Journal of Taibah University Medical Sciences (2024) 19(3), 687–695

Taibah University

Journal of Taibah University Medical Sciences

www.sciencedirect.com

Original Article

Human gingival mesenchymal stem cells-lyosecretome attenuates adverse effect of hydrogen peroxide-induced oxidative stress on osteoblast cells

Banun Kusumawardani, PhD [a,*], Muhammad Nurul Amin, PhD [a], Yani C. Rahayu, drg [b], Desi S. Sari, PhD [c], Morin I. Altariq [d], Arini H. Putri [d], Amara Kanya [d], Chiquita Prahasanti, PhD [e] and Mohammed A. Aljunaid, MDS [f,g]

[a] *Department of Biomedical Sciences, Faculty of Dentistry, University of Jember, Indonesia*
[b] *Department of Oral Biology, Faculty of Dentistry, University of Jember, Indonesia*
[c] *Department of Periodontics, Faculty of Dentistry, University of Jember, Indonesia*
[d] *Undergraduate Program of Dental Medicine, Faculty of Dentistry, University of Jember, Indonesia*
[e] *Department of Periodontics, Faculty of Dental Medicine, Airlangga University, Indonesia*
[f] *Doctoral Program of Dental Medicine, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia*
[g] *Department of Dental Medicine, Faculty of Medicine, Taiz University, Taiz, Yemen*

Received 9 October 2023; revised 19 March 2024; accepted 3 May 2024; Available online 13 May 2024

الملخص

أهداف البحث: تهدف الدراسة لتحديد محتوى البروتين الكلي، ونشاط مضادات الأكسدة، والقدرة الوقائية لمفرزات الخلايا الجذعية الوسيطة اللثوية البشرية في نموذج الإجهاد التأكسدي الناجم عن بيروكسيد الهيدروجين.

طريقة البحث: تمت زراعة الخلايا الجذعية الوسيطة اللثوية البشرية للحصول على وسط مشروط (مفرزات)، ثم تم تجفيفها بالتجميد لإنتاج مفرزات مجففة بالتجميد. تم تحديد البروتين الكلي عن طريق فحص حمض البيكينكونيك والرحلان الكهربائي لهلام بولي أكريلاميد دوديسيل كبريتات الصوديوم لتحسين قياسات البروتين. تم قياس تركيز مضادات الأكسدة بواسطة اختبار "أ بي تي اس"، في حين تم تحديد القدرة الوقائية للمفرزات المجففة بالتجميد ضد الإجهاد التأكسدي من خلال النشاط الأيضي لخلايا بانية العظم. تم تقسيم مجموعة الدراسة إلى مجموعة مراقبة (مستنبت) ومجموعة علاج المفرزات المجففة بالتجميد.

النتائج: تحتوي المفرزات المجففة بالتجميد على تركيز بروتين يبلغ 2086.00 ± 0.20 ميكروغرام/مل، مع وزن جزيئي يبلغ 174، 74، 61، 55، و26 كيلو دالتون، والتي يعتقد أنها تسهل هجرة الخلايا، فضلا عن ربط السيتوكينات وعوامل النمو. كما قدمت المفرزات المجففة بالتجميد أعلى نشاط مضاد للأكسدة بنسبة 93.51% بتركيز 4.8 مجم/مل، مع قيمة تركيز مثبط50 تبلغ 2.08 مجم/مل. وقد ظهر أعلى نشاط استقلابي للخلية في مجموعة العلاج بالمفرزات المجففة بالتجميد 1.25 ملغم/مل. جميع تركيزات المفرزات المجففة بالتجميد للخلايا الجذعية الوسيطة اللثوية البشرية - تخفف من التأثير الضار للإجهاد التأكسدي الناجم عن بيروكسيد الهيدروجين.

الاستنتاجات: يمكن أن تحافظ المفرزات المجففة بالتجميد التي تم الحصول عليها من الخلايا الجذعية الوسيطة اللثوية البشرية على النشاط الأيضي في خلايا بانيات العظم كحماية ضد إجهاد بيروكسيد الهيدروجين.

الكلمات المفتاحية: الخلايا الجذعية الوسيطة اللثوية؛ بيروكسيد الهيدروجين؛ المفرزات المجففة بالتجميد؛ الإجهاد التأكسدي؛ الخلايا العظمية

Abstract

Objective: To determine total protein content, antioxidant activity, and protective ability of lyophilized human gingival mesenchymal stem cells (hGMSCs)-secretome in hydrogen peroxide (H_2O_2) induced oxidative stress model.

Methods: Human GMSCs were cultured to obtain a conditioned medium (secretome), then lyophilized to produce lyosecretome. Total protein was determined by bicinchoninic acid assay (BCA) and SDS-PAGE to improve protein measurements. Antioxidant concentration was measured by ABTS assay, while the protective

* Corresponding address: Department of Biomedical Sciences, Faculty of Dentistry, University of Jember, Jalan Kalimantan 37 Jember, 68121, Indonesia.
E-mail: banun_k.fkg@unej.ac.id (B. Kusumawardani)

Peer review under responsibility of Taibah University.

Production and hosting by Elsevier

 https://doi.org/10.1016/j.jtumed.2024.05.002

ability of lyosecretome against oxidative stress was determined by the metabolic activity of osteoblast cells. The study group was divided into a control group (culture medium) and a lyosecretome treatment group (0.0; 0.157, 0.313, 0.625, 1.25, 2.5, 5, and 10 mg/mL + H_2O_2).

Results: Lyosecretome had a protein concentration of 2086.00 ± 0.20 μg/ml, with a molecular weight of 174, 74, 61, 55, and 26 kDa, which are thought to facilitate cell migration, as well as bind cytokines and growth factors. Lyosecretome also provided the highest antioxidant activity of 93.51% at a concentration of 4.8 mg/ml, with an IC_{50} value of 2.08 mg/ml. The highest cell metabolic activity (79.53 ± 2.41%) was shown in the 1.25 mg/ml lyosecretome treatment group. All concentrations of hGMSC-lyosecretome attenuate the adverse effect of H_2O_2-induced oxidative stress.

Conclusion: Lyosecretome obtained from hGMSCs can maintain metabolic activity in osteoblast cells as protection against H_2O_2 oxidative stress.

Keywords: Gingival mesenchymal stem cells; Hydrogen peroxide; Lyosecretome; Osteoblast cells; Oxidative stress

Introduction

Gingival tissue is a source of human gingival-derived mesenchymal stem cells (hGMSCs), which are easy to collect with less morbidity when harvesting gingival tissue. GMSCs are a subpopulation of tooth-derived mesenchymal stem cells that exhibit characteristics of mesenchymal stem cells (MSCs), such as high proliferation, multipotent differentiation, and immunomodulation. Thus, hGMSCs represent a unique source of MSCs for potential applications in tissue engineering and regenerative therapy. In recent years, many studies have focused on novel cell-free therapies with secretomes obtained from hGMSCs.[1–5] Several bioactive molecules, such as cytokines, proteins, growth factors, chemokines, and nucleic acids released by MSCs, are involved in cell-to-cell communication and cross-communication that mediate the therapeutic effects of MSCs.[6–8] In addition, the secretome of MSCs has a dual role in triggering senescence of other MSCs, and in contributing to the pathological stages of ischemic organ changes and disorders.[9]

Cell-free therapies with secretomes have several advantages over cell-based therapies, including ease of storage and handling, lower immunogenicity, and lower costs to ensure utility and feasibility in the clinic.[10,11] However, handling hGMSC-secretome products is a formidable challenge, and further investigations are needed to elucidate their composition and properties of the secretome. Therefore, characterization of hGMSC-secretome products is necessary to ensure the reproducibility of hGMSC-secretome product handling procedures and to evaluate the efficacy of hGMSC-secretome products in periodontal tissue engineering and regenerative therapy.

Secretome is a thermolabile biotechnology product, so it cannot be dried using thermal methods because it can degrade its bioactive components. The lyophilization process using the freeze-drying method is expected to maintain the functional and structural integrity of the hGMSC-secretome. The lyophilization process is helpful in reducing the rate of degradation and maintaining the quality of secretome products so that secretome products have a longer shelf life.[12] However, the freeze-drying method has weaknesses because the pressure during freezing and drying can change the stability of biological products, so it is necessary to add cryoprotectants to protect proteins from damage to ice crystals formed during the freezing stage. In this case, mannitol is used as a cryoprotectant because it provides controlled and complete crystallization during the freeze-drying process, resulting in a strong crystal structure, which can maintain the stability of the secretome during storage.[12–14]

The secretome is known to be less immunogenic compared to MSCs, so it can be used as a cell-free therapy that can promote functional recovery of various tissue injuries. However, the protective effect against oxidative stress-induced bone tissue injury remains to be explored. This oxidative stress can result in damage to various cellular components of osteoblasts, contributing to conditions like osteonecrosis. Additionally, it plays a vital role in initiating impaired osteoblastic bone formation.[15] Notably, hydrogen peroxide (H_2O_2) acts as a potent oxidizing agent capable of inducing cells to generate large amounts of reactive oxygen species (ROS).[16] These ROS are typically produced within the mitochondria as part of normal cellular metabolism. Elevated levels of ROS are associated with oxidative stress, apoptosis, and cellular dysfunction.[17] Furthermore, H_2O_2 can regulate cell function and induce cell death by penetrating cell membranes and functioning as a secondary messenger within signal transduction pathways.[18–20] This study aimed to determine the total protein content, antioxidant activity, and protective ability of lyophilized hGMSC-secretome in the H_2O_2-induced oxidative stress model in vitro.

Materials and Methods

Study design

This research was an experimental study, and all research was conducted at the Molecular Medicine Laboratory, Center for Development of Advanced Science and Technology, Jember University.

Human-GMSCs (Code: hGMSCs-P5) and rat calvarial osteoblast cells (Code: rOb-P3) were provided by the Regenerative Dentistry Research Group. These cells were characterized, cryopreserved, and stored for more than two years in the Molecular Medicine Laboratory, Center for Development of Advanced Science and Technology, Jember University.[2,3,8,21]

The study group was divided into a control group (culture medium), treatment group A1 (lyosecretome 0.0 mg/mL + H_2O_2), treatment group A2 (lyosecretome 0.157 mg/mL + H_2O_2), treatment group A3 (lyosecretome 0.313 mg/mL + H_2O_2), treatment group A4 (lyosecretome 0.625 mg/mL + H_2O_2), treatment group A5 (lyosecretome 1.25 mg/mL + H_2O_2), treatment group A6 (lyosecretome 2.5 mg/mL + H_2O_2), treatment group A7 (lyosecretome 5 mg/mL + H_2O_2), and treatment group A8 (lyosecretome 10 mg/mL + H_2O_2).

Cultivation of human-gingival mesenchymal stem cells (hGMSCs)

In passage 5 (coded as hGMSCs-P5), hGMSCs were cultured using Dulbecco's modified Eagle's Medium (DMEM) in T75 culture flasks. The culture medium was enhanced with 10% fetal bovine serum (FBS), 0.5% Amphotericin B, and 2% Penicillin-Streptomycin. Human-GMSCs were incubated in 5% CO_2 at 37 °C. The culture medium was replaced every three days until the cells reached a of 70–80% confluency level.

Preparation and purification human-GMSCs-secretome

After reaching 80% confluency, hGMSCs were washed with phosphate-buffered saline (PBS). Subsequently, cells were treated with DMEM and 10% FBS, and incubated for 24 h in a 5% CO_2 and at 37 °C. The culture medium (supernatant) was collected in a conical tube and centrifuged at 173×*g* (3,000 rpm) for 10 min. This process was to remove cell debris and apoptotic bodies, and to obtain secretome. The secretome was filtered with a 0.22 μm filter, purified with a 5 kD centrifugal filter ultrafiltration tube, and centrifuged at 5,000×*g* at 4 °C for 40 min.

Lyophilization of hGMSCs-secretome

Lyophilization, also known as freeze-drying, is performed to obtain secretome powder. Mannitol was dissolved in the purified secretome for a final concentration of 0.5% (w/v). Next, the solution was frozen at a temperature of −80 °C, and freeze-drying was carried out at conditions of 80×10^{-1} mbar and a temperature of −50 °C for 72 h. Lyosecretome products were stored at −80 °C until required for use.

Determination of total protein

The total protein of secretome and lyosecretome was determined by bicinchoninic acid (BCA) Assay. The lyosecretome was dissolved in a basal medium with the same concentration as the secretome. The dissolved sample was placed in 25 μl of a 96-well plate. Concentration absorbance calibration curves were prepared using Bovine Serum Albumin (BSA) standards. The reagent solution was added to each sample or standard (ratio 1:1) as much as 200 μl. The plate was shaken for 30 s and incubated at 37 °C for 30 min. After incubation, the absorbance value of 562 nm was read with a microplate reader.

Determination of protein molecular weights

The protein molecular weights of secretome and lyosecretome were determined by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method. The lyosecretome was dissolved in a basal medium with the same concentration as the secretome. Samples with a concentration of 40 μg/μl were placed into the Eppendorf tube as much as 50 μl for each sample. All samples were heated to denature the protein at 95 °C for 3 min. Buffer solution (50 μl) was added to each sample. Samples and protein ladder markers were inserted into each well on the gel. The gel is put into the electrophoresis apparatus with a voltage of 50–80 V until the sample moves to the bottom of the gel. Next, the gel was soaked in Coomassie Brilliant Blue (CBB) dye. The gel was washed with distilled water and de-stained with 20% methanol and 10% acetic acid until the CBB dye only coloured the protein markers.

Antioxidant analysis

The free radical scavenging activity of lyosecretome was performed by 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay. ABTS (19.2 mg) was dissolved in 5 mL of distilled water (aquadest) to obtain a 7 mM solution. Additionally, 3.31 mg of potassium persulphate was dissolved in 5 mL of aquadest to form a 2.45 mM potassium persulphate solution. The ABTS and potassium persulphate solutions were mixed and incubated in darkness at room temperature for 14 h to prepare the ABTS reagent. Furthermore, lyosecretome was diluted to various concentrations (0.8, 1.6, 2.4, 3.2, 4.0, and 4.8 mg/ml). The ABTS reagent and the lyosecretome sample solution were mixed in a cuvette at a volume ratio of 1:4. The mixture was incubated at room temperature. After 10 min, the absorbance was measured at 734 nm using a spectrophotometer. The antioxidant activity of lyosecretome towards ABTS·+ was measured using the formula ABTS·+ scavenging effect (%) = ((AB−AA)/AB) × 100. AB is the absorbance of ABTS radical + methanol; AA is the absorbance of ABTS radical + sample extract/standard. Ascorbic acid was used as a standard substance. The results are presented in IC_{50} value (mg/ml) corresponding to inhibition concentration at which 50% radicals are scavenged.

Induction of oxidative stress in rat calvarial osteoblast cells

Rat calvarial osteoblast cells at passage 3 (Code: rOb-P3) were cultured in T75 culture flasks until they reached 80% confluency. Subsequently, these cells were harvested and seeded onto a 96-well plate at a density of 10,000 cells per square centimeter (10,000 cells/cm^2). The culture medium used for the cells consisted of DMEM media supplemented with 10% FBS, 0.5% Amphotericin B, and 2% Penicillin-Streptomycin. The seeded cells were then placed in an incubator with an environment of 5% CO_2 at a temperature of 37 °C for 48 h.

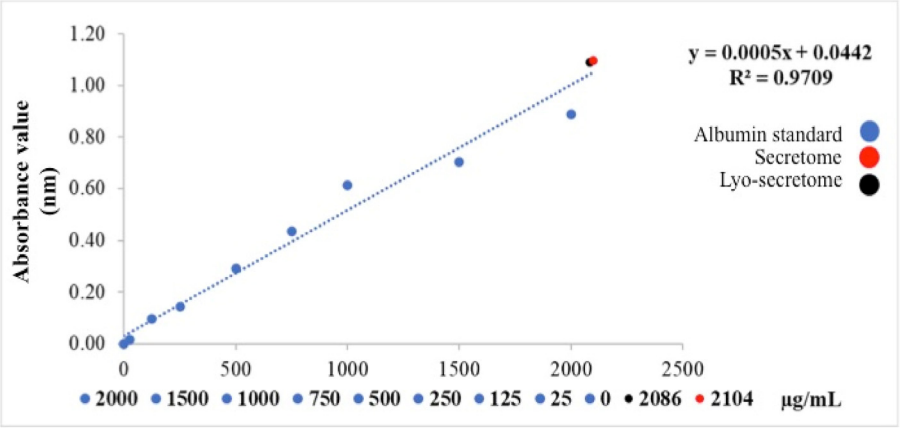

Figure 1: Absorbance curve of total protein concentration. The total protein concentration absorbance curve was calibrated using BSA standard.

Table 1: Total protein concentration in secretome and lyosecretome of human-GMSCs.

Sample	Total protein ± SD (µg/ml)	Significance (*P*)
Secretome	2104.00 ± 0.21	0.971
Lyosecretome	2086.00 ± 0.20	

Following this incubation period, the culture medium was discarded, and 100 µl of basal medium containing hGMSCs-lyosecretome at various concentrations (0.157; 0.313; 0.625; 1.25; 2.5; 5; and 10 mg/ml) was added to the cells. The cells were then subjected to another 24-h incubation in the same 5% CO_2 and 37 °C environment. Subsequently, the culture medium was removed and replaced with 100 µl of H_2O_2 solution at a concentration of 1 mM, mixed with the culture medium to induce oxidative stress in one group of cells. The other group, serving as a control, did not receive H_2O_2 induction. Both groups of cells were then incubated for an additional 24 h under the same conditions. The morphology of rat calvarial osteoblast cells was observed with an inverted microscope.

Measurement of metabolic activity in rat calvarial osteoblast cells

Metabolic activity in rat calvarial osteoblast cells was measured by MTT assay. Initially, 10 µl of MTT solution was added to each well and incubated for 4 h. Subsequently,

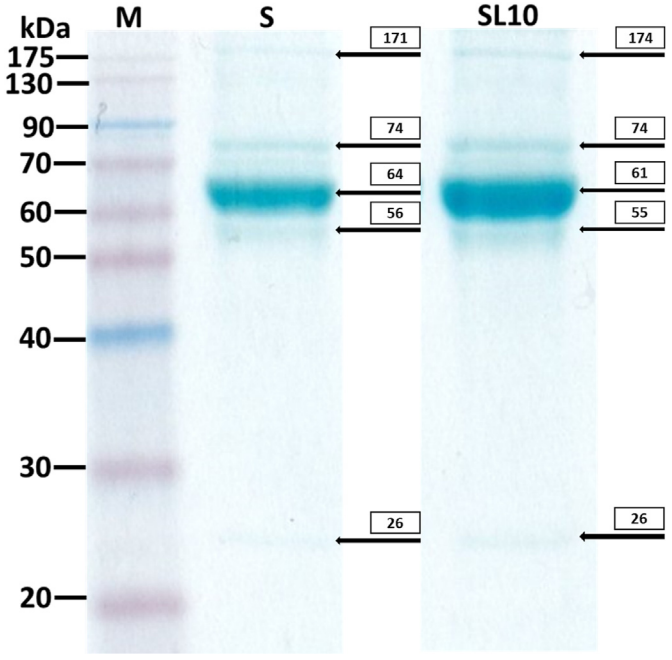

Figure 2: Protein profiling of secretome and lyosecretome. Proteins obtained from secretome (S) and lyosecretome (SL10) were separated by SDS-PAGE gel, and characterized based on their molecular weight in kilo Daltons (kDa).

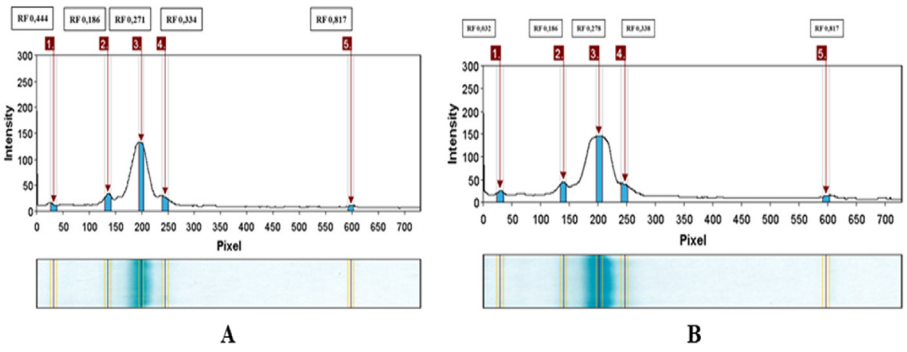

Figure 3: Densitometric analysis of protein bands intensity in secretome and lyosecretome. A band's Retention factor (Rf) value is calculated based on the peak index's position in relation to the profile length. Next, the molecular weight of each band is analyzed from their Rf values.

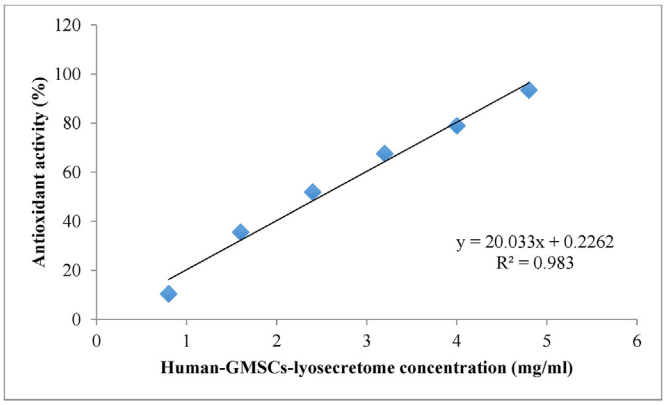

Figure 4: Linear correlation between lyosecretome and antioxidant activity. The correlation coefficients (R^2) of hGMSCs-lyosecretome with ABTS assays are 0.98297, confirming that hGMSCs-lyosecretome had strong contribution to radical scavenging activity.

100 µl of SDS-HCL solution was added to each well and incubated at 37 °C for an additional 4 h. The absorbance was then measured at a wavelength of 570 nm using a microplate reader. This measurement allowed the determination of optical density for both the treatment and control groups. The percentage of metabolic activity in rat calvarial osteoblast cells was calculated based on the formula for cell metabolic activity (%) = 100 × (ODs/ODc). Where the ODs represent the average optical density of the tested samples, and ODc represents the average optical density of the control samples.

Table 2: Protective ability of hGMSCs-lyosecretome in osteoblast cells against H_2O_2 oxidative stress.

hGMSCs-lyosecretome (mg/ml)	Cell metabolic activity ± SD (%)	Significance (*P*)
Control	100 ± 2.74[b]	0.000
0	70.86 ± 1.21[a]	0.000
0.157	74.83 ± 0.49[a]	0.213
0.313	79.24 ± 2.55[a,b]	0.014
0.625	79.49 ± 2.64[a,b]	0.011
1.25	79.53 ± 2.41[a,b]	0.011
2.5	78.38 ± 6.44[a,b]	0.024
5	78.67 ± 1.69[a,b]	0.020
10	76.26 ± 3.26[a]	0.095

[a] Significantly different to the control group.
[b] Significantly different to the treatment group A1 (lyosecretome 0 mg/mL + H_2O_2).

Data analysis

Data on total protein in the secretome and lyosecretome of hGMSCs were analyzed using the Paired Sample t-test. Protein molecular weight was analyzed with Gel Analyzer 19.1 Software. Furthermore, data on the percentage of metabolic activity in rat calvarial osteoblast cells was expressed as the mean ± standard deviation (SD) and analyzed by one-way analysis of variance, with a significance of <0.05.

Results

Total protein

Based on the BCA assay, the secretome and lyosecretome of hGMSCs had no significant difference in total protein concentration ($P = 0.971$). The total protein concentration

absorbance curve was calibrated using the BSA standard Figure 1. The total protein concentration in the secretome was 2104.00 ± 0.21 μg/ml and in the lyosecretome was 2086.00 ± 0.20 μg/ml Table 1.

Protein molecular weights

Proteins were separated by the SDS-PAGE and characterized based on their molecular weight in kilo Daltons (kDa). SDS-PAGE showed blue protein bands on acrylamide gel Figure 2, which were then analyzed with Gel Analyzer 19.1 Software Figure 3. The protein in the secretome has five bands with a molecular weight of 171, 74, 64, 56, and 26 kDa, while the protein in lyosecretome also has five bands with a molecular weight of 174, 74, 61, 55, and 26 kDa.

Antioxidant activity

The antioxidant activity of lyosecretome was determined from the oxidation of potassium persulphate with ABTS salt. The principle of the ABTS method is the ability of antioxidant compounds to stabilize free radical compounds by providing proton radicals, which is characterized by a reduction in the blue color to a colorless state. Lyosecretome had the highest antioxidant activity of 93.51 ± 0.37% at a concentration of 4.8 mg/ml, with an IC_{50} value of 2.08 mg/ml. The antioxidant activity of lyosecretome towards $ABTS^{\cdot+}$ exhibited in a concentration-dependent manner Figure 4. Furthermore, the activity of lyosecretome against ABTS radicals was significantly lower compared to the antioxidant activity of ascorbic acid ($P < 0.05$). Ascorbic acid had the highest antioxidant activity of 99.82 ± 0.09% at a concentration of 0.006 mg/ml, with an IC_{50} value of 0.0018 mg/ml.

Cell metabolic activity

The protective ability of hGMSCs-lyosecretome in osteoblast cells against H_2O_2 oxidative stress was determined by cell metabolic activity. Lyosecretome with a concentration of 0.157; 0.313; 0.625; 1.25; 2.5; 5 and 10 mg/ml were administered to osteoblast cells before H_2O_2 induction. The treatment group A1 (lyosecretome 0 mg/mL + H_2O_2) had lower cell metabolic activity (70.86 ± 1.21%) than the control group (100 ± 2.74%). All concentrations of hGMSCs-lyosecretome attenuate the adverse effect of H_2O_2-induced oxidative stress ($P = 0.000$). The cell metabolic activities of the lyosecretome treatment groups (0.157; 0.313; 0.625; 1.25; 2.5; 5; and 10 mg/ml) were higher than the treatment group A1 (lyosecretome 0 mg/mL + H_2O_2). The lowest cell metabolic activity (74.83 ± 0.49%) was shown in the lyosecretome treatment group at a concentration of 0.157 mg/ml. In comparison, the highest metabolic activity (79.53 ± 2.41%) was shown in the 1.25 mg/ml lyosecretome treatment group Table 2.

Discussion

MSCs-secretome stimulates tissue-resident MSCs to proliferate and express higher levels of osteoblast markers, resulting in new tissue mineralization with the formation of new trabeculae.[22,23] With standardized manufacturing procedures, MSCs-secretome is easily isolated from cell culture supernatants and formulated into freeze-dried pharmaceutical preparations.[24,25] Therefore, this study investigated the capacity of the hGMSCs-secretome, known as lyosecretome to protect rat calvarial osteoblast cells from the adverse effects of H_2O_2-induced oxidative stress. In addition, the proteomic level and antioxidant activity of hGMSCs-lyosecretome were also determined.

Secretome and lyosecretome contain biomolecules, especially proteins, which are very sensitive to physical and chemical influences, resulting in a denaturation process that results in changes to their structure and activity.[26] Therefore, secretome-containing protein must be stored at low temperatures ranging from −20 °C to −80 °C or stored in freeze-dried preparations with a lyophilization process to maintain the protein content without losing its function.[27] The lyophilization process changes the secretome to lyosecretome with the addition of cryoprotectant, then freeze-dried.[28] Mannitol, as a cryoprotectant agent, plays a role in preventing damage to protein particles during the lyophilization process.[24] This study has proven that lyophilization of secretome obtained from hGMSCs into lyosecretome is able to maintain its protein content in freeze-dried powder preparations, with a mass loss of only 0.85%. The protein concentration in the hGMSCs-secretome was 2104.00 ± 0.21 μg/ml, and in the hGMSCs-lyosecretome was 2086.00 ± 0.20 μg/ml (Table 1). These data are in accordance with previous studies that the lyophilization process is considered successful if the mass loss in lyosecretome is not more than 1% of its initial content.[29]

SDS/PAGE is a protein analysis technique used to separate proteins based on their size in a polyacrylamide gel matrix. The molecular weight of the protein in the secretome and lyosecretome obtained from hGMSCs was determined by the size of the protein bands on SDS-PAGE. The thickest band showed the highest intensity projection. Overall, the molecular weight of the secretome and lyosecretome proteins has almost the same band thickness, except that the lyosecretome protein band with a molecular weight of 61 kDa has a wider thickness than the secretome with a molecular weight of 64 kDa. The thickness of the protein bands produced by SDS-PAGE is affected by the quantity of protein, not by the size of the protein molecular weight contained therein. Proteins with the same molecular weight are not necessarily the same type of protein because each protein has differences in its structure.[30]

Previous studies reported that most of the proteins in lyosecretome obtained from adipose-derived mesenchymal stem cells were vesicles/exosomes and annotated extracellular matrix. Functionally, this lyosecretome plays a role in various biological processes such as the immune response, cell metabolism, stress response, and cytoskeleton maintenance. Nonetheless, the protein families most implicated are heat shock proteins (HSPs), structural proteins, and endopeptidase inhibitors, as well as proteins involved in oxidative stress responses.[24] In this study, proteins in secretome and lyosecretome obtained from hGMSCs are thought to have protein types such as α-2-macroglobulin, Fa1p, peptide binding proteins 72/74, α-(1,4)-glucosidase, heat shock cognate 71 kDa protein, extracellular matrix protein-1, α-tubulin, and chymotrypsinogen.[31–37] For instance, α-2-

macroglobulin functions as an extracellular macromolecule with a broad-spectrum protease inhibitory role. It serves as an ideal substrate for various catalytic types of endopeptidases. In addition to its role in regulating extracellular proteolysis, Alpha-2-macroglobulin also contributes to diverting proteolysis towards smaller substrates. Moreover, it aids in facilitating cell migration and binding growth factors, cytokines, and damaged extracellular proteins.[38]

Hydrogen peroxide is a strong oxidizing agent, which can induce cells to produce large amounts of ROS.[16] Abnormal H_2O_2 levels inhibit cell differentiation and lead to cell apoptosis or necrosis.[39,40] Oxidative stress, resulting from excessive ROS formation, has been shown to damage various cellular components of osteoblasts and has been suggested as an essential initiating factor for impaired osteoblastic bone formation.[41] An in vitro study showed that ROS decreased bone formation by inhibiting the processes of osteoblastic cell proliferation, differentiation, and calcification.[42]

In order to determine the survival/defense response of osteoblasts to oxidative stress, this study evaluated the protective ability of hGMSCs-lyosecretome in osteoblast cells against H_2O_2 oxidative stress based on cell metabolic activity. This study revealed that hGMSCs-lyosecretome significantly prevented H_2O_2-mediated alterations in osteoblasts. All concentrations of hGMSCs-lyosecretome attenuate the adverse effect of H_2O_2-induced oxidative stress in osteoblast cells Table 2. These cytoprotective properties are thought to involve the ability of hGMSC-lyosecretome to directly scavenge free radicals, enhance endogenous antioxidant defenses, immunomodulate through suppression of reactive oxygen species, alter mitochondrial bioenergetics, and contribute functional mitochondria to osteoblast cells damaged by H_2O_2 induction.

In addition, h-GMSCs-lyosecretome is thought to also inhibit H_2O_2-induced osteoblast dysfunction, possibly by increasing mitochondrial membrane potential and decreasing markers of mitochondria-mediated apoptosis. Oxidative stress plays a critical role in cellular apoptosis through multiple pathways, including mitochondria, death receptors, and endoplasmic reticulum stress. Additionally, it triggers the activation of key cellular components such as nuclear factor κB, protein kinase, and caspases.[43,44] H_2O_2 serves as a secondary messenger, penetrating cell membranes to participate in signal transduction pathways.[19] ROS are a normal product of cell metabolism; an excess of ROS, particularly from mitochondria, can cause cellular function and induce apoptosis.[17] However, early autophagy through the endoplasmic reticulum stress pathway can reduce oxidative damage to osteoblasts.[45] Autophagy protects cells against various cytotoxic stimuli by decreasing damaged organelles at an early stage. Nevertheless, an overabundance of autophagy can ultimately result in cell death.[46,47]

Changes in the composition of the MSC secretome that occur during senescence can have a negative impact on body health, so a healthy MSC secretome is needed for tissue repair and homeostasis. During senescence, the MSC secretome undergoes marked modifications in its composition and develops a senescence-associated secretory phenotype (SASP). Therefore, analysis of the MSC secretome is essential before use in secretome therapy because the MSC secretome can lose its beneficial functions and also exert negative pro-inflammatory and pro-aging activities when MSCs become senescent.[48]

Lyosecretome from hGMSCs can be exploited as an attractive alternative to cell-based therapies. Therefore, molecular characterization and physicochemical characterization at the proteomic level are needed to control the quality of hGMSCs-lyosecretome. A key aspect of hGMSCs-lyosecretome production is the isolation of secretome from the hGMSCs supernatant by a validated, scalable, and good manufacturing practice (GMP)-compliant production process, thereby obtaining a standardized medicinal product suitable for clinical application.

Conclusions

In conclusion, this study confirmed that hGMSCs-lyosecretome had antioxidant activity of 93.51 ± 0.37%, and hGMSCs-lyosecretome with a concentration of 1.25 mg/ml was able to counteract H_2O_2-induced oxidative stress damage in osteoblast cells. Therefore, hGMSCs-lyosecretome has the potential as a secretome-based therapy for treating several bone diseases. Future studies are needed to validate the effect of hGMSCs-lyosecretome in osteogenic tissue by using a mouse oxidative stress model.

Source of funding

This study was supported by the Directorate General of Higher Education, Research and Technology, Ministry of Education, Culture, Research and Technology, Republic of Indonesia (127/E5/PG.02.00.PL/2023).

Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

This research was approved by the Health Research Ethics Commission, Faculty of Dental Medicine, Jember University (No. 2272/UN25.8/KEPK/DL/2023, Approval Date: 08 September 2023).

Authors contributions

BK, MNA, and YCR carried out the research and collected the data. **DSS** designs the study, supervises, visualizes and validates, acquires funding, and reviews draft material. The data was organized, analyzed, and interpreted by **MIA and AHP**, who also reviewed the article. **AK, CP, and MAA** organized, analyzed, and interpreted the data and revised the article. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

References

1. Grawish ME. Gingival-derived mesenchymal stem cells: an endless resource for regenerative dentistry. **World J Stem Cells 2018**; 10:116. https://doi.org/10.4252/WJSC.V10.I9.116.

2. Kusumawardani B, Robin DMC, Puspitasari E, Savitri IJ, Suendi DAP. Cultivation and expansion of mesenchymal stem cells from human gingival tissue and periodontal ligament in different culture media. **Dent J 2021**; 54: 39–45. https://doi.org/10.20473/j.djmkg.v54.i1.p39-45.
3. Kusumawardani B, Rachmawati D, Ajeng D, Suendi P, Savitri IJ. Slow freezing cryopreservation in combination with cryoprotectants preserve gingival mesenchymal stem cells. **Malays J Med Health Sci 2023**; 19: 2636–9346.
4. Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. **Nat Med 2005**; 11: 367–368. https://doi.org/10.1038/NM0405-367.
5. Soudi A, Yazdanian M, Ranjbar R, Tebyanian H, Yazdanian A, Tahmasebi E, et al. Role and application of stem cells in dental regeneration: a comprehensive overview. **EXCLI J 2021**; 20: 454. https://doi.org/10.17179/EXCLI2021-3335.
6. Konala VBR, Mamidi MK, Bhonde R, Das AK, Pochampally R, Pal R. The current landscape of the mesenchymal stromal cell secretome: a new paradigm for cell-free regeneration. **Cytotherapy 2016**; 18: 13. https://doi.org/10.1016/J.JCYT.2015.10.008.
7. Crivelli B, Chlapanidas T, Perteghella S, Lucarelli E, Pascucci L, Brini AT, et al. Mesenchymal stem/stromal cell extracellular vesicles: from active principle to next generation drug delivery system. **J Control Release 2017**; 262: 104–117. https://doi.org/10.1016/J.JCONREL.2017.07.023.
8. Kusumawardani B, Purnamasari J, Ajeng D, Suendi P. Determination of total protein and calcium in gingival mesenchymal stem cell-conditioned medium. **Insisiva Dent J: Majalah Kedokteran Gigi Insisiva 2021**; 10: 65–70. https://doi.org/10.18196/DI.V10I2.13096.
9. Lyamina S, Baranovskii D, Kozhevnikova E, Ivanova T, Kalish S, Sadekov T, et al. Mesenchymal stromal cells as a driver of inflammaging. **Int J Mol Sci 2023**; 24. https://doi.org/10.3390/IJMS24076372.
10. El Andaloussi S, Mäger I, Breakefield XO, Wood MJA. Extracellular vesicles: biology and emerging therapeutic opportunities. **Nat Rev Drug Discov 2013**; 12: 347–357. https://doi.org/10.1038/NRD3978.
11. Mocchi M, Dotti S, Del Bue M, Villa R, Bari E, Perteghella S, et al. Veterinary regenerative medicine for musculoskeletal disorders: can mesenchymal stem/stromal cells and their secretome be the new frontier? **Cells 2020**; 9: 1–26. https://doi.org/10.3390/CELLS9061453.
12. Smith RKW, Werling NJ, Dakin SG, Alam R, Goodship AE, Dudhia J. Beneficial effects of autologous bone marrow-derived mesenchymal stem cells in naturally occurring tendinopathy. **PLoS One 2013**; 8. https://doi.org/10.1371/JOURNAL.PONE.0075697.
13. Franzé S, Selmin F, Samaritani E, Minghetti P, Cilurzo F. Lyophilization of liposomal formulations: still necessary, still challenging. **Pharmaceutics 2018**; 10. https://doi.org/10.3390/PHARMACEUTICS10030139.
14. Kumar KN, Mallik S, Sarkar K. Role of freeze-drying in the presence of mannitol on the echogenicity of echogenic liposomes. **J Acoust Soc Am 2017**; 142: 3670. https://doi.org/10.1121/1.5017607.
15. Baek KH, Oh KW, Lee WY, Lee SS, Kim MK, Kwon HS, et al. Association of oxidative stress with postmenopausal osteoporosis and the effects of hydrogen peroxide on osteoclast formation in human bone marrow cell cultures. **Calcif Tissue Int 2010**; 87: 226–235. https://doi.org/10.1007/S00223-010-9393-9.
16. Callaway DA, Jiang JX. Reactive oxygen species and oxidative stress in osteoclastogenesis, skeletal aging and bone diseases. **J Bone Miner Metab 2015**; 33: 359–370. https://doi.org/10.1007/S00774-015-0656-4.
17. Vela L, Contel M, Palomera L, Azaceta G, Marzo I. Iminophosphorane-organogold(III) complexes induce cell death through mitochondrial ROS production. **J Inorg Biochem 2011**; 105: 1306–1313. https://doi.org/10.1016/J.JINORGBIO.2011.06.004.
18. She C, Qing Zhu L, Fang Zhen Y, Dong Wang X, Rong Dong Q. Activation of AMPK protects against hydrogen peroxide-induced osteoblast apoptosis through autophagy induction and NADPH maintenance: new implications for osteonecrosis treatment? **Cell Signal 2014**; 26: 1–8. https://doi.org/10.1016/J.CELLSIG.2013.08.046.
19. Hara-Chikuma M, Watanabe S, Satooka H. Involvement of aquaporin-3 in epidermal growth factor receptor signaling via hydrogen peroxide transport in cancer cells. **Biochem Biophys Res Commun 2016**; 471: 603–609. https://doi.org/10.1016/J.BBRC.2016.02.010.
20. Tafazoli Moghadam E, Yazdanian M, Alam M, Tebyanian H, Tafazoli A, Tahmasebi E, et al. Current natural bioactive materials in bone and tooth regeneration in dentistry: a comprehensive overview. **J Mater Res Technol 2021**; 13: 2078–2114. https://doi.org/10.1016/J.JMRT.2021.05.089.
21. Darjanki CM, Prahasanti C, Fitria A E, Kusumawardani B, Wijaksana IKE, Aljunaid M. RUNX2 and ALP expression in osteoblast cells exposed by PMMA-HAp combination: an in vitro study. **J Oral Biol Craniofac Res 2023**; 13: 277. https://doi.org/10.1016/J.JOBCR.2023.02.007.
22. Bari E, Roato I, Perale G, Rossi F, Genova T, Mussano F, et al. Biohybrid bovine bone matrix for controlled release of mesenchymal stem/stromal cell lyosecretome: a device for bone regeneration. **Int J Mol Sci 2021**; 22. https://doi.org/10.3390/IJMS22084064/S1.
23. Eichholz KF, Woods I, Riffault M, Johnson GP, Corrigan M, Lowry MC, et al. Human bone marrow stem/stromal cell osteogenesis is regulated via mechanically activated osteocyte-derived extracellular vesicles. **Stem Cells Transl Med 2020**; 9: 1431–1447. https://doi.org/10.1002/SCTM.19-0405.
24. Bari E, Perteghella S, Di Silvestre D, Sorlini M, Catenacci L, Sorrenti M, et al. Pilot production of mesenchymal stem/stromal freeze-dried secretome for cell-free regenerative nanomedicine: a validated GMP-compliant process. **Cells 2018**; 7. https://doi.org/10.3390/CELLS7110190.
25. Bari E, Scocozza F, Perteghella S, Sorlini M, Auricchio F, Torre ML, et al. 3D bioprinted scaffolds containing mesenchymal stem/stromal lyosecretome: next generation controlled release device for bone regenerative medicine. **Pharmaceutics 2021**; 13. https://doi.org/10.3390/PHARMACEUTICS13040515.
26. Masson P, Lushchekina S. Conformational stability and denaturation processes of proteins investigated by electrophoresis under extreme conditions. **Molecules 2022**; 27: 6861. https://doi.org/10.3390/MOLECULES27206861.
27. Abla KK, Mehanna MM. Freeze-drying: a flourishing strategy to fabricate stable pharmaceutical and biological products. **Int J Pharm 2022**; 628. https://doi.org/10.1016/J.IJPHARM.2022.122233.
28. Gaetani P, Torre ML, Klinger M, Faustini M, Crovato F, Bucco M, et al. Adipose-derived stem cell therapy for intervertebral disc regeneration: an in vitro reconstructed tissue in alginate capsules. **Tissue Eng Part A 2008**; 14: 1415–1423. https://doi.org/10.1089/TEN.TEA.2007.0330.
29. Bari E, Ferrarotti I, Di Silvestre D, Grisoli P, Barzon V, Balderacchi A, et al. Adipose mesenchymal extracellular vesicles as alpha-1-antitrypsin physiological delivery systems for lung regeneration. **Cells 2019**; 8. https://doi.org/10.3390/CELLS8090965.
30. Matsumoto H, Haniu H, Komori N. Determination of protein molecular weights on SDS-PAGE. **Methods Mol Biol 2019**; 1855: 101–105. https://doi.org/10.1007/978-1-4939-8793-1_10.

31. de Oliveira Neto M, de Freitas Fernandes A, Piiadov V, Craievich AF, de Araújo EA, Polikarpov I. SAXSMoW 3.0: new advances in the determination of the molecular weight of proteins in dilute solutions from SAXS intensity data on a relative scale. **Protein Sci 2022**; 31: 251−258. https://doi.org/10.1002/PRO.4227.
32. Matafora V, Bachi A. Secret3D workflow for secretome analysis. **STAR Protoc 2020**; 1. https://doi.org/10.1016/J.XPRO.2020.100162.
33. Norheim F, Raastad T, Thiede B, Rustan AC, Drevon CA, Haugen F. Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. **Am J Physiol Endocrinol Metab 2011**; 301. https://doi.org/10.1152/AJPENDO.00326.2011.
34. Ejazi SA, Bhattacharyya A, Choudhury ST, Ghosh S, Sabur A, Pandey K, et al. Immunoproteomic identification and characterization of *Leishmania* membrane proteins as non-invasive diagnostic candidates for clinical visceral leishmaniasis. **Sci Rep 2018**; 8: 1−11. https://doi.org/10.1038/s41598-018-30546-y.
35. Rustini NL, Ariati NK. Identification of active antioxidant compounds from ungu leaf ethanol extract. **J Health Sci Med 2018**; 2: 9−12.
36. Andrade A, Dall'Agnol M, Newton S, Martinez MB. The iron uptake mechanisms of enteroinvasive Escherichia coli. **Braz J Microbiol 2000**; 31: 199−204. https://doi.org/10.1590/S1517-83822000000300009.
37. Finst RJ, Kim PJ, Griffis ER, Quarmby LM. Fa1p is a 171 kDa protein essential for axonemal microtubule severing in Chlamydomonas. **J Cell Sci 2000**; 113(Pt 11): 1963−1971. https://doi.org/10.1242/JCS.113.11.1963.
38. Vandooren J, Itoh Y. Alpha-2-Macroglobulin in inflammation, immunity and infections. **Front Immunol 2021**; 12:803244. https://doi.org/10.3389/FIMMU.2021.803244/BIBTEX.
39. Arakaki N, Yamashita A, Niimi S, Yamazaki T. Involvement of reactive oxygen species in osteoblastic differentiation of MC3T3-E1 cells accompanied by mitochondrial morphological dynamics. **Biomed Res 2013**; 34: 161−166. https://doi.org/10.2220/BIOMEDRES.34.161.
40. Li J, Stouffs M, Serrander L, Banfi B, Bettiol E, Charnay Y, et al. The NADPH oxidase NOX4 drives cardiac differentiation: role in regulating cardiac transcription factors and MAP kinase activation. **Mol Biol Cell 2006**; 17: 3978. https://doi.org/10.1091/MBC.E05-06-0532.
41. Kiffin R, Bandyopadhyay U, Cuervo AM. Oxidative stress and autophagy. **Antioxid Redox Signal 2006**; 8: 152−162. https://doi.org/10.1089/ARS.2006.8.152.
42. Marques-Carvalho A, Kim HN, Almeida M. The role of reactive oxygen species in bone cell physiology and pathophysiology. **Bone Rep 2023**. https://doi.org/10.1016/J.BONR.2023.101664.
43. Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. **EMBO Rep 2006**; 7: 880−885. https://doi.org/10.1038/SJ.EMBOR.7400779.
44. Manna SK, Sarkar S, Barr J, Wise K, Barrera EV, Jejelowo O, et al. Single-walled carbon nanotube induces oxidative stress and activates nuclear transcription factor-kappaB in human keratinocytes. **Nano Lett 2005**; 5: 1676−1684. https://doi.org/10.1021/NL0507966.
45. Yang YH, Li B, Zheng XF, Chen JW, Chen K, Jiang SD, Jiang LS. Oxidative damage to osteoblasts can be alleviated by early autophagy through the endoplasmic reticulum stress pathway−implications for the treatment of osteoporosis. **Free Radic Biol Med 2014**; 77: 10−20. https://doi.org/10.1016/J.FREERADBIOMED.2014.08.028.
46. Liu SY, Chen CL, Yang TT, Huang WC, Hsieh CY, Shen WJ, et al. Albumin prevents reactive oxygen species-induced mitochondrial damage, autophagy, and apoptosis during serum starvation. **Apoptosis 2012**; 17: 1156−1169. https://doi.org/10.1007/S10495-012-0758-6.
47. Younce CW, Kolattukudy PE. MCP-1 causes cardiomyoblast death via autophagy resulting from ER stress caused by oxidative stress generated by inducing a novel zinc-finger protein, MCPIP. **Biochem J 2010**; 426: 43−53. https://doi.org/10.1042/BJ20090976.
48. Siraj Y, Galderisi U, Alessio N. Senescence induces fundamental changes in the secretome of mesenchymal stromal cells (MSCs): implications for the therapeutic use of MSCs and their derivates. **Front Bioeng Biotechnol 2023**; 11. https://doi.org/10.3389/FBIOE.2023.1148761.

How to cite this article: Kusumawardani B, Nurul Amin M, Rahayu YC, Sari DS, Altariq MI, Putri AH, Kanya A, Prahasanti C, Aljunaid MA. Human gingival mesenchymal stem cells-lyosecretome attenuates adverse effect of hydrogen peroxide-induced oxidative stress on osteoblast cells. J Taibah Univ Med Sc 2024;19(3):687−695.