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Developing a Multichannel Bioreactor with a Collagen Scaffold, ECM, and Cryoprecipitate to Significantly Produce Platelets from Umbilical Cord Blood Stem Cells

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ABSTRACT

Background: Platelets play a key role in the treatment of thrombocytopenia. Nowadays, platelets (PLTs) are only obtained through blood donation. However, due to the limitations in their preparation and storage, they are produced in laboratories, especially through bioreactors that convert megakaryocytes from stem cells into large-scale injectable PLTs.

Materials and Methods: In this study, the CD34 cells isolated from cord blood were differentiated into megakaryocytes. A 6-chamber bioreactor with a two-layer collagen scaffold, several ECM factors, and human cryoprecipitate were used to simulate the structure of the bone marrow. After the addition of megakaryocytes to the scaffold, PLTs were produced due to the flow pressure and the interaction between the scaffold structure and the ECM factors.

Results: CD41 + cells were expanded 100 times as much as CD34 + cord blood stem cells. The mean PLT release from one megakaryocyte in the pure collagen scaffold was 17.42 PLTs. Once fibrin, fibronectin, hyaluronic acid, and cryoprecipitates were added to collagen, the mean PLT release was 21.4, 22.4, 23.9, and 27.37, respectively. With the simultaneous addition of three factors to collagen (CFFH) and then four factors (CFFHC), the number of PLTs reached 30.52 and then 34.

Conclusion: Functional PLTs can be produced on a large scale with a multi-chamber bioreactor using a combination of ECM and cryoprecipitate with collagen scaffolding.

Keywords: Bioreactor; Scaffolds; Cryoprecipitate; Platelet; Umbilical cord blood stem cell

INTRODUCTION

Platelets (PLTs) emerge as the most important cells when damaged arteries begin to bleed. They are produced by Bone Marrow Stem Cells (BMSCs). These cells differentiate into megakaryoblasts, megakaryocytes, pro-PLT cells, and finally adult PLTs. The fragmentation of megakaryocytes, contacts with different physical factors such as the extracellular

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matrix (fibronectin, hyaluronic acid, fibrinogen, and Laminin) of bone marrow, Von Willebrand factor (VWF), cell-cell interactions with stromal cells, and vascular endothelial cells concert the production of PLTs. Eventually, adult platelets enter the peripheral blood[1–3]. PLTs primarily integrate into the arterial wall and participate in the first phase of coagulation during arterial damage. They live in blood for ten to fourteen days[4,5].

Thrombocytopenia is one of the major life-threatening challenges that can occur as a complication in conditions such as aplasia, leukemia, chemotherapy, radiotherapy, and immunological diseases. In these cases, PLT product transfusion is considered the only known solution to prescribe[6–8].

Apart from PLT consumption for patients with thrombocytopenia, the use of PLT-Rich Plasma (PRP) and PLT lysate in regenerative medicine and cell therapy as a treatment is currently expanding. Limited PLT sources, short life of PLTs, product storage challenges, and immunological reactions to PLT injections have led researchers to consider producing PLTs in the laboratory. Most conventional culture methods fail to produce enough PLTs, which is because PLT production needs sheer stress as well as physical interactions between cells and matrix materials[7,9–15]. Therefore, three-dimensional (3D) environments and biological scaffolds are essential for PLT production from bone marrow. Recently, different models of bioreactors have widely been used. Different scaffolds have been used in bioreactors to provide optimal conditions for cell surface receptors. Also, extracellular matrix interactions are used for maximum PLT release[4,7,14,16–18]. PLT production is performed *in vitro* from BMSCs, peripheral blood, Umbilical Cord Blood Stem Cells (UCBSCs) and Induced Pluripotent Stem (IPS) cells[14,19–21]. Easy preparation, low antigen diversity, and convenience are the key advantages of UCBSCs[3,20–22]. However, due to the low ploidy of UCBSC cell-derived megakaryocytes, PLT production from these cells is low, and the use of ploidy enhancers has adverse effects on PLT function[19,23].

In this study, to produce functional PLTs from UCBSCs, a multi-chamber bioreactor was utilized along with collagen scaffolds and multiple extracellular matrix agents including fibrin, fibronectin, hyaluronic acid, and, for the first time, human cryoprecipitate (which in rich in VWF, fibrinogen, and fibronectin) [24,25]. These materials are significantly effective in releasing PLTs from natural megakaryocytes in the body and are the most useful factors for evaluating PLT production in terms of quantity and quality [7]. Two-layer collagen scaffolds were integrated to a novel multi-chamber (6-chamber) bioreactor culture system, whereby one side of the chamber would allow the seeding of the cultured UCBSCs-derived MKs into the scaffold cross-flows and expose them to shear forces to induce platelet release into a parallel collecting flow. The scaffold was decoded to contain MKs within its porous structure while allowing the clothing selection of platelets by the outlet flow. It would take advantage of the discrepancy in size between the MKs and their platelet progeny. Thus, there is a combination of novel engineered scaffold structures, multiple extracellular matrix agents, human cryoprecipitate, and a multi-chamber bioreactor with a highly efficient UCBSCs culture protocol to bring functional in vitro platelet production closer to clinically relevant levels. In this study, for the first time, a multi-chamber bioreactor was used to increase platelet production in each stage. The effective combination of extracellular matrix factors and cryoprecipitate with a collagen scaffold, especially for UCBSCs-derived megakaryocytes, was used to increase the production of platelets. The produced PLTs were examined for the normality of the cell structure and their functional abilities.

MATERIALS AND METHODS

Multi-chamber bioreactor design

A multi-chamber (6-chamber) bioreactor was made with polycarbonate, which had low interaction with PLTs. The main compartment of the bioreactor was designed in the form of two complementary plates each of which had six chambers with a diameter of 5 mm. The chambers were connected through narrow channels (1 mm in diameter). These compartments were custom-made from polycarbonate to provide a flexible polymer surface with low platelet reactivity. Each plate had an inlet and an outlet. In the upper plate, the inlet and outlet were connected to a culture medium tank through a PVC pipe, and the

pipe was connected to a peristaltic pump to apply a flow. The inlet of the lower plate was connected to the tank of the culture medium, and its outlet was connected to the blood bag. The fixtures and fittings were made of medical-grade polypropylene and PVC tubing to reduce the exposure of cells to charged surfaces or cell reaction surfaces. The flow in the channels before and after the bioreactor was externally regulated away from the flow chamber by the addition of pinch valves and restrictions. Six two-layered collagen scaffolds of 5x5x3 mm were attached to a PVC holding frame in the bioreactor system (with 6x6 mm diaphragms on the upper and lower surfaces). Thus, the upper layer of collagen was the part with large pore size to seed megakaryocytes in this layer and the lower layer of the scaffold where very small pore size only allows PLTs to pass through. This frame was placed between two complementary plates in a way that the upper and lower chambers were connected through the collagen scaffold and secured by two screws. A 10-cm graft of PDMS tubing was placed before and after the chamber to provide a gas exchange. This bioreactor design creates a stable parallel flow and, as a result, the shear stress on the surfaces of the collagen scaffold.

Collagen scaffolds fabrication

A two-step freezing method was used to obtain the porous bilayer scaffold. Approximately, 3 ml of a %1 collagen suspension was filtered (0.41 um Wattman filter), poured into a 24-well plate, and frozen at -20°C for an hour. Then, for partial melting, the plate was incubated at Room Temperature (RT) for 5 minutes. Afterwards, the second collagen layer was poured up to 3-mm thickness above the first layer and frozen at -40°C for an hour. Then, the plate was freeze-dried for 24 hr[26]. Collagen crosslinking was done by EDC/NHS for an hour, and the scaffold was washed three times with Distilled Water (DW) for 15 minutes[14,27]. Finally, the plate was freeze-dried for 24 hour and stored at -20°C until testing. SEM studies were performed based on three different layers including a top layer, a bottom layer as well as top and bottom surfaces.

To set up the bioreactor and evaluate its performance, after the six scaffolds of collagen were installed, a suspension of a normal donor's PLTs and the peripheral blood mononuclear cells (PBMC) of acute monoblastic and acute monocytic leukemia (AML M5) patients was diluted with the culture medium solution and entered the main path of the bioreactor. The blasts were of approximately the same size as the culture-derived megakaryocytes (up to 30µ). They were available and had good resistance to physical contacts with the scaffold. Generally, they are very homogeneous in size and can be isolated in large numbers in peripheral blood[7,19,28]. After half an hour of bioreactor activity, the upper path fluid deposition was evaluated and the culture medium solution was collected from the lower pathway. In general, the cells that were rejected from the scaffold were only PLTs, and the upstream sediment was a mixture of mononuclear cells and PLTs.

$CD34^+$ cells separation and megakaryocytic differentiation

Human Umbilical Cord Blood (UCB) specimens were collected from normal deliveries into sterile tubes containing heparin. The samples were collected under written informed consent and in agreement with the Declaration of Helsinki. This study was approved by the Committee of Ethics of Tabriz University of Medical Sciences (TUOMS) (code of ethics: IR.TBZMED.REC. 2018.610). From each UCB sample, 15-20 ml was diluted 1:2 in a Phosphate Buffer Saline (PBS) supplemented with 5% Fetal Bovine Serum (FBS [Gibco]). For Mononuclear Cells (MNCs) isolation, the samples were slowly poured on 15 ml of a Ficoll solution (P = 1.077 g/ml; Sigma, St. Louis, MO), and then the tubes were centrifuged at 850 g (acceleration 4, without brake) at 4°C for 25 minutes. Subsequently, the MNC layer (buffy layer) was collected and washed twice with PBS, and the viable cells were counted by trypan blue staining on a hemocytometer. Approximately, $10x10^6$ MNCs were collected from 50-80 ml of the UCB samples. The positive selection Magnetic-Activated Cell Sorting (MACS) system was applied to enrich $CD34^+$ cells according to the manufacturer's instructions (Mini MACS, Miltenyi Biotech, Auburn, CA). At this step, $1x10^4$/ml UCB $CD34^+$ cells were cultured in a complete Gibco RPMI-1640 medium in 24-well

culture plates, along with 50 ng/ml thrombopoietin (TPO) (Bio-Techne) and 5 ng/ml Stem Cell Factor (SCF) (Gibco). The complete RPMI with 50 ng/ml TPO was re-added on the eighth day. The concentration of the differentiated cells was raised to $1x10^6$ cells and then cultivated for 3-4 days[23]. The morphological evaluation of the differentiated megakaryocytes on day 11 and the pro-platelets on day 13 of culturing as well as the bioreactor platelets was performed using the Giemsa staining method (Figures 3D and 5A).

Induction and production of PLTs in a bioreactor

The designed bioreactor consists of six chambers each containing a piece of a two-layer collagen scaffold, 5 mm in diameter, 3 mm in thickness, and cut by a specific cutter. Each chamber is separated by a gasket to prepare a channel for a medium flow. The upper chamber is connected to the medium reservoir, and the CO2 pump and the lower pump are connected to a blood bag containing Citrate Phosphate Dextrose Adenine (CPDA-1). The device contains a bag for harvesting the released PLTs through propylene pipes (Figure 3). Before starting, the bioreactor chambers were thoroughly washed three times with 5 ml PBS, then washed with RPMI supplemented with 1% BSA for 30 minutes, and finally three times with RPMI. About $1x10^4$ $CD41^+$ cells were seeded on the upper layer of each scaffold, and 5 ml RPMI was slowly added to each chamber. Thereafter, the chamber screw was closed, and the bioreactor started working for one hour at 37°C and the flow rate of 30-70 µl/sec with 5% CO2.[14] Thrombopoiesis under the shear stress in the bioreactor took place in a short period, and there was no need to cultivate for many days. In previous studies, the activity of bioreactors took 20 minutes to two hours. In this study, however, a good result was obtained in one hour [14,29–31]. The final harvested PLTs were counted either with a hemocytometer or an automatic cell-counting system (Sysmex kx21). To evaluate the effect of the extracellular matrix and cryoprecipitate on the PLT production rate, 10 µl of 2% fibronectin (25 µg/ml), 2% hyaluronic acid, and human cryoprecipitate were used separately in the bioreactor and gently injected into the collagen scaffold. To evaluate the effect of fibrin, 10 µl of 20% human citrated plasma was added to the scaffold, and 5 µl of tissue thromboplastin was added and incubated at 37°C for 10 minutes to form fibrin filaments in the collagen cavity. Afterward, the excess serum was removed from the scaffold.

Evaluating the differentiation of megakaryocytes and platelets

To evaluate the megakaryocytic differentiation, 1×10^5 cells were washed with PBS and incubated with 10 ul of FITC conjugated anti-CD41. After an hour, they were washed with PBS and re-suspended in 500 ul of 5% PBS containing BSA. The differentiation of megakaryocytes and PLTs was evaluated by the BD FACS Caliber flow cytometry system (BD, USA). For the harvested PLTs, 400 ul of CPDA-1 blood bag suspension was incubated with 10 ul of anti-CD41-FITC and 10 ul of anti-CD42-PE antibodies (Becton Dickinson). Then, it was washed and re-suspended in a 5% BSA-PBS solution and evaluated as previously described[10,32]. Giemsa staining (Sigma-Aldrich) was also done to investigate the morphology of megakaryocytes, proPLTs, and PLTs. The cell smear was stained with diluted Giemsa stain (1:20, vol/vol) for 20 minutes (Figures 5A and 5D).

Evaluations of the function of the bioreactor-derived platelets

Two types of tests had to be used to check the function of the produced platelets. The first was the platelet aggregation test conducted with an aggregometer, and the second was the immunofluorescence test of the interaction between the bioreactor-derived platelets and normal donor platelets. The aggregation profiling of the PLTs so as to present the PLTs activation materials such as ADP, Arachidonic acid, Ristocetin, and collagen was done through aggregometry (chronology model 700 x, Havertown, Pennsylvania, PA) based on the standard methods[10,33,34] (Figure 5C). The second test to investigate the interaction of bioreactor PLTs in aggregation with natural PLTs was the immunofluorescence test. Normal donor PLTs were stained with DIOC6 (green), and bioreactor PLTS were stained with Cell Tracker™ (red). Then, they were stimulated by passing through a high-pressure

collagen-coated microtubule. Correspondingly, it was examined with an immunofluorescence microscope and photographed with a camera[14,17,35](Figure 5E).

Statistical analysis

The normality of the collected data was investigated using the Shapiro-Wilk test. Statistical significance was determined using either analysis of variance or Tukey post-hoc test. Also, $p < 0.05$ was decided to be the significance level. The analyses were performed with the SPSS software version 24.

RESULTS

Characterization of the collagen scaffold and application of the multi-chamber bioreactor

A two-stage freezing process resulted in a collagen scaffold with different pore sizes between the top and the bottom parts. In terms of both mean pore size and porosity percentage, the top part had a large pore structure (up to 50 mm in diameter), while the pore size in the bottom part was much smaller. At the upper and lower levels, the pore sizes were completely different (Figure 1).

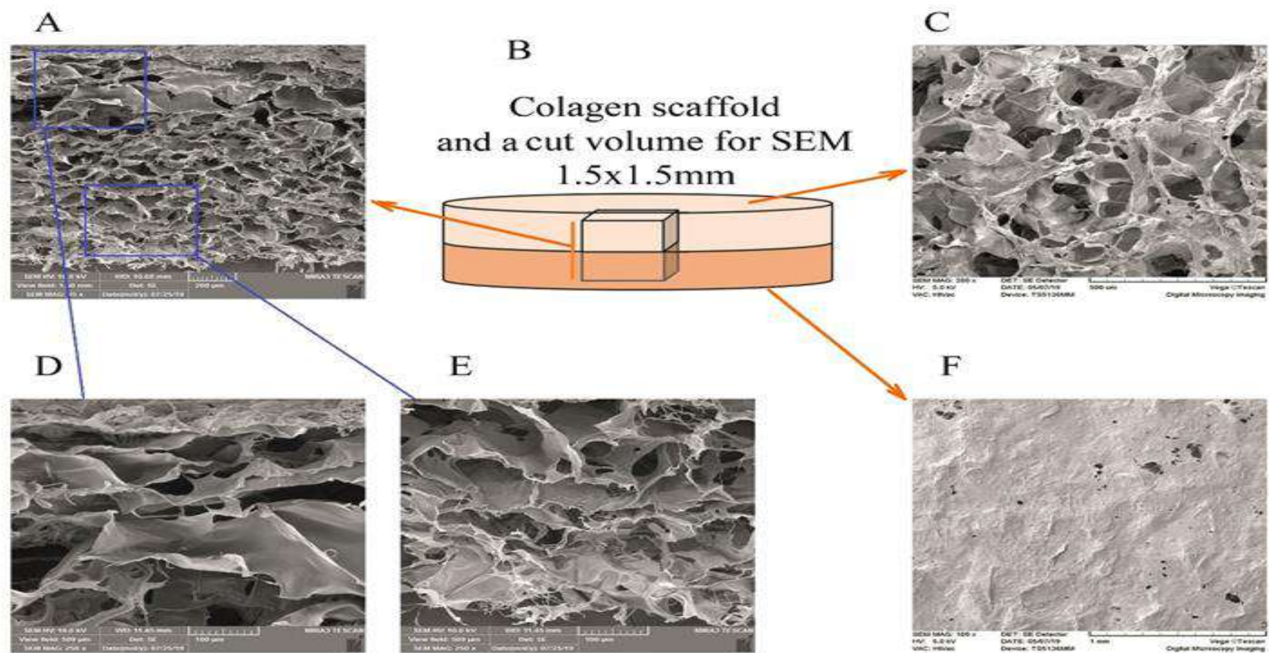

Figure 1. Electron microscopy of two layers of collagen scaffold: **(A)** Scanning both upper and lower phases of the scaffold, **(B)** The two–layer model and the section selected for scanning, **(C)** SEM photo from the top of the scaffold where there are very large pores for megakaryocytes to pass, **(D)** SEM photo of the upper side of the scaffold where the large pores for megakaryocytes are located, **€** SEM photo of the lower side of the scaffold where tiny pores block the passage of megakaryocytes, **(F)** SEM photo from the bottom of the scaffold where very small cavities only allow PLTs to pass through

Megakaryocytes differentiated and expanded efficiently

The purity of the enriched $CD34^+$ cells after a single MACS column pass was evaluated by flow cytometry and found to be 71.2 ± 8%. On average, during 11 days, 18.6 ± $2.5x10^6$/ml cells expanded from the initial cell concentration of $1.5x10^5$. The expression of $CD41^+$ in the expanded cells was about 64.4 ± 5%. Accordingly, the cell expansion increased 80 times in the presence of SCF and TPO (Figure 3C).

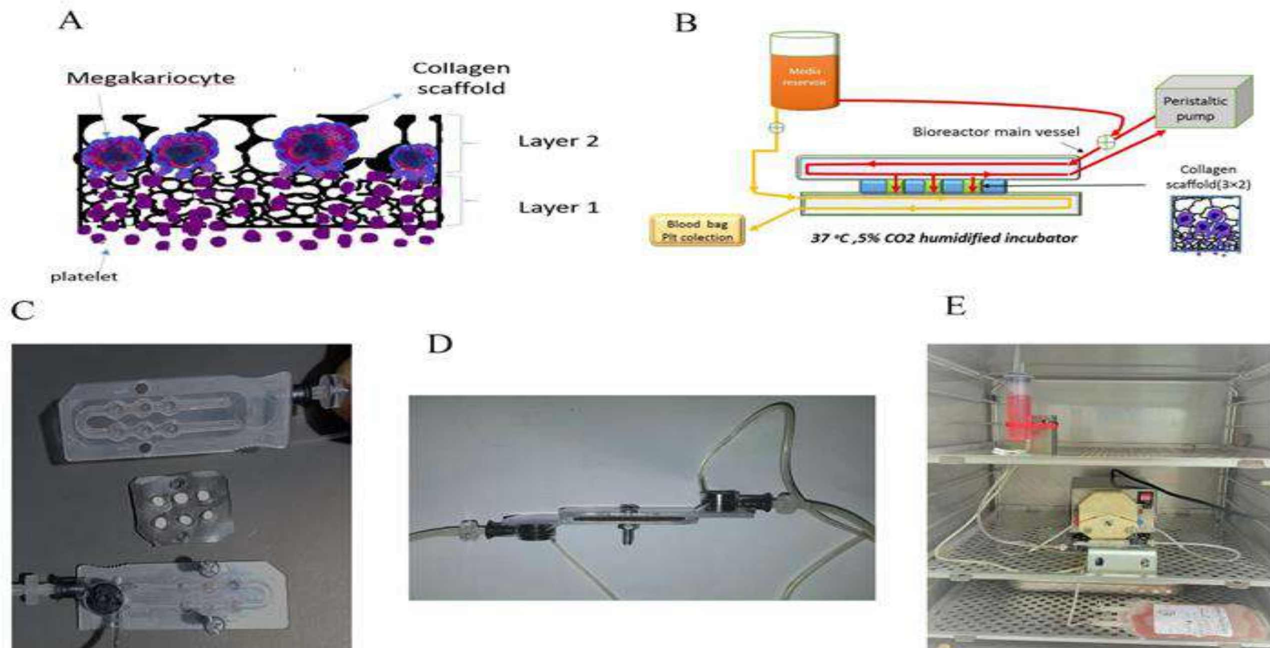

Figure 2. The designed bioreactor has six chambers containing a scaffold and a system for transferring the culture medium into the scaffold and the PLT collection path. **(A)** Schematic diagram of how PLTs are produced from megakaryocytes in two scaffold layers, **(B)** Schematics of the bioreactor activity, **(C)** Two plates containing the culture medium transfer duct and six open chambers connected to the scaffolds (the top one is connected to the culture medium tank and the pump, and the bottom one is connected to the culture medium and the platelet collection bag) which are placed in elastic washers, **(D)** How to connect two plates with two screws after mounting the collagen, **(E)** The complete bioreactor system for PLT collection inside an incubator

The role of the bilayer collagen scaffold in simulating a bone marrow microvasculature and allowing the platelet flow

A bone marrow microenvironment-like scaffold produced from two layers of collagen was used to produce PLTs from megakaryocytes. The upper layer with an open structure of about 50 µm served to preserve megakaryocytes, and the lower layer with a smaller size (10 to 20 µm) was used for PLT transfer and separation from megakaryocytes. The harvesting rate was also enhanced with 6-chamber plates, where the upper and lower parts were separated with a PVC filter. About $1x10^4$ cells were seeded in each chamber at the top layer of the scaffold, and the bioreactor ran at a 30-70 ml/min rate for one hour. The flow rate through the outlet pipe was set at 0.1 ml/min. In no-flow conditions, the flow of the medium was spontaneous and no PLT particle isolation was observed. The produced PLTs were collected into a gas-permeable collection bag containing acid citrate dextrose (ACD) as an anticoagulant (Figure 3). The stained PLTs with anti-CD41 and anti-CD42b antibodies had physical parameters similar to those of human peripheral blood PLTs. The mean number of the collected $CD41^+$ and $CD42^+$ platelets was $1.045 \pm 0.0735 \times 10^6$ in each 3D tissue perfusion system (Figure 4B).

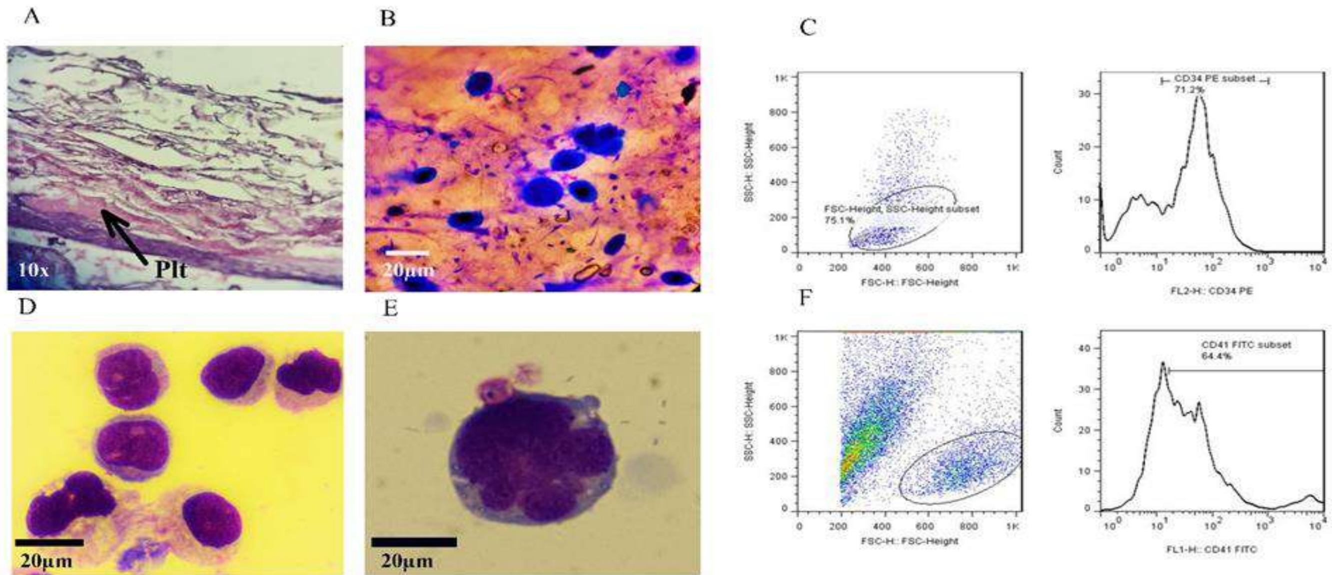

Figure 3. UCBSC-derived megakaryosts and how megakaryocytes and PLTs are located in the scaffold and flow cytometric response: **(A)** Accumulation of PLTs (red) in the lower layer of the scaffold in pathology sections and hematoxylin staining (magnification of 10), **(B)** Megakaryocytes trapping in the upper layer of Giemsa staining scaffold (scale bar = 10 μm), **(C)** Flow cytometric analysis of stem cells isolated from cord blood, **(D)** Morphology of megakaryocytes produced on day 11 of culture (scale bar = 20 μm), **(E)** Morphology of mature polyploid megakaryocytes produced on day 13 of culture (scale bar = 20 μm), **(F)** Flow cytometric analysis of UCBSC-derived megakaryosts

Increase of pro-PLT formation and PLT release by cryoprecipitate and ECM

The use of ECM and cryoprecipitate increased the mean number of the collected PLT. By the addition of fibrin, fibronectin, and hyaluronic acid to the scaffold collagen, $1.286 \pm 0.0587 \times 10^6$, $1.346 \pm 0.0756 \times 10^6$, $1.428 \pm 0.1010 \times 10^6$ PLTs were produced, respectively. Moreover, as the cryoprecipitate was added to collagen, $1.641 \pm 0.1152 \times 10^6$ PLTs were released. The combination of fibrin, fibronectin and hyaluronic acid (CFFH) with the collagen scaffold resulted in $1.83 \pm 0.0896 \times 10^6$ PLTs, and the combination of fibronectin, fibrin, hyaluronic acid and cryoprecipitate (CFFHC) with the collagen scaffold produced $2.04 \pm 0.0894 \times 10^6$ platelets. The number of PLTs released from each megakaryocyte in the pure collagen scaffold was about 17.42. After the addition of fibrin, fibronectin, hyaluronic acid, and cryoprecipitate to collagen 21.4, 22.4, 23.9, and 27.37 PLTs were produced, respectively. The collagen produced about 30.52 PLTs in the presence of CFFH and 34 in the presence of CFFHC.

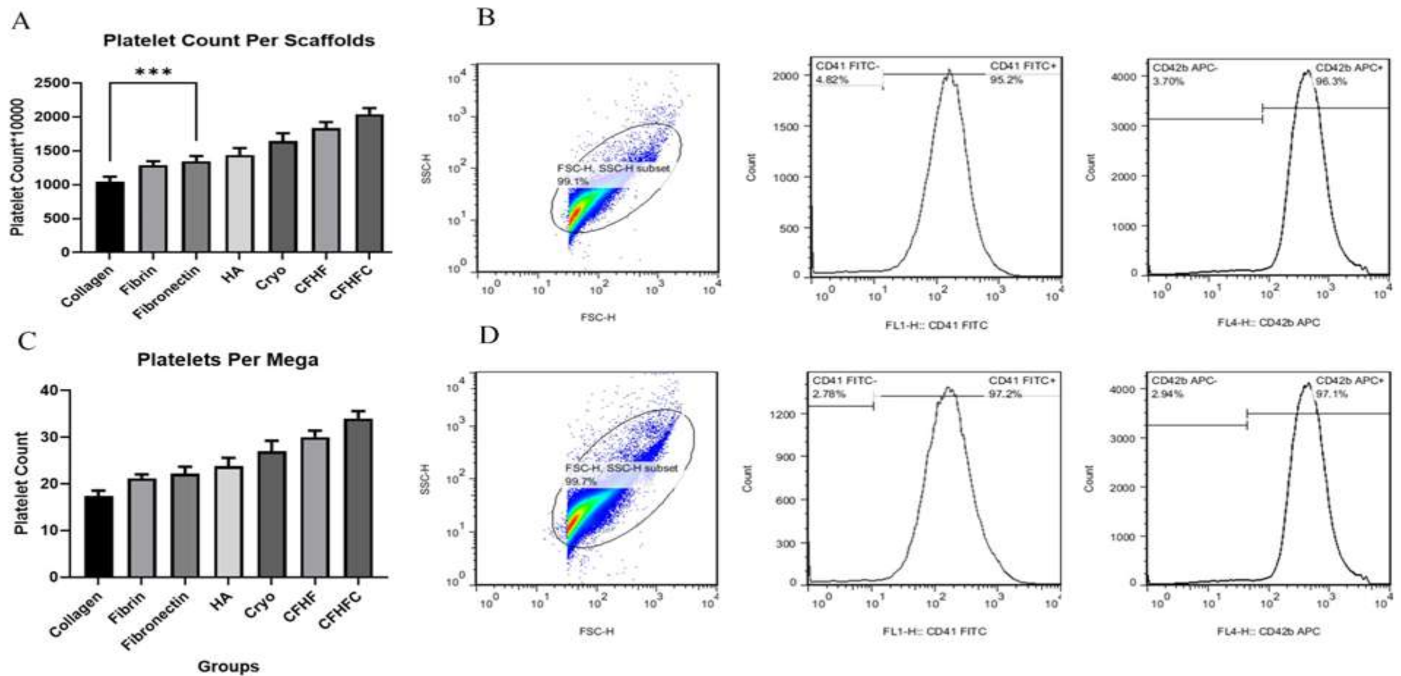

Figure 4. PLTs produced by the bioreactor under different combination conditions and the flow cytometric results for the PLTs obtained from the bioreactor: **(A)** Diagram of PLT production (×1000) from 6×10^6 megakaryocytes in different ECM biomaterials used together with scaffold collagen in a bioreactor: The analysis of variance showed that the mean number of PLTs (in 1000) was different in EC factor groups ($p - value$ <0.001). The results of the Tukey post-hoc test showed that the mean numbers of PLTs (per 1000) in different groups of factors were significantly different (p < 0. 0.001). The greatest difference was between the means in collagen-CFHCF groups (994.8), and the least mean difference was between Cryo-CFHF groups (192.20). **(B)** Flow cytometric results of anti-CD41 and anti-CD42 markers for the PLTs produced in the bioreactor, **(C)** Diagram of the platelets produced from one megakaryocyte: The analysis of variance showed a statistically significant difference between the mean numbers of PLTs (in one-per-meg) in different factor groups (p < 0.001). The results of the Tukey post-hoc test showed that the mean numbers of PLTs (one-per-meg) in different groups of factors were significantly different (p < 0. 0.001). The greatest mean difference was in collagen-CFHCF groups (16.58), and the least difference was between the means in Cryo-CFHF groups (3.15). (**D**) Flow cytometric results of anti-CD41 and anti-CD42 markers for the positive control of normal donor PLTs

Functionality of exvivo-produced platelets and their contribution to clot formation

The expression of CD41 and CD42 surface markers of the produced PLTs was evaluated by flow cytometry. It was found to be 95.2% and 96.3%, respectively. Based on Giemsa staining, the morphology (size, structure, and granularity) of the produced PLTs was normal (Figure 5A). The PLTs function was evaluated by aggregometry, and the stimulation by ADP (21%), Arachidonic Acid (11%), collagen (25%) and Restocetin (19%) was reported with the positive platelet response to the platelet activators (Figure 5C). To investigate the interaction of the bioreactor-derived platelets and the normal donor platelets, the bioreactor platelets were stained with Cell Tracker™ Red CMTPX and added to whole blood containing PLTs pre-stained with DiOC6 (green). The PLTs were suspended into Tyrode's buffer containing a tissue factor, calcium ionophore, and VWF. They were also perfused over immobilized type I collagen at a shear rate of 1000 s–1 for PLT aggregation. The results showed that the bioreactor PLTs participated in clot formation. Also, the result of immunofluorescence microscopy revealed that bioreactor and normal PLTs are involved in clot formation (Figure 5E).

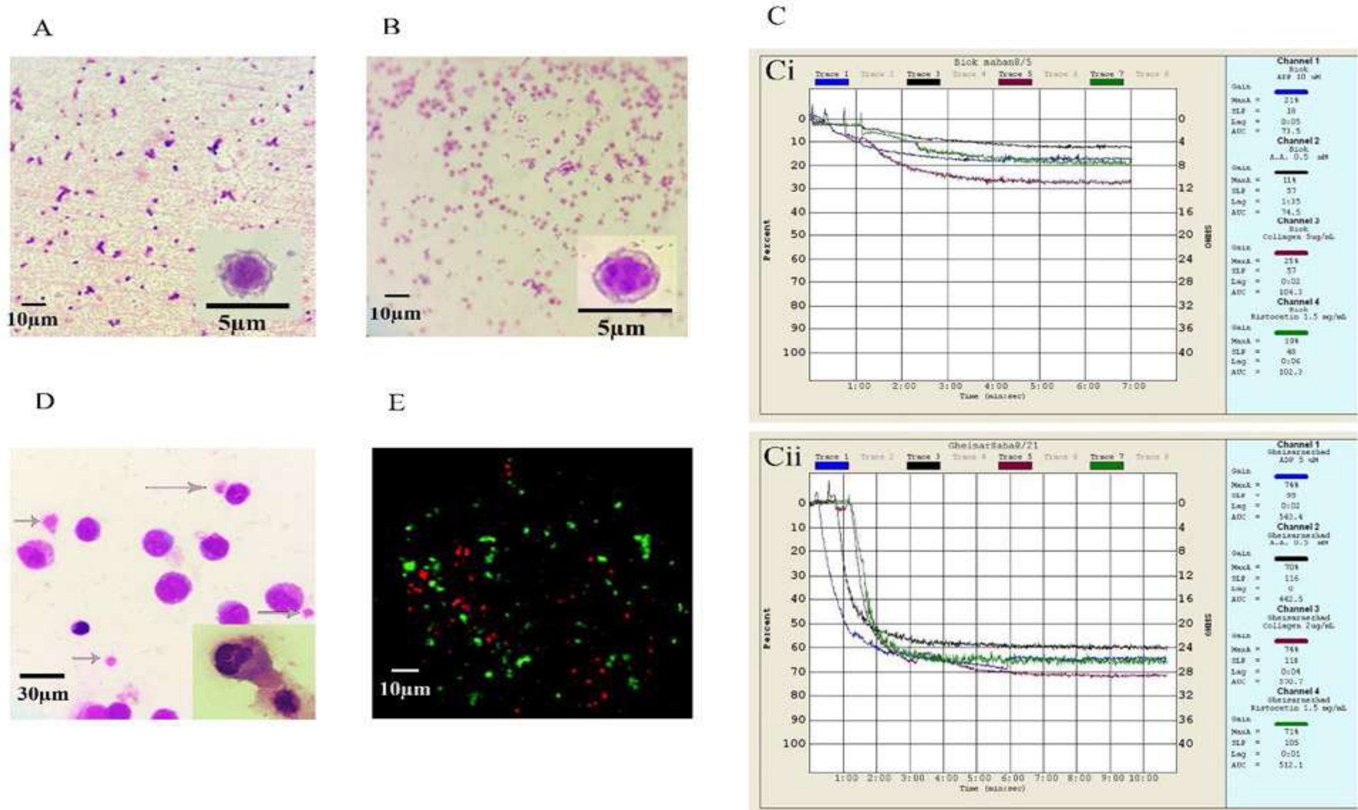

Figure 5. The morphology of PLTs and pro-PLTs produced by bioreactor and the PLT activity: **(A)** Bioreactor PLTs (Giemsa staining) (scale bar = 10 µm), **(B)** Normal donor PLTs (Giemsa staining) (scale bar = 10 µm), **(C)** Platelet aggregation In an Impedance Aggregometry test measuring the ability of various platelet agonists (ADP, Arachidonic Acid, collagen, and Restocetin) to induce in vitro activation and platelet-to-platelet activation, **(Ci)** The results yielded by the aggregometer about bioreactor PLTs aggregation in response to platelet activators, **(Cii)** The results yielded by the aggregometer about PLT aggregation from normal donors in response to PLT activators, **(D)** Morphology of pro-PLTs produced on day 13 of culture (scale bar = 30 µm), **(E)** The involvement of PLTs from the bioreactor (green) in the thrombus formation together with normal donor PLTs (red) examination by immunofluorescence microscopy (scale bar = 10 µm).

DISCUSSION

In the present study, an attempt was made to increase PLT production in the laboratory up to an injectable level. This was motivated by the daily need of patients for a large quantity of PLTs. Three-dimensional environments, biological scaffolds and extracellular matrix agents are essential for PLT production from bone marrow. Also, BM modeling is essential for PLT production from megakaryocytes.

The present study sought to use bone marrow-like materials through certain mechanisms to produce PLTs. This was done by the use of a bilayer structure of collagen and different extracellular matrix factors such as fibrin, fibronectin, hyaluronic acid, and human cryoprecipitate, which is abundant in VWF, fibrinogen, and fibronectin. The circulatory system in the bone marrow vessels was simulated by means of a multi-channel bioreactor with shear stress that helped to facilitate platelet separation. UCBSCs were utilized due to their ease of access and low level of immunization. However, the low ploidy of megakaryocytes from the UCBSCs limited their use. Since PLT production is proportional to the ploidy of megakaryocytes, UCBSCs are not efficient enough in PLT production when compared to bone marrow hematopoietic stem cells (BM-HSCs).[23]

Different research teams have simulated bone marrow structures with different scaffolds and ECM to produce PLTs with a bioreactor. Pallotta et al. (2011)[36] designed a silk-like structure using silk, through which megakaryocytes produced pro-PLTs. This model served to increase the number of PLTs to 10 per megakaryocyte. As an important point, the researchers suggested that PLTs formed in 3D systems would perform better than those generated in 2D settings, but the activating nature of the silk would limit the quality of the product. Avanzi et al. (2016)[23] used differentiated umbilical cord blood megakaryocytes that increased ploidy with RHO kinase and latrunculin. They also used a bioreactor designed with nanofiber membranes sandwiched between two flow chambers. They enhanced PLT production by increasing ploidy, but RHO kinase and letroncholine hurt PLT performance due to impaired actin polymerization. Buduo et al. (2015) [17] tried to produce PLTs in a bioreactor through silk scaffolding and using collagen, fibrinogen, fibronectin, and laminin. They achieved good results, but the activating nature of silk limited the quality of the product. In the study by Shepherd et al. (2018)[14], PLTs were produced in collagen scaffolds from IPSs, without any other ECM material, but the potential dangers of these cells restricted their application in clinical trials. Currao et al. (2016)[18] created a similar BM function with Hyaloranan and showed a significant increase in PLT count, but the use of hyaluronan without other structures in the bone marrow matrix limited its efficiency in thrombosis. Saville et al. (2022)[37] used a mathematical model to design the bioreactor and changed the length and diameter of connections and stress flow to produce platelets through the collagen scaffold. Although this study is helpful in the design of new bioreactors, these findings are in the use of other factors. Like extracellular matrix factors, it can have different results.[37]

In this investigation, it was found that UCBSCs possess many merits including low immunogenicity, availability, and ease of preparation. The Ploidy depletion of megakaryocytes can also be compensated with an increase in the number of bioreactor channels and the use of stimulant factors; ploidy enhancers such as actin polymerization inhibitors and latrunculin and Rho-kinase inhibitors are shown to have adverse effects on megakaryocytes and PLTs[38]. Moreover, ECM materials such as fibronectin, fibrin, hyaluronic acid, and human cryoprecipitate were used. It is the first study in which cryoprecipitate was used to produce PLTs in bioreactors. Cryoprecipitate contains fibrinogen, VWF and fibronectin, which can simulate the bone marrow structure and stimulate megakaryocytes to increase PLT production[24,25]. The VWF factor binds to megakaryocytes via the Glycoprotein Ib GPIb receptor and serves as a key factor to stop megakaryocytes and release PLTs in the blood circulation[39]. The interaction of primary megakaryocytes with fibrinogen, VWF, hyaluronic acid and fibronectin stimulates pro-PLT formation[39-41].

The significant number of the PLTs produced with these factors, compared to collagen and other factors, makes the proposed system similar to the PLT production in the bone marrow. Indeed, it provides PLTs with whatever factor or property required. Using more MK cells, increasing the number of chambers in bioreactors, and using extracellular matrix agents and cryoprecipitate may help to increase the number of PLTs to an injectable level.

CONCLUSION

In this study, a porous collagen scaffold with a two-layer structure was developed. The top layer with large cavities holds megakaryocytes, and the bottom layer with small cavities produces PLTs from megakaryocytes as well as ECM and human cryoprecipitate, which abundant contain the factors influencing thrombopoiesis for PLT production. Moreover, a bioreactor with several chambers was designed to produce large quantities of PLTs without any ploidy manipulation of megakaryocytes. This resulted in both functionally and structurally active platelets. If these scaffold, extracellular matrix and cryogenic agents are used in large bioreactors with hundreds of chambers, PLT production may increase to a reasonable amount for injection. To increase PLT production, it is recommended for future studies to use cord blood stem cells with endothelial cells and adhesion molecules like VEGF and VCAM-1.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

Data availability statement

The dataset created and/or analyzed during the current study will be provided if requested from the corresponding author.

Funding statement

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Ethical approval statement

This research project with the ethical code of IR.TBZMED.REC.1397.610 was approved in Tabriz University of Medical Sciences and conducted in terms of the ethical principles of research.

Patient consent statement

In this study, human umbilical cord (UCB) blood samples were collected from women with normal deliveries. The blood collection was done with written informed consent and in agreement with the Helsinki Declaration. The study was approved by the Ethics Committee of Tabriz University of Medical Sciences (TUOMS) (code of ethics: IR.TBZMED.REC.2018.610).

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