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Research Report

Comparative in vitro study of the cytotoxicity of gelatine and alginate to human umbilical cord mesenchymal stem cells

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ABSTRACT

Background: *Mesenchymal stem cells (MSCs) and scaffold combination constitute a promising approach currently adopted for tissue engineering. Umbilical cord-derived mesenchymal stem cells (hUC-MSCs) are easily obtained and non-invasive. Gelatine and alginate constitute a biocompatible natural polymer scaffold. At present, a cytotoxicity comparison of gelatine and alginate to hUC-MSCs is not widely conducted.* ***Purpose:*** *This study aimed to compare the cytotoxicity of gelatine and alginate in hUC-MSCs in vitro.* ***Methods:*** *Isolation and culture were performed on hUC-MSCs derived from healthy full-term neonates. Flow Cytometry CD90, CD105 and CD73 phenotype characterization was performed in passage 4. 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was performed to measure the cytotoxicity. The three sample groups were: (T1) hUC-MSCs with α-MEM (alpha-minimum essential medium) solution as control; (T2) hUC-MSCs with gelatine; (T3) hUC-MSCs with alginate.* ***Results:*** *Flow cytometry of hUC-MSCs displayed positive CD90, CD105 and CD73 surface markers. Gelatine and alginate had no effect on the viability of hUC-MSCs and no statistically significant difference ($p>0.05$) of cytotoxicity between gelatine and alginate to hUC-MSCs.* ***Conclusion:*** *Gelatine and alginate proved to be non-toxic to hUC-MSCs in vitro.*

Keywords: *alginate; gelatine; mesenchymal stem cells; umbilical cord*

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INTRODUCTION

Stem cell research has increased due to the realisation that stem cell-based therapies have the potential to repair bone or tooth loss caused by trauma, fractures, surgery, tumour resection, congenital malformation, dental implant failure-associated osteoporosis and periodontitis in dentistry.[1] Tissue construction which consists of stem cells and scaffold combination represents a promising approach to bone tissue engineering. Mesenchymal stem cells (MSCs) have considerable potential for the field of regenerative medicine due to their self-renewing capacity, multilineage differentiation potential and immunosuppressive properties.[2] Although human bone marrow mesenchymal stem cells (hBM-MSCs) are the most common and best characterized stem cell source, umbilical cord derived stem cells (hUC-MSCs) provide a novel source of MSCs[3] and, recently, hUC-MSCs have been shown to possess significant osteogenic differentiation potential.[4] Isolation of hBM-MSCs requires an invasive procedure that may cause aspiration site morbidity, while hUC-MSCs are easily obtained through a non-invasive process that does not result in morbidity.[5] Moreover, hUC-MSCs can be less immunogenic than hBM-MSCs.[3]

The ideal scaffold is able to facilitate adhesion, migration, proliferation and cellular organization in three-dimensional fashion from a cell population required for tissue engineering. High porosity and ideal pore size facilitate the diffusion of nutrients, oxygen and waste products from cellular metabolism.[6,7] Biodegradability allows scaffold to be absorbed by the body. The time required for degradation ideally matches that of new engineered tissue formation.[6] Biocompatible and non-toxic properties represent prerequisites to avoiding an inflammatory reaction and toxicity.[7]

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Biomaterial scaffold is broadly divided into two categories: natural polymer such as gelatine, alginate, dextran, chitosan and synthetic polymer of which poly (lactic) acid (PLA), Poly(glycolic) acid (PGA), Poly (urethanes) are examples. The biocompatibility of natural polymer is greater.[8] However, synthetic polymer demonstrate superior mechanical properties, but induce an inflammatory response in the body of the host, both acute and chronic.[9]

Gelatine and alginate are natural polymers both of which can be processed into injectable scaffold that easily fill any irregularly-shaped defect.[10] Alginate originates from algae which requires an extensive purification process in order to avoid an immune response after implantation. The advantages of alginate are those of lower low toxicity and higher biocompatibility. Nevertheless, the mechanical strength and biodegradability of this material is low and less capable of accommodating cell adhesion.[8] Disadvantages of using alginate include large batch-to-batch variations, the high cost of biosynthesis and the hydrophilic properties that render it ineffective in protein adsorption. Therefore, alginate scaffold must be modified to carry out cellular function.[11]

Gelatine, the result of protein denaturation from partial hydrolysis of collagen, is considered a choice polymer that can ideally be used in bone tissue engineering. This material is non-toxic, biocompatible and biodegradable both in vitro and in vivo. As a collagen derivative, gelatine contains cell binding motifs such as arginine-glycine-aspartic acid sequences (RGD) which play a role in the processes of adhesion, proliferation, cell differentiation and Matrix metalloproteinase (MMP) which influences biodegradation.[10]

Gelatine is cost effective and can be processed to resemble the structure of collagen as the major organic protein of the bone matrix. Particulate leaching, gas foaming and freeze drying all represent processing methods that have been adopted in the preparation of gelatine porous scaffold. The majority of the fabrication methods are simple and economical.[12] It is hoped that gelatine and alginate possess properties non-toxic to hUC-MSCs in order that one can replace the other if either is unavailable.

The major challenge to the development of optimum bone scaffold is its biocompatibility. Our previous study demonstrated that 2% of gelatine solvent was non-toxic for hUC-MSCs in vitro.[13] An in vivo study showed that 2% of alginate was safe for bone marrow mesenchymal stem cells.[14] Cytotoxicity comparison of gelatine and alginate to hUC-MSCs has not been widely studied. The aim of the research reported here was, therefore, to compare the cytotoxicity of gelatine and alginate to hUC-MSCs in vitro.

MATERIALS AND METHODS

A Caesarean section was performed on a healthy full-term neonate. Ethical approval was granted by the Research Ethics Committee of Soetomo Public Hospital in Surabaya. (547/Panke.KKE/IX/2017). This isolation and culture procedure was performed using stem cell laboratory protocols at the Stem Cell Research and Development Centre, Universitas Airlangga, Surabaya, Indonesia.

The section of umbilical cord was cut to a length of approximately 1 cm., with the artery, vein and adventitia being separated. Wharton's Jelly was subsequently immersed in a tube containing 0.25% Trypsin at 37ºC for 40 minutes and centrifuged in order to separate the supernatant. Samples were immersed in Phosphate Buffered Saline (PBS) (1X, pH 7.4), containing 0.75 mg/ ml of Collagenase Type IV (Sigma-Aldrich, St. Louis, MO, USA) and 0.075 mg/ mL of DNase I (Takara Bio, Shiga, Japan) prior to incubation at 37°C for 60 minutes. Filtering was carried out using a cell strainer. One cc of Fetal Bovine Serum (FBS) was added and agitated for 10 minutes after which the samples were filtered using sterile gauze on Becker glass and centrifuged for ten minutes at 1600 rpm. The resulting pellets were resuspended in Dulbecco's Modified Eagle's Medium (DMEM). Solutions containing the single cell were transferred to a petri dish and incubated at 37ºC and in 5% CO_2. Replacement of the medium was performed every three days, with passage being carried out after confluence had occurred for approximately 21 days. Cells from passage 4 were harvested and evaluated for phenotypic characterization.

Characterization of MSCs phenotype in hUC-MSCs cultures was performed by means of flow cytometry. In passage 4, hUCMSCs were seeded in wells with Alpha Minimum Essential Medium (αMEM) (Sigma-Aldrich, St. Louis, MO, USA) before being washed with PBS (1X, pH 7.4) and fixed with 10% formaldehyde for ten minutes. The cells were then incubated at 37°C using the Human MSCs Analysis Kit (BD Bioscience, USA) with the addition of a CD90, CD105 and CD73 and negative CD45 cocktail of primary antibodies and washed with PBS (1X, pH 7.4). The primary antibody was labelled using Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 minutes. The cells were subsequently viewed and analysed by Fluorescence Assisted Cell Sorting (FACS) using a Calibur Flow Cytometer (BD Bioscience, USA).

This study used 2% gelatine (Rousselot, VION company, Guangdong, China) dissolved in a solution of 0.15 M sodium chloride and 25 M HEPES buffer solution (Sigma-Aldrich, St. Louis, MO, USA) at pH 7.0 which had been sterilized by autoclaving (at 121°C for 15 minutes), a process described by Hendrijantini *et al*.[13]

A 2% alginate solution, as described earlier by Wang,[14] was prepared by dissolving sodium alginate (Sigma-Aldrich, St. Louis, MO, USA) in distilled water at room temperature with vigorous agitation continuing until complete uniform dispersion had been achieved. The dispersion was heated to 80°C in a water bath and maintained at this temperature for 30 minutes. Hydrochloride acid of 0.1 M was used to adjust the solution to pH 7.0. A 15-minute autoclaving process at 121°C was then undertaken.[14]

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Colorimetric assay of 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was performed to measure the cytotoxicity of the gelatine and alginate solvent against hUC-MSCs. Three sample groups were prepared: (T1) hUC-MSCs with α-MEM solution; (T2) hUC-MSCs with 2% of gelatine and (T3) hUC-MSCs with 2% of alginate. Each group consisted of three samples with all groups being prepared in 96-well plates containing a final volume of 200µl and density of 5000 cells per well. After incubation, 10 µl of the MTT reagent was added to each well and subsequently serially diluted and incubated for 2-4 hours at 37°C. The living cells converted the MTT into purple formazan crystals, the sum of which was calculated using an Elisa Reader at a wavelength of 595 nm.

The data obtained was described as a mean value and standard deviation. The data underwent statistical analysis using a Shapiro Wilk test to obtain the distribution of data and followed by a Mann-Whitney test to identify the differences between groups using R. Version 3.4.0 software. (GNU, Auckland, New Zealand). A value of $p<0.05$ were considered statistically significant.

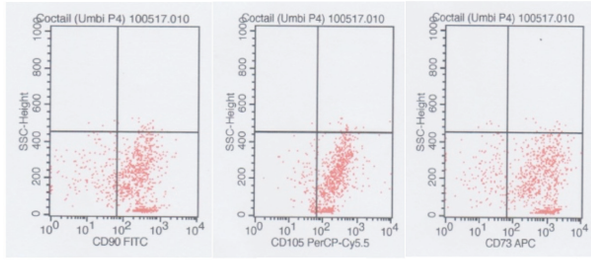

Figure 1. Flow Cytometry CD90, CD105 and CD73 of hUC-MSCs.

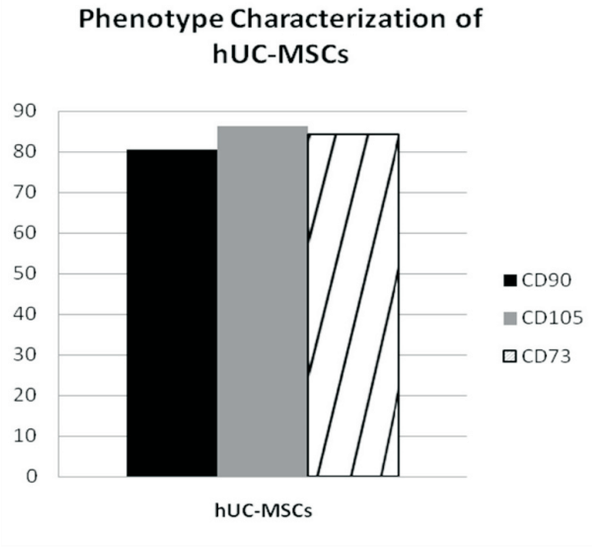

Figure 2. Phenotype characteristic expression in hUC-MSCs.

RESULTS

In passage 4, hUC-MSCs expressed 80.48 % CD90, 86.33% CD105 and 84.34% CD73. The result of flow cytometry is shown in Figure 1, while the phenotype characteristics of hUC-MSCs can be seen in Figure 2.

The photograph of MTT assay of hUC-MSCs on gelatine and alginate can be seen in Figure 3. The optical density was then calculated and is shown in Table 1. Based on statistical analysis, it can be concluded that the gelatine and alginate solvent did not affect the viability of hUC-MSCs and that no significant statistical difference ($p>0.05$) of cytotoxicity existed between gelatine, alginate and hUC-MSCs.

DISCUSSION

In this study, umbilical cord-derived stem cells were considered to be mesenchymal stem cells (MSCs) due to having positive surface antigen for MSCs (CD90, CD105 and CD73).[15] An immunosuppressive mechanism was demonstrated by MSCs, while the immunoregulation molecule of MSCs constituted HLA (human leucocyte antigen) class 1 which suppresses T cell proliferation. This molecule was able to inhibit lysis of MSCs mediated by NK (natural killer) cells, as well as their secretion of IFN-γ (interferon gamma). The addition of MSCs to mixed lymphocyte culture suppressed the production of Immunoglobulins (IgM, IgG and IgA) in vitro.[16] In a xenograft model, T-cell proliferation as adaptive immunity was effectively suppressed by hUC-MSCs as seen in the negative expression of CD40, CD80 and CD86 which played a role in T-cell activation. Humeral immune

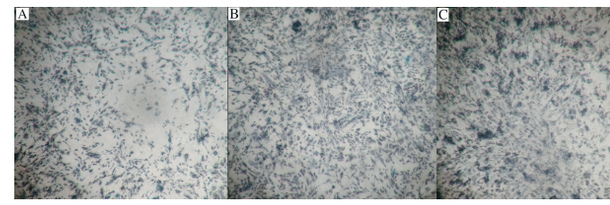

Figure 3. MTT assay on control (A), gelatine (B), and alginate (C) in hUC-MSCs.

Table 1. MTT assay hUC-MSCs on gelatine and alginate

MSCs source	Group	Mean	SD	p value
hUC-MSCs	Control	0.616	0.009	0.11
	Gelatin	0.578	0.0147	
	Alginate	0.626	0.578	

Data presented as mean ± SD (n=12)

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response and B cell proliferation were inhibited by hUC-MSCs.[3,15] Human leukocyte antigen HLA-G6 that inhibits Natural killer NK cells cytolytic activity was produced by hUC-MSCs as well as anti-inflammatory cytokines.[15] These features of hUC-MSCs might have contributed to cell viability in the study reported here.

The MTT Assay results proved that the alginate and gelatine were non-toxic to hUC-MSCs. This finding was consistent with studies conducted on both gelatine and alginate scaffold cytotoxicity to hUC-MSCs. A previous study of MTT assay demonstrated that gelatine solvent was non-toxic for hUC-MSCs.[13] Alginate scaffold provides an environment that supports cellular activity and the viability of hUC-MSCs.[17] The results of this study of alginate cytotoxicity in hUC-MSCs were similar to those of a previous one indicating that hUC-MSCs capsulated in alginate-fibrin microbeads significantly enhanced cell viability.[18] Alginate and chitosan combined scaffold showed strong cytocompatibility features in hUC-MSCs in vitro using MTT assay.[19] Furthermore, hUC-MSCs cultured on scaffold consisting of gelatine, alginate and beta-tricalcium-phosphate demonstrated cell viability, metabolic activity and proliferation.[20]

This study revealed no significant statistical difference in MTT assay results between gelatine and alginate to hUC-MSCs. This may be due to the hydrophilic nature of gelatine and alginate as biomaterial scaffold that facilitates cell attachment and water absorption in order to provide cell nutrition and metabolism activity.[21,22] Another factor that influenced cell metabolism in both biomaterial scaffolds was the pore size of the porous scaffold. A pore size of 100-300nm provided an environment conducive to cell metabolism.[23] Gelatine solvent possessed a pore size between 58nm and 475nm.[24] A pore 5-200nm in diameter was identified in the alginate solvent.[18]

Gelatine contained high levels of amino acids such as glycine 26-34% and arginine 8-9%. Glycine signalling reduced cell apoptosis.[25] Arginine was consumed by human MSCs during cell culture to maintain cellular metabolis[26], thereby implying that gelatine supports cell viability. Moreover, proliferation of MSCs was enhanced by arginine.[27]

Alginate contained blocks of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues. High M-block content alginates were immunogenic and more potent in inducing production of cytokine compared to high G-block alginates. Alginate extracted from *Laminaria spp.* contained 60% G-block.[22] Therefore, this study confirmed that alginate was non-toxic to hUC-MSCs.

The cell culture condition in hUC-MSCs influenced cell viability. The cell culture in this study was carried out at standard neutral pH 7. The acidity of cell culture significantly inhibited cell proliferation, increased cell apoptosis and decreased cell viability.[28] Finally, it can be concluded that gelatine and alginate scaffold were non-toxic to hUC-MSCs in vitro.

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