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Development of an Ex-Vivo Organ Culture Model of the Femoral-Tibial Joint

Natalie Fox[a], Martin Stanley[b], Daniel Thomas[a], John Fisher[b] & Eileen Ingham[a]
Institute of Medical & Biological Engineering, [a]Schools of Biomedical Science and [b]Mechanical Engineering, University of Leeds, UK.

There is interest in, and a clinical need for, the development of more effective cartilage substitution therapies that restore hyaline cartilage function. Research and development of novel cartilage repair and regenerative solutions would be greatly enhanced by the capacity to pre-clinically evaluate potential new therapies in physiological biotribological models of viable natural articulations, such as the femoral-tibial joint, in order to evaluate their effects on the biology and tribology of the adjacent and opposing cartilage. Towards this long term goal, we have developed methods for organ culture of osteochondral plugs. The aims of this study were to further develop these methods for the organ culture of whole femoral condylar and tibial osteochondral tissues and establish culture conditions that maintained chondrocyte viability and extracellular matrix integrity.

Porcine knees were obtained within 4 hours of slaughter. For whole "joint" tissue culture, the femoral condyle and tibial plateau were dissected using stringent aseptic technique. The majority of the cancellous bone was removed by reaming to leave the intact cartilage with a layer of cortical bone (circa 5 mm thick). Osteochondral (OC) plugs, 9 mm diameter were removed from porcine knee condyles. Blood and bone marrow were removed from the underlying cortical bone using a dental water flosser (Waterpik) before incubating the tissues overnight in HBSS plus 12.5 $U.ml^{-1}$ heparin and antibiotics. Tissues were then cultured in defined medium (DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1% (v/v) ITS Pre-mix (BD Biosciences), 50 $mg.ml^{-1}$ ascorbic acid, 0.1 μM dexamethasone and antibiotics. The" whole joint" tissues were cultured in culture pots bubbled with 5% CO_2 in air at 37°C. OC plugs were cultured in 24-well plates in an atmosphere of either 20%, 10% or 5% oxygen and 5% CO_2.

The viability of the cartilage immediately after dissection (day 0) and after 8 or 14 days of culture was assessed by XTT assay and LIVE/DEAD staining. Following LIVE/DEAD staining of the tissues, sections were viewed by confocal microscopy (Zeiss LSM510 Meta). The proportion of living/dead cells in different zones of the cartilage and the surface zone of the cartilage (where the majority of dead cells were located) was determined using Image J.

Samples of tissues were fixed [10% (v/v) neutral buffered formalin for 48 h] and processed for histology. Sections (5μm) were stained with haematoxylin & eosin and alcian blue. H&E stained sections were used to determine the total number of cells in the different zones of the cartilage .Glycosaminoglycan (GAG) levels were quantified in the cartilage at day 0, 8 and 14 days of culture and also in the culture medium throughout the culture period using the dimethylene blue assay.

Data are expressed as the mean (n≥3; different animals) ± 95% confidence limits. Data were analysed using Student`s t-test or ANOVA followed by Sidak`s multiple comparisons test ($p< 0.05$).

The conversion of XTT (an indicator of tissue viability) of the OC plugs in culture in a 20% oxygen environment reduced significantly between day 0 and day 8 (Fig 1a) but there was no significant change between day 8 and day 14 and the GAG levels in the OC plug cartilage did not change throughout the culture period (Fig 1b). Similar results were obtained when the cultures were performed in a 5% or 10% oxygen environment. Alteration of the osmolality of the culture medium from 330 mOhms to a more physiological level (equivalent to synovial fluid at 400 mOhms) was also tested (data not shown) with no enhancement in tissue viability.

LIVE/DEAD cell staining at all time points showed that the majority of the chondrocytes in the mid and deep cartilage zones were alive. There was, however, band of mainly dead cells in the surface zone, even at day 0. Since XTT conversion is a measure of mitochondrial activity it was postulated that the chondrocytes in the freshly isolated cartilage increased metabolic activity to recover from dissection and that a period of time in culture was required for the tissue to adjust to the organ culture conditions.

Due to practicalities of performing the "whole joint" tissue experiments it was not possible to set up both 8 day and 14 day cultures simultaneously and hence the data for these tissues is compared with its own control day 0 tissue in Figs (2) and (3). The femoral condyle and tibial cartilage also showed a reduction in XTT conversion between days 0 and 8 but the difference in XTT conversion was only significant for the femoral cartilage (Fig 2).. The data at day 14 indicated that the whole joint tissues behaved in a similar manner to the osteochondral plugs in culture, with little change in the viability of the cartilage from day 8 to day 14. LIVE/DEAD cell staining of the whole joint tissues again showed that the majority of the cells remained alive in the mid and deep zones of the cartilage during the 14 day culture period. At day 0, 80% and 88% and at day 14, 72% and 70% of the chondrocytes were viable in the femoral and tibial tissues respectively and the differences were non-significant. Again, similar to the OC plugs, there was a band of mainly dead cells in the surface zone. The depth of the zone of dead cells was measured and was shown to increase with time in culture from circa 80 μm (femoral and tibial) at day 0 to 200 μm (femoral) and 180 μm (tibial) at day 14.

The reduction in XTT conversion by the tissues could have been due to reduced mitochondrial function of the chondrocytes or to cell death and a loss in cells number. The numbers of chondrocytes in the different zones of the cartilage were estimated from H&E stained sections. There was a trend towards reduced cell numbers following 8 and 14 days of culture, and in agreement with the LIVE/DEAD cell staining this was due to a loss of cells in the surface zone of the cartilage, with a significant ($p< 0.05$) reduction from 60 to 25 cells per field of view and 50 to 30 cells per field of view for femoral and tibial cartilage respectively after 8 days of culture.

There was no significant loss of GAGs from the whole joint cultures over the 14 day culture period (Fig 3). This was supported by alcian blue staining of tissue sections.There was no significant difference in the levels of GAGs released into the culture medium over time for both the femoral and tibial tissues.

These studies have shown that it is possible to maintain large cuts of femoral and tibial osteochondral tissues in organ culture for an extended period of time. Overall the data indicated that the chondrocytes in the mid- and deep zones of the cartilage remain viable. However, the chondrocytes in the surface zone of the cartilage lost membrane integrity rapidly either following death of the animals or during the initial dissection process and there was further loss of viable chondrocytes in the surface zone of the tissues during organ culture. It is hypothesised that physiological loading of opposed femoral and tibial tissues in a novel physically interactive bioreactor will improve the viability of the surface zone chondrocytes in the tissues and this will be the focus of future studies.

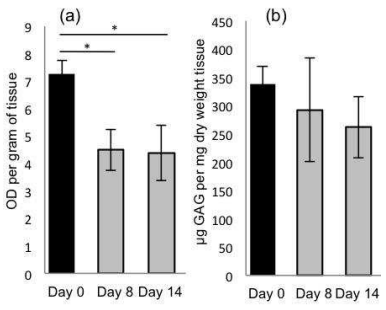

Fig (1). Viability (a) and GAG content (b) of osteochondral plugs
Data are presented as the mean (n=6-7) ± 95% CL.
* indicates significant difference ($p< 0.05$; ANOVA)

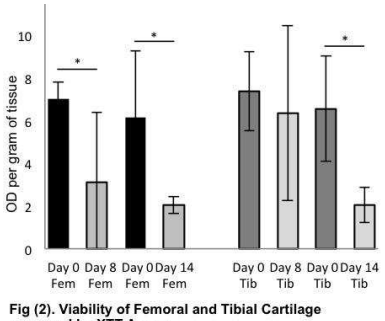

Fig (2). Viability of Femoral and Tibial Cartilage assessed by XTT Assay
Data at days 8 &14 of culture matched with day 0 controls.
Data are presented as the mean (n=3-5) ± 95% CL.
* indicates significant difference ($p<0.05$;Student`s t-test)

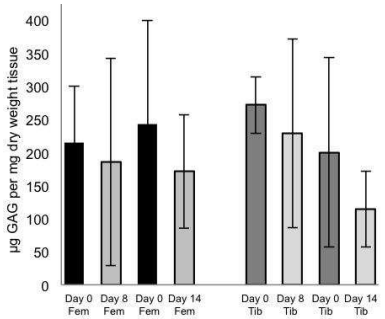

Fig (3). GAG content of Femoral and Tibial Cartilage
Data at days 8 &14 of culture matched with day 0 controls.
Data are presented as the mean (n=3-5) ± 95% CL

The research and development of more effective cartilage substitution therapies would be greatly enhanced by the capacity to pre-clinically evaluate novel therapies in in vitro physiological tribological models of natural tissue articulations, for example of the natural femoral-tibial joint under dynamic loading. In order to develop such advanced physiological model systems it will be necessary to develop systems for the organ culture of osteochondral tissues.