Preparation of Cell-Loaded Microbeads as Stable and Injectable Delivery Platforms for Tissue Engineering

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Abstract

Cell transplants in therapeutic studies are not preserving their long term functional inside the donor body. In mesenchymal stem cells transplants, transplanted cells disperse through body and easily degraded by immune cells after transplant process. Various strategies such as usage of the immunosuppressive drug to eliminate allograft rejection are designed to increase efficiency of the cell therapy. The other hand strategy is the construction of biomimetic encapsulates via using polymeric materials which preserve of stem cells from environmental effect. In this study, we hypothesize that L929 cell lines and mesenchymal stem cell (MSCs) might continue their viability and functionality inside the alginate microbeads during the 12 days of in vitro conditions. For that purpose, uniform, and injectable size (<200 µm) of cell-loaded microbeads were constructed by electrostatically assisted spraying techniques. Our results showed that both L929 and MSCs cell lines continue their metabolic activity inside the microbeads and cells during the incubation periods. Glucose consumption and lactic acid production level of both groups of the cell lines were consistently observed. Released cell number at day 12 was increased compared to day 0. Protein expression of both groups of the cells was increased day by day with doubling of the cell number. In this work, we illustrate that mesenchymal stem cells and L929 cells might be used in 3D cell culture models.

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Key words: Cell therapy, Cell encapsulation, Cell loaded microbeads, Alginate microbeads

Introduction

Mesenchymal stem cells (MSCs) are a component of the tissue construct, secrete growth hormone or factors, and regulate the signalling pathway via secretion of the various cytokines and growth factors such as vascular endothelial growth factors, and transforming growth factor (TGF-β). Injection of the mesenchymal stem cells into the degenerative tissue provides a secretion of therapeutic proteins, construction, and regeneration of the tissue . Mesenchymal stem cells were used to provide effective treatment to diseases such as cardiovascular disease, orthopedic disease, rheumatologic disease, endocrine disease, and neurodegenerative disease (Kim et al., 2019). Encapsulation of the mesenchymal stem cells via various polymeric material enhance the therapeutic efficiency of the mesenchymal stem cell transplantation without applying immunosuppressive drugs . A recent study illustrated that rat adipose-derived stem cells (ASCs) express the osteogenic factors in vitro and regenerate the bone fractures in vivo .

Mesenchymal stems cells (MSCs) disperse through the tissue after transplantation of the cells. Injection of the MSCs induces an immunological response that decreases the cellular activity of transplanted cells at damaged tissue . Suspended formation of the MSCs limit differentiation of the cells and expression of the regenerative factors. For that reason, mesenchymal stem cells require a vascularized 3D construct for more efficient cell therapy. Mesenchymal stem cell (MSCs) loaded alginate microbeads provide 3D construct, large surface area to cell proliferation and attachment. Encapsulation of mesenchymal stem cells might eliminate the dispersion of the cells inside the tissue after the injection procedure.

Alginate, a known negatively charged polysaccharide, is used in various applications such as biotechnological industries, pharmaceutical, food, and textile , .A sequence of the alginate containing α-l-guluronic acid (G) and β-d-mannuronic acid side (M)characterize the functionality and gelation formation of alginate. On the other hand, alteration of the environmental factor such as ionic strength of the medium, the concentration of the gelling ions, pH of the solution regulates gelation form of alginate. For example, deprotonation of the carboxylic acid group in a sequence of the alginate at a certain value of the pH increases the dissolving rate of the alginate in the solution. Soluble form of alginic acid in waters such as sodium alginate depends on the pH value of the solvent. Sodium alginate might become an insoluble formation and then precipitates as an alginic acid into the solution via increasing the pH value. A three-dimensional network of the alginate is obtained by ionic bonding via diffusion of the multivalent cations into the alginate solution. Similarly, ionic bonding between the multivalent cations and alginate might break down with releasing of the multivalent cations by pH of the microenvironment and change the formation of the alginate .

Various encapsulation strategies such as drop generation by gravity, electrostatically assisted spraying, aerodynamically assisted jetting systems are used to construct microbeads formation. Injectable size of adipose mesenchymal stem cell-loaded alginate microbeads might be constructed with electrostatically assisted spraying system. Adipose mesenchymal stem cell-loaded alginate microbeads by electrostatically assisted spraying system regenerates bone fraction post-transplant week 2.Allogenic islet transplantation is a procedure for the regulation of the insulin hormone level inside the body. Islet-loaded alginate microbeads continues their functionality in type 1 diabetic patients at post-transplant week 1. Moreover, co-encapsulation of the islet cells with mesenchymal stem cells increases the nitric oxide production rate and secretion of immunomodulatory cytokines inside the tissue. The immunomodulatory activity of the mesenchymal stem cells enhances the efficiency of islet transplantation .

Various physiological (pH, enzymatic activity, redox potential, and glucose concentration) and external stimuli (mechanical forces, light, temperature, and magnetic field) control the degradation rate of the polysaccharide. Alteration of the pH value significantly affects polymeric structures. Alginates stabilize their structure at pH values ranging from 5 to 10. Alginate degradation by environmental pH values has occurred with two processes such as via β-alkoxy-elimination(pH>10) and proton catalyzed by hydrolysis (pH<5). Poly-

mers might be naturally degraded by the upregulation of the enzymes on the polymeric side. Alginate is naturally degraded by alginate lysates and alginate polymerases which are not synthesis inside the human body. This mechanism was introduced by the β-elimination mechanism. The other degradation mechanism of the alginate microbeads isthe non-enzymatic breakdown of bonding between the chains via chelating of the Ca^{2+} molecules by chemical reagents such as EDTA and sodium citrate. Calcium content inside the alginate microbeads is not preserved in vitro condition because of the cell proliferation inside the microbeads and diffusion of cellular metabolites (lactate, phosphate, and citrate) from inside of microbeads to the environment. Releasing the calcium from the microbeads also affects the mechanical property of the alginate microbeads.

In this work, we prepare cell-loaded microbeads via an encapsulator for obtaining a more efficient delivery platform for living cells in cellular therapy-based applications. We investigated the effect of concentration of L929 cell and human mesenchymal stem cell-loaded alginate microbeads on cell proliferation, alginate microbeads morphology, and metabolite production rate. For that purpose, L929 and mesenchymal stem cells loaded alginate microbeads were constructed with an electrostatically assisted spraying system. Constructed cell-loaded microbeads were monitored under the fluorescence microscope during the 12 days of incubation periods. Released cell number from microbeads, viability of the released cells, metabolite (glucose consumption, lactic acid production, and protein production) production rate of the cells were analyzed during 12 days of periods.

Materials and Methods

Materials

Alginate (%50 mannuronate units, Sigma-Aldrich, low viscosity, A1112) solution was prepared with %4 concentration in saline (0.9% isotonic sodium chloride/ Polypharma/ Polyfleks) solution and overnight stirring at room temperature. Calcium chloride (75 mM) was used as a crosslinking solution. Nozzles (0.35 mm) were incubated inside sodium citrate solution (85 mM) to prevent clogging of the system. Encapsulation systems were sterilized with and ethanol (%70). The system was treated with UV (2 hours) before the cell culture experiment.

Preparation and Optimization of Microbeads

Ethanol (%70) was run before the operation of the system (NiscoEncapsulator VAR V1 LIN-0043, Nisco Engineering AG, Zurich, Switzerland). UV sterilized research-grade alginate (UV sterilized) was loaded on 50 mL syringe, and the syringe pump was set up 5 mL/hour flow rate. Stirrer speed was adjusted to the desired level. Operation arm and nozzle tip (0.35 mm) were installed inside to system. Then the system was run with alginate solution until loading of the system (cables and tip). The electrode was dipped inside the crosslinking solution and voltage was adjusted after dripping of alginate solution inside the crosslinking solution. Alginate microbeads were fabricated by applying of desired electrostatic force (6 kVa) which is an optimum for microbeads production (L. Wang et al., 2013). The voltage and syringe pump were closed after 30 minutes of the running of the alginate solution. Various concentrations of the microbeads group (5000, 2500, 1250, 500, and 250) were incubated in a 1 mL MSCs nutrient medium. The medium was exchanged before the measurement of the microbeads diameter at each time point.

Morphological evolutions of microbeads

Microbeads were visualized with a fluorescence microscope (Zeiss Axio Vert.A1 inverted microscope) for advanced routine and the diameter of the microbeads was measured with Zeiss Program (Carl Zeiss Microscope). The diameter value of each group was graphed using Microsoft Excel Program.

Stability of microbead in various conditions

Morphological structure and also size of microbeads is controlled at various environmental condition (Acetate solution, Medium solution (Nutrient Free Medium (Biological Industries)), Phosphate Buffer Solution (PBS,

Gibco)). Acetate solution (100 mM) was prepared with powder sodium acetate solution and desired Ph (Ph: 5.5) value was arranged with HCl and NaOH stock solution.

After 1 and 5 days of the experiment, microbeads were visualized with a fluorescence microscope (Zeiss Axio Vert.A1 inverted microscope).

Preparation of cell loaded microbeads

Fibroblast cells (L929, ATCC, NCTC clone 929) were cultured in MSCs nutrient-free medium (Biological Industries). After thawing of the L929 cells according to the manufacturing procedure, cells (50,000 cells/cm^2) were seeded in a T-25 flask andincubated at 37 C and 5% CO_2. The waste medium was removed, and a fresh medium was added every 48 hours. When cells were reached %80 confluences of the flask, cells were trypsinized and centrifuged at 300 RCF5 min. Cells were collected in ringer lactate solution (%0.5 Human Serum Albumin Solution) and calculated with a cell counter device (BioRad).

Adipose stem cells were cultured in MSCs nutrient-free medium (Biological Industries) with 100 IU/ml penicillin, 100 µg/ml streptomycin. After thawing of the mesenchymal stem cells, cells (20,000 cells/cm^2) were seeded in a T-25 flask. Tissue culture dishes were incubated at 37 C and 5% CO_2. The waste medium is removed, and a fresh medium was added every 48 hours. When cells were reached %80 confluency of the flask, cells were trypsinized and centrifuged 300 RCF for 10 minutes. Cells were collected in ringer lactate solution (%0.5 Human Serum Albumin Solution) and calculated with a cell counter device (BioRad).

Fibroblast cells (L929) and mesenchymal stem cells (MSCs) were trypsinized from a petri dish and counted with a cell counter (BioRad cell counter). L929 (20 million cells) and Mesenchymal stem cells (10 million cells) were used to load them into alginate microbeads. For sterilization of the system, ethanol (%70) was run before the operation of the system (NiscoEncapsulator VAR V1 LIN-0043, Nisco Engineering AG, Zurich, Switzerland). The system was treated with UV (2 hours) before the cell culture experiment. Cells were diluted into 4 million cells/mL (L929) (48 cells in 1 microbeads) and 2 million cells (MSCs) (24 cells in 1 microbeads) with UV sterilized alginate polymer and put into the syringe (Becton Dickinson (BD)).

Then the system was run with a cell-alginate mixture until loading of the system (cables and tip (0.35 mm)) with the mixture. The electrode was dipped inside the crosslinking solution and voltage was adjusted after dripping of alginate solution inside the crosslinking solution. Cell-loaded alginate microbeads were fabricated by applying of desired electrostatic force (6 kVa) which is an optimum for microbeads production (L. Wang et al., 2013). The voltage and syringe pump were closed after 30 minutes of the running of the alginate solution.

Microbeads were two times washed with saline (0.9% isotonic sodium chloride/ Polypharma/ Polyfleks) solution. Various concentrations of the microbeads were cultured in a mesenchymal stem cell medium (MSCs nutrient-free medium, Biological Industries). Each group (Mesenchymal stem cell (400,200,100, and 50), L929 (1000, 500, 250, 125)) were transferred to (2*T25 flask) flask for the cultivation of the cell loaded microbeads.

Morphological evaluation of cell loaded microbeads

Bright Field Microscope

Culture medium was discarded, and fresh medium was added in microbeads environment at each time point of the experiment. Microbeads were visualized with fluorescence microscope after adding of fresh medium (MSC NutriStem® XF Medium) and the diameter of the microbeads was measured with Zeiss Program (Carl Zeiss Microscope). The diameter value of each group was graphed using GraphPad Prism 9.

Fluorescence Microscope

Culture medium was exchanged, and fresh medium was added in microbeads environment each time point of experiment and then, microbeads were visualized with fluorescence microscope (Zeiss Axio Vert.A1 inverted

microscope for advanced routine). GFP signal was measured using the ImageJ program and intensity values of the microbeads were graphed with GraphPad Prism 9.

Glucose and lactic acid measurements

The metabolic activity of the cells inside the microbeads was tracked during 12 days incubation times. Glucose consumption and lactic acid production inside the cultivation environment were measured and results give information about the cellular metabolic activity. Cell medium (500 µL sample for glucose and 500 µL sample lactic acid) is collected 12 days periods. The collected cultivation medium was analyzed with the ADVIA® 1800 Clinical Chemistry System. In this system, reagents were loaded into the system before their usage. The concentration of the glucose and lactic acid level inside the solution was measured with ADVIA® 1800 Clinical Chemistry System .Glucose consumption and lactic acid production values of the cell inside the microbeads were graphed with a GraphPad Prism 9.

Cell viability in culture medium

Cell culture medium was collected at the end of the 12 days. Attached cells to the flask surface were trypsinized and centrifuged at 300 RCF 10 minutes. Cells were collected in ringer lactate solution (%0.5 Human Serum Albumin Solution) and calculated with a cell counter device (BioRad). The viability of the cell number was assessed with Trypan blue staining protocol.

Total protein and mRNA concentration in culture medium

BCA Assay

Cell medium (1 mL sample cultivation medium) was collected during the incubation time of the microbeads. The total protein amount in the solution was measured with a BCA assay kit (Takara). BCA assay was performed according to the manufacturing procedure. After incubation of the reagent with the sample, the concentration of the total protein in the medium was measured with a microplate reader at 562 nm spectrophotometer. Measurement of the absorbance value was graphed with the GraphPad Prism 9.

Nanodrop Measurement

Cell medium (1 mL sample cultivation medium) was collected during the incubation time of the microbeads. The amount of total mRNA in the sample tube was measured by (Thermo Scientific Nanodrop One.). Measurement of the absorbance value was graphed with the GraphPad Prism 9.

SDS PAGE

Protein samples in cell culture medium was collected at the end of the 12 days. Sample were loaded on SDS Page (%10). SDS Page were stained with Coomassie brilliant blue dye. Images were observed and analyzed with BIO-RAD ChemiDoc XRS+ Molecular Imager with Lab Software.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software. Obtained results were analyzed by running an unpaired t-test from the averaged data obtained from 3 independent experiments using GraphPad Software. $P < 0.05$ from 3 independent experiments using GraphPad Software.

Result

Microbeads Fabrication

Preparation and optimization of microbeads

Alginate microbeads were produced with an encapsulator device (NiscoEncapsulator VAR V1 LIN-0043, Nisco Engineering AG, Zurich, Switzerland) and cultured in Mesenchymal Nutrient Free Medium (Biological Industries)) during 14 days in medium (Figure 1).41.600 microbeads were produced in 30 minutes and each group (5000 microbeads/well, 2500 microbeads/well, 1250 microbeads/well, 500 microbeads/well, 250 microbeads/well) were incubated in a 1 mL cell culture medium. The diameter of the microbeads for 12 days

was recorded fluorescence microscope. The diameter of the microbeads is increased from 190 to 280 µm day by day for each group of experiments. Diameter of the microbeads is negatively correlated with number of the microbeads per mL as given Figure 1.

Stability Analysis of Microbeads

The morphologic structure of the microbeads was tested in various incubation conditions (100 mM pH:5.5 Acetate Solution, Medium, Phosphate buffer saline (PBS), and saline @ 37 ^{o}C). The diameter and morphology of the microbeads were compared between each group of the experiment (Figure 2). There was no significant change observed on diameter of microbeads in various incubation conditions. In various environmental conditions, salt formation around the microbeads was observed on day1 (Figure 2.C) and then removed via medium and saline wash of the microbeads (Figure 2.E, and F).

Morphological evaluation of cell loaded microbeads*Bright Field Microscope*

Cell (L929 and MSCs) stability and proliferation were tested with various amounts of the microbeads (1000, 500, 250, 125 and 400, 200, 100, 50 microbeads/mL) (Figure 3. A and Figure 4. A). The diameter of cell-loaded microbeads was recorded (visualized) using a fluorescence microscope. Diameters between each group of the experiment were analyzed with each other. Cell release from the alginate microbeads was monitored during the incubation period. Diameter of the microbeads was similar at day 0. The following incubation days, diameter of the microbeads were altered at each group of experiment. Non-uniformal distribution of the microbeads and cell release were observed under the microscope.

Fluorescence Microscope

Green fluorescent protein (GFP) signal of mesenchymal stem cell (MSCs) was recorded by fluorescence microscope (Zeiss Axio Vert.A1 inverted microscope) for 12 days (Figure 5. A). Alteration of the green fluorescence particle (GFP) intensity was observed at each group of microbeads day by day until Day 12(Figure 5. B). There is no correlation between the microbeads number and GFP signal intensity. Fluctuation of the GFP signal is observed each group of the experiment.

Glucose and lactic acid concentration

Metabolites in the cell culture medium are essential elements for tracking cellular growth. Both cell lines (L929 and MSCs) seem to consume glucose and produce the lactate inside the 3D architecture. While glucose consumption level both cell lines is sharply decreased, and lactic acid production rate consistently is increased in 2 days incubation time. A consistent plot between glucose consumption and lactic acid production in L929 cell media and MSC media was observed following incubation periods(Figure 6).

Viable cells in a cell culture medium were observed on day 12.

Total released cell numbers of both cells inside the medium and attached to the tissue culture dishes were calculated using a Bio-Rad cell counter device (TC20^{TM} Automated Cell Counter). The cell number of both cell lines (L929, and MSCs cell lines) inside the medium was decreased with decreasing of the microbeads numbers in cell culture media. The concentration of the cells in alginate solution was 4 million cells/mL and 2 million cells/mL. Cell numbers of the L929 and MSCs cell-loaded microbeads were 48 cells (L929) per microbeads, and 24 cells (MSC_s) per microbeads. These numbers have also consisted of each group of the experiment (Figure 8). Percentage of the cell viability (L929 cell lines) was gradually decreased with decreasing of the microbeads numbers in cell culture media (500, 250, 125 microbeads number in following orders percentage of the L929 cell viability %67.5, %54, %60). Percentage of the cell viability (MSCs cell lines) was stable with decreasing of the microbeads numbers in cell culture media (400, 200, 100, 50 microbeads number in following orders percentage of the MSCs cell viability % 42.5, % 45.5, % 46.5, %66).

Total protein and mRNA concentration in culture medium

BCA Assay

Total protein concentration inside the medium sample was evaluated during 12 days of periods and alteration of the total protein contents in a cultured medium of L929 and MSCs cell lines were compared to each group of the experiment. BCA assay was used for quantification of the total protein amount in a biological assay. The total protein amount inside the medium is gradually increased with an increase in the number of cell-loaded microbeads (Figure 7).

mRNA measurement

Alteration of the mRNA level inside the culture medium of L929 and MSCs was observed between each group of experiments. mRNA concentration of L929 and MSCs culture medium was not significantly changed during the incubation period. The concentration of the mRNA level inside the culture medium of mesenchymal stem cell (MSCs) was decreased.

SDS PAGE

Protein distribution of the culture medium were observed during the incubation of the cell loaded alginate via SDS-Page. Alteration of the protein content shows that cells not only continue their metabolic activity during the incubation periods but also secrete metabolic proteins into the cell culture medium. Secreted proteins by cells from inside of the microbeads to culture medium aggregated during the incubation periods. Aggregation profile of each sample mask to show expression of the low abundant protein on SDS-PAGE. Consistent result between glucose consumption and protein content inside the culture medium were observed in L929 cells. There was no difference observed in MSCs cells because of the low proliferation rate which is consisted with glucose consumption results.

DISCUSSION

Mesenchymal stem cells were currently used for regeneration of the various tissue types, . Stability and controlled release of the mesenchymal stem cell in target area is important for effective therapy and tissue regeneration. The previous study showed that alginate microbeads are stable *in vitro* condition during 12 days of periods and 2 weeks *in vivo* study. Leslie and coworkers fabricate injectable alginate microbeads and control enzymatic degradation of alginate microbeads by various concentration of alginate lysate enzyme. However, they did not consider of alginate lysate stability at the injection side of the damaged tissue. Mixture of stem cell loaded microbeads and alginate lysate might not stay together after injection at target area. In this research, we evaluated non-enzymatical degradation of alginate by the diffusion of the calcium to environment and production of the metabolites by cell loaded alginate microbeads.

An anionic polymer such as alginate has a net negative charged group. The morphology of the alginate microbeads is affected by environmental pH. Alginate is not only degraded by enzymatic cleavage but also by ion exchange. This process mainly occurs by environmental pH effect on the hydrophilicity of the carboxyl groups in the anionic polymer. Ionizable carboxylic groups of alginates are turned into negatively charged carboxylate ions in high pH conditions . Salt contents were observed in presence of the calcium content after a 1-day incubation with phosphate buffer solution. Similarly, salt content is formed in the medium condition on day 5. Calcium ions in alginate solution diffuse into the medium and phosphate buffer saline (PBS) solution and calcium ions form a calcium salt inside the medium and PBS. The salt content in acetate conditions was not observed because of the effect of the H-bonding in alginate polymer.In cell culture media conditions, alginate microbeads swell day by day because of the presence of the chelators, monovalent ions, and non-crosslinking divalent cations like Mg^{2+}. Moreover, the concentration of the $CaCl_2$ (75 mM $CaCl_2$) content in the hardening solution is much higher than the concentration of the $CaCl_2$ content in the medium condition (1.8 mM $CaCl_2$). For this reason, the high-water content outside of the microbead swell the microbeads day by day. Size of the microbeads should be lower than the 500 μm for injectable applications. Scientist in previous workfabricated400-micrometer microbeads. In this study, alginate microbeads were around the200-micrometer.

Main problem of the cell viability in 3D cell culture research is the poor oxygen penetration inside the 3D architecture and absence of metabolites for cellular growth which is caused by absence of the vascular

structure in 3D structure. Micro-scale 3D architecture might solve this problem because of the low oxygen gradient from inside to outside. In this research, cells inside the microbeads continue their viability during the 12 days incubation period. L929 cell lines in each group of the experiment were proliferated inside the microbeads for 7 days and then release into the environment because of the swelling of the alginate microbeads. The non-uniformly distribution of microbeads increased day by day because of the swelling and cell proliferation as a spheroid form inside the microbeads. Concentration of cell in alginate solution effect on interaction of the cells, cell proliferation and cultivation time inside the microbeads during the incubation period . Although mesenchymal stem cell (MSCs) stays stable inside the microbeads during 12 days of periods, cell release and cell debris were observed in 400 microbeads/mL samples at day 12. The viability and proliferation number of the mesenchymal stem cell was monitored by expression of green fluorescence tagged proteins (GFP). GFP signal intensity is preserved during the incubation period of the cell. Fluctuations of the GFP signal intensity are related to the cell doubling and cell release from the microbeads during the culturing of the cells. Proliferated cells increase the diameter of the microbeads and spheroids formation of the L929 cells line was observed under the light microscope on day 12. Spheroid's formation of the L929 cell lines indicates that L929 cells were also proliferated inside the alginate microbeads. Cell number and cell viability between each group of the experiment was consistent.

Glucose is an essential element for the metabolic activity of the cell. Glucose concentration affects metabolic process inside the cell. Lactic acid is the metabolic waste product that is produced after the cellular respiration process of the cell. Glucose consumption and lactic acid production level indicate metabolism of the cells *in vitro*conditions. Normaxia, hypoxia, and hyperoxia conditions determined according to soluble oxygen content in water alter the metabolic activity of the stem cells. Glucose consumption of the cells is significantly increase during the adaptation of the cells to the environment especially hypoxia conditions such as 3D cell culture. Similarly, glucose consumption of the stem cell loaded microbeads significantly increased on day 2. After adaptation of the cells to the environment, glucose consumption and lactic acid production were gradually linearized like 2D cell culture . Cell number after 8 days, glucose consumption and lactic acid production is gradually increased. On the other hand, alginate degradation enhanced by large amount of the chelators, monovalent ions, and non-crosslinking divalent cations like Mg^{+2} in culture medium restrict the concentration and cultivation time of microbeads in vitro conditions. Proliferation of the cells increase the pH of the environment by metabolic waste and oxygen demand by the cells. High acidic condition cause cell death during the incubation of the cell loaded microbeads might be monitored by glucose consumption and lactic acid production level. During the incubation of the 1000 microbeads/mL group, glucose consumption is significantly dropped, and lactic acid production is significantly decreased because of the environmental conditions such as pH or large amount of metabolic waste. This result has not seemed in other groups (500, 250, 125 microbeads/mL).

Various hormone, growth factors, and proteins were used to regulate differentiation and proliferation of the cell number in culture media. Metabolic activity of the cell are not only depends on cultivation condition but also effected by protein expression of the cells . Cells induces protein expressionto adapt environment and continue metabolic activity during the cultivation of the cells. Protein paths in this research show that protein concentration inside the culture medium increase day by day, but glucose consumption and lactic acid production showan approximately linear curve. These results indicate that protein expression inside the microbeads was not regulated by the glucose consumption and lactic acid production of the cells.

Mesenchymal cell-loaded alginate microbeads have a great potential to applicate not only regenerative therapy but also various disease and therapeutic approaches. Biodegradability of the alginate microbeads causes not only releasing of the cells from the microbeads to environment and but also releasing of the therapeutic proteins to the degenerative side. According to result, released cells were remain viable at 12 days of periods in all the groups of the experiment. Cellular activity inside the microbeads construct is preserved during 12 days of periods.

Conclusion

In this study, we successfully constructed injectable cell-loaded microbeads as stable and efficient delivery

platforms for *in vitro* cell culture experiments. This platform is potentially applicable to cell therapeutic approaches. We performed comprehensive optimization study for both L929 cell line and mesenchymal stem cells, and prepared their alginate microbead loaded versions for *in vitro* conditions. Metabolic activity of these cells was tracked by various experimental methods. During the incubation period, L929 and mesenchymal stem cells constitute spheroid formation inside the microbeads. Both cell lines seem to preserve their viability, cellular functioning, and metabolic activity at a higher level for 12 days periods. By this way, cells not only stabilized in the microbead structure for the initial incubation time but also released into the environment afterwards by the degradation of polymeric wall of microbeads. Additionally, the protein expression level was observed as consistently increasing with the proliferation of cells encapsulated inside. These findings point out to a very significant end where microencapsulation of living cells with the optimized conditions has a strong impact on providing a powerful tool for ensuring the survival and metabolic activity of cells to be delivered to the injured or damaged tissue part for cellular therapy purposes.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Data availability statement: Data available on request from the authors

References

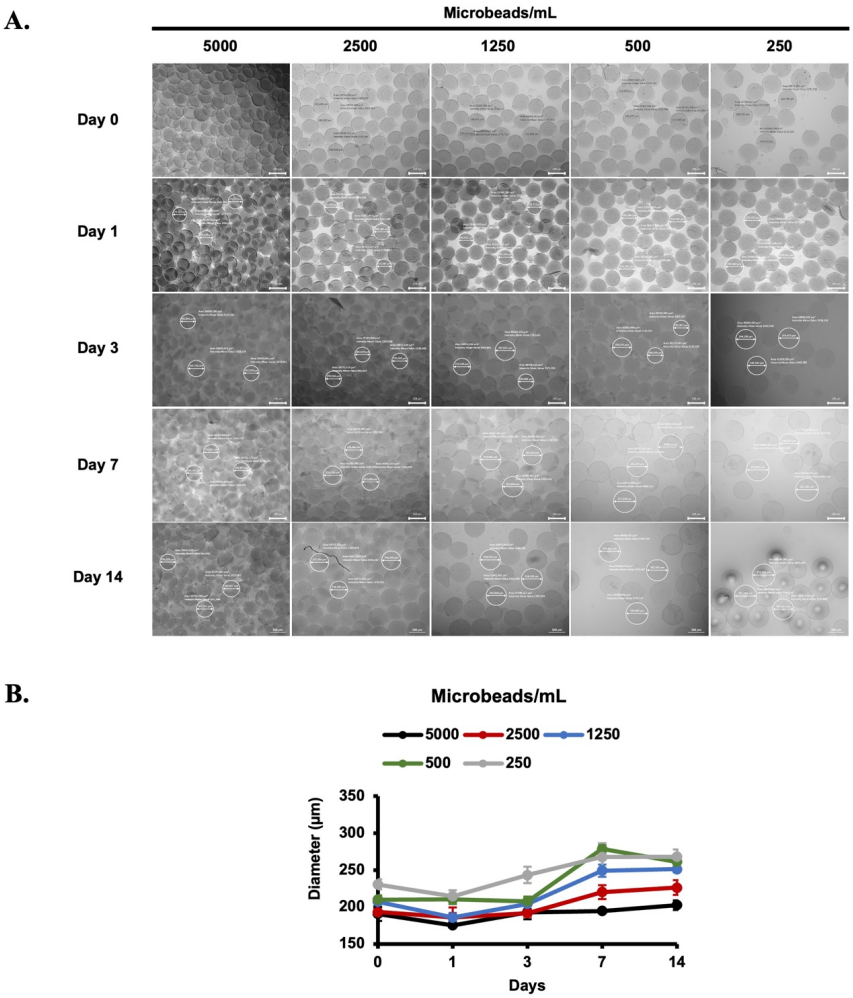


Figure 1. Diameter changes between each group (5000, 2500, 1250, 500, and 250 microbeads/mL) of the experiment are visualized during 14 days of periods under the light microscope (A). Diameter changes between each group (5000, 2500, 1250, 500, and 250microbeads/mL) of an experiment are graphed during 14 days of periods under the light microscope (B).

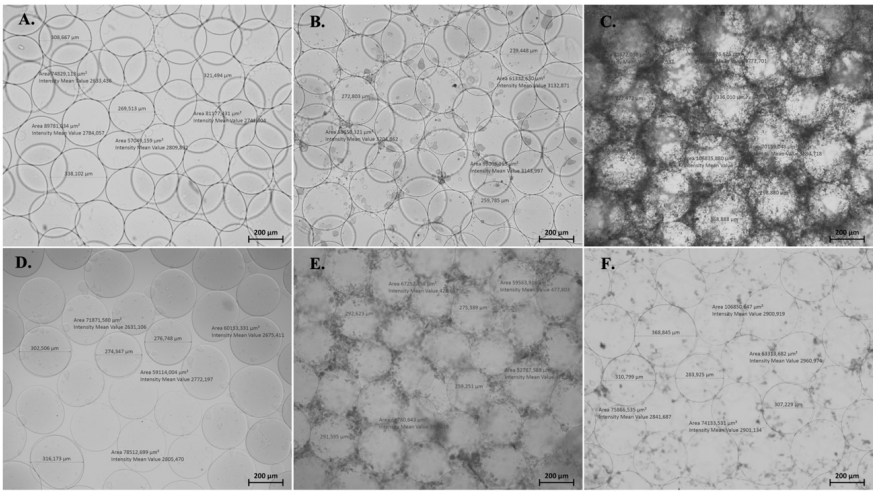


Figure 2. Morphological structure and size of microbeads at 37 ^{o}C in various incubation conditions (Acetate solution after 1 day (A), Medium condition after 1 day (B), Phosphate buffer saline (PBS) condition after 1 day (C), Acetate solution after 5 days (D), Medium incubation after 5 days (E), PBS incubation after 5 days (F).

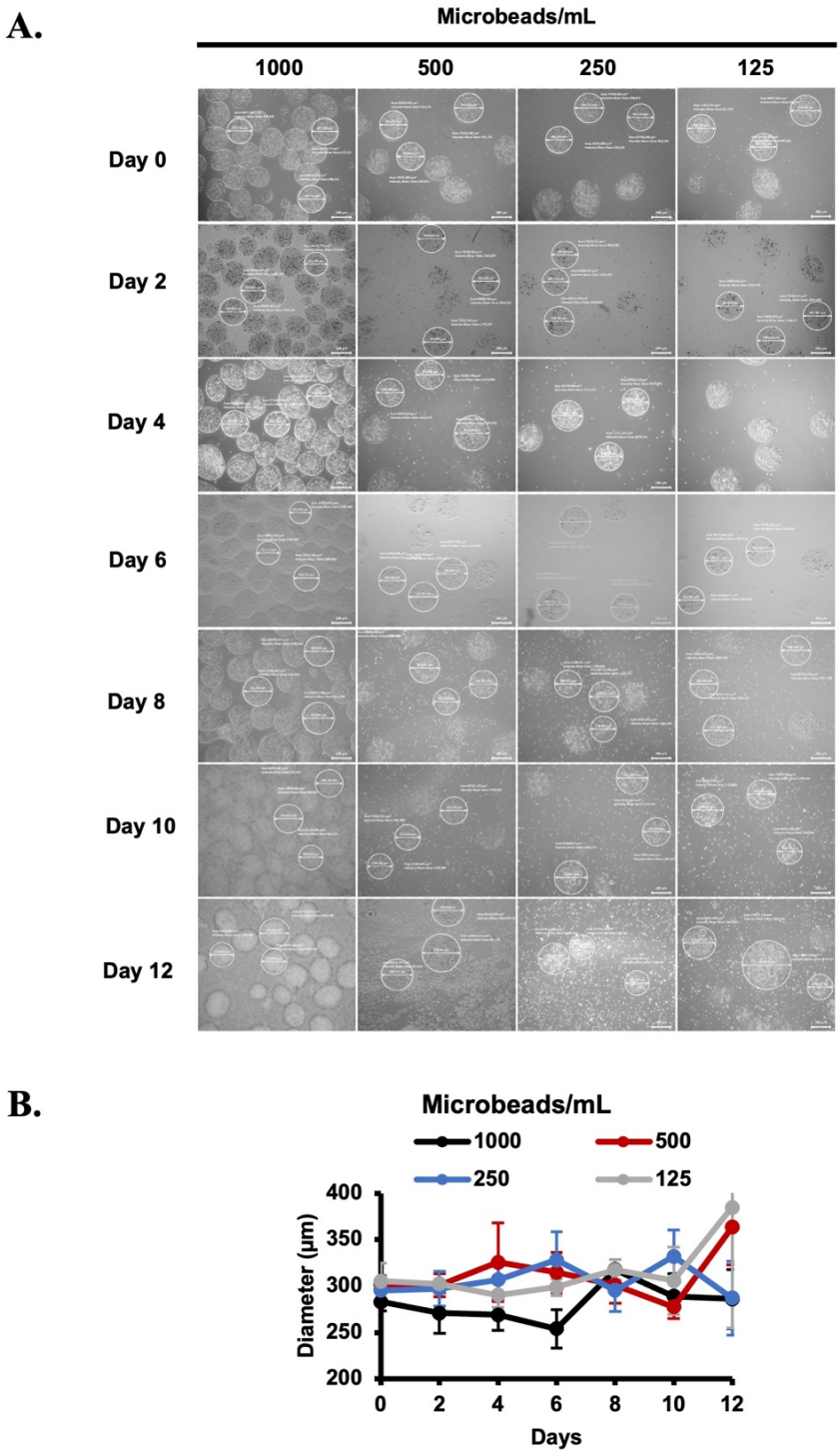
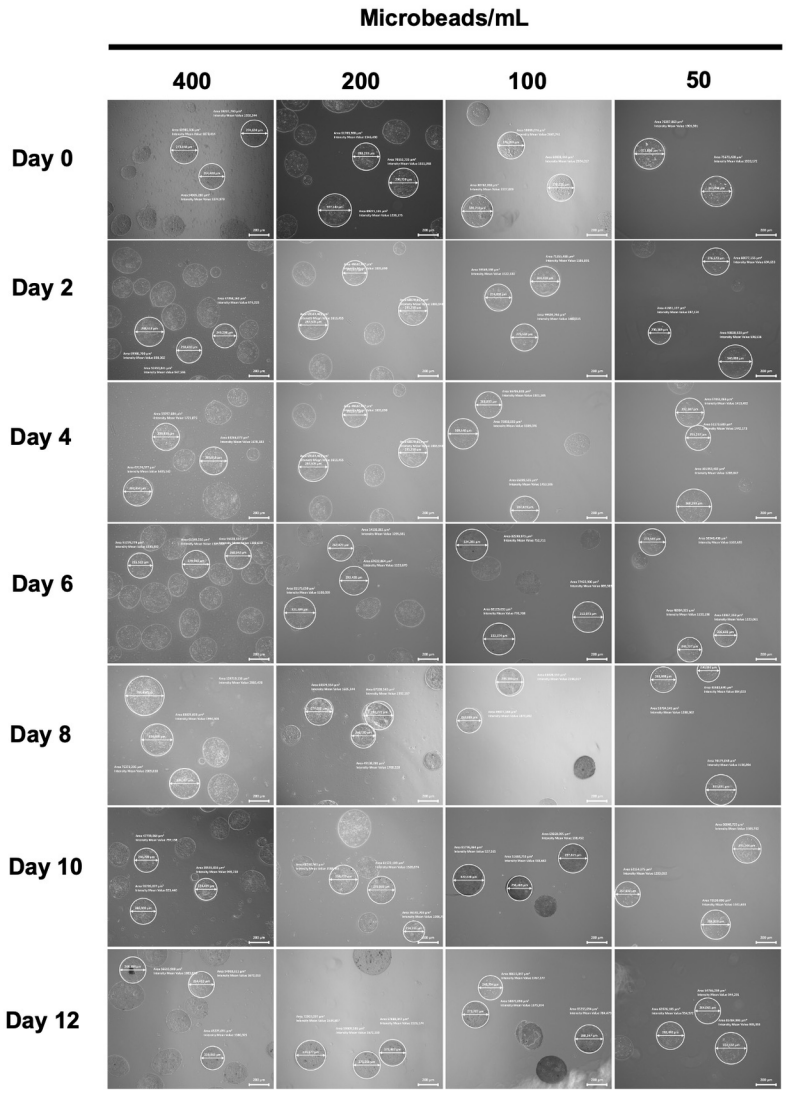


Figure 3. Visualization of L929 release from microbeadstime-dependent manner under the fluorescence microscope (5X) (A).Diameter of L929 loaded beads in MSCs nutrient medium during 12 days period (B).

A.



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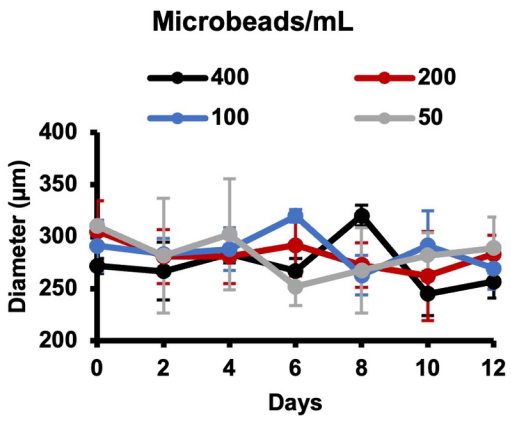

Figure 4. Visualization of Mesenchymal Stem Cell (MSCs) release from microbeads in a time-dependent manner under fluorescence microscope (5X) (A). Diameter of MSCs loaded beads in MSCs nutrient medium during 12 days period (B).

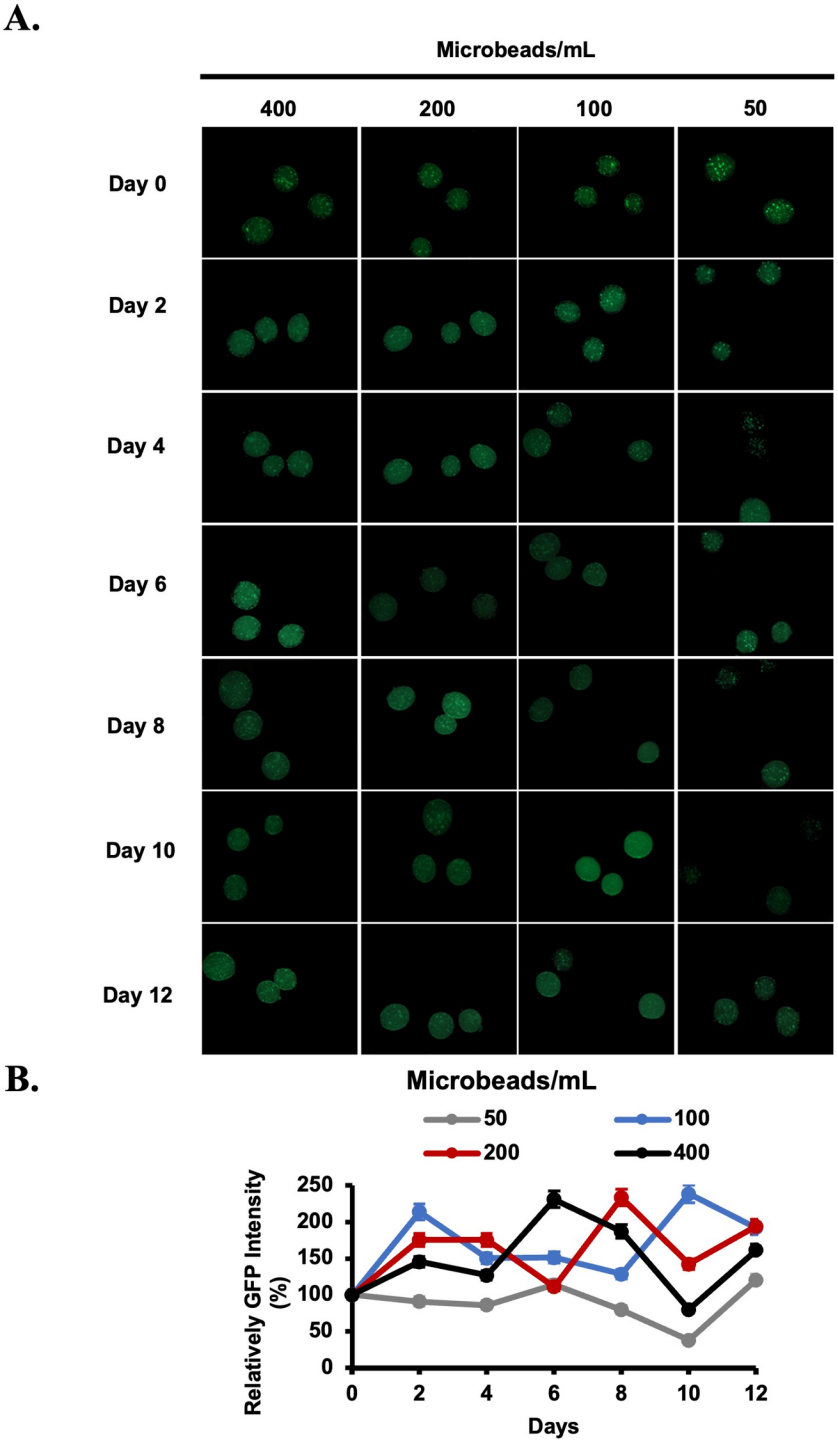

Figure 5. Visualization of green fluorescence protein secretion by Mesenchymal Stem Cell (MSCs) inside microbeads in a time-dependent manner under fluorescence microscope (5X) (A). Green fluorescence protein intensity by inside mesenchymal stem cells (MSCs) loaded microbeads in a time-dependent manner under fluorescence microscope (5X) (B).

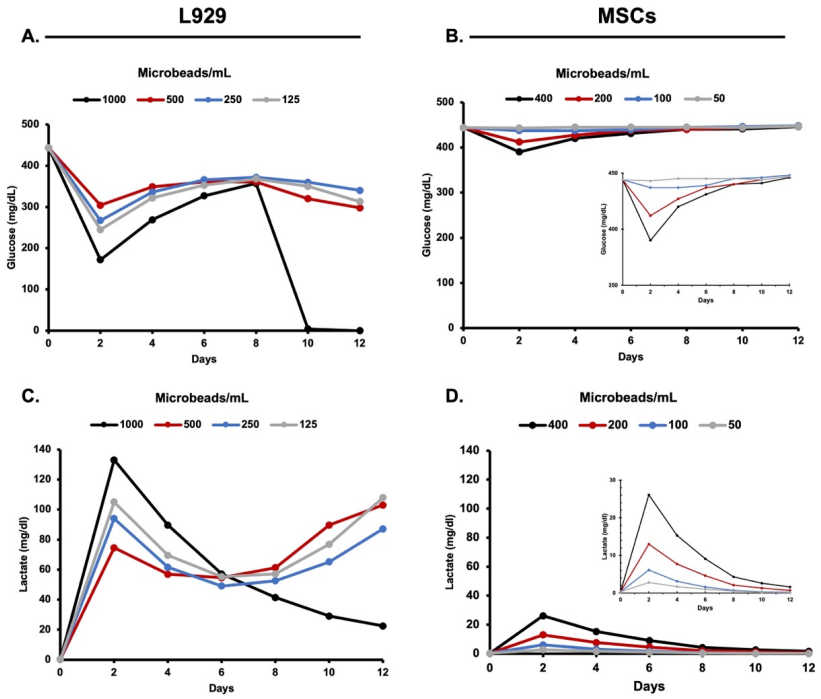

Figure 6. Glucose consumption and lactic acid production of cell line during12 days. (A) Glucose consumption of L929, (B) lactic Acid production of L929, (C) glucose consumption of MSCs, and (D) lactic acid production of MSCs.

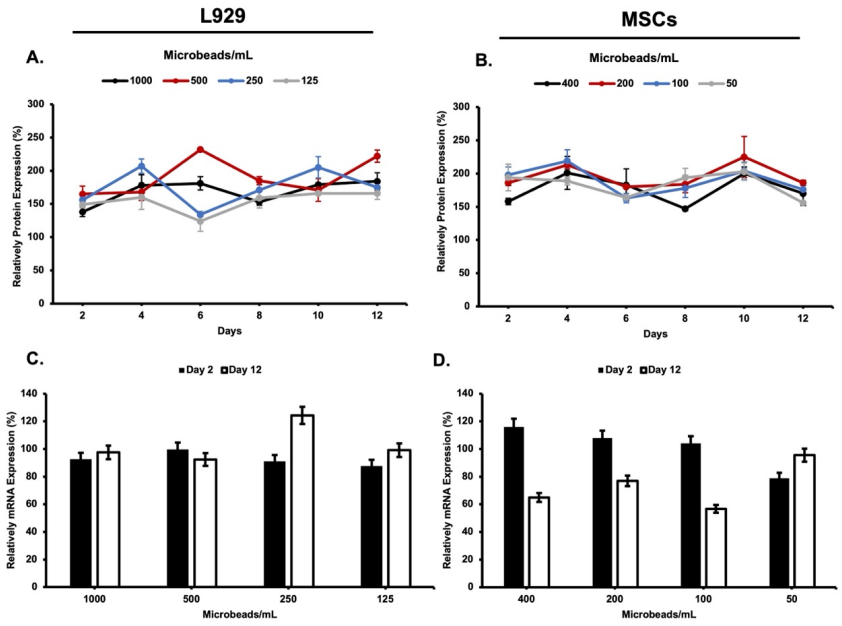

Figure 7. Protein concentration of cultured medium solution both L929 (**A**) cell line and Mesenchymal Stem Cell (MSCs) (**B**) cell line.mRNA concentration *in* medium solutions of (**C**) L929 cell line and (**D**) Mesenchymal Stem Cell (MSCs).

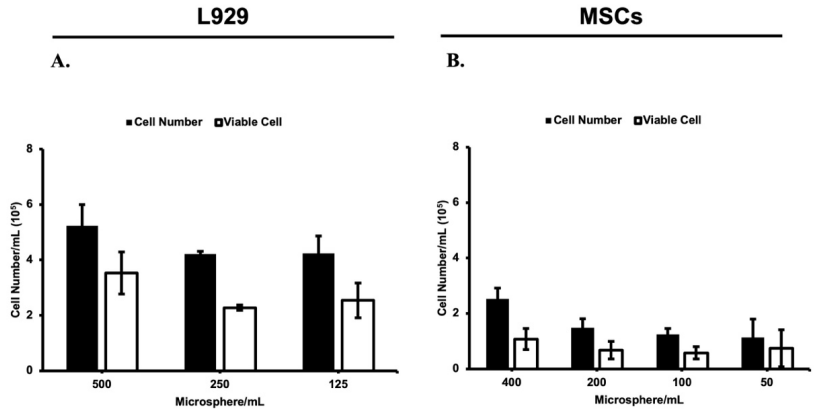

Figure 8. Cell viabilityof released L929 (A) and MSCs (B) cell lines in cell culture medium at day 12.

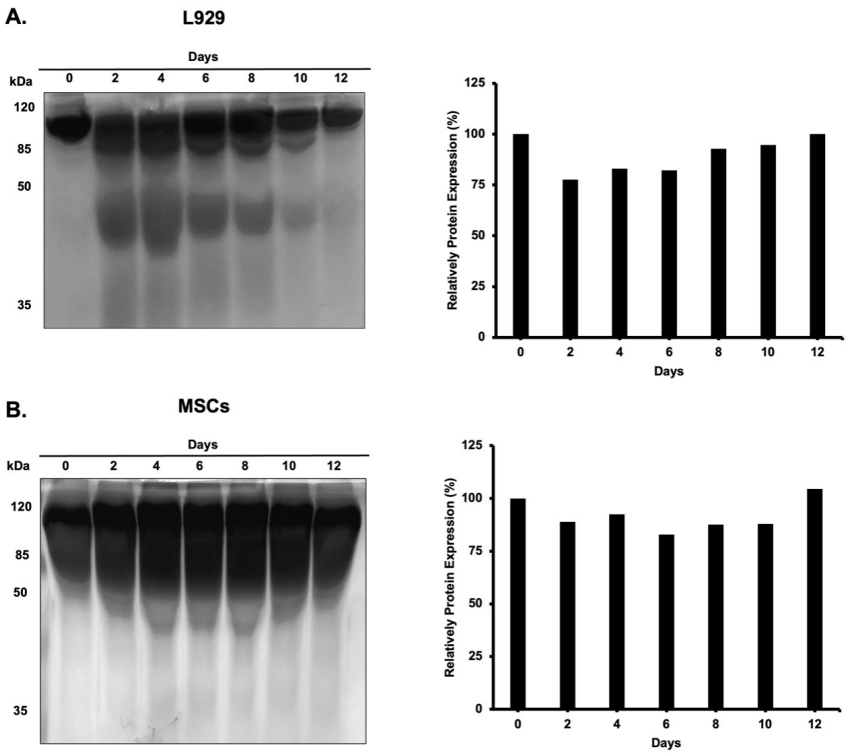

Figure 9. Protein distribution of the cell culture medium of L929 (1000 microbeads/mL) (A) and MSCs (400microbeads/mL) (B) during the 12 days.