Tuesday, 29 July 2014

Dear Professor Xiu-Xia Song,

We would like to thank you and the reviewers for the useful comments forwarded to us. Please find below replies to the queries raised. The corrections/suggestions are highlighted in the revised manuscript.

We hope that the revised manuscript would satisfy the editorial team of the journal.

Thank you in advance.

Yours Sincerely,

Ziad Dahabreh, Michalis Panteli, Ippokratis Pountos, Mark Howard, Peter Campbell, Peter V. Giannoudis

REVIEWER 1

Q1: This manuscript describes, in a clearly written style, a series of experiments in vivo attempting to define which, out of a panel of commercially available materials used as bone graft substitutes, performs best with respect to colonization by osteogenic cells and expression of a differentiation marker (alkaline phosphatase). The study was carried out in a system which minimizes the relevance of mechanical factors on the outcome, and care was taken to normalize alkaline phosphatase activity relative to the number of cells in the same, as estimated from the DNA content. However, it seems that these materials were highly heterogeneous, not only in chemical composition, but in degree of porosity, consistency, stability in the medium for a period of weeks, and so on. Therefore, best performance in this study does not necessarily relate to an easily identifiable property, or even a combination of properties. One of the risks of this situation is that one product that performs much better than the rest in this assay may receive strong endorsement without any clear explanation, and without evidence that this best performance will be accompanied by better clinical results in the in vivo situation, where mechanical factors are decisive. There is no doubt about the practical relevance of the issues involved, and the authors are to be commended for careful design of the quantitative experiments with alkaline phosphatase.

Answer: We would like to thank the reviewer for their comments. We have revised the discussion/limitations section to include the points made.

Q2: Other aspects, however, are very difficult to quantify, and this applies to the procedures used to "enhance" the homogeneity of contact of the seeded cells with the materials, which vary from granular powders to soft solids, and which are not necessarily mixed with the cells to the same extent, no matter how much you stir the plates. I also find it difficult to see how volume of these very different preparations could be adjusted with the necessary precision, with the help of a beaker.

Answer: The cells were mixed with the graft material as well as it would be physically possible in a consistent and reproducible manner. We decided to use a standardised volume to mimic a clinical situation, in which the bony-defect size is not a variable. As such, the comparison of different graft materials against a standardised volume rather than weight or other variables would be practically applicable. We agree that the consistency of the cells/Graft units might not have been the same, however, this could be an inherit property of the graft material i.e. it's structural composition to facilitate the homing of the cells and allow them to proliferate and differentiate. Even in the best clinical case scenario absolute control of this issue cannot be guaranteed.

Q3: Most importantly, much of the conclusions depend on images (Figure 1) which can be interpreted in different ways, and are certainly not quantitative. I am especially concerned about the disparity between cell staining (left) and scanning electron microscopy (right) for the same materials (see 1c and 1d, for instance). Cells are plentiful in the left panels, and undetectable in the right panels, at least in some cases. Also, totally different electron microscopy aspects are offered for the same material, when one compares the colonization by freshly harvested vs in vitro expanded cells (again, compare 1c to 1d, for instance). I find it hard to accept that the structure of the material to which no cell is attached becomes radically different as a consequence of different sources of the same cell type being present in the same culture. I think these issues need to be addressed in order to make their conclusions more solid.

Answer: We have revised the figures to address the points raised by the reviewer. Cell staining and microscopy were performed initially to confirm viability (qualitative) before further quantitative assessments were undertaken.

Q4: An additional issue (which may or may not be trivial) concerns the fact that the research is supported by an educational grant from an organization which has the same name as the manufacturer of most products tested. I understand only one product from that supplier performed exceptionally well, and others from the same source were not outstanding. This may simply reflect the objective findings of the authors, but it may raise doubts in the minds of commercial competitors, who did not have a comparable material for testing, especially if the funding is ultimately shown to come from the same source as the test material. I would recommend that the authors clarify whether this outstanding material is only available from this specific source, or can be obtained from more than one supplier, and if so, whether it also performs exceedingly well in their hands.

Answer: We appreciate the anxiety shown by the reviewer. However, this study was partly funded by the industry in the form of an educational grant. We can confirm that the industry had no influence on the design, conduct or results of the study. Similar materials can be obtained from other suppliers as currently there are a number of generic based products with the same properties.

Reviewer 2

Q1: This study should check the Alizarin Red S staining, bone-relative gene expression and in vivo assay to confirm their conclusion.

Answer: We would like to thank the reviewer for these comments. Alizarin Red S stains calcium therefore we decided not to include it for potential nonspecific staining of the graft itself. We have included these comments in the limitations section of the revised manuscript.

Reviewer 3

Q1: The major concerns: 1) The unquantified, incomplete live/dead staining &SEM data. Fig 1 shows only unquantified data from 3 (out of 7) BGSs. These data are inconclusive.

Answer: We would like to thank the reviewer for these comments. Figures have been revised.

Q2: The unacceptable interpretation of ALP data. The authors used ALP as the only osteogenic marker in this manuscript without mention any potential alternative interpretation the data. In fact, ALP is expressed by a variety of cells, including MSC.

Answer: We agree with the reviewer that ALP is expressed in osteoblasts as well as MSCs under specific conditions. In our study, nucleated cells from bone were used. Therefore, both MSCs and committed cells of the osteoblastic lineage were included. When osteoblastic media were used (MSCs forced to differentiate towards osteoblasts). Therefore ALP activity would be a valid marker in this instance.

Q3: Completely lack of in vivo data. In vitro data alone could be misleading.

Answer: This is purely an in-vitro study studying the potential of the studied materials to support the osteoprogenitor cells. We agree with the reviewer that in-vivo data would be useful and this point has been included in the discussion section.