A novel sonic microfragmentation approach increases the osteogenic potential of decellularized human bone particles.

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Disclosures: none

INTRODUCTION: Human decellularized bone particles (dbPTs) are commonly used in the clinical practice as a support to allograft surgeries. dbPTs retain both the mineral and the organic components of the extracellular matrix. These include growth factors and various non-collagenous proteins such as fibronectin, heparan sulfate, dermatan sulfate, chondroitin sulfate and hyaluronic acid, ideally suited to trigger the osteogenic response in the host tissue. However, the biological responses to allograft are limited by the procedures required to ensure biological safety and prolonged shelf-life, namely freezing and freeze-drying methods. Interestingly, early work in periodontics provided anecdotical evidence that the size of bone substitutes chips may critically influence the extent of osteogenic differentiation and bone regeneration. However, a reproducible and scalable procedure for bone chips fragmentation has never been described and previous work was based on a limited range of size (above 100 μm). In this work, we hypothesized that allograft material can be fragmented through a controlled process to achieve a precise dimensional characterization and an optimal biological response by bone cells in order to be useful for clinical applications.

METHODS: Cortical bone shafts were produced starting from long bones (harvested from a unique human cadaver donors) by personnel of the accredited public non-profit Musculoskeletal Tissue Bank (hereafter BTM) of IRCCS Istituto Ortopedico Rizzoli (Bologna, Italy; EU TE code: IT000096). Upon decellularization, a novel micro-fragmentation method was developed based on sequential steps involving ultracentrifugal milling, freeze drying, sieving, ball milling and ultrasonication. A Response Surface Method (RMS) was used to determine the influence of several parameters of the ultrasonication on the dbPTs dimensions, chemical structure, and the biological responses. The power level of the ultrasonicator (Factor A), the duty cycle (Factor B) and processing time (Factor C) were considered as continuous factors. 12 different trials of bone particles were generated by different combination of these 3 factors. Each trail was subjected to structural analysis by ATR-FTIR and to morphological and dimensional analysis using SEM and confocal microscopy. Moreover, by dispersing these samples in a Sil-MA solution and exposing them to UV radiation, we developed a composite material in which the dbPTs were entrapped into a Sil-MA hydrogel matrix, suitable for biological assays (shown in Figure 1). To investigate the effect of the different protocols of sonication on cell proliferation and osteogenic differentiation, we employed commercial adipose-derived stem cells (ADSCs, Lonza, Switzerland). *In vitro* assays were performed to measure proliferation (Alamar Blue assay, Biorad) and osteogenic response (ALP activity assay, Abcam) within 14 days of culture in osteogenic medium. A correlation matrix of the collected dataset was computed as a summary of the overall collected data.

RESULTS: 12 different trials of dbPTs were generated upon different combination of ultrasonic power, duty cycle and processing time. Descriptive statistics was elaborated for the area and diameter distribution within each trial, showing a median area ranging from 4.6 to 13 μm^2 and a median diameter ranging from 1,5 to 4 μm. A rough calculus of the exposed surface available after each treatment was done by considering a unit of bone volume of 1 μm^3 and dividing it in particles with a volume equal to the mean or the median volume calculated based on the equivalent mean or median diameter of the particles. Compared to the untreated, all the sonication treatments increased the exposed area. In particular, the treatment number 11 was the most effective with a total surface area 5 times higher than the reference. Analyses of SEM images revealed that an organic phase was exposed after the sonication in dbPTs (Figure 1). Cell proliferation assays were performed by seeding ADSCs (n=3) on composite scaffolds obtained by each of the 12 sonication treatments after 2 and 14 Days in culture. Interenstingly, at the early time point of 48 hours after seeding, cell proliferation did not significantly differ between the different sonication treatments of dbPTs and from the unsonicated dbPT. However, after 14 days in culture, all the sonicated dbPTs showed higher proliferation rates than the reference (unsonicated sample); moreover, sample number 11, characterised by the lowest mean area of dbPTs, showed a significantly ($p<0.01$) greater cell proliferation than other samples. Moreover, test of osteogenic differentiation revealed that ALP activity at the end of the experiment (day 14) was markedly affected by micro fragmentation of bone particles, as all the values from dbPTs samples were higher than the control, non-sonicated sample. In particular, higher values of ALP activity were measured in samples with harsher protocols characterized by smaller size of bone particles (11 and 12). To verify the hypothesis of a higher bioactivity after the sonic micro-fragmentation process, a correlation matrix of the collected dataset was computed as a summary of the overall collected data. The correlation between couples of variables should be considered significant if r^2 is lower than *-0.4* or higher than *0.4*. The hierarchical clustering of the matrix by the average method revealed that the biological responses were all in some extent inversely correlated with the mean, and median area of the particles (Mean A and Median A) and directly correlated with the total superficial area.

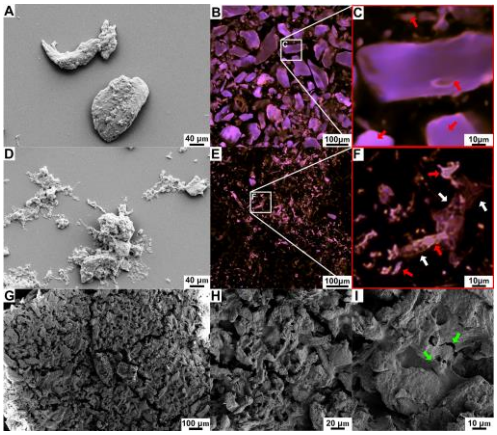

Figure 1:SEM and confocal images taken on (A, B, C) the unsonicated powder and (D, E, F) the sonicated powder. (C) and (D) Magnified regions of interest of the confocal images. In these images, red arrows show the fragment of bone while white arrows show the exposed organic phase (visible as filaments). The treatment resulted to be effective in revealing the internal organic phase. (G, H, I) SEM images at different magnification of the Sil-MA/fragmented dbPTs composite material. dbPTs resulted to be well dispersed while the fractures present were probably due to interfacial problems between the matrix and the particles. Fractures were highlighted by green arrows.

DISCUSSION: The size of bone particles used in allograft procedures may critically influence the extent of osteogenic differentiation and thus bone regeneration. In this work, we developed a method based on ultrasonication to reduce the dimension of human bone particles through a controlled and scalable process, which allowed us to model the relationship between size and the resulting osteogenic differentiation. A clear correlation, not proven before, was identified between the particle size and the biological response as measured in vitro using osteoprogenitor cells.

SIGNIFICANCE: this is the first report to propose a model linking a workflow for processing allograft tissue, the size of bone particles and the resulting osteogenic response by osteoprogenitor cells. Implications of this work may extend to bone tissue banks and manufacturers to produce bone derived products with improved biological performances. Moreover, these results provide novel insights into the use of decellularized bone matrix in procedures of bone tissue regeneration as particle size should be regarded as an important parameter for achieving the optimal grade of tissue regeneration which can be exploited in novel composite scaffolds for bone regeneration.