



## Research Article

# Determination of Total Protein and Calcium in Gingival Mesenchymal Stem Cell-Conditioned Medium

*Determinasi Total Protein dan Kalsium pada Gingival Mesenchymal Stem Cell-Conditioned Medium*

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## Abstrak

*Gingival mesenchymal stem cell-conditioned medium (GMSC-CM)* merupakan media terkondisi hasil kultur gingiva mesenchymal stem cell. Keuntungan GMSC yaitu mudah diisolasi dari jaringan gingiva, selain itu jaringan gingiva mudah diperoleh dengan teknik invasif yang minimal. Di sisi lain, CM mengandung protein, sitokin, kemokin dan faktor pertumbuhan yang berperan penting dalam proses diferensiasi osteogenik. Penelitian ini bertujuan untuk mendeterminasi total kadar protein dan kalsium pada GMSC-CM. GMSC dikultur pada media kultur dengan tambahan 10% FBS. Media GMSC-CM diperoleh dari media yang telah disaring menggunakan filter 0,22µm dan CM dipekatkan dengan sentrifugal filter untuk mendapatkan konsentrat GMSC-CM. Konsentrasi total protein pada GMSC-CM dan konsentrat GMSC-CM dilakukan dengan *bicinchoninic acid protein assay*. Uji kadar kalsium dilakukan dengan metode spektroskopi serapan atom. Hasil penelitian menunjukkan GMSC-CM mempunyai konsentrasi total protein 2502±0.06µg/ml dan konsentrat GMSC-CM sebesar 1.912±0.08µg/ml. Kadar kalsium pada GMSC-CM yaitu 0,013% dan pada konsentrat GMSC-CM 0,009%. Kesimpulan penelitian ini adalah GMSC-CM mempunyai konsentrasi total protein dan kalsium yang tinggi. Parameter ini digunakan untuk mengembangkan bioproses yang meningkatkan produksi GMSC-CM, yang akan mendukung penerapan *cell-free therapy* untuk regenerasi jaringan.

**Kata Kunci:** *Cell-free Therapy; Gingival Mesenchymal Stem Cell-conditioned Medium; Kalsium; Protein Total*

## Abstract

*Gingival mesenchymal stem cell-conditioned medium (GMSC-CM)* is a conditioned medium obtained from cultured gingival mesenchymal stem cells. GMSCs are easily isolated from gingival tissue, whereas gingival tissue is easily obtained by minimally invasive techniques. On the other hand, CM contains proteins, cytokines, chemokines and growth factors that play an important role in osteogenic differentiation. This study aims to determine the total protein and calcium levels in GMSC-CM. GMSCs were grown in culture media with 10% of FBS. CM of GMSC was obtained from the media collection process with a 0.22 µm filter and concentrated with a centrifugal filter to obtain a concentrated GMSC-CM. The concentration of total protein was performed by bicinchoninic acid protein assay on GMSC-CM and concentrated GMSC-CM. Calcium level was performed by atomic absorption spectroscopy method. The results are that GMSC-CM had a total protein concentration of 2502±0.06 µg/ml, and the concentrated GMSC-CM was 1.912±0.08 µg/ml. The calcium level of GMSC-CM was 0.009% and concentrated GMSC-CM was 0.009%. It can be concluded that GMSC-CM had a high concentration of total protein and calcium. These parameters were used to develop bioprocesses to enhance the production of GMSC-CM, which will support the implementation of *cell-free therapy* for tissue regeneration.

**Keywords:** *Calcium; Cell-free Therapy; Gingival Mesenchymal Stem Cell-conditioned Medium; Total Protein*

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## INTRODUCTION

Gingival mesenchymal stem cells (GMSCs) are spindle-shaped cells isolated from gingival tissue, with multipotent differentiation capacity *in vitro*. GMSCs are easily isolated from gingival tissue, whereas gingival tissue is easily obtained by minimally invasive techniques without tooth extraction or other surgical procedures. Due to their self-renewal and differentiation capacity, GMSCs have been used in regenerative medicine and have been developed as a promising therapy towards restoring the structure and function of periodontal tissues due to periodontal disease.<sup>1</sup> However, several cases reported that the implanted MSC did not last long, so it is suspected that the success of MSC therapy may be influenced by various bioactive factors produced by MSC.<sup>2</sup> These bioactive factors, referred to as MSC secretomes or conditioned media (MSC-CM), will modulate endogenous cell behavior, thereby contributing to new tissue formation.<sup>3</sup>

The use of MSC-CM has several advantages over MSCs as it does not require cell implantation. In this case, there is no need to match donors and recipients to avoid rejection problems. In addition, the absence of allogeneic cell replication in MSC-CM would improve patient safety.<sup>4</sup> The concentration of bioactive factors contained in MSC-CM is usually relatively low, so the use of MSC-CM does not cause a severe inflammatory response.<sup>5</sup> Furthermore, MSC-CM can be produced, dried, packaged and transported more easily than MSCs. The ease with which this potentially therapeutic chemical can be stored offers the foundation for its cost-effective distribution.<sup>6</sup>

Optimization and standardization of GMSC-CM processing are required to avoid changes in the protein secretion profile and ensure effective quality control. These qualities and conditions will determine the quantity, quality, and type of biomolecules released by GMSCs in the

conditioned media. It is very important to understand these parameters and develop bioprocesses that enhance GMSC-CM derivative products.

After conditioning, the cell supernatants were collected and centrifuged or filtered to remove non-adherent cells. CM can be further processed in a way that abundant serum protein can be depleted, or the media can be concentrated. The concentration of protein secreted in CM is usually low, and the protein is in a soluble form. Methods that do not involve precipitation are used to maintain protein concentration, such as centrifuge filtration, dialysis, or evaporation. However, centrifuge filtration with a micro-concentrator will result in protein binding to the membrane, and the desired protein may be lost.<sup>7</sup> Based on these, this study focused on developing an optimized strategy to provide total protein and calcium concentrations of GMSC-CM and concentrated GMSC-CM.

## MATERIAL AND METHOD

Gingival mesenchymal stem cells (GMSCs) with fourth passage character (GP-4) were obtained from the GMSCs collection inventory at the Laboratory of Molecular Medicine - CDAST, University of Jember, Jember, Indonesia.<sup>1</sup> The research procedure was approved by the Health Research Ethics Commission, Faculty of Dentistry, University of Jember (No.1245/UN25.8/KEPK/DL/2021). GMSCs were grown in culture media consisting of DMEM with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2.5 g/mL amphotericin B. Cells were cultured in a 10 cm tissue culture dish and harvested using 0.25% trypsin-EDTA.

After GMSC reached 80-90% confluency, cells were washed with phosphate-buffered saline (PBS) 5 times. Cells were then treated with DMEM basal medium with 10% FBS without adding penicillin-streptomycin and incubated for 24 h in 5% CO<sub>2</sub> at 37°C. The supernatant

from GMSC was collected and centrifuged at 173xg (3,000rpm) for 10 min, then filtered with a 0.22µm filter to obtain GMSC-CM. Next, GMSC-CM was concentrated 100-fold using an ultrafiltration centrifugal tube (Ultra-15 10 kD centrifugal filter, EMD Millipore, Billerica, MA, USA) centrifuged at 5,000xg at 4°C for 40 minutes. GMSC-CM and concentrated GMSC-CM were packaged and stored at -80°C.

Total protein content in GMSC-CM was determined by bicinchoninic acid (BCA) protein assay. The BCA protein assay procedure began by preparing standard bovine serum albumin (BSA) protein in DMEM basal media. Furthermore, 25 µL of BSA protein standard, GMSC-CM, and concentrated GMSC-CM were placed on a 96-microwell plate. Working reagent (solution A + B) in 200 µL was added to each group. The plate was shaken with a plate shaker for 30 seconds, incubated for 30 minutes, and the absorbance was measured with a microplate reader at 562 nm.

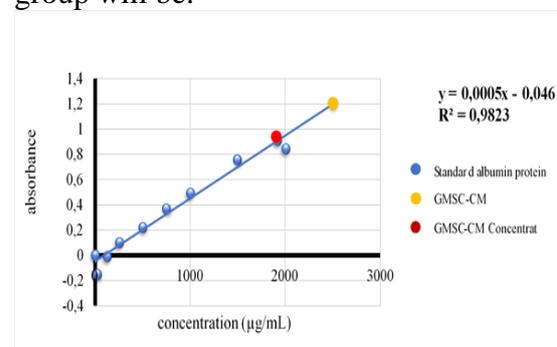
The calcium level test was carried out by preparing 1 mL of 20,000 ppm potassium chloride and 1 mL of nitric acid added to each tube containing 1 mL of GMSC-CM and concentrated GMSC-CM. The prepared sample (1 mL) was diluted 1:7 in 4 mL of distilled water so that the total volume of each sample was 5 mL. The calcium content of the sample was measured by atomic absorption spectroscopy.

All experiments were carried out in triplication. Data were expressed as mean ± standard deviation (SD). Differences between means were evaluated by Student's t-test (SPSS software). The *p*-value ≤ 0.05 was considered statistically significant.

**RESULT**

The absorbance values of BCA protein assay in each group of GMSC-CM and concentrated GMSC-CM showed the standard curve regression line equation, namely  $Y=0.0005x-0.046$  (Fig. 1). Based

on this equation, the total protein concentration in GMSC-CM was  $2.502\pm0.06$  µg/ml, and the concentrated GMSC-CM was  $1.912\pm0.08$  µg/ml. The total protein concentration of GMSC-CM was higher than that of concentrated GMSC-CM (Table 1). The total protein concentration was obtained from the equation with a correlation coefficient of 0.9823. The value of  $R^2=0.9832$  indicated a strong positive relationship between X (concentration) and Y (absorbance), that the higher the absorbance value is, the higher the total protein concentration of the group will be.



**Figure 1.** Bicinchoninic acid protein test. The absorbance value of the BCA protein test results showed that GMSC-CM had a higher protein concentration than the concentrated GMSC-CM, which was obtained from the equation  $y = 0.0005x - 0.046$  with a coefficient value of  $R^2 = 0.98$

**Table 1.** Protein concentration of GMSC-CM and concentrated GMSC-CM

Group	Protein Concentration ± SD (µg/ml)
GMSC-CM	2,502 ± 0.06
Concentrated GMSC-CM	1,912 ± 0.08

**Table 2.** Calcium level of GMSC-CM and concentrated GMSC-CM

Group	Calcium level ± SD (%)
GMSC-CM	0.013 ± 0.00
Concentrated GMSC-CM	0.009 ± 0.00

Calcium levels obtained by atomic absorption spectroscopy can be seen in

Table 2. The calcium level of each sample was derived from the standard curve. Calcium levels for GMSC-CM (0.013%) were slightly higher than concentrated GMSC-CM (0.009%).

## DISCUSSION

GMSC-CM has been known to have advantages as a ready-to-use material that can be used for regenerative medicine without isolation and culture of GMSC. However, several issues must be addressed before its clinical application. One of the most important is the limitation of information regarding standards for MSC-CM-based bioprocesses and therapeutic procedures.<sup>8</sup> Several challenges need to be overcome to develop a procedure that utilizes MSC-CM as a cell-free therapy. MSC-CM differ depending on MSC origin and culture conditions.<sup>9,10</sup> Hence, this study suggests basic procedures in the preparation of conditioned medium, which optimizes the development of GMSC-CM-based products.

The most common way to make GMSC-CM is to centrifuge the culture medium to remove cell debris, then use the supernatant directly or in concentrated preparations. This study has successfully developed a CM processing procedure based on total protein concentration and calcium level in GMSC-CM and concentrated GMSC-CM. The low concentration of protein and calcium in concentrated GMSC-CM is considered due to the centrifuge filtering procedure with an ultra-concentrator, resulting in protein binding to the membrane and even protein loss.

The total protein concentration in GMSC-CM was  $2,502 \pm 0.06 \mu\text{g/ml}$  higher than the total protein concentration in the concentrated GMSC-CM, which was  $1,912 \pm 0.08 \mu\text{g/ml}$ . The GMSC-CM did not undergo a filtering process so that the protein in the CM produced from GMSC was still complete, both micro and macro proteins. At the same time, the concentrated GMSC-CM was produced through a

filtering process with a predetermined size; thus, it affected the total protein concentration. Proteins secreted by MSCs in CM play an important role in regulating physiological processes through paracrine and autocrine mechanisms and acting as potential therapeutic biomarkers in various diseases.<sup>11</sup> Proteins also carry out signaling functions in the form of cellular functions such as cell proliferation and differentiation.<sup>12</sup> The high concentration of total protein in MSC-CM can support the occurrence of this function and can also alter the genome of recipient cells.<sup>13</sup> Thus, concentrations of the substance can cause allergic and toxic reactions in cells and tissues.<sup>14</sup>

Different secretome signatures have been discovered after proteomic analyses of MSC secretions from different tissue origins. Only 124 of the 1533 proteins found in MSCs produced from bone marrow, adipose tissue, and tooth pulp was found in all three sources.<sup>15</sup> The proteins secreted by MSCs are functional factors associated with the biological effects of MSCs. MSCs from Wharton's jelly release more cytokines, proinflammatory proteins, and growth factors, whereas MSCs from adipose tissue secrete more extracellular matrix (ECM) proteins and metalloproteinases and have an angiogenic profile.<sup>16</sup>

Furthermore, calcium is a nutrient that is associated with the formation and metabolism of bone formation. Therefore, the calcium level test on GMSC-CM is very necessary. GMSC-CM has calcium content (0.015%), which is not much different from concentrated GMSC-CM (0.009%). High calcium levels contained in CM can promote proliferation, migration and differentiation of MSC. Therefore, calcium content is very important in regulating extracellular matrix mineralization.<sup>17</sup>

GMSC-CM is a promising candidate for cell-free therapy. It will be able to be used in regenerative medicine through several methods that are possible to be injected into tissue or circulation directly, mixed with a hydrogel, or coated onto a

scaffold using a fibrin gel. Therefore, standard procedures for purification and quantification are required to utilize GMSC-CM in therapy. It is necessary to standardize long-term storage methods to maintain the function of GMSC-CM and minimize variability in the stability and composition of GMSC-CM. It would increase the possibility of administering GMSC-derived products as cell-free therapy rather than implanting GMSCs themselves.

## CONCLUSION

Gingival mesenchymal stem cell-conditioned medium (GMSC-CM) had a higher concentration of total protein ( $2502 \pm 0.06 \mu\text{g/ml}$ ) and calcium (0.015%) than the total protein concentration ( $1,912 \pm 0.08 \mu\text{g/ml}$ ) and calcium (0.009%) of the concentrated GMSC-CM. These parameters were used to develop bioprocesses that enhanced the production of GMSC-CM, which would support the implementation of cell-free therapy for tissue regeneration.

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