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Study of Human Amniotic Membrane Mesenchymal Stem Cells Using Gelatin and Alginate as Nontoxic Scaffolds

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Abstract

Perinatal mesenchymal stem cells (MSCs), for example, from amniotic membrane, have advantages over adult sources, such as bone marrow, in terms of ease of availability, cell naivety, tissue stem cell abundance, high capacity of proliferation, and less donor site morbidity, because it does not require invasive procedures. Natural polymer scaffolds, such as gelatin and alginate, are biocompatible. Combination of stem cells from amniotic membrane (hAMSCs) and gelatin or alginate as scaffold can be promising. However, cytotoxicity comparison of gelatin and alginate to hAMSCs has not been widely studied. This study was aimed to compare cytotoxicity of gelatin and alginate on hAMSCs *in vitro*. Isolation and culture were performed on hAMSCs of the healthy full-term pregnancy. In passage 4, Flow Cytometry CD90, CD105, and CD73 phenotype characterization was done. Colorimetric assay of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was performed to measure the cytotoxicity. There were three sample groups: (control group) hAMSCs with alpha-minimum essential medium (α-MEM) solution as control; (gelatin group) hAMSCs with gelatin; (alginate group) hAMSCs with alginate. Each group consisted of 12 samples. Flow cytometry of hAMSCs expressed 28.78% CD90, 36.95% CD105, and 44.41% CD73 surface markers. No sample depicted toxicity in either gelatin or alginate group, and this is indicated by the average percentage of living cells in gelatin 97.26% and in alginate 98.43%. No statistically significant difference (p=0.057) of cytotoxicity was found between gelatin and alginate to hAMSCs. Gelatin and alginate were nontoxic to hAMSCs *in vitro.*

Keywords: Gelatin; Alginate; Amniotic membrane; Mesenchymal stromal stem cells.

1. INTRODUCTION

The alveolar bone quality is essential for placement of osseointegrated implant. Alveolar bone defects can be caused by chronic inflammatory diseases, injury, congenital abnormalities, resorption of alveolar bone after tooth loss, and osteoporosis, and they can decrease the patient's quality of life [1]. According to the prevalence and distribution of bone loss rates 57.8% in men, 42.2% in women. Moreover, the severity of bone defects increases with age [2].

The golden standards for the bone loss treatment have always been autogenous bone grafting due to its osteogenic, osteoconductive, and osteoinductive properties. However, there are weaknesses in autogenous bone graft; for example, they are more expensive. Moreover, the required complex procedure depends on the location and size of the defect [3]. These donors are still unable to overcome the morphological and functional demands of three-dimensional structure, as well as the complicated construction of periodontal equipment, or the challenging features of peri-implant and alveolar bone defects [4]. Therefore, it is expected to obtain alternative methods of bone grafting that do not result in morbidity at the donor site. Donors should have unlimited availability and have the same bone regeneration capacity as autogenous bone grafts [3].

Several measures have been undertaken to fulfill the requirements of materials that must be compatible, osteoconductive, and osteoinductive. Natural and synthetic materials have been used as scaffolds for implantation in defects or seeded with cells [5]. Currently, bone marrow mesenchymal stem cell (BMMSC) is a choice that is often used, but invasive procedure is needed in patients for cell isolation [6]. Many perinatal mesenchymal stem cells (MSCs), for example, amniotic membranes,

chorion plates, decidua parietal, and umbilical cord, have advantages over adult sources, such as bone marrow, in terms of ease of availability, lack of donor site morbidity, naivety of cells, tissue stem cell abundance, and high capacity of proliferation [7]. The amniotic membrane is a highly proliferated postnatal organ that is discarded after birth, and cell collection does not require invasive procedures, which are generally considered an ethical problem [8]. hAMSC was isolated and cultured from a healthy woman at the 38th week of her pregnancy. Examination of hAMSC culture showed cells expressing vimentin and cytokeratin. Cells expressing cytokeratin have a distinctive MSC phenotype, namely expression of CD90, CD105, and CD73.

Gelatin has been used for many years in pharmaceutical, cell culture, and tissue engineering because of its excellent biocompatibility, easy processing, and low cost. During the last decade, gelatin has been extensively evaluated to function as a carrier of cells, bioadhesives, and bioartificial grafts. For irregular defects, scaffold from alginate can be used for stem cell-based bone engineering. However, the ability of alginate is lacking in cell adhesives and mechanical properties [9].

There have been studies on biocompatibility and osteogenic differentiation potential of human umbilical cord mesenchymal stem cells (hUCMSCs) with gelatin solvent. The cytotoxicity evaluation of gelatin solvent showed that it has minimum toxicity [10]. As well as studies of alginate for periodontal ligaments and gingival mesenchymal stem cells encapsulated in alginate microbeads oxidized and thoroughly investigated for cellular feasibility and osteogenic differentiation. The results obtained were better nontoxic scaffolds with better differentiation of osteogenic and adipogenic stem cells compared to the *in vitro* control group. The results of this study also show that the use of alginate scaffold suits tissue engineering. Alginate is a carrier of specific tissue regeneration and has successfully deposited the mineral matrix and maintains bone regeneration [11]. However, cytotoxicity comparison of gelatin and alginate to hAMSCs has not been widely studied.

2. METHOD(S)

This study was an *in vitro* laboratory-based experimental study using hAMSCs of the healthy full-term pregnancy. It was granted ethical approval by The Research Ethics Committee, Dr. Soetomo General Hospital, Surabaya.

2.1. Isolation and Culture of hAMSCs

Part of the human amniotic membrane was cut into 8×8 cm-sized sections and transferred to 0.25% Trypsin (Gibco BRL, Gaithersburg, MD, USA) and then proceeded with 40 min of incubation. The solution was removed and replaced with 0.75 mg/ml Collagenase Type IV (Sigma-Aldrich, St. Louis, MO, USA) and 0.075 mg/ml DNase I solution (Takara Bio, Shiga, Japan). Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 (1:1) (Gibco BRL, Gaithersburg, MD, USA) was added. A medium containing cells was then transferred into a petri dish and incubated at 37°C and 5% carbon dioxide (CO_2). Cell growth was observed daily, with the medium being replaced every 3 days; on reaching confluence, the passage was also performed. The isolation procedure was performed using stem cell laboratory protocols at the Stem Cell Research and Development Centre, Airlangga University.

2.2. Flow Cytometry Phenotypic Characterization

Characterization of hAMSCs phenotype was performed using flow cytometry. Cells were seeded in well with alpha-minimum essential medium (αMEM) (Sigma-Aldrich, St. Louis, MO, USA) in passage 4. Afterward, they were fixated with 10% formaldehyde and incubated using the Human MSC Analysis Kit (BD Bioscience, USA) with the addition of a CD90, CD105, and CD73 and negative CD45 cocktail primary antibodies. The primary antibody was labeled using Fluorescein isothiocyanate (FITC)-conjugated goat antimouse antibody (Sigma-Aldrich, St. Louis, MO, USA). Cells were then viewed and analyzed by fluorescence-assisted cell sorting (FACS) Calibur flow cytometer (BD Bioscience, USA).

2.3. Gelatin and Alginate Scaffold Preparation

A 2% gelatin (Rousselot, VION company, Guangdong, China) was dissolved in a 0.15 M of sodium chloride solution and 25 M of HEPES buffer solution (Sigma-Aldrich, St. Louis, MO, USA). This process was performed at pH 7.0 and then sterilized at a temperature of 121°C for 15 min by autoclaving.

A solution of 2% alginate was prepared by dissolving sodium alginate (Sigma-Aldrich, St. Louis, MO, USA) in distilled water at room temperature under vigorous stirring, and continued until uniform dispersion was obtained. The dispersion was heated to 80°C in a water bath and kept at this temperature for 30 min. Hydrochloride acid of 0.1 M was used to adjust the solution to pH 7.0. Autoclaving process was performed thereafter at 121°C for 15 min.

2.4. Gelatin and Alginate Toxicity Measurement to hAMSCs

Colorimetric assay of 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was performed to measure the cytotoxicity of the gelatin and alginate solvent against hAMSCs. Three sample groups were prepared: (control group) hAMSCs with α-MEM solution; (gelatin group) hAMSCs with gelatin; (alginate group) hAMSCs with alginate. Cells were distributed into 96-well plates containing the final volume of 100 μl per well and then incubated. A 10 μl of the MTT reagent was added at each well. The living cells would convert the MTT into purple formazan crystals. Optical density changes at a wavelength of 595 nm using spectrophotometer were performed to measure the formazan quantity.

2.5. Data Analysis

The data obtained were presented in the form of average value and standard deviation. The data underwent statistical analysis using Kolmogorov–Smirnov normality test, and independent *t* test was used to evaluate the differences in the mean between groups. A value of $p<0.05$ was considered statistically significant.

3. RESULTS

3.1. Phenotype Characteristics of hAMSCs

Amniotic membrane-derived MSC expressed 28.78% CD90, 36.95% CD105, and 44.41% CD73 in passage 4.

The result of flow cytometry is shown in Figure 1, and the phenotype characteristics of hAMSCs can be seen in Figure 2.

Figure 1: Flow cytometry images and scale bar CD90, CD105, and CD73 result of hAMSCs.

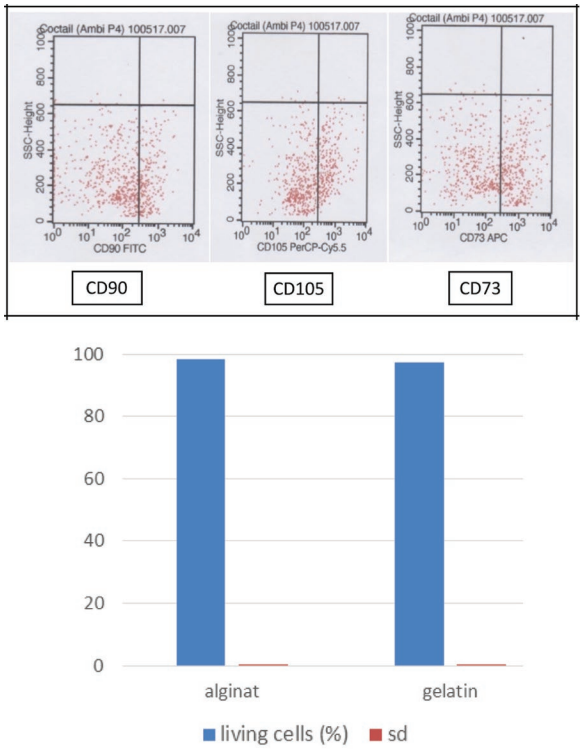

Figure 2: Phenotype characteristics expression of hAMSCs.

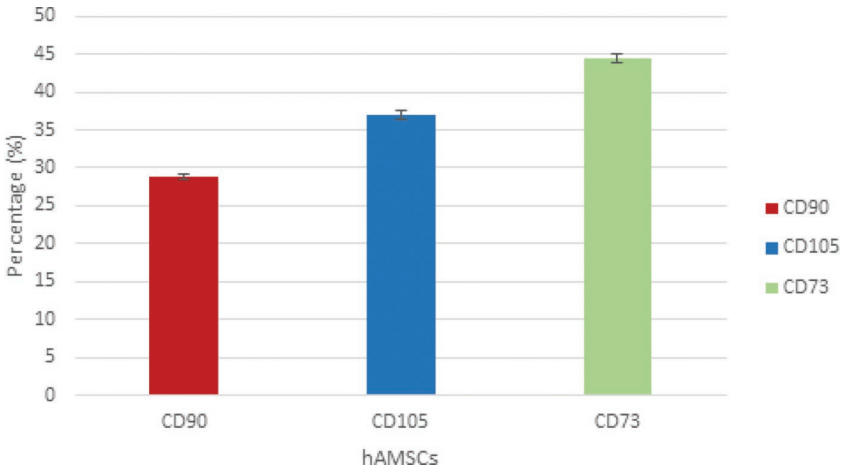

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Figure 3: Microscopic images and scale bar MTT assay on control (A), gelatin (B), and alginate group (C) to hAMSCs.

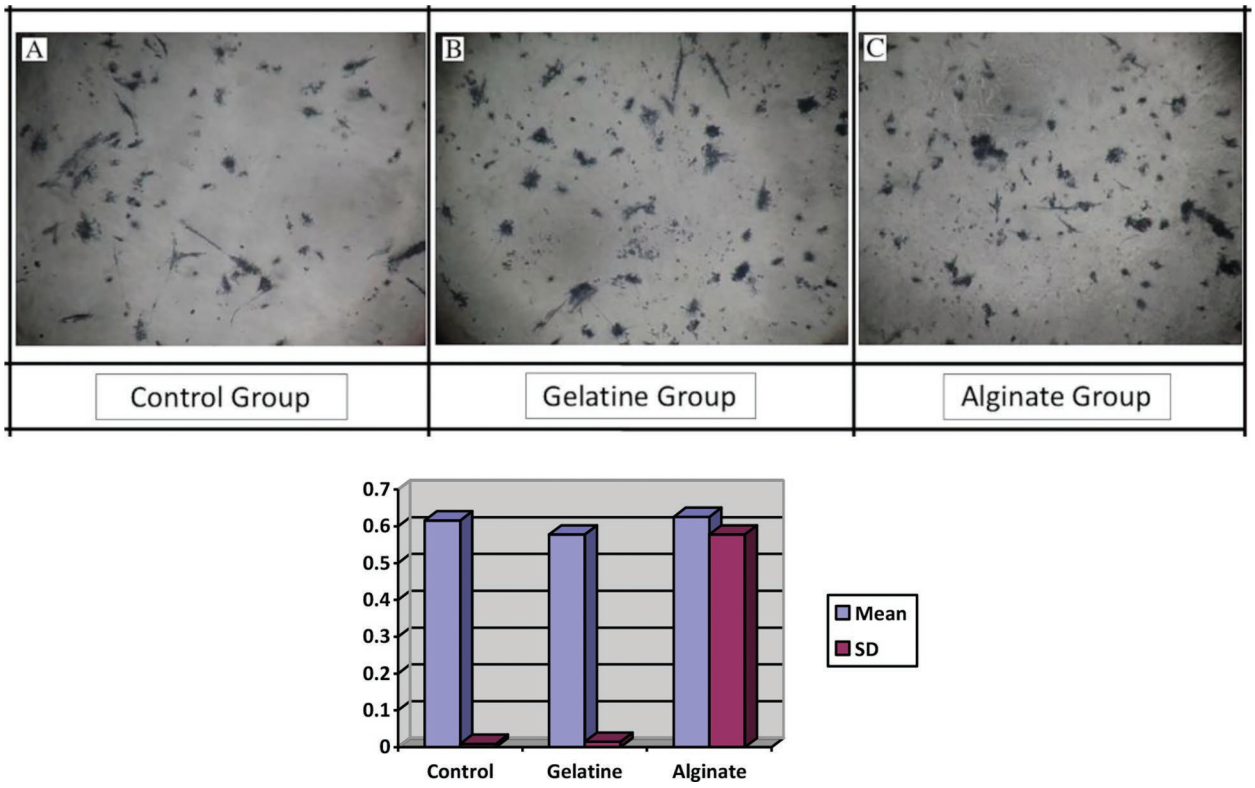

Table 1: Optical density of hAMSCs on gelatin and alginate group.

MSC source	Group	Mean	SD
hAMSCs	**Control**	0.616	0.009
	Gelatin	0.578	0.0147
	Alginate	0.626	0.578

Data presented as mean±SD (n=12).

Table 2: MTT assay of cytotoxicty of gelatin and alginate to hAMSCs.

MSC source	Group	Mean	SD	*p* value
hAMSCs	**Gelatin**	**97.26%**	0.085	0.057
	Alginate	**98.43%**	0.061	

Data presented as mean±SD of living cells percentage (n=12).

Figure 3 described the photograph of hAMSCs MTT assay on gelatin and alginate. The optical density was then calculated and shown in Table 1. The statistical analysis revealed that gelatin and alginate solvent did not affect the viability of hAMSCs, and no significant statistical difference (p=0.057) of cytotoxicity was found between gelatin and alginate to hAMSCs as shown in Table 2.

4. DISCUSSION

Studies have shown that gelatin and alginate were nontoxic materials. The research of biocompatible gelatin evaluation scaffold increased by MSC for bone tissue regeneration showed that gelatin scaffold has no toxicity and gelatin is biocompatible [12]. Gelatin is used as a gel-forming agent that can be thermally reversed for encapsulation, and it has been used in many tissue engineering applications because of its biocompatibility and ease of gelation [13]. Gelatin protects the sequential information

signal of arginine–glycine–aspartic acid, which promotes cell attachment, proliferation, and stem cell differentiation. In a recent analysis, a block has proven the use of gelatin-coated flask for the success of MSC from BMMSC or nonamniotic placental stem cells [14]. Gelatin is a fraction dough composed entirely of amino acids joined by a peptide relationship to create polymers that animate in a molecular mass from 15.000 to 40.000 and contain 8–13% moisture and have a relative density of 1.3–1.4. As gelatin is a protein, it proves the antique chemical properties of these materials, namely amino acids that have a functional group -COOH, -NH2 (for example, gelatin is hydrolyzed by the side of a proteolytic system to manifest amino elements). This causes the possibility of the gelatin fraction factor containing these amino acids to provide nutrients to the cell [15]. Gelatin, a highly biocompatible material with a surface anchor point, shows stable growth and adhesion to the surface of the matrix, and nothing is a clear influence of microsphere diameter [13].

The research showed that hydrogel alginate is a promising scaffold for dental-derived stem cells; an *in vitro* study showed that alginate is a promising candidate as a nontoxic scaffold for periodontal ligament stem cells (PDLSCs) and gingival mesenchymal stem cells (GMSCs). Previous studies show that the mechanical properties of alginate hydrogel have an influence on both the structure of the fabricated scaffold and the proliferation and metabolism of encapsulated cells. It also has the ability to differentiate these stem cells from osteogenic and adipogenic tissues. Alginate hydrogel is a three-dimensional carrier and can be biodegradable for new types of stem cells [16].

Alginate is a family of natural, anionic, and biocompatible polysaccharides derived from brown algae. Alginate contains blocks of (1–4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers [17]. Alginate is a biomaterial that is widely used for cell encapsulation due to low toxicity, high biocompatibility with hosts and with closed cells, and the fact that alginate can form relations with divalent cations under mild conditions and form egg-box models. Egg-box models composition is highly suitable for encapsulation of bio-macromolecules and living cells, but alginate quality can be ensured [17].

There exists a negative relationship between cell viability and alginate. Negative concentrations relationship between cell viability and microsphere diameter, which matches the equation. Changes in process parameters have little effect on cell viability as long as the diameter and microsphere solution have the same concentration. These results show how, in the process of cell encapsulation, surface loading induced by microspheres is the main factor affecting cell viability rather than mechanical pressure. Increased microsphere diameter will produce significant cell viability. Higher concentrations of alginate solutions have a higher surface tension, thereby reducing cell viability in the resulting microspheres. The lower concentration of alginate supports better cell proliferation, especially in the following period. That may be because lower concentrations of alginates show a larger average pore size and therefore interconnectivity is higher and facilitates better supply nutrition, metabolic waste exchange, and cell migration growth [13].

Alginate has mechanical integrity for clinical applications: a balance must be made between mechanical properties and their effect that hydrocolloid viscosity can have the viability of encapsulated cells. As the concentration of alginate increases to 2% and 5%, dead cells are mostly found in the matrix hydrogel. The higher the concentration of the alginate solution damages cells because they show higher viscosity, the higher they exert shear stresses on cells when they are homogenized. Membrane cells are highly sensitive to shear forces, and mixing can cause cell death. Interestingly, although cell death in the gel matrix was seen after 1 day of culture, the cell population seemed to recover quickly and the cell viability level $>$90% was reported in all subsequent conditions. This might indicate that cells are able to multiply rapidly into the space left by dead cells in the alginate matrix [18]. In gelatin test, cytotoxicity and cell viability *in vitro* showed excellent cell concentration of cytocompatibility and cell viability of up to 25%, while higher extract concentrations induced fewer toxic cell effects compared to positive and negative controls [19].

One reason that tends to cause nontoxic results is that hAMSCs have a number of immunosuppressive activities. It is true that they are limiting the proliferation of T and B cells, immersing inflammatory properties, macrophages, dendritic cells, neutrophils, and natural killer cells, by increasing cell induction with regulatory functions, such as with regulatory T cells and anti-inflammatory M2 macrophages. These properties are the basis for their use for the treatment of inflammatory disorders, and they have been obtained from various types of preclinical disorders that are exacerbated by inflammation [20]. This research was conducted at pH buffer (pH$=$7). The effect of pH on triple-helix content, thermal stability, and mechanical properties of gelatin was investigated. The gelatin test is conducted for different pH solutions from gelatin type A pigskin and is mechanically determined. A pH higher than 9 and lower than 5 in the cross-linked sample proves neat cell proliferation and viability. In other words, changes in pH do not affect the cytotoxicity of gelatin [21]. Different from gelatin, alginate modified at neutral and alkaline pH values tends to be eroded more easily than under acidic conditions. Alginic acid is one of the mucoadhesive biomaterials that is highly investigated because of its very neat cytocompatibility and biocompatibility, biodegradation, sol–gel transition characteristics, and chemical elasticity that gives rise to further alteration to adapt its properties [22].

This study uses the fourth passage, as a result of Early-passage hAMSCs (E-hAMSCs), which are interpreted as having a doubling time (DT) of 200 h—more complex and having a fibroblast shape, while passage of hAMSCs (L-hAMSCs) with DT is more from 400 days—has a flat and hypertrophic phenotype. As a result of the amount of passage accumulating, the proliferative properties decrease and the expanded number of cells decreases when the DT, which is 130 h, accumulates about 3.7 times with the Late (L-hAMSCs). The proportion of cell stage identified in the average hAMSCs was 78.26% in phase G0/G1, 11.61% in phase S, and 10.13% in phase G2/M. Change in the L-hAMSCs cell cycle showed an 11% increase in the G1 phase relative to E-hAMSCs, indicating that the reduce proliferation growth is due to a decreased phase S and enhanced control of G1 checkpoints [23]. This concluded that the possibility of good nontoxic/viability may be due to DT factors.

hAMSCs possess senescence that is addressed by the disruption of cell progress and the mechanisms associated with ageing. Senescence of the baby's cells is a natural physiological way that occurs throughout pregnancy and is especially visible at term pregnancy. This same characteristic also occurred when hAMSCs is developed [24]. hAMSCs had burst the marker expression in senescence [25]. This property is also the possibility of suppressing cytotoxicity from the results of this study. Several prospective surface markers of MSC or possible markers related to stem cells, namely Stro-1, SSEA-4, CD271, and CD146 sequences, have been proposed so far; however, there are large differences in their expression in various MSC sources.

This study shows that gelatin, as well as alginate, is a nontoxic material. Gelatin as a scaffolding material encourages cell attachment, proliferation, and differentiation of stem cells. Gelatin, a highly biocompatible substance with such a surface anchor point as well as alginate hydrogel, affects both the structure of the manufactured scaffold and the proliferation and metabolism of encapsulated cells. Alginate hydrogel is just a three-dimensional carrier for current stem cell types. In conclusion, gelatin and alginate are good scaffold choices to carry hAMSCs.

5. CONCLUSION

Gelatin and alginate were nontoxic to hAMSCs *in vitro.* No significant statistical difference of cytotoxicity was found between gelatin and alginate to hAMSCs.

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Author Contributions
This work was conducted with the collaboration of all authors. NH was responsible for the experiment design, data analysis, literature search, and preparation of the research report. In addition, PH, HS, and CKH performed the sample collection and literature search. RPD and FB performed statistical analysis. CKH, RPD, and FB contributed for research report and literature search work.

Conflict of Interest
None.

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