Rheological properties, biocompatibility and in vivo performance of new hydrogel-based bone fillers

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Three different heterologous substitutes for bone regeneration, manufactured with equine-derived cortical powder (CP), cancellous chips (CC) and demineralized bone matrix granules (DBM), were compared in in vitro and in vivo settings. We tested: a commercially available bone paste (Osteoplant-Activagen™, consisting of aqueous collagenous carrier, CP, DBM; named A); a second-generation injectable paste (20 kDa polyethylene glycol/hydroxypropyl-methyl cellulose-based hydrogel, CP, DBM; B); a pre-formed bone filler (400 kDa polyethylene oxide/hydroxypropyl-methyl cellulose-based hydrogel, CP, CC, DBM; C). Vitamin C acted as a visco-modulator during C and B β-rays sterilization, modifying graft injectability. For each filler, we examined dissolution in culture medium, gene expression of the substitute-exposed osteogenically-induced human bone marrow stromal cells (hBMSC), and performance in a rabbit bone defect model. A dissolved after 1 h, while fragmentation of B peaked after 8 h. C remained unaltered for 2 days, but affected the microenvironmental pH, slowing the proliferation of exposed cells. B-exposed hBMSC overexpressed bone sialoprotein, osteocalcin and RUNX2. For all fillers histological results evidenced bridged lesion margins, marrow replenishment and bone-remodeling. However, B-treated lesions displayed a metachromatic type II collagen-rich matrix with prehypertrophic-like cells, matching the in vitro expression of cartilage-specific markers, and suggesting a possible application of B/C double-layer monolithic osteochondral plugs for full-thickness articular defects.

Introduction

Bone defects in humans may be caused by a number of different factors, including – but not limited to – traumas, inflammation, degenerative diseases and surgical treatment of tumors; since bone provides the organism with structural stability, protection and acts as a physiological reservoir of hematopoietic and mesenchymal progenitor cells, these defects can be problematic. The need for bone grafts is constantly growing, with a forecast market of $2.3 US billion dollars in 2017 (see: http://www.prweb.com/releases/bone_grafts/standard_bone_allografts/prweb8953883.htm). In order to restore bone defects, several composites have been developed1 and, depending on their formulation, they can be used to repair damaged tissues and to impart desirable biological and/or mechanical properties.2 Among the known bone repair materials and void fillers is the autologous cancellous bone, which is osteoinductive/conductive and non-immunogenic and is currently considered the gold standard for bone defect repair.3 Unfortunately limited availability and variable quality, hematoma, infection, increased operative time and bleeding, donor site morbidity, and additional costs add to the limitations of the autologous cancellous bone.4–6 The use of an alternative allograft bone also shows several drawbacks, such as a reduced osteogenic capacity and a reduced revascularization at the defect site, a higher resorption rate and a greater immunogenic response, along with limited commercial availability of an appropriate graft material.7 At the same time synthetic materials may suffer from unacceptable workability, handling and/or setting parameters, insufficient density, non-physiological absorption rates and from inability to impart adequate construct stability with time.8 Because of all the above mentioned limits, the expanding need for bone reconstruction is paired by the growth of interest in bone substitutes of animal origin, also known as heterologous or xenogeneic grafts.9 Xenografts represent a theoretically unlimited supply of available materials if they can be processed for a safe trans-
plantation in humans. They are obtained at competitive costs, they show osteoinductive/conductive properties, and can be developed to mimic the physical and mechanical nature of human tissue to be substituted.12 Xenogeneic bone substitutes, such as equine-derived biomaterials, can be manufactured to reproduce the three-dimensional characteristic of the autologous tissue while sustaining cell proliferation onto the construct.11 It is also worth noting that the putative threats posed by transmissible spongiform encephalopathies, in this respect, do not represent an issue, due to the chemical stability of equine prions. Nonetheless, the complete absence of potential immunogenicity of xenogeneic materials in humans has to be carefully examined; for this reason specific guidelines (ISO 10993) must be accounted for prior to the applicative use of these materials. However, along with xenogeneic substitutes, there is also a need for bone defect fillers that display proper stability, rate of absorption, workability and cohesiveness,14 to completely fill the lesion void for a sufficient amount of time while promoting bone growth. The development of injectable and moldable bone pastes or putties has long been foreseen as a practical approach to enhance the adaptability of these materials to bone defects of irregular geometry. Several injectable/moldable bone grafts are currently available,15 and some of them have been tested for the inclusion of different bone particulates or mineral granules.16 The visco-elastic properties of hydrogels, their high tissue-like water content, and ability to homogeneously incorporate conductive matrices, make them suitable substrates to carry powders and granules and render them even more appealing from the commercial point of view.17,18 Other than improving bone substitute cohesion and acting as thickening excipients or carriers of osteoconductive granules, hydrogels also provide a physical support, a degradable hydrated three-dimensional matrix which influences the cell–scaffold interactions, physically and biologically modulating both cell invasion and colonization into the construct, ultimately guiding actions, physically and biologically modulating both cell invasion and colonization into the construct, ultimately guiding bone tissue regeneration.19,20 Along with several natural polymers used in hydrogels for tissue engineering approaches, such as collagen, hyaluronan, alginate, chitosan or fibrin,21 an additional wide range of synthetic hydrogels has shown suitable physical and chemical properties for regenerative medicine applications; these materials include poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(propylene fumarate) (PPF), cellulose derivatives (e.g., hydroxypropyl methyl cellulose, HPMC), Pluronic F-127 and polypeptides. In the attempt to combine the most suitable properties of bone particulate and hydrogel composites, then, we focused our attention onto three different injectable or pre-formed bone fillers, obtained by combining equine bone-derived particles with two different kinds of gels: one based on an aqueous carrier and natural equine Achilles’-derived tendon type I collagen, the other on a synthetic polyethylene glycol/oxide (PEG/PEO) and hydroxypropyl methyl cellulose (HPMC) hydrogel. Indeed, hydrogels based on several PEO or PEG/HPMC formulations have been used for years as cell scaffolds, adhesive medical applications, and delivery vehicles with promising results.22 The study was then designed to evaluate the physicochemical characteristics, and the in vitro and in vivo performances of the chosen fillers, to confront the eventual osteopromotive effect of the different polymeric gels on the bone forming ability of equine particles.

Results

Rheological and chemical–physical analyses of second-generation hydrogels

Non-sterile LMW and HMW hydrogel samples showed an average pH of 7.8 and 8.6, respectively. Viscosities of sterile and non-sterile LMW samples were alike: non-sterile and sterile LMW gel exhibited average values of 5.5 and 6 Pa s respectively, at a shear rate of 7 s−1 at 25 °C. This result highlighted the anti-polymerizing effect of the concentrations of vitamin C higher than 0.5 mM. Vitamin C is able to limit intra- and inter-molecular rearrangement of PEG and HPMC polymeric chains originating as a consequence of sterilization, thus maintaining the visco-elasticity of gels and injectability of bone fillers nearly unaltered. Non-sterile HMPW samples showed higher viscosity than LMW, in particular, they returned an average value of 12.7 Pa s, at a shear rate of 7 s−1 at 25 °C. The visco-elasticity of sterile HMW hydrogel, instead, was not detectable by using the Rheolab QC rheometer, due to a significant change of its physical status, which switched from a gel-like to a silicone-like behavior, owing to the interactions of polymeric radicals. A vitamin C concentration lower than 0.5 mM, then, was unable to significantly prevent molecular rearrangement of the polymers, providing pre-formed bone fillers characterized by higher physical stability. The results of FT-IR analysis on the LMW hydrogels show that, after sterilization, there are no evident changes in the molecular structure of the polymeric species (Fig. 1, panel A). The spectra of the LMW gels coincide when performed before and after sterilization, both in terms of absorption wavelengths of the specific bond of the polymers, and in the molar extinction coefficients. In contrast, the IR spectra of the HMW hydrogels significantly differ in the molar extinction coefficients of the bands attributable to the vibrational wavelengths of polyethylene glycol bonds (Fig. 1, panel B). The sterilized gel, in fact, is in a higher state of polymerization than the unsterilized sample; this results in lowering of the intensity of the absorption bands in the spectrum between 1700 and 600 cm−1.

In vitro dissolution study

Specimens generated to perform the dissolution analysis displayed the same size, with no significant statistical difference between samples of the various fillers (Fig. 2, panel A); on average they occupied 37% of the area of the visual field of the culture well. Once culture medium was added to the wells, the materials started to dissolve, gradually distributing smaller size particles over the well area. While the number of large particles (area > 1000 pixels, relative to the image acquisition settings) resulted essentially constant among all materials and were limited to a few (less than 50 per each material), most
identified particles displayed areas smaller than 500 pixels; these fragments contributed to 85% of the total number of fragments at any time-point, for the A and B fillers, but only to 69% for the C filler, which compensated this reduction with the presence of a class of medium-size particles, comprised within 500 and 1000 pixels. Relative to the small-size particles, their number peaked at different timings, due to specific dissolution kinetics of each material. The largest amount of fragments was identified at 1 h, 8 h and 1 day after medium exposure for A, B and C, respectively. The medium-size class identified during the C dissolution co-peaked at 8 h (data not shown). Moreover, their absolute numbers were grossly different among the three filler types (170 ± 30 for A, 129 ± 42 for B and 20 ± 13 for C; Fig. 2, panel B). The progressive reduction of the number of small-size fragments upon medium exposure, which accounts for half of their number for any tested filler within the experimental timings, calls for their complete dissolution in the liquids. The C filler, however, maintained its structural integrity for at least a day; its partial fragmentation was in fact achieved only (and limitedly) afterwards, from the second day of medium exposure, on.

material partially absorbed liquids from the medium, thus slightly increased in size at early timings after medium exposure.

Fig. 1 IR spectra of non-sterile and sterile samples of LMW (A) and HMW (B) hydrogels. The fingerprint regions of infra-red spectra are depicted, and used to identify changes in chemical bonds within the polymers molecules, as a consequence of sterilization by beta-irradiation. Assignment of the absorption bands of spectra: 1086 cm$^{-1}$ $\nu$(C–O–C), 1253–1300 cm$^{-1}$ $\delta$(CH$_2$), 1349 cm$^{-1}$ $\nu$(C–C) and $\delta$(CH$_2$) where $\nu$, $\tau$ and $\delta$ are indicating stretching, twisting and bending vibration mode respectively.

Fig. 2 Substitutes dissolution studies. (A) This panel depicts a selection of representative images acquired during the dissolution of each biomaterial at different timings. Tested fillers are indicated in the top row (A, B and C); timings are indicated on the left column; not all assessed time-points are presented. All images were acquired with the same settings. White bars: 4 mm. (B) Time-dependent distribution of the number of fragments obtained at the different time-points for each material under testing. For each filler, data points represent the average number of fragments, derived from images acquired from 4 separate specimens, at the specified time-point. Curves are the best-fit interpolation obtained from raw data. Error bars depict standard deviations. X-axis categories not to scale.
Influence of different fillers on cell proliferation

No significant differences in the growth kinetic profiles could be observed among the tested hBMSC, once exposed to A or B, with respect to control cultures (Fig. 3, panel A); all these cells performed an average of 4.35 ± 0.43 duplications within the two weeks of culture. In contrast, C-exposed cells performed only 2.93 ± 0.54 doublings, as testified by their slower growth rate (Fig. 3, panel A). This effect was exerted both on uninduced and on osteoinduced cells, and it was due to a diminished proliferative potential; this loss can in fact be ascribed to the remarkable reduction of the mRNA expression level for the proliferation marker Ki67, both in control and in osteoinduced hBMSC (Fig. 3, panel B). In addition, C-exposed cells would regain their proliferation standards if cultured for one additional week, indicating that the effect was transient (data not shown). In the attempt to pinpoint the possible cause of such a proliferative loss, we hypothesized that C could either release or uptake something into or from the growth medium.

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**Fig. 3**  *In vitro* cultures of substitute-exposed hBMSC. (A) Growth kinetics of hBMSC in Transwell cultures in the absence (control cultures, CN) or in the presence of the fillers under testing (A, B and C). Data depicts the average absorbance values obtained among the three different primary cultures, each derived from four independent determinations at each time-point. Error bars depict standard deviation (SD) values; for clarity CN, A and B SD values are omitted. (B) Assessment of the expression profile of Ki67 in control uninduced (Unind) or in osteoinduced (Osteo) hBMSC cultures, in the absence or in the presence (+C) of the C filler in Transwell cultures. Histograms depict the average gene expression levels, and ±SD values, normalized to the endogenous expression of GAPDH and to control uninduced cultures, of quadruplicate analysis performed on two different primary hBMSC cultures. (C) The graph depicts the time-dependent variations of pH values once the different fillers are placed in a 24-well culture plate filled with sterile PBS solution. Data is the average ± SD values of three independent experiments performed on two different primary hBMSC cultures. (D) Assessment of cell growth kinetics in control cells (CN), or in C-exposed cells, with (C post-PBS or C post-FCS) or without (C) previous washing of the filler specimens for 12 hours either in sterile PBS or in 10% FCS-containing standard growth medium. The analysis was performed in triplicate with the Alamar Blue™ method, as described under the Experimental section.
In this light, we first assessed the pH values of a standard sterile phosphate buffered saline solution (having a nominal pH value in between 7.4 and 7.8), once in contact with either C or B. The presence of both fillers caused the alkalization of the solution, although the C-induced effect was much more consistent in amplitude and duration; the pH level reached an average value of 8.75 which was maintained up to 36–48 hours (Fig. 3, panel C). Consequently we pre-washed C-filler specimens either in 10% FCS-containing growth medium or in PBS, for 12 h and then re-performed a cell growth kinetic study by the Alamar Blue™ test. Indeed, the 40% loss of proliferation was fully recovered by this simple procedure using PBS, but was instead only partially restored by washes with standard growth medium (Fig. 3, panel D).

**Gene expression studies**

We then concentrated on the possible effects and/or interferences of the materials under testing in an in vitro osteogenic differentiation setting. The retrieved transcript samples evidenced a doubled level for RUNX2, the most relevant osteogenic transcription factor, in A- and B-exposed hBMSC cultures (Fig. 4). Similar results, but limited only to the B filler, can be ascribed to the BSP and to the osteocalcin mRNAs levels, respectively, reaching a 4- and a 2-fold increase, when compared to control cells. Although the increase in RUNX2 expression is not statistically relevant, its effects may still be reflected in the expression of some downstream targets. Indeed this is suggestive of a pro-osteogenic stimulus –

![Fig. 4](image_url)
partially provided in A and more strongly sustained in B – that preferentially enhances mid and late mineralization markers, such as BSP and OC, both under control of RUNX2. At the same time, however, a significant reduction of the OP transcript levels was detected for all experimental conditions. Moreover, possibly owing to the limited proliferative activity of the osteoinduced cells, we did not notice any significant alterations in the mRNA levels for type I collagen, in any of the culture conditions, with respect to control cells (Fig. 4).

**In vivo studies**

Preliminary assays on a limited number of experimental animals were performed to evaluate the *in vivo* outcomes of the implanted fillers. The chosen rabbit model heals spontaneously within 8 weeks from the intervention; in this light, while no significant differences were noted in the A- or C-implanted animals versus the sham operated controls (Fig. 5, panel A), B-implanted rabbits displayed an interesting re-organization of the regenerative tissue within the lesion boundaries. One month post-op, the lesion margins – although closer than at the time of the intervention – had not yet fully joined, leaving areas devoid of cortical bone (Fig. 5, panel B, asterisk). The inner portion of the lesion presented evidences of bone remodeling (Fig. 5, panel B (i)) and of active marrow presence (Fig. 5, panel C), thus testifying the viability of the tissue, as well as its response to exogenous materials (Fig. 5, panel B, (ii and iii) and panel D). Interestingly, though, an extended area, encompassing the middle portion of the lesion, displayed a hypertrophic-like cartilaginous appearance, with large cells and a relatively diffused metachromatic matrix (Fig. 5, panel B, iv and panel E). Immunoreactivity of this region to an anti-type-II collagen antibody confirmed the presence of this typical articular cartilage marker in a non-canonical site along with unreactive residual polymer/DBM granules inside the lesion, and was detected only in B-implanted animals. Within the planned 8 weeks, control and experimentally-treated lesions had bridged the initial gap in cortical bone, although to a very limited thickness and non-homogeneous distribution, particularly in B samples (Fig. 6). Independently of the biomaterial used, trabecular bone deposition and marrow replenishment was seen in all samples, although the persistence of a relatively large amount of fibrotic tissue, surrounding the regenerating bone area, could still be detectable in A sections (Fig. 6). Owing to the appearance of cartilage-like tissue within the lesion boundaries in B-implanted animals, we were then prompted to re-assess some *in vitro* samples for the expression of cartilage marker genes, upon B-exposure of cultured hBMSC in Transwell cultures. Interestingly, the relative expression of SOX9, a master transcription factor for cartilage differentiation, and of aggrecan, an abundant component of the articular cartilage, were strongly upregulated, reaching a several-fold increase in transcript levels, as compared to control cells (3.03 ± 0.71 and 59.21 ± 10.94, respectively; Fig. 7).

**Discussion**

Several materials have been developed to treat bone lesions and voids; these preparations are meant to provide a suitable conductive surface for osteoprogenitor cells, and to fit defects with irregular geometry. To these purposes ceramic particulates are often used in these preparations. However its substitution with a natural bone-derived particulate may ease the cell-substrate recognition and matrix deposition, since it provides a naturally microstructured scaffold, a well-known requirement for proper matrix deposition. The nowadays accessible equine raw material and a patented deantigenation process render this substitution feasible and economically sustainable, and we adopted this solution in our bone fillers. In order to achieve malleability and proper direct contact with the bone lesion boundaries, binders or gels are also added to putty preparations. Water-soluble cellulose derivatives, such as HPMC, and polyethers, such as PEG and PEO, have been used since long in food, pharmaceutical, biomedical and cosmetic industries, due to their non-toxicity and low cost, and represent well-tested, safe and easily available components. Binder or gel controlled clearance should be optimized according to the requirements posed by the lesion site, to couple new bone deposition with scaffold degradation and to allow proper nutrient and vascular invasion of the lesion site. In this perspective, the availability of suitable and specific concentrations of vitamin C within the hydrogels provides two relevant advantages: (i) sustains collagen deposition throughout the entire lesion volume, vitamin C being a requested co-factor for the activity of prolyl- and lysyl-hydroxylases, essential enzymes for collagen fibril assembly; (ii) allows modulation of the viscoelastic properties of the resulting filler, exploiting its antioxidant properties and interfering with the β irradiation-ignited polymerization processes. Hence biomechanical properties can be modulated, in our fillers, by the concentration of a required nutrient while undergoing sterilization by β-irradiation, all in one single step; moreover this approach avoids invasive sterilization procedures (such as gamma irradiation), more burdensome on the physicochemical properties of the filler, and eases very much the storage and handling procedures of the filler for the putative clinical applications, providing a ready-to-use material. Clearly the different formulations of the fillers affected their behavior, in accordance with the FT-IR analysis: the tested hydrogels dissolved at different rates *in vitro*. The number of larger particles (area per particle > 1000 pixels) that did not undergo dissolution, which amounts to less than 50 per each material, can be ascribed to the DBM granules and/or chips used and is equally present in all materials. The filler C, however, also released a small subset of medium size particles (500 < area per particle < 1000 pixels), due to its larger polymer component and its longer-lasting degradation requirements. Interactions between the proposed fillers and marrow stromal cells, among which bone precursor cells are known to reside, were the objective of the subsequent analyses. No significant alterations in the cell proliferation rates were observed when exposing cells to either
A or B, with respect to control cells, although a donor’s dependent variability can be observed among the different primary cultures used. In spite of this consideration, the proliferation of the hBMSC exposed to C was exceedingly different from the other tested fillers, with 40% reduction of the cell number after 5 days of exposure. Since no direct contact was involved in our experimental system between cells and fillers, we concentrated onto growth medium alterations. Indeed, changes in

**Fig. 5** Histological results. (A) Lateral and frontal view radiograms (lw and fw, respectively) of the lesion generated in the rabbit distal portion of the femur; in the enlargements the dashed lines outline the lesion boundaries. The representative images refer to a sham operated control animal. A calibrator scale is also visible in whole images. The panel also depicts a representative image of the operated femur whole section (ws), 1 month after surgery, stained with hematoxylin; the superimposed rectangle identifies the area of the B implant. (B) Multiple image reconstruction of part of the lesion area in a B-treated animal, 1-month after implantation; white bars: 50 μm. Dotted lines indicate unresolved areas. Arrows depict the inner bottom side of the generated lesion; arrowheads mark the outer lesion margins, while the asterisk identifies the fibrotic region yet to be joined, at the cortical bone level, to completely resolve the lesion. Circled areas are enlarged in the corresponding in-sets: (i) signs of bone remodeling ad rearrangement are evident in the line-up of putative osteoclasts on the surface of pre-existing trabeculae with osteocytes, in the marrow cavity of the lesion; residual polymer (ii) and DBM/polymer granules (iii) are visible throughout the entire lesion depth and outside the lesion margin area; an extended area, embracing the mid portion of the lesion, displays a hypertrophic-like cartilaginous appearance (iv), with large cells and scarcely organized matrix; white bars (i–iv): 20 μm. (C) Representative section of the lesion area in B-treated animals, evidencing bone marrow presence and signs of active vascular invasion (arrows). (D) Megakaryocyte-like cells (arrows) can be detected in the vicinity of residual polymer/DBM granules. (E) Metachromatic response of the cartilaginous-like area of the inner portion of the lesion. (F) Immune-positivity of the extracellular matrix in the cartilaginous-like portion of the lesion challenged with the anti-type-II collagen antibody (arrows). Arrowheads indicate unresponsive residual polymer/DBM granules in the area. (G) Articular cartilage section of the same B-implanted animal, challenged with anti-type-II collagen antibody. The image is presented as a positive control to panel F. White bars (panels C–F): 10 μm.
the extracellular pH are known to affect the milieu of cultured cells: alkalization of the extracellular medium may sustain the enrichment of the cell fraction in specific phases of the cycle, such S, G and M, and an improved osteoblasts functionality. This effect, however, was suppressed above pH 7.8, identified as the limit for the optimal functionality for viable osteoblasts. Both B and C fillers determined the alkalization of the medium used, plausibly related to the release of polymeric residuals; however, alkalization of PEO-based C fillers was more significant than PEG-based B samples, both in amplitude and duration. The C-induced transient increase in the medium pH values, well above pH 8.5, may have caused a temporary cytostatic effect on the exposed cells, although a cytotoxic action cannot be ruled out either. Nonetheless, permeation of the culture medium into the C filler, testified by its slight dimensional enlargement and its partial loss in consistency (both within 24–48 hours) can be accounted for the complete buffering of any residual alkalizing component of the dissolving C filler, and for the subsequent resuming of cell growth. The absence of correlation of the C effects to the differentiation status of the exposed cells, and the lack of pH and cell growth alterations upon pre-washing of C samples, keep in line with the above mentioned explanation, and support the hypothesis that components of the C filler, such as mainly remnants of PEO, may be released into the growth medium during its slower dissolution, rather than being sequestered from it. We then assessed the possible interference due to the filler presence after the osteoinduction protocol. It should be reminded that progenitor cells are forced to differentiate in the absence of a physiological extracellular matrix under a substantial bi-dimensional condition and devoid of any vascularization, thus in a rather hostile environment. Nonetheless, the chemical osteogenic stimulation of the cultured hBMSC ignites the expression rates of specific marker genes. In this respect, all materials under testing performed in line with the osteoinduced control cultures. The expression levels for RUNX2, a master transcription factor for osteogenic differentiation, remains slightly up-regulated only upon A and B exposure. Notably, RUNX2 expression is normally returned to control levels soon after osteogenic commitment or during tissue regenerative events. However its effects may be sustained once and if material B is present in the cell cultures, as testified by the enhancement of downstream mRNA levels for other classical markers of osteogenesis, such as bone sialoprotein and osteocalcin. Both these proteins are well-known pre-requisites for matrix deposition and mineralization. Osteocalcin, exclusively produced by osteoblasts, has been recently identified also as a bone endocrine mediator, while bone sialoprotein is a nucleator of matrix mineralization and contributes to the vascular invasion of the newly deposited bone as well as to its osteointegration. Peculiarly, RUNX2 was previously demonstrated to play a relevant role in BSP transcriptional control, particularly during cell osteogenic differentiation. BSP and OC overexpression could then be interpreted as a direct consequence of the B-driven RUNX2 transcriptional up-regulation and osteogenic differentiation.
In contrast, no significant alterations were noted, for any culture conditions, in the expression of type I collagen mRNA; this suggests that, after two weeks of osteoinductive culture conditions, B affects only the mineralization events of the complex osteogenic differentiation mechanism. A relevant reduction in osteopontin transcript was instead detected for all filler/DBM-treated cultures. Osteopontin, an abundant non-collagenous protein expressed in a variety of tissues, early expressed during osteogenic differentiation, accumulates at the interfaces of cells and matrix in bone and contributes to mineralization and bone deposition and remodeling metabolism. A reduced expression in OP could be ascribed to several causes, among which a reduced stemness, particularly in osteoinduced cells. This loss of stemness potential, however, should not be considered as a functional impairment for the cells. Evidence of functional overlap between BSP and OP has been already presented; hence it could be speculated that a partial reduction in the expression of one of these two cognate proteins could be compensated by the increased expression of the other. This aspect can thus be maximized in B-exposed cells, where BSP expression is preserved above the control values. When transferred into an in vivo experimental system, all fillers allowed a partial recovery of the lesion tissue, since bridging of the defect margins displayed a reduced thickness of the cortical layer, at least within the experimental timeframe. With all tested fillers, though, evident signs of active remodeling, marrow replenishment and vascular invasion could be detected, indicating a substantial equivalence in efficacy, as also in line with the in vitro results related to type I collagen expression. However, in the inner portions of the lesion in B-treated animals, we evidenced large areas colonized by cells with a pre-hypertrophic chondrocyte-like appearance, both at 1 and 2-month post operative. Their location, number and phenotype call for their identification as bone marrow mesenchymal progenitor cells, differentiating according to the endochondral ossification process. Their differentiation stage, though, is delayed with respect to the local requirements of bone regeneration, and is suggestive of their switch towards an articular chondrogenic phenotype; this hypothesis is supported by a substantial positivity to type II collagen, which is specific of articular cartilage, and by the significant B-driven up-regulation, in vitro, of the expression of aggrecan and SOX9, a marker and a master-gene of articular cartilage, respectively. Possibly, then, the dissolving components of the B filler may have interfered with the cell response in vitro, although, in that setting, osteogenic medium and the stiff substrate (i.e. the plastic of the culture well) were sufficient per se to sustain elevated RUNX2 mRNA levels. In contrast, this softer B filler could drive, in vivo, the differentiation of the resident marrow mesenchymal cells toward chondrogenesis, undertaking this lineage as a specific response of the cell mechanosensitivity to its 3D softer environment, a functional mechanism already proven in other settings.

As a whole these results suggest the possible combined use of B and C, on the chondral and on the osseous side of a monolithic but bi-phasic filler, respectively; the described pro-chondrogenic properties of B could be exploited in the cartilaginous portion of the osteochondral plug, while C would provide the more bone-supportive properties on the opposite side. The generation of these monolithic/bi-phasic constructs should be easily achievable, considering the PEG-PEO/HPMC chemistry and its polymerization requirements; moreover, their use could possibly avoid the risks posed by the integration of two compartments, by the presence of glues or of interfacing materials in the same construct, often encountered in biomaterial-mediated treatments of osteochondral lesions.

**Experimental section**

**Composition of the bone fillers**

This study was designed to compare one commercially available bone substitute and two novel xenografts under development as class III medical devices. All biomaterial samples were obtained in syringes directly from the manufacturer (Bioteck SpA, Vicenza, Italy) and evaluated without further manipulation. Equine-derived cortical powder (CP, particle size <0.35 mm), cancellous chips (CC, particle size 0.5–1.0 mm) and demineralized bone matrix granules (DBM, particle size <0.35 mm) were used as heterologous bone particles. Materials were obtained through Zymo-Teck®, a physical–chemical enzymatic deantigenation patented method, which guarantees grafts with preserved biological and biomechanical properties, finely characterized in terms of physico-chemical, morphological and topographical properties (P. Fattori, Patent IT VI20120209_A, February 11th, 2014). This processing is performed at physiological temperature (37 °C), and removes completely the tissue antigenic components without changing the native quaternary conformation of collagenous matrix molecules, which are therefore totally preserved. In order to obtain DBM, deantigenated particles were subjected to a 90%-demineralization process, exposing type I collagen and bone morphogenetic proteins (BMPs) of the extracellular matrix (ECM) to exert osteopromotion and to create an environment physiologically and biologically favorable for bone regeneration. Three formulations were tested: a commercially available injectable bone paste consisting of collagenous aqueous carrier, CP and DBM (Osteoplast-Activagen™, henceforward named A); two second-generation bone substitutes, in particular, an injectable paste consisting of polyethylene glycol/hydroxyl-propyl methyl cellulose-based hydrogel (PEG/HPMC) with CP and DBM, named B; and a pre-formed bone filler consisting of polyethylene oxide/HPMC-based hydrogel (PEO/HPMC), with CP, CC and DBM, named C. Control of the polymerization reactions and of crosslinking density provides good flexibility for these hydrogels to avoid granule dispersion and loss during surgery, and to assure complete filling and direct contact with the tissue surrounding the defect, maximizing bone repair. HPMC, PEG and the chemically similar PEO hydrogels undergo a polymerization reaction by physical steri-
lization, which can be modulated by introducing very limited amounts of anti-oxidant molecules. A subsidiary amount of vitamin C was added to hydrogels, acting as a visco-modulator agent (M. Fiorini, Patent WO_2015_107502_A, July 23rd, 2015). Sterile bone fillers were prepared either in injectable or in shapeable pre-formed substitutes. The hydrogels used to manufacture B and C second-generation bone fillers were identified as LMW (low molecular weight) and HMW (high molecular weight) gels, respectively. LMW consisted of 20 kDa PEG (Sigma-Aldrich, Steinheim, Germany), high viscosity HPMC (Benecel™, Ashland, Covington, KY, USA) and phosphate buffered saline (PBS pH 7.4, Amresco, Solon, OH, USA) containing vitamin C (Sigma-Aldrich, Steinheim, Germany) at a concentration >0.5 mM. HMW consisted of 400 kDa PEO (Sigma-Aldrich), high viscosity HPMC (Benecel™, Ashland) and PBS pH 7.4, containing vitamin C at a concentration <0.5 mM. For A and B injectable bone fillers, the gel component was about 70% w/w, while the amount of DBM particles was up to 22% w/w. For the C pre-formed bone substitute, the gel component was 50% w/w, while DBM particles were added at 12% w/w. For each formulation, bone particles were mixed with the respective gel to obtain an injectable and moldable paste. Once ready, the materials were placed in 1 cc plastic syringes and sealed with syringe caps. The second-generation LMW and HMW hydrogels alone and the bone fillers were then sterilized using β-rays (25 kGy) and stored at room temperature until used for further studies. All described materials (A, B and C) are produced in compliance with ISO 10993 guidelines and therefore meet the requirements to exclude cytotoxicity, genotoxicity, carcinogenicity, reproductive toxicity, skin sensitization, intracutaneous irritation, delayed-type hypersensitivity, system toxicity and local effects after implantation inside suitable tissue.

Rheological and chemical–physical analyses of second-generation hydrogels

Kinematic viscosity of both LMW and HMW hydrogels was investigated using a Rheolab QC rheometer (Anton Paar, Graz, Austria) equipped with a measuring cup model C-CC27/ QC-IM. Viscosity was measured from 1 to 100 s\(^{-1}\) of the shear rate at 25 °C. The chemical structure of hydrogels before and after sterilization by beta irradiation was investigated using a Fourier transform infrared (FTIR) spectroscopy measurement method with a FT-IR Cary 630 instrument (Agilent Technologies, CA, USA) equipped with an ATR (Attenuated Total Reflectance) module. Hydrogels were first freeze-dried in a LIO2000 Liofilizator (5 Pascal, Milan, Italy) to remove water and to allow the right acquisition of the sample with the ATR module. Method parameters were set as follows: spectral range between 4000 and 650 cm\(^{-1}\), resolution 4 cm\(^{-1}\), gain 222.

In vitro dissolution study of bone fillers

For each filler type, four cylindrical specimens of the same thickness were aseptically extruded from a 1 mL sterile syringe and transferred into a multiwell plate. Specimens were covered with standard Coon’s modified F12 medium (Biochrom A.G., Berlin, Germany) and maintained in culture until disaggregation occurred. At fixed intervals (i.e. immediately after medium addition; 5 or 30 min; 1, 2, 4, 8 or 12 hours; 1, 2 or 4 days; 1 or 2 weeks afterwards) each specimen was photographed with a Nikon Digital Sight DS-5Mc camera, mounted on a Nikon SMZ1000 binocular microscope, with the same settings. Images were processed with the National Institutes of Health Image J freeware (release 1.38X; http://rsb.info.nih.gov/ij/). The procedures used to perform the analysis allowed one to: (a) set the same threshold for each image; (b) apply the “circularity” function to identify contour-defined particles within the image; (c) apply the “measure” function to all the identified particles in each image to determine their number, area in pixels and integrated density; (d) numeric data was then used to evaluate the area of each image occupied by the biomaterial (defined as the sum of the areas of all single assessed particles in each picture), to eliminate from calculations areas smaller than 25 pixels (corresponding to less than 0.003% of the total image area), thus reducing noise fluctuation, and to define the number and the class distribution of fragments of various sizes, for each material and time-point.

Cell culture

Three different primary cultures of human bone marrow-derived stromal cells (hBMSC) were performed. Cells, acquired from Lonza (Walkersville, MD, USA), were derived from three separate donors and expanded in standard Coon’s modified F12 medium (Biochrom A.G., Berlin, Germany) with 10% fetal calf serum (FCS) and 1% L-glutamine, for two weeks. Cells were then detached by tripinization, counted and seeded for the subsequent analysis. When needed, sub-confluent cultures were osteoinduced by substituting the standard culture medium with a differentiation factors-enriched medium (F12 medium with 10% FCS, 2.5 × 10\(^{-4}\) M ascorbic acid, 1.0 × 10\(^{-2}\) M β-glycerophosphate, 1.0 × 10\(^{-7}\) M dexamethasone) for two additional weeks.

Growth kinetics

To evaluate the possible effects on the cell growth kinetics of each filler, 1 × 10\(^4\) hBMSC per well were seeded in 10% FCS standard medium in 24-well Transwell plates and maintained in F12 medium for an additional 24 h. Non-attached cells were removed. Fillers were placed in Transwells, avoiding direct contact with the cells but allowing dissolution of the material inside the culture medium of each Transwell culture; four separate specimens were used for each material and each primary culture under testing. Medium was changed twice a week. Cell growth was evaluated by the Alamar Blue™ assay (Invitrogen, Milano, Italy) as indicated by the manufacturer. Briefly, at fixed time-points (0, 2, 4, 7 and 12 days after plating) cells, under all culturing conditions, were exposed to a complete medium containing 10% of the vital dye for 4 hours. Supernatants were then collected and spectrophotometrically evaluated at 600 and 570 nm. Whenever needed, cell doublings were calculated as a function of the absorbance values at a specified time-point vs. the absorbance of the cells at plating.
Absorbance values were plotted vs. time to express the growth rate as a function of the culture conditions. Results were compared to filler-free cultured hBMSC (CN). When needed, filler specimens were pre-washed with 6 mL of either sterile PBS or 10% FCS-containing standard growth medium for 12 hours (the 6 mL volume is equivalent to the total volume of medium changed every week per well of a 24-well plate). Monitoring of the pH values of cultured filler specimens was performed assessing 1 mL of the culture solution of three samples per tested filler, at each time-point, using a Crison microPH 2000 pH meter.

Gene expression analysis

Sub-confluent cultures were osteoinduced for two additional weeks, in Transwell cultures containing the fillers under testing, and total messenger RNA was extracted from each culture condition according to the instructions of the Perfect Pure RNA Cultured Cell Kit (5-Prime GmbH, Hamburg, Germany); generation of the cDNA pools, for each sample, was carried out by using the SuperScript™ III First-strand synthesis system for the RT-PCR Kit (Invitrogen). Primer sets for each gene (glyceraldehyde-3-phosphate dehydrogenase, (GAPDH), Ki67, Runx2, osteopontin (OP), osteocalcin (OC), bone sialo-protein I (BSP), type I collagen (Coll I), Sox9 and aggrecan gene (AGG)) were derived from published sequences57,58 or purposely designed (Ki67 forward: 5′-TCACTTGCCCTATGACTTCTGG). Relative expression of each gene of interest was assessed by sybr-green real time quantitative RT-PCR. Equal amounts (5 μg) of purified mRNAs (per primary culture/per culture condition) were retrotranscribed and 1:20 dilutions of the recovered cDNAs pools (per primary culture/per culture condition used) were amplified with the RealMasterMix SYBR ROX 2.5X (5′-Prime) in an Eppendorf Mastecycler Realplex2 apparatus, performing quadruplicate reactions for each sample as follows: 95 °C for 3 minutes; 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s, and a final step at 72 °C for 7 min. Gene expression in each sample was normalized to the endogenous control gene GAPDH, and versus osteogenically-induced hBMSC in filler-free cultures. For each gene the specificity of the reaction products was counterchecked by melting curve analysis.

In vivo experiments

Male KBL SPF/VAF rabbits (average weight 3.00–3.25 kg; acquired from Charles River Laboratories Italia s.r.l., Italy) were used for the in vivo experimental settings. All procedures involving animals were performed under the supervision of the Ethical Committee for Animal Experimentation (CSEA) of the ICCRS-AOU San Martino-IST, Genova, Italy, where the stabling facility is located, within the activities planned for the animal use project #362, and in compliance with the current standards of FELASA and of the Italian Ministry of Health (D.M.S. no. 146/2009A). Once the quarantine had ended, animals were anesthetized (diazepam 1 mg kg⁻¹ i.p., ketamine 35–50 mg kg⁻¹ i.m., xylazine 5–10 mg kg⁻¹ i.m.) and operated.

A lesion (5 mm in diameter, 8 mm-deep) was drilled in the lateral distal portion of the femur, in the middle of the epiphyseal neck. The lesion was cleared of debris, rinsed with sterile saline solution and filled with the filler under testing. Two animals were implanted per each filler under testing; two additional control animals were sham operated and their lesions left empty. Animals were X-rayed post-operatively (Alpha ROC-MGF 110-HQ; Instrumentarium IMAGING; Milwaukee; WI, USA; settings at 30 kW, 20 mA). Animals underwent a standard antibacterial treatment (enrofloxacin (Baytril) 10 mg kg⁻¹ i.m., once per day for 5 days) and were caged with a 12/12 h dark/light cycle for the following 8 weeks; water and food were provided ad libitum.

Histology and immunohistochemistry

At set timings (4 and 8 weeks) animals were euthanized; the distal femoral heads were recovered from each experimental animal, cutting the bones at the epiphyseal neck, clearing them of soft tissues and washing them with PBS, pH 7.2. A 4-day prolonged fixation was performed in 4% paraformaldehyde at 4 °C; subsequently specimens were rinsed for 3 h in PBS, then in 70% ethanol and in water. Decalcification was performed for 6 days in Osteotec (Bio-Optica Milano Spa, Milano, Italy), providing abundant fresh solution every day. Specimens were then washed twice in water and in 70% ethanol for 2 h, and cut into halves along a plane passing through the diameter of the cylindrical lesion and parallel to the femur major axis. Samples were then dehydrated and processed for paraffin inclusion. Sections (5 μm thickness) of whole samples were stained with hematoxylin–eosin to evidence tissue morphology, according to standard protocols. Images of stained sections were acquired with a Nikon Digital Sight DS-5Mc camera, mounted on an Olympus BX5 1 fluorescence microscope, using the Nikon imaging software NIS-Elements F, release 2.20. Alternatively sections were dewaxed, rehydrated and challenged with an anti-type II collagen antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA; CIIC1; 1 : 200 in PBS). Immunopositivity was revealed by further challenge of the sections with a biotinylated-conjugated secondary antibody (1 : 500 in PBS) and the StreptABCComplex/AP (DakoCytomation Denmark A/S; DK-2600 Glostrup, Denmark).

Statistical evaluation

The Mann–Whitney test was used. Whenever indicated *: 0.05 < p ≤ 0.01; **: 0.01 > p > 0.001 and ***: p < 0.001.

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