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STUDY ON CREATING PERIPHERAL BLOOD DERIVED FIBRIN GEL AS SCAFFOLDS FOR HUMAN DENTAL PULP STEM CELLS

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ABSTRACT

In the recent years, regeneration of damaged pulp tissues has been considered a significant research direction and has attracted research attempt from many developed countries throughout the world. In Vietnam, researches in this field have limited access. Our study was conducted with the aim to evaluate the proliferation of human dental pulp stem cells (hDPSCs) in fibrin gel towards the application of hDPSC-fibrin gel complex in pulp tissue regeneration. Fibrin gel was prepared from the combination of human blood derived fibrinogen solution and 0.8 M $CaCl_2$ solution (with ratio 40:1). Porous architecture and fiber size were characterized by H&E staining and SEM, respectively. Degradation of fibrin gel in human plasma was determined by measuring the gel weight everyday for 7 days. Then, fibrin gel was placed onto the cultured human fibroblast surface to assess acute toxicity toward the cells. Human dental pulp stem cells were cultured in fibrin gel with the density of 10^4 cells/cm^3. Cell proliferation was investigated by MTT assay everyday for 11 days consecutively. Results showed that fibrin gel was created from plasma after 20 minutes with 100 % efficiency, pore size ranged 30-60 µm, and fiber diameter was about 0.5 µm. Fibrin gel was still remain in plasma for 7 days and did not cause acute cytotoxicity to human fibroblasts. Dental pulp stem cells grew well in fibrin gel until the 9^{th} day and reduced until the 11^{th} day, cell density on the 9^{th} day was approximately 12 times more than the first day. The obtained results showed that the fibrin gel can be used as the scaffold supporting dental pulp stem cells for applications in restorative dentistry.

Keywords: fibrin gel, scaffold, human dental pulp stem cell, restorative dentistry.

1. INTRODUCTION

Oral diseases, including tooth decay, periodontal pulp diseases, etc. are very common and increasing through years. Although Vietnam is classified into the group that have the highest rate of oral diseases, the communities pay less attention to this manner. Nowadays, root canal filling and implant are commonly used to treat a tooth which has damaged pulp. These two methods are successful in saving the tooth, however, the damaged pulp is not restored. Moreover, after a period of time, these methods can lead to some unintended consequences, for instance, tooth becoming darker in colour, jawbone atrophy, etc. [1].

Recently, the studies of hDPSCs have led to new opportunities for restoration of damaged pulp tissue. hDPSCs can be obtained from baby teeth, orthodontic tooth or wisdom teeth. These cells strongly proliferate, and are capable of differentiating into many dental cell types such as odontoblasts and ameloblasts [2, 3]. hDPSCs are seeded into an appropriate scaffold, then the scaffold/cells complex is transplanted into treated pulp. Presently, fibrin has been widely investigated as one of the autologous materials for transplantation. Fibrin is characterized as protein fibers involving in coagulation. In blood, fibrin exists as an inactive precursor fibrinogen, when coagulation factors present, fibrinogen are converted into fibrin which then form a fibrin network by polymerization, and seal blood clots. By mimicking this mechanism, autologous fibrin was prepared from peripheral blood of patient, and serve as a source material in various forms such as blocks, sheet, gel for further research in the biomedical application [4, 5].

2. MATERIALS AND METHODS

2.1. Materials

Human peripheral blood; human dental pulp stem cells; human fibroblasts; cell culture medium.

2.2. Fibrin gel preparations

The human peripheral blood was centrifuged at 3000 rpm for 10 minutes. After centrifugation, the top layer plasma was aspirated into a tube, and repeat the centrifugation steps once more time, the plasma was stored at 4 °C until using In order to prepare fibrin gel, plasma is poured into a shaped container, followed by the addition of 0.8 M $CaCl_2$ solution (Sigma) with the ratio of 40 : 1, and incubated at room temperature for 20 minutes.

2.3. Evaluation of fibrin gel structure and degradation

Fibrin gel structure was determined by H&E staining and scanning electron microscope (SEM).

Degradation of fibrin gel was evaluated by incubating the gel in autologous plasma. Plasma was harvested by centrifugation human blood at 3000 rpm for 10 minutes. Next, fibrin gel was incubated in plasma for 7 days. The residual weight of fibrin gel was determined everyday.

2.4. Evaluation of acute toxicity of fibrin gel

Acute toxicity of fibrin gel towards human fibroblasts was quantified according to ISO 10993. Fibroblasts were isolated from human skin, and cultured in DMEM/F12 supplemented with 10 % FBS (Fetal Bovine Serum) (Sigma). When the cells were approximately 80 % confluent, fibrin gel was placed on the cell surface. After 1 day, effect of fibrin gel to the cultured cells was determined.

2.5. Evaluation of proliferation of hDPSCs in fibrin gel

hDPSCs was obtained according to the previous protocol established by the same authors [6]. Teeth were collected from volunteers, and stored in PBS (Phosphate Buffered Saline)

(Gibco) containing Penicillin/Streptomycin (Sigma). A tooth was cut into 2 pieces in order to collect the pulp tissues. Pulp tissue was cut into small pieces of 1×1 mm^2, then cultured in DMEM/F12 supplemented with 10 % FBS for cell isolation. When the cells grown to 80 – 90 % confluence, cells were detached from the culture surface using Trypsin/EDTA (Sigma) and moved into the new roux. hDPSCs underwent serials of secondary expansion until Passage 4 (P4) generation (after 4 times of subculture) was obtained.

hDPSC at P4 were detached from the culture surface, resuspended in plasma at a density of 10^3 cells/ml, and aliquoted 0.5 ml each well in a four-well plate. Then, $CaCl_2$ solution was added in to plasma with the ratio 40:1 on order to form fibrin gel containing cells. Fibrin gel containing cells was cultured in DMEM/F12 containing 10 % FBS for 11 days.

Cell growth was evaluated by MTT assay from day 1 to day 15. Every two days, old medium was aspirated, followed by addition of fresh medium supplemented with 5 mg/ml MTT (Sigma) at a ratio 1:10 (v/v) to initiate the formation of formazan crystals. After 4 hours, supernatant in each well was discarded, DMSO (Sigma) was added to dissolve the crystals, followed by measuring OD at 450 nm wavelength.

3. RESULTS AND DISCUSSION

3.1. Fibrin gel preparations

Blood was centrifuged at 3000 rpm for 10 minutes. After 2 times of centrifugation, the obtained plasma is usually yellow, plasma volume was 40 % approximately to total blood volume (Figure 1).

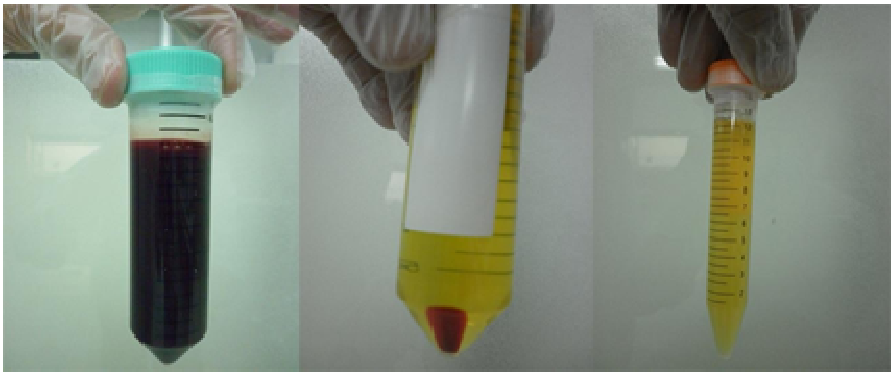

Figure 1. Plasma obtained from centrifugation.

Yellow colour of plasma added with $CaCl_2$ changed into opaque yellow. After 20 minutes, the fibrin gel was formed, with the shape of the container (Figure 2).

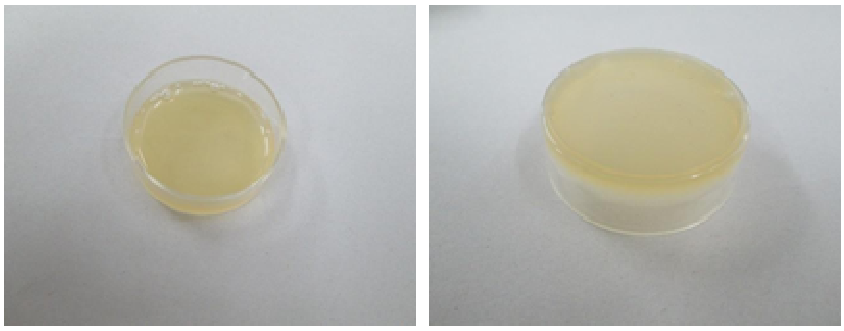

Figure 2. Fibrin gel was formed in round plastic plate.

3.2. Fibrin gel structure

Fibrin gel structure was determined by HE staining and SEM. HE staining showed that fibrin gel structure was homogeneously porous with 30 - 60 µm pore size (Figure 3A). SEM image showed that fibrin was a porous structure, formed by interlacing protein fibers. These protein fibers were approximately 0.5 µm in diameter (Figure 3B). This result was consistent with the result about the *in vivo* generation of fibrin fibers to prevent bleeding and some of published studies by other authors [7, 8, 9].

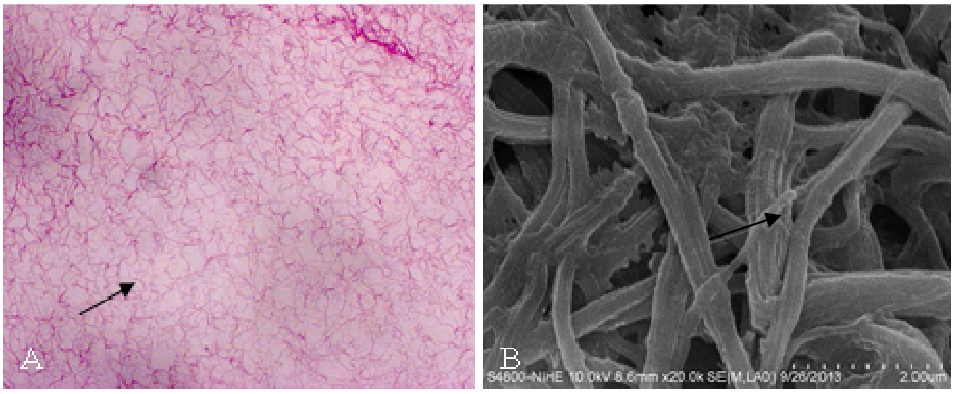

Figure 3. Fibrin gel structure. A: HE staining (×100), B: SEM image (×20000)
(Fibrin fibers were indicated by the arrow).

3.3. Fibrin gel biodegradation

In the living body, there are existing enzymes that can degrade fibrin. This enzyme system helps improve blood flow when the thrombosis is initiated, and provides favorable conditions for wound healing process after inflammation or swelling. The most important enzyme that can degrade fibrin is plasmin. Plasmin presents on human plasma and have ability to degrade fibrin into dissolve products.

In this experiment, autologous human plasma (collected from patients themselve for fibrin gel preparation) was used to evaluate fibrin gel degradation. Blood was centrifuged at 3000 rpm for 10 minutes to separate plasma containing autologous plasmin. Result of fibrin degradation in autologous human plasma showed that fibrin gel steadily degraded during 7 days of incubation in plasma. After 7 days, fibrin gel preserved 25% of the initial weight, average weight loss of

fibrin gel on each day was 10.49% compared with the first day (Figure 4). This result showed that fibrin gel was *in vitro* biodegradable in human plasma, and that it can probably remain longer than 7 days in the same condition as above.

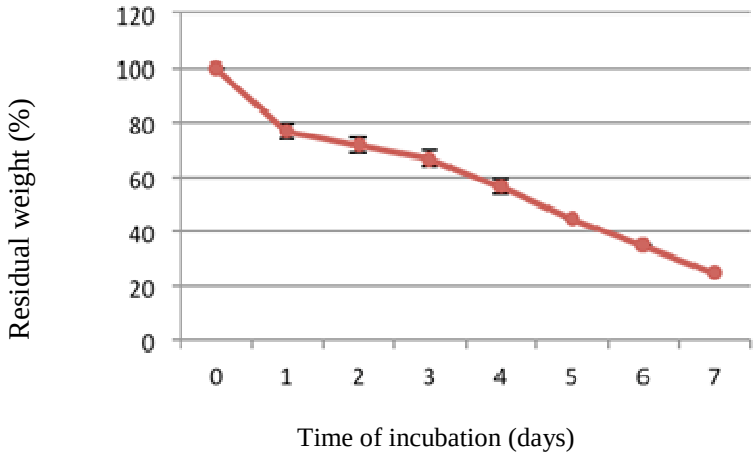

Figure 4. Diagram of fibrin degradation upon percentage of residual weight.

3.4. Acute toxicity of fibrin gel

Acute toxicity of fibrin gel was examined according to the effects of fibrin gel on human fibroblasts during a short time of exposure. Fibroblasts were cultured until a homologous layer (95%) was formed on culture surface of the plate (Figure 5). Fibrin gel was then placed on the cell layer. Acute toxicity of fibrin gel towards fibroblasts was evaluated after 1 day of incubation. Latex was used as a positive control.

After 1 day, when cultured in the present of Latex, fibroblasts were found to be deformed, the cells became round, formed clusters and completely detached from the culture surface (Figure 6A). This occurred due to the fact that Latex causes toxicity to cells. Latex is widely used as a positive control for toxicity studies. This result is consistent with above studies.

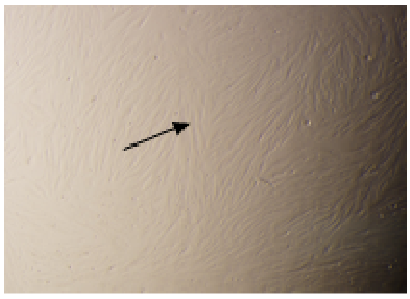

Figure 5. Morphology of fibroblasts (×100) as indicated by the arrow.

Regarding to the culture plate containing fibrin gel, there as no sign of cell death and detachment. Fibroblasts still maintained their growth after 1 day incubating in the present of fibrin gel. It was found that the cells proliferated well, and tended to be closer to each other (Figure 6B). This result showed that fibrin did not cause acute toxicity to fibroblasts.

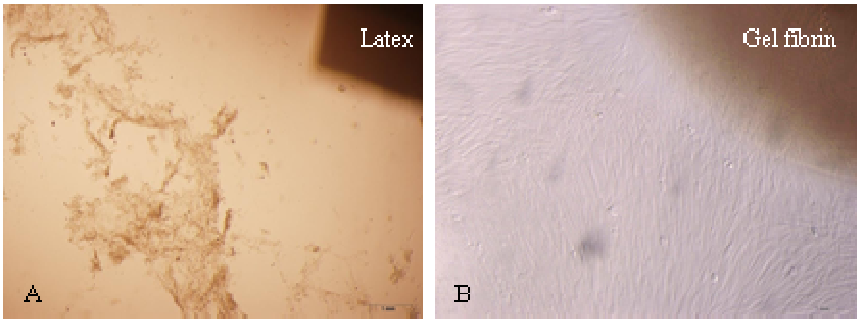

Figure 6. Fibroblasts were cultured in the present of different materials. A: latex, B: fibrin gel (x100).

3.5. Proliferaton of hDPSCs on fibrin gel

3.5.1. Observation of cells under optical microscope

hDPSCs were isolated and cultured as previous study published by the same authors [6]. Morphology of hDPSCs at P4 generation was characterised as spindle-shaped, 80 - 100 µm in length, and 15 - 20 µm in width, which was similar to the previous results (Figure 7).

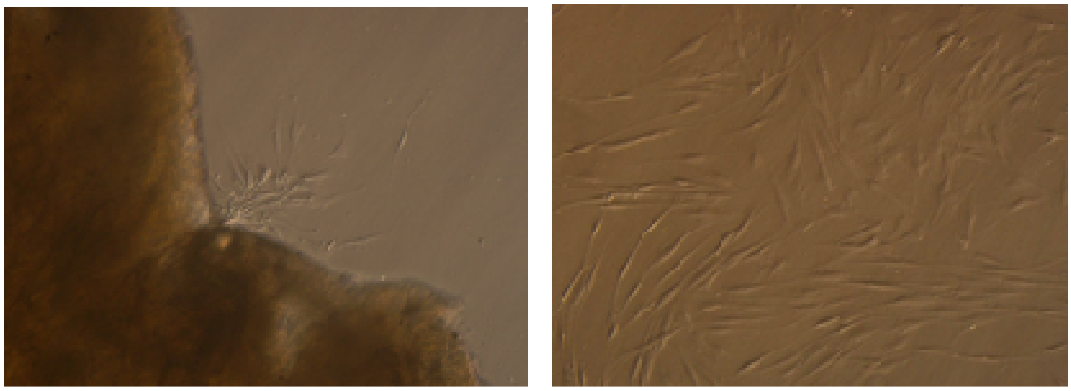

Figure 7. Human dental pulp stem cell. A: Primary culture cells (×100); B: Cells at P4 generation (×200).

hDPSCs were seeded in fibrin gel when fibrin gel was in liquid form. Accordingly, the cells were suspended in fibrin solution. After around 20 minutes, the gel form of fibrin was obtained, and captured the cells inside it.

After culturing for 1 day, cells inside fibrin gel were observed to be elongated shape with spreading branches. On the following days, cell growth was detected inside fibrin gel (Figure 8A). SEM image illustrated morphology of hDPSCs on the surface of fibrin gel. On the 11^{th} day, fibrin gel containing cells was found to disintegrate into small pieces. This degradation continued in the following days, however, fibrin gel did not completely degrade after 15 days, and degradation was not observed in the fibrin gel containing cells. This result showed that fibrin was a biodegradable material, and its degradation can be stimulated by the cells inside.

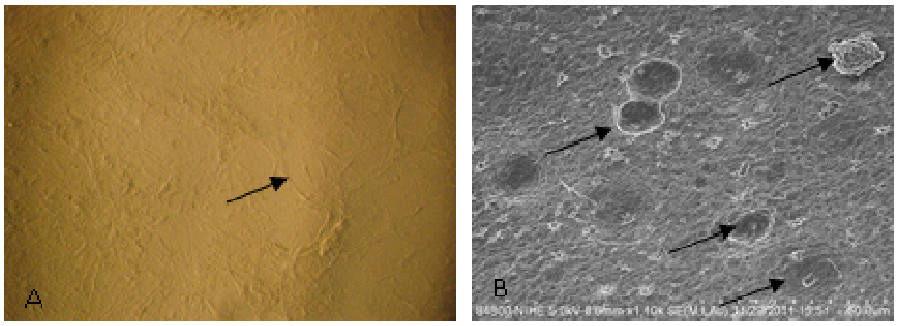

Figure 8. Morphology of hDPSCs when cultured in fibrin gel (×100).
A: optical microscope obsevation, B: SEM image.

3.5.2. Evaluation of cell proliferation in fibrin gel

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow substrate. In living cell, MTT is oxidized by mitochondrial enzyme, and formed formazan crystals. In dead cells, crystals are not formed. Formazan crystals are soluble in DMSO, and optical density (OD) of the final content can be measured at a wavelength of 595 nm. The more crystals are formed, the higher optical density is detected, which indicates there are more living cells.

After adding MTT solution into culture medium, purple crystals are formed inside the cells. After 4 hours, the crystals break cell membrane and have similar shape of cells. From the first day to the 11th day, the number of crystals increased in fibrin gel, but decreased from the 11th day to the 15th day. This result is shown in Figure 9 and Figure 10.

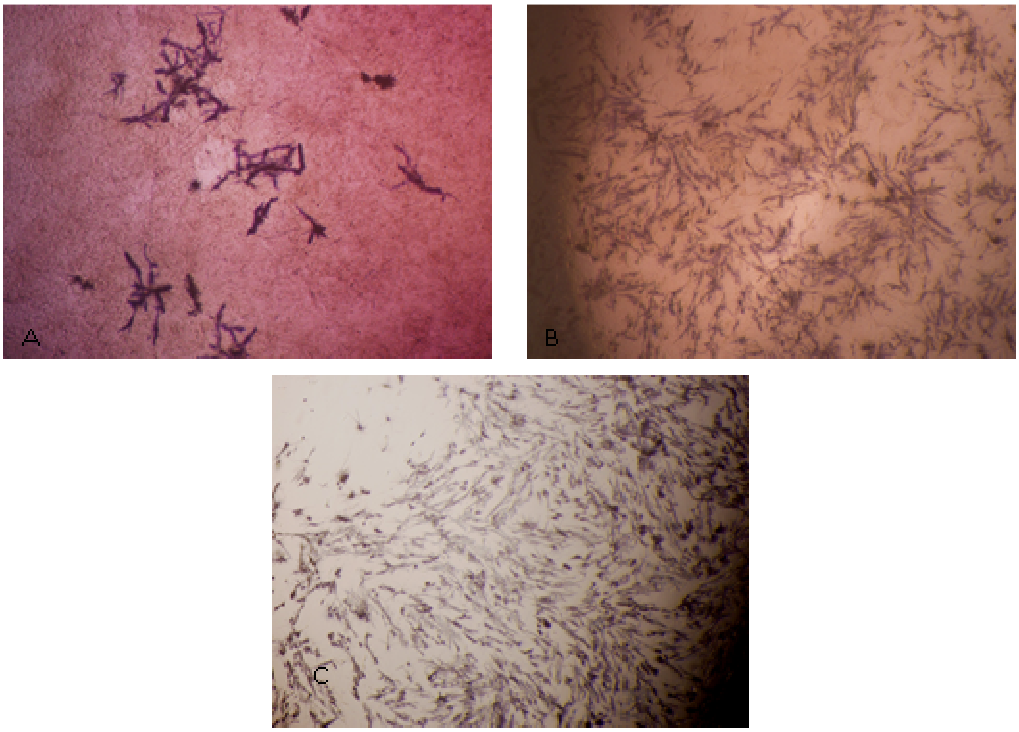

Figure 9. MTT crystals formed in the cell cultured in fibrin gel during days of investigation.
A: the first day, B: the 7th day, C: the 11th day (×100).

Result from the diagram (Figure 10) shows that:

OD of day 3 was higher than OD of day 1, however this was not a big change, because the cells needed a time to recovered after being treated with enzymes and centrifugation, therefore cell division was not signifcant, growth rate remained slow.

From day 3 to day 9, the number of cells increased by day (statistically significant difference), which proved that cells proliferated strongly during the time from day 3 to day 9. This period of time was determined as the log phase of cell population when cell division and metabolism process occurred the most powerful. Furthermore, three-dimensional structure of fibrin provided advantages of special aspect and appropriate self-supplied nutrients for rapid cell proliferation.

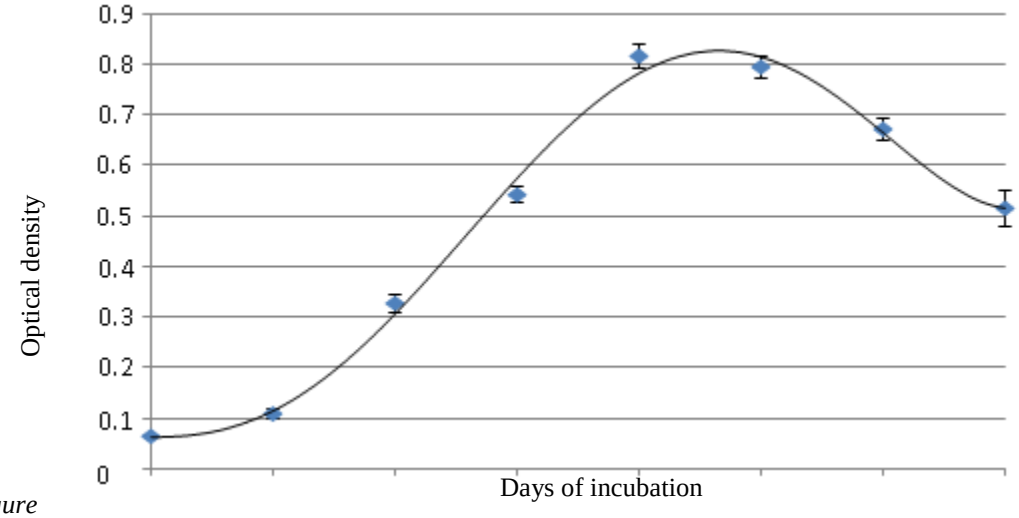

Figure 10. Diagram of Optical density (OD) conducted when culturing cells in fibrin gel.

From day 9 to day 11, proliferation threshold was determined, cell population entered the balance phase, cell division and metabolism rate started to decrease. OD values reduce from day 11, and a sharp drop of OD value was found on the 15th. The reason was that culture space no longer satisfied the outgrowth of cells, resulting in initiation of contact inhibition. This was the decline phase of cell population.

Fibrin gel was capable of maintaining and providing appropriate conditions for cell proliferation. hDPSCs was able to grow well in the fibrin scaffold, and peaked about on day 9, which was considered to be similar with the studies of Kolehmainen and Ahmed K [10, 11].

4. CONCLUSIONS

Fibrin gel can be prepared from a combination of autologous plasma and 0.8 $CaCl_2$ into various designed shapes, its pore size was 30-60 µm, diameter of fibrin fiber was determined as 0.5 µm. Fibrin gel did not cause acute toxicity towards human fibroblasts, and provided a good support for the proliferation of hDPSCs. Therefore, fibrin gel is potential to be applied in restorative dentistry.

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TÓM TẮT

NGHIÊN CỨU TẠO GEL FIBRIN TỪ MÁU NGOẠI VI LÀM KHUNG CHỨA TẾ BÀO GỐC TUỶ RĂNG NGƯỜI

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Nghiên cứu các phương pháp phục hồi tủy răng tổn thương là hướng nghiên cứu có ý nghĩa thực tế và đang được quan tâm mạnh mẽ tại các quốc gia phát triển trên thế giới trong

những năm gần đây. Tại Việt Nam, các nghiên cứu trong lĩnh vực này vẫn còn khiêm tốn. Nghiên cứu này được tiến hành nhằm đánh giá sự tăng trưởng của tế bào gốc tủy răng trên gel fibrin hướng tới việc ứng dụng phức hợp tế bào gốc tuỷ răng - gel fibrin trong phục hồi tủy răng. Dung dịch fibrinogen được thu nhận từ máu người hiến tặng, được kết hợp với $CaCl_2$ 0,8 M (tỉ lệ 40 : 1) để tạo thành gel fibrin. Gel fibrin được xác định cấu trúc lỗ bằng nhuộm HE, kích thước sợi bằng kính hiển vi điện tử quét, khả năng phân hủy trong huyết tương bằng cách đo khối lượng mỗi ngày trong 7 ngày. Sau đó, gel fibrin được đặt trong môi trường nuôi nguyên bào sợi để đánh giá độc tính cấp tính đối với tế bào. Tế bào gốc tủy răng được nuôi trong gel fibrin với mật độ 10^4 tế /cm^3. Sự phát tăng sinh tế bào được đánh giá bằng phương pháp MTT mỗi ngày và tiến hành liên tục trong 11 ngày. Kết quả cho thấy gel fibrin đã được tạo ra từ huyết tương sau 20 phút với hiệu quả 100 %, kích thước lỗ khoảng 30 - 60 µm, đường kính sợi 0,5 µm, gel fibrin có khả năng tồn tại trong huyết tương 7 ngày và không gây độc cấp tính cho tế bào. Tế bào gốc tủy răng tăng trưởng tốt trong gel fibrin cho tới ngày 9 và giảm dần cho tới ngày 11, mật độ tế bào ngày cao nhất cao gấp khoảng 12 lần so với mật độ ban đầu. Những kết quả thu nhận được cho thấy gel fibrin có thể được sử dụng làm khung chứa tế bào gốc tuỷ răng nhằm ứng dụng trong nha khoa phục hồi.

Từ khoá: gel fibrin, khung, tế bào gốc tủy răng người, nha khoa phục hồi.