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Comparison of IL-10, TGF-β1, and VEGF Levels in The Secretome of Limbus, Adipose, and Bone Marrow Mesenchymal Stem c3lls

Dicky Hermawan[1], I Ketut Sudiana[2], Evelyn Komaratih3, Deya Karsari[4], Igo Syaiful Ihsan[5]

[1] Doctoral Program of Medical Science, Faculty of Medicine, Universitas Airlangga, Indonesia; Stem Cell Research and Development Center; Department of Ophthalmology, Faculty of Medicine, Universitas Airlangga,[2] Department of Anatomical Pathology, Faculty of Medicine, Universitas Airlangga, [3] Department of Ophthalmology, Faculty of Medicine, Universitas Airlangga, [4]Stem Cell Research and Development Center Universitas Airlangga, [5]Stem Cell Research and Development Center; The Faculty of Health, Medicine and Natural Sciences, Universitas Airlangga
[1]dicky.hermawan@fk.unair.ac.id,[2] i-ketut-s@fk.unair.ac.id,
[3]evelyn.komaratih@fk.unair.ac.id, [4]deyakarsari@staf.unair.ac.id,
[5]syaiful.ihsan@fikkia.unair.ac.id

Objective: In this study, we investigated whether the levels of IL-10, TGF-1, and VEGF in the secretome of limbal (LMSCs), adipose (AMSCs), and bone marrow (BMMSCs) mesenchymal stem cells are similar.
Methods: LMSCs, AMSCs, and BMMSCs were isolated from a healthy 3-month-old New Zealand White Rabbit weighing 2.5–3.5 kg. The isolation of LMSCs from corneoscleral tissue was based on the protocol developed by Chen et al. and modified by Komaratih et al. Stem cell culture and secretome production were carried out according to the Stem Cell Research and Development Center, Universitas Airlangga protocol. Passage-5 MSCs were cultured using the hanging drop technique. MSCs are characterized using positive markers (α-SMA, CD-200, and Vimentin), while negative markers use CD-34 and CD-45. Examination of IL-10, TGF-β1, and VEGF levels in the secretome of LMSCs (LMSC-S), AMSCs (AMSC-S), and BMMSCs (BMMSC-S) using the ELISA method
Results: The mean levels of IL-10 in the LMSC-S, AMSC-S, and BMMSC-S were 697.51 ± 57.12 ng/L, 692.94 ± 61.85 ng/L, and 645.73 ± 65.08 ng/L, there was no significant difference in IL-10 levels (ρ = 0.31). The mean levels of TGF-β1 in the three groups were 876.84 ± 70.75 pg/mL, 768.54 ± 38.12 pg/mL, and 798.82 ± 110.02 pg/mL, there was no significant difference in TGF-β1 levels (ρ = 0.62). The mean levels of VEGF in the three groups were 31.36 ± 3.95 ng/L, 31.93 ± 6.76 ng/L, and 34.33 ± 6.86 ng/L. There was no significant difference in VEGF levels (ρ= 0.63).
Conclusions: The levels of IL-10, TGF-1, and VEGF in the LMSC-S, AMSC-S, and BMMSC-S were the same.

Index Terms— IL-10, limbal, mesenchymal stem cell, secretome.

I. INTRODUCTION

Mesenchymal stem cells (MSCs) have emerged as a cell-based therapy for various diseases. Bone marrow mesenchymal stem cells (BMMSC) were first discovered in 1968 by Friedenstein et al.[4], who described a fibroblast-like population capable of secreting growth factors and cytokines relevant to hematopoiesis and other processes. Mesenchymal stem cells have been isolated from different adult tissues, such as adipose tissue[5] and the corneal limbus[6]. The corneal limbus is a transition zone between the peripheral cornea and the anterior sclera. The corneal limbus plays a vital role in supplying corneal stem cells.[7]

Although MSCs are present in many tissues, their total number in the body is rare. Cell therapy protocols generally require hundreds of millions of MSCs for treatment. Therefore, in vitro cell expansion is needed for about ten weeks before implantation.[8], [9] Several studies have suggested that the time of MSC implantation is too short to have an effective effect. [10], [11], [12], [13], [14] It has been reported that less than 1% of MSCs survive for more than one week after systemic administration.[15], [16], [17], [18], [19] This suggests that paracrine mechanisms may mediate the primary effects of MSCs.[20] Recent studies have also drawn attention to the diverse bioactive factors produced by MSCs, which may play essential roles in regulating various physiological processes.[21] Therefore, the secretome of MSCs has attracted much attention due to its potential use in tissue repair and regeneration. [20], [22], [23], [24], [25]

This study is part of the Repair Mechanism of Corneal Endothelium Damage Post-phacoemulsification with the Administration of Limbus Mesenchymal Stem Cell Secretome. It has obtained Ethical Clearance from the Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine, Universitas Airlangga.

The eye organ has a specificity where the inflammatory response is attempted to be suppressed or avoided. The eye organ has a high interleukin 10 (IL-10) content.[26] IL-10 is an anti-inflammatory cytokine that functions to suppress the inflammatory reaction that occurs. Transforming growth factor β (TGF-β) and acting as an immune modulator also play a role in scar tissue formation. However, the role in scar tissue formation is not expected in the eye organ; the role of an immune modulator is vital. In the eye organ in the wound healing process, new blood vessel tissue is not expected to form; in this case, the role of vascular endothelial growth factor (VEGF) is not too expected. Limbal mesenchymal stem cells (LMSC), one of the sources of stem cells in the eye organ, are expected to contain the same cytokines and growth factors in their secretome as in the secretome of other MSCs, like BMMSC. Eye donors as a source of LMSC are indeed not easy to obtain, so if the levels of IL-10, TGF-β, and VEGF in the secretome of adipose mesenchymal stem cells (AMSC) are the same as those of LMSC, AMSC has the potential to be used as a substitute for LMSC, especially for the repair of corneal endothelial cells which are of interest in our study.

MATERIALS AND METHODS

A. Limbal, adipose, and bone marrow tissue collection

Corneoscleral, adipose, and bone marrow tissues were obtained from one healthy 3-month-old rabbit weighing 2.5–3.5 Kg. The collection was performed by aseptic surgery in The Experimental Animal Laboratory of the Stem Cell Research and Development Center, Universitas Airlangga, based on the protocol developed by Chen et al. (2011).[1] The rabbit

was anesthetized with ketamine and xylazine injection (ketamine 40 mg/mL, xylazine 20 mg/mL) intramuscularly.

The sedated rabbit was placed on the operating table and covered with sterile cloth. The eyeball was disinfected with 5% povidone-iodine and rinsed with basal salt solution (BSS). A 360^0 conjunctival peritomy was performed, followed by the collection of 360^0 full-thickness corneoscleral tissue consisting of 1 mm of the peripheral cornea and 3 mm of the limbus. The adipose tissue was collected from abdominal fat in the inguinal region, and the 10-15 mL bone marrow was extracted from the femoral bones by cavity puncture in the supracondylar under sterile conditions. The limbal and adipose tissue that has been taken is then washed using phosphate buffer saline (PBS) 3 times and stored in a transport medium consisting of Dulbecco's modified eagle medium (DMEM) + 200 U/mL penicillin + 200 U/mL streptomycin at a temperature of 4^0C.[1]

B. Isolation and culture of limbal, adipose, and bone marrow mesenchymal stem cells.

LMSC was isolated from corneoscleral tissue using a protocol developed by Chen et al. (2011) and modified by Komaratih et al. (2017).[1], [2] The entire isolation process was performed aseptically on a laminar flow clean bench. The cornea was separated from the limbus using corneal scissors, and the limbus was then cut to a size of 2 x 2 mm and washed with PBS 3 times to remove residual blood. Tissue pieces were placed in sterile centrifuge tubes containing 2 mg/mL dispase and incubated at 37^0C for 30 minutes. Thirty minutes after incubation, the tissue pieces were centrifuged at 250 G for 5 minutes. The supernatant was then discarded, and the tissue pieces were resuspended with collagenase A type I (0.2 mg/mL) and incubated for 16-18 hours at 37^0C until the tissue was completely digested. Complete growth media: minimum essential medium alpha (α-MEM) + penicillin 200 U/mL + streptomycin 200 U/mL + 1% amphotericin B + non-essential amino acid (NEAA) 100 nm. + fetal bovine serum (FBS) 10% was added to the digested tissue to continue centrifugation at 250 G for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended with complete growth media.[1]

Cell culture was carried out according to the Stem Cell Research and Development Center, Universitas Airlangga protocol. The cell pellet was cultured in a 60 mm diameter culture petri dish with complete growth media and incubated at 37^0C and 5% CO_2 for 24 hours. The growth media was replaced 24 hours after incubation, and then the growth media was replaced every three days until the cells reached 80-90% confluence. Confluent cells were subcultured using the warm trypsinization method using 0.25% trypsin ethylene-diaminetetraacetic acid (EDTA). The growth media was discarded, and then 2 ml of 0.25% trypsin EDTA was added to the culture petri dish and incubated at 37^0C for 3 minutes. The culture petri dish was observed under an inverted microscope to ensure that all cells were detached from the bottom of the petri dish. Four milliliters of complete growth media were added to the culture petri dish to stop the action of trypsin. The cell suspension was put into a centrifuge tube and centrifuged at 250 G for 5 minutes. The supernatant was discarded, the cell pellet was resuspended with complete growth media and cell counting was performed. The cells were then re-cultured in a 100 mm diameter culture petri dish for cell expansion. Isolation and culture of AMSC and BMSC stem were carried out using the same process as the LMSC process above.

Bone marrow mesenchymal isolated from femur bones collected in a transport medium containing 3% PSN (penicillin-streptomycin). The bones are washed twice with PBS

containing 1% PSN to flush away the blood cells and the residual soft tissues. Then, bones are transferred into a new 100-mm sterile culture dish, with A 21-gauge needle attached to a 5 mL syringe to draw 10 mL complete α-MEM, and then the needle is inserted into the bone cavity. The marrow is slowly flushed, leaving the solid mass in the medium, and the dish is incubated at 37°C in a 5% CO_2 incubator. The growth media was replaced every three days until the cells reached 80-90% confluence.

C. Characterization of limbal, adipose, and bone marrow mesenchymal stem cells.

Limbal mesenchymal stem cells, AMSCs, and BMSCs, were characterized by immunohistochemical examination. Cells that had been monolayered were made into single cells by the trypsinization process. After adding trypsin, centrifugation was carried out at 1600 rpm for 5 minutes. The cell pellet was added with 1 ml of α-MEM growth medium, resuspended, and planted on a special object glass of as much as 20 µl. The object glass was placed in a box containing wet paper and then incubated at 37^0 C for one hour. Furthermore, fixation was carried out with 4% paraformaldehyde for 15 minutes at room temperature. After that, it was washed with PBS 4 times, dried, blocked with PBS containing 1% serum for 15 minutes at room temperature, then washed with PBS 4 times and dried. In each sample, alpha-smooth muscle actin (α-SMA), CD-200, and Vimentin antibodies were added for positive markers, and CD-34 and CD-45 for negative markers (labeled with FIT-C) were incubated at 37^0C for one hour. After that, it was washed with PBS 4 times, and then the water was dried around the object glass with tissue paper. Furthermore, 50% glycerin was dropped on the object glass, and the results were immediately viewed with a fluorescence microscope at 40x magnification.

D. Procedure for making LMSC, AMSC, and BMSC secretome.

Limbal mesenchymal stem cells, AMSCs, and BMSCs passage-4 were trypsinized and cultured in 3D spheroids to form embryoid bodies using the hanging drop technique according to the protocol developed by Rungarunlert et al. (2009).[3] Limbal mesenchymal stem cells, AMSCs, and BMSCs passage-4, which had reached 90% confluence, were harvested using the warm trypsinization technique. The cells were then resuspended with growth media with the composition α-MEM + 1% NEAA + 1% penicillin-streptomycin + 1% amphotericin B. A total of 25 µL of cell suspension was aspirated with a micropipette and then dropped onto the bottom of a 60 mm diameter petri culture vertically to form a drop (the number of stem cells per drop for LMSC was 148,500 cells/25 µl, AMSC was 156,000 cells/25 µl, and BMSC was 65,100 cells/25 µl). The cell suspension was dropped until it filled the bottom of the petri dish with 2 mm between drops. The petri culture was then stored in an inverted position in a 37^0 C, 5% CO_2 incubator, namely the bottom of the petri dish above (hanging drop), for 24 hours. After 24 hours, the petri culture was removed from the incubator, then turned over, and added growth media to make a secretome. The petri culture was stored at 37^0 C, 5% CO_2 in an incubator. Limbal mesenchymal stem cells, AMSC, and BMSC secretome were collected every 24 hours. The collected secretome was filtered with a 0.22 µm Millipore and stored at -80^0 C until ready to use.[3]

E. Measurement of IL-10, TGF-β1, and VEGF levels in the LMSC, AMSC, and BMSC secretome.

Examination of IL-10, TGF-β1, and VEGF levels in the secretome of LMSC, AMSC, and BMSC using the ELISA method with two examinations (duplo) for each secretome sample measured. The ELISA kit used in this study was the rabbit ELISA Kit (E0004Rb, E0025Rb, E0026Rb) from the Bioassay Technology Laboratory (BT-Lab), Shanghai Korain, China.

F. Statistical analysis

The collected data were analyzed using SPSS 27. The normality test used the Shapiro-Wilk test. The homogeneity of variances test used the Levene test. Mean difference test between groups using the ANOVA test followed by the Bonferroni test if the data is normally distributed and homogeneous, or the Brown-Forsythe test followed by the Games-Howell test if the data is normally distributed but not homogeneous. If the data is not normally distributed, use the Kruskal-Wallis test, followed by the Mann-Whitney test. The test is carried out with a 95% confidence level.

II. RESULTS

A. Characterization of LMSC, AMSC, and BMSC.

When the characteristics of MSCs (fig. 1) in the three groups were examined, positive results were obtained for α-SMA, CD-200, and Vimentin, and negative results were obtained for CD-34 and CD-45.

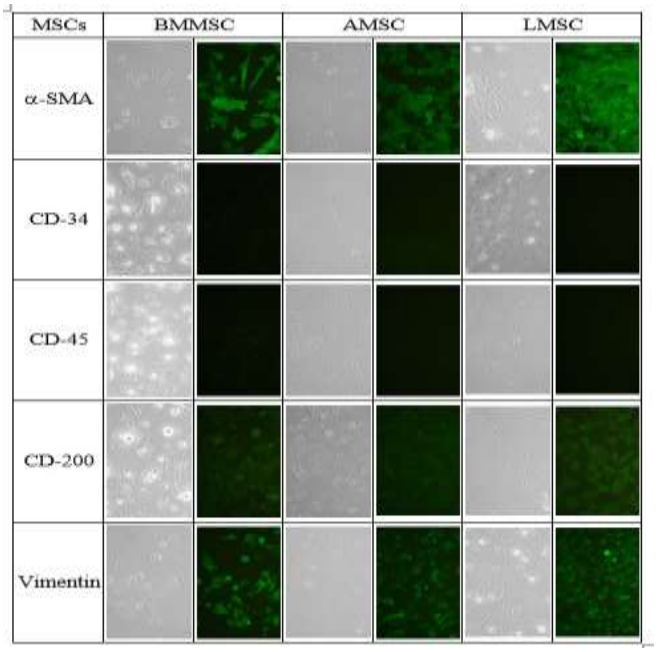

Figure 1. Characterization of LMSC, AMSC, and BMSC with α-SMA, CD-200, Vimentin for positive markers and CD 34, CD 45 for negative markers.

B. IL-10 levels.

The average levels of IL-10 in LMSC secretome (LMSC-S), AMSC secretome (AMSC-S), and BMMSC secretome (BMMSC-S) were 697.51±57.12 ng/L, 692.94±61.85 ng/L, and 645.73±65.08 ng/L (table 1).

Table 1. IL-10 levels in the secretome of LMSC-S, AMSC-S, and BMMSC-S.

Group	N	Mean ± SD ng/L	Median	Min	Max	p Shapiro-Wilk	p Kruskal-Wallis
LMSC-S	7	697.51 ± 57.12	703.80	595.30	769.30	0.66	0.31
AMSC-S	7	692.94 ± 61.85	672.80	631.80	809.30	0.18	
BMMSC-S	7	645.73 ± 65.05	670.30	509.80	692.30	0.13	

The highest IL-10 levels were in LMSC-S and the lowest in BMMSC-S. Statistically, there was no significant difference between the average IL-10 levels in LMSC-S, AMSC-S, and BMMSC-S ($p = 0.31$).

C. TGF-β1 levels.

The average levels of TGF-β1 in LMSC-S, AMSC-S, and BMMSC-S were 876.84±70.75 pg/mL, 768.54±38.12 pg/mL, and 798.82±110.02 pg/mL (table 2).

Table 2. TGF-β1 levels in the secretome of LMSC-S, AMSC-S, and BMMSC-S.

Group	N	Mean ± SD pg/mL	Min	Max	p Shapiro Wilk	p Levene	p Brown-Forsythe
LMSC-S	7	876.84 ± 70.75	758.60	983.60	0.85	0.02	0.62
AMSC-S	7	768.54 ± 38.12	721.10	830.50	0.86		
BMMSC-S	7	798.82 ± 110.02	626.10	895.50	0.14		

The highest TGF-β1 level was in LMSC-S and the lowest in AMSC-S. Statistically, there was no significant difference between the average TGF-β1 level in LMSC-S, AMSC-S, and BMMSC-S ($p = 0.62$).

D. VEGF levels

The average level of VEGF in LMSC-S, AMSC-S, and BMMSC-S were 31.36±3.95 ng/L, 31.93±6.76 ng/L, and 34.33±66.86 ng/L (table 3).

Table 3. VEGF levels in the secretome of LMSC-S, AMSC-S, and BMMSC-S.

Group	N	Mean ± SD	Min	Max	p	p Levene	p ANOVA

		ng/L			Shapiro Wilk		
LMSC-S	7	31.36 ± 3.95	27.20	39.30	0.12		
AMSC-S	7	31.93 ± 6.76	24.20	44.00	0.64	0.35	0.63
BMMSC-S	7	34.33 ± 6.86	28.60	47.40	0.09		

The highest VEGF level was in BMMSC-S and the lowest in LMSC-S. Statistically, there was no significant difference between the average VEGF level in LMSC-S, AMSC-S, and BMMSC-S ($p = 0.63$).

III. DISCUSSION

In this study, MSCs can be isolated from corneal limbal tissue.[6], [27] The cornea has limbal stem cells (LSCs) found in the limbus, the border area between the cornea and conjunctiva.[6] Limbal stem cells are the source of mesenchymal stem cells.[27]

In this study, tissue preparation for LSCs isolation followed the method used in the study of Chen et al. (2011),[1] tissue isolation and culture using the method used by Komaratih et al. (2017),[2] using the hanging drop culture method as conveyed by Rungarunlert (2009)[3] for passage-5 culture. The hanging drop culture method is a basic method for producing embryoid bodies (EBs) from embryonic stem (ES) cells. It produces uniform EB sizes by removing the same number of ES cells, offering the same environment to form individual EBs in each drop through cell aggregation caused by gravity. This culture technique has been used to produce many types of cells.[3]

In passage 4, by hemocytometer counting, the number of LMSCs was 5.94×10^6/ml, AMSCs was 6.24×10^6/ml, and BMMSCs was 2.60×10^6/ml. Contamination occurred during the culture of BMMSCs, so the culture process was repeated. This likely caused the number of BMMSCs per milliliter to be less than other MSCs.

In this study, IL-10, TGF-1, and VEGF were found in the secretomes of LMSCs, AMSCs, and BMMSCs. This proves that the LMSC-S also contains pro-inflammatory, anti-inflammatory, and growth factor cytokines that are the same as those in the secretomes of other MSCs.[22] In our study, the average levels of IL-10 in the secretome of the LMSCs, AMSCs, and BMMSCs were 697.51 ± 57.12 ng/L, 692.94 ± 61.85 ng/L, and 645.73 ± 65.08 ng/L. The IL-10 content in the secretome of LMSCs was like AMSCs, with a difference of only 5 ng/L. The difference in IL-10 levels from the secretome of BMMSCs with LMSCs and AMSCs was approximately 50 ng/L, with the IL-10 levels of BMSCs-S being lower. The mean levels of TGF-β1 in the secretome of LMSCs, AMSCs, and BMMSCs were 876.84 ± 70.75 pg/ml, 768.54 ± 38.12 pg/ml, and 798.82 ± 110.02 pg/ml. The levels of TGF-β1 in the secretome of LMSCs were the highest compared to AMSC-S and BMMSC-S. The mean levels of VEGF in the secretome of LMSCs, AMSCs, and BMMSCs were 31.36 ± 3.95 ng/L, 31.93 ± 6.76 ng/L, and 34.33 ± 66.86 ng/L. The highest levels of VEGF were in the secretome of BMSCs.

The study of Jahandideh et al. (2024) aimed to clarify whether trimetazidine (TMZ) and diazoxide (DZ) could enhance the immunomodulatory effects of human embryonic stem cell-derived mesenchymal stem cell (hESC-MSCs) secretome on peripheral blood mononuclear cells (PBMCs) induced by LPS. Cells cultured from frozen human MSCs (RH6-MSCs) did not use the hanging drop method. After the cell culture reached 80% confluency, the cells were incubated with 50 μM TMZ for 6 hours and 100 μM DZ for 30 minutes. IL-10, TNF-1, and IL-1 levels were measured using an ELISA kit from the PBMCs' supernatants treated with TMZ-MSC-CM and DZ-MSC-CM (Thermo Scientific). IL-10 levels in the TMZ group were 446.0 ± 64.6 pg/mL, and in the DZ group were 668.8 ± 108.2 pg/mL. In the supernatant of hESC-MCs, neither TMZ nor DZ groups were detected.[28] Different from our study, in the study of Jahandideh et al., the measurement of cytokine levels was not from hESC-MSCs secretome but from PBMCs supernatant, while in our research, LMSC-S, AMSC-S, and BMMSC-S secretome. The ELISA kits used were also different. Jahandideh et al. used an ELISA kit from Thermo Scientific, while we used Bioassay Technology Laboratory.

Raj et al.'s (2021) study aimed to measure the growth factors and cytokines levels in the dental pulp mesenchymal stem cell secretome (DPMSC-S) and assess their effects on oral cancer cell proliferation. The IL-10 levels were 10.37 pg/mL, TGF-β1 8.1 pg/mL, and VEGF 413.56 pg/mL.[29] There were differences in the growth factor and cytokine levels results with our study, possibly because the source of the mesenchymal stem cell secretome and the measurement methods used differed. In the study by Raj et al., growth factor levels were measured using the LEGENDplexTM Human Growth Factor panel (13-plex; Biolegend, San Diego, CA, USA; Cat. No. 740180), and cytokine levels were measured using the LEGENDplexTM Human Essential Immune Response Panel (13-plex; Biolegend, San Diego, CA, USA; Cat. No. 740929).

In the study by Villatoro et al. (2019), one of the objectives of the study to isolate and measure cytokines and growth factors contained in the secretome of mesenchymal stem cells, higher IL-10, TGF-β and VEGF-A content was obtained from the secretome of BMMSC compared to AMSC from canine species. The study measured the concentration of 15 substances contained in the secretome, including chemokine Monocyte Chemoattractant Protein-1 (MCP-1), cytokines (Interleukins: IL-2, IL-6, IL-8, IL-10, IL-12p40, Tumor Necrosis Factor-alpha: TNF-α, Interferon-gamma: IFN-γ), immune-mediators (Prostaglandin E2: PGE2, Nitric Oxide: NO, Indoleamine 2,3-dioxygenase; IDO) and growth factors (Beta-nerve grown factor: NGF-β, stem cell factor: SCF, Transforming Growth Factor beta: TGF-β, Vascular Endothelial growth factor A: VEGF-A). Eleven substances contained were measured with a commercial Luminex kit canine cytokine 11-plex assay (Thermo Fisher Scientific), while the concentrations of TGF-β and PGE2 were measured with an ELISA kit (R&D).[30]

Our study and those of Jahandideh et al., Raj et al., and Villatoro et al. confirm that the secretome of mesenchymal stem cells contains growth factors, pro-inflammatory and anti-inflammatory cytokines. The results of our study provide information on the levels of IL-10, TGF-β1, and VEGF, which are similar in LMSC-S, AMSC-S, and BMMSC-S, so that we can replace the need for LMSC-S with AMSC-S which is relatively more straightforward to obtain and has been mass-produced than LMSC-S which requires young human eye donors which are challenging to obtain.

CONCLUSION

Mesenchymal stem cells can also be isolated from limbal tissues, such as adipose tissue and bone marrow. IL-10, TGF-β1, and VEGF are found in the secretome of limbal, adipose, and bone marrow mesenchymal stem cells. IL-10, TGF-β1, and VEGF levels in the secretome of limbal, adipose, and bone marrow mesenchymal stem cells do not differ.

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Authors' contributions
1. Dicky Hermawan: conceptualization, methodology, formal analysis, investigation, data curation, original draft preparation, review, and editing. 2. I Ketut Sudiana: methodology, investigation, and review 3. Evelyn Komaratih: methodology, investigation, and review 4. Deya Karsari: isolation processing, culture, and secretome production of stem cells. 5. Igo Syaiful Ihsan: prepare space in the animal laboratory, provide animals, and take adipose tissue and bone marrow.

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Declarations

Ethics approval and consent to participate
The research ethic was approved by the Faculty of Veterinary Medicine, Universitas Airlangga's ethics committee under Code of Ethics No. 2.KEH.122.09.2022, dated September 13, 2022. All applicable international, national, and institutional guidelines for the care and use of animals are followed. All procedures carried out in research involving animals complied with the ethical standards of the Faculty of Veterinary, Universitas Airlangga, Surabaya, Indonesia.

Competing interests
The authors declare no competing interests.

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