ISSN 1948-0210 (online)

World Journal of *Stem Cells*

World J Stem Cells 2012 September 26; 4(9): 94-100

Baishideng Publishing Group
www.wjgnet.com

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World Journal of Stem Cells

A peer-reviewed, online, open-access journal of stem cells

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2009-2013

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World Journal of Stem Cells

Contents

Monthly Volume 4 Number 9 September 26, 2012

World Journal of Stem Cells
Volume 4 Number 9 September 26, 2012

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NAME OF JOURNAL
World Journal of Stem Cells

ISSN
ISSN 1948-0210 (online)

LAUNCH DATE
December 31, 2009

FREQUENCY
Monthly

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http://www.wjgnet.com

PUBLICATION DATE
September 26, 2012

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1948-0210/g_info_20100313165700.htm

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Stem Cells

Online Submissions: http://www.wjgnet.com/esps/
wjsc@wjgnet.com
doi:10.4252/wjsc.v4.i9.94

World J Stem Cells 2012 September 26; 4(9): 94-100
ISSN 1948-0210 (online)

ORIGINAL ARTICLE

Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) supports adhesion and migration of mesenchymal stem cells and tenocytes

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Received: September 21, 2011 Revised: March 1, 2012
Accepted: March 25, 2012
Published online: September 26, 2012

Abstract

AIM: To establish the potential of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) as a material for tendon repair.

METHODS: The biocompatibility of PHBHHx with both rat tenocytes (rT) and human mesenchymal stem cells (hMSC) was explored by monitoring adhesive characteristics on films of varying weight/volume ratios coupled to a culture atmosphere of either 21% O_2 (air) or 2% O_2 (physiological normoxia). The diameter and stiffness of PHBHHx films was established using optical coherence tomography and mechanical testing, respectively.

RESULTS: Film thickness correlated directly with weight/volume PHBHHx (r^2 = 0.9473) ranging from 0.1 mm (0.8% weight/volume) to 0.19 mm (2.4% weight/volume). Film stiffness on the other hand displayed a biphasic response which increased rapidly at values > 1.6% weight/volume. Optimal cell attachment of rT required films of ⩾ 1.6% and ⩾ 2.0% weight/volume PHBHHx in 2% O_2 and 21% O_2 respectively. A qualitative adhesion increase was noted for hMSC in films ⩾ 1.2% weight/volume, becoming significant at 2% weight/volume in 2% O_2. An increase in cell adhesion was also noted with ⩾ 2% weight/volume PHBHHx in 21% O_2. Cell migration into films was not observed.

CONCLUSION: This evaluation demonstrates that PHBHHx is a suitable polymer for future cell/polymer replacement strategies in tendon repair.

Key words: Mesenchymal stem cell; Tenocytes; Polyhydroxyalkanoates; Hypoxia; Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)

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Lomas AJ, Chen GGQ, El Haj AJ, Forsyth NR. Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) supports adhesion and migration of mesenchymal stem cells and tenocytes. *World J Stem Cells* 2012; 4(9): 94-100 Available from: URL: http://www.wjgnet.com/1948-0210/full/v4/i9/94.htm DOI: http://dx.doi.org/10.4252/wjsc.v4.i9.94

INTRODUCTION

Polyhydroxyalkanoates (PHA) are a family of biopolymers consisting of polyesters of many different hydroxy-

carboxylic acid molecules. Micro-organisms produce PHAs as an energy storage molecule when exposed to unbalanced growth conditions during culture; for instance, excess lauric acid, limited nitrogen and limited phosphorous supply[1]. Originally viewed as replacements for traditional petrochemical-derived polymers, PHAs are now largely redundant as everyday materials due to the prohibitive cost of large quantity production[1]. There is now increased interest in these polymers from the medical device sector where the earlier prohibitive costs are reduced due to the reduced scale of operations. In addition, PHAs display relatively high immunotolerance, low toxicity and biodegradability which are all crucial for the medical device sector[2].

PHBHHx is the designation of molecules consisting of random co polymers of 3-Hydroxybutyrate and 3-Hydroxyhexanoate[3]. It is one of the few PHA molecules that can currently be produced on a large enough scale for use in both scientific research and medical device construction[4]. PHBHHx has a melting temperature of 111.7 ℃, a glass transition temperature of -0.67 ℃, a tensile strength of 4.1 MPa, an elongation at break of 103.8%, and a Young's modulus of 130.4 MPa, making it potentially useful for widespread biomaterial applications and different cell types[5,6].

Tendons form the bridge between muscle and bone. They are typically slow to repair after injury or disease, have a poor blood supply and are relatively acellular when compared to other tissues[7]. Tendon is composed mainly of collagen type I fibrils arranged in a hierarchical structure surrounded by a layer of endotenon[8]. These fascicles come together to form larger and larger subunits, eventually forming the complete tendon. The arrangement of collagen I fibrils give the tendon its strength in tension. Tenocytes are the major cell group present in tendons, making up around 95% of the cellular mass[9]. They are a highly specialized form of fibroblast and are responsible for the maintenance of tendon extracellular matrix (ECM) [collagen I (the major component of tendon), collagen Ⅲ, collagen Ⅴ, glycosaminoglycans, elastin and fibronectin] and for the repair of tendon tissue, either after injury or as part of normal physiological process[10,11]. Under normal physiological conditions, tenocytes are found in small numbers spread between the collagen fibrils[10].

Human mesenchymal stem cells (hMSCs) are viewed as a candidate cell source for tendon tissue engineering as, unlike tenocytes, they can be readily sourced, isolated and expanded *in vitro*. The exposure of hMSCs to external tensile forces and/or supplementation with additional growth factors can induce differentiation into cells that resemble tenocytes in physiological activity and marker expression profile have been produced[12,13].

The objective of this investigation was to monitor and quantify the interaction (attachment) and monitor the migration of tenocytes and hMSCs with PHBHHx polymer films of a variety of weight:volume ratios to characterize the optimal ratios for use in tissue engineering application. Here we show that both cell types adhere to PHBHHx films, with tenocytes preferring a thicker, stiffer scaffold, whereas MSCs adhered to all PHBHHx films tested, with greater adhesion noted in physiological oxygen conditions. Migration across, but not into, PHBHHx films was also apparent for both rat tenocytes (rT) and hMSC. Taken together, this demonstrates that PHBHHx is a suitable biomaterial for tendon tissue engineering.

MATERIALS AND METHODS

Cells

Tenocytes were isolated from the Achilles tendon of 8 wk old male Wistar rats. The tendon was dissected, minced into 1 mm sections, and placed onto a dry Petri dish. The tendon was allowed to adhere for 1 h before careful addition of 5 mL pre-warmed media [DMEM (4.5 g/L glucose) (Lonza, UK), 10% Fetal bovine serum (FBS) (Lonza, UK), 1% L-Glutamine (L-Glut) (Lonza, UK), 1% non essential amino acids (NEAA) (Lonza, UK)], taking care not to dislodge tissue pieces. These were then incubated under standard conditions for 7 d, during which time cells migrated from the tendon tissue onto the petri dish. Cells were then expanded in T-75 flasks in either 21% O_2 or 2% O_2 using previously described methodologies[14,15].

hMSC were isolated from human bone marrow *via* an adhesion method described elsewhere[16]. hMSC were maintained on 10 ng/mL Fibronectin (Lonza, UK) coated flasks in DMEM, 5% FBS, NEAA, and L-Glut and incubated at 37 ℃, 7% CO_2 and either 21% O_2 or 2% O_2. Nitrogen gas was supplied using an N_2 generator supplied by Peak Scientific. Culture media was changed twice weekly. hMSCs were passaged at 90%-95% confluency using Trypsin/EDTA. During experimentation, passage numbers of 4 or less were used.

Polymer

PHBHHx [87.9% Hydroxybutyrate (HB), 12.1% Hydroxyhexanoate (HHx)] was dissolved in 10 mL chloroform (Sigma Alrich, UK), at varying weights (0.08-0.24 g/10 mL) at room temperature in a sealed, clean glass tube. Once dissolved, 3.2 mL was poured into an open 60 mm glass Petri dish and left overnight to ensure complete evaporation. Films were then transferred into non-adherent 60 mm Petri dishes (Sterilin, UK), one film/dish. Films were then washed in 3 mL 70% Ethanol/ 30% distilled H_2O for 3 h, before washing with sterile PBS (Lonza, UK) 3 times. The films were dried for 1 h before use.

Polymer characterization

Polymer thickness was measured using a home built Optical Coherence Tomography (OCT) system according to a previously published method[17]. OCT generated images (laser wavelength interference patterns) were taken at random areas of 3 different films, with 3 images taken of each film. Image J analysis software was then used to determine thickness.

Stiffness was measured using a BOSE ElectroForce

3200 system. Samples were cut to 22 mm × 5 mm ribbons and placed into the grips, with 10 mm of the polymer in each grip, leaving a 2 mm initial sample length. This was then deformed by 0.5 mm in a uniaxial direction and the force required measured. Stiffness was calculated with the equation: k = F/δ, where k = stiffness, F = force and δ = displacement in single direction of freedom (i.e., direction the force acts in).

Cell attachment

Following preparation, PHBHHx films were immersed in 3 mL media containing 3 × 10^4 cells/mL in non-adherent, 6 well plates (Costar, UK). After 24 h incubation in either 21% O_2 or 2% O_2, films were removed from the dishes, gently washed in PBS, placed in a 15 mL centrifuge tube and immersed in pre-warmed Trypsin/EDTA (Lonza, UK) for 5 min, before quenching with excess media and removing the film. The surface of the non-adherent dish was also washed once with PBS and exposed to 1 mL Trypsin/EDTA for 5 min, before quenching with excess media. After centrifugation, cell pellets were re-suspended and cell counts established from both film and non-adherent well by hemocytometer counts of Trypan Blue (Sigma Alrich, UK) positive cells only. A control group where cells were seeded into wells containing no polymer film was also performed. The combined film and well cell counts were treated as 100% and used to establish percentage attachment.

Cell migration

Cell migration was measured by labeling cells with DiO (Vybrant Multicolor Cell Labeling Kit, Invitrogen, UK) and inoculating them as described earlier onto 2% PHBHHx films. These were then incubated in a 21% O_2 or 2% O_2 incubator for 24 or 72 h, after which time the media was removed, the well washed with PBS, fixed with 4% Paraformaldehyde (Sigma Alrich, UK) for 5 min, and then re-immersed in PBS. Confocal microscopy (Olympus Fluoview, Olympus IX71) was performed to determine if cells had migrated into polymer films, by creating a "z-stack" representation of the polymer cross section, giving a fluorescent signal where cells are located and allowing for a plane of reference to be made from the images.

Statistical analysis

Results were deemed to be significant if $P \leqslant 0.05$, or as indicated in figure legends using a 2-tailed, paired, Students *T*-test.

RESULTS

Polymer characterization

Films were first characterized by determining both thickness and stiffness. Thickness was determined using OCT and Image J analysis software. The thickness of polymer films correlated directly with the initial polymer input (r^2 = 0.947). The 0.8% weight/volume films had an average thickness 0.10 ± 0.009 mm, while the 2.4% weight/volume films had a measured thickness of 0.19 ± 0.018 mm (Figure 1A). We next sought to determine the stiffness of the polymer films examined above. Stiffness was determined with mechanical testing with the Bose ElectroForce 3200 system as described in Materials and Methods. The calculated stiffness (resistance to elongation) values ranged from 153 ± 42 N/m (0.8% weight/volume PHBHHx) to 1706 ± 371 N/m (2.4% weight/volume PHBHHx). A biphasic increase in stiffness was observed between ⩽ 1.6% weight/volume (N/m = 135.24 * weight/volume, r^2 = 0.9605) and ⩽ 1.6% weight/volume (N/m = 570 * weight/volume, r^2 = 0.9657) A significant increase in stiffness was observed between films ⩾ 2% weight/volume when compared to ⩽ 1.6% weight/volume ($P \leqslant 0.024$) (Figure 1B).

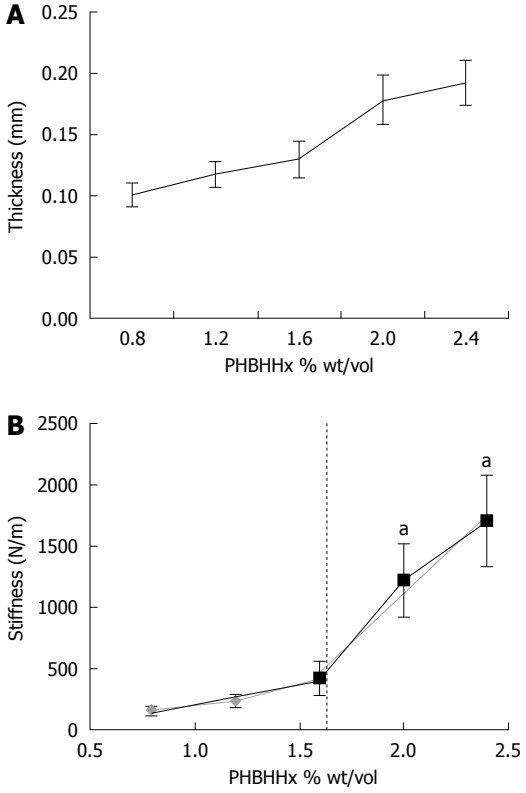

Figure 1 Characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) films. A: Optical Coherence Tomography and Image J software analysis were used to determine poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) film thickness. Average ± 1SD shown on graph, *n* = 9; B: PHBHHX film stiffness was measured with the BOSE ElectoForce 3200 system. Average ± 1 SD shown on graph, *n* = 3. [a]Indicates significant increase compared to ⩽ 0.6% weight/volume PHBHHx. Trend lines are indicated by hatched red lines.

Cellular attachment

rT seeded onto PHBHHx films in 21% O_2 displayed a significant increase in film-adherence between 0.8% weight/volume (3.87 × 10^4 ± 2.73 × 10^4 cells/film) and ⩽ 2.0% weight/volume (⩽ 9.47 ± 4.46 cells/film, $P \leqslant 0.02$). Similarly, the percentage of cells attached to the film in relationship to the overall number of cells in each demonstrated a significant increase between 0.8% weight/volume (19.36% ± 4.98%) and ⩽ 1.6% weight/

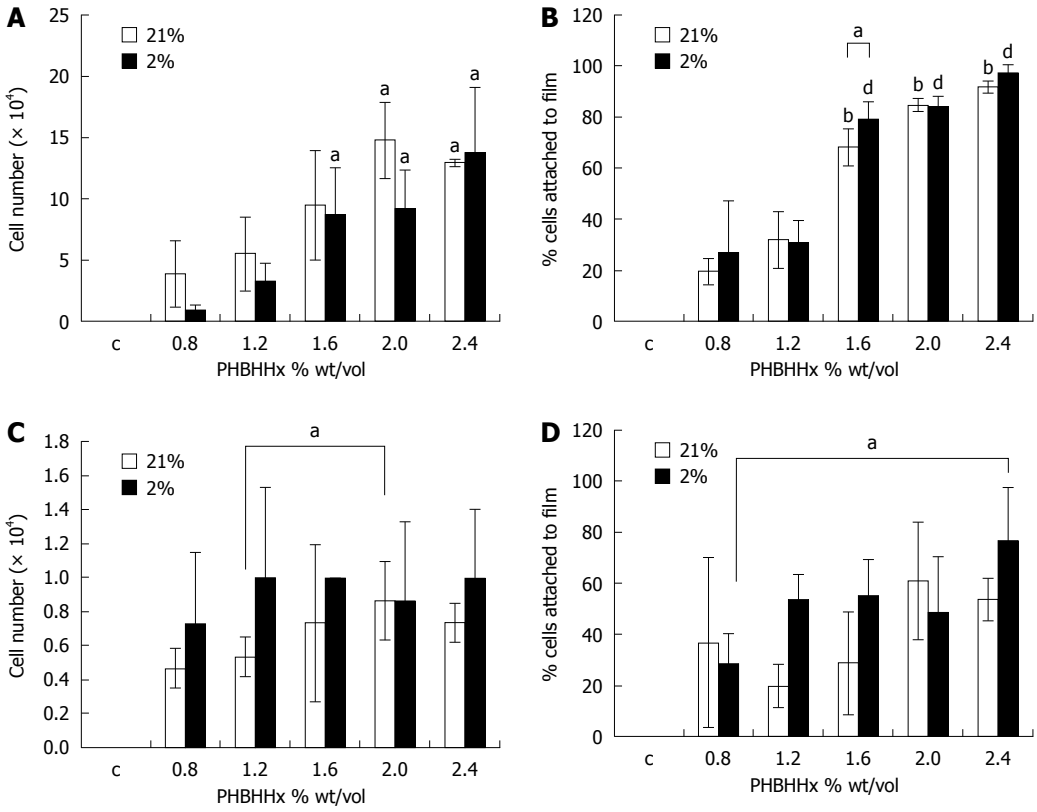

Figure 2 Cell attachment to poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) films. A: Number of tenocytes attached to poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) films of varying % weight/volume polymer concentration; B: Tenocyte attachment to films of varying % weight/volume polymer concentration as a percentage of total cell number in the well; C: Number of human mesenchymal stem cells (hMSCs) attached to varying % weight/volume concentration of polymer; D: hMSCs attachment to films of varying % weight/volume polymer concentration as a percentage of total cell number in the well. Axes are as labeled, error bars indicate one standard deviation. [a]Indicates $P \leqslant 0.05$, [b]$P \leqslant 0.02$, [d]$P \leqslant 0.01$ *vs* ≤ 0.8% weight/volume PHBHHx or as indicated.

volume (≤ 68.38% ± 7.31%, $P \leqslant 0.02$) (Figure 2B). The use of physiological oxygen (2% O_2) in rT PHBBHx film adherence and percentage attachment studies yielded similar results to above. Significant increases in adherence were noted between films of 0.8% weight/volume and 1.6% weight/volume ($0.87 \pm 0.42 \times 10^4$ *vs* $8.67 \pm 3.84 \times 10^4$ - $13.73 \pm 5.36 \times 10^4$, $P \leqslant 0.05$) (Figure 2A), demonstrating that rT cells adhere better to substrates with a stiffness ⩾ 420 N/m. Significant increases in percentage cell attachment were also noted between films of 0.8% weight/volume (34.37% ± 8.27%) and ⩽ 1.6% weight/volume (≤ 84.36% ± 3.98%, $P \leqslant 0.01$) (Figure 2B). Direct comparison of attachment profiles in 21% O_2 and 2% O_2 revealed a significant increase in cell attachment to 1.6% weight/volume films in 2% O_2 *vs* 21% O_2 ($P = 0.05$) (Figure 2B).

hMSC adherence to PHBHHx films with varying weight/volume ratios was relatively consistent in 21% O_2 across all films tested although a significant increase was noted between films of 1.2% weight/volume ($0.53 \times 10^4 \pm 0.12 \times 10^4$ cells/film) and 2% weight/volume ($0.88 \times 10^4 \pm 0.23 \times 10^4$ cells/film) ($P = 0.04$) (Figure 2C). When expressed as a percentage of total cells present in the dish (film and well), considerable variability was noted. Qualitative increases in cell attachment were noted between 1.2% weight/volume (19.7% ± 8.4%) and 2% weight/volume (61.1% ± 22.9%) ($P = 0.059$) (Figure 2D), suggesting that hMSCs require a stiffer substrate than rT cells for optimal attachment. Reducing atmospheric oxygen to 2% O_2 created a qualitative rise in cellular attachment between 0.8% weight/volume ($0.73 \times 10^4 \pm 0.41 \times 10^4$) and 1.2% weight/volume ($1.0 \times 10^4 \pm 0.53 \times 10^4$) ($P = 0.63$) (Figure 2C). Little variation was seen between films where weight/volume ⩾ 1.2%. When expressing values as a percentage of total cells present, non significant increases were seen between 0.8% weight/volume (28.7% ± 11.8%) and 1.2% weight/volume (53.8% ± 9.7%); however, a significant increase is observed when 0.8% weight/volume (28.7% ± 11.8%) and 2.4% weight/volume (77.1% ± 20.6%) ($P = 0.03$) (Figure 2D) are compared. Taken together, this indicated that hMSC cultured in physiological oxygen display an adherence preference for PHBHHx films with stiffness of 240 N/m (*vs* 1220 N/m in 21% O_2).

Cellular migration

Our final investigation was intended to determine if cells rapidly migrated into PHBHHx films. Based on our previous observations, we used a 2% PHBHHx film throughout. No tenocyte or MSC migration was observed into the polymer film after either 24 or 72 h in either O_2 concentration in either x-z or y-z directions (Figure 3).

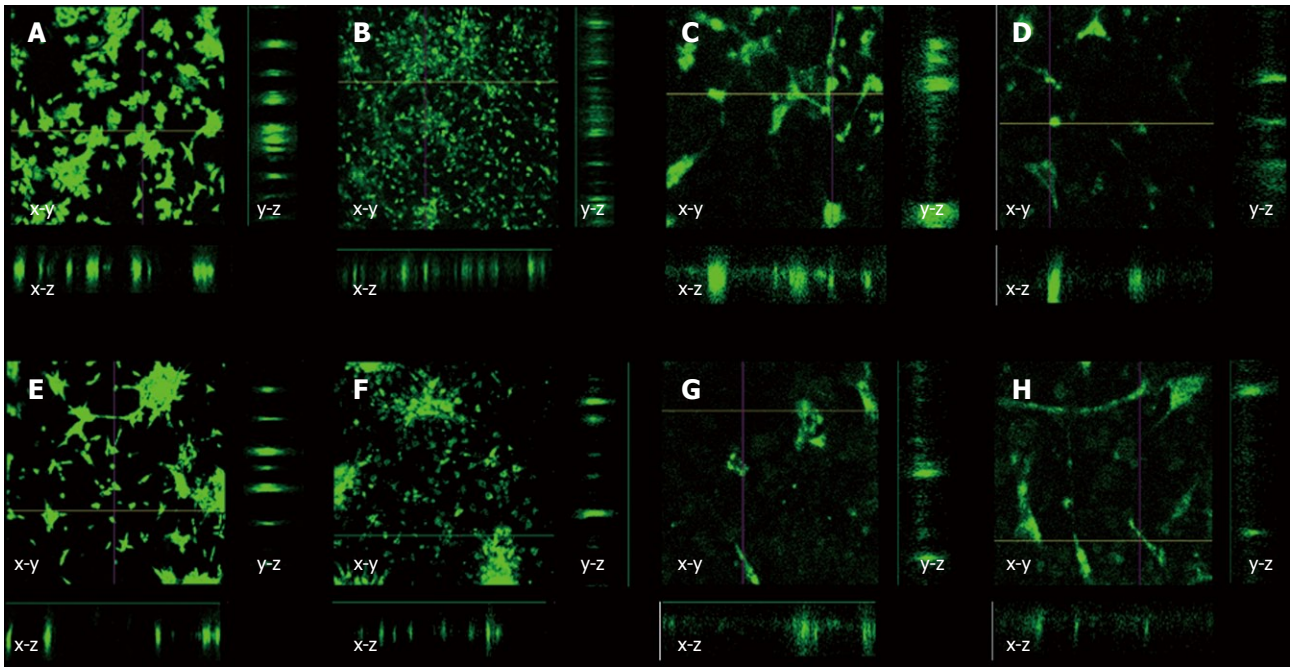

Figure 3 Representative images showing surface and cross section views through poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) films. A: tenocytes 21% O_2, 24 h; B: tenocytes 21% O_2, 72 h; C: Human mesenchymal stem cells (hMSCs) 21% O_2, 24 h; D: hMSCs 21% O_2, 72 h; E: Tenocytes 2% O_2, 24 h; F: Tenocytes 2% O_2, 72 h; G: hMSCs 2% O_2, 24 h; H: hMSCs 2% O_2, 72 h. All images were taken at 10 × magnification. x-y indicates surface view, x-z and y-z indicate reconstructed cross section views.

Substantial spreading across the surface of the PHBHHx was apparent after 72 h, indicating its high compatibility with tenocytes and hMSCs (Figure 3F).

DISCUSSION

This study demonstrates for the first time that tenocytes will adhere to and spread across PHBHHx polymer films with a preferred rigidity of > 420 N/m. This supports the assertion of PHBHHx as a candidate material for tendon tissue engineering and adds to the body of literature supporting the biocompatibility of PHBHHx.

PHBHHx has been previously used to culture many different cell types. A previous report demonstrated that hMSC adherence was greatly improved on PHBHHx films when compared to both tissue culture plastic and other PHA molecules[6]. Adipose derived MSCs were also successfully cultured in 3D PHBHHx scaffolds before being stimulated into chondrogenic differentiation[18]. The PHBHHx scaffold provided the cells with a suitable matrix for support of growth and differentiation *in vitro* and when implanted *in vivo* as evidence by the production of cartilaginous ECM, a key requirement of cartilage tissue engineered constructs. ECM is also the key component in tendon as it is this, rather than the cellular component, that sustains the mechanical load[8]. Therefore, PHBHHx can be used as a 3D scaffold to support cells while they express and develop an ECM. This support capacity is not limited to chondrocytes, as demonstrated by the osteogenic differentiation of rabbit bone marrow derived stem cells on PHBHHx providing additional demonstration of the polymers applicability for orthopedic application use[3].

The proportion of HHx in the polymer has been proposed as an important modulator of cell behavior. Proliferative capacity of rat smooth muscle cells was enhanced by 20% HHx although attachment at seeding was optimal with 12% HHx[19]. In our study we also found robust attachment of hMSC and rT to 12% HHx polymer films when used at the optimal weight/volume ratio.

Investigation into rT attachment to PHBHHx films as a total of all cells present in the well demonstrates that when weight/volume ratios of ⩾ 1.6% were used, virtually all cells adhered to the film in preference to the untreated plastic surface. This can in some part be explained by the increase in stiffness of the polymer film between 1.6% and 2.0% weight/volume. In other words, increased polymer rigidity promoted increased tenocyte adhesion. This reinforces a number of previous studies which have demonstrated that material stiffness affects cellular behavior in many ways, including adhesion[20-22]. hMSCs have previously been shown to adhere to PHBHHx and many other different surfaces with differing mechanical properties[3,23,24], explaining why little difference was found between polymer concentrations. As cell fate was not investigated in this study, it is not known what, if any, effect on differentiation potency this had. Ongoing 3-D tissue engineering experimentation will address these questions.

The tendon is poorly vascularized and has a low mean oxygen concentration[25]. We therefore performed our investigation in both room oxygen (21% O_2) and tendon

tissue normoxia (2% O_2). Previous studies into the effects of different oxygen concentrations on cells have also demonstrated enhanced proliferation, enhanced clonogenicity, reduced karyotypic abnormalities, reduced spontaneous differentiation, altered transcriptional profiles, and altered FTIR profiles across numerous cell types, including hMSCs[14,15,26-28]. When comparing 2% O_2 with 21% O_2, only small differences were found between cell number or percentage attachment at the same PHBHHx concentration for either cell type. A qualitative increase was observed in tenocytes ($\geqslant$ 1.6% weight/volume) in 21% O_2 over 2% O_2; however, this was not significant. For reasons we do not fully understand, we observed large standard deviations in a number of 2% O_2 sample groups, which could be contributing to this. It should be noted that little difference in the percentage of cells attached to the polymer were observed between the differing oxygen conditions, demonstrating that oxygen tension was not effecting cellular attachment to the films *per se* but was rather reducing the population of cells available for attachment. hMSCs were generally noted to adhere better in hypoxic conditions to all polymer film compositions; however, no significant rises were found, possibly due to large inter-group deviations. To our knowledge, this is the first study looking into the *in vitro* effects of oxygen tension on the interaction of primary mammalian cells with polyhydroxyalkanoate scaffolds.

Cell spreading was monitored across polymer surfaces in the absence of mechanical stimuli or directional forces over a period of 72 h by marking cells with fluorescent tracker dye (DiO). This method was essential due to polymer opacity. After 24 h, the cells were clumped together on the surface of the polymer. This behavior is not uncommon in cell culture and can be explained by the cells not being separated sufficiently when re-suspending after centrifugation. After 72 h, the cells were seen to move apart from each other, filling the available space on the polymer. Yang *et al*[29] found that mouse islet cells showed increased metabolic activity when cells were cultured on PHBHHx when compared to tissue culture plastic and Poly Lactic Acid. This investigation also looked into cell migration across a PHBHHx film surface, finding that cells were moving from an even distribution to clump together to start to form functional units. These findings agree with this work that cell locomotion is possible across PHBHHx surfaces.

DiO and other similar dyes used for tracking cells can be expelled by the ABCG2 multi-drug transporter pathway[30]. hMSC retained dye more efficiently than tenocytes although both had undergone reductions in intensity after 72 h, suggesting that the dye had been exocytosed. Migration into the polymer surface was measured with confocal microscope generated z-stacks, allowing for cross sectional views to be created in both the x-z and y-z directions. Cells were always found to be in one plane of view, with no further fluorescent signatures being seen above or below the single plane. This indicated that the cells remained on the surface of the polymer as opposed to migrating into it, indicating that localized polymer degradation had not occurred over the time period tested. This observation is reinforced by previous reports which state that PHBHHx is broken down *in vivo*[31] and *in vitro*[32] at very slow rates *via* hydrolysis. Further investigation of cell migration into and across PHBHHx surfaces could potentially form the basis of a mathematical modeling study; however, this is beyond the scope of this investigation.

This investigation demonstrates that tenocytes and hMSCs can adhere to and spread across PHBHHx films over 24 and 72 h time periods. Film scaffolds fabricated with $\geqslant$ 1.6% weight/volume polymer/solvent, with a stiffness $\geqslant$ 420 N/m are the most effective in supporting this activity with rT cells; however, hMSCs displayed a capacity for adhesion to all polymer films of stiffness $\geqslant$ 240 N/m. Physiological normoxia increased hMSC adhesion to most PHBHHx films; however, no significant differences were seen due to large intergroup variation and little effect was observed on rT cell adhesion. PHBHHx can now be considered to be a potential material for use in future tendon tissue engineering application.

ACKNOWLEDGMENTS

We are grateful to Miss Samantha Wilson and Dr. Ying Yang for their assistance in OCT operation.

COMMENTS

Background

Tendon injury is an increasing problem in medical science due to the very slow rate at which damaged tendon repairs. Current surgical repair techniques can be ineffective in some cases. As a result, tissue engineering is seen as a viable option in tendon repair. Previous studies into the effectiveness of alternative replacement materials to tendon are starting to show good results; however, a perfect solution is yet to be found. Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) has been shown to support several cell types, but as yet tendon cells have not been investigated.

Research frontiers

Current research into tendon tissue engineering focuses on finding materials that can support cellular adhesion while at the same time being able to withstand the high mechanical forces transmitted through tendons, restoring function at a faster rate than would otherwise be possible.

Innovations and breakthroughs

This investigation has for the first time looked into the interaction of PHBHHx with rat tenocytes (rT) and the effect of PHBHHx scaffold stiffness on human mesenchymal stem cells (hMSCs). It has also looked at how atmospheric oxygen effects PHBHHx/cell interaction.

Applications

This research provides the basis for further investigation of Polyhydroxyalkanoate polymer molecules in the field of tendon tissue engineering. This is an exciting development, as PHA molecules, specifically PHBHHx, are renowned for their long term mechanical integrity and biocompatibility *in vivo*.

Terminology

PHBHHx: is a natural polymer produced as an intracellular energy storage molecule by bacteria in certain conditions. hMSC: are pre curser cells to many different tissue types in the body, including bone, cartilage, skeletal muscle and tendon. rT: Rat Tendon cell (tenocyte) are cells isolated from tendon from adult rats. OCT: Optical Coherence Tomography is a laser based system used for investigating thin sections of materials or the upper layers of block materials.

Peer review

The manuscript is a brief research communication reporting mainly attachment

of MSC and tenocyte to poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (or PHBHHx) films. The adhesive properties of both cell types were examined in relationship to varying weight/volume ratios of PHBHHx and O_2 tension. The manuscript by Lomas *et al* demonstrates that rT and human MSCs adhere to and migrate on PHBHHx. The work is noteworthy due to the fact that PHBHHx is one of the few polymers that can be produced on a large scale and also has properties suitable for medical use.

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World Journal of Stem Cells

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World J Stem Cells 2012 September 26; 4(9): I
ISSN 1948-0210 (online)

Acknowledgments to reviewers of *World Journal of Stem Cells*

We acknowledge our sincere thanks to our reviewers. Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of our World Series Journals. Both the editors of the journals and authors of the manuscripts submitted to the journals are grateful to the following reviewers for reviewing the articles (either published or rejected) over the past period of time.

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World J Stem Cells 2012 September 26; 4(9): I
ISSN 1948-0210 (online)

Events Calendar 2012

January 22-27, 2012
Keystone Symposia: Cardiovascular Development and Regeneration
Taos, NM, United States

February 2-3, 2012
Stem Cells 2012
San Diego, CA, United States

February 16, 2012
The 2012 London Regenerative Medicine Event
London, United Kingdom

February 23, 2012
CiRA Symposium: Advances in Nuclear Reprogramming and Stem Cell Research
Kyoto, Japan

February 26 - March 2, 2012
Gordon Research Conference: Reprogramming Cell Fate
Galveston, TX, United States

March 9, 2012
Cell Culture Technology: Recent Advances, Future Prospects
London, United Kingdom

March 11-16, 2012
Keystone Symposia: The Life of a Stem Cell: From Birth to Death
Olympic Valley (Lake Tahoe), CA, United States

March 25-30, 2012
Keystone Symposia: Advances in Islet Biology
Monterey, CA, United States

March 28-29, 2012
Single Cell Analysis Europe
Edinburgh, United Kingdom

April 1 - 6, 2012
Keystone Symposia: Mechanisms of Whole Organ Regeneration, joint with Regenerative Tissue Engineering and Transplantation
Breckenridge, CO, United States

April 25-28, 2012
3rd International Congress on Responsible Stem Cell Research
Aula Nuova del Sinodo
Vatican City, Vatican City

April 27-29, 2012
2nd Institute of Advanced Dental Sciences & Research International Conference 2012: Fundamentals of Conducting and Reporting Research--Biological, Pharmaceutical, Medical & Dental Sciences
University of the Punjab, Lahore, Pakistan

April 29 - May 2, 2012
3rd International Conference on Stem Cell Engineering (ICSCE)
Co-organized by the Society for Biological Engineering (SBE) and the ISSCR
Seattle, WA, United States

April 30, 2012
Stem Cells to Tissues
Boston, MA, United States

April 30, 2012
Regenerative Biology: From Stem Cells to Tissues
The Joseph B Martin Conference Center, Harvard Medical School
Boston, MA, United States

April 30-May 2, 2012
Till & McCulloch Meetings
Montreal, QC, Canada

May 18, 2012
The 2012 Stem Cell Discussion Forum
London, United Kingdom

May 21-22, 2012
Driving Stem Cell Research Towards Therapy.
Edinburgh, United Kingdom

June 5-8, 2012
18th Annual International Society for Cellular Therapy Meeting
Washington, DC, United States

June 13-16, 2012
International Society for Stem Cell Research 10th Annual Meeting
Yokohama, Japan

June 25-27, 2012
The Stem Cell Niche - development and disease
Copenhagen, Denmark

June 27-28, 2012
Bioprocessing & Stem Cells Europe
London, United Kingdom

June 28-29, 2012
Origins of Tissue Stem Cells
Edinburgh, United Kingdom

July 9-11, 2012
Stem Cells in Cancer - 2nd annual Cambridge Stem Cell International Symposium
Cambridge, United Kingdom

July 15-18, 2012
39th Annual Meeting & Exposition of the Controlled Release Society
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World J Stem Cells 2012 September 26; 4(9): I-V
ISSN 1948-0210 (online)

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Name of journal

World Journal of Stem Cells

ISSN

ISSN 1948-0210 (online)

Editor-in-Chief

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Indexed and Abstracted in
PubMed Central, PubMed, Digital Object Identifier, and Directory of Open Access Journals.

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Baishideng Publishing Group Co., Limited

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

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