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Characterization of Microencapsulation Human Adipose Tissue Mesenchymal Stem Cells (hAT-MSCs) and Nanoparticle of Conditioned Medium of AT-MSCs

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Abstract

This study aims to characterize the microencapsulated type of human Adipose Tissue Mesenchymal Stem Cells (hAT-MSCs) and nanoparticles containing a conditioned medium of hAT-MSCs (CM-hATMSCs). The hAT-MSCs microencapsulation was measured the cells viability on 1st, 7th, and 14th days, by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, stability test was performed using magnetic stirrer at a speed of 300, 700, 1100, and 1200 rpm (revolutions per minutes) and applied on hAT-MSCs microencapsulation. The particle size of CM-hATMSCs nanoparticles were measured by particle size analyzer (PSA) and the growth factor levels namely Epidermal Growth Factor (EGF), Interleukin-6 (IL-6), Vascular Endothelial Growth Factor (VEGF), Insulin-Like Growth Factor-1 (IGF-1) were investigated using ELISA method. In the 14th day showed decreasing of cell viability percentage from 90 - 100% to 50% in both of 1.5×10^5 and 3×10^5 cells hAT-MSCs microencapsulation. CM-hATMSCs microencapsulation are quite stable and has a fairly good mechanical resistance when tested for stability using rotation at a speed of 1,200 rpm only damages 10 % of it. The CM-hATMSCs nanoparticles collected from hAT-MSCs growth medium in PSA test have an average particle size of 141 nm. Based on measurement of growth factor level, hAT-MSCs microencapsulation (3×10^5 cells) and CM-hATMSCs nanoparticles showed secretion of growth factors of EGF, IL-6, VEGF, and IGF-1 compared to positive controls. In summary, hAT-MSCs microencapsulation formulation using alginate showed good product based on the viability, stability, structure, and growth factor secretion ability. The characterization results showed that microencapsulated hAT-MSCs and CM-hATMSCs nanoparticles were successfully synthesized with the appropriate criteria.

Keywords: Conditioned medium, Human adipose tissue mesenchymal stem cell, Microencapsulation, Nanoparticles

Introduction

Recently, cell therapy using stem cells has the potential to accelerate the healing of chronic wounds such as burns and diabetic wounds [1,2]. Adipose Tissue Mesenchymal Stem Cells (AT-MSCs) are stem cells derived from fatty tissue that have the ability to differentiate into other cells that are osteogenic, myogenic, neurogenic, and hematopoietic. Human AT-MSCs (hAT-MSCs) are known to have an important role in the healing process of wounds on the skin both directly and indirectly [3,4]. The hAT-MSCs can be easily isolated from subcutaneous fatty tissue in large quantities and without causing pain. The hAT-MSCs also do not cause excessive immunological reactions when used in cell therapy so they are widely used in the medical world as an alternative for healing wounds on the skin [5].

The hAT-MSCs secrete a broad variety of cytokines, growth factors, and cells adhesion molecules such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and transforming growth factor (TGF)-β well known as Conditioned Medium (CM) that hAT-MSCs and CM of hAT-MSCs (CM-hATMSCs) widely applied to accelerate wound healing [6]. Various studies state that growth factors secreted by hAT-MSCs and CM-hATMSCs can stimulate collagen synthesis and migration of dermal fibroblasts and reduce skin wrinkles to accelerate the wound-healing process [7]. Another factor that is also secreted by hAT-MSCs is the angiogenic factor. Angiogenic factors can induce proliferation as well as differentiation of endothelial cells by *in vitro* study [8,9]. Various studies have shown that CM-hATMSCs by *in vitro* study can repair wounds in cutaneous. The CM-hATMSCs can stimulate the migration of dermal fibroblasts by *in vitro* study [6]. Dermal fibroblast cells (NBL-6) can migrate, and cover wounds faster cultured in CM of equine MSCs (CM-EMSCs) than controls (without CM-EMSCs) [9].

In the implantation process of MSCs, there is often a distribution in non-target tissues [10]. The transplantation of hMSC is influenced by environment and inflammation, which can trigger unwanted differentiation of cells [11]. To overcome this problem, it is necessary to develop a delivery approach with the immobilization of hMSCs. Cell immobilization systems can develop the administration of hMSCs and can simultaneously be protective of tissues. The encapsulated microenvironment suggests that it can retain the secretory function of hMSCs, including secretions that play a role in growth. Cells that have been encapsulated can be transplanted without causing an excess immunity reaction and inflammation [12]. By utilizing the inflammatory agents TNF-α, and IFN-γ, micro alginate 1.7 and 2.2 % can increase the secretion of active hMSCs cultures [13]. Microencapsulation has many advantages, namely resistance to mechanical stress, no inhibition of the diffusion and osmosis processes in cells and is easier when injected in the implantation region [14].

Nanoparticles are used as drug delivery systems to control particle size, surface properties, and release of active substances to obtain a drug-specific action pharmacologically at its dosage regimen. Nanoparticles control and release the therapeutic material slowly during distribution, modify the distribution of the therapeutic material in the organs of action, and slow down the clearance of the medicinal material, thereby minimizing side effects [15]. Controlled release and particle degradation characteristics can be modulated by the selection of matrix constituents. The loading of the drug is relatively high and the drug can be applied into the system without chemical reaction; this is an important factor for maintaining drug activity.

Based on the problems above, it is necessary to develop an approach to the delivery of hAT-MSCs immobilization through production of hAT-MSCs microencapsulation and CM-hATMCS nanoparticles, but the research that conducts complete tests does not yet exist. Therefore, this study aimed to characterize hAT-MSCs microencapsulation and CM-hATMSCs nanoparticles derived from hATMSC.

Materials and methods

hATMSCs isolation and cell culture

The hAT-MSCs were isolated from 3 donors healthy liposuction patients. These cells were characterized by immunophenotyping according to standards for characteristics of human mesenchymal stem cells. The cells characterization was done by our previous study, showed that the cells have characteristics of mesenchymal stem cells which was CD44+, CD73+, CD90+, and CD105+, and negative lineage (CD11b−, CD19−, CD34−, CD45− and HLA−DR−) [16].

The hAT-MSCs were cultured on culture disks with a plastic surface with 4 mL of medium. The hAT-MSCs culture medium was prepared by mixing 10 % Fetal Bovine Serum (FBS, Gibco, 16170078), 1 % Antibiotic-Antimycotic (ABAM, Biowest, L0010), Minimum Essential Medium α (MEM α) (Gibco, 12571063), 1% Amphotericin B (Biowest, L0009-050), 1 % Nanomycopulitine (Biowest, LX16-010) and 0.01% Gentamicin (Gibco 15750078) to 100 % of the total volume in a 50 mL tube. Cells were centrifuged at 1600 rpm for 5 min. The supernatant was discarded, then the pellet was resuspended with 4 mL of culture medium. The cell suspension was placed in the T25 flask (SPL, 70025). Cells were incubated in a 5% CO_2 incubator (Thermo IH3543), at 37 °C [17].

Producing of microencapsulated hATMSCs

As much as 4 mL of sterile 1.75 % Alginate and 1 mL of hAT-MSCs cell suspense are alternately inserted into 1.2 % $CaCl_2$ solution while being distilled at a speed of 500 - 700 rpm. Once the beads are formed, the solution was washed with PBS. After washing, it is then dripped with chitosan 0.2 % (5 - 10 min). Then the solution is rewashed using PBS. The last is the re-addition of 0.15 % alginate for 3 min. The cells already present in the microencapsulation were put into 6 well plates and the medium were added. Scanning Electron Microscopy/Energy Dispersive X-Ray Spectroscopy (SEM/EDS) was used to observe the calculation and size scale of AT-MSC microencapsulation [18].

Cell viability test of microencapsulated hAT-MSCs

Cell viability tests were performed to see the percentage of living cells microencapsulated. Viability tests are carried out on microencapsulation with difference hAT-MSCs cells density of 0, 1.5×10^5, and 3×10^5 to observe the viability of hAT-MSCs microencapsulation based on 3 different cells number in separated well. Observations were carried out on the first day after microencapsulation, the 7[th] day (first week), and the 14[th] day (second week). Each of the tests was performed with duplo replication. After treatment, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyl phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega G3580) was added to each well and incubated for 3 h at 37 °C, 5 % CO_2. Absorbance was measured using spectrophotometry read at a wavelength of 490 nm [19-21].

Stability test of microencapsulated hAT-MSCs

The microencapsulation mechanical stability tests were carried out by observation of the presence of damage to the alginate beads. The stability test was carried out by casting microencapsulation using a magnetic stirrer at speeds of 300, 700, 1100, and 1200 rpm [22,23].

Producing of CM-hATMSCs nanoparticles

One gram of chitosan was dissolved in 100 mL of 1 % glacial acetic acid using a magnetic stirrer so that a chitosan concentration of 1 % was obtained. A total of 15 mL of conditioned medium was added to the solvent mixture (20 mL propylene glycol: 20 mL ethanol 70: 20 mL dimethyl sulfoxide (DMSO) 10 %)) and 100 mL aquadest. Briefly, a 1 % chitosan solution of 40 mL was added so that the chitosan concentration becomes 0.2 %. The mixture was stirred using a magnetic stirrer for 10 min. The 20 mL of 0.4 % Na-TPP was dripped at a rate of 1 drop/3 s with a burette and in a magnetic stirrer with 300 rpm until nanoparticles were formed and characterized by homogeneous turbidity. Then stay above the magnetic stirrer for 15 min so that a stable solution of conditioned medium nanoparticles was obtained. Then observed the stability of the nanoparticle solution in 5 d including color, turbidity, and precipitate [24].

Particle size analyzer of CM-hATMSCs nanoparticle

A total of 2 mL of CM-hATMSCs nanoparticle were analyzed using the Particle Size Analyzer (PSA) Beckman coulter tool [25].

Measurement of EGF, IL-6, VEGF, IGF-1 level in hAT-MSCs microencapsulated and CM-hAT-MSCs nanoparticles

Some growth factors that are present in nanoparticle CM-hATMSCs were measured using ELISA Assay (Elabscience, E-EL-H0059 (EGF); E-EL-H0102 (IL-6); E-EL-H0111 (VEGF); E-EL-H0086 (IGF-1)). The experimental group including I: Negative control (hAT-MSCs); II: Positive control (hAT-MSCs microencapsulated); III; 1.5×10^5 microencapsulated hAT-MSCs; IV: 3×10^5 hAT-MSCs microencapsulated; V: CM-hATMSCs nanoparticle. Briefly, 100 µL sample and standard working solution were prepared approximately in each well and then incubated for 90 min at a temperature of 37 °C. The biotinylated detection Ab working solution (100 µL) was added at a temperature for which the solution was already removed, and incubated at 37 °C, for 60 min. Then, the solution is aspirated from each well and a wash buffer (350 µL) is added, soaked for 1 - 2 min, repeated 3 times. Briefly, 100 µL HRP conjugate working solution was added followed by 30 min, at 37 °C. After incubation, 350 µL of wash buffer was used to wash the solution in 5 times. Briefly, 90 µL of substrate reagents are added and then incubated at 37 °C (protected from light) for 15 min until the color turns blue. Next, the solution is added with 50 µL of the solution stopped (the solution changes color to yellow). Microplate reader (Multiskan Go, Thermo Scientific) is used to measure absorbance with a wavelength of 450 nm [20,26-28].

Statistical analysis

Statistical analysis was performed using SPPS 20.0. One-way variance analysis (ANOVA) was used to compare the differences between groups with $p < 0.05$ considered significant, along with the independent T-test and Tukey post hoc test and 95 % confidence interval for comparing between two treatments. Data are presented as mean and standard deviation [6,29].

Results and discussion

Cell therapy using stem cells such as hAT-MSCs began to be widely researched for many treatments. However, in the hAT-MSCs implantation process, it is often distributed in non-target tissues, and hMSCs transplants are often affected by the environment, and inflammation so that cells can differentiate into unwanted cells. The hAT-MSCs microencapsulation was produced to coat cells so that cells can be transplanted without causing excessive immunity and inflammatory reactions but still allow cells to exchange nutrients and oxygen between the encapsulated cells and the environment outside the cell because the capsule material is semipermeable. The nanoparticles as a delivery system of CM-hATMSCs were formulated to control the particle size, surface properties, and release of the active substance in order to obtain pharmacological effects with specific action at its dose regimen.

Producing of microencapsulated hAT-MSCs

Figure 1 shows the results of hAT-MSCs microencapsulation with a light microscope. **Figures 1B** and **1C** show that the cells were successfully added to the alginate capsule matrix, marked by the presence of white dots of cells attached to the matrix. **Figure 2** shows the SEM/EDS results of microencapsulated hAT-MSCs. The smallest microencapsulation scale has the smallest average size of 1,150 μm and the largest is 1,653 μm. **Figure 2** showed that in the alginate matrix which was added 3×10^5 hAT-MSCs cells, there were cell points in which cells attached to the alginate capsule matrix. The alginate matrix is different compared to the matrix in microencapsulation without hAT-MSCs cells, there are no white dots attached to the matrix. The results showed that hAT-MSCs microencapsulation was successfully generated in the matrix of the alginate capsule shown in **Figures 1** and **2** marked with the white point of the cell attached to its matrix. The use of alginate as a capsule ingredient has been widely studied in sufficient detail to qualify as safe for human applications [30].

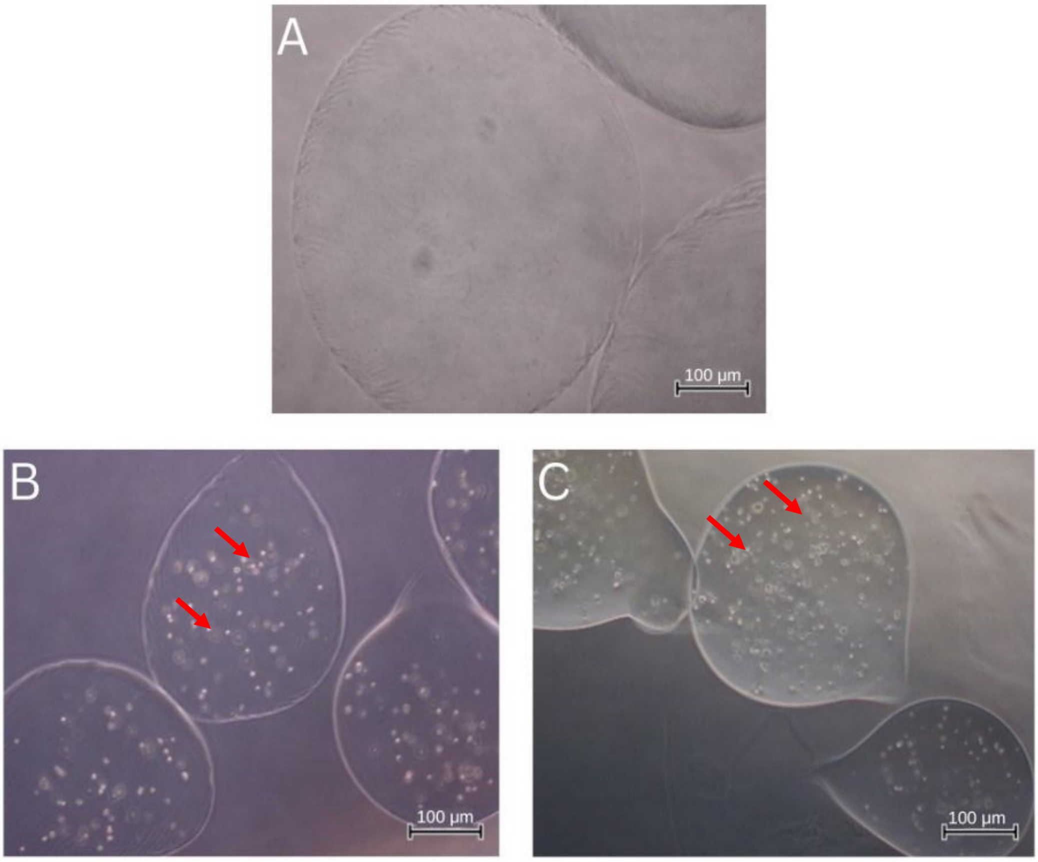

Figure 1 hAT-MSCs microencapsulation morphology by light microscopy. A) 0 hAT-MSCs cells, B) 1.5×10^5 AT-MSC cells, C) 3×10^5 AT-MSC cells. The presence of attached cells is indicated by white dots on the alginate capsule matrix (red arrow).

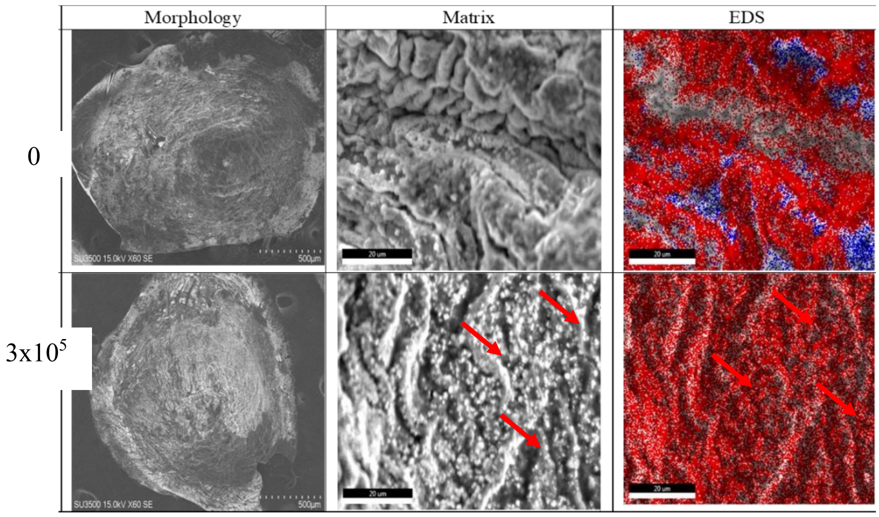

Figure 2 Microencapsulated hAT-MSCs Morphology and Matrix with SEM/EDS. The presence of attached cells that bound to the surface of the alginate matrix are indicated by white dots (red arrow).

Cell viability test

The microencapsulated AT-MSCs in alginate after being tested for cell viability for 14 d decreased viability to 50 % in each cell count (**Figure 3**). In **Figure 3** it can be seen the percentage of cell viability on different numbers and incubation periods. On the 1st day after microencapsulation, 3×10^5 microencapsulated hAT-MSCs had almost 100 % viability and in 1.5×10^5 hAT-MSCs had almost 90 % viability. On the 7th day (first week), the cell viability at 3×10^5 cells decreased to 90 % and at 1.5×10^5 cells decreased to 60 %. On the 14th day (2nd week), the viability cells at 3×10^5 hAT-MSCs decreased to 50 % and at 1.5×10^5 cells decreased to 50 %. Viability is the number of living cells estimated as a measure of the concentration of cells in the product. Stable viability shows good resistance to environmental influences. This is necessary to ensure that the cells are still viable in the microencapsulated during storage. Cell death is possible due to the reduced permeability of microencapsulation. Microencapsulation permeability is a major factor in the success of cell encapsulation technology because the survival of encapsulated cells will ultimately depend on the optimal balance of microencapsulation permeability that determines the supply of essential nutrients and eliminates toxic metabolites [31].

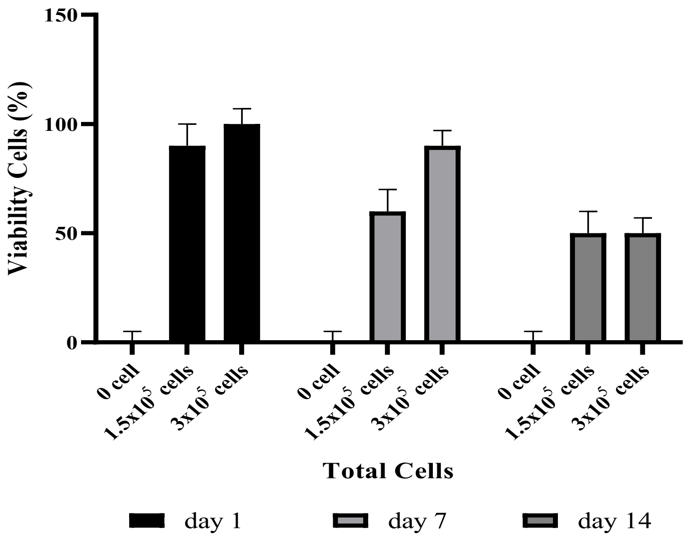

Figure 3 Cells Viability of Microencapsulated hAT-MSCs. The histogram represents the average cell viability of microencapsulated hAT-MSCs. 0 cells: non-microencapsulated hAT-MSCs as control, 1.5×10^5 cells: 1.5×10^5 microencapsulated hAT-MSCs, and 3×10^5 cells: 3×10^5 microencapsulated hAT-MSCs. Data shows mean ± standard deviation.

Stability test

Stability test to measure the mechanical resistance based on the damage to the beads after being rotated at difference speeds. Based on **Figure 4**, it can be seen that the mechanical resistance reached 100 % at a speed of 300 - 1100 rpm and was damaged at 1200 rpm but only 10 %. This microencapsulation with alginate is quite stable and has a fairly good mechanical resistance when tested for stability using rotation at a speed of 1,200 rpm only damages 10 % of it (**Figure 4**). This means that the alginate capsule generated had a strong crosslink so that the cells in it were not damaged when facing mechanical shocks [31]. The production of conditioned medium nanoparticles showed positive results that nanoparticles formed were characterized by turbidity in the solution. The addition of tripolyphosphate to the solution mixture helped chitosan to cross-link because the positive charge of chitosan will interact with the negative charge of tripolyphosphate and the cell could be trapped in a cross-linked chitosan matrix. The PSA results showed that a particle size of 141 nm had met the nanoparticle requirements.

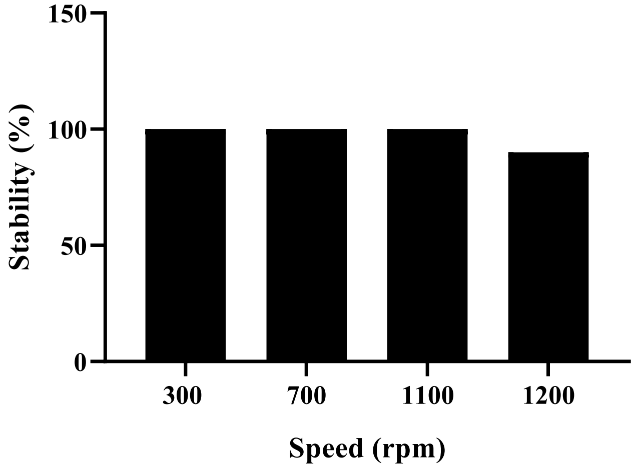

Figure 4 Stability Test of Microencapsulated hAT-MSCs. All data are expressed as the mean ± standard deviation. The experiments were performed at once test.

Producing of CM-hATMSCs nanoparticles

Alginate is a natural unbranched binary copolymer constituted by (1,4)-linked β-Dmannuronic acid (M) and α-L-(1,4)-linked guluronic acid (G) that can be extracted from polysaccharides in bacteria like *Azotobacter vinelandii* and Pseudomonads or from algae like *Ascophyllum nodosum* and *Sargassum spp*. Alginate is dropped into the CaCl2 solution during the creation of microcapsule gel in order to create non-homogeneous microcapsules. Alginate-polymers are crosslinked with calcium ions between α-L-(1,4)-linked guluronic acid and α-L-(1,4)-linked guluronic acid (GG) as well as (1,4)-linked β-Dmannuronic acid and α-L-(1,4) linked guluronic acid (MG) to create gels [32].

The results of the CM-hATMSCs nanoparticles taken from hAT-MSCs growth medium dissolved in ethanol, propylene glycol, and DMSO solvents have not been able to completely dissolve the extract so a filtering process is needed to remove the insoluble part which is a nonpolar-compound. Tripolyphosphate was added to a mixture of extract solution and chitosan solution with the aim of forming nanoparticles using the ionic gelation method. Chitosan will be cross-linked with the help of tripolyphosphate because the positive charge of chitosan will interact with the negative charge of tripolyphosphate. Meanwhile, the extract components will be trapped in a cross-linked chitosan matrix.

PSA of CM-hATMSCs nanoparticle

In the PSA test, CM-hATMSCs nanoparticles have an average particle size of 141 nm, this indicates that the nanoparticles from the conditioned medium of ATMSCs included in nanoparticle size criteria with a range of 1 - 1000 nm [33].

Measurement of growth factor level in hAT-MSCs microencapsulated and CM-hATMSCs nanoparticles

The hAT-MSCs secretes a variety of growth factors and cytokines that contribute to wound healing and tissue regeneration. The microencapsulated hAT-MSCs in alginate as well as nanoparticles of CM-hATMSCs showed good secretion of growth factors EGF, IL-6, VEGF, and IGF-1 (**Figure 5**). **Figure 5** shows the levels of EGF, IL-6, VEGF, and IGF. Based on the results, hAT-MSCs microencapsulated with alginate still secreted growth factors EGF, IL-6, VEGF, and IGF-1. In **Figure 5**, 3×10^5 microencapsulated hAT-MSCs showed high secretion of growth factors EGF, VEGF, IGF-1 and it has a low levels IL-6, compared to positive control. Similar to CM-hATMSC nanoparticles that showed the secretion of growth factors EGF, VEGF, and IGF-1 compared to positive controls.

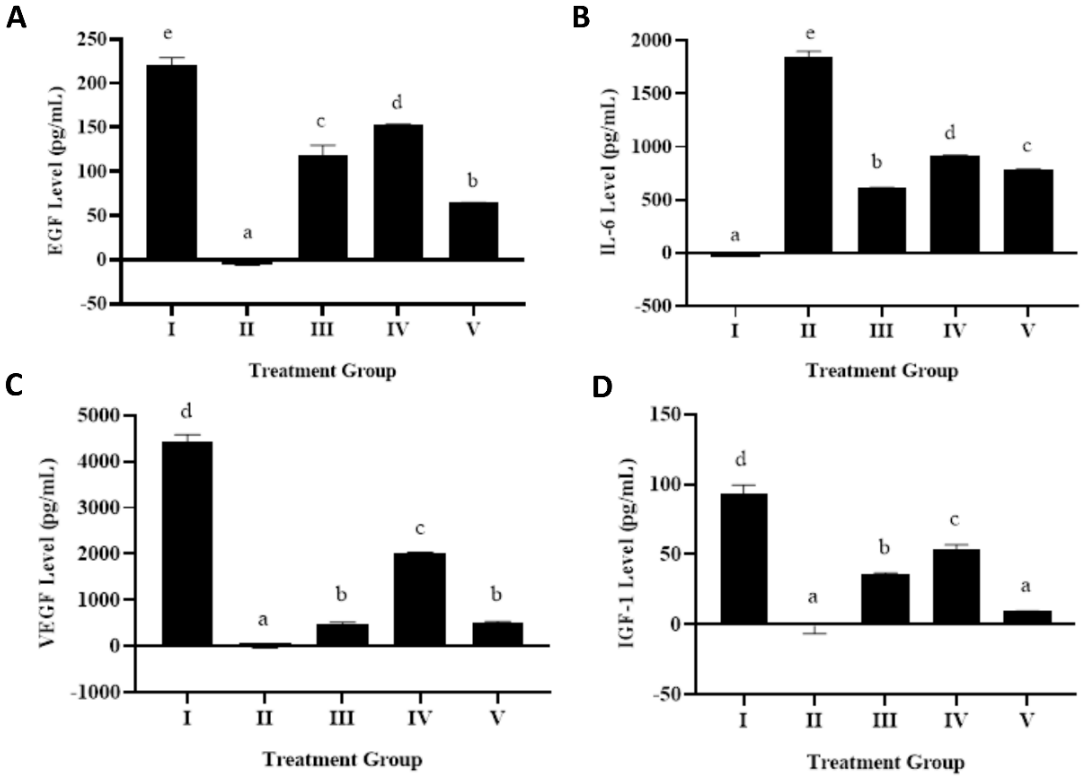

Figure 5 Effects of hAT-MSCs Microencapsulated and CM-hAT-MSCs nanoparticles towards the level of EGF, IL-6, VEGF, IGF-1. A) and B). Different letters (a, b, c, d, e) shows significant differences among treatment. C) and 5) Different letters (a, b, c, d) shows significant differences among treatment based on One-Way ANOVA and Tukey Post Hoc Test at $p < 0.05$. *Data presented in means ± standard deviation. I: Negative control (7×10^5 hAT-MSCs); II: Positive control: Microencapsulation non hAT-MSCs; III: 1.5×10^5 microencapsulated hAT-MSCs; IV: 3×10^5 microencapsulated hAT-MSCs; V: CM-hATMSCs nanoparticles. The different letter shows a significant difference among treatment based on One-Way ANOVA and Tukey Post Hoc Test at $p < 0.05$.

The encapsulated microenvironment indicated the secretory function of growth factors. Based on this study, the growth factor (EGF, VEGF and IGF-1) have a high levels concentration compared to the positive control but it's low when compared to hATMSC cells only. The low levels of growth factor are due to the microencapsulation performed on the cells. hAT-MSCs cells generally have a lot of growth factors, but the growth factors secreted by the cells are trapped in the microencapsulation layer that protects the cells. Therefore, the levels are lower than the hAT-MSCs only but are still detected as having growth factors. Microencapsulation can protect the compounds secreted by the cells on their way to the therapeutic target, this technique can also be used to manage their release to target particular areas or to enhance their flow and organoleptic qualities. Others have proposed that microencapsulation, in which the cell product is shielded by alginate polymer derivatives, allowing for higher diffusion rates [34,35].

Encapsulation techniques still allowed cells to carry out the exchange of nutrients and oxygen between the encapsulated cells and the environment outside the cell [13]. Furthermore, the hAT-MSCs microencapsulation using alginate has a potential to be used as stem cell therapy and conditioned medium nanoparticles can potentially be an effective drug delivery to control the release of active substances so as to obtain pharmacologically specific drug action at the dosage regimen.

Conclusions

In this study, hAT-MSCs microencapsulation formulation using alginate showed good results, based on the viability, stability, structure, and growth factor secretion ability. The characterization results showed that microencapsulated hAT-MSCs and CM-hATMSCs nanoparticles were successfully fabricated with the appropriate criteria. The hAT-MSCs microencapsulation in alginates could potentially be used as stem cell therapy, and conditioned medium nanoparticles could potentially be an effective drug delivery to control the release of active substances.

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