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Chapter 2. ADSC DIFFERENTIATION INTO OSTEOBLASTS IN THE PRESENCE OF TISSUE SCAFFOLDS OBTAINED FROM CORE-SHELL MICRO- AND NANOFIBERS WITH ADDITION OF Cu

2.1. Introduction

Biomaterials in regenerative medicine have a variety of use, but the most common is to serve as scaffolds. They are providing physical support, delivery of cells and mobilization of endogenous cells to repair tissues. Thanks to this they play a key role in the reconstruction and replacement of hard to regenerate tissues lost due to trauma or disease [1].

Amongst the most challenging to heal injuries are large bone defects as they are beyond bone's capacity to completely regenerate without medical ingerence. Biomaterials in those defects provide required stabilization and support allowing bones to restore their function. Currently, the gold standards of treatment are bone autografts and allografts. However, they are burdened with numerous disadvantages, such as limited sources, risk of immune rejection or disease transmission [2]. To increase the benefit/risk ratio for the recipient currently used graft could be replaced with polymer scaffolds. In addition to reducing the risk of immune response, lowering the cost of procedure and their overall conveniency they bring more treatment strategies. They allow for performing autologous cell stimulations, stem cell implantation and differentiation [3].

One of the most widely use biomaterial in tissue engineering is polycaprolactone (PCL). It is synthetic polymer characterized by high strength, relatively long

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degradation time, good biocompatibility, and relatively low cost of production. Its properties make it a great candidate to produce improved cell scaffolds. Moreover, with carefully selected additives its features can be easily enhanced making the therapy more successful and convenient for patient [4].

Lately copper has caught the attention of many scientists as a potential additive to biomaterials. Its antibacterial properties may prevent post implant sepsis, which also leads to limitation of antibiotics use during the recovery period. Furthermore, multiple studies show that copper promotes angiogenesis. It might contribute to the copper ability to stimulate osteogenesis, which was also show in some studies. Taking in consideration those properties copper seems to be a good candidate for addition to biomaterials especially those dedicated for bone regeneration [5, 6].

2.2. Material and methods

2.2.1. Biomaterial production

Biomaterials used in this study were produced in two stages. First the solutions for nanofibers were mixed, starting with combining tetrahydrofuran and dimethylformamide with 300 nm copper nanowires in concentration of 1% or 5% in ultrasonic homogenizer. To this solution polycaprolactone was added to obtain PCL/Cu solution. Further, obtained solution was combined with 17% polycaprolactone solution to obtain a core-shell nanofibers called PCL//PCL/Cu/1% or PCL//PCL/Cu/5%.

In the second stage obtained solutions were used to produce fibers using electrospinning technique. Fibers obtained in an electrostatic field falling on the collector surface formed a non-woven fabric. During the works, the conditions for the production of micro and nanofibers were established/optimized. The following manufacturing conditions were used in the preparation of the fibers: (i) solution flow: 10 ml/h; (ii) collector type used: rotary, (iii) collector rotational speed 20 rpm, (iv) voltage: 0.95-1.00 kV/cm; (v) temperature of the solution 24°C, (vi) temperature of the gas (atmospheric air) flowing through the working chamber: 23°C; (vii) gas humidity in the working chamber: 35%.

2.2.2. SEM pictures

The SEM Scanning Electron Microscope was used to study the structure of micro and nanofibers. The samples were tested at a magnification of 5000 to 10000 x. As the tested samples are mostly made of polymers, before testing, the samples were placed in a sputtering machine where, after creating a vacuum, an electrically conductive silver coating with a thickness of several nm was applied. After the sputtering process, the samples were placed in the SEM microscope and the structure research was started.

2.2.3. Cell culture

Experiments were conducted using Adipose Derived Stem Cells (ADSC) which were cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza 12-604F/U1) with 10% Fetal Bovine Serum (FBS, Corning 35-079-CV) and 1% Antibiotic-Antimycotic (AA, Gibco 15240-062) and incubated at 37°C, 5% CO_2.

2.2.4. Biomaterial extracts preparation

Biomaterials were sterilized by 1h incubation in 70% ethanol, which was followed by rinsing with Phosphate Buffered Saline (PBS, Lonza 0000708833). Biomaterials were left to completely dry for 2-3 h and then incubated for 24 h in standard cell culture medium (DMEM 10% FBS 1% AA) at 37°C, 5% CO_2.

2.2.5. MTT Assay

For MTT test ADSC were seeded in 96-well plate and incubated for 24 hours. After the time cells medium was replaced with fresh portion of the standard medium, biomaterial extracts in concentration of 100% and 50% and 5% DMSO (Corning, 25-950-CQC) solution. The next day media was changed to MTT solution with which cells were incubated for 2 hours. The MTT solution has been prepared by dilution of 3 mg Thiazozyl Blue Tetrazolium Bromide (Sigma, MKCK7253) in 3 ml DMEM without phenol red (Gibco, 2036286). The resulting formazan crystals were then dissolved with isopropanol. The absorbance was measured spectrophotometrically at 570 nm.

2.2.6. ADSC differentiation into osteoblasts

ADSC differentiation into osteoblasts was carried out by seeding cells into 12-well cell plate and incubation till the cell's confluency reached 80%. Then the medium was changed to Induction Medium with or without biomaterial extracts. Cell media was changed for fresh portions every 3-4 days for 3 weeks. The Induction Medium consist of: DMEM, 0.05 mM L-Ascorbic Acid 2-phosphate sesquimagnesium salt hydrate (Sigma, A8960), 0.1 μM Dexamethasone (Sigma, D4902), 10 mM β-glycerophosphate disodium salt hydrate (Sigma, G9422), 10% FBS, 1% AA. Cell morphology was observed daily.

2.2.7. Alizarin Red staining

To perform Alizarin Red staining cells were fixed with 2% formaldehyde and incubated with Alizarin Red solution (Millipore, 2003999) for 0.5 h, which was followed by rinsing with distillated water. Cells were observed using light microscope. To obtain quantitative results, Alizarin Red dye was extracted from cells by incubating them with acetic acid, scraping from the bottom of the well, which was followed by incubation at 80°C. Meanwhile the Alizarin Red standards were prepared. The absorbance was measured at 405 nm.

2.2.8. Statistical analyzes

The statistic evaluation was performed using R-studio. The data normality was tested by Shapiro-Wilk test, based on the obtained results Wilcoxon signed-rank test or Student's t-test were carried out.

2.3. Results

2.3.1. PCL//PCL/Cu/1% and PCL//PCL/Cu/5% fabrics

Uniform microfibers of PCL//PCL/Cu/1% and PCL//PCL/Cu/5% were obtained, which formed non-woven fabric (Fig. 1).

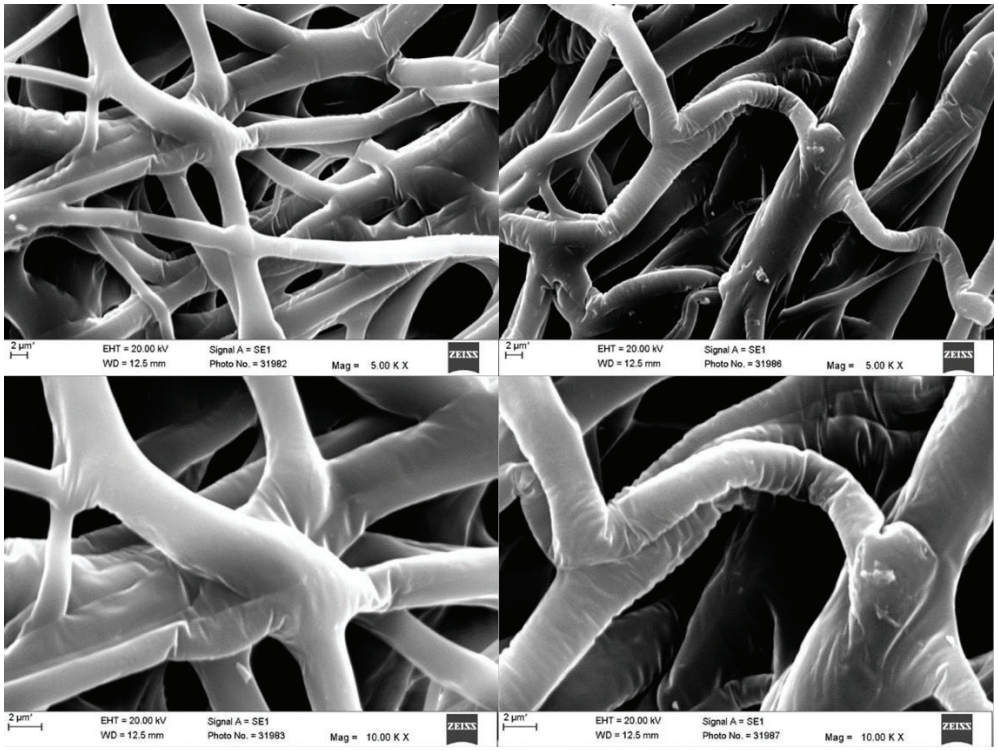

Fig. 1. The structure of the non-woven fabric. Images were taken using a scanning electron microscope (SEM)

Rys. 1. Struktura włókniny. Obrazy wykonano przy użyciu skaningowego mikroskopu elektronowego (SEM)

2.3.2. Biocompatibility of biomaterials extracts

The viability of cells incubated in biomaterials extracts was not markedly affected as at least 94.11% of cells remained viable upon testing (Fig. 2). In other variants of biomaterial extracts ADSC viability was higher than for cells incubated in standard cell culture medium reaching up to 119.13% for 50% PCL//PCL/Cu/5% extract and 111.7% for 100% PCL//PCL/Cu/1% extract.

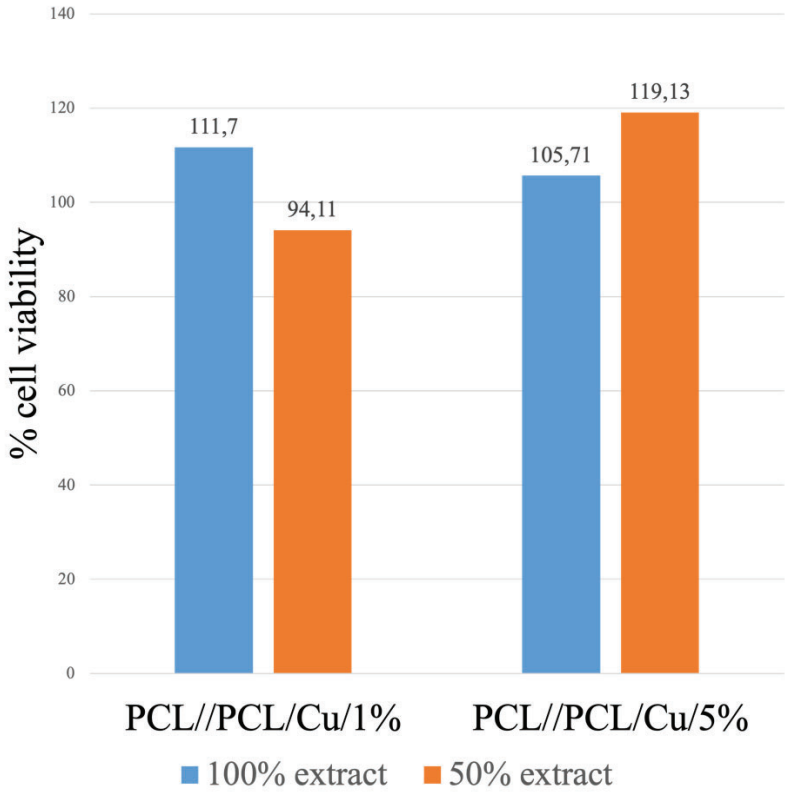

Fig. 2. Biocompatibility of PCL//PCL/Cu/1% and PCL//PCL/Cu/5% extracts
Rys. 2. Biokompatybilność ekstraktów z PCL//PCL/Cu/1% i PCL//PCL/Cu/5%

2.3.3. ADSC differentiation into osteoblasts

As a result of Alizarin Red staining, more stained calcium deposits were observed in the well, where cells were grown in the induction medium with the addition of biomaterials extracts than in wells where cells were incubated in induction medium without extracts (Fig. 3). For cells incubated with PCL//PCL/Cu/5% extract the amount of stained calcium deposits was higher than for cells incubated with PCL//PCL/Cu/1% extract. In well where cells were incubated in DMEM 10% FBS 1% AA no calcium deposits were observed.

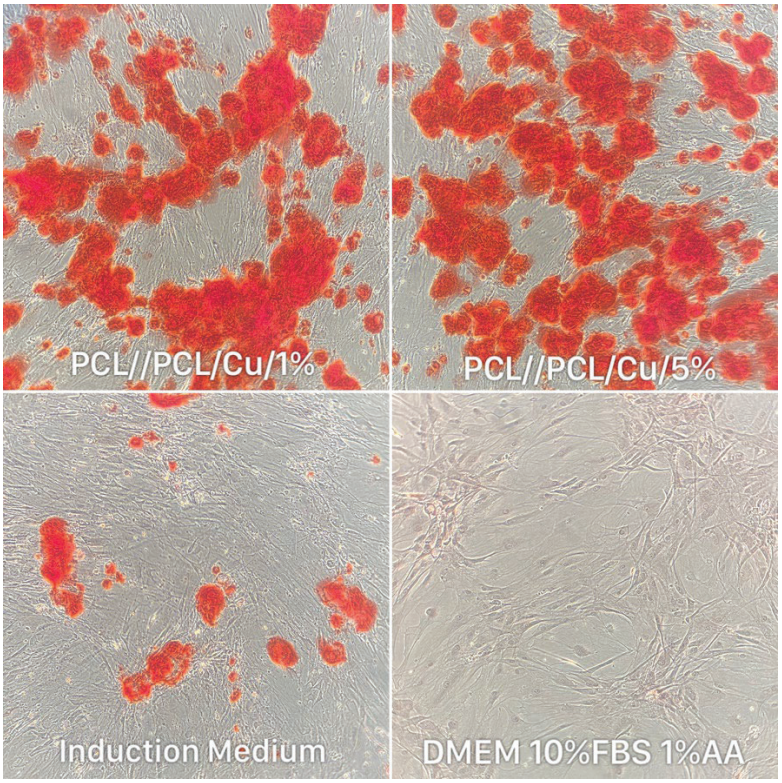

Fig. 3. Calcium deposits stained with Alizarin Red
Rys. 3. Złogi wapnia wybarwione Alizarin Red

The presence of copper significantly (p-value < 0.001) positively affected the differentiation ADSC cells into osteoblasts, as the staining intensity of Alizarin Red was three to four times higher for cells incubated with the addition of Cu-doped biomaterial extracts than for cells incubated without. Moreover, higher Alizarin Red dye concertation was observed in cells incubated with addition of PCL//PCL/Cu/5% extracts than for cells incubated with addition of PCL//PCL/Cu1% extract (Fig. 4).

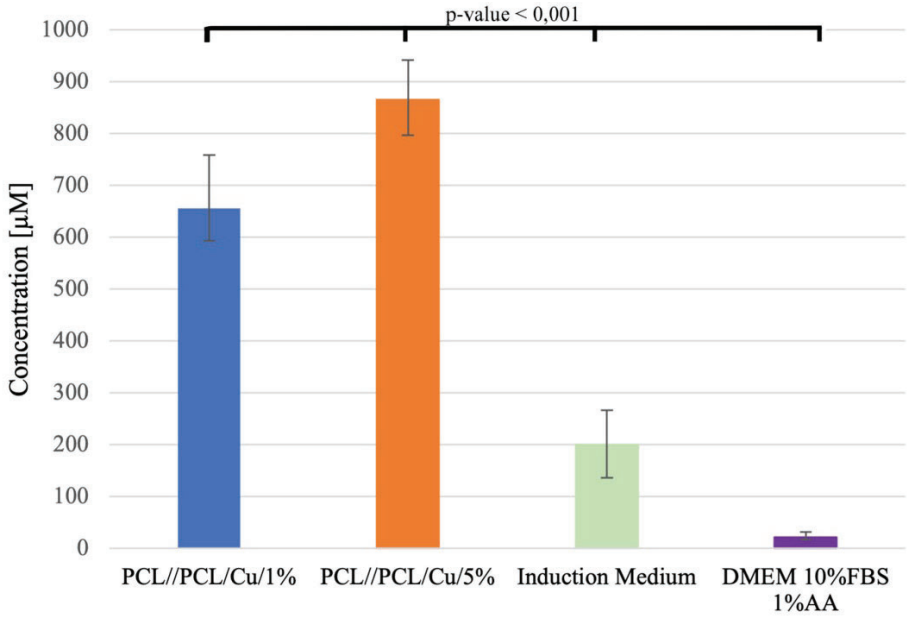

Fig. 4. The intensity of Alizarin Red staining which semi-quantitatively reflects upon the transdifferentiation of ADSC into osteocytes
Rys. 4. Intensywność barwienia Alizarin Red, która w sposób półilościowy odzwierciedla transdyferencjację ADSC do osteocytów

2.4. Discussion and concussion

Polycaprolactone is a well-known biomaterial that thanks to its desired properties found a variety of usage in regenerative medicine. Nonetheless, each new variant of it created by combining it with any additive must be tested for biocompatibility and changes in properties.

Based on performed analyzes it can be concluded that produced PCL//PCL/Cu/1% and PCL//PCL/Cu/5% extracts have no toxic effect on ADSC. Furthermore, the observed increased viability of cells suggests that the addition of copper may stimulate the proliferation of cells. This assumption can therefore be confirmed by the data available in the literature, where authors show that copper stimulates the proliferation of different cell types. Burghardt et al. found that copper in a specific concentration range stimulates the proliferation of mesenchymal stem cells [7]. Alizadeh et al., have shown that copper nanoparticles enhance endothelial cell proliferation and Philips et al. presented that copper cause an increase in dermal fibroblasts proliferation [8, 9].

ADSC differentiation into osteoblasts was performed based on methodology, which was established by combining two methods in order to achieve sufficient efficiency

[10, 11]. Obtained results suggest that the addition of biomaterial extracts significantly stimulates osteogenic differentiation. Taking into consideration numerous reports from the literature on the stimulating effect of copper on osteogenesis it can be concluded that an increased number of calcium deposits are caused due to the presence of copper [12–14]. Observed stimulation may be occurring because of copper's ability to enhance the alkaline phosphatase enzyme activity and osteogenic gene expressions [15, 16].

Taking into consideration all performed analyses it can be concluded that Cu-doped PCL may serve as a promising proto-material for the development of tissue structures facilitating the differentiation of ADSC into osteoblasts and it may find potential application in the production of scaffolds for bone regeneration.

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Abstract

Polycaprolactone (PCL) is a biodegradable polymer widely used in medicine as a biomaterial for tissue regeneration. This biomaterial is characterized by high strength, relatively long degradation time and good biocompatibility. Copper in pure form is highly cytotoxic, but at the same time, it is an element with very good electrical and thermal conductivity. In this study, the PLC polymer was combined with copper nanowires and transformed into core-shell fibers upon application of electro-spinning. The end-product formed a highly porous sheet-like structure suitable for serving as, an artificial fibrous cell scaffold. Obtained fibrous scaffolds were examined for the influence on the differentiation of ADSC into osteoblasts.

Here we show shown that: (i) the viability of cells incubated in biomaterial extracts was not markedly affected as at least 94.11% of cells remained viable upon testing, (ii) the presence of copper significantly (p-value < 0.001) positively affected the differentiation ADSC cells into osteoblasts, (iii) the staining intensity of Alizarin Red was three to four times higher for cells incubated with the addition of Cu-doped biomaterial extracts than for cells incubated without.

Based on the above results, we conclude that Cu-doped PLC may serve as a promising proto-material for the development tissue-structures facilitating the differentiation of ADSC into osteoblasts.

Keywords: polycaprolactone, differentiation, core-shell fibers, copper