

# Establishment of A Three-Dimensional Culture Condition for The U266 Cell Line Based on Peripheral Blood Plasma-Derived Fibrin Gels

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## Abstract

**Objective:** The study of pathophysiology as well as cellular and molecular aspects of diseases, especially cancer, requires appropriate disease models. *In vitro* three-dimensional (3D) structures attracted more attention to recapitulate diseases rather than *in vitro* two-dimensional (2D) cell culture conditions because they generated more similar physiological and structural properties. Accordingly, in the case of multiple myeloma (MM), the generation of 3D structures has attracted a lot of attention. However, the availability and cost of most of these structures can restrict their use. Therefore, in this study, we aimed to generate an affordable and suitable 3D culture condition for the U266 MM cell line.

**Materials and Methods:** In this experimental study, peripheral blood-derived plasma was used to generate fibrin gels for the culture of U266 cells. Moreover, different factors affecting the formation and stability of gels were evaluated. Furthermore, the proliferation rate and cell distribution of cultured U266 cells in fibrin gels were assessed.

**Results:** The optimal calcium chloride and tranexamic acid concentrations were 1 mg/ml and 5 mg/ml for gel formation and stability, respectively. Moreover, the usage of frozen plasma samples did not significantly affect gel formation and stability, which makes it possible to generate reproducible and available culture conditions. Furthermore, U266 cells could distribute and proliferate inside the gel.

**Conclusion:** This available and simple fibrin gel-based 3D structure can be used for the culture of U266 MM cells in a condition similar to the disease microenvironment.

**Keywords:** Blood Plasma, Fibrin, Multiple Myeloma, Three-Dimensional Culture

**Citation:** Jomehpour M, Khosravi M, Janfada M, Abroun S, Vahdat S. Establishment of a three-dimensional culture condition for the U266 cell line based on peripheral blood plasma-derived fibrin gels. *Cell J.* 2023; 25(4): 229-237. doi: 10.22074/CELLJ.2023.562849.1138.

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## Introduction

Multiple myeloma (MM) is one of the most common type of blood cancer worldwide after lymphoma (1). MM is slightly more common in men, and the average age at diagnosis is 65 years. The overall five-year survival rate for patients with MM is 56% (2). Despite the recent introduction of various drugs and treatment strategies for MM (2), relapses occur in most patients (3), and MM remains predominantly incurable (4). Therefore, it is required to study the mechanisms involved in MM pathogenesis and evaluate the effectiveness of novel drugs in a more similar condition to the MM microenvironment.

Most myeloma research is conducted *in vitro* due to the lack of suitable animal models that accurately mimic the disease (5). In this regard, various cell lines, such as OPM-2, MM.1s, RPMI-8226 and U266, have been grown in two-dimensional (2D) cell culture systems and used for

drug screening and disease research. Among them, U266 is a well-known and frequently-utilized myeloma cell line (6) that expresses IL-6, a crucial component for the maintenance and proliferation of myeloma cells (7, 8).

Although accessibility, simplicity and low cost have made 2D cell culture one of the most widely used culture conditions, this type of cell culture has numerous limitations. Some examples of limitations are the inability to simulate the natural microenvironment of cells, three-dimensional (3D) cell-cell and cell-matrix interactions in tissue or tumor mass, tumor heterogeneity and 3D aspects of the tissues and tumors, including oxygen and nutrient gradients (9); therefore, fabrication of various kinds of tissue- or tumor-like 3D structures has been developed and introduced in recent years. The fabrication of fibrin gels using the plasma fraction of bone marrow (BM) aspirate is an important example of the development

Received: 02/October/2022, Revised: 30/January/2023, Accepted: 14/February/2023

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of 3D structures for BM tissue (1, 10). Fibrin gels as natural substrates would be safe and suitable matrices for a wide range of cell-based applications (11), with less inflammatory response induction and acceptable cell adhesion capacity, suitable for cell differentiation, and similar to the natural extracellular matrix of some types of cells in the human body (12).

One approach for the generation of fibrin gels is the use of commercial fibrin samples and a mixture of fibrinogen and thrombin, which is expensive and carries the risk of viral transmission (13). As another approach, patients' BM sample-derived plasma can be used, which appropriately could recapitulate the BM microenvironment. However, as BM aspiration for plasma derivation is a time-consuming, complicated invasive procedure, and it is not available for all research, the development of fibrin gels using an alternative to patient-derived BM plasma samples can advance this field.

In this study, it was tried to produce fibrin gels from the peripheral blood plasma samples as an alternative to BM plasma-derived gels in order to generate simple and available patient sample-independent 3D culture conditions for the U266 cell line. Moreover, the effect of plasma freezing on the generation and stability of gels was evaluated.

## Materials and Methods

### Study design

In this experimental study, fibrin gels derived from fresh or frozen peripheral blood plasma samples were generated in the presence of calcium chloride and tranexamic acid for the culture of U266 cells in 3D structures (Fig.1). To this end, different concentrations of calcium chloride and tranexamic acid were added to plasma samples to find the optimum concentrations for further experiments. Plasma samples in the absence of calcium chloride and tranexamic acid were used as control groups for gel formation and stability experiments, respectively. Moreover, in order to assess the proliferation rate and viability of cultured

cells in the generated structures, U266 cells without gels were considered the control groups. All experiments were performed using at least three independent replicates.

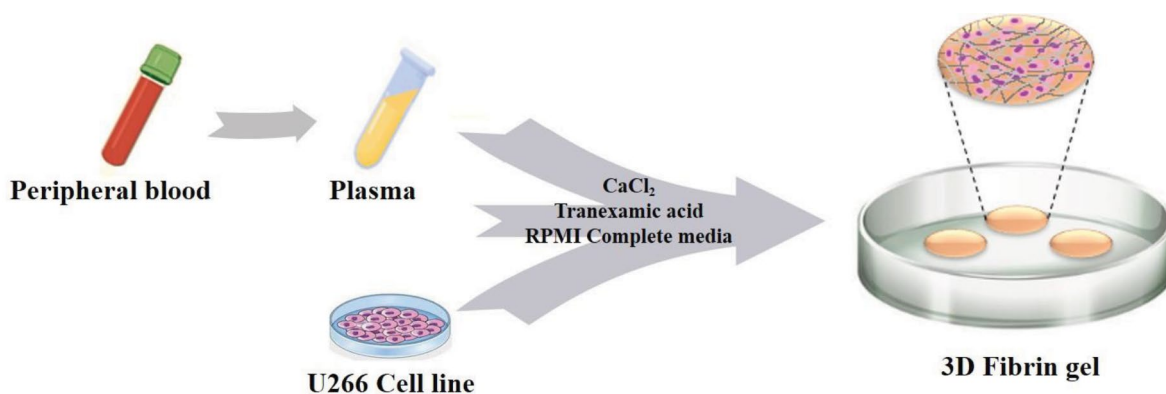
### Plasma derivation from peripheral blood

Plasma samples were derived from the peripheral blood of three volunteer individuals who were negative for viral contamination [human immunodeficiency virus (HIV) and hepatitis B virus (HBV)]. Each donor provided two milliliters of blood in EDTA anticoagulant. The blood plasma fraction was separated by centrifugation at 1500 rpm for 10 minutes at 22°C. In order to remove the donor-specific factors affecting the generation of gels, the separated plasma samples were pooled. A fraction of plasma was frozen at -20°C to evaluate the effect of plasma freezing on gel generation and stability.

### Generation of fibrin gels

Fibrin gels were fabricated using 40 µl of freshly isolated or frozen plasma samples, calcium chloride (Cat. No.: C7902, Sigma-Aldrich, Darmstadt, Germany) (to induce gelation), and tranexamic acid (14) (Caspian Tamin Pharma.Co., Rasht, Iran) (which generates more stable fibrin gels), in a total volume of 100 µl in RPMI-1640 medium (Cat. No.: 035-51800, Gibco, USA) (1). Different concentrations of calcium chloride (1-5 mg/ml) were used to find the optimum concentration for gelation. Gel formation was examined at subsequent 15-minute intervals for two hours at 37°C. Microtubes were inverted and tapped for assessment of gelation.

Moreover, in order to increase the stability of fibrin gels, different concentrations of tranexamic acid (1-6 mg/ml) were used to determine the optimum concentration with the least amount of gel degradation over seven days. For this purpose, the percentage of weight changes of fibrin gels over seven days was considered a measure of their stability (1). Gels were placed at 37°C and 50 µl of RPMI-1640 medium was added on top of the gels to prevent them from drying.



**Fig.1:** The schematic of our study design. A simple and cost-beneficial 3D culture condition was developed using peripheral blood-derived plasma, 1 mg/ml calcium chloride and 5 mg/ml tranexamic acid for the culture of U266 multiple myeloma cells in order to recapitulate a condition closer to the disease microenvironment.

## Gel-free culture of U266 cells

The U266 MM cell line was provided from the Iranian National Center for Genetic and Biologic Resources. Cells were cultured in the complete culture medium, including RPMI-1640 medium supplemented by 1% l-glutamine, 10% fetal bovine serum (FBS, Cat. No.: BI-1201, Bioidea, Tehran, Iran) and 1% penicillin/streptomycin (Cat. No.: BI-1203, Bioidea, Tehran, Iran) at 37°C, 5% CO<sub>2</sub> and 95% humidity (15).

## Flow cytometry

Flow cytometry was used to assess the expression of CD138 surface marker on U266 cells. Three days post-culture, U266 cells were centrifuged to remove culture medium and washed with PBS. Thereafter, 10<sup>5</sup> cells were incubated with CD138 antibody (Cat. No.: IQP-153F, IQ Products, Netherlands) at 4°C for 30 minutes. After two washes, CD138 expression was analyzed using flow cytometer system (BD FACSCanto II, BD Biosciences, USA). Data analysis was performed with Flowing Software 2.5.1.

## MTT assay

In order to find the appropriate cell seeding count, MTT assay was performed. For this purpose, we cultured different cell seeding counts of U266 cells (10<sup>4</sup>, 2 × 10<sup>4</sup>, 3 × 10<sup>4</sup>, 4 × 10<sup>4</sup>, 8 × 10<sup>4</sup>, 1.6 × 10<sup>5</sup>) in 2D condition (without gel). One, two and three days post-culture, tetrazolium salt solution (Cat. No.: 88417, Sigma-Aldrich, Darmstadt, Germany) was added to each well of a 96-well plate and was incubated at 37°C for 3 hours. Then, the plate was centrifuged at 400 g for 5 minutes. The supernatant was removed and replaced with a dimethyl sulfoxide (DMSO) solution to solubilize the sediment. The absorbance of the solution is then quantified with an ELISA reader (Labsystems Multiskan MS, Artisan Technology Group) at 570 nm.

## Culture of U266 cells within the 3D fibrin gels

In order to culture U266 cells in fibrin gels, suspended cells in complete culture media were mixed with plasma, and thereafter, calcium chloride and tranexamic acid were added to initiate gelation. Using complete culture medium, the total volume was increased to achieve the density of 3 × 10<sup>4</sup> cells per 100 μl. Then, each 100 μl of cell/gel mixture was placed in non-adherent culture plates in the form of droplet-like structures (16) and transferred to an incubator at 37°C. The complete culture medium was added to the generated droplet-like structures after gelation.

## Enzymatic digestion of fibrin gels

After three days, the cultured U266 cells were isolated from fibrin gels by incubating and digesting the gels with different concentrations of collagenase type I (1, 5 and 10 mg/ml) (Cat. No.: 17100-017, Gibco, USA). For this regard, the generated droplet-like cell/gel structures were washed and digested with collagenase type I enzyme at 37°C. The enzyme was diluted after the complete digestion of gels using culture medium and removed by centrifugation at 1500 rpm for 5 minutes.

## Cell viability assessment

Trypan blue staining (Cat. No.: BI-1803, Bioidea, Tehran, Iran) was used to evaluate cell proliferation and viability. For this purpose, 10 μl of the cell suspension and 10 μl of 0.4% trypan blue were mixed, and thereafter, 10 μl of the mixture was loaded on a Neubauer hemocytometer slide for examination. Cell viability was calculated as the ratio of live cells without color to all cells. Moreover, the cell expansion fold in fibrin gel was measured as the ratio of the cell count at day three of culture to the seeding cell count (17).

## Histological assessment

In order to evaluate cell distribution inside the gels, fibrin gels containing U266 cells three days post-culture were fixed in 1% formaldehyde (Cat. No.: HT501128, Sigma-Aldrich, Darmstadt, Germany) for 24 hours at 4°C. Then, each of the created fibrin gels was placed in a 2% agarose solution to facilitate the cutting and embedding process. A microtome was used to section paraffin-embedded samples (5-μm thick). Finally, the sections were stained by hematoxylin and eosin (H&E) (18).

## Statistical analysis

The experiments were carried out with at least three independent replications. Statistical analysis was performed by the t test method between two groups and by the ANOVA (Tukey post hoc) method between at least three groups. SPSS software (version 26, IBM, United States) was used for statistical analysis. The significance of differences between groups was assessed using the P ≤ 0.05 significance level. In the graphs, the data is presented as mean ± standard deviation.

## Ethical considerations

The procedure performed in this study was approved by the Ethics Committee of Tarbiat Modares University (IR.MODARES.REC.1399.229).

## Results

### Fibrin gels were generated using peripheral blood-derived plasma

To develop a 3D structure suitable for culture of the U266 cell line, fibrin gels were formed using peripheral blood plasma (Fig.1). As presented in Figure S1 (See Supplementary Online Information at [www.celljournal.org](http://www.celljournal.org)), among different groups with different calcium chloride concentrations (1-5 mg/ml), gelation was observed in plasma samples incubated with 1 mg/ml and 2 mg/ml calcium chloride; however, other calcium chloride concentrations (3, 4 and 5 mg/ml) could not induce gel formation even after two hours of incubation. Therefore, concentrations of 1 mg/ml and 2 mg/ml calcium chloride were used for further experiments.

### Tranexamic acid increased gel stability

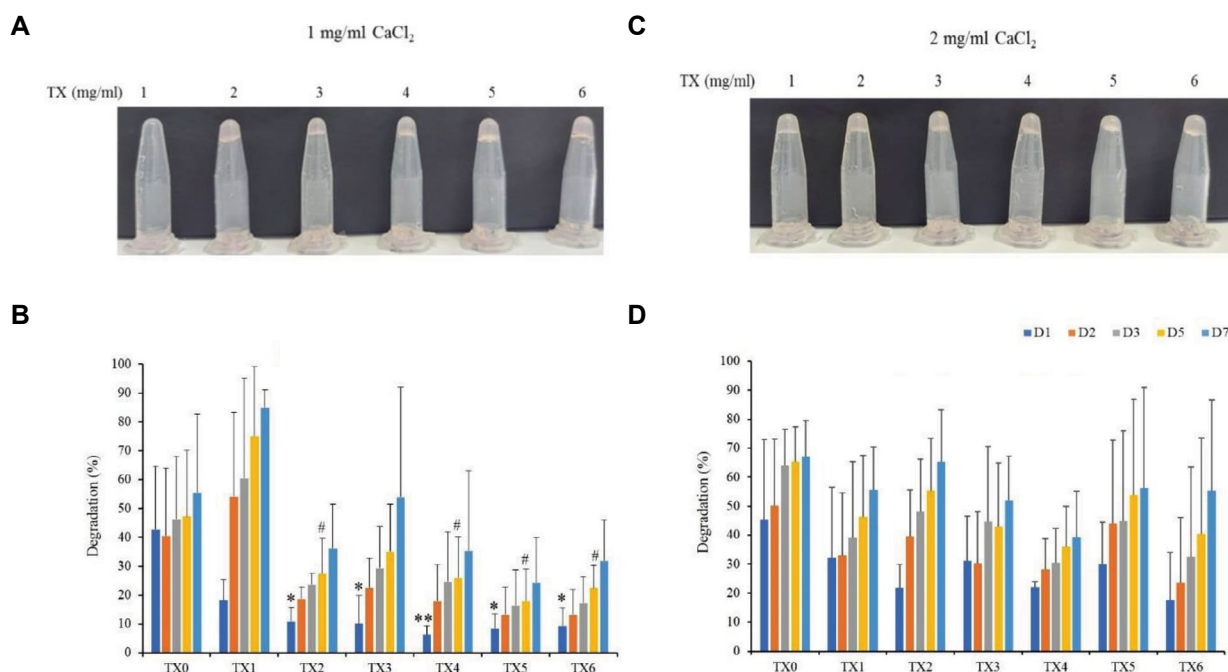
To determine the optimal tranexamic acid concentration with minimum gel degradation in seven days, the

percentage of weight changes was calculated in the presence of 1 mg/ml (Fig.2A, B) or 2 mg/ml (Fig.2C, D) calcium chloride as well as different concentrations of tranexamic acid (1-6 mg/ml). As shown in Figure 2B, although degradation was observed in all generated fibrin gels containing 1 mg/ml calcium chloride over seven days, the overall degradation rate was lower in tranexamic acid-treated gels compared to gels without tranexamic acid (TX0). Moreover, gel degradations in 2-6 mg/ml tranexamic acid-treated groups were significantly less than those in the TX0 group, one day after gel formation (the P values of the differences of the TX2, TX3, TX4, TX5 and TX6 groups compared to the TX0 group were 0.02, 0.02, 0.00, 0.01 and 0.01, respectively). Additionally, degradation rates in groups with 2, 4, 5 and 6 mg/ml tranexamic acid were significantly less than in the TX1 (treated with 1 mg/ml tranexamic acid) group five days after gel formation (the P values of the differences of the TX2, TX4, TX5 and TX6 groups compared to the TX1 group were 0.03, 0.03, 0.01 and 0.02, respectively). However, as shown in Figure 2D, the degradation rates of gels generated with 2 mg/ml calcium chloride were not significantly affected and decreased in the presence of different concentrations of tranexamic acid when compared to the TX0 control group over a seven-day period.

**Plasma freezing did not affect the gel stability**

To evaluate the effect of plasma freezing on gel formation and stability, thawed plasma samples were used for fibrin gel formation using 1 mg/ml (Fig.3A, B) or 2 mg/ml (Fig.3C, D) calcium chloride and different

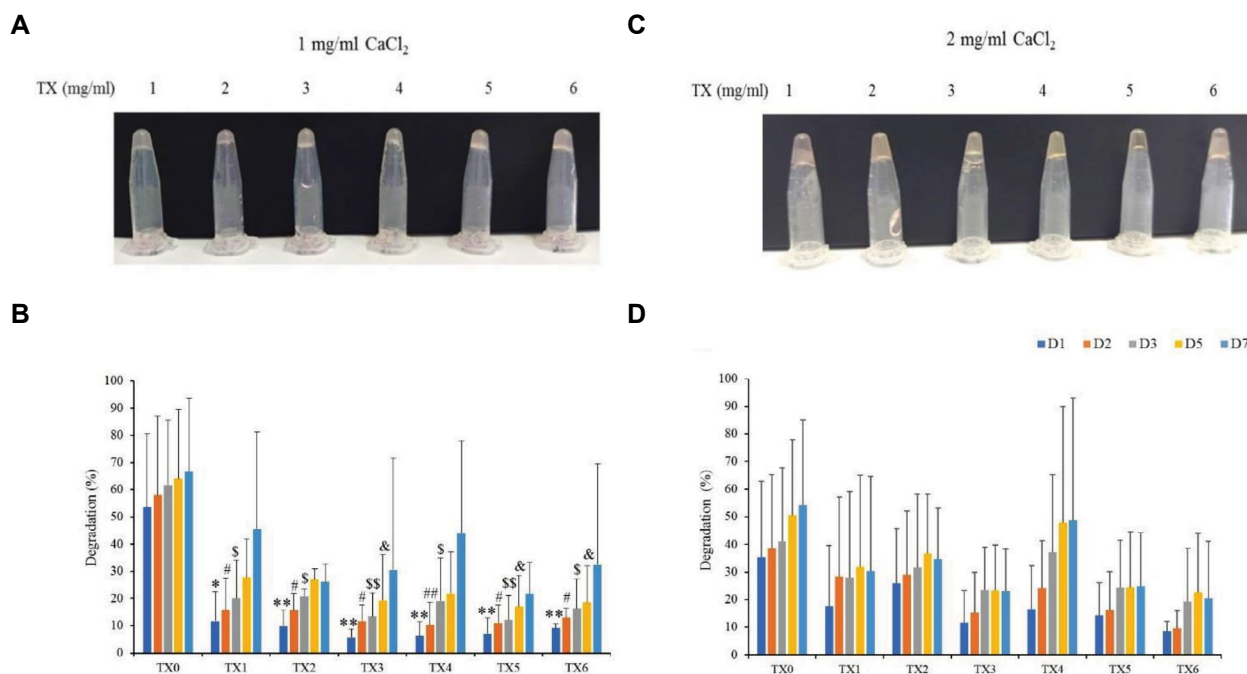
concentrations of tranexamic acid (1-6 mg/ml). Two hours after mixing the mentioned items, fibrin gels were formed in all samples, showing no impact of plasma freezing on gel formation (data not shown). Thereafter, the weight changes were measured for all gels during the following seven days. As presented in Figure 3, similarly as when using fresh plasma, degradations were observed in frozen plasma-derived gels during subsequent days, and the degradation rates were decreased by utilizing tranexamic acid. Moreover, the degradation rates of gels formed by 1 mg/ml calcium chloride and 1-6 mg/ml tranexamic acid during the first three days were significantly less than those of the TX0 control group (Fig.3B) (the P values of the differences of the TX1, TX2, TX3, TX4, TX5 and TX6 groups compared to the TX0 group at day 1 were 0.01, 0.00, 0.00, 0.00, 0.00 and 0.00, respectively, at day 2 were 0.02, 0.02, 0.01, 0.00, 0.01 and 0.01, respectively and at day 3 were 0.02, 0.02, 0.00, 0.02, 0.00 and 0.01, respectively). Additionally, the degradation rates of gels formed by 3, 5 and 6 mg/ml tranexamic acid at day five were significantly less than those of the TX0 control group (Fig.3B) (the P values of the differences of the TX3, TX5 and TX6 groups compared to the TX0 group at day 5 were 0.03, 0.02 and 0.03, respectively). However, similarly as observed in fresh plasma-derived gels, the degradation rates of gels formed by 2 mg/ml calcium chloride and different concentrations of tranexamic acid were not significantly affected compared to the TX0 control group (Fig.3D).



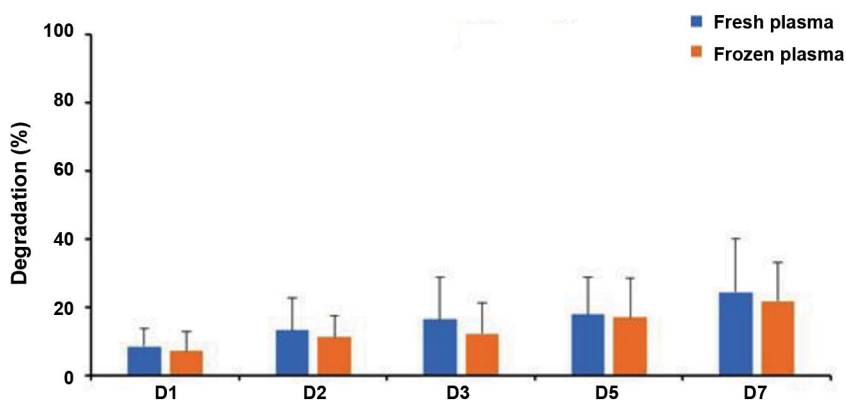
**Fig.2:** The effect of different concentrations of tranexamic acid on the stability of fibrin gels formed by fresh plasma and 1 or 2 mg/ml calcium chloride. **A.** Fibrin gels formed by 1 mg/ml calcium chloride and different concentrations of tranexamic acid at day five. **B.** The percentages of weight changes as a measure of gel degradation in the formed gels using different concentrations of tranexamic acid and 1 mg/ml calcium chloride during seven subsequent days. **C.** Fibrin gels formed by 2 mg/ml calcium chloride and different concentrations of tranexamic acid at day five. **D.** The percentages of weight changes as a measure of gel degradation in the formed gels using different concentrations of tranexamic acid and 2 mg/ml calcium chloride during seven subsequent days. The mean ± standard deviation is used to present all data. The statistically significant differences compared to the group without tranexamic acid (TX0) at day one and compared to the group with 1 mg/ml tranexamic acid (TX1) at day five are displayed with \* and #, respectively. \*, #; P<0.05 and \*\*, #; P<0.01. The exact numbers of P values are presented in the text. Statistical analysis was performed by ANOVA (Tukey post hoc) method with three experimental replicates. TX stands for tranexamic acid, and D stands for day. Numbers displayed alongside TX indicate different concentrations (mg/ml) of tranexamic acid.

According to the obtained results, 1 mg/ml calcium chloride was selected as the optimum concentration for further steps. Furthermore, based on the data shown in Figure 3, the degradations of the gels formed in the presence of 3, 5 and 6 mg/ml tranexamic acid were significantly less than those of the TX0 group during five days, and the variation of gel degradation at day seven was smaller in the gels formed in the presence of 5 mg/ml tranexamic acid compared to the other groups,

although the differences were not statistically significant. Therefore, the 5 mg/ml tranexamic acid concentration was selected for the next experiments. Moreover, as shown in Figure 4, the degradation percentages of gels formed by frozen plasma samples in the presence of 1 mg/ml calcium chloride and 5 mg/ml tranexamic acid were not statistically different from those of gels formed using fresh plasma samples, 1 mg/ml calcium chloride and 5 mg/ml tranexamic acid.



**Fig.3:** The effect of different concentrations of tranexamic acid on the stability of fibrin gels formed by frozen plasma and 1 or 2 mg/ml calcium chloride. **A.** Fibrin gels formed by 1 mg/ml calcium chloride and different concentrations of tranexamic acid at day five. **B.** The percentages of weight changes as a measure of gel degradation in the formed gels using different concentrations of tranexamic acid and 1 mg/ml calcium chloride during seven subsequent days. **C.** Fibrin gels formed by 2 mg/ml calcium chloride and different concentrations of tranexamic acid at day five. **D.** The percentages of weight changes as a measure of gel degradation in the formed gels using different concentrations of tranexamic acid and 2 mg/ml calcium chloride during seven subsequent days. The mean  $\pm$  standard deviation is used to present all data. The statistically significant differences between groups with different concentrations of tranexamic acid compared to the gels without tranexamic acid (TX0) at days one, two, three and five are displayed with \*, #, \$ and &, respectively. \*, #, \$, &;  $P < 0.05$  and \*\*, ##, \$\$;  $P < 0.01$ . The exact numbers of P values are presented in the text. Statistical analysis was performed by ANOVA (Tukey post hoc) method with three experimental replicates. TX stands for tranexamic acid, and D stands for day. Numbers displayed alongside TX indicate different concentrations (mg/ml) of tranexamic acid.



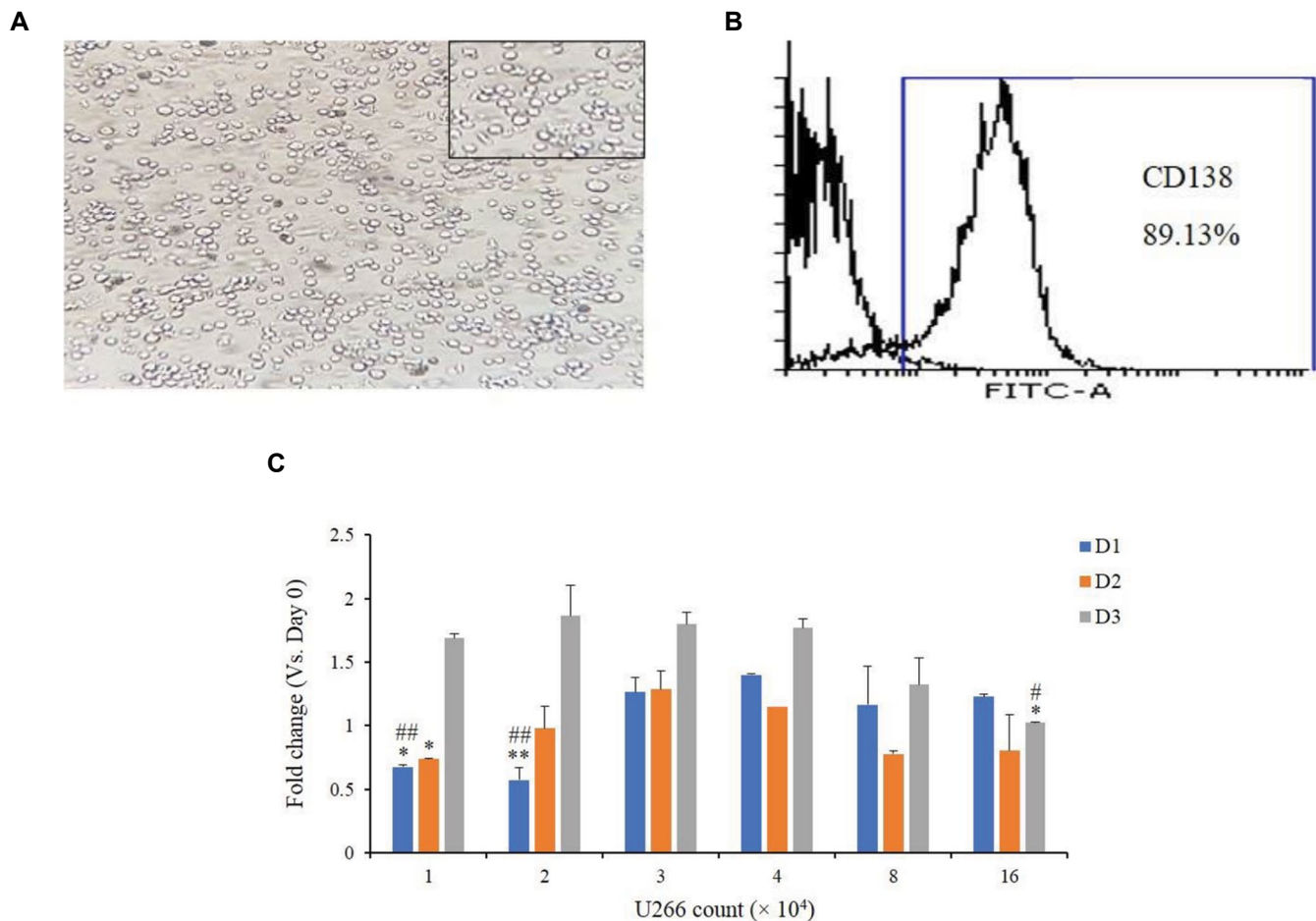
**Fig.4:** The degradation rates for the gels formed with fresh or frozen plasma samples in the presence of 1 mg/ml calcium chloride and 5 mg/ml tranexamic acid over the course of seven days. The data is presented in the form of mean  $\pm$  standard deviation. Statistical analysis was performed by ANOVA (Tukey post hoc) method with three experimental replicates. D stands for day.

**The fibrin gel-based 3D culture condition could support the culture of U266 cells**

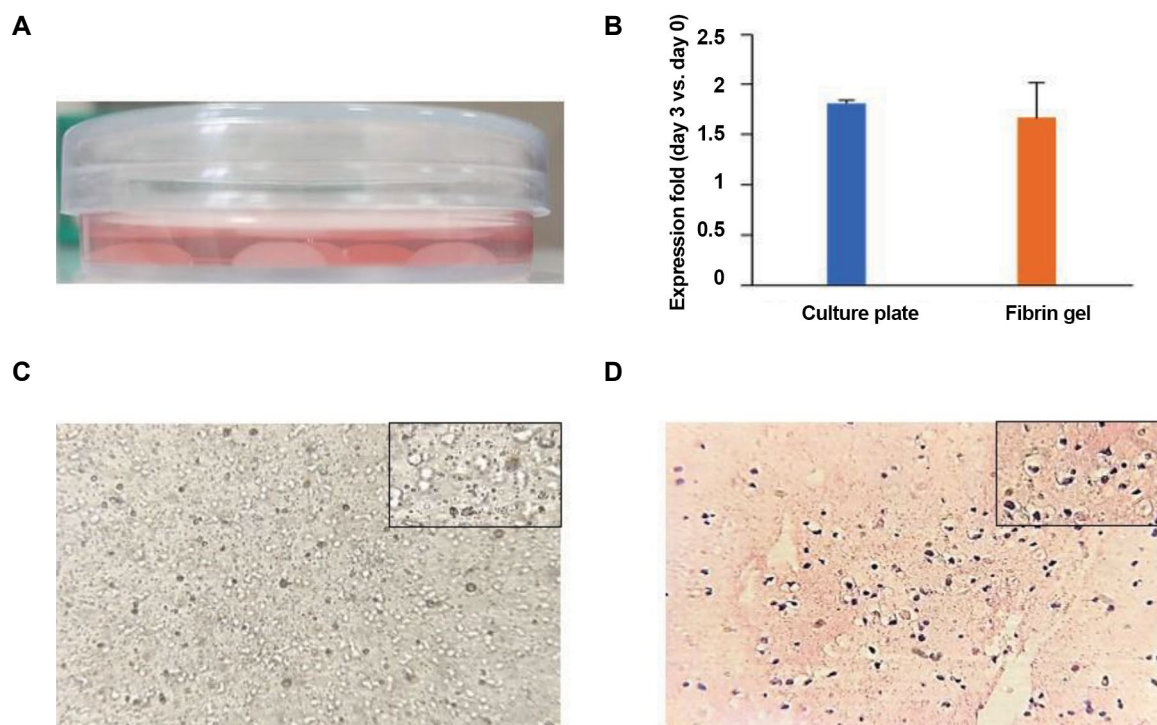
In order to evaluate the feasibility of using fibrin gels formed by peripheral blood-derived plasma samples as 3D culture structures, U266 MM cells were used. The morphology of U266 cells in the gel-free culture condition is presented in Figure 5A. As shown in Figure 5B, U266 cells were positive for the CD138 myeloma marker. When  $3 \times 10^4$  U266 cells were cultured in 100  $\mu$ l of complete culture media, the cell expansion fold was significantly higher than when other cell seeding densities were cultured, which was evaluated by MTT assay in gel-free cultures of U266 cells three days post-culture (Fig.5C) (the P values of differences of  $3 \times 10^4$  seeding density compared to  $10^4$  seeding density at days 1 and 2 were 0.01 and 0.03, respectively, compared to  $2 \times 10^4$  seeding density at day 1 was 0.00 and compared to  $1.6 \times 10^5$  seeding density at day 3 was 0.01; however, the P values of differences of  $4 \times 10^4$  seeding density compared to  $10^4$  and  $2 \times 10^4$  seeding densities at day 1 were 0.00 and 0.00, respectively, and compared to  $1.6 \times 10^5$  seeding density at day 3 was 0.01). As a result,  $3 \times 10^4$  cells/100  $\mu$ l was selected as the optimal

cell seeding density for 3D culture of U266 within the gels (Fig.6A). Thereafter, different concentrations of collagenase type I (1 mg/ml, 5 mg/ml and 10 mg/ml) were used to isolate cultured U266 cells from the gels three days post-culture. Accordingly, although different concentrations of collagenase type I did not significantly affect the cell viabilities, the viability of isolated cells was higher after treatment with 5 mg/ml collagenase (Fig.S2, See Supplementary Online Information at [www.celljournal.org](http://www.celljournal.org)) and this concentration was selected for further steps.

To evaluate the expansion fold of cultured U266 cells in fibrin gels, isolated cells from the gels were counted three days after culture. As presented in Figure 6B, the U266 expansion fold after three days of culture in fibrin gels was similar to the cultured cells in the 2D gel-free condition, and the differences in expansion folds between these two types of cell culture conditions were not statistically significant. Moreover, U266 cells were distributed through the gels, which was assessed using phase contrast imaging (Fig.6C) as well as H&E staining of sectioned paraffin-embedded gels (Fig.6D).



**Fig.5:** Gel-free culture of U266 cells. **A.** The morphology of U266 cells at day three of culture ( $\times 20$ ). **B.** Flow cytometry analysis of CD138 marker expression on U266 cells. **C.** The effect of different seeding densities on the U266 cell fold after three days of culture versus day zero. The data is presented in the form of mean  $\pm$  standard deviation. The statistically significant groups compared to the  $3 \times 10^4$  and to  $4 \times 10^4$  seeding density groups are presented by \* and #, respectively, at the same corresponding days post-culture. \*, #;  $P < 0.05$  and \*\*, ##;  $P < 0.01$ . The exact numbers of P values are presented in the text. Statistical analysis was performed by ANOVA (Tukey post hoc) method with three experimental replicates. D stands for day.



**Fig.6:** Culture of U266 cells within the fibrin gels. **A.** The droplets generated by the mixture of U266 cells and fibrin gel components, after gelation. **B.** Expansion fold of U266 cells at day three post-culture compared to day zero in two different culture conditions: in culture plates and in fibrin gels. Statistical analysis was performed by the t test method with three experimental replicates. **C.** Phase-contrast image of U266 cells in fibrin gels ( $\times 20$ ). **D.** Distribution of U266 cells in fibrin gels using H&E staining ( $\times 20$ ).

## Discussion

In this study, it was tried to generate a simple, available, cost-beneficial and reproducible 3D structure for the culture of the MM cell line in order to have culture conditions more similar to the 3D BM microenvironment for further applications. In this regard, fibrin gels were generated as native substrates for the culture of the U266 cell line. Fibrin gels are widely used as cell injection substrates, cell culture plate coatings, and tissue-engineered structures (19, 20). In comparison with synthetic polymers, fibrin gel had widespread advantages such as a controllable degradation rate, non-toxic degradation products, and excellent biocompatibility. In addition, the morphology, mechanical properties, and stability of fibrin gels can be regulated by controlling precursor materials and ionic strength (21). Moreover, fibrin gels have shown high cell culture efficiency and enhanced adhesion properties (22). The fibrin-based 3D generated structures have been introduced and applied for disease modeling of various types of diseases, including MM (10, 23, 24).

Most fibrin gel-based structures for MM disease modeling have been generated using BM-derived plasma samples (1, 10, 25). However, this type of structure may be affected by patient-specific variables and is not accessible to all researchers. Therefore, we aimed to use peripheral blood plasma samples as alternatives for BM blood plasma samples. It has been shown that there is no significant difference between fibrinogen levels in fibrin

clots derived from peripheral blood and BM (26). Several studies used peripheral blood plasma to generate 3D structures in order to model various diseases (1, 23, 24). On the other hand, it is possible that in the process of plasma derivation, platelets remained in the plasma fraction and affected the gel properties as well as the growth and fate of the cultured cells. It has been shown that platelets contain abundant growth factors, including platelet-derived growth factor (PDGF), transforming growth factor (TGF)- $\beta 1$ , insulin-like growth factor (IGF)-1, and bone morphogenetic protein (BMP)-2, and entrapped platelets can progressively release these cytokines during fibrin matrix remodeling (27). Moreover, integrin  $\alpha \text{IIb}\beta 3$  is expressed abundantly, with an estimated 80,000 copies per platelet. The integrin binds fibrinogen and other plasma proteins. Thrombin promotes the signaling processes by increasing platelets  $\text{Ca}^{2+}$  levels, activating integrins  $\alpha 2\beta 1$  and  $\alpha \text{IIb}\beta 3$ , and exposure of phosphatidylserine. Extensive fibrin formation can also be observed on coated platelets and relies on transglutaminase (FXIII) activity and  $\alpha \text{IIb}\beta 3$  binding. It should be noted that platelets from MM patients are activated with disease progression, and MM cell lines activate platelets. Moreover, the results of xenograft MM models have shown that platelets contribute to the tumor cell engraftment of MM cell lines *in vivo* (28, 29).

In the first step, the effects of different factors, including calcium chloride concentration, tranexamic

acid concentration, and long-term plasma preservation, on gel formation and stability were evaluated. It was revealed that 1 mg/ml calcium chloride was the optimum concentration for gelation of fresh and thawed frozen plasma samples. Similarly, de la Puente et al. evaluated the effect of different concentrations (0-4 mg/ml) of calcium chloride on fibrin gel formation. They showed that 1 mg/ml calcium chloride was the best concentration with the least gelation time (1). On the other hand, we showed that different concentrations of tranexamic acid were able to stabilize fresh and frozen plasma-derived gels for seven days. However, minimum variations in gel degradation rates during seven days were seen when 5 mg/ml of tranexamic acid was used, especially in the case of frozen plasma samples (although the differences were not statistically significant at day seven). Consistently, several studies have evaluated the impact of different concentrations of tranexamic acid on the stability of fibrin gels. They showed that 4 mg/ml of tranexamic acid had the least gel degradation rate (1, 13, 30). The observed differences between optimal tranexamic acid concentrations may be attributed to batch variations and the purity of products.

In practice, the availability and reproducibility of fresh plasma samples are two challenging issues that may be overcome by the development of plasma banks (31). This required the evaluation of the impact of long-term plasma cryopreservation on gel formation and stability. It was revealed that plasma cryopreservation did not statistically affect the gelation procedure and gel degradation during the seven days. Similarly, it has been reported that freezing has little effect on fibrinogen levels (32). Moreover, the level of fibrinogen remains stable in twice-thawed and refrozen fresh frozen plasma (33). In order to more precisely evaluate the effect of plasma cryopreservation on gel properties, assessment of the concentration of plasma components such as coagulation factors before and after freezing is recommended to be considered in future studies.

In order to assess the impact of cell culture in 3D fibrin gels on the viability and proliferation of MM cells, the U266 cell line was selected. In the first step, the density of  $3 \times 10^4$  cells per 100  $\mu$ l was selected to be cultured in both 2D and 3D culture conditions (gel-free and inside the fibrin gel, respectively). The selected cell seeding density was in line with another study that has used this cell density for the culture of other MM cell lines in fibrin gels (1). U266 cells counted three days after culture in fibrin gels showed an expansion fold similar to cells cultured in 2D gel-free condition, indicating that the generated gel might have no toxic effects on the viability and proliferation of U266 cells. Similarly, other studies using calcium chloride and tranexamic acid for gel formation did not report any toxic effects of these two compounds on cultured cells (1, 10, 24, 25). For instance, the impact of tranexamic acid on the viability of myeloma cell lines was tested by MTT assay, and no toxic effect on cell viability was found at any of the tested concentrations (1-10 mg/ml) (1). However, a

more precisely assessment of the probable toxic effects of these two compounds on U266 cells necessitates additional testing to detect any mutations and specific cell damage. On the other hand, it has been reported that after eight days of culture, the viability of conjunctiva mesenchymal stem cells-derived photoreceptor cells cultured in fibrin gel, which was composed of fibrinogen and thrombin, was enhanced compared to the cells cultured in tissue culture plates (34). Moreover, another study showed that after three days of culture, the viability of induced pluripotent stem cells cultured in fibrin gel was similar to cells cultured in tissue culture plates (35). Furthermore, the proliferation rates of U266 cells in both 2D and 3D culture conditions were similar to the reported proliferation rates of the RPMI8226 MM cell line (36).

It should be noted that more evaluations, such as apoptosis and necrosis assessments of cultured cells using flow cytometry, are required to highlight the suitability of fibrin gels for the survival and proliferation of cultured U266 cells. Moreover, our used enzymatic digestion protocol might affect the viability and the calculated expansion fold of cells cultured in fibrin gels; therefore, histological assessments of cells in 3D structures would overcome the negative effects of enzymatic digestion protocols on cells and be recommended for future studies. H&E staining exhibited the distribution of cultured U266 cells in the gel, which may increase their 3D interactions. The observed pattern of U266 cell distribution in the gel was similarly reported by other studies culturing different types of cells (13, 30, 36), which may highlight the importance of 3D culture of MM cells for the development of more physiologically relevant *in vitro* disease models (37, 38).

## Conclusion

We have developed a 3D culture condition based on fibrin gels derived from peripheral blood plasma samples for the culture of the U266 MM cell line. The gels could be generated from cryopreserved plasma samples, which makes them accessible and suitable for a wide range of applications. U266 MM cells cultured in peripheral blood plasma-derived gels maintained their proliferation rate and were distributed throughout the gel. This culture system may be an appropriate substitute for BM-derived fibrin gels for 3D culture of MM cells to study the disease and drug effectiveness.

## Acknowledgments

The authors would like to express their appreciation to the peripheral blood donors. This work was supported by a grant from Tarbiat Modares University and a grant from the Iran National Science Foundation (INSF) to Sadaf Vahdat with grant number 99024778. The authors have no conflict of interest.

## Authors' Contributions

Ma.J., M.K., Me.J.; Contributed equally to Methodology, Formal analysis, Visualization and Writing original draft.



S.A.; Conceptualization and Study consultation, Review and Editing the manuscript. S.V.; Conceptualization and Study supervision, and Writing, Review and Editing the manuscript. All authors read and approved the final version of manuscript.

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