ORIGINAL ARTICLE

Biocompatibility Assessment of Mesenchymal Stem Cell-loaded Alginate-Chitosan Microcapsules by Intrabone Marrow Implantation in Sprague Dawley Rats

Retno Wahyu Nurhayati[1,2,3], Ahmad Jabir Rahyussalim[3,4,5], Sinha Haelamani[6], Astari Dwiranti[6], Gita Pratama[5,7], Chintya Mutiara Sari[4], Rio Wikanjaya[4], Kamila Alawiyah[8], Dinda Shezaria Hardy Lubis[6]

[1] Departement of Chemical Engineering, Faculty of Engineering, Universitas Indonesia, Depok, West Java 16424 Indonesia
[2] Research Center for Biomedical Engineering, Faculty of Engineering, Universitas Indonesia, Depok, West Java 16424 Indonesia
[3] Stem Cells and Tissue Engineering Cluster, Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia, Jakarta 10430 Indonesia
[4] Department of Orthopaedic Traumatology, Faculty of Medicine, Universitas Indonesia - Dr. Cipto Mangunkusumo General Hospital (RSCM), Jl. Diponegoro No 71, Jakarta 10430, Indonesia
[5] Integrated Service Unit of Stem Cell Medical Technology (IPT-TK Sel Punca), Dr. Cipto Mangunkusumo General Hospital (RSCM), Jl. Diponegoro No 71, Salemba, Jakarta Pusat 10430, Indonesia
[6] Department of Biology, Faculty of Mathematics dan Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia
[7] Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Indonesia - Dr. Cipto Mangunkusumo General Hospital (RSCM), Jl. Diponegoro No 71, Salemba, Jakarta Pusat 10430, Indonesia
[8] Department of Biology, Faculty of Mathematics and Natural Sciences, Sriwijaya University, Palembang, 30128, Indonesia

ABSTRACT

Introduction: Microencapsulation is a promising technology for protecting valuable cells and promoting secretome delivery in regenerative medicine. The current study evaluated the biocompatibility of alginate-chitosan microcapsules, loaded with xenogeneic cells, in Sprague–Dawley rats. **Materials & Methods:** The xenogeneic cells, human umbilical cord-derived mesenchymal stem cells, were entrapped in alginate beads (50 mg/ml) and then coated with chitosan (10 mg/ml). The microcapsules were then implanted at the intrabone marrow site, and the post-implantation effect was evaluated after 14 days. **Results:** The results showed that xenogeneic cell-loaded microcapsule-implanted and non-implanted rats had no significant differences in recovery post-surgery, as assessed from wound healing, body weight, and leukocyte ratios. Flow cytometric analysis of the leukocyte ratios indicated that the lymphocyte and granulocyte percentages (64.20±8.72%; 14.57±6.25% in the control group and 63.87±5.64%; 14.73±4.25% in the treatment group) were in the normal range (37.4-91.9% for lymphocytes and 5.4-64.4% for granulocytes). However, the monocyte percentages (21.22±2.48% and 21.40±1.39% in the control and treatment groups, respectively) were higher than the normal range (1.1-5.7%), which might indicate chronic inflammation in rats postoperatively. **Conclusion:** The abovementioned results suggested that chronic inflammation was caused by invasive surgical procedures rather than implanted microcapsules. To our knowledge, this study is the first to report the intrabone marrow implantation of biopolymer-based microcapsules in a rat model.
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Corresponding Author:
Retno Wahyu Nurhayati, PhD
Email: retno.wahyu01@ui.ac.id
Tel : +6221-7863516

INTRODUCTION

Stem cells are rare and unique cells in the human body that have the powerful ability to generate various mature cells through differentiation as well as to produce identical daughter cells. Currently, stem cell therapy is a promising approach to the treatment of terminal and degenerative diseases. Autologous or allogeneic stem cells are typically delivered intravenously; thus, it is not guaranteed that the injected cells will be transported efficiently to the desired site. Therefore, the delivery system of valuable stem cells must be improved to promote therapeutic effects.

Biopolymer-based microencapsulation is a promising technology for improving stem cell delivery. Cell-loaded microcapsules can be used to deliver stem cells or their therapeutic metabolites to specific target sites. Stem cell microencapsulation can also reduce immune rejection and promote cell survival in allogeneic transplantation. The selection of hydrogel materials plays a crucial role in cell encapsulation. Hydrogels can create viscoelastic cell microenvironments that mimic the dynamic mechanical properties of the extracellular matrix (ECM). Hydrogel-based strategies can enhance tissue engineering and regenerative medicine applications by providing a more accurate model for studying cell-ECM interactions and optimizing cellular responses (3). Stem cells can be encapsulated in hydrogels based on biopolymers for tissue regeneration using various methods, including hanging drops, extrusion, and 3D bioprinting. The hanging drop method is a cheap and feasible technique for encapsulating cells. Temperature- or pH-responsive materials such as collagen can be used for cell microencapsulation via the hanging drop method. Extrusion-based cell encapsulation offers advantages such as scalability, high cell density, and the ability to incorporate various biomaterials. The method can be performed manually or using an automated extrusion machine. However, challenges include maintaining cell viability during the extrusion process and optimizing the mechanical properties of the printed structures to support cell growth and function over time. The latest bioprinting technique allows the precise placement of cells within biomaterials, enabling the formation of complex tissue-like architectures (4). These encapsulated cells maintain their functionality, promote cell-to-cell interactions, and facilitate nutrient and waste exchange. However, bioprinting requires more complex operation and higher cost than hanging drop and extrusion methods.

Previously, we developed double-layered alginate-chitosan microcapsules that can be used to protect valuable $CD34^+$ progenitor cells. The microcapsules were nontoxic to other cells in vitro. Based on a co-culture assay, it was found that the encapsulated cells could improve proliferation and maintain the stemness of bare cells. The current study was intended to evaluate the biocompatibility of human stem cell-loaded alginate-chitosan microcapsules in rat models. Biocompatibility assessment is important for ensuring that the microcapsules are safe to the recipient body. The assessment parameter is the postthaw inflammatory response, which can be characterized based on leukocyte observation. The increase in the number of leukocytes can be detected by using flow cytometry, a powerful tool for analyzing and measuring the multi-characteristics of a single particle or cell by flowing cells to pass through certain rays quickly and accurately. Biocompatibility evaluations can be performed i*n vivo* using transplantation. The microcapsules developed in our study were intended to deliver paracrine to bone marrow; therefore, intrabone marrow implantation was selected as a suitable method to assess the biocompatibility of alginate-chitosan microcapsules.

MATERIALS AND METHODS

The *in vitro* and *in vivo* protocols performed in this study have been reviewed and approved by the Faculty of Medicine Universitas Indonesia – Dr. Cipto Mangunkusumo General Hospital Ethical Committee (KEPK FKUI-RSCM).

Umbilical Cord Mesenchymal Stem Cells (UC-MSCs) Isolation and Culture

MSCs were isolated from the umbilical cord as described elsewhere (13). Briefly, umbilical cord from non-infectious subjects was soaked in a povidone-iodine solution for 10 min and washed 3 times with phosphate-buffered saline (PBS). The blood vessels were removed and then Wharton jelly-rich tissue was placed in a multi-well plate. Complete media containing Dulbecco's Minimum Essential Medium, 10% Fetal Bovine Serum (FBS), and Antibiotic-Antimycotic. Spent media were changed every 2-3 days. Sprouting cells from explants were detached by an enzymatic method with 0.25% trypsin-EDTA and seeded into a T-flask. The cells were passaged at least twice before being used for further experiments. Cell culture was conducted in a fully humidified CO_2 incubator at 37°C. Otherwise stated, all reagents for cell culture and chemicals were purchased from ThermoFisher Scientific (USA) and Merck (USA), respectively.

MSC Characterization using Flow Cytometry

The human MSC Analysis Kit (BD Biosciences, USA) was used to stain 10^5 cells in accordance with the manufacturer's instructions. After being stained for 30 minutes in the dark, the cells were washed with PBS. The stained cells were loaded into a flow cytometer (FACS Aria III, BD Biosciences, USA) to examine the expression of surface markers (CD73, CD90, CD105, and Lin).

Alginate-Chitosan Microcapsules Preparation

Alginate-chitosan microcapsules were prepared as described in our published work (7,8,14) with modification. In brief, the alginate solution was prepared by dissolving sodium alginate into PBS (w/o Ca^{2+} Mg^{2+}) with a final concentration of 50 mg/ml. Chitosan solution was prepared by dissolving chitosan into acetic acid with a final concentration of 10 mg/ml. The MSCs were mixed with alginate solution in a ratio of 1: 4. About 10 µL of the mixture solution was dropped into the stirred $CaCl_2$ solution. The alginate microcapsules were then coated with chitosan to form double-layered alginate-chitosan microcapsules.

Microscopic observations

Inverted and fluorescence microscope can be used to

observe the cells in a microcapsule. The cells were stained using DNA-specific dye (propidium iodide) to distinguish cells from other objects according to our previous report (9). In brief, the microcapsules were submerged in paraformaldehyde (Sigma-Aldrich, USA) for 15 minutes. After washed with PBS w/o Ca/Mg, the gels were submerged in cold methanol for one hour. The gels were treated for 30 minutes with RNAse (Sigma-Aldrich, USA), and then were stained with propidium iodide (Wako Pure Chemicals, Japan) for 15 minutes. The gels were examined using a fluorescence microscope (Zeiss, Germany).

Biocompatibility Evaluation of Alginate-Chitosan Microcapsules

Animal care was conducted in Animal Research Facility Laboratory, Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia. The animal models used in this study were 3-month-old healthy *Rattus norvegicus*-Sprague Dawley strain with no physical disabilities (Badan Pengawas Obat dan Makanan/ National Agency of Drug and Food Control, Indonesia). The biocompatibility evaluation was carried out in vivo using with intra-bone marrow transplantation method. The rat models were divided into control and treatment groups (each n = 3). After being anesthetized with ketamine-xylazine, the femur bones of the control and treatment groups were drilled (bor diameter 1.55 mm). MSC-loaded microcapsules (1 microcapsule/femur) were implanted in treatment group, then the wound was covered. In the case of rats in the control group, the wound was covered directly after the femur bone was drilled.

Blood Analysis using Flow Cytometry

The rats were terminated after 2 weeks of surgery(15). Cardiac blood was collected immediately, and blood clotting was prevented with heparin addition. Isolated blood was centrifuged at 3,000 rpm to remove plasma. The plasma-depleted blood cells were diluted with PBS solution (1:9). The diluted blood was then loaded into a flow cytometry (16,17). The percentage of each cell type population from total leukocytes was then calculated using the formula below:

$$\frac{n_e}{n_t} \times 100$$

where n_e is the number of each type of leukocytes; n_t is the number of total leukocytes.

RESULT

Characteristics of UC-MSCs

Explant method is a feasible technique to isolate MSCs from wharton jelly of umbilical cord. The cells will start sprouting from umbilical cord explant within 2 weeks after explantation. After 3 times passage culture, the high purity cells can be collected and represents the MSC characteristics, such as fibroblastic cell morphology and expressing specific surface markers (CD73/CD90/CD105) (16). From harvested cells (Fig. 1), the percentage of CD73, CD90 and CD105 positive cells were more than 95% and the lineage negative (CD45, CD34, CD11b, CD19 and HLA-DR) expressing cells were less than 5% (Fig. 2)

Fig. 1 Microscopic observations of cultured UC-MSCs. Scale bar indicates 50 micrometers

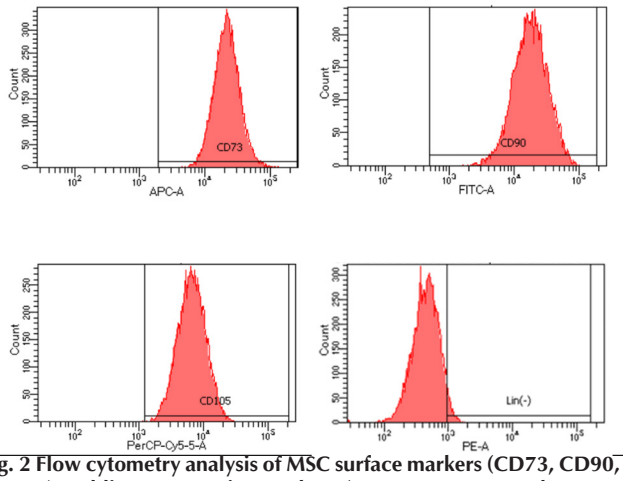

Fig. 2 Flow cytometry analysis of MSC surface markers (CD73, CD90, CD105) and lineage negative markers (CD45, CD34, CD11b, CD19 and HLA-DR)

Alginate-Chitosan Microcapsules

Alginate-chitosan microcapsules consisted of two layers, cell-loaded alginate as the core and chitosan as the outer layer (Fig. 3). Alginate microcapsules are formed due to cross-linking between Ca^{2+} ions from $CaCl_2$ solution and alginate polymer. The distribution of cells in the microcapsule can be observed by the help of a common DNA dye including propidium iodide. As can be seen at Fig. 4, the cells scattered evenly in alginate microsphere. Alginate is a great material for supporting cell entrapment, however, the calcium-crosslinked alginate gel can be easily degraded by bivalent cation or chelators. As reported in our previous study (9), a secondary coating is a suitable technique to improve protecting ability of alginate-based microcapsules.

Biocompatibility Evaluation of Alginate-Chitosan Microcapsules

The double layered alginate-chitosan employed in the current study survived in the intrabone marrow for at least 2 weeks without compromising its sphere integrity (data not shown). After 2 weeks post implantation, the

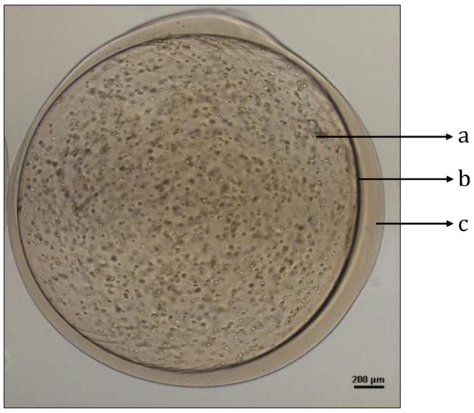

Fig. 3 Alginate-chitosan microcapsule: (a) human MSCs; (b) alginate layer; and (c) chitosan layer

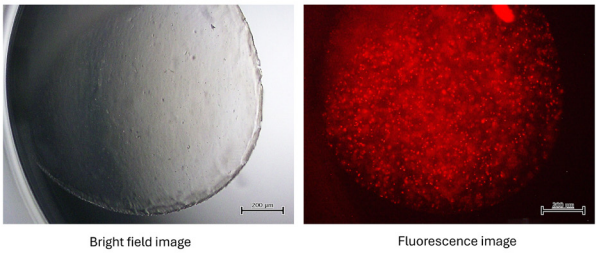

Fig. 4 Visualization of encapsulated cells after propidium iodide staining

cardiac bloods from non-implanted (control group) and microcapsule-implanted (treatment group) rats. In treated group, microcapsules were loaded with xenogeneic human cells as described in Method section. The collected cardiac blood from both of control and treatment rat groups were then analyzed using flow cytometry method. In the histogram data, the leukocytes and erythrocytes populations were gated based on the forward and side scattering plots (Fig. 5).

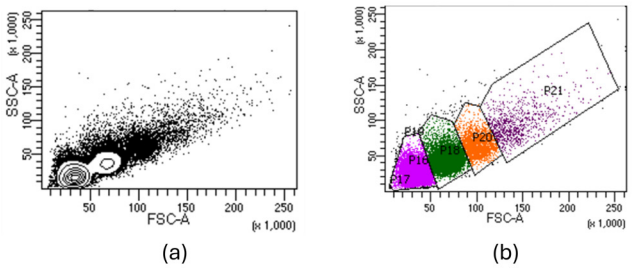

Fig. 5 Representative flow cytometric histograms of rat cardiac blood cells. (a) Non-gated histogram; (b) Gated histogram based on forward (FSC) vs side scatters (SSC). P16, P18, P20, P21 were erythrocyte, lymphocyte, monocyte, and granulocyte, respectively.

Based on the morphological analysis in the control and treatment group, it was seen that the wound due to the transplant process in the rat healed after 14 days and there was no inflammation sign in the rat such as swelling and redness (Fig. 6). The body weights (in grams) of controls and treatments groups were 193.33±25.01 and 185.33±16.77 on day 0 (before surgery). After 14 days of recovery, the body weights were increased 1.4 folds in both groups which were 279.33±26.56 grams (control group) and 260.00±17.44 grams (treatment group).

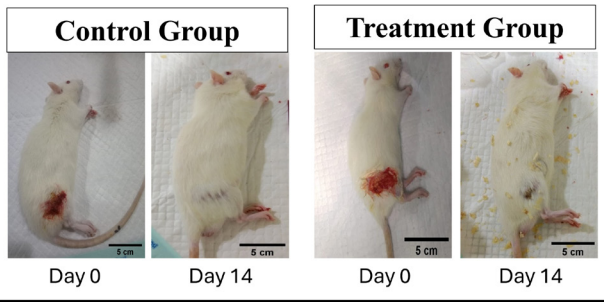

Fig. 6 Wound recovery of control and treatment groups on day 0 and day 14 post surgery

The results of leukocytes percentage analysis in the rat can be seen in Fig 7. It was shown that percentage of lymphocytes, monocytes, and granulocytes were 64.20±8.72; 21.22±2.48; 14.57±6.25 in control group and 63.87±5.64; 21.40±1.39; 14.73±4.25 in treatment group. The ANOVA test results of lymphocytes, monocytes, and granulocytes percentage between the control and treatment group rats showed a p value > 0.05 (nonsignificant difference). Based on a reported study, the normal leukocytes percentage in the rat are 37.4-91.9% for lymphocytes, 1.1-5.7% for monocytes, and 5.4-64.4% for granulocytes (16). These results suggested that only the percentage of monocytes of control and treatment groups were higher than that of non-operated rats, meanwhile the percentages of lymphocytes and granulocytes were considered within normal range.

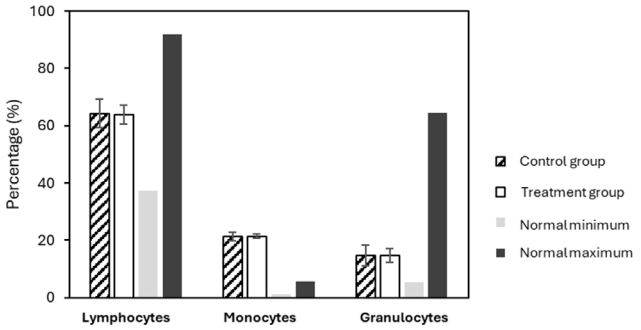

Fig. 7 The percentage of leukocyte components from control and treatment groups. Normal minimum and maximums data from a reported study(19). The student t-test of all leukocyte-type ratios between control and treatment groups showed non-significant difference (p > 0.05).

DISCUSSION

Biocompatibility evaluations of alginate-chitosan microcapsules were performed by transplanting the microcapsules into the bone marrow of test animals found in the femur. Rattus norvegicus Sprague-Dawley rats were used as models. The use of rats in the Sprague–Dawley strain has several advantages, including large body size and disease resistance. The alginate-chitosan microcapsule biocompatibility was evaluated based on rat morphology and leukocyte percentage using flow cytometry.

Inflammatory processes in the body are divided into

two types, namely acute and chronic inflammation. Acute inflammation is defined as inflammation that occurs over a short period, such as a few hours or days. This inflammation is characterized by an increase in body granulocytes, such as neutrophils, basophils, and eosinophils. Acute inflammation is the initial response to a stimulus that causes inflammation. Neutrophils are a type of white blood cell that first attacks the inflammatory agent via phagocytosis within 1–4 days after inflammation. This causes an increase in neutrophils in the acute inflammatory process. Chronic inflammation is defined as inflammation that occurs over a long period of time, about weeks, months, or even years. In chronic inflammation, monocytes and lymphocytes are increased. Monocytes are not only phagocytotic inflammatory agents but also inducers of fibrosis and angiogenesis.

Our study showed that xenogeneic cell-loaded microcapsule-implanted rats and non-implanted rats had no significant differences in recovery postoperatively, as assessed by wound healing, body weight, and leukocyte ratios. Further analysis of the leukocyte ratios indicated that the lymphocyte and granulocyte percentages in the control and treatment groups were in the normal range. However, the monocyte percentages in the control and treatment groups were higher than the normal range, which may indicate chronic inflammation in rats postoperatively. Monocytes are a type of leukocyte that play an important role in inflammation, so the monocyte percentage can be used as a parameter for inflammation. This indicates that chronic inflammation in the animal models was not caused by the transplantation of alginate-chitosan microcapsules. Chronic inflammation in rats is caused by invasive surgery involving the intra-bone marrow transplantation method. Tissue damage caused by femoral dissection and drilling can trigger chronic inflammation.

CONCLUSION

The alginate-chitosan microcapsules loaded with xenogeneic MSCs were implanted on the intrabone marrow site to evaluate the biocompatibility of microcapsules in animal models. The xenogeneic cell-loaded microcapsule-implanted and non-implanted rats did not differ significantly in terms of recovery post-surgery, as assessed from wound healing, body weight, and leukocyte ratios. The results showed that chronic inflammation was caused by invasive surgical procedures, and the comparative data with the control suggested that microcapsules did not induce additional inflammation in the rat model. To our knowledge, this study is the first to report the intrabone marrow implantation of biopolymer-based microcapsules in a rat model.

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