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Ability of polyurethane foams to support placenta-derived cell adhesion and osteogenic differentiation: preliminary results

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Abstract In bone tissue reconstruction, the use of engineered constructs created by mesenchymal stem cells (MSCs) that differentiate and proliferate into 3D porous scaffolds is an appealing alternative to clinical therapies. Human placenta represents a possible source of MSCs, as it is readily available without invasive procedures and because of the phenotypic plasticity of many of the cell types isolated from this tissue. The scaffold considered in this work is a slowly degradable polyurethane foam (EF PU foam), synthesized and characterized for morphology and in vitro interaction with chorion mesenchymal cells (CMCs). These cells were isolated from human term placenta and cultured onto the EF PU foam using two different culture media (EMEM and NH osteogenic differentiation medium). Synthesized EF PU foam showed homogeneous pore size and distribution, with 89% open porosity. In vitro tests showed CMCs scaffold colonization, as confirmed by Scanning Electron Microscopy (SEM) observations and hematoxylin–eosin staining. Alizarin Red staining revealed the presence of a small amount of calcium deposition for the samples treated with the osteogenic differentiation medium. Therefore, the proposed EF PU foam appears to stimulate cell adhesion in vitro, sustaining CMCs growth and differentiation into the osteogenic lineage.

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1 Introduction

The clinical therapies currently available for bone regeneration are based on the use of autologous or heterologous demineralized bone or bone substitutes, although these approaches have several drawbacks. Autologous bone is scarcely available due to the lack of donor sites and its harvesting often requires painful invasive surgery. On the other hand, transplant from a donor can be rejected and expose the patient to infective pathogens. Therefore, for the reconstruction of bone defects, a great benefit could be achieved from alternative sources and, in particular, from engineered constructs that can be integrated into the surrounding tissues.

To obtain a bone-engineered tissue, scaffolds should meet a number of essential requirements, and of primary importance is the presence of interconnected porosity, with pores of adequate size to allow chemotaxis, cell proliferation and differentiation [1]. The use of synthetic or natural polymers for bone regeneration is extremely appealing in the clinical field, since they can be easily fabricated into three-dimensional (3-D) structures that fit well the defect size, presenting a large surface for cell adhesion and migration, and a controlled porosity that allows for an adequate diffusion of nutrients and waste products. Among the natural polymers, the most frequently used for tissue regeneration are alginate, collagen, hyaluronic acid and gelatin [2–4], while synthetic biodegradable polymers for bone tissue regeneration include poly(α-hydroxyesters), polydioxanone, polyorthoesters, polyanhydrides and some polyurethanes [5–12]. However, an adequate balance

between in vivo scaffold degradation and tissue regeneration is not easily achievable, because of a number of different variables that may occur in clinical conditions, such as the geometry of the bone defect to be filled, the different volumes of the material required and the functional loading, which affects bone apposition and remodeling. Therefore, biointegration is an appealing alternative to biodegradation, and it can be achieved by use of polymeric scaffolds with a very slow degradation rate, that can be designed to fulfill all the requirements of the specific application. Following this approach, scaffolds could be used effectively when there is a need to substitute bone defects, preventing tissue collapse and sustaining newly forming tissue. In this respect, the range of mechanical and morphological properties that can be obtained with polyurethanes (PU) is significantly larger than with commonly used medical-grade biodegradable polymers [13–15]. In the last 10 years we have set up a process to obtain crosslinked PU foams with slow degradation rate and with a controlled range of pore size, open porosity and mechanical properties [13, 16]. PU foams with different hydrophilicity [17], surface-modified by a protein coating [18], and composites [17] have also been developed and characterized.

Concerning the cells, stem cells will undoubtedly play a key role in the development of such strategies, due to their ability to differentiate into multiple cell phenotypes. Adult stem cells, in particular, have attracted considerable research interest because they pose few ethical dilemmas and limitations in terms of availability, in comparison to embryonic stem cells. Among them, mesenchymal stromal cells (MSCs) are particularly appealing because of their demonstrated tolerogenic properties and differentiation potential into osteoblasts, chondrocytes, myocytes, tenocytes, adipocytes and endothelial cells [19, 20]; in addition, they have been widely investigated in musculo-skeletal tissue regeneration. MSCs have been isolated from several tissues, including bone marrow (BM) [21], umbilical cord blood [22, 23], peripheral blood [24] and adipose tissue [25, 26]. While MSCs isolated from these tissues appear promising for clinical applications [24, 27], in some cases limitations exist in terms of access to, and use of these sources. In particular, the procedures required to obtain the tissues for the isolation of the cells may be invasive, the cells number obtained can be low, and the differentiation potential may be dependent on the age of the donor [28, 29]. In this perspective, the attention of the researchers has been turned to the human term placenta as possible source of progenitor/stem cells [30]. The fact that placental tissues originate during the first stages of embryological development supports the possibility that these tissues may contain cells which have retained the plasticity of the early embryonic cells. Furthermore, as the placenta is generally discarded after birth, it is available in large supply, the isolation of cells from this tissue does not involve any invasive procedure for the donor, and their use does not pose any ethical problem [31]. These aspects make cells isolated from the fetal membranes of the placenta, in particular from amniotic and chorionic membranes, good candidates for possible use in cell therapy and tissue engineering approaches, with the possibility of providing cells that are capable of differentiating into multiple different cell types, and which also display immunological properties that would allow their use in an allo-transplantation setting.

This study was aimed at investigating the adhesion and cell viability of chorionic mesenchymal cells (CMCs), isolated from human term placenta, and their potential to differentiate toward the osteoblastic phenotype when cultured on PU-foamed scaffolds.

2 Materials and methods

2.1 Polyurethane foam (EF PU foam) synthesis

The base chemical formulation of the synthesized EF PU foam is similar to one used for a non porous component of a commercial hemodialyzer. The EF PU foam was synthesized with a previously described [13, 32] one step bulk polymerization method, using water as expanding agent and iron-acetylacetonate (FeAA) as the catalyst. Briefly, to a polyether-polyol mixture (EF, a modified ElastoFlex LF2946 formulation, Elastogran, Villanova d'Asti, AT, Italy; OH = 4.0998 mmol $\times$ g^{-1}), FeAA (0.001% w/w_{polyol}) and water (2% w/w_{polyol}) were added and mixed with a mechanical stirrer. The necessary amount of methylene diphenyl diisocyanate (MDI) prepolymer (Lupranate MP102, BASF, Ludwigshafen, Germany, NCO = 5.24 mmol $\times$ g^{-1}) to obtain the stoichiometric ratio of OH/NCO = 100/73 was then added. The reaction mixture was stirred for 60 seconds and then poured into a custom-made poly(methylmethacrylate) mold ($V = 500\ cm^3$). The mold was firmly closed and the expanding reaction was allowed to take place at room temperature (RT). The foam was extracted from the mold after 72 h and the superficial compact skin was removed to obtain a homogeneous porous structure. Finally, the foam was post-cured at RT for 7 days. The synthesized PU foam was purified by a 48 h immersion in absolute ethanol at RT, and subsequently carefully dried in air at RT before following characterization.

2.2 EF PU foam characterization

Density analyses were performed on cylindrical specimens ($\Phi = 15$ mm, $h = 10$ mm) cut out of the foam with a

mechanical die. Density was analyzed according to EN ISO 845 standard practice, by weighing and measuring the specimens ($n = 5$) after conditioning for 24 h at 25°C.

Porosity, average pores size and pores size distribution were evaluated by micro CT analysis using a 1172 micro CT imaging system (Skyscan, Aartselaar, Belgium) at 4.9 μm voxel resolution, 173 μA X-ray tube current, and 60 kV voltage without any filters. The specimens were rotated through 180° around the long axis of the sample, with a rotation of 0.4°. The projection radiographs of the sample were reconstructed to serial coronal-oriented tomograms using a 3D cone beam reconstruction algorithm, setting the beam hardening to 20% and the ring artifact reduction to 12. Tridimensional reconstruction of the internal pore morphology was carried out using axial bitmap images and analyzed by CTan and CTvol softwares (Skyscan, Aartselaar, Belgium). The grey scale threshold was set between 45 and 255, removing all objects smaller than 400 voxels and not connected to the 3D model. In order to eliminate potential edge effects, the cylindrical volume of interest (VOI) was selected in the center of a scaffold ($\Phi = 2.5$ mm, $h = 2$ mm). Scaffold porosity was then calculated as:

$$\text{Porosity} = 100\% - \begin{array}{l}\text{vol\% of binarised object} \\ \text{(scaffold materials) in VOI}\end{array} \quad (1)$$

The mean pore diameter distribution was determined by measuring the material thickness on the inverse model, generated by setting grey scale threshold between 0 and 45. The 3D models were generated through the algorithm adaptive rendering.

2.3 Chorionic mesenchymal cells (CMCs) isolation

As previously described [31], human term placentas obtained after maternal consent, according to the guidelines of the Ethical Committee of the Catholic Hospital (CEIOC), were generally processed immediately after birth as follows. First, the decidua parietalis was removed by careful scraping. The chorion was then manually separated and washed extensively in phosphate-buffered saline (PBS, Sigma, St Louis, MO, USA) containing 100 U/mL penicillin and 100 μg/mL streptomycin (P/S; both from Euroclone, Whetherby, UK), before being cut into small pieces (3 × 3 cm). Chorion fragments were subjected twice to 9 min incubation in Hank's Balanced Salt Solution (HBSS, Lonza), containing 2.4 U/mL dispase at 37°C, separated by a resting period of 5–10 min in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Lonza), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma). The stromal and trophoblastic layers of the chorion were then separated from each other and digested separately with 0.75 mg/mL collagenase (Roche, Mannheim, Germany) and 20 μg/mL DNAse (Roche) for approximately 3 h at 37°C. Mobilized cells from the stromal layer, called chorionic mesenchymal cells (CMCs), were passed through a 100 μm cell strainer (BD Falcon, Bedford, MA) and the cells were collected by centrifugation at 200 RPM for 10 min and then resuspended in the culture medium.

2.4 In vitro interaction of EF PU foam with CMCs

EF PU foam samples ($\Phi = 6$ mm; $h = 2$ mm; $n = 4$) were placed in 48-multiwell culture plate, disinfected with 70% ethyl alcohol in distilled water for 30 min, and then carefully washed with sterile water. The samples were then sterilized with UV light for 10 min. Before cell seeding, EF PU foam samples were immersed in Eagle's Minimum Essential Medium (EMEM, Lonza) for 72 h at 37°C with 5% CO_2.

CMCs, isolated from human term placenta as previously described, were suspended in culture medium at a density of 5×10^6 cell × ml^{-1}. The cell suspension (100 μL $well^{-1}$) was seeded onto each EF PU foam specimen, placed in an incubator (5% CO_2, 37°C) and maintained under static culture conditions for 20 days, replacing the culture medium every 3 days. Two different culture media were used, namely, EMEM and Nonhematopoietic Osteo-Diff Medium (NH OsteoDiff Medium, NH, Miltenyi Biotec, Bergisch Gladbach, Germany), to evaluate the influence of the culture medium on the osteogenic differentiation onto EF PU foam samples. As an internal control, culture and osteogenic differentiation of the placenta derived cells were performed on tissue culture polystyrene wells (TCPS), at the same seeding density, following the protocols previously reported [31].

EF PU foam samples kept in culture with CMCs, using the two culture media, were observed by SEM (TM-1000 Tabletop, Hitachi), to evaluate cell morphology and distribution on the foam surface. After 20 days of culture EF PU foam specimens were fixed with 1.5 vol% glutaraldehyde solution buffered in 0.1 M sodium cacodylate (pH 7.2), dehydrated through a series of ethyl alcohol solutions (from 20 to 100 vol% ethyl alcohol in distilled water) and air dried. The specimens were sputter-coated with gold and examined at an accelerating voltage of 15 keV in a secondary electrons mode.

Scaffold colonization and cell distribution were evaluated by hematoxylin–eosin staining, while calcium deposits, characteristic of the in vitro osteogenic differentiation, were visualized by Alizarin Red S staining. EF PU foam samples, kept in culture with CMCs, were fixed in formalin for 24 h, dehydratated in a graded series of ethanol and embedded in paraffin. Sections 3-μm-thick were deparaffinized with xylene 100%, rehydratated through a series of ethyl alcohol solutions and stained with hematoxylin–eosin

(Bio-Optica, Milan, Italy) or with Alizarin Red S (Sigma) staining.

For Alizarin Red S staining the rehydratated sections were stained for 3-5 minutes with 58 mM Alizarin Red S (pH 4.1 - 4.3) and washed for 2 min with distilled water. The slides were then dehydratated in acetone, and after that in an acetone – xylene (1:1) solution, cleared in xylene and mounted with Eukitt quick-hardening mounting medium (Sigma).

3 Results and discussion

3.1 EF PU foam characterization

Results of the physical and morphological characterization are reported in Table 1.

The EF PU foam presents high porosity, and a value of average pore size adequate for cell ingrowth and scaffold colonization [33–35].

The 3D model generated by micro CT analysis (Fig. 1a) demonstrates a homogeneous morphology of the foam and regular pore size, shape and distribution. Micro CT analyses also allowed to investigate the average pore size distribution (Fig. 1b); most of the foam pores have diameter size in the range between 150 and 400 µm, resulting adequate for applications in bone tissue engineering [33].

Therefore, it is possible to state that pore size, pore volume fraction and resulting surface area available for cell attachment are well controlled with the proposed scaffold manufacturing method. The set up foaming process allows production of scaffolds with homogeneus structure and morphological properties able, in principle, to support cells ingrowth, scaffold colonization and nutrients and waste metabolism products exchange.

Table 1 Physical and morphological properties of EF PU foam

Density (g/cm^3)	Average pore size (µm)	Porosity %
0.127 ± 0.003	268.46	89.67

3.2 In vitro interaction of EF PU foam with CMCs

To in vitro evaluate the ability of EF PU foam scaffolds to support CMCs homing and osteogenic differentiation, CMCs were cultured in the presence of the NH osteoinductive culture medium. The cells behavior under these conditions was compared to that showed by the cells cultured in EMEM medium. At 20 days post-seeding, good CMCs adhesion to the pore surface of the EF PU foam was observed, both when cultured with EMEM and with the osteoinductive medium (Fig. 2). The pore surface of the foam was well colonized by the cells, which showed a flattened and well spread morphology (Fig. 2c, d). These results demonstrate the absence of cell suffering and indicate a good in vitro cells interaction of the tested PU foam. The cells were detected both on the scaffolds surface and within the foam pores (Fig. 2b). In addition, the presence of extracellular matrix (ECM) synthesized by the cells, either when cultured with EMEM or with NH culture medium, was observed on the samples (Fig. 2a, c).

SEM results were confirmed by histological analysis performed through the hematoxylin–eosin staining (Fig. 3a, b), which showed that the cells were also present in the inner part of the scaffolds, in particular in the case of cells cultured with EMEM medium (Fig. 3a).

The Alizarin Red staining allowed visualization of cells calcium deposition and evaluation of cells differentiation towards the osteoblastic phenotype. The histological images of the samples treated with NH culture medium revealed the presence of a small amount of calcium deposition (Fig. 3d), which cannot be observed in the case of CMCs cultured with EMEM (Fig. 3c), even though the scaffolds appear to show higher levels of cell colonization, as already detected through the hematoxylin–eosin staining.

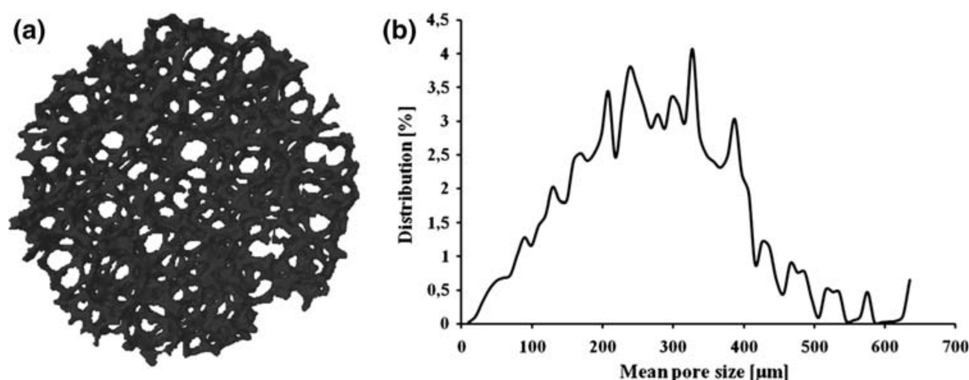

Fig. 1 3D model of the EF PU foam generated through the micro CT algorithm adaptive rendering (**a**) and trend of the foam average pore size distribution as evaluated by micro CT (**b**)

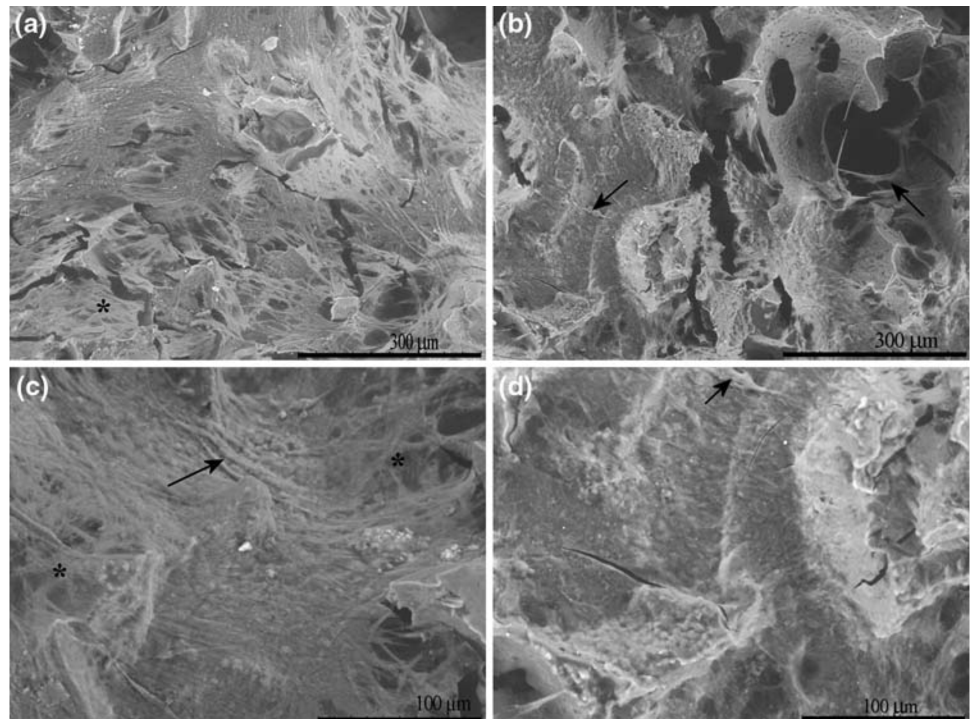

Fig. 2 SEM images of EF foam 20 days after CMCs seeding in EMEM medium (**a**, **c**) and in NH medium (**b**, **d**). The arrows indicate the cells while the extracellular matrix (ECM) is marked with *

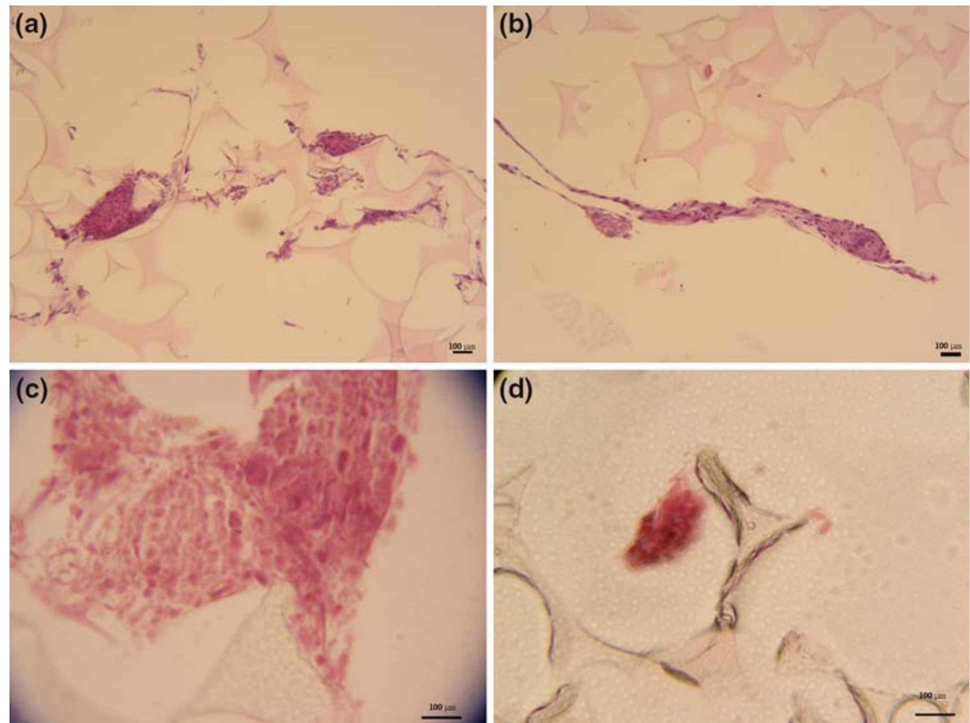

Fig. 3 Representative histological images of hematoxylin–eosin staining (100× magnification) of CMCs cultured on EF PU foam with EMEM (**a**) and with the osteogenic culture medium (**b**) and of Alizarin Red staining (200× magnification) of CMCs cultured on EF PU foam with EMEM (**c**) and with the osteogenic culture medium (**d**). Scale bar = 100 μm

These results fit well with previous in vitro cytocompatibility tests carried out on PU foams [13, 18, 32, 36]. The good in vitro cytocompatibility of the material has already been demonstrated with different cell lines (i.e. MG63, Saos-2). An in vitro study was also performed on PU foams synthesized with a polyether-polyol mixture with lower hydrophylicity to investigate the ability of human bone marrow MSCs to proliferate and possibly differentiate towards the osteoblastic phenotype [32]. The obtained results demonstrated that the PU foams were able to provide MSCs with the necessary support to proliferate and to undergo osteogenic differentiation, and these conclusions correlate well with the results obtained in the present study.

To increase cell proliferation and PU scaffold calcification, Fassina et al. studied calcified extracellular matrix (ECM) production on PU foams by Saos-2 cells cultured in a perfusion bioreactor [36] or under electromagnetic stimulation [37]. Under these dynamic culture conditions, Authors observed an increase of cell proliferation, gene expression and extracellular matrix production in comparison with static conditions [36]. The dynamic culturing approach could therefore prove useful to improve calcium deposition by the cells, and CMCs differentiation into osteoblasts.

Another promising result was recently obtained by an in vivo study performed into the dorsal subcutaneous tissue of male rats. The obtained results (data not published) indicated the good in vivo interaction of PU foams with the surrounding tissue; in fact, the tested materials evoked an inflammatory physiological response without any fibrous encapsulation [38].

4 Conclusions

In this work, for the first time in the literature, the differentiation of MSCs from human placenta has been evaluated by culturing the cells onto scaffolds, and not only on tissue culture polystyrene wells (TCPS). In conclusion, the good morphological properties of the tested EF PU foam make this material a valid scaffold to support adhesion and differentiation of chorionic mesenchymal cells from human placenta into osteoblasts. Due to the ability to stimulate cell adhesion, scaffold colonization and osteoblast differentiation, these foamed PU scaffolds appear good candidate as bone graft substitutes. Further improvements in the performance of placenta-derived mesenchymal cells on PU-foamed scaffolds can be given by the incorporation of CaP fillers or coatings. According to this approach, additional investigations are now in progress. Furthermore, cell culture under dynamic conditions (perfusion chambers and bioreactors) and electromagnetic stimulation will be tested to improve calcium deposition and, consequently, cell differentiation.

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