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PLLA/HA Nano composite scaffolds for stem cell proliferation and differentiation in tissue engineering

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A B S T R A C T

Due to their mulitpotency, Mesenchymal stem cells (MSCs), have the ability to proliferate and differentiate into multiple mesodermal tissues. The aim of this study was to isolate MSCs from human Umbilical Cord (hUCMSCs) to determine their osteogenic potential on nanofibrous scaffolds. To this end, Poly (L-lactic acid) (PLLA)/Nano hydroxyapatite (HA) composite nanofibrous scaffolds were prepared by electrospinning. The structure and morphology of the scaffolds were investigated using scanning electron microscopy. Human mesenchymal stem cells (MSCs) were isolated from the umbilical cords and cultured in the PLLA/HA scaffold. The viability and proliferation of the cells was then determined by an MTT assay. Cellular adhesion, proliferation and osteogenic differentiation were assessed in these constructs using a range of histological and microscopic techniques. The osteogenesis assays indicated the superiority of nanofibrous scaffolds in supporting MSCs undergoing bone differentiation. Collectively, the bone construct prepared with PLLA/HA scaffold and proliferated MSCs would be a suitable candidate for use in bone regenerative medicine.

Key words: hUCMSCs; Nanofibrous scaffolds; Hydroxyapatite; PLLA; Cell proliferation; Bone differentiation.

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INTRODUCTION

The main goal of tissue engineering is to repair or replace the function of faulty or damaged tissues or organs by delivering functional cells, henceforth referred to as stem cells to the exact zone of need. Hence, in this field of engineering, one of the key factors is the creation of a scaffold for the cellular attachment, proliferation and differentiation [1]. Since cells are living in a complex medium of an extracellular matrix (ECM) in a nanometer-scale [1-3], a nanofibrous scaffold with three dimensional interconnected pores and large ratio of surface area to the volume is needed to enhance the cell growth and function [1].Currently there are three commen methods for manufacturing Nanofibrous Membranes (NFM) as tissue engineering scaffolds, namely, phase separation, self-assembly and electrospinning (ES) [4, 5]. ES is the most common method which can produce the polymer NFM under a high voltage electrostatic field operated by the metallic nozzle of a syringe and a metallic collector. Projected jets of polymer solution are randomly deposited in the form of a non-woven fabric onto the target collector [6]. This is used to fabricate scaffolds comprising non-woven, three-dimensional, porous and NF-based matrices. The characteristics of NF, such as high surface area to volume ratio and similar structural morphology to the fibrillar extracellular matrix (ECM), suggest that they may serve as effective tissue engineering scaffolds. In order to expand the use of electrospun NF in biomedical fields, their surfaces have been modified with bioactive molecules and cell recognizable ligands, which subsequently provide bio-modulating or biomimetic microenvironments for the cells' attachment, growth and proliferation [3,6,7].

Composites of polymers and bioactive ceramics have attracted increasing attention as promising biomaterials for bone tissue engineering as well [8]. Three dimensional (3D) porous composite scaffolds can induce the ingrowths of a cell to the desired shape and may facilitate the vascularization of new generated tissue. PLLA has been widely investigated in tissue engineering, thanks to its good biocompatibility and its amenability to be modified with inorganic materials in order to improve its biological properties [9, 10].

Hydroxyapatite (HA) has been widely used as an implant for biomedical applications and bone regeneration, especially due to its bioactive, biodegradable and osteoconductive properties. However, the application of pure HA particle is very limited for its brittleness. Thus, much attention has been focused on the preparation of polymer/HA composites recently. Among them, Poly (L-lactide) (PLLA)/HA composite has been widely investigated due to the good Osteoconductivity and osteoinductivity of HA and the biodegradability of PLLA in the composites [10, 11].

It was found recently that MSC with osteogenic potential can be isolated from prenatal tissues such as umbilical cord, umbilical cord blood, amniotic fluid and fetal blood [12, 13]. The umbilical cord is composed of a special embryonic mucous tissue, called Wharton's Jelly, lying between the covering amniotic epithelium and the umbilical vessels. Stromal cells of Wharton's Jelly have been shown to possess multipotent properties between embryonic and adult stem cells and have thus fulfilled the criteria for mesenchymal stem cells published by the International Society for Cellular Therapy [12].The umbilical cord (UC) has gained great interest in the last years since it is a postnatal organ discarded after birth whose isolation of cells is noninvasive, painless and harmless for mother and child. The capability of stem cells from the umbilical to easily

expand and to be cryogenically stored for years without losing their viability, has attracted much attention and produced a vivid impression on the sight [14].

In the present study, attempt was made to establish a new method for isolating human MSCs derived from the human umbilical cord which was capable of differentiating into other tissues in a Nano environmental condition. The electrospinning method was used to fabricate PLLA/HA composite scaffolds. The pore structure and morphology of the prepared scaffolds and cells attachment, growth capacity and differentiation of hUCMSCs into osteoblasts were investigated.

MATERIALS AND METHODS

Isolation of human mesenchymal stem cells (MSCs) from umbilical cord Wharton's Jelly: In brief, to remove cord blood, fresh umbilical cords were prepared and rinsed twice by Hanks' balanced salt solution (HBSS), (Merck, Germany). The washed cords were cut into ca. 1cm sized pieces and Wharton's Jelly was scratched out carefully and floated in Dulbecco's modified Eagle's medium with low glucose (DMEM-LG, Bio-IDEL) containing 10% FBS (GIBCO®), 5% penicillin and streptomycin (GIBCO®). The pieces of cord were subsequently incubated at 37^{o}C in humid air with 5% CO_2. The medium was replaced every 3 days after the initial plating, and non-adherent cells were removed by washing. Fibroblast-like cells appeared after 10 days of cultures [15].

Preparation of PLLA/HA Scaffolds: PLLA/HA scaffold was prepared by using the electrospinning technique [16]. The materials used for the fabrication of the nanofibrous scaffolds were PLLA (intrinsic viscosity =1.0 dl g−1, Sigma-Aldrich), Chloroform (Merck, Germany), DMF (N, N-dimethyl formamide, Merck, Germany), nHA particles (Merck, Germany) and the electrospinning machine (Semi industrial Es). Chloroform and DMF were used as the solvents to prepare PLLA/HA polymer solutions (the ratio of PLLA and HA was 10:1 in PLLA/HA polymer solution). The obtained suspension was transferred into a glass syringe, actuated by an infusion pump and fed through the needle, which was kept at a high DC voltage (20kV) and the distance of electric field was fixed at 20cm. Plasma treatment of hydrophobic polymers was one of the approaches used for providing micro and nanometric alterations of the surface architecture to improve protein and cell adhesion [17]. The scaffold was plasma treated in reactive O_2 plasma for 4min.

Cell culture: hUCMSCs (MSCs) were harvested from human umbilical cords. After being subcultured for the third passage, the cells were trypsinized and mixed with DMEM-LG. The nanofibrous scaffolds (PLLA/HA) were carefully cut into small rectangles ($1\times1cm^2$) and were then sterilized with 70% ethanol overnight and ultraviolet light for 2h and then rinsed three times with PBS before being placed in 24-well culture plates. Then they were put into 24-well plates and immersed in PBS for 3h. Afterwards, a cell suspension with a cell density of 5×10^4 cells.ml^{-1} was placed on the scaffold with a micropipette and allowed to adhere to the surface within 3h before adding the culture

medium. Cells were grown in the 24-well plate. The cell culture medium was replaced every 2 days [18].

Determination of Cellular Viability with MTT Assay: The viability and proliferation of fibroblast-like stem cells were determined by 3-[4, 5-Dimethylthiazol-2-yl]-2, 5 Diphenyltetrazolium Bromide (MTT) assay. The assay reflected the activity of a mitochondrial dehydrogenase that transforms light yellow MTT into dark blue Formazan. The intensity of the resulting color was determined photometrically [19]. The cell-seeded scaffolds were maintained at 37^{o}C under 5% CO_2 for different time periods. During the test, the culture medium in the disks was removed and rinsed by PBS three times and then 200µ RPMI1640 (Bio-IDEL) was added to each well. 20µ of MTT solution (5 mg/mL) were freshly added to the culture wells and incubated at 37°C and 5% CO2 for 4h. The upper medium was removed carefully and the intracellular Formosan was solubilized by adding 100µl of DMSO (Merck, Germany) to each well, incubating at 37◦C and 5% CO2 for 10min. The absorbance of the produced Formosan was measured at 570nm with an ELIZA reader (URIT-660, China). All experiments were performed in duplicate, and the relative cell viability was expressed relative to the control cells [20]. The experiment was repeated three times, the results of which are presented as means.

Scanning electron microscopy (SEM): The surface morphology of the scaffolds and cell scaffold constructs were examined using SEM. The cell loaded scaffold specimens from day-3 culture were prepared for SEM. For this purpose the MSCs-loaded scaffolds were fixed in a 4% PFA (Sigma-Aldrich) solution at 30ºC for 45 min, followed by washing with dH_2O, drying and coating with gold. The specimens were examined with SEM (LEO1430VP) at an acceleration voltage of 10 kV.

Differentiation of hUCMSCs into osteoblasts on scaffolds: Passage 3 hUCMSCs were used for the seeding into the PLLA/HA scaffolds. Prior to seeding, the scaffolds were sterilized with 70% ethanol overnight and ultraviolet light for 2h and then placed into 24-well cell culture plates and treated with PBS. The well-plate was left in the incubator at 37^{o}C with 5% CO_2, for 3h. A cell suspension (20µl) with a cell density of 2×10^5 cells.ml^{-1} was seeded evenly into the scaffolds with a micro-pipette. The Osteogenesis medium (growth medium with the addition of 0.1 mol L^{-1} dexamethasone, 0.05 mmol L^{-1} ascorbic acid-2-phosphate and 10 Mmol L^{-1}–glycerophosphate, Bio-IDEL) was applied 24 hours after plating [21]. Medium changes were performed every 2 days. Osteogenesis was assessed on day 21.

Von Kossa staining: The von Kossa stain is used to quantify mineralization in cell culture and tissue sections. Briefly, the cell scaffold was washed with PBS and fixed in 4% PFA solution for 45 min and cell-nanofibrous scaffolds were stained with 1% silver nitrate (Merck, Germany) for 45 minutes under ultraviolet light, followed by 2.5% sodium thiosulfate (Merck, Germany) for 5 minutes. Then, cell- nanofibrous scaffolds were rinsed 3 times with distilled water, checked by an inverse microscope and photographed [22].

Alizarin red staining: To confirm the presence of differentiated mesenchymal cells, it was necessary to stain the cells after culture with dyes used specifically for bone cells (day 21) [23]. In brief, the cell-nanofibrous scaffolds were fixed in 4% formaldehyde for 45 min followed by staining with alizarin red (Merck, Germany) for 8 min. After washing five times with distilled water, they were examined under a microscope.

RESULTS

Isolation and culture of MSCs from umbilical cords: After the initial 9 days of primary culture, hUCMSCs adhered to the Culture flasks' surface and presented a small population of single cells with spindle shapes. On days 12-14 after initial plating, the cells looked like long spindle-shaped fibroblastic cells, began to form colonies and became confluent (Figure 1). During the second passage, the cells presented a homogeneous population of spindle fibroblast-like cells.

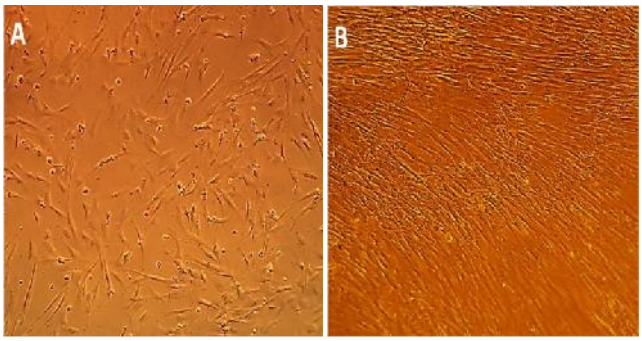

Figure 1.Adhered cells in primary cultures of hUCMSCs. The morphology and growth of cells withan MSC-like phenotype after (A) first passage. (B) Third passage (Photos provided by inverted microscope with Original magnification, × 200 for both panels)

Fabrication PLLA/HA scaffolds: For tissue engineering application, distribution of the HAp in-and-outside the fibers would further influence cytocompatibility of the scaffolds, such as cell adhesion and proliferation. Therefore, plasma treatment using oxygen as a reactive gas was used as an effective method to introduce oxygen containing groups (such as carboxyl and hydroxyl groups) onto the polymer surface to improve the hydrophobicity and introduce carboxyl groups onto the PLLA nanofiber surface.

Scaffold morphology and cell culture on scaffold: The morphology of the nanofibrous scaffold was demonstrated by SEM micrographs. Figure 2 shows a well-fabricated nano\fibrous porous PLLA/HA matrix, similar to those of natural ECM. The nano-fiber exhibits a large surface area, which benefits the good in-growth of cells [17]. Moreover, Figure 2 shows the MSCs attachment and growth on PLLA/HA electrospun membranes day 3 of seeding. From the SEM micrographs it can be observed that the cells

adhered and spread on the surface of the polymer nanofibers. This is evidence for the cells' good interaction and integration with the surrounding fibers.

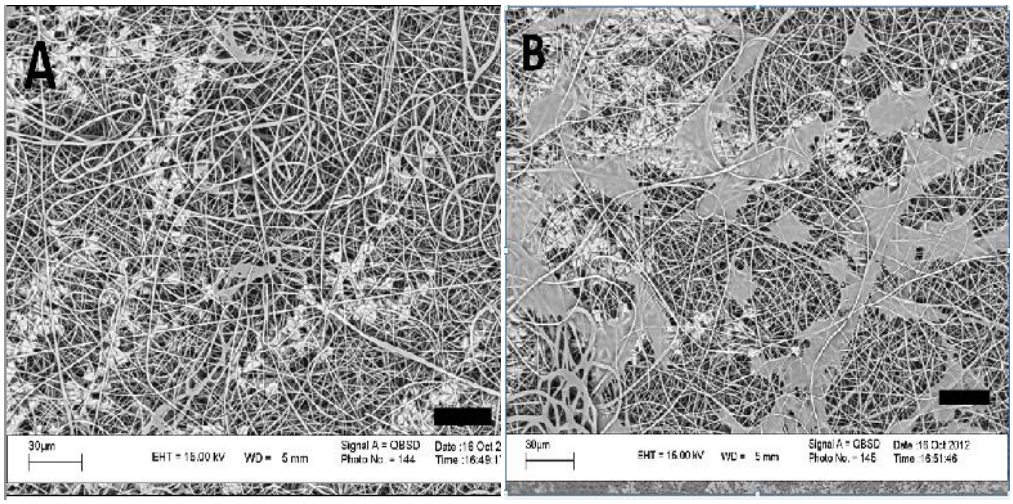

Figure 2. SEM image of (A) PLLA/HA scaffold, scale bare is 30 µm. (B) Cell morphology of hUCMSCs on the scaffold (three days after seeding). Scale bar is 30µm.

MTT assay: After cell culturing for 3, 5 and 7 day periods, the viability and proliferation of fibroblast cells were determined by MTT assay. The test showed that more viable cells existed in scaffolds a week after culture, making them quite suitable for MSCs growth. After day-7 of the culture, the value of absorbance was higher than that of the others days. This indicated that the MSCs shows much better viability on the PLLA/HA scaffold than the control cells (Figure 3).

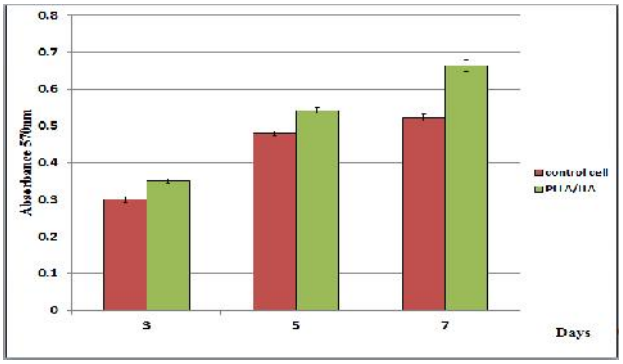

Figure 3. MTT–tetrazolium assay of hUCMSCs cultured in PLLA/HA scaffold for different times. Formosan absorbance is expressed as a function of scaffolds. (Error bars represent standard deviation).

Osteogenic differentiation: In order to analyze the mineralization capability of MSCs, the cells from the second confluence were seeded on the PLLA/HA scaffold, and cultured for 21 days in the osteogenic medium. At the end of that time, the formation of mineral nodules in cultures on PLLA/HA was assessed by Alizarin red and von Kossa staining (Figure 4).

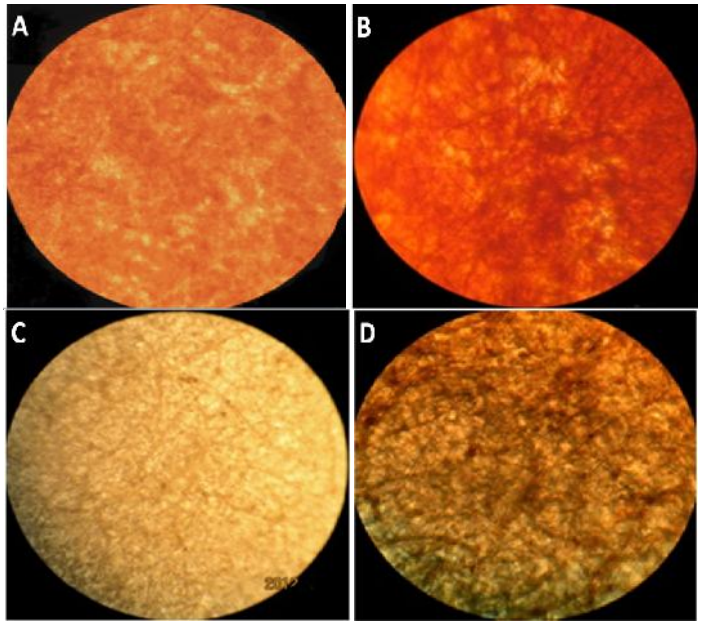

Figure 4. Microscopic image of (A and C) PLLA /HA scaffold cultured with hUMSCs in basal medium for 21 days. (B and D) PLLA /HA scaffold cultured with hUMSCs in osteogenic medium for 21 days. (Photos provided by inverted microscope with Original magnification, × 200, for all panels)

The differentiation of human mesenchymal stem cells seeded in PLLA/HA nanofiber scaffolds are shown in Figure 4A and C. hUCMSCs cultured in PLLA/HA without osteogenic differentiation showed negative staining to Alizarin red and von Kossa. Figure 4B and D show seeded hUCMSCs in PLLA/HA scaffold treated with the osteogenic medium. The images show positive staining to Alizarin red and von Kossa. The result suggests that hUMSCs differentiates into osteogenic cells with the presence of mineral deposition.

DISCUSSION

Umbilical cord MSCs may play an important role in applications and experimental research of adult human MSCs. The basic requirements for skeletal engineering are appropriate sources of cells, optimal biochemical conditions and a biocompatible scaffold conducive to cell attachment and maintenance of cell function. Mesenchymal stem cells

from the human umbilical cord represent a promising source for skeletal regeneration due to their capability for osteogenic differentiation through a well-defined pathway. The optimal stem cell/biomaterial hybrid mimics the physical and biological functions of the native extracellular matrix (ECM) [24]. Cell attachment on the scaffold surfaces is the prerequisite to their proliferation and differentiation. The behavior of cells on biomaterial depends on the scaffold's surface characteristics including roughness, topology, damp absorbance, electrical charge and chemistry and energy of the surfaces [25, 26].

It is noteworthy that umbilical cord MSCs isolation has already established and characterized as a new human MSC cell line [14]. In the present study, MSCs were isolated without collagens which may affect the proliferation and differentiation of MSC. Our results indicated that the isolation method is significantly different from the methods reported by others [24]. In this study, cell attachment and proliferation were determined using SEM observations and MTT assays respectively. The results showed that the surface of the scaffolds were compatible with MSC attachment and proliferation. Moreover, in the present study, we demonstrated that hUCMSCs were capable of osteogenic differentiation *in vitro* in a 3D fashion environment, based on evidence of osteoblast differentiation. For hUCMSCs seeded in PLLA/HA nanofibers and treated with osteogenic medium, Alizarin red and von Kossa were positive for 21 days of culture, indicating potential mineral deposition. In comparison, hUCMSCs seeded in PLLA/HA nanofibers without osteogenic differentiation were mostly negative for Alizarin red and von Kossa. In summary, the osteogenic differentiation of hUCMSCs in a 3D biomaterial-based environment provides the foundation for future applications of this new cell source for bone tissue engineering.

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Conflict of interest statement: None

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