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Investigation of human mesenchymal stem cells culture on nanofibrous polyurethane scaffolds and films

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ABSTRACT

Purpose: of this study was to evaluate whether electrospun porous nanofibrous scaffold of polyurethane (PU) with low and high beads accommodate the viability and growth of human bone marrow mesenchymal stem cells (hBM MSCs) in comparison with flat surface (Polypropylen).

Design/methodology/approach: To our knowledge, the influence of the beads density on nanofibrous scaffold has never been investigated. For this purpose, we electrospun PU to fabric two porous nanofiber scaffolds with less and high density beads to enhance cells attachment and proliferation of hBM MSCs. Moreover, those surfaces were compared to a flat surface (PP). The samples were studied using scanning electron microscopy (SEM), Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) and static contact angle measurement.

Findings: The characterization of the samples revealed that hydrophilic surface of high quantity nanofiber with fewer beads scaffolds (LBNF-PU) had less nanofiber with higher quantity of beads that were overlapped on each other firmly compared to low quantity nanofiber with more beads scaffolds (HBNF-PU). MSCs cell morphology on both HBNF-PU and LBNF-PU nanofibrous scaffolds and flat surface was different; it was observed elongated cell shape for LBNF-PU and flat surface and rounded cell shape for HBNF-PU. Live/dead studies confirmed cell viabilities on flat and nanostructured surfaces. Cells expansion on Polypropylen and nanofibrous scaffolds were increased until 7 days of culture.

Research limitations/implications: The randomly nanofiber scaffold limited the growth of human bone marrow mesenchymal stem cells (hBM MSCs). The aligned nanofiber scaffold will be evaluated at next investigation.

Originality/value: Nanofibrous scaffold have recently draw attention for potential applications in small vascular replacement. Human bone marrow mesenchymal stem cells (hBM MSCs) growth on porous nanofibrous scaffolds is a promising strategy for tissue engineering. The influence of the beads density on nanofibrous scaffold has never been investigated. For this purpose, we electrospun PU to fabric two porous nanofiber scaffolds with less and high density beads to enhance cells attachment and proliferation of hBM MSCs.

Keywords: Nanomaterials; Biomaterials; Mesenchymal stem cells; Polyurethane; Electrospinning

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MATERIALS

1. Introduction

In vascular tissue engineering polymeric vascular grafts such as non-absorbable PET (woven fibres), PU and Polypropylen (PP) were using successfully in replacement of large diameter blood vessels (inner diameter > 6 mm) and they do not elicit cytotoxicity. However, polymeric grafts (inner diameter < 6 mm) expressed poor patency due to thrombosis and hyperplasia [12]. The cell culture of endothelial cells onto the luminal surface of the polymeric graft seems a potential way to improve the patency of polymeric grafts (inner diameter < 6 mm) [14]. In addition the new materials called nanofibrous scaffold (NFS) could be an alternative for replacement of small vascular grafts. The main advantage of nanofibres is that they offer higher surface area compared with rivals [23]. Nanofibre scaffolds have been shown to enhance cell adhesion, proliferation, and extracellular matrix synthesis compared with microfiber scaffolds [19]. Electrospinning allows the production of randomly oriented or aligned nanofibres from a variety of polymer materials, including synthetic and natural polymers [1,5,20,25]. The technique consists of applying an electric field between the injection device tip and the collector wherein fibrous jets travel to form the nanofibrous scaffold on the ground. This scaffold had a porous structure with a fibre diameter of several hundred nanometres [1,5,8].

To date, numerous synthetic NFS polymers such as polycaprolactone (PCL), Poly-L-lactide (PLLA), Poly(glycolic acid) (PGA), Polyethylene terephthalate (PET) and Polyurethane (PU) are widely studied as support of smooth muscle cells (SMCs) and endothelial cells growth [10,11,13,14,17,22]. For example, collagen coated poly(L-lactic acid)-co-poly(ε-caprolactone) P(LLA-CL) nanofiber mesh increased the attachement, viability and spreading of human coronary artery endothelial cells [11].

Stem cells are defined by their ability for self-renewal and differentiation. Mesenchymal stem cells (MSCs) that are known as adherent cells (fusiform and cuboidal structure), are able to differentiate themselves into other cell types including; adipocytes, chondroblasts, cardiomyocytes, osteoblasts and other connective tissues when exposed to an appropriate stimulus [7]. MSCs possess also immunophenotype characteristics as well as specific cell-surface markers, and are positive for markers such as CD105, CD73 and CD90 but negative for hematopoietic markers CD3, CD34, CD45 [3]. The interaction of polyurethane-MSCs has been investigated from researchers. It has been demonstrated that polyurethane morphology in nanoscale dimension can influence cell structure and function [9]. Zahedmanesh et al. showed that fibrin-polyurethane composite scaffolds support chondrogenesis of human bone marrow (hBM) derived MSCs [24]. Similar supporting effect of PU (meniscal-shaped scaffold) on hBM MSCs in bioreactor system has been reported by Liu et al. [16]. Niu et al. highlighted the good mouse BM MSCs derived cardiomyocyte growth on PU compared to other polymer, such as polypropylene carbonate [18].

To our knowledge, the influence of the beads density on nanofibrous scaffold has never been investigated. For this purpose, we electrospun PU to fabric two porous nanofibres scaffolds with less and high density beads to enhance cells attachment and proliferation of hBM MSCs. Moreover, those surfaces were compared to a flat surface (PP). The samples were studied using scanning electron microscopy (SEM), Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) and static contact angle measurement.

2. Materials and methods

2.1. Materials

The granules of polyurethane (PU) and N-dimethylformamide were purchased from Inovenso Ltd (İstanbul, Turkey) in order to prepare the polymeric solution for electrospinning. The polypropylene (PP) film biaxally oriented with thickness of 0.03 mm is obtained from Goodfellow Ltd. (Huntingdon, England)

2.2. Production of nanofibres

Two types of PU nanofibres mats were produced with electrospinning technique; high quantity nanofibres with

fewer beads scaffolds (LBNF-PU) and low quantity nanofibres with more beads scaffolds (HBNF-PU). For this purpose, the polymer solution of 15 wt. % PU and 85 wt. % N-dimethylformamide (DMF) were purchased from Innovenso is prepared. Then the solution was fed into a 5 ml standard syringe set on a micro pump (KDS 100, KD Scientific, Holliston, MA) ready to spin. High voltage was applied between the tip (stainless steel needle) and collector (aluminium foil) to electrospin the polymeric solution. To obtain HDF mats a 15 kV and a flow rate of 1.25 ml/h and LDF a 25 kV and 1.5 ml/h were applied. Nanofibres mats collected on aluminium foil were dried overnight under vacuum.

2.3. Characterisation of nanofibres and film

First static contact angle measurements were achieved on samples by means of the NRL Contact Angle Ganiometer, (Model No: 100-00). The scanning electron microscopy (SEM; model LEO 1430 VP) was used to analyse the morphology of mats and cell adhesion on the samples. Prior to analyse, the samples were coated with gold using sputter coating. The images of SEM were analysed to determine the diameter of fibres and beads with ImageJ software (free edition) (National Institutes of Health, USA). In order to probe the chemical structure of the samples Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR-Model: MIDAC) spectroscopic analysis was performed in a range of 500-4500 cm^{-1} at a resolution of 4 cm^{-1}.

2.4. Human bone marrow mesenchymal stem cell culture

Human mesenchymal stem cells (hMSCs) obtained from whole bone marrow (BM) cells (StemCell Technologies, Vancouver, Canada). They were cultured in DMF10 (60% Dulbecco's Modified Eagle's Medium – Low glucose (DMEM-LG, Biochrom, Berlin, Germany) + 40% MCDB-201 (Sigma, St-Louis, MO, USA) supplemented with 10% FBS (Biochrom), 1% penicillin and streptomycin (Sigma-Aldrich, St-Louis, MO, USA) and were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 10 days then the culture medium was changed once in every 2-3 days. At 80-85% confluence, adherent MSC cells were trypsinized with Trypsin solution (Gibco Invitrogen), and cell viability was checked by trypan blue dye exclusion. In the present study, passage 3 (P3) BM-MSCs were used.

2.5. Flow cytometry analysis

Flow cytometric analyses of P3 BM-MSCs were performed on a FACS Aria flow cytometer (Becton, Dickinson Biosciences) to evaluate BM-MSCs in terms of expression of main MSC surface markers CD73 (BD Biosciences), CD90 (BD Biosciences), CD105 (eBioscience) and lack of expression of hematopoietic stem cell markers CD34 (BD Biosciences). All markers were conjugated with either fluorescent isothiocyanate, allophycocyanin or phycoerythrin. BM-MSCs were trypsinized and washed with PBS. To evaluate BM-MSCs marker profile, 1.5 x 105 cells were suspended in 100 mL PBS-BSA-Na azide with 2 mL of each flow cytometry antibody in a separate tube and incubated for 30 min. in the dark. At the end of incubation, cells were washed twice with PBS and finally diluted in 200 mL PBS-BSA-Na azide. The analysis of cells was performed according to 10,000 event count with the FACS Aria. The acquired data was analyzed by using BD FACS Diva Software v6.1.2 (Beckon Dickinson Biosciences).

2.6. Cell proliferation and Live/Dead cell viability assay

Cell proliferation were carried out with hBM MSCs that were seeded at the density of 15.000 cells per samples (PP film, LBNF-PU and HBNF-PU nanofibrous scaffolds) and were incubated in 12-well tissue culture plates. Uncoated coverslips were used as control. Cultures were carried on for 7 days. BM-MSCs adherence onto the plastic surface of the culture flask and fibroblast like morphology were investigated under an inverted light microscope at days 3 and 7. Cells cultured on nanofibers and control were analyzed at days 3 to 7 with scanning electron microscopy. Cell viability was assessed using the LIVE/DEAD Assay Kit (Calbiochem-Merck Millipore) at days 3 and 7. Live cells were stained with a cell-permeable green fluorescent Cyto-dye and dead cells were stained with propidium iodide. Stained live and dead cells was visualized by fluorescence microscopy using a band-pass filter which detects FITC and rhodamine. Viable cells were stained only with the Cyto-dye, fluorescing green, whereas the dead cells were stained with both Cyto-dye (green) and propidium iodide (red), resulting in a yellow fluorescence.

3. Results

3.1. Characterisation of nanofibrous scaffolds and film

Recent years, number of novel scaffolds developed for tissue engineering increased sharply. The most commonly investigated materials remain nanofibrous scaffolds. Porous NF scaffolds with high or less beads can influence the cell adhesion, migration and proliferation. At to date, the effects of beads were not investigated in the literature. SEM images of electrospun nanofibrous scaffolds revealed porous, nanoscaled fibrous with beads and randomly oriented fibres (Figure 1a). The fibre and beads diameters of LBNF-PU were in the range of 150 nm ± 50 nm and 2.2 µm ± 0.5 µm, respectively obtained. This fibre diameter of PU for cell culture was equivalent to other studies [10,12]. Moreover, the beads were not firmly connected or overlapping to each other. The rival, HBNF-PU had fewer fibres in the range of 250 nm ± 25 nm with higher beads on the surface.

The beads were overlapping on each other firmly and connected with fibres. The average of beads diameter was found approximately 3.8 µm ± 0.5 µm (Figure 1a). The wettability of scaffolds is one of the factor that influence cell behaviour such as adhesion and proliferation. LBNF-PU scaffold were found highly porous and hydrophobic with a contact angle of 110° ± 5. However, with increasing beads density, HBNF-PU scaffolds became less hydrophobic with a contact angle of 65° ± 5. This difference may be explained by that HBNF-PU has a high quantity beads with low nanofibres that possibly diminished surface area to volume ratio compared to LBNF-PU and make it more hydrophilic. For PP, the surface was found more hydrophilic than NF with a contact angle of 35° ± 5 (Figure 1c). We further confirmed the difference between LBNF-PU and HBNF-PU by using ATR-FTIR. Figure 1b shows the ATR-FTIR spectra of NF scaffolds and flat surface. The NF scaffolds and PP infrared spectrum revealed similar absorption between 2500-3500 cm^{-1} that can be attributed to C-H alkenes stretching. The peak observed at 2359 cm^{-1} for HBNF-PU corresponding to excess of OH that was not observed for LBNF-PU. 1700 and 1526 cm^{-1} are typical band of the stretching C=O and N-H bonds [21]. For PP film, the peak observed at 2968 cm^{-1} was attributed to stretching vibration asymmetrical CH_3 while at 1375 cm^{-1} corresponding to deformation vibration symmetrical of CH_3. In overall, the infrared spectra identified the organic functions and chemical bonds in the tested materials for cell adhesion and growth.

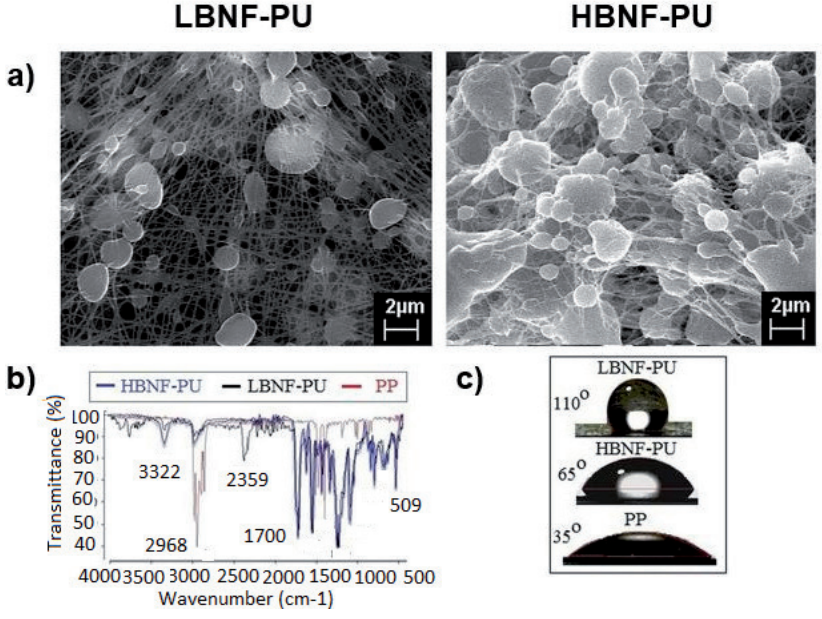

Fig. 1. Nanofibrous scaffolds characterization: a) SEM images of LBNF-PU and HBNF-PU scaffolds, b) ATR-FTIR spectra of LBNF-PU, HBNF-PU scaffolds and PP film, c) water contact angle measurement of LBNF-PU and HBNF-PU scaffolds and PP film

3.2. Characterization of hBM MSCs

Undifferentiated BM MSCs maintain spindle shape morphology (Figure 2a). Flow cytometric phenotyping of hBM MSCs cultured until passage 3 revealed positivity for typical mesenchymal markers CD73, CD105 and CD90 and negativity for representative hematopoietic marker CD34 (Figure 2b).

3.3. Cell morphology on nanofibrous scaffolds

Cells were seeded on the coated surfaces and their morphologies were checked at different time points (first, third and seventh day of culture). Uncoated coverslips were used as control. Representative images of cultures at different time after seeding are shown in Figure 3. Cells showed adhesion and expansion on the surfaces coated with control, PP and HBNF-PU. The typical spindle shaped morphology and the gradual.

Formation of a monolayer without substantial difference in comparison to the uncoated control surface was evident (Figure 3). Due to complex structures of LBNF-PU and HBNF-PU, cells could not be detectable with confocal microscopy. To assess the response of the BM MSCs toward the nanofibres, cells were analysed with SEM.

3.4. SEM analysis

After 3 and 7 days of culture, the samples were analysed with SEM and the results were presented at Figure 4. It was observed that the hBM MSCs were apparently attached to samples and associated with fibres in all directions. Moreover, they penetrated into the pores of NF scaffolds (Figure 4).

Also, the dimension of cells was higher on NF scaffolds than flat surfaces (data not shown). On the surface of NF scaffolds, elongated cell shape and at some area rounded cell shape was observed, confirming that the seeded hBM MSCs attached to both nanofibres scaffolds (Figure 4, white arrow). Here, it can be state that the density of beads on nanofibres scaffold affect clearly the morphology and the nanostructure performed better growth of hBM MSCs. It was also demonstrated that the hAMSCs cultured on PLLA nanofibres scaffold were more rounded compared to flat films [12].

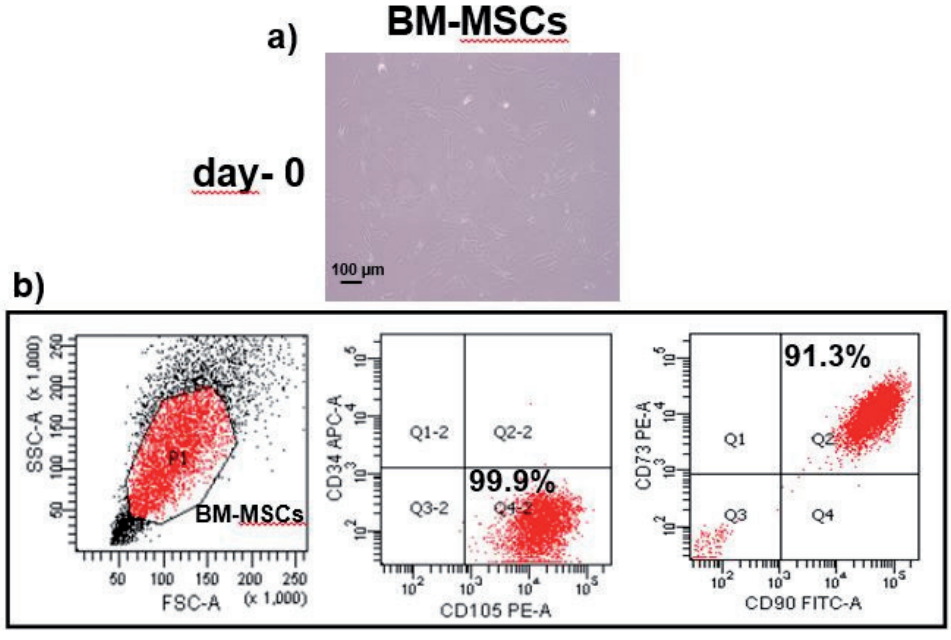

Fig. 2. hBM MSCs characterization: a) Morphology of hBM MSCs, scale bar 100 µm, b) Flow cytometry characterization of human bone marrow mesenchymal stem cells (hBM MSCs) passage 3, with the percentage of positive cells for each marker

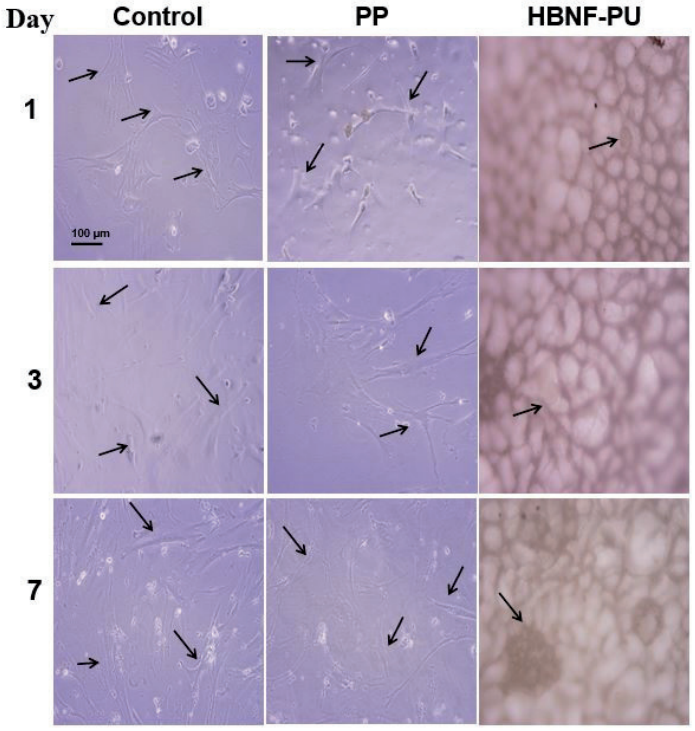

Fig. 3. hBM MSCs cultures on nanofibrous scaffolds: Morphology of hBM MSCs on three substrates control, PP and HBNF-PU at 1, 3 and 7 days, scale bar 100 µm

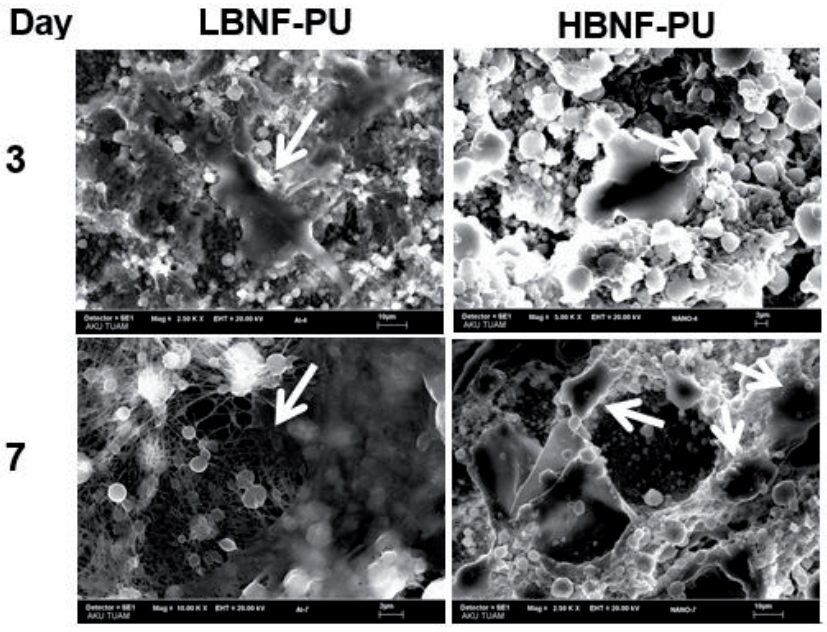

Fig. 4. hBM MSCs morphology on electrospun nanofibrous scaffolds: Scanning electron microscopy (SEM) images of hBM MSCs on LBNF-PU and HBNF-P U at 3 and 7 days

3.5. Live/Dead assay

After confluence hBM MSCs were trypsinized, suspended and seeded on PP film, PU nanofibres and uncoated surfaces (control) for 7 days. Live and dead assay revealed that hBM MSCs on uncoated surfaces and PP films were 90-95% viable during seven days. Although complex structure of nanofibrous scaffolds we observed cell viabilities on both LBNF-PU and HBNF-PU.

In this study, hBM MSCs were cultured on electrospun nanofibrous scaffolds (LBNF-PU and HBNF-PU) to determine the effect of beads density on nanofibrous scaffold on cell viability, morphology and proliferation. The porosity and wettability of materials are the characteristics that influence cell behaviour. It was reported that the maximum adhesion and growth of endothelial cells and fibroblasts on PE was around a contact angle of 50-55° [2].

We previously showed that hBM MSCs cultured on human elastin like polypeptide (with less contact angle value; 19.0 ± 1.9) coated surfaces had higher cell viability compared to that observed for human elastin like polypeptide 1 (with high contact angle value; 24.2 ± 0.9) coated surfaces [6]. According to our contact angle results and previous work, it can be deducted that HBNF-PU with a contact angle of 65° should outperformed compared to rivals. SEM images of our electrospun nanofibrous scaffolds revealed porous, nanoscaled fibrous with beads and randomly oriented. Bashur et al. mentioned the importance of electrospun PU fibre diameter (from 0.28 to 2.3 mm) and the degree of fibre alignment for BM stromal cells expansion [4]. They discussed that electrospun fibre meshes consisting of smaller and more aligned fibres are attractive over larger unaligned fibre for ligament tissue engineering applications [4]. Lee et al. demonstrated the hydrophilic electrospun polyurethane nanofibres matrices provided an excellent environment for hMSCs growth and proliferation [15]. It was observed that the hBM MSCs were apparently attached to samples and associated with fibres (LBNF-PU and HBNF-PU) in all directions. Moreover, they penetrated into the pores of NF scaffolds (Figure 5). This phenomena was not new, it was also observed by different study that cells can be localised at underneath of fibres or deeper [10]. Here, it can be state that the density of beads on nanofibres scaffold affect clearly the morphology and the nanostructure performed better growth of hBM MSCs. Our data revealed no significant differences regarding the biocompatibility of electrospun nanofibres compared to standard TCP or PP, indicating that both scaffolds are able to sustain viable cultures of MSCs. Overall, our data indicate that nanofibrous scaffolds provided a suitable environment for hBM MSC, and no evident cytotoxicity was observed.

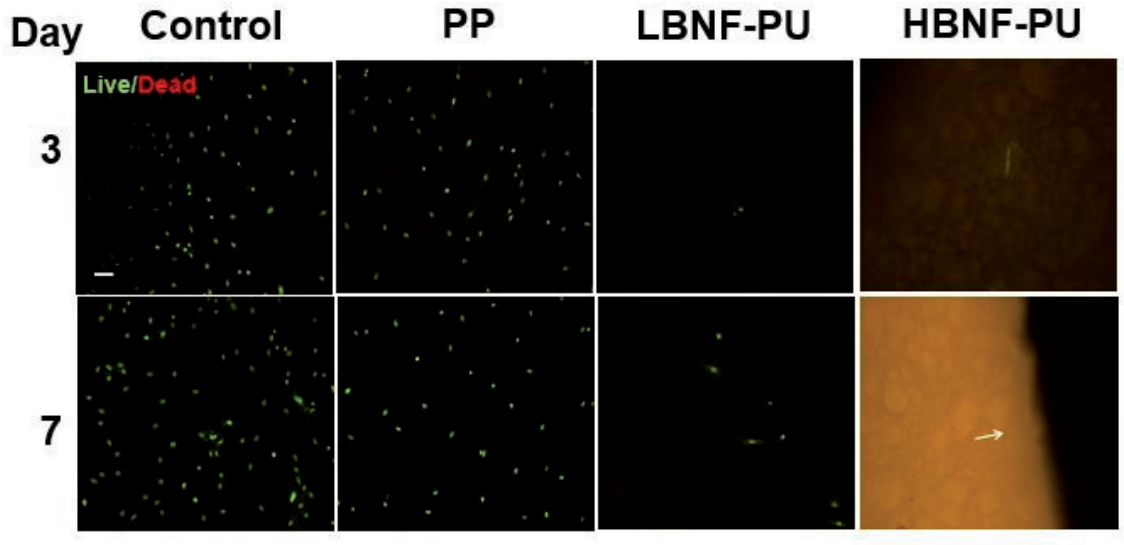

Fig. 5. Live/Dead cells imaging: Fluorescent microscopic analysis of live (green) and dead (red) hBM MSCs on PP, LBNF-PU and HBNF-PU at 3 and 7 days

4. Conclusions

The findings found above highlight the effect of low and high beads density in nanofibrous scaffold formed during electrospun process upon hBM MSCs growth. The characterisation of these samples revealed that hydrophilic surface of HBNF-PU had less nanofibres with higher quantity of beads that were overlapped on each other firmly compared to LBNF-PU. HMSCs cell morphology on both HBNF-PU and LBNF-PU nanofibrous scaffolds and flat surface was different; it was observed elongated cell shape for LBNF-PU and flat surface and rounded cell shape for HBNF-PU. Finally, the results have demonstrated that the beads influence clearly the HMSCs growth as well as morphology of cells.

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