medium (Gibco, US) containing 20% heat-inactivated fetal bovine serum (Gibco, US) were added into the tube to neutralize the Type I collagenase activity. Samples were filtered afterward by pouring in beaker glass covered by 3 layers of sterile gauze. The supernatant of the filtration was centrifuged 2000 rpm in 5 min, disposed, and resuspension of the pellet by adding PBS. Centrifugation was repeated and disposed off again the supernatant remaining the pellet. This pellet then be resuspended within the complete medium. Those suspensions were cultivated on the culture plate and kept on the incubator 37°C, 5% CO₂.^[17]

Isolation of peripheral blood mononuclear cells

The peripheral blood sample was collected from a healthy volunteer that had been screened for HIV and Hepatitis-B. The blood sample was slowly poured into conical tube 50cc and added with PBS (ratio 1:1) until homogenous suspensions then were slowly poured through the wall of conical containing Ficoll 5cc and centrifuged 1600 rpm for 30 min in 26°C. After separated into 4 layers, the second layer with buffy coat form was slowly poured into the conical tube and washed using PBS 10cc. Subsequently, the conical was centrifuged 1600 rpm for 5 min. The pellet then was cultured in the medium.^[17,18]

Sensitization of peripheral blood mononuclear cells

PBMCs suspension was divided into 4 groups that would be the control group, sensitized with secretome, sensitized with GM-CSF, also the combination of secretome and

GMCSF (ratio 1:1:1). All groups then were incubated in 37°C for 2 h.

Co-cultivation of osteosarcoma-mesenchymal stem cells with peripheral blood mononuclear cells

OS-MSCs monolayer cells were trypsinized as done until being a single cell. OS-MSCs then were put into coverslip within M24 until 5.10^4 cells. The cells were incubated in 37°C temperature and CO₂ 5% for 24 h. PBMCs sensitized with secretome, GMCSF, and those combinations then were added into each coverslip according to the classified group with ratio 1:1. All of the samples then were incubated in 37°C temperature and CO₂ 5% for 48 h.

Enzyme linked immunosorbent assay, immunocytochemistry, and immunohistochemistry analysis

Enzyme Linked Immunosorbent Assay (ELISA) model was used to measure Interleukin-2 (IL-2) level was sandwich ELISA Elabscience (E-EL-H0099). Immunocytochemistry was used to evaluate Cluster of Differentiation (CD)-133 and STAT-3 expressions using a fluorescent microscope. Caspase-3 expressions were evaluated using immunohistochemistry analysis.

Statistical analysis

One-way analysis of variance test and Tukey Honestly Significant Difference was used to evaluate differences between groups ($P < 0.05$).

RESULTS

The histopathological examination confirmed the OS chondroblastic type [Figure 1a]. Positive expression result of CD-133 was detected [Figure 1b].

The highest level IL-2 results were obtained for PBMCs that sensitized by secretome and GMCSF. Nonsensitized PBMCs as the control group had the lowest levels of IL-2 secretion [Table 1]. The PBMCs culture and results IL-2 level in PBMCs after sensitization in Figure 2a and b.

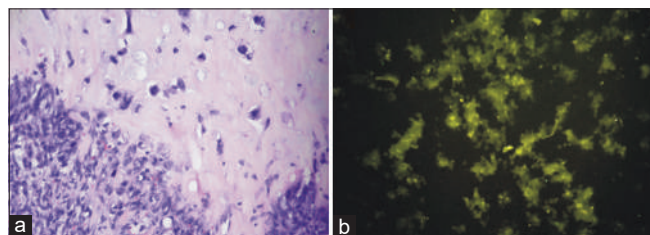


Figure 1: (a) Histopathological examination showed chondroblastic type osteosarcoma tissue (b) Cluster of Differentiation-133 marker results show positive immunocytofluorescence with magnification $\times 200$

Microscopic evaluation of cultivation from between groups can be seen in Figure 3a OS-MSCs cultivation; 3b OS-MSCs and pure PBMCs cultivation; 3c co-cultivation of OS-MSCs and PBMCs sensitized with secretome; 3d OS-MSCs co-cultivated with PBMCs activated by GMCSF; 3e Co-cultivation of OS-MSCs and PBMCs activated by GMCSF and secretome. The greatest IL-2 level was found in OS-MSCs + PBMC + Secretome group [Table 2]. There was no significant different of IL-2 level between groups [Figure 3f].

The STAT-3 expression as shown in Figure 4a OS-MSCs Cultivation; 4b OS-MSCs and PBMCs Cultivation sensitized with secretome; 4c OS-MSCs cultivation and PBMCs sensitized with GMCSF; 4d The OS-MSCs co-cultivated with PBMCs activated by GMCSF and secretome; The greatest expression of STAT-3 was found in OS + PBMC + GMCSF group [Table 3]. There was no significant different of STAT-3 expression between groups [Figure 4e].

The caspase-3 expression as shown in Figure 5a OS-MSCs only; 5b OS-MSCs co-cultivated with PBMCs sensitized by secretome; 5c OS-MSCs cultivated with PBMCs sensitized by GMCSF; 5d OS-MSCs co-cultivated with PBMCs activated by GMCSF and secretome. The greatest expression of Caspase-3 was found in OS-MSCs + PBMC + Secretome + GMCSF

Table 1: The interleukin-2 level in peripheral blood mononuclear cells sensitized by secretome, granulocyte macrophage colony stimulating factor and combination

Group	Mean \pm SD	ANOVA
PBMCs	0.06145 \pm 0.00247	0.011*
PBMCs + secretome	0.06545 \pm 0.00106	
PBMCs + GMCSF	0.06260 \pm 0.00127	
PBMCs + secretome + GMCSF	0.07495 \pm 0.00318	

*Significant at $P < 0.05$. GMCSF: Granulocyte macrophage colony stimulating factor, PBMCs: Peripheral blood mononuclear cells, SD: Standard deviation

Table 2: Interleukin-2 level in osteosarcoma -mesenchymal stem cells co-cultivated with peripheral blood mononuclear cells sensitized by secretome, Granulocyte macrophage colony stimulating factor and combination

Group	Mean \pm SD	ANOVA
OS-MSCs	0.0622 \pm 0.00335	0.083
OS-MSCs + PBMCs	0.06670 \pm 0.00945	
OS-MSCs + PBMCs + secretome	0.08150 \pm 0.1127	
OS-MSCs + PBMCs + GMCSF	0.06976 \pm 0.00567	
OS-MSCs + PBMCs + secretome + GMCSF	0.07083 \pm 0.00318	

OS-MSCs: Osteosarcoma -mesenchymal stem cells, GMCSF: Granulocyte macrophage colony stimulating factor, PBMCs: Peripheral blood mononuclear cells, SD: Standard deviation

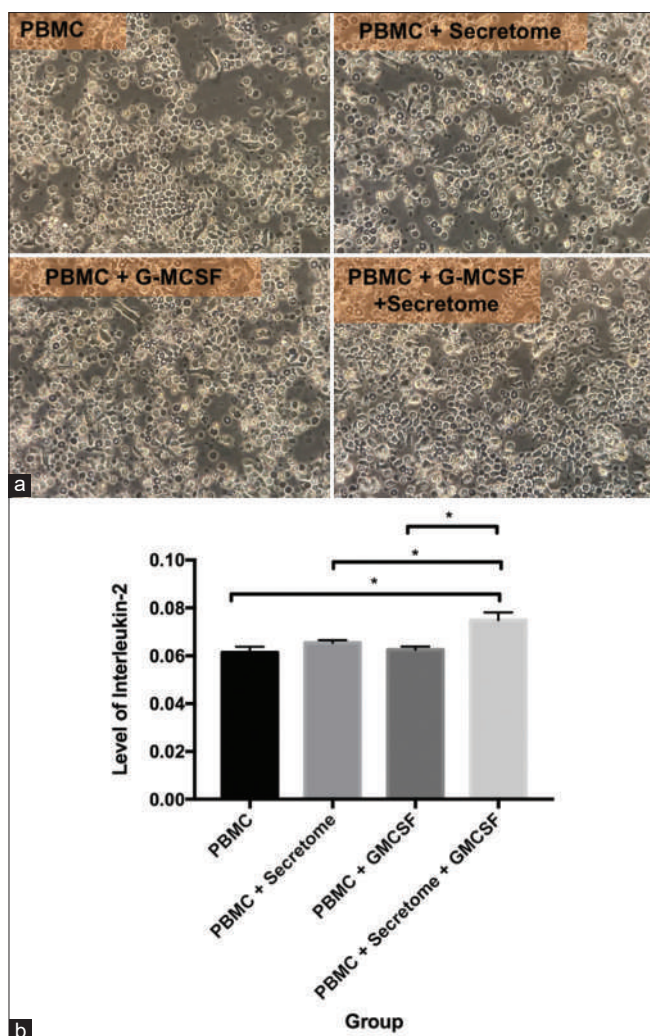


Figure 2: (a) Cultured peripheral blood mononuclear cells in each group with $\times 200$ magnification (b) the Interleukin-2 level of peripheral blood mononuclear cells sensitized by secretome, granulocyte macrophage colony stimulating factor, and combination

group [Table 4]. There was significant different of Caspase-3 expression between groups [Figure 5e].

DISCUSSION

In this study, isolation of OS-MSCs was obtained from an open biopsy sample of an OS patient that have been histopathologically confirmed through CD-113 marker.^[19-21] Most PBMC's is found as naïve cells which possess no function as the effector.^[9,22] Sensitized PBMC's with secretome and GMCSF before co-cultivation with OS-MSCs hopefully able to increase the potential of PBMCs. PBMCs sensitized with secretome and GMCSF showed a higher amount of secreted IL-2 compared to all the groups. The amount of IL-2 level reflects the capability of PBMCs as immunotherapy agent. IL-2 may act directly as a cytokine which trigger the apoptotic process while also activate Natural killer-Cell which is a crucial cellular immunity in againts cancerous cells.^[23]

Table 3: Signal transducers and activators of transcription-3 expression in osteosarcoma -mesenchymal stem cells co-cultivated with peripheral blood mononuclear cells sensitized by secretome, granulocyte macrophage colony stimulating factor and combination

Group	Mean \pm SD	ANOVA
OS-MSCs	16.230 \pm 2.420	0.978
OS-MSCs+PBMCs+secretome	17.243 \pm 4.785	
OS-MSCs+PBMCs+GMCSF	17.580 \pm 5.495	
OS-MSCs + PBMCs + secretome + GMCSF	17.566 \pm 4.231	

OS-MSCs: Osteosarcoma -mesenchymal stem cells, GMCSF: Granulocyte macrophage colony stimulating factor, PBMCs: Peripheral blood mononuclear cells, SD: Standard deviation

Table 4: Caspase-3 expression in Osteosarcoma -mesenchymal stem cells co-cultivated with peripheral blood mononuclear cells sensitized by secretome, Granulocyte macrophage colony stimulating factor and combination

Group	Mean \pm SD	ANOVA
OS-MSCs	8.250 \pm 1.892	0.001 *
OS-MSCs + PBMCs	8.500 \pm 2.081	
OS-MSCs + PBMCs + secretome	12.750 \pm 2.986	
OS-MSCs + PBMCs + GMCSF	15.000 \pm 4.966	
OS-MSCs + PBMCs + secretome + GMCSF	20.500 \pm 3.316	

*Significant at $P < 0.05$. OS-MSCs: Osteosarcoma -mesenchymal stem cells, GMCSF: Granulocyte macrophage colony stimulating factor, PBMCs: Peripheral blood mononuclear cells, SD: Standard deviation

The expression of STAT-3 and the level of IL-2 showed insignificant result. However, caspase-3 expression in this study showed significant results regarding the superiority of apoptotic potential in OS-MSCs cultivated with PBMCs activated using GMCSF and secretome. Caspase-3 expression is heavily associated with the process of cell apoptosis through the dependent caspase pathway given its role as an effector component in the final stages of apoptosis.^[7] PBMCs that were sensitized with secretome and GMCSF have the highest apoptotic triggers potential in co-cultivation with OS-MSCs; and PBMCs that are sensitized with GMCSF alone can be an alternative to be the choice of immune-based therapy regimens for OS.

The GMCSF has a role in the differentiation, maturation, and activation of the PBMCs component in anti-cancer management.^[24] In addition, secretome can modulate lymphocyte proliferation and resemble the role of MSCs in the treatment of several diseases.^[25,26] However, if used separately, PBMCs sensitization with GMCSF has statistically better results than sensitization with secretome

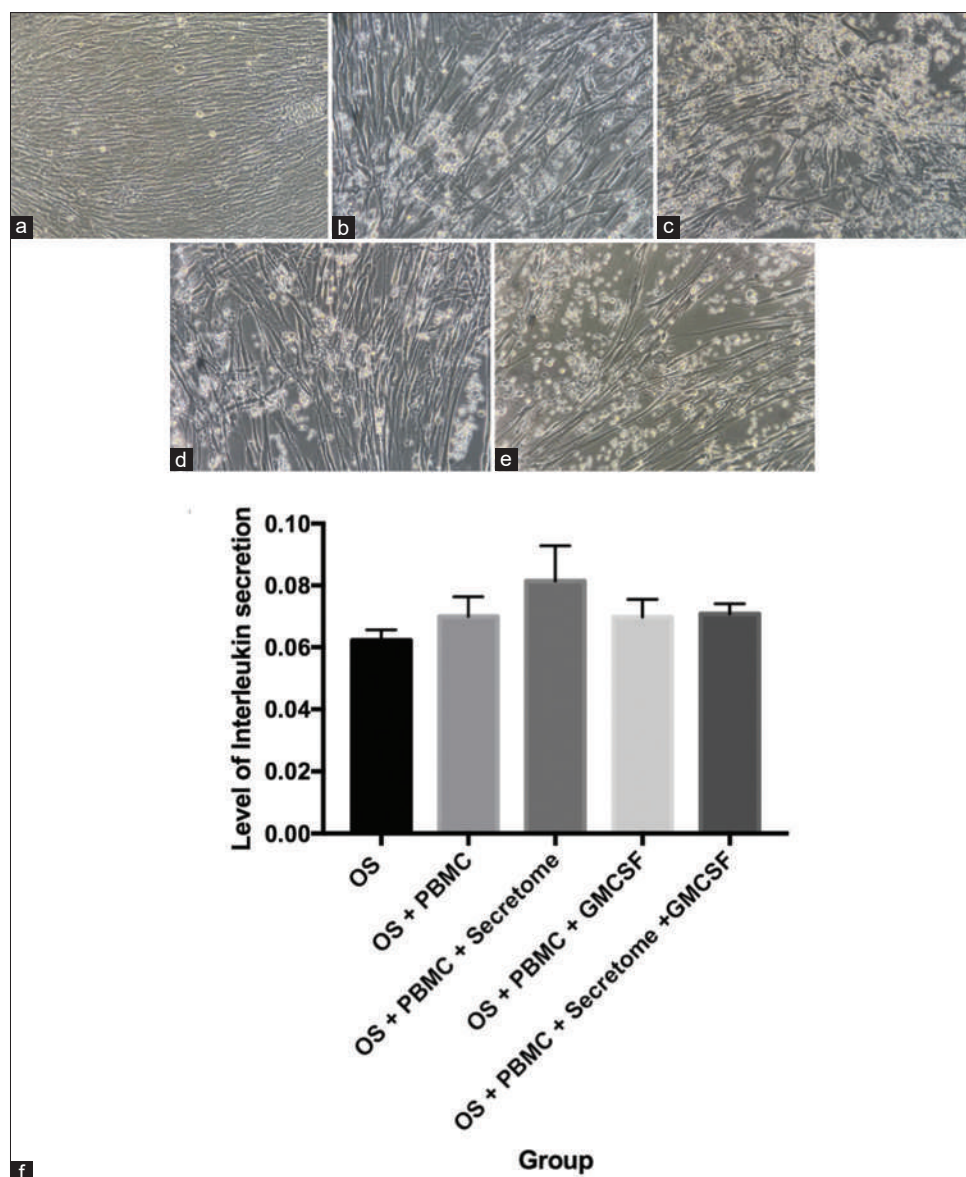


Figure 3: Microscopic evaluation of cultivation (a) osteosarcoma-Mesenchymal stem cells only (b) osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cells (c) osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cell sensitized with secretome (d) osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cell sensitized with granulocyte macrophage colony stimulating factor (e) osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cell activated using granulocyte macrophage colony stimulating factor and secretome (f) Interleukin-2 levels in osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cells sensitized by secretome, granulocyte macrophage colony stimulating factor, and combination

in caspase-3 expression as the originator of apoptosis OS-MSCs.

This study investigates OS-MSCs apoptosis by the evaluation of intrinsic pathway components including IL-2, STAT-3, and caspase-3 through a process in the mitochondria.^[27,28] The process was initiated by IL-2 in cytokine receptors that activate JAK and STAT-3 and STAT-5. This activation will inhibit the function of Bcl-xL and stimulate Cytochrome-C activation that increase in APAF-1 triggers caspase-9

activation and caspase-3 stimulation resulted in OS stem cell apoptosis.^[29] The ability to enhance the apoptosis of OS-MSCs through co-cultivation with PBMCs activated using secretome and GMCSF can be the basis of therapy with less immunological impact compared to chemotherapy.

CONCLUSION

OS-MSCs co-cultivated with PBMCs activated using by secretome and GMCSF has the greater ability enhancing

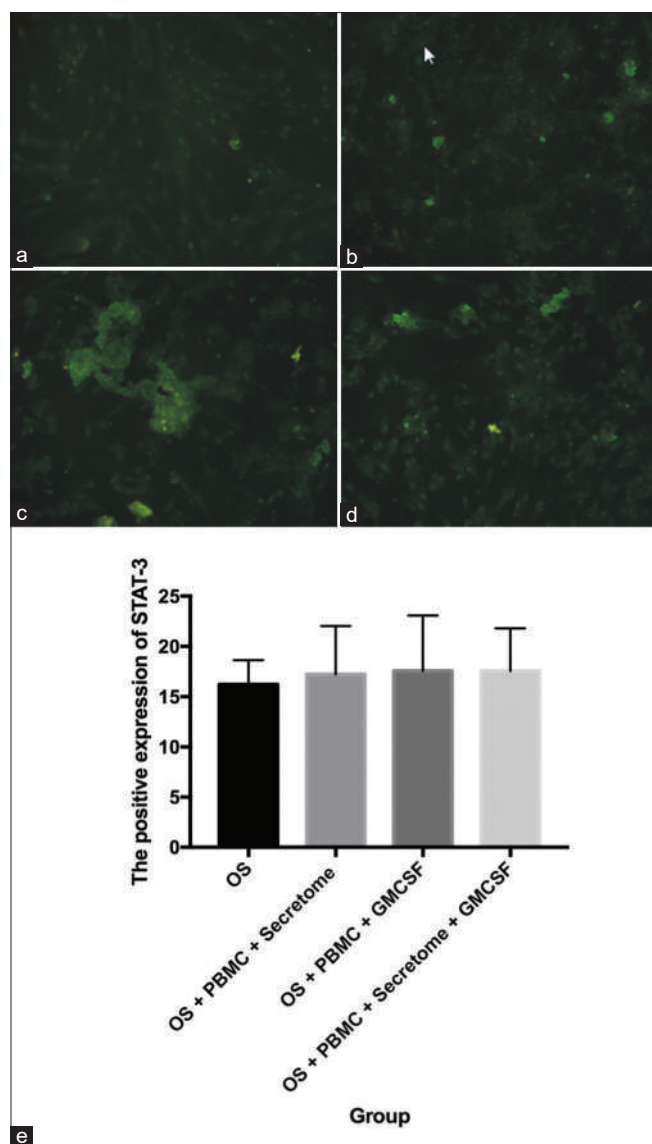


Figure 4: The positive expression number of Signal Transducers and Activators of Transcription-3 (a) osteosarcoma-Mesenchymal stem cells only, (b) osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cells sensitized by secretome (c) osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cells sensitized by granulocyte macrophage colony stimulating factor (d) osteosarcoma-Mesenchymal stem cells cultivated with Peripheral blood mononuclear cells activated using granulocyte macrophage colony stimulating factor and secretome (e) Signal Transducers and Activators of Transcription-3 expressions in osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cells sensitized by secretome, granulocyte macrophage colony stimulating factor, and combination

OS-MSCs apoptosis. The further investigation before clinical trial on human is necessary.

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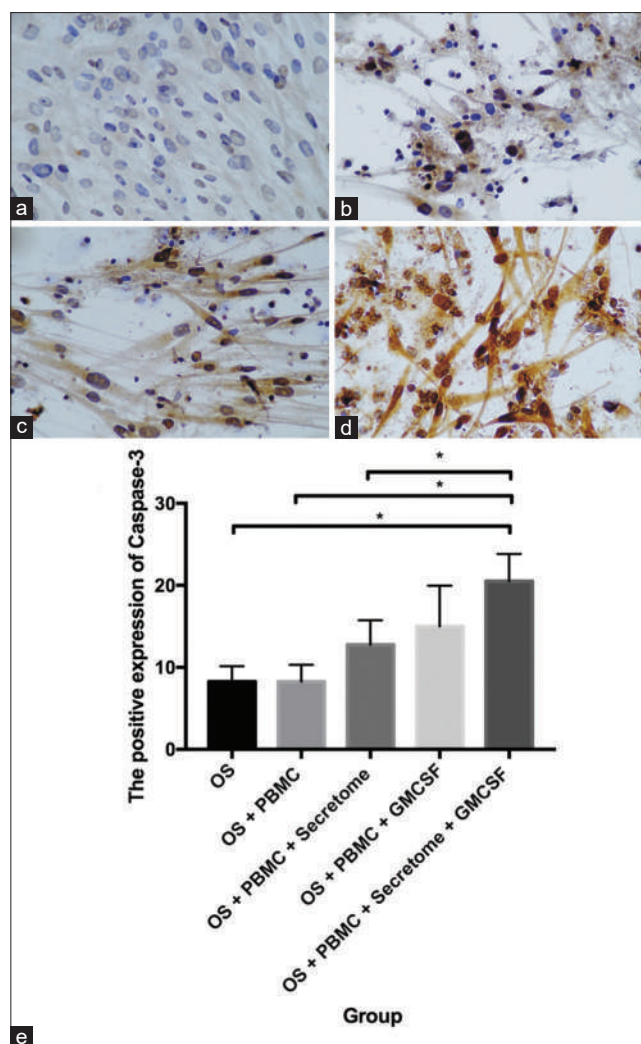


Figure 5: The positive expression of caspase-3 in (a) osteosarcoma-Mesenchymal stem cells only, (b) osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cells sensitized by secretome (c) osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cells sensitized by granulocyte macrophage colony stimulating factor (d) osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cells activated using granulocyte macrophage colony stimulating factor and secretome (e) The caspase-3 expression in osteosarcoma-Mesenchymal stem cells co-cultivated with Peripheral blood mononuclear cells sensitized by secretome, granulocyte macrophage colony stimulating factor, and combination

Conflicts of interest

There are no conflicts of interest.

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**KOMITE ETIK PENELITIAN KESEHATAN
RSUD Dr. SOETOMO SURABAYA**

**KETERANGAN KELAIKAN ETIK
(" ETHICAL CLEARANCE ")**

1089/KEPK/IV/2019

**KOMITE ETIK RSUD Dr. SOETOMO SURABAYA TELAH MEMPELAJARI
SECARA SEKSAMA RANCANGAN PENELITIAN YANG DIUSULKAN, MAKA
DENGAN INI MENYATAKAN BAHWA PENELITIAN DENGAN JUDUL :**

**" Potensi Peripheral Blood Mononuclear Cells (PBMCs) sebagai Pencetus Apoptosis
Jalur Sinyal Sitokin pada Stem Cell Osteosarkoma: Penelitian Eksperimental in Vitro "**

PENELITI UTAMA : Dr. Ferdiansyah, dr., Sp.OT (K)

**PENELITI LAIN : 1. Mouli Edward, dr., M.Kes., Sp.OT (K)
2. Prof. Dr. Fedik A Rantam, drh., DVM
3. Hizbillah Yazid, dr**

UNIT / LEMBAGA / TEMPAT PENELITIAN : RSUD Dr. Soetomo

DINYATAKAN LAIK ETIK

Berlaku dari : 04/04/2019 s.d 04/04/2020

Surabaya, 4 April 2019



**(Dr. Elizen Hanindito, dr., Sp.An, KIC, KAP)
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****) Sertifikat ini dinyatakan sah apabila telah mendapatkan stempel asli dari Komite Etik
Penelitian Kesehatan***