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Cyclic hydrostatic pressure stimulates enhanced bone development in the foetal chick femur *in vitro*

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ABSTRACT

Mechanical loading of bone and cartilage *in vivo* results in the generation of cyclic hydrostatic forces as bone compression is transduced to fluid pressure in the canalicular network and the joint synovium. It has therefore been suggested that hydrostatic pressure is an important stimulus by which osteochondral cells and their progenitors sense and respond to mechanical loading *in vivo*. In this study, hydrostatic pressure regimes of 0–279 kPa at 0.005–2 Hz were applied to organotypically cultured *ex vivo* chick foetal femurs (e11) for 1 hour per day in a custom designed bioreactor for 14 days and bone formation assessed by X-ray microtomography and qualified by histology. We found that the mineralised portion of the developing femur cultured under any cyclic hydrostatic pressure regime was significantly larger and/or denser than unstimulated controls but that constant (non-cycling) hydrostatic pressure had no effect on bone growth. Further experiments showed that the increase in bone formation was directly proportional to stimulation frequency ($R^2 = 0.917$), but independent of the magnitude of the pressure applied, whilst even very low frequencies of stimulation (0.005 Hz) had significant effects on bone growth. Expression of Type-II collagen in both epiphyses and diaphysis was significantly upregulated (1.48-fold and 1.95-fold respectively), together with osteogenic genes (osteonectin and osteopontin) and the osteocyte maturation marker CD44. This work demonstrates that cyclic hydrostatic pressure promotes bone growth and mineralisation in a developmental model and supports the hypothesis that hydrostatic forces play an important role in regulating bone growth and remodelling *in vivo*.

Introduction

Hydrostatic forces play an important role in many biological systems, transducing mechanical forces in hydrated or fluid-filled tissues such as bone, cartilage and joint synovium into dynamic changes in pressure. Compressive loading and unloading of these tissues during movement causes the pressure of the interstitial fluid to increase and decrease at various rates and magnitudes, creating dynamic shear and hydrostatic forces which have been theorised to be detectable by cells [1–4].

As a potential route by which cells may sense and respond to the external mechanical environment, dynamic hydrostatic pressure has been investigated by previous authors using a variety of custom-designed apparatus and either osteochondral explants or cells isolated from these tissues. Whilst results have been varied, a general finding is that cyclic pressure changes have a positive effect on bone development *in vitro*, particularly when these pressures are relatively low. Indeed, a marked distinction can be made between the high physiological pressures found in the joints (e.g. femoral head synovium during exercise is ~18 MPa [5]) and the low pressures in other tissues such as cerebrospinal fluid (1.2 kPa [6]), interstitial fluid (0.27 kPa [7]) and blood pressure (8–24 kPa [8]), and the range of experimentally applied pressures has been diverse.

Perhaps most relevantly to orthopaedic tissue engineering, it has been calculated that the pressure experienced by osteocytes in the canalicula-lacuna network of load-bearing bones is in the order of 270 kPa [9]; higher than blood pressure but still an order of magnitude lower than synovial pressure changes, and with a relaxation time for the network estimated at 4.9 ms allowing for rapid pressure changes to be experienced. The frequency of pressure changes are broadly similar in many physiological processes, e.g. human pulse rate (1–2 Hz) and locomotive bone/joint compression (0.3–3 Hz) under normal circumstances, whilst many other pressure fluctuations in tissues are biologically linked to these first two processes (e.g. fluid flow in the canaliculi and capillary pressures in the muscles).

In order to further assess the role of pressures at these magnitudes on bone mechanotransduction, we have designed and built a custom bioreactor in collaboration with Tissue Growth Technologies (MN, USA) which uses compressed air to provide dynamic gas pressures to a sealed chamber accommodating a standard cell culture plate (Fig. 1). We utilised the embryonic day 11 *ex vivo* foetal chick femur recently described by Kanczler and Smith et al. [10,11] as a model system for studying the response of organotypically cultured developing whole bones to dynamic pressures. This tissue is primarily composed of osteoblasts and progenitor

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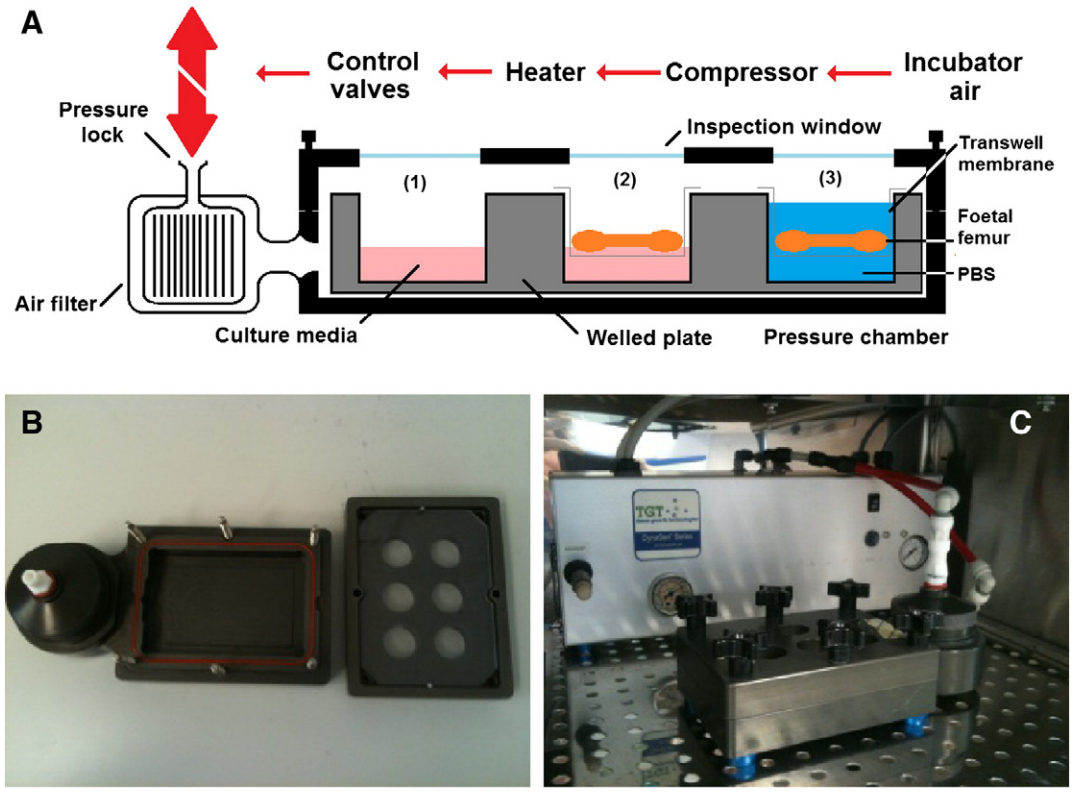

Fig. 1. A. Schematic cross-section of the bioreactor chamber, showing how the environment *in vitro* is pressurised *via* compressed incubator air. The chamber is shown set up for (1) cells in monolayer cultured on the base of the well, (2) the normal organotypic method for culturing the femurs at the media–air interface and (3) the femur submerged under PBS during the application of the hydrostatic stimulation regime. B shows the actual bioreactor chamber used in the investigation (l), with removable lid and acrylic inspection windows (r). C shows the bioreactor chamber attached to the ancillary apparatus that supplies warmed, compressed, recycled incubator air to the chamber at dynamic pressures which are computer-controlled using TGT's GrowthWorks software *via* a system of valves and internal sensors.

cells with a number of encased osteoblasts maturing into osteocytes and negligible numbers of osteoclasts. At this stage of development, the onset of primary mineralisation is underway in the region surrounding the diaphysial shaft (bone collar) allowing the progress of bone maturation to be observed by X-ray microtomography in response to experimental treatments. Vascular invasion has not yet occurred in the organ, although pores in the mineralising collar exist to admit ingrowth of blood vessels to the endosteum.

In this investigation, chick femurs were exposed to hydrostatic pressures of between 0 and 279 kPa (the presumed range experienced by osteocytes as modelled by Zhang et al. [9]) at frequencies of 0.005 Hz to 2 Hz. Hydrostatic pressure was applied to *ex vivo* bones organotypically cultured on transwell membrane supports and submerged in liquid media during stimulation (transduced hydrostatic pressure). The effects of cyclic versus static pressure, and magnitude and frequency of stimulation were investigated as was the response of femurs at different stages of development. We also evaluated the ability of the bioreactor to transduce pressures to materials under a layer of aqueous media using fluorescein-releasing alginate hydrogels.

The chief advantages of this bioreactor over preceding devices for providing hydrostatic pressure *in vitro* are the selection of physiologically relevant pressure ranges similar to those predicted to be sensed by osteocytes *in* vivo, and fine computer control of both the magnitude and frequency of force that can be applied. By compressing the gas phase above the media containing the tissue, the effects of hydrostatic pressure can be studied without introducing undue shear forces. Our overall aims were to determine the effect of hydrostatic pressure on developing bone and evaluate the relative importance of static or cyclic stimulation and frequency of loading.

Materials and methods

Chick foetal femur culture

Intact femurs were removed from freshly killed Dekalb white chick foetuses after 11 days incubation and carefully cleaned of all muscle tissue by rolling on sterile tissue paper. Femurs measured approximately 7 mm in total length on isolation and were maintained *ex vivo* on porous polycarbonate membrane inserts in 6-well cell culture plates over 1 ml α-modified Eagle's medium (alpha-MEM) containing 1% penicillin-streptomycin and 150 μg ml^{-1} ascorbic acid as recently described by Kanczler and Smith et al. [10,11]. In most experiments, osteogenic media was used which was the same formulation as above with the addition of 2 mM sodium β-glycerophosphate and 10^{-7} M dexamethasone (both Sigma, UK). The femurs were maintained at 37 °C and at 5% CO_2 in a humidified incubator, with culture media being completely replaced every 24 hours. In initial experiments one of each femur pair was used as an untreated paired control.

Hydrostatic pressure bioreactor

Cyclic hydrostatic pressures were applied to the femurs using a custom-made bioreactor designed and built in a collaboration between Professor Alicia El Haj (ISTM, Keele University, UK) and Tissue Growth Technologies (Minnetonka, MN, USA). The bioreactor chamber is a sealed, anodised aluminium vessel accommodating a standard-sized cell culture plate (with the lid removed) allowing pressure changes to be transferred by the ancillary equipment to the gas phase above the culture plate. Compressed recycled incubator air is fed from a