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Induced-Coagulated Plasma-Fibrin Gels as a Biological Scaffold for Cell Attachment and Proliferation of Umbilical Cord-Derived Mesenchymal Stem Cells (UC-MSC)

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Abstract

Fibrin gels are an ideal natural biological scaffold for tissue engineering because they are biocompatible, biodegradable, and have many biological surface markers. However, most research on fibrin gels used commercial fibrin kits that could be costly and limited in some areas. In this study, fibrin gels were made by inducing blood coagulation by adding a common diagnostic kit to assess the time for blood to clot, called activated partial thromboplastin time (aPTT). This induced coagulated plasma (iCoplas)-fibrin gels was evaluated for its ability to enhance biological activity of umbilical cord-derived mesenchymal stem cell (UC-MSC), which were cell attachment and proliferation. Fibrinogen concentration had influence on cell attachment, where only 50% of the cells could attach to 77 mg/dl fibrinogen gels whereas 93% cells adhered to 154 mg/dl fibrin gels. There were no significant differences in cell proliferation on polysterene culture dish and fibrin gels ($p>0.05$). These results showed that iCoplas-fibrin gels could be used as a fibrin-based scaffold, yielding no significant difference than polysterene-tissue culture dish cultures in cell attachment and cell proliferation on 154 mg/dl fibrinogen concentration.

Keywords: Fibrin gels, mesenchymal stem cells, activated partial thromboplastin time, cell attachment and proliferation.

Introduction

Bone defects could be caused by disease, degenerative process, or trauma. Treatment using cells or materials from the patient and implant it to the damaged tissue are called autologous treatment. Autologous bone grafts are often used to treat bone defects. Although this method can produce good results, it has drawbacks such as second site morbidity, pain, increased risk of surgical infection, and limited graft supply. Bone substitution method is an alternative method that can be used to treat bone defects. Physical characteristic of bone substitutes; such as porosity, pore size, and surface availability, should be considered so that the substitute could imitate the condition and characteristic of the bone. These characteristic is essential for cell attachment, proliferation, and differentiation of the cell to generate new bone tissues (Castelas *et al.,* 2006).

Tissue engineering is an interdisciplinary field that applies the principle of engineering and life science, in the purpose of developing a biological substitute that can restore, maintain, or improve tissue function or a whole organ. Tissue engineering consist into three components; cells, scaffold, and signal molecules (Ohba *et al.,* 2009). Cells that are usually used in tissue engineering are stem cells. Stem cells are undifferentiated cells that can be differentiated into many other

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cells. Stem cells also have the ability to self renew (Meirelles *et al.,* 2009) making it as an advantage for tissue engineering.

Mesenchymal stem cells are adult stem cells, commonly found in the bone marrow and umbilical cord (Docheva *et al.,* 2008). Adult mesenchymal stem cells are suitable for clinical use as they could attach to plastic, proliferate, and differentiate well *in vitro*, and have suitable properties for implantation by having low immunogenic and high immunosupressive properties (Fosset and Khan, 2011). Umbilical cord-derived mesenchymal stem cells are much harder to isolate but had a superior proliferation potential and more suppressive effects on peripheral blood mononuclear cell proliferation than bone marrow-derived mesenchymal stem cells (Arufe *et al.*, 2011).

Scaffolds are tailored made matrix to mimic natural environment for the cell, giving it an optimal condition to attach, proliferate, and differentiate (Baker and Chen, 2012). Fibrin, a natural occurring biodegradable matrix, is reported to be biocompatible, biodegradable, and has many biological surface marker making it an ideal natural biological scaffold (Shaikh *et al.,* 2008). Several researches on tissue engineering have explored the potency of commercial fibrin kit to make fibrin-based scaffolds. Although benefits in efficiency, using commercial fibrin kit could be costly and are not always available in some places. Activated Partial Thromboplastin Time (aPTT) Kit is a coagulation diagnostic kit to assess the prothrombin time or the time for blood to clot in clinical setting (Korte *et al.*, 2000). Using aPTT kit to fabricate induced coagulated plasma (iCoplas)-fibrin gel scaffolds, opens the possibility to use the patient's own blood as autologous scaffolds which could decrease the potential risk of body reaction (Ye *et al.,* 2000). In this study, we observe cell attachment and proliferation characteristic of umbilical cord-derived mesenchymal stem cells in iCoplas-fibrin gels.

Materials and Methods

Ethical Acceptance from the Etchical Committee Board

This study had been approved by the Institutional Review Board at the Faculty of Medicine, Universitas Gadjah Mada (UGM), Yogyakarta for collection and study with human umbilical cord tissue.

Sample Criteria

Umbilical cord was taken from aterm babies (32 until 38 weeks old) born in hospitals or clinics that cooperate with this research. Inform consent was informed and signed by the parents. Samples are stored in a sterile 50 ml centrifuge tube containing Phosphate Buffer Saline (PBS) (Biobasic, Toronto, Canada) supplied with penicillin (Gibco, 15140, Invitrogen Corporation, NJ, USA) and fungizone (Gibco, 15140, Invitrogen Corporation, NJ, USA). Samples are processed immediately or stored in 4 ^{o}C for a maximum 24 hours.

Plasma Collection and Fibrinogen Concentration Measurement

Whole blood for plasma source were obtained from healthy donor, collected in citrate vacutainer vacutainer (BD Vacutainer, NJ, USA) and processed by centrifugation at 2000 rpm for 10 minutes at room temperature. Fibrinogen concentration in the collected plasma citrate was measured by using turbidimetry method in Clinical Pathology Laboratory, Sardjito Hospital, Yogyakarta.

Icoplas-fibrin Gel Fabrication

Plasma citrate was diluted with double distilled water to obtain 154 mg/dl and 77 mg/dl fibrinogen concentration. Fibrin gels were fabricated by adding 150 µl of plasma and 100 µl TriniCLOT aPTT HS (Tcoag, Ireland) into a 24-wells plate. To homogenize the suspension, the plate was agitated gently then incubated in 37 ^{o}C for 5 minutes then the solution was added by 150µl of warm 0.1 M $CaCl_2$ and incubated at room temperature for 30 minutes. Fabricated fibrin gels were rinsed

with phosphate buffered saline and sterilized by ultraviolet (UV) radiation for 1 hour.

Mesenchymal Stem Cell Preparation and its Characterization

Isolation from umbilical cord by Explant Method

Cells was isolated using the explant method, according to Koliakos *et al*. (2011) without any addition in enzymatic reaction. Umbilical cord samples were cut into 1 cm^3 pieces and washed with sterilize PBS twice. Each explant pieces were cut again into 3 mm^3 explants and washed with *Dulbecco's Modified Eagle Media low glucose* (DMEM-LG) (Gibco, 25200, Invitrogen Corporation, Canada). Explants were placed into 60 mm culture dish and was incubated in room temperature for 3 minuets. The cells were cultured with DMEM-LG medium supplemented with *Fetal Bovine Serum* (FBS) 10% (v/v) (FBS Heat Inactivated, Caissonlabs, USA), penisilin streptomisin 1% (v/v) , dan fungizone 0.5 %. Cell culture was incubated in 37 ^oC, 5% CO_2 with 90% humidity. Culture medium was changed every two days and the cells were not used past passage 4. Upon reaching 100% confulency, cultured cells were trypsinize and subcultured.

Characterization of umbilical cord-derived cells by CD45 and Stro-1 expression

a. **Detection of CD45 expression by immunoflowcytometry**

Approximately 10^5 umbilical cord-derived cells were centrifuged at 2000 rpm for 5 minutes. Pellet cells then were added with 2.5 µl anti-CD45 (BD Bioscience, San Jose, CA 95131, USA) and vortex for 2 minutes. Cells were then incubated in dark at room temperature. Pellet cells were then washed with 1 ml of wahing buffer and centrifugated at 2 000 rpm for 5 minutes. Pellets cells were then resuspended with 100 ml *Facsflow*. Cell suspension then transfered to a flowcytometry tube and analyzed using FacsCalibur.

b. **Detection of STRO-1 expression by immunocytochemistry (ICC)**

Cells with a density of $5x10^4$ cells/ml was seeded on a cover slip in a 35mm TCD and incubated until 70% confluence. Cells on the cover slip were fixed with cold methanol and 0.3% H_2O_2. Cover slip were then washed with PBS and added with *background snipper* and incubated in room temperature for 10 minutes. The STRO-1 primary antibody were added to the cover slip and incubated for 10 minutes. Cover slip were washed again with PBS and added with 100 µl biotinylated universal secondary antibody (Novostain Universal Detection Kit NCL-RTU, Novocastra Lab LTD, UK) and incubated for 10 minutes. Cover slip were washed again with PBS and added with Betazoid DAB chromogen and incubated for 10 minutes. Cover slip were washed with aquadest and added 2 µl of counter-stain MayerHaematoxylin (Dako, Denmark) and incubated for 30 seconds. Cover slip were then washed with aquadest and dip in xylol then alcohol before incubating it in room temperature to dry. The dried cover slip is then mounted on the object glass and observed under the microscope. Cells with STRO-1+ will be colored dark brown as for negative cells will be blue.

Cell attachment assay on PS-TCD and fibrin gels

Approximately 10^4 cells are cultured in a 24 well plate with or without fibrin gels. Cells were supplemented with growth medium and incubated in 37 ^oC for 1 and 3 hours. Cells were then trypsinized and cell count was done by using hematocytometer.

Cell proliferation characteristic using MTT assay

Approximately 10^4 cells are cultured in a 24 well plate with or without fibrin gels. Cells were then incubated in 37 ^oC for 1, 3, 5, and 7 days. Cell proliferation was investigated using the MTT assay (refer to Song *et al.,* 2013). Medium was changed in every 2 days.

Osteogenic diferentiation of UC-MSC

Approximately $3x10^4$ cells are cultured in a 24 well plate. Cells were then supplemented with growth medium and incubated in 37 ^oC. On the second day, growth medium was changed with osteogenic medium which contained DMEM medium, 10% FBS, 150 µg/ml ascorbic acid, 10 mM β-glycerophosphate and10 nM dexamethasone (Silla-Asna *et al*., 2007).

Alizarine Red S staining

Mineralization staining was done by using Alizarine Red S staining (Sigma-aldrich, St. Louise, MO63130, USA).

Statistical analysis

All results represent replicates (n=3). Statistical analysis was performed using *one-way Analysis of Variance* (ANOVA) followed by *Fisher's paired Least Significance Difference* (PLSD) and analyze with *Student t-test*; a *p* value <0.05 was considered to indicate statistical significance.

Results and Discussion

Umbilical cord-derived cells, obtained by explant method, express STRO-1 marker and did not express the hematopoietic marker, CD45

Fibroblast-shaped cells were isolated from the umbilical cord using the explant method. Cell surface markers were investigated to characterize the isolated cells. STRO-1, a proven specific mesenchymal stem cell marker (Bobis *et al*., 2006), was expressed in cultured cells using the immunocytochemistry staining as shown by stained brown cells (Figure 1B). CD45, a well known hematopoietic marker, was also investigated using immunoflowcytometry (Docheva *et al*., 2008). There were 98.24% cells that did not express the CD45 marker.

Mesenchymal stem cells are a well investigated stem cell due to their unique characteristic. However, there are no definite specific marker for the identification of MSC (Docheva *et al*., 2008). To avoid uncertainty, in 2006, the International Society for Cellular Therapy had proposed minimal criteria to define MSC. First, MSC must have the ability to attach to plastic in standard culture conditions. Second, MSC must express CD73, CD90, and CD105 and do not express CD11b, CD14, CD19, CD34, CD45, and HLA-DR surface markers. Third, MSC must be able to differentiate into osteoblast, adipocyte, and chondrocyte *in vitro* (Dominici *et al.,* 2006). Fibroblast-like cells were obtained from umbilical cord explant. The cells adhered to plastic and were expand until 5 passages. Immunoflowcytometry using CD45 antibody showed 98.24% of the cells did not express the CD45 surface marker.

STRO-1 antigen is a well known MSC marker (Lin, *et al.,* 2011; Zomorodian and Eslaminejad, 2011). Although it still has unknown function, STRO-1^- cells are not capable in forming colony-forming-units-fibroblast (CFU-F) (Dazzi *et al* 2006; Kolf *et al.,* 2007). Umbilical cord-derived cells form CFU-F once they adhered to the plastic and proliferate until confluence (Figure 1C). Immunocytochemistry staining using STRO-1 antibody showed the cultured cells were positive for STRO-1 marker.

The ability to differentiate into osteoblast was also evaluated by mineralization Alizarine Red Staining. Cells culture showed change in morphology and increase mineralization after 14 days of incubation in osteogenic medium (Figure 1D). Mineralization was an indicator for osteoblast maturation and is considered as a osteogenic differentiation test (Silas-asna *et al.,* 2007). These results indicate that the umbilical cord-derived cells, isolated by explant culture method, could possibly be MSC.

iCoplas-fibrin gel could be degraded after 4 weeks in basal medium

Icoplas-fibrin gels were successfully fabricated using the aPTT kit (Figure 2A). Fibrinogen concentration from plasma citrate from a single donor was evaluated. Citrate plasma from the donor had 154 mg/dl of

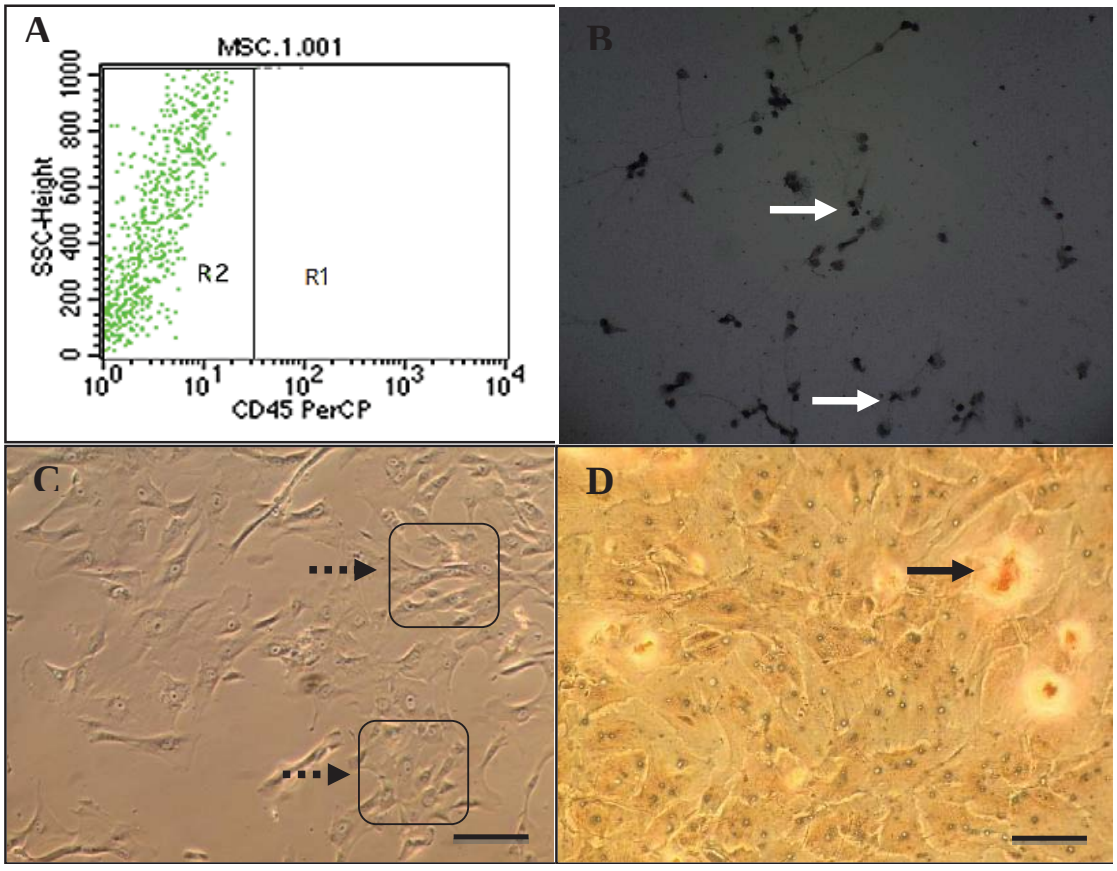

Figure 1. Identification of umbilical derived-cells. (A) Immunoflowcytometry using CD45 antibody showed 98.24% cells are negative; (B) Immunocytochemistry using STRO-1 antibody gave STRO-1+ cells a dark brown colour pointed by white arrows. (C) Colony forming unit fiboblast (CFU-F) forms in 3 day cultures pointed by dotted arrows; (D) Alizarine staining culture in osteogenic medium showed mineralization by formation of red nodules pointed by black arrows. Bars : 50 µm.

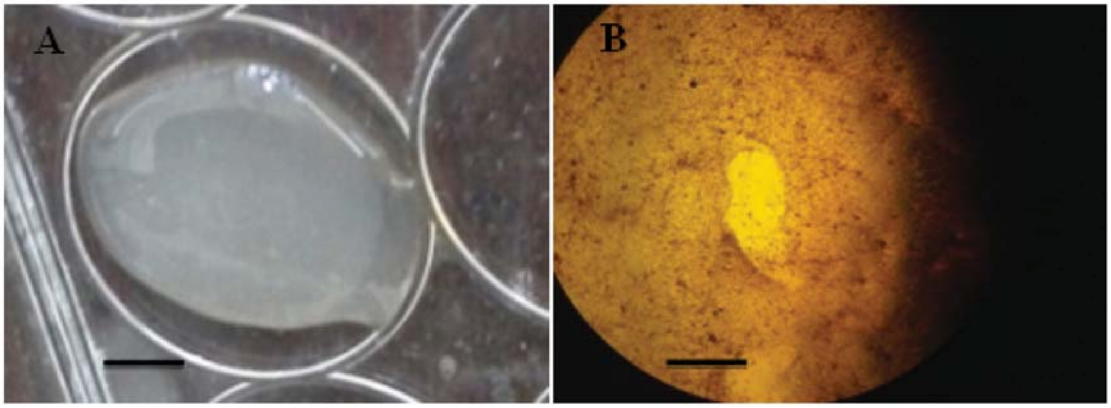

Figure 2 . Fibrin gel made by using aPTT Kit. (A) Fibrin gels forms by adding plasma citrate, aPTT HS, and CaCl2. (B) Fibrin gel degradation, with 77 mg/dl fibrinogen concentration, visualized using inverted microscope after 10 to 14 days of incubation with growth medium. Bars : 50 mm.

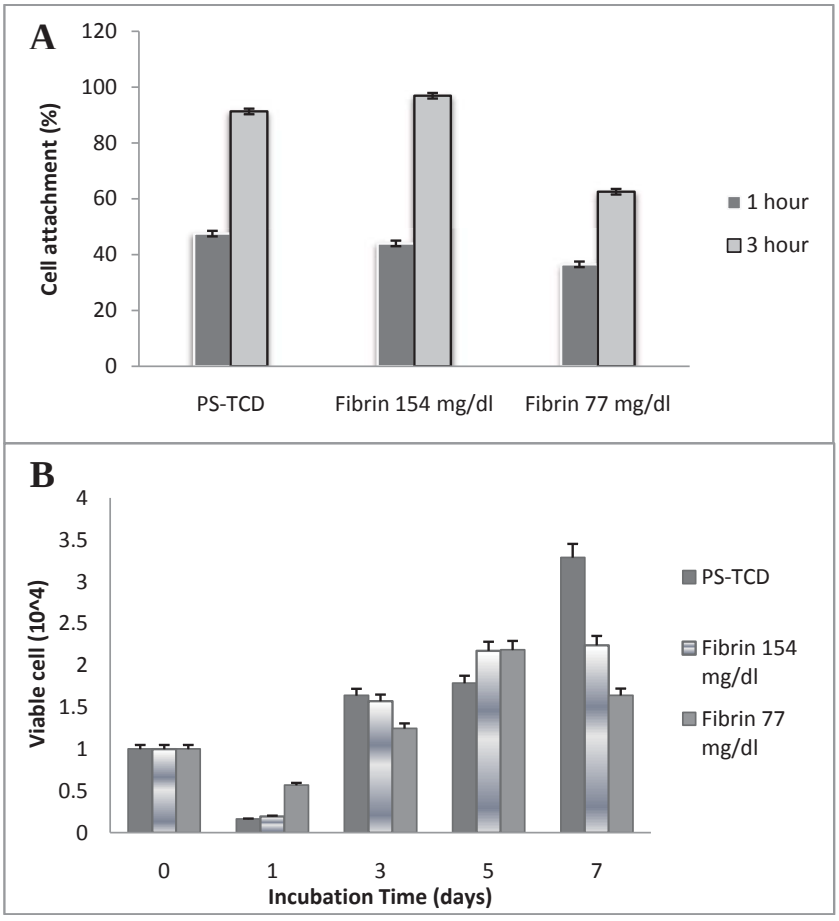

Figure 3. Mesenchymal stem cell characteristic on fibrin gels. (A) Cell attachment assay, cell attachment on fibrin gels are influenced by fibrinogen concentration; and (B) Proliferation assay showed no significant difference in PS and fibrin gel cultures.

fibrinogen concentration and is used for the main concentration to fabricate the fibrin gels. In 2008, Ho and coworkers, state that fibrinogen concentration of fibrin gels could influence cell proliferation. To evaluate this matter, plasma citrate was diluted with aquabidest to 77 mg/dl concentration. Fibrin gel was formed after incubation of citrate plasma, aPTT HS, and CaCl2 0.1M. Incubation time for clot formation were 7 minutes and 40 seconds and 12 minutes and 35 seconds for fibrin gel with a concentration of 154 mg/dl and 77 mg/dl, respectively.Fibrin gel degradation assay was conducted by incubating fibrin gels in growth medium and was observed visually under the microscope. The 77 mg/dl fibrin gel showed fibrin degradation after 10 to 14 days of incubation in basal medium as for the 154 mg/dl fibrin gel showed no signs of degradation after 4 weeks of incubation in basal medium (Figure 2).

Higher fibrinogen concentration took longer time to degrade. Fibrin gel degradation is a natural process, which conclude restriction of cross-linking bonds and monomer release. In the body, fibrin gels or fibrin clots are broken down by plasmin, an enzyme produced by the liver, resulting in the release of fibrin degradation products (FDP) (Cesarman-Maus and Hajjar, 2005; Ho *et al*., 2006). *In vitro* cell culture fibrin degradation could be cause by unstable or low forming cross-linking bonds. Low

concentration of fibrinogen could form fewer cross-linking bonds resulting in a shorter time of degradation.

Fibrinogen concentration influence cell attachment

Cell attachment was measured by cell count after 1 and 3 hours of cell seeding into the fibrin gel with 154 mg/dl and 77 mg/dl fibrinogen concentration. Polysterene tissue culture dish (PS-TCD) was used as a control as mesenchymal stem cell would attached to plastic surface (Ogura *et al*., 2004). After 1 hour of seeding, 50%, 43%, and 37.5% cells were attached to PS-TCD, fibrin gel 154 mg/dl, and fibrin gel 77 mg/dl, respectively. Cells attached after 3 hours of incubation statistically, showed no significant difference in PS-TCD, 87.5%, and fibrin gel 154 mg/dl, 93%. As for fibrin gel 77 mg/dl showed much less cell attachment with only 50% cells were attached to the scaffold.

Mesenchymal stem cells adhered to plastic and to fibrin gels. Morphologically there are differences in the PS-TCD and fibrin gel cultures. In PS-TCD, the cells form a more flattened shaped and in the fibrin gels the cells forms a thinner spindle liked shaped. Cell attachment onto extracellular matrix (ECM) will form focal adhesions bridging the ECM and the cytoskeleton of the cell (Tam *et al.*, 2012). Focal adhesion is an integrated center for cell signaling, mechanosensing, and force transduction to mediate cell attachment, spreading, and motility in response to ECM (Fraley *et al*., 2010). They consist of multi-protein complexes (e.g. talin, vinculin, and paxillin), and transduce the mechanical response to dynamic remodeling of actin cytoseleton. Variation in composition and physical characteristic of the matrix could influence cell shape and function due to this mechanical feedback from the ECM to cell behavior (Tam *et al*., 2012; Trappmann *et al*., 2012).

Cell attachment assay showed fibrinogen concentration in fibrin gels could influence cell attachment. Fibrin possess a site-specific sequence, R-G-D (arginin-glycine-aspartate acid) which could be recognize by specific cell receptors called integrin (Lee *et al*., 2004; Ogura *et al*., 2004). This pairing of site specific motif and integrin will mediate cell attachment and the formation of focal adhesion (Frantz *et al*., 2010). Lower cell attachment could be cause by lower concentration of fibrinogen, limiting the availability of site-specific sequences, which are significant for cell attachment.

Cell proliferation did not give significant difference in PS-TCD and fibrin gel scaffolds

Cell proliferation was measured day 1, 3, 5, and 7 in a 24-well plate. Starting density was 5 $x10^3$ cell/cm^2. Proliferation characteristic in PS-TCD and fibrin gels gave no significant differences. All cultures showed similar patterns in cell proliferation. Day 1, there were a decrease of viable cells in all cultures. In fibrin gels viable cell number decrease until 80.4% and 43.16% in 154 mg/dl and 77 mg/dl fibrin gels, respectively. Huang and co-workers (2010), proposed that trypsin induce proteome alteration during cell subculture. Furthermore, they found out that 36 proteins were expressed differently on trypsinized cells, where proteins involve in cell metabolism, growth regulation, mitochondrial electron transportation, and cell adhesion are down-regulated and proteins involve in cell apoptosis are up-regulated. Cells that were transported using the trypsinize method would undergo apoptosis mechanism resulting in a decrease of viable cells.

An increase in cell proliferation was observed from day 3 until day 7 for PS-TCD and fibrin gel 154 mg/dl cultures. Fibrin gel 77 mg/dl cultures had increase in viable cell until day 5, and decreases on day 7 (Figure 3).

On day 7, cells cultured on PS-TCD showed more viable cells than fibrin gel cultures. Variation of the ECM properties such as topography, elasticity, and stiffness could influence cell behavior (Tam *et al*., 2012).

Evans and co-workers (2009) conclude that cell growth, proliferation and differentiation were all increased as a function on ECM stiffness. PS-TCD cultures have higher stiffness than fibrin gel cultures, giving it an advantage in cell growth and proliferation. However, there are more parameters that need to be considered for tissue cell culture.

Dimensionally, fibrin gels forms a 3-dimensional (3D) structure giving it dissimilarity characteristics than the 2-dimensional (2D) PS-TCD. Baker and Chen (2012) proposed that the difference in dimensional structure of 3D and 2D could effect on soluble gradients, polarity, matrix properties, adhesion molecules, and matrix stiffness which all then could influence on cell attachment, growth, proliferation, and differentiation.

Conclusion

STRO-1^{+}/CD45^{-} mesenchymal stem cell was isolated from the umbilical cord using the explant method. These fibroblast-shaped cells could attached to plastic and differentiate into osteoblast cells. Icoplas-fibrin gels could be fabricated using the aPTT kit with citrate plasma with fibrin concentration of 154mg/dl and 77 mg/dl. Cell attachment was influenced by fibrinogen concentration as for higher fibrinogen concentration (154 mg/dl) gave no significant difference then PS-TCD culture. ECM plays a role in cell proliferation, PS-TCD culture with higher stiffness showed higher proliferation although did not gave significant difference. These results showed that iCoplas-fibrin gels could be used as a fibrin based scaffold yielding no significant difference than PS-TCD cultures in cell attachment and cell proliferation on 154 mg/dl fibrinogen concentration. It is exciting to see rather or not iCoplas-fibrin gels share differentiation potential than PS-TCD. Further research on this matter could lead to the possibility of develop an autologous treatment for bone engineering.

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