Report to the BSPED – October 2011

Development of a novel *ex vivo* femoral slice model for the study of osteonecrosis and other bone diseases

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Recipients of a BSPED Research Award - November 2008 - £15,000

Introduction

Bone mass and quality is maintained by the co-ordinated activities of many different cell types, and a breakdown of such co-ordination results in bone pathology. The processes involved in maintenance of healthy bone are complex, and involve the synchronisation of bone resorption by osteoclasts, and osteoblast-mediated bone formation. Osteocytes are osteoblasts which have become embedded within the bone matrix and may be involved through mechano-sensing in the regulation of bone formation and resorption.

A large number of studies investigating bone pathologies use *in vivo* animal models, but these use large numbers of animals with significant economic cost. Most bone related studies that do not involve animals use monolayer cell cultures, or humans *in vivo*. Monolayer cell cultures are mostly single cell types in isolation, and these rarely behave as they would in tissue where cells communicate, either on, or in an extracellular matrix. This communication is essential for the initiation and processing of normal bone function and reparative responses. *In vivo* human studies on the other hand, of necessity, are indirect by nature, severely limited by ethical considerations and subject to a range of confounding factors (*e.g.* age, sex, disease stage, treatment regimen).

Since survival rates from childhood acute lymphoblastic leukaemia (ALL) are now very high, there is increasing interest in the long-term quality of life. Osteonecrosis is a potentially devastating bone disorder which is being increasingly recognised as a complication of the acute treatment of childhood ALL. It is a disabling bone pathology leading to pain, deformity, loss of mobility and fractures, sometimes necessitating total joint replacement. Weight-bearing joints are affected most frequently. The pathogenetic basis of osteonecrosis is poorly understood, but is thought to include suppression of osteoblasts, apoptosis of osteocytes, and adverse effects on nutrient arteries leading to thrombosis and fat embolism. Aetiological factors may include glucocorticoids, asparaginase, high-dose methotrexate and cyclophosphamide. Because of the immunosuppressive effect of chemotherapy, children undergoing treatment for ALL are at greater risk of a systemic infection than normal, and this infection may contribute to the development of osteonecrosis. Interestingly, animal models of osteonecrosis have recently been induced using a single low-dose lipopolysaccharide (LPS; the principal component of the outer membrane of Gram-negative bacteria) injection and subsequently three injections of high-dose methylprednisolone. Our group has actively researched this subject area during the last few years but have been restricted by the availability of model systems and have thus used monolayer cultures of a single cell type for the work to date.

Our proposal planned to extend our work on osteonecrosis in children with ALL by developing a novel bone ‘slice’ culture method. Such a system would be more closely aligned to the *in vivo* state

than traditional single-cell culture systems, and would allow cell/cell interaction between differing cell types. We thus used the monies awarded to pay for consumables, as well as salary (4 months) for an experienced technician to develop these techniques. We then planed to use these preliminary results in applications to further fund our work on osteonecrosis and other bone pathologies.

Results

The work successfully enabled the maintenance of rat femoral bone slices in culture for up to 14 days (Figs 1 and 2). We investigated the use of different thicknesses (1.5, 2 and 2.5 mm) of bone slices as well as those from different regions of the femur e.g. distal epiphysis and diaphysis. Different culture media were also investigated at early stages. We concluded that 1.5 mm slices in α-MEM containing 10% fetal calf serum was the best combination to proceed with. Viability tests indicated that this methodology adequately maintained the slices for up to 14 days, although some cell types had disappeared by the end of this period. However, the osteocytes seemed to be viable throughout this period and we currently think that these slices provide a novel method of studying osteocytes *in situ* and encased in mineralised matrix.

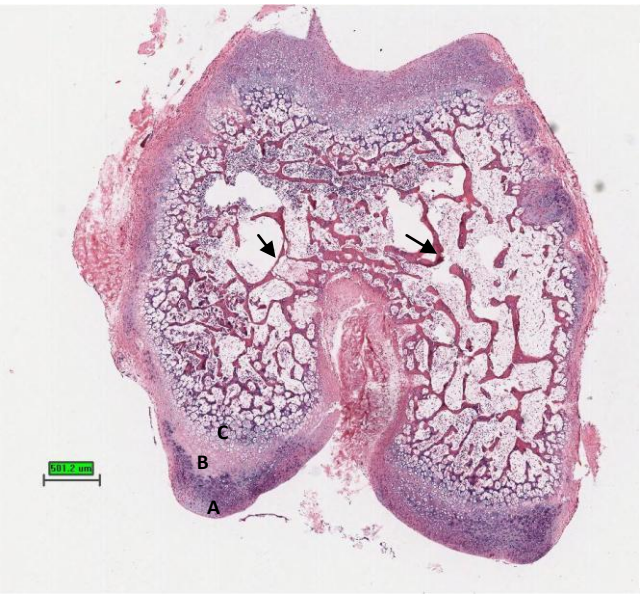

Fig 1. Image of slice from the distal epiphyseal femur after 7 days in culture. A large quantity of trabeculae (examples indicated by the black arrows) could be observed throughout the tissue. (A), (B) and (C) represent the proliferative, hypertrophic, and ossification zone of the epiphyseal plate, respectively. Magnification: ×20.

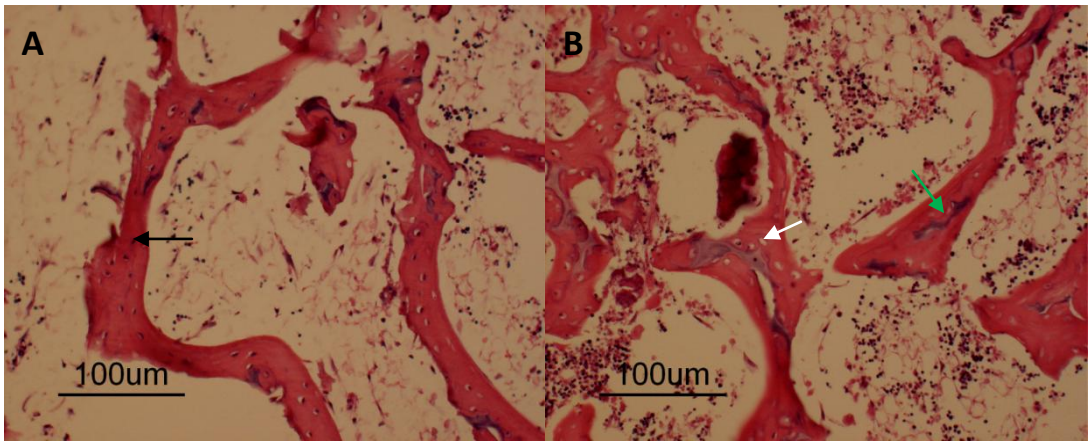

Fig 2. H&E images for a femoral slice after 7 days in culture. More osteocytes (black arrow) and fewer empty lacunae (white arrow) were observed in the control group (A) than in the LPS treated

group (B). Calcified cartilage (green arrow) was also discernible within the trabeculae. Magnification: ×400.

In other experiments slices were treated with LPS to mimic infection and the numbers of osteocytes, identified by DMP-1 immunohistochemistry (IHC), counted. The concentrations of IL-6 produced by the slices were also quantified. A significant reduction in osteocyte number was seen after 7 days (Fig 3), and IL-6 concentrations were also significantly changed in the presence of LPS (results not shown).

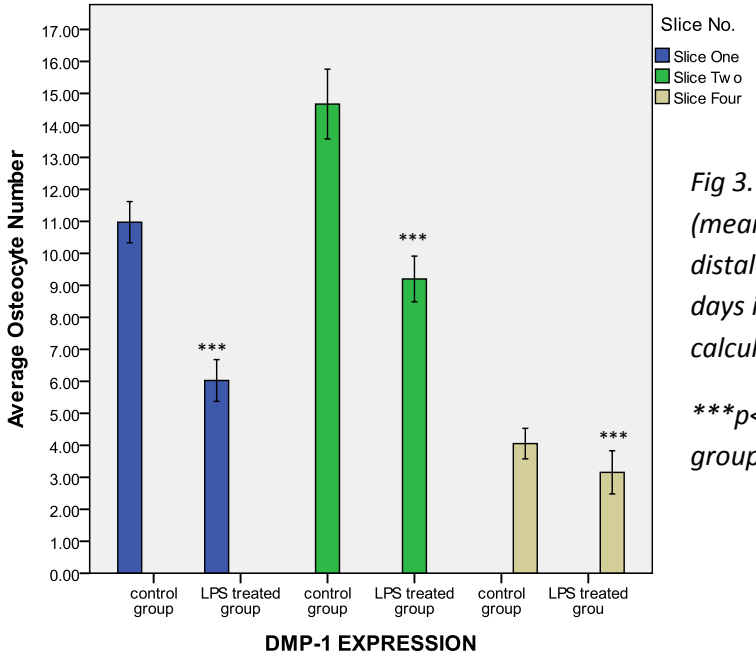

Fig 3. Trabecular osteocyte number (mean +/- 2 SE) per 100µm^2 area of the distal epiphyseal femoral slices after 7 days in culture with or without LPS, and calculated from the DMP-1 IHC images.

****p<0.001: in comparison to the control group from the same level of distal femur*

In summary, the monies awarded enabled us to successfully develop a novel method of culturing rat bone slices *in vitro*. The results have been included in several of our grant applications (see below), and the work has enabled us to move towards our ultimate goal of being able to maintain human bone slices *in vitro,* and thus be able to study human bone cells in their natural 3-D environment.

Other outcomes

1. Preliminary results from this work were used in an application for funding from the Dr Hadwen Trust. This was awarded and the work started in October 2011
   'Development of new methodologies for the 3-D in vitro study of bone disease' £83,060 (2010-2012). Evans BAJ (PI)**,** Mason DJ, Sloan AJ, Ralphs J, Gregory JW.
2. Preliminary results from this work were also used in an application for funding for a PhD studentship from The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) – the outcome is pending
3. The work was continued by a Cardiff Institute of Tissue Engineering and Repair MSc student – she was awarded 72% for her dissertation