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Effect of platelet extract on proliferation and migration of fibroblast cell

Abstract

Nowadays, one of the most important cells involved in wound healing are fibroblasts, which play a key role in promoting the healing process. Hence, the present study aims to investigate the effect of different concentrations of platelet extract derived from umbilical cord blood on the proliferation and migration of fibroblast cells. The present study is a case-control study that used mature human fibroblast cells. Platelet extract derived from umbilical cord blood plasma was prepared and then fibroblasts were treated with different concentrations (UCB-PL). The results of the present laboratory have shown that 10% concentration is the highest effective concentration in cell migration. Also, the results of the present study revealed that there was no increase in proliferation with the increase in concentration, meaning that in 20% and 15% concentrations in 3 hours of 24, 48, and 72 hours, a decreasing trend in proliferation was also observed. In the case of the negative control, the obtained results show that this group has a significant difference with all groups except the 20% concentration group ($p \leq 0.05$), and in the case of the UCB-PL/FBS group, an increase in proliferation compared to the negative control and positive control groups has been shown in the 3 mentioned times ($p \leq 0.05$). The results revealed that platelet extract, derived from umbilical cord blood, can proliferate and migrate the fibroblast cells, which are key functions in the wound healing process.

Keywords: *Platelet Products, Umbilical Cord Blood, Peripheral Blood, Fibroblast*

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Introduction

Nowadays, one of the most important cells involved in wound healing are fibroblasts, which play a key role in advancing the healing process by cell proliferation and migration and by initiating intracellular signaling pathways related to wound healing. Over the pas

t years, one of these products prepared for this purpose is products derived from platelets, which can be called platelet-rich plasma products, platelet gel, and platelet extract products (1). All platelet products are rich in growth factors and cytokines. In this regard, due to the special conditions of the platelet extract, growth factors are released and their access to the cells of the wound area is facilitated. These factors include vascular endothelial growth factor, platelet-derived growth factor, fibroblast growth factor, hepatocyte growth factor, and beta-transforming growth factor. Each of these factors initiates various signaling pathways within the cell and causes different cell responses such as proliferation, migration, and differentiation (2). In the study of the treatment of keratinocyte cells with platelet extract derived from peripheral blood, a significant difference between cell proliferation and cell migration has been observed in the treated cells compared to the control samples (3).

Additionally, this significant difference was also observed in another study that investigated the effect of platelet extract derived from peripheral blood on the proliferation and migration of fibroblasts. Since these studies have referred to the advantages of platelet gel derived from umbilical cord blood and according to the results of these studies on growth factors in platelet gel derived from umbilical cord blood, it has been determined that both vascular endothelial growth factor and the platelet-derived growth factor have high levels in platelet gel derived from umbilical cord blood compared to platelet gel derived from peripheral blood (4).

Ranzato et al. (2019) examined the effect of platelet extract derived from peripheral blood on the proliferation and migration of fibroblasts and a significant difference was also observed (3). Umbilical cord blood-derived platelet-rich plasma (UCB-PRP) including growth factors has attracted attention for regeneration in medical science. cryopreserved umbilical cord blood-derived platelet-rich plasma has growth factors like PDGF-BB, TGF-β, and VEGF. This research determined the contents of growth factors (i.e., PDGF-BB, TGF-β1, and VEGF) in UCB-PRP that was cryopreserved for 3 years an attempt to examine the capability of these growth factors to induce to osteoblast cell differentiation of UC-MSCs(mesenchymal stromal cells). These researchers suggest potential clinical application to regenerative medicine of UCB-PRP.(5). PRP is an important material in body which contains some growth factors. Also, it has been used as a cell scaffold. PRP can be used in existing cellular and regenerative medical treatment. Salkin et.al showed that PRP significantly increases proliferation in hADSCs (human adipose-derived mesenchymal stem cells), reducing total apoptosis and senescence. (6). Platelet extract are efficient cells in mediators able to positively affect cell activity in treat healing. (7). the most abundant macromolecules in ECM is collagen. the only enzymes capable of degrading the collagen are matrix metalloproteinases (MMPs). This resistance of collagen is involved in several biological processes. Successful healing in tissues is related to the regeneration of the extracellular MMPs. (8).

The different effects of PL on various types of cells indicate that the growth factors and cytokines in PL are the main

coordinators of the wound healing pathway. In the injured tissue, as soon as the injury begins, a cascade of events such as inflammation, new tissue formation, and new tissue replacement occurs through a pattern of signaling pathways. These mediators include growth factors and cytokines that stimulate intracellular activities, known as the regeneration process (9). In the healing process, fibroblast cells gather at the injury site and play a major role in the healing process. Based on mentioned materials, this study aims to investigate the effect of different concentrations of platelet extract derived from umbilical cord blood on the proliferation and migration of fibroblast cells.

Materials and Methods

This study was a case-control study conducted at Royan Research Institute in Isfahan. Adult human fibroblast cells were used in this study. The mentioned cells were obtained from the cell bank of Royan Research Institute. Platelet extract derived from umbilical cord blood plasma was prepared and then fibroblasts were treated with different concentrations (UCB-PL).

1-Preparation of cell culture medium: In this study, Dulbecco's Modified Eagle's Medium (DMEM) was used to culture the desired cells. 10% fetal bovine serum, 1% Penicillin streptomycin antibiotic, streptomycin, 1% L-glutamine, and 1% non-essential amino acids were added to this medium before use. Then, to eliminate the growth of all types of bacteria, the culture medium was filtered by a 0.2 filter (which does not allow the passage of particles larger than 0.2 micrometers). It should be noted that after adding a serum, the medium should be kept at a temperature of 4°C for a maximum of two weeks.

2. Cell passage: During cell passage, cells are transferred from one culture vessel to another culture vessel to increase their number. To separate the cells from the bottom of the flask, 1 to 4 ml of trypsin was added in proportion to the size of the flask. Trypsin breaks the cell connections and causes the cells to separate from the bottom of the flask and float. Then, the flask containing the cells was placed in the incubator for 1 to 3 minutes and finally, all the cells were separated from the bottom of the flask by tapping. After separating the cells from the bottom of the flask with three times the amount of trypsin used, the medium containing serum was added to inactivate the trypsin. The contents of the flask were transferred to a 15 ml test tube. The cells were centrifuged for 5 minutes at 1500 rpm and a temperature of 25°C. After the centrifugation, the supernatant was poured out and the cell sediment was dissolved in 1 ml of medium. After counting the cells, the desired number (about 600000 cells for a 75 cm^2 flask, 2000 cells for a 25 cm^2 flask) was added to the flask and the rest of the cells were frozen.

3- Cell count: To count cells from the homogenized medium containing cells, 5 µl of the medium was taken and mixed with 5 µl of trypan blue 0.4% dissolved in ion-free phosphate-buffered saline. Then, using a new bar slide under a light microscope, the number of cells placed under every 4 squares of the Nicobar slide was counted and the number of cells was calculated using the following formula.

Formula 1 $$\text{percentage of viable cells} = \frac{\textit{total number of cells} - \textit{number of died cells}}{\textit{total number of cells}} \times 100$$

Formula 2

$$\text{Total number of cells} = \frac{\text{Total number of cells}}{4} \times 2\text{dilution}$$

coefficient (if the cell is diluted) fluid volume of the main tube containing the cell $104\times$ percentage of viability of cells

4. Freezing and storage of cells: To freeze the cells, 1 ml of freezing medium was added for every 2000000 cells, which contains 40% fetal bovine serum, 50% Dulbecco's Modified Eagle's Medium, and 10% dimethyl sulfoxide. Dimethyl sulfoxide is a potent solvent that prevents the formation of ice crystals and damage to cells in the process of cell freezing. Then, they were kept for 24 hours in a freezer at -18°C and then in a freezer at -70°C for 24 hours. Then, the samples were transferred to the nitrogen tank. This operation should be done in such a way that the cells spend the shortest possible time outside the freezer.

5- Thawing frozen cells: To re-cultivate (thaw) frozen cells, the cryovial tube containing frozen cells is transferred from the nitrogen tank to Bain-Marie at 37°C so that only a small particle of ice remains in it. It is crucial to be careful when removing the vial, since if the vial is not completely closed, liquid nitrogen may penetrate the vial and cause an explosion during thawing. After thawing the contents inside the cryovial, it was transferred to a falcon and 4 to 5 ml of culture medium was added to it. As a result, dimethyl sulfoxide is diluted and cannot damage the cells. Then, to settle the cells, centrifugation was performed for 5 minutes, 1500 rpm, and 25°C temperature. After centrifugation, the supernatant was poured out and it was dissolved in 1 ml of medium, and transferred to a flask containing fresh culture medium.

6- Preparation of platelet extract product derived from umbilical cord blood: preparation of platelet extract was done through the protocol available at Royan Research Institute. For this purpose, umbilical cord blood samples were transferred into transfer bags (with a volume of 50+450 ml). Then, the transfer bag was centrifuged at room temperature for 22 minutes. The upper yellowish liquid is called platelet-rich plasma (PRP). Platelet-rich plasma PRP was introduced into another transfer bag using a plasma extractor.

Then, the collected platelet-rich plasma was centrifuged at 5000×g and at room temperature for 15 minutes. The supernatant liquid is called low-platelet plasma. This low-platelet plasma was transferred into another transfer bag, and some PPP was used for viral-mycoplasma-bacterial and endotoxin tests. Only 50 or 100 ml of plasma remains on the platelet concentrate, which was suspended on the shaker and counted by the platelet cell counter. If the count of platelets in the platelet concentrate is 1 x 109/ml, it will be suitable for the preparation of platelet extract. The platelet concentrate was kept at -70°C freezer for 24 hours and was quarantined until the negative results of the screening tests were confirmed. Then, the platelet concentrate was fully thawed in Bain-Marie at 37°C. To remove platelet debris, the thawed concentrate was centrifuged at 3000 g for 30 minutes at room temperature. The supernatant solution after centrifugation is called platelet extract. The platelet extract obtained at this stage was divided into the desired volume (5-10 ml) and stored in a freezer at -70°C until use.

7. Examining the effect of platelet extract on proliferation (fibroblast): In this study, 50000 cells for each well of 6-well plates were poured and cultured. Fibroblast cells in DMEM culture medium containing concentrations of 5%, 8%, 10%, 15%, and 20% of platelet extract derived from umbilical cord blood along with the positive control group containing 10% FBS and the negative control devoid from both (FBS) and UCB-PL) were cultured. Also, the UCB-PL/FBS group, which included equal proportions of platelet extract derived from umbilical cord blood and FBS, was used. In the cultures where the UCB-PL product was used, 1 µl of heparin was added for every 1 ml of the culture medium to prevent coagulation or gelling of the medium. After 24, 48, and 72 hours, they were stained with trypan blue, and the cells were counted using an inverted microscope, and the difference in proliferation was calculated at different hours in different concentrations.

8. Measuring the rate of cell migration by scratch assay: Scratching test was used to measure the effect of UCB-PL on the rate of cell migration. To perform this test, 6-well plates were used and 1 x 105 cells were added to each well with 4 ml of DMEM medium containing 10% FBS. After 3 days, the bottom of the plate was covered with cells and their medium was replaced with the same previous medium. After 24 hours, a diagonal scratch was made in the middle of each cell using a crystal tip and a ruler. Then, the medium of each cell was drained and washed 3 times with PBS to remove the created cell debris, and this time, 4 ml of DMEM medium containing different concentrations of platelet lysate product derived from umbilical cord blood 8%-10% and UCB-PL/FBS group (equal proportion of each) as well as medium without platelet lysate (positive control containing 10% FBS and negative control without FBS and UCB-PL) were poured for each house. Then, each sample was photographed at the scratch site at 0, 6, 12, and 24 hours after the scratch by a camera connected to a microscope. The rate of cell migration into the scratch space was calculated with Cell science software. Accordingly, the distance between two edges of the scratch was measured by several lines by the software. Then, the mean of the measured lines was calculated and put in the wound closure formula, and the degree of closure of each scratch was quantitatively calculated. Data were analyzed using SPSS-16 Software and ANOVA parametric test.

Results

Examining the cell proliferation in the laboratory has indicated the highest increase in fibroblast proliferation is related to the concentration of 10% in the 3 mentioned times. Also, the results of the present study revealed that there was no increase in proliferation with an increase in concentration, meaning that in the concentration of 20% and 15% in 3 times of 24, 48, and 72 hours, a decreasing trend in proliferation was also observed. Also, in the case of the negative control, the obtained results show that this group has a significant difference with all groups except the 20% concentration group ($p \leq 0.05$), and in the case of the UCB-PL/FBS group, an increase in proliferation compared to the negative control and positive control groups was shown in the 3 mentioned times ($p \leq 0.05$) (Figure 2). According to Figure (1), the proliferation of fibroblast cells in concentrations of 8%-10% at 24 hours is more significant than in other groups.

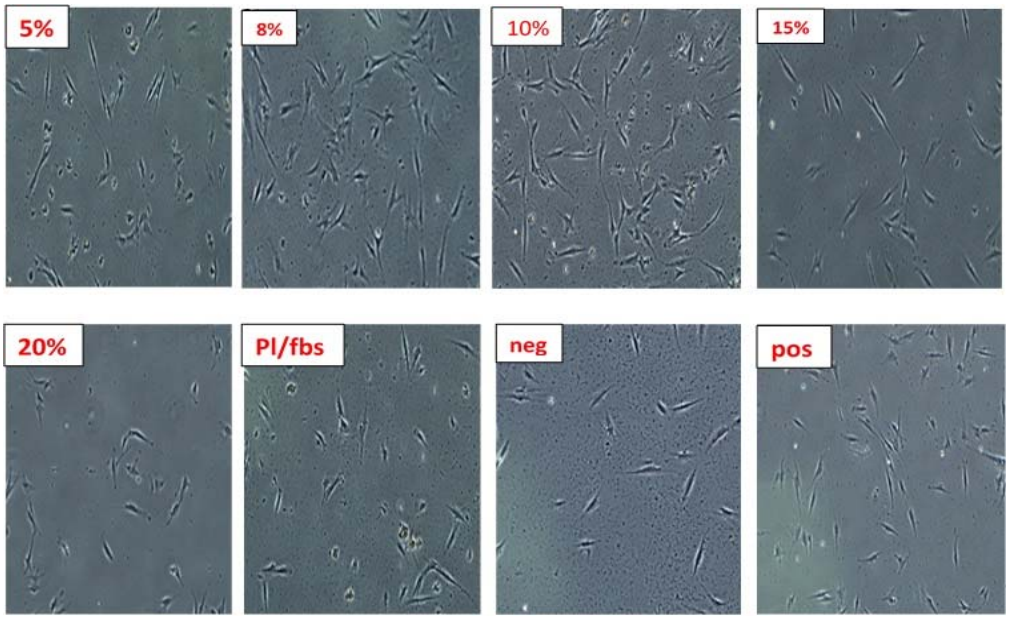
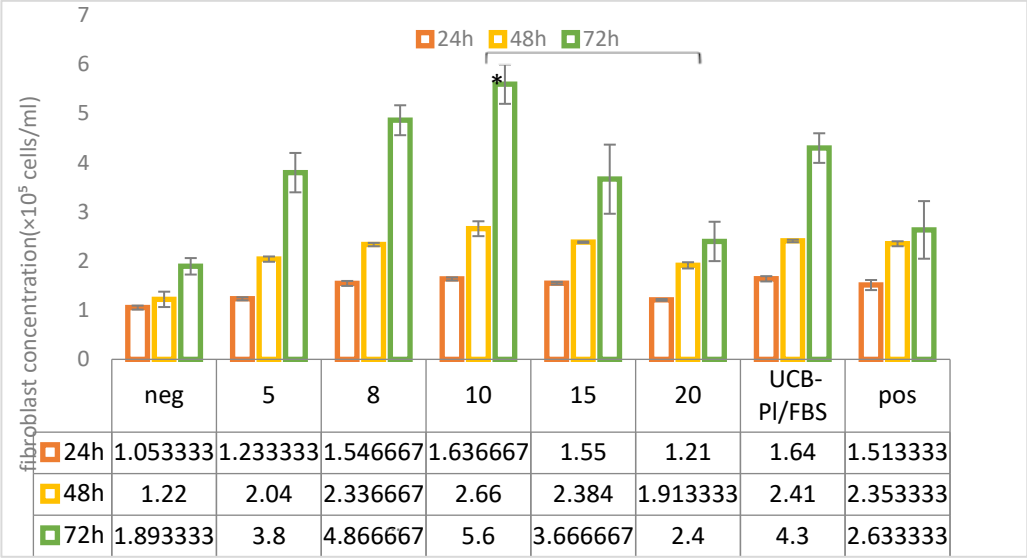


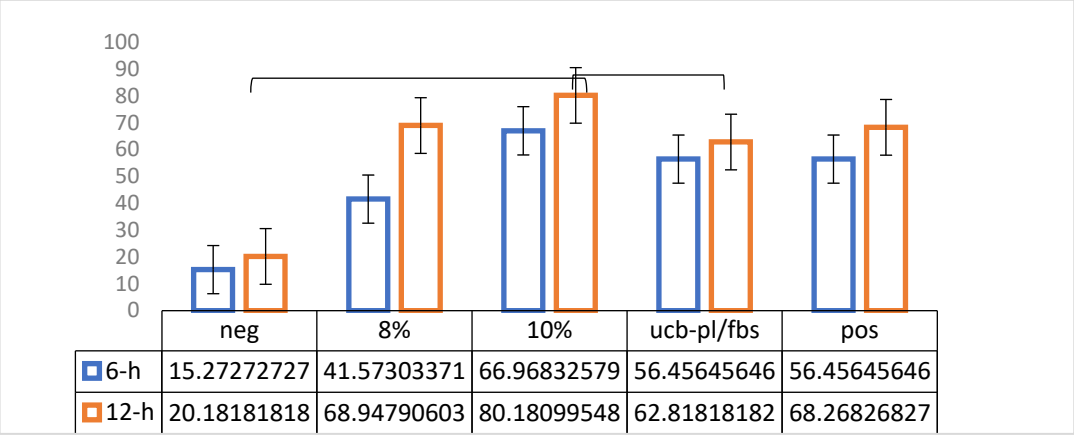
Figure 1: Examining the increase in the proliferation in different concentrations of platelet extract derived from umbilical cord blood compared to control groups in 24 hours



	neg	5	8	10	15	20	UCB-Pl/FBS	pos
24h	1.053333	1.233333	1.546667	1.636667	1.55	1.21	1.64	1.513333
48h	1.22	2.04	2.336667	2.66	2.384	1.913333	2.41	2.353333
72h	1.893333	3.8	4.866667	5.6	3.666667	2.4	4.3	2.633333

Figure 2: The effect of different concentrations of UCB-PL on fibroblast proliferation.
In this Chart, * means a significant difference between the 10% group and other groups ($p \leq 0.05$).

To examine the effect of different concentrations of platelet extract derived from umbilical cord blood on the migration of fibroblast cells, the viability percentage of UCB-PL/FBS concentrations of 8%-10% was selected for the rest of the tests because these concentrations did not have a toxic effect on fibroblast cells. This experiment was performed with the mentioned concentrations in 3 replications. The results showed that they had a significant increase in the rate of cell migration (Figure 3). Also, based on the results of Figure 4, at 8% concentration in 24 hours, the scratch area is not completely covered by cells.



	neg	8%	10%	ucb-pl/fbs	pos
6-h	15.27272727	41.57303371	66.96832579	56.45645646	56.45645646
12-h	20.18181818	68.94790603	80.18099548	62.81818182	68.26826827

Figure 3: The chart related to the effect of different concentrations of UCB-PL on fibroblast migration ($p<0.01$)

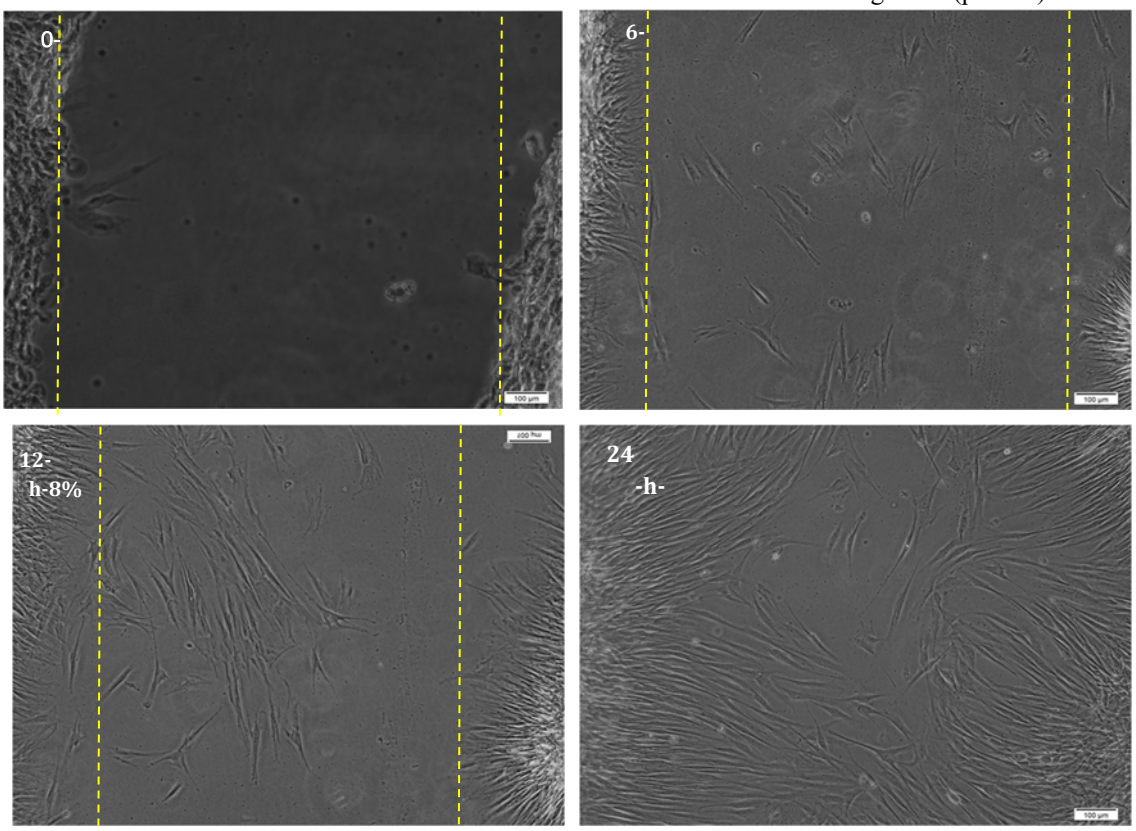


Figure 4: Measuring the effect of 8% UCB-PL on the rate of cell migration in fibroblast cells by scratching method at 4 times of 0, 6, 12, and 24 hours after scratching

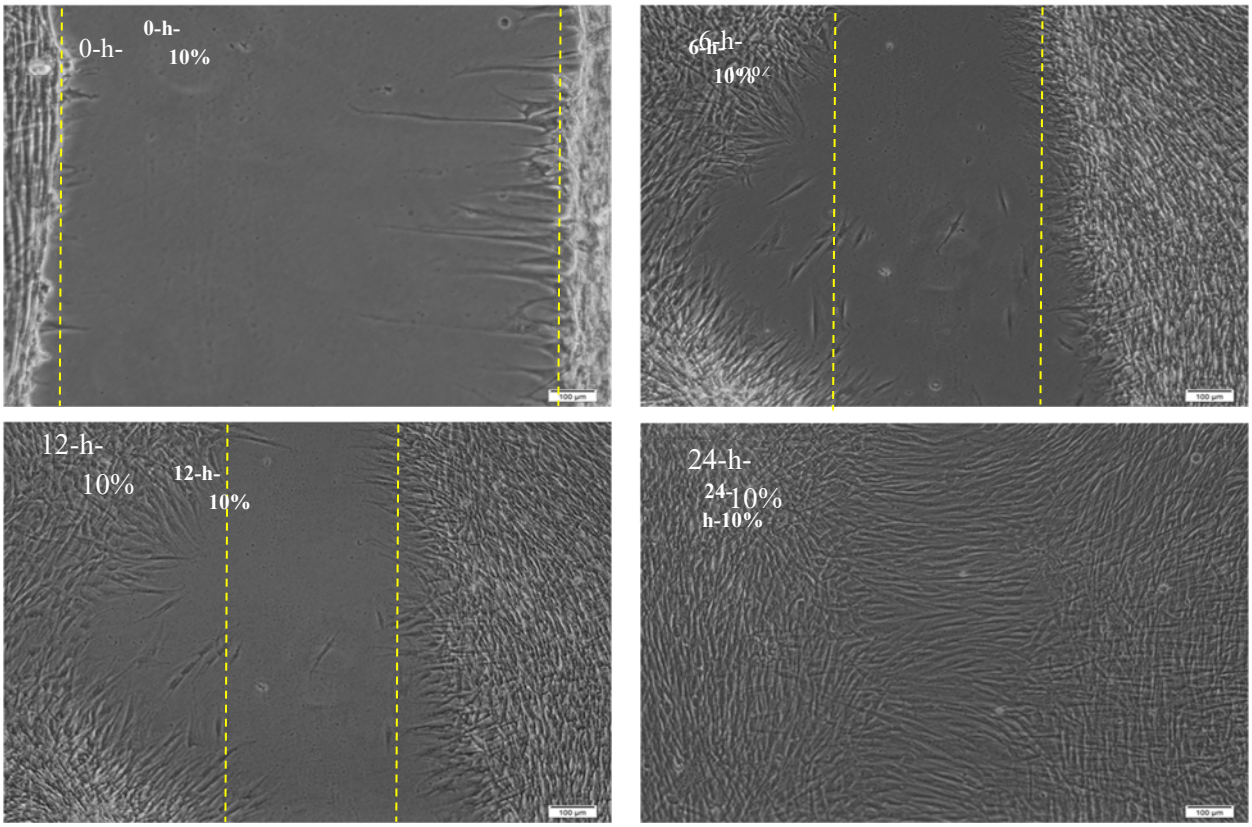


Figure 5: Measuring the effect of 10% UCB-PL on the rate of cell migration in fibroblast cells by scratching method at 4 times of 0, 6,12, and 24 hours after scratching

Based on Figure 5, at a concentration of 10% 24 hours after scratching, the space between the two edges is completely covered and the scratched area is filled with cells. A concentration of 10% has shown as the highest effective concentration in cell migration.

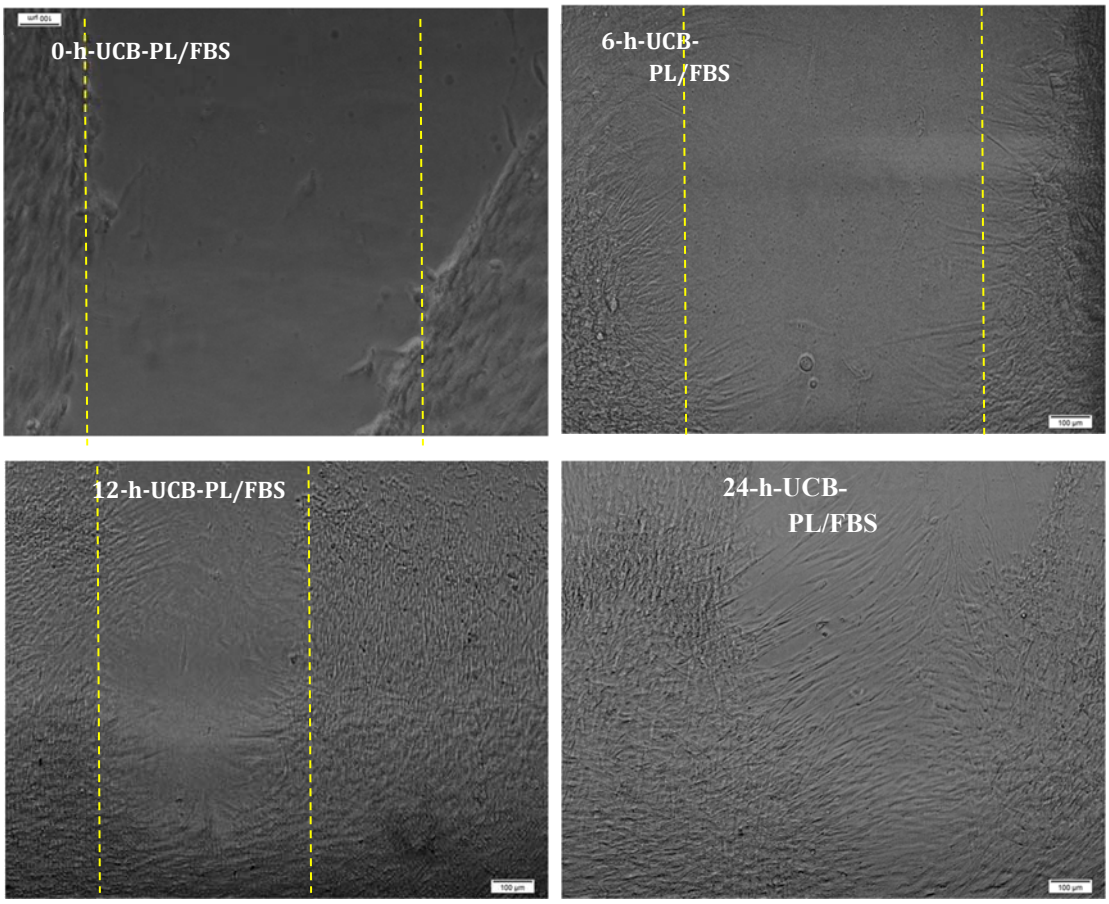


Figure 6: Measuring the effect of UCB-PL/FBS on the rate of cell migration in fibroblast cells by scratching method at 4 times of 0, 6,12, and 24 hours after scratching

Figure 6 shows that at this concentration, the scratch area was not completely covered by cells at 24 hours.

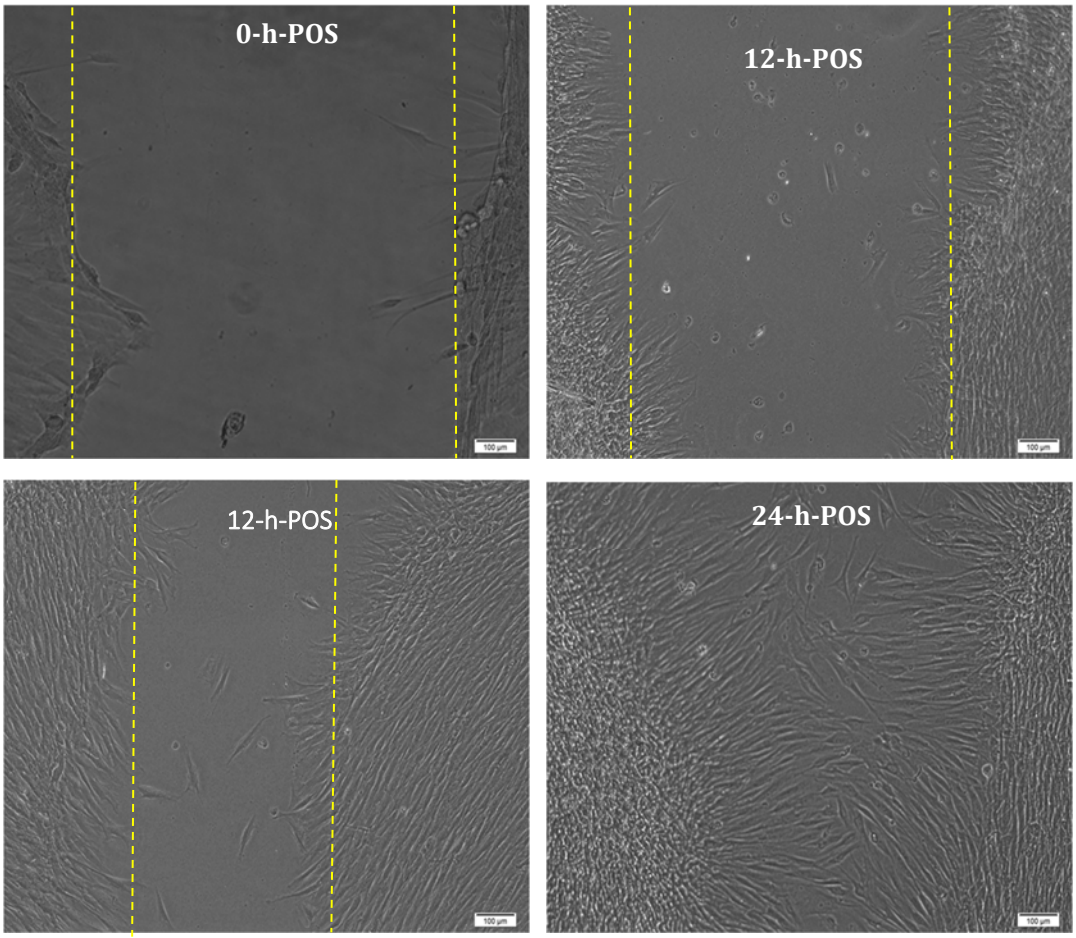


Figure 7: Measuring the effect of 10% FBS on the rate of cell migration in fibroblast cells by scratching method at 4 times of 0, 6, 12, and 24 hours after scratching

As can be seen in Figure 7, the results of the current laboratory indicate that a concentration of 10% is the highest effective concentration in cell migration.

Discussion

The present study aimed to investigate the effect of different concentrations of platelet extract derived from umbilical cord blood on the proliferation and migration of fibroblast cells. Examining the cell proliferation in the laboratory has shown that the highest increase in fibroblast proliferation is related to the concentration of 10% in the 3 mentioned times. Additionally, the results of the studies suggest that there is no increase in proliferation with an increase in concentration, meaning that in the concentration of 20% and 15% in times of 24, 48, and 72 hours, a decreasing trend in proliferation was also observed. Also, in the case of the negative control, the obtained results show that this group has a significant difference with all groups except for the concentration group of 20% ($p \leq 0.05$), and in the case of the UCB-PL/FBS group, an increase in proliferation compared to the negative control and positive control groups has been shown in the 3 mentioned times. ($p \leq 0.05$).

In the studies conducted by Filova et al., indicated the favorable effects of the PVA–PL (Poly vinyl alcohol Platelet lysate) nanomats on the three cell types involved in the wound healing process, and established PVA–PL nanomats as a promising candidate for further evaluation with respect to in vivo experiments. (10). However, the results of the present study were on the proliferation of fibroblast cells as a result of treatment with platelet extract derived from umbilical cord blood, in which the effective concentration was determined to be 10%. Also, in the study conducted by Ranzato in the evaluation of the toxicity of the extract-derived peripheral blood platelet on keratinocyte cells, the concentration of 30% and higher in fibroblast cells and 50% in endothelial cells were considered as a toxic effect (11), while the present study has shown a concentration of 20%. The difference in the effect of

effective concentration in our results was obtained with a significant difference of $p<0.5$.

Paparazzi showed the difference between the rates of platelet gel factors derived from umbilical cord blood and the platelet gel derived from peripheral blood (12). It can be concluded that due to the presence of more growth factors in platelet extract derived from umbilical cord blood compared to peripheral blood, the effectiveness of this product is greater so that in the platelet extract derived from umbilical cord blood at a concentration of 10%, a higher fibroblast proliferation was observed, but in the platelet extract derived from peripheral blood at a concentration of 19%, the highest value was observed. The difference in the results indicates that the platelet extract derived from umbilical cord blood with a lower concentration than peripheral blood can cause higher proliferation. The difference in the results may be due to the presence of more cytokine growth factors in UCB-PL than in PL.

Cell migration is one of the important and key functions of cells in the wound healing process since the cells cause the damaged area to be covered by this response in the wound healing process. Based on the results obtained in the study carried out by Ranzato et al to measure the effect of PL on the migration of keratinocytes, fibroblasts, and endothelial cells, the concentration of 20% was reported as the effective concentration on cell migration (13). The results of the present study have shown the concentration of 10% as the highest effective concentration in cell migration. As seen in the photos obtained from this examination, it is qualitatively confirmed that in 10% concentration, 24 hours after scratching, the space between the two edges is completely covered and the scratched area is filled with cells. However, in other concentrations except for the negative control group, the scratch area was not completely covered by cells, and in the negative control group, it was shown that there is no sign of complete wound closure in this hour, and after 24 hours, the first migrating cells have reached each other from both sides of the scratch edge.

The results of a quantitative and qualitative study of migration emphasize that the platelet extract derived from the umbilical cord blood has a higher effect on the migration of fibroblast cells than the peripheral blood platelet extract due to its richer bone growth factors. Ranzato et al. (2011) have reported that platelet extract products derived from peripheral blood control the expression of MMP 2 and MMP9 genes, which play an important role in the degeneration of the extracellular matrix and wound healing and cell migration (14). The above results in the migration of fibroblasts can be one of the reasons for the effect of the blood-derived platelet lysate product on the expression of genes related to cell migration such as MMP 2 and MMP9. Since proliferation and migration are events that require cellular and molecular mechanisms, studies have been carried out to understand these mechanisms. Ranzato E (2000) examined the effect of PL on treated fibroblasts in ERK1/2-P38-PI3K/AKT signaling pathways (3). By measuring the expression level of P ERK1/2-P38-P AKT P proteins compared to ERK1/2-P38-AKT proteins under the influence of PL by western blot technique, it was shown that this extract has an effect on all these pathways, but in the case of the PI3K/AKT pathway, this effect is more significant. Also, for keratinocyte cells, only the two ERK1/2-P38 pathways were examined and the result was the P38 pathway. As mentioned, the UCB-PL product contains many growth factors that trigger various signaling pathways.

Conclusion

Despite the difference between the results of the present study in the proliferation and migration of fibroblasts treated with platelet extract derived from umbilical cord blood and the results of previous studies on fibroblasts treated with platelet extract derived from peripheral blood and also since proliferation and the rate of viability and migration is one of the most important and key functions of the wound healing process, this product can be used as a medicine to accelerate and improve the wound healing process.

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Conflict of interest

None.

Financial support

None.

Ethics statement

None

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