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Beverage waste derived biomaterials for tissue engineering

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S1. MATERIALS AND METHODS:

The materials used in this work were prepared by modifying the chemical nature of the material derived from beer bagasse (BB) in order to optimise the behaviour of the final solid as a bone substitute. Three different modifications were carried out, as indicated below.

Optimisation of BB was done by treatment with base (0.5, 1 or 3N NaOH) on BB46 for an hour at 100 ºC. The materials were subsequently washed with hot water (80 ºC), until the eluent reached pH = 7, dried at 150 ºC during 4 hours (RT-100 ºC, 5 ºC/min), calcined at 600 ºC during 4 hours in air, structured as pellets pressed at 4 tons/cm^2 (1.3 cm diameter by 3 mm depth) and calcined in air at 700 ºC. The compositions of original and base treated materials are collated in Table 1.

In order to produce structured materials capable of acting as bone substitutes, the structuration of the powdered materials was carried out by using inverse templating. For this procedure the powdered materials BB46 (dried 6 hours at 150ºC, milled in a rotary mill, to below 120 μm and calcined at 600 ºC for 4 hours), BB46 3N NaOH were suspended in an aqueous solution of polyvinyl alcohol (4 % PVA) with stirring for 4 hours at room temperature for homogenisation, with a solid/liquid ratio = 1/1. Polyurethane sponges of densities between 10 and 80 kg/m^3 were tested, cut into cubes of 1 cm, immersed in the BB/PVA/water slurry and after impregnation dried at room temperature for 72 hours and then calcined at 700, or 1000 ºC (1 ºC/min up to the final temperature which was maintained for 3 hours). The results obtained with the polyurethane of 70 kg/m^3 are shown in the article due to the similarity in its porosity to healthy bone.

Characterisation of biomaterials

The composition of the BB derived materials was studied with inductively coupled plasma atomic emission spectroscopy (ICP) in a Perkin-Elmer Optima 3300DV apparatus. X-ray diffraction (XRD) patterns of samples were analysed on a PANalytical X'Pert PRO X-ray diffractometer using Cu Kα radiation (45 kV, 40 mA) and parallel beam optical geometry at 2 θ = 15–85 ° with a step size of 0.02 ° and a counting time of

10 s per step. The crystalline phases were identified by reference to JCPDS diffraction file data 4-90 °, 0.04 °/pass 20 seconds and the results were analysed with the X´Pert HigScore Plus program. The inter and intraparticulate pore volumes were determined by mercury intrusion porosimetry (MIP) using a Fisons Pascal 140/240 apparatus on samples, previously dried overnight at 150 °C using the recommended mercury contact angle of 141 º and surface tension of 484 mNm^{-1}. The mechanical strength of the conformed materials was determined by applying pressure on the external surface of the pieces until breakage with a Chatillon dynamometer (LTMC) with a 0.9 mm test head, ten discs of each material were tested to ensure the precision of the results. The micrometric morphological and structural study was carried out in a Scanning Electron Microscope (SEM) Hitachi Tablet Microscope TM-1000 coupled to an EDX analyser (SwiftED, Oxford Instruments).

X-ray photoelectron spectroscopy was used for surface chemical compositions assessment of materials using a VG ESCALAB MkII spectrometer equipped with a 5-channeltron detection system, with a standard Al K_α excitation source (hν= 1486.6 eV), with materials positioned at the electron take off angle normal to the surface with respect to the analyser, which was set to constant pass energy E_p =50 eV. The binding energy (BE) scale was calibrated by measuring the reference peak of C 1s (BE = 285 eV) due to surface contamination with an accuracy of ±0.2 eV. A least square peak fitting routine was used for the analysis of XPS spectra, and the relative concentrations of chemical elements were calculated by the standard quantification routine using their Schofield values.

Cell cultures

Osteoblast-like murine MC3T3-E1 cells were cultured in α-MEM (Gibco) supplemented with 10 % foetal bovine serum and 1 % penicillin-streptomycin (basal medium) in a humidified atmosphere at 37 °C and at 5 % CO_2. To induce differentiation, cells were placed in osteogenic media: basal medium supplemented with 10 mM β-glycerophosphate and 50 µg/ml ascorbic acid and incubated under the same conditions described above for 15 days.

Cell viability

To evaluate the cytocompatibility of the materials obtained from beer bagasse, an LDH assay was performed. Hydroxyapatite (HA), a synthetic calcium phosphate ceramic that mimics the natural apatite composition of bones and teeth and has been described as a potential material to coat scaffolds for promoting osteoblast differentiation, was used as a reference material. MC3T3-E1 cells were seeded on bagasse-derived materials and on HA, structured as tablets, at an initial density of 2 x 10^4 cells/cm^2 in 24-well culture plates. Cells were cultured for 24 hours under standard conditions in basal and osteogenic medium; the culture medium was then collected and the lactate dehydrogenase (LDH) released to the medium was quantified using the Cytotoxicity Detection kit (Roche), following the manufacturer's instructions. Briefly, culture supernatants were collected and centrifuged at 1500 rpm for 5 minutes to remove cells. Cell-free supernatants were incubated with the kit´s substrate mixture. LDH activity was determined in a coupled enzymatic reaction; during this reaction, the tetrazolium salt is reduced to formazan. The formazan dye was quantified spectrophotometrically at 490 nm using an ELX808 microplate reader (BioTeK). LDH released by cells permeabilized in 0.2 % Triton was taken as 100 % cell death.

To evaluate the MC3T3-E1 cell viability and proliferation rates, cells were grown on BB and HA derived materials structured as tablets. Cells (2 x 10^4 cells/cm^2) were seeded on the materials and incubated for 1-, 3- and 7-days in basal medium, as described above. Cell proliferation was determined using the Cell Proliferation Assay kit (AppliChem) following the manufacturer's instructions, this Cell Proliferation Assay employs 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT). In living cells, mitochondria have the capability to reduce XTT to form an orange coloured, water soluble dye. Therefore, the concentration of this dye is proportional to the number of metabolically active cells. The absorbance of each well was measured spectrophotometrically at 450 nm using an ELX808 microplate reader (BioTeK). For comparative reasons the absorbance values were normalized according to the surface area of each sample.

Cell differentiation

MC3T3E1 cells were seeded on BB and HA derived materials structured as tablets. Cells (2×10^4 cells/cm^2) were seeded on the materials and incubated for 15-days in osteogenic medium, as described above. After incubation the cells were rinsed with PBS and then lysed into PBS containing 0.2 % Triton X-100. Cell lysates were centrifuged and the soluble fraction was used for enzyme assay. Samples were incubated with an assay mixture of *p*-nitrophenyl phosphate (*p*NPP) (Sigma). Cleavage of *p*NPP in a soluble yellow end product, *p*-nitrophenol, which absorb at 405 nm, was used to assess ALP activity. The optical density of *p*-nitrophenol at 405 nm was determined spectrophotometrically and ALP activities were normalised to total protein content using the bicin-choninic acid (BCA) method. ALP activity was calculated as nanomoles of *p*-nitrophenol liberated per microgram of total cellular protein per hour and expressed as percentage of control cells (cells grown on HA tablets). For comparative reasons the absorbance values were normalized according to the surface area of each sample.

Cell morphology

After 3 days culture on the BB and HA derived materials the MC3T3-E1 cells were prepared for cytoskeletal examination by fluorescence microscopy. Cells seeded on polystyrene plates were used as controls. Samples were rinsed three times with PBS and then fixed with 4 % formaldehyde for 20 min at 37 ºC, and washed again with PBS. The fixed cells were permeabilized in 0.2 % Triton X-100 (Sigma) for 15 min and washed three time in PBS. The cells were incubated with TRITC-phalloidin (1.2 ug/mL, Sigma) for 1 h at 37 ºC to determine cytoskeletal organization. After three washes in PBS, nuclei were counterstained with Hoechst 33258 (Molecular Probes; 0.2 mg/ml in PBS) for 10 min. The samples were mounted under coverslips using Vectashield (Vector Labs) and were then visualized using a Leica, DMLB fluorescence microscope.

Statistical analysis

The results are shown as the mean +/- standard error of the mean of data from four experiments. The data were analysed by single factor analysis of variance (ANOVA)

followed by the post hoc Tukey's honestly significant difference test. A significance level of $p < 0.05$ was chosen, and Statistica 7.0 (StatSoft Inc, Tulsa, OK) software was used for all statistical tests.

In vivo **studies**

All animal handling and experimental procedures were approved by the Animal Care and Usage Committee of Universidad Complutense according to the guidelines for ethical care of experimental animals of the European Community (RD 53/2013).

Muscle tissue model

Healthy male Wistar rats (Harlan, weighing 300 - 400 g) were housed in standard laboratory conditions (temperature 24 °C and humidity 60 % with 12 hour day, 12 hour night cycles). Animals were fed with standard laboratory diet and water ad libitum. Surgical process was performed in anesthetized animals using a mix of Isofluorano^{R} with 30 % O_2. A 3 cm long incision was performed on the back of the animals following the line of the spinal column. One intramuscular pocket was made in each side of the spinal column in the back quarters at 50 mm from the incision and 3 cm from tail root. Two different samples were located in each animal using 18 animals (n = 4). The muscle and skin tissue were sutured with vicryl 2.0 (Ethicon). After three weeks post implantation animals were sacrificed using intravenous injection of Pentobarbital (300 μL).

Histology

Before starting histological treatment, removed samples were evaluated to observe macroscopic signs in adjacent tissue and sample integration. Afterwards, the removed

specimens were fixed in PFA 4 % (Sigma) during 2 days. Samples were decalcified using nitric acid solution (5 %), followed by a dehydration process by an increasing of ethanol solutions and embedded in paraffin blocks. Tissue sections were obtained using microtome (Leika RM2155) of 4-6 μm thickness, stained with Hematoxylin Eosin (H/E) and finally, tissue slices were evaluated by light microscopy (Olympus IX51).