THE EFFECT OF POLY-E-CAPROLACTONE NANOFIBERS WITH PLATELETS ON CELL PROLIFERATION AND VIABILITY

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Abstract

Platelets are effective source of growth factors and other bioactive molecules. They can be combined with an appropriate scaffold where the mechanical properties of the scaffold and bioactive properties of platelets modulate cell behavior in the site of the injury. In this study, we compared different concentrations of platelets adhered on PCL nanofibers and evaluated their effect on mouse 3T3 fibroblasts. Platelets in maximum concentration significantly stimulated the viability when it is compared with control. Proliferation of fibroblasts was significantly higher than in the control group cultured on the plain scaffolds. Cells were well- spread on the scaffolds with adhered platelets, whereas on the plain scaffolds, the cells created clusters. The effect of platelets was dose-dependent. It is obvious, that the combination of bioactive substances and PLC nanofibres significantly stimulates cells growth and metabolic activity of the tested cell type.

Keywords: Platelets, nanofibers, cell culture

1. INTRODUCTION

Proper wound dressings should enhance cell adhesion and stimulate cell proliferation and viability. Nowadays, lot of efforts is being made to create biocompatible and biodegradable scaffolds which mimic the natural microenviroment of the tissue of interest. This scaffold serves as a physical support for cell adhesion and it is subsequently degraded by the cells for the formation of the new extracellular matrix where they will grow and cooperate. Scaffold can be enriched by bioactive substances which affect cellular fate.

Platelets are small anucleated fragments derived from megakaryocytes and contain various growth factors and bioactive molecules such as PDGF, VEGF, EGF and IGF [1]. These mediators can influence cell behavior and are involved in the early stage of wound healing and regenerative processes. Several types of platelet products including platelet rich plasma, platelet lysate, and platelet rich fibrin can be fabricated. Platelet rich plasma is defined as an autologous platelet concentrate of suspended in a small amount of plasma [2]. It is a cost-effective and easy way of delivering the bioactive substances.

Electrospinning is a unique technique to create polymeric nanofibers. The fibers have nanoscale diameter and thus can mimic the microarchitecture of extracellular matrix. They are highly porous with interconnected pores, enabling the exchange of gases and nutrients [3]. Moreover, compared to micrometer-sized surfaces, nanofibers have a higher rate of protein adsorption support an adhesion of proteins [4].

Polycaprolactone is a biodegradable, biocompatible, linear, aliphatic polyester, which is widely used in tissue engineering applications of bone [5], cartilage [6] and heart and vessels [7]-[9]. It was approved by the FDA as sewing material and material for drug delivery [10].

This study was focused on comparison of the viability and proliferation of fibroblast cell line cultured on composite PCL nanofibers scaffolds enriched with different concentrations of platelet.

2. MATERIALS AND METHODS

2.1. Scaffold fabrication

Scaffolds were prepared from poly-ε-caprolactone by needleless electrospinning method. 24 % (w/v) solution of PCL (45 000 Da, Sigma Aldrich, MO, USA) was resuspended in chlorophorm:ethanol (ratio 9:1 v/v). Samples were electrospun by Nanospider NS500 (Elmarco, Czech Republic) using wire needleless electrode. The applied voltage range was 80-100 kV. The temperature was 24 ± 2°C and relative humidity 55 ± 10 %. The distance between the wire electrode and the collector was 20 cm.

2.2. Composite scaffold preparations

Samples with diameter of 0.6 mm were punched out from the electrospun mat. Subsequently, they were sterilized using 70 % ethanol for 30 minute and washed thrice with PBS. Human deleucotized platelet concentrate in plasma solution derived from apheresis was purchased from Blood Transfusion Service (UHKT, Czech Republic). Three concentrations of platelets were prepared (TAD max - maximum concentration, $915*10^9$ platelets/L; TAD 1/2 - half the maximum concentration, $457.5*10^9$ platelets/L; TAD ¼ - quarter the maximum concentration, $228.75*10^9$ platelets/L). Each concentration was adhered on the sterile nanofibrous scaffolds. Samples were incubated for 2 hours at 22°C and then were rinsed with PBS. The plain scaffolds served as a control.

2.3. Cell culture and seeding

Mouse 3T3-A21 cell line was purchased from Sigma-Aldrich, Munich, Germany. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % FBS and treated with penicillin/streptomycin (100 IU/mL, 100 µg/mL). Cells were kept in a humidified incubator (37°C, 10 % CO_2 and 80-90 % RH) and medium was changed every 3-4 days. Subconfluent cells were washed with PBS containing 0.02 % (w/v) ethylenediaminetetraacetic acid (EDTA) and treated with trypsin/EDTA solution. The detached cells were counted using a Bürker chamber. The scaffolds were seeded with fibroblasts with a density 8,850 cells/cm^2. All groups were supplemented with 5 % FBS.

2.4. Cell metabolic activity measurement

The metabolic activity of the fibroblast was determined using the MTS assay (CellTiter96® AQueous One Solution Assay; Promega, Madison, WI, USA). On the experimental days, the scaffolds were transferred to the new wells. To each scaffold, 20 µL of MTS solution within 100 µl fresh medium were added. After 2 hours of incubation, the absorbance of the metabolized solution (100 µL) was detected at 490 nm using Tecan Infinite® PRO 200 series reader (Tecan, Männedorf, SUI). The background absorbance (690 nm) was subtracted from the measured data, as well as the absorbance of the plain media.

2.5. Cell proliferation

To determine the cell proliferation, a fluorescence-based kit (Quant- iT™ PicoGreen® dsDNA Assay Kit; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used. The samples used for the MTS assay were transferred to 500 µL of cell lysis buffer (10 mmol L−1 Tris, 1 mmol L−1 EDTA, 0.2 % v/v Triton X-100). Then, three freeze/ thaw cycles were done to facilitate of the release DNA from the samples. Between each step, the samples were thoroughly vortexed. Fluorescence intensity was detected using Tecan Infinite® PRO 200 series reader Tecan (λex = 485 nm, λem = 528 nm) and the DNA content was determined using calibration curve of standards from the kit.

2.6. Confocal microscopy visualization

Samples were fixed with frozen methanol and subsequently washed with PBS and stained with DiOC (to 1 µg/mL in PBS, 30 minute) to visualize the cellular membranes and propidium iodide (5 µl/mL) to visualize the

cell nuclei. Between the incubations the samples were rinsed with PBS. A Zeiss LSM 510 DUO confocal microscope was used for imaging (λex = 488 nm, λem = 501 nm for DiOC6; λex = 536 nm,λem = 617 nm for propidium iodide).

2.7. Scanning electron microscopy visualization

Samples were fixed with 2.5 % glutaraldehyde ((Sigma-Aldrich, MO, USA) for 2 hours in 4°C. Afterwards the samples were dehydrated with the ethanol changes (35 %, 48 %, 70 %, 96 % and 100 %). Hexamethyldisilazane (Sigma-Aldrich, MO, USA) was used to remove any residual water from the samples. The samples were sputter-coated with gold (app. 30 nm) in the Quorum Q150R (Quorum Technologies, United Kingdom) and visualized using Vega 3 SBU microscope (Tescan, Czech Republic).

2.8. Statistical analysis

Quantitative data are presented as mean ± standard deviation. The data were evaluated using SigmaStat 3.5 software. Statistically significant differences between the groups with normal distribution were evaluated with ANOVA method and Tukey test for post hoc analysis. The data with abnormal distribution were tested with Kruskal-Wallis test and Dunn's method for post hoc analysis. The statistically significant level was accepted as 5 %

3. RESULTS

Metabolic activity of the seeded cells was evaluated using MTS metabolic assay on day 1, 3, 8, 10 and 14 (**Figure 1**). PCL nanofibers functionalized with the maximum concentration of platelets (TAD max) exhibited a significantly higher viability of cells in comparison to the control group (PCL). On day 1 and 3, there was no significant difference between the groups. From day 8 up to the day 14, it was obvious that with increasing concentration of platelets, the metabolic activity of the cells increased. Proliferation of the cells were measured by PicoGreen assay (**Figure 2**). The results showed increase of DNA on all the scaffolds functionalized with platelets from day 3. All groups with platelets exhibited gradually increasing number of cells in a dose-independent manner. On the control group, the DNA content was very low and oscillated during the experiment. It is obvious that the scaffolds functionalized with platelets stimulated cell proliferation in vitro. Results from both assays are in concordance with the results of Vocetkova et. al., where platelets in additive solution adhered on PCL scaffold were used and seeded with dermal cells. Fibroblast exhibited better proliferation and metabolic activity when seeded on the functionalized scaffolds [3]. Diaz-Gomez et. al. cultured human adipose-derived mesenchymal stem cells on PCL nanofibers functionalized with platelets. These functionalized scaffolds increased cell adhesion and proliferation [11].

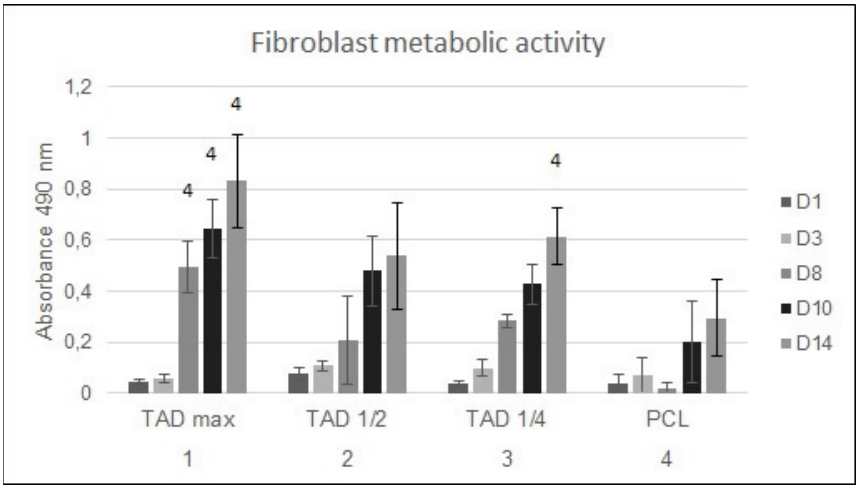

Figure 1 Metabolic activity of 3T3 fibroblast

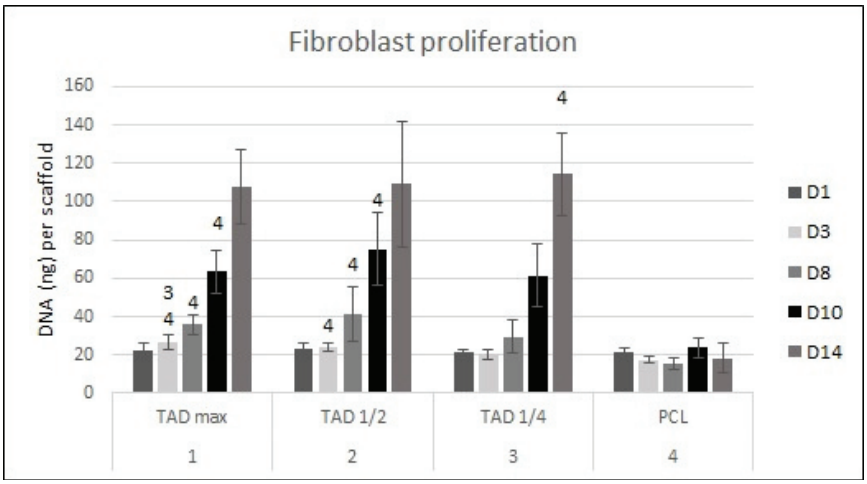

Figure 2 Proliferation of fibroblast

Confocal microscopy was used to visualized the cells seeded on the scaffolds (**Figure 3**). Cells seeded on the functionalized scaffolds were well spread and did not form the clusters as on the PCL control scaffolds. These clusters indicated unfavorable cultering conditions.

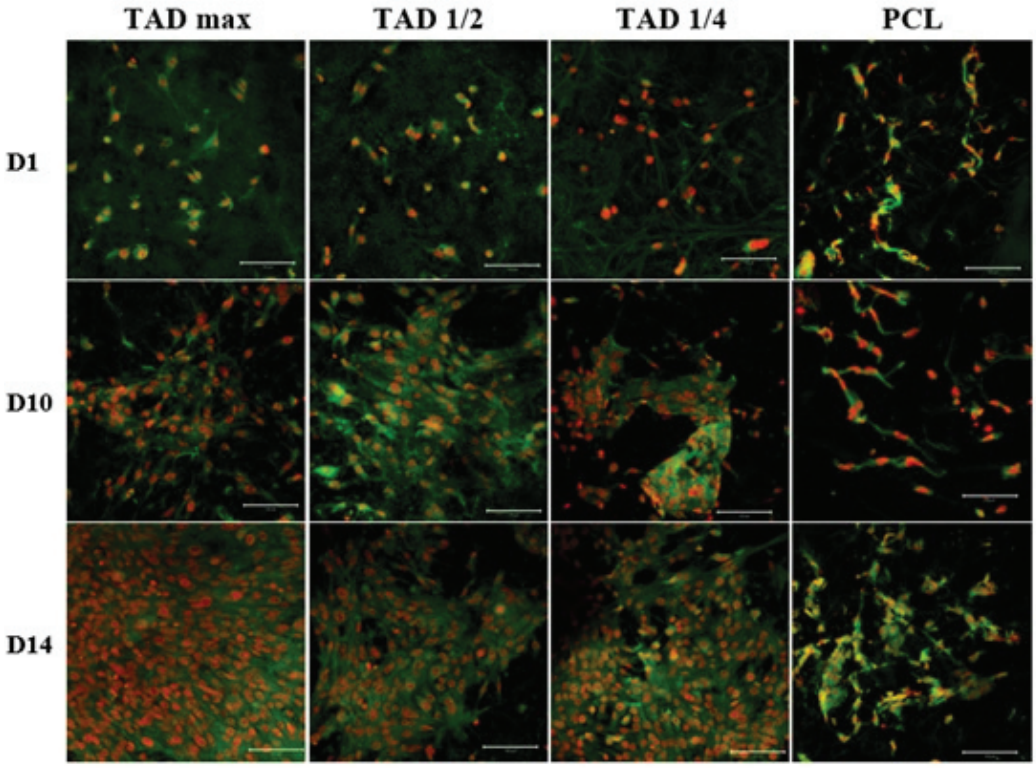

Figure 3 Confocal microscopy visualization of fibroblasts seeded on the scaffolds (magnification 200x, scale bar 100 μm)

To visualize the surface of the nanofibers with platelets and cells, scanning electron microscopy was used (**Figure 4**). The fibrin network was visible in all the samples with platelets. Lesczak et al. tested different types of surfaces to observe blood compatibility. They found that platelet/leukocyte complexes were formed and specific markers for platelet activation were expressed on PCL surfaces [12].

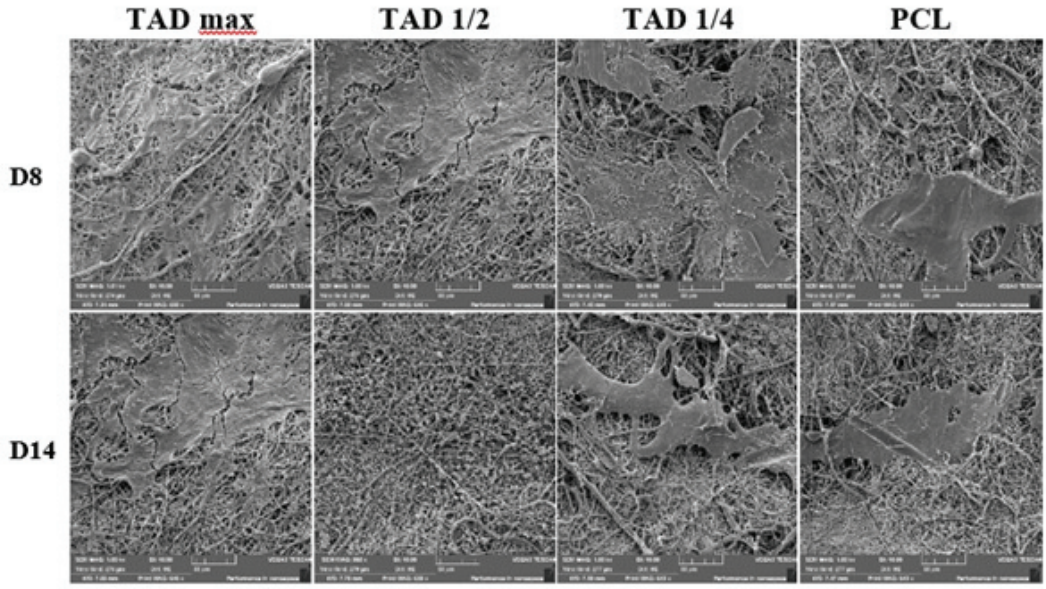

Figure 4 SEM visualization of fibroblasts on scaffolds (magnification 1000x, scale bar 50 μm)

4. CONCLUSION

Nanofibrous scaffold from poly-ε-caprolactone is, thanks to its properties, a suitable material for tissue engineering applications. The scaffold can be combined with bioactive molecules which affect cellular fate. We have previously tested the effect of platelets in additive solution, adhered on PCL nanofibers prepared by centrifugal spinning, on MG-63 cell lines. In the current study, we used pooled platelets in plasma adhered on PCL nanofibers prepared by electrospinning method. As a model for *in vitro* testing, 3T3 fibroblasts were used. It was observed that platelets and plasma had synergistic effect and positively influenced cell proliferation and viability. Our results clearly demonstrated that all concentrations of platelets in plasma are sufficient to ensure cells growth.

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