NON-DESTRUCTIVE MONITORING OF VIABILITY IN AN *EX VIVO* ORGAN CULTURE MODEL OF OSTEOCHONDRAL TISSUE

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Abstract

Organ culture is an increasingly important tool in research, with advantages over monolayer cell culture due to the inherent natural environment of tissues. Successful organ cultures must retain cell viability. The aim of this study was to produce viable and non-viable osteochondral organ cultures, to assess the accumulation of soluble markers in the conditioned medium for predicting tissue viability. Porcine femoral osteochondral plugs were cultured for 20 days, with the addition of Triton X-100 on day 6 (to induce necrosis), camptothecin (to induce apoptosis) or no toxic additives. Tissue viability was assessed by the tissue destructive XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide tetrazolium salt) assay method and LIVE/DEAD® staining of the cartilage at days 0, 6 and 20. Tissue structure was assessed by histological evaluation using haematoxylin & eosin and safranin O. Conditioned medium was assessed every 3-4 days for glucose depletion, and levels of lactate dehydrogenase (LDH), alkaline phosphatase (AP), glycosaminoglycans (GAGs), and matrix metalloproteinase (MMP)-2 and MMP-9. Necrotic cultures immediately showed a reduction in glucose consumption, and an immediate increase in LDH, GAG, MMP-2 and MMP-9 levels. Apoptotic cultures showed a delayed reduction in glucose consumption and delayed increase in LDH, a small rise in MMP-2 and MMP-9, but no significant effect on GAGs released into the conditioned medium. The data showed that tissue viability could be monitored by assessing the conditioned medium for the aforementioned markers, negating the need for tissue destructive assays. Physiologically relevant whole- or part-joint organ culture models, necessary for research and pre-clinical assessment of therapies, could be monitored this way, reducing the need to sacrifice tissues to determine viability, and hence reducing the sample numbers necessary.

Keywords: Organ culture, apoptosis, necrosis, viability monitoring, glucose, lactate dehydrogenase, matrix metalloproteinase, glycosaminoglycan.

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Introduction

There is a clinical need to develop more effective biomaterial and regenerative therapies for the repair and replacement of cartilage in articulating joints. Currently, there are many treatment options. These include hyaluronic acid injections, to supplement lubrication (Ayhan *et al.*, 2014); subchondral drilling, microfracture and abrasion arthroplasty to stimulate a fibrocartilage repair by bone-derived multipotent stromal cells (Pridie, 1959; Steadman *et al.*, 2001; Goyal *et al.*, 2013a; Johnson, 1986; Steinwachs *et al.*, 2008); autologous chondrocyte transplantation or matrix-enhanced chondrocyte implantation (Brittberg *et al.*, 1994; Gillogly *et al.*, 1998; Goyal *et al.*, 2013b); and autologous or allogeneic osteochondral (OC) transplant (Goyal *et al.*, 2014; Sherman *et al.*, 2014). However, none of these treatments result in full restoration of hyaline cartilage function (Orth *et al.*, 2014).

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 tibio-femoral 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 OC tissues, coupled with methods that allow on-line monitoring of the physiological status of the tissues.

Ex vivo organ culture is increasingly being used in both fundamental and applied biomedical research, as it is much more representative of natural physiological cellular behaviour than simple monolayer culture. Organ culture has been employed in studies of meniscal tissue (Dai *et al.*, 2013; Narita *et al.*, 2009), articular cartilage (Jacoby and Jayson, 1976; Hollander *et al.*, 1991; Fay *et al.*, 2006; Tchetina *et al.*, 2006), OC explants (Amin *et al.*, 2009a; Seol *et al.*, 2014), and whole porcine patellae (Ashwell *et al.*, 2008; Ashwell *et al.*, 2013). However, organ culture is extremely challenging, as conditions in these cultures must be able to maintain tissue viability and stable matrix composition. In order to monitor tissue viability in organ culture, tissues are sacrificed for analysis throughout the culture period, significantly reducing the sample number at each time point compared to the *in situ* monitoring situation where numbers are preserved.

Cell viability is usually defined by plasma membrane integrity and/or the presence of metabolic activity. For cells in monolayer or suspension culture, dye exclusion such as Trypan blue (Pappenheimer *et al.*, 1917) is commonly used, but this is not applicable to tissues. Cell tracker dyes such as 5-chloromethyl fluorescein diacetate (CMFDA) and propidium iodide (PI), calcein and ethidium homodimer, or

fluorescein diacetate and PI have been shown to be useful for monitoring cell viability, by labelling live and dead cells in tissues or engineered tissues, and localising them using confocal microscopy, but this is limited to tissues up to 4 mm in thickness (Amin *et al.*, 2009b; Dai *et al.*, 2013; Breuls *et al.*, 2003).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and XTT (2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide tetrazolium salt) reduction assays, which measure cellular oxidative metabolism, also provide an accurate measure of tissue metabolism, but the process requires the destruction of the tissue (Mosmann, 1983; Roehm *et al.*, 1991).

Other methods of viability assessment include the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) apoptosis assay (Mueller-Rath *et al.*, 2007; Narita *et al.*, 2009; Seol *et al.*, 2014), and histological analysis of cells and extracellular matrix (ECM) (Naqvi *et al.*, 2005; Mueller-Rath *et al.*, 2007; Dai *et al.*, 2013), but these techniques require the tissues to be fixed and sectioned prior to the assay protocols. Apoptosis can also be assessed by measuring caspase 3/7 in whole tissue lysates, but again this requires destruction of the tissue (Dudli *et al.*, 2012).

Non-invasive, non-destructive methods to assess tissue viability have been reported, such as measurement of oxygen consumption by optical oxygen sensing (O'Riordan *et al.*, 2000; Deshpande and Heinzle, 2004; Deshpande *et al.*, 2004), and determination of carbon dioxide production (Yang and Balcarcel, 2004). However, these methods require the cultures to be sealed from ambient gases so that those gases cannot dissolve into the culture medium. Such sealing would be prohibitive to tissue survival in organ and tissue culture.

Lactate dehydrogenase (LDH) release from damaged cells into the surrounding medium has been used to monitor cell death in culture as a non-invasive method of cell viability assessment (Racher *et al.*, 1990; Wagner *et al.*, 1991; Legrand *et al.*, 1992). Similarly, online glucose monitoring has also been used to assess viable cell numbers in cell culture, with reduced consumption of glucose indicating reduced metabolism and cell death (Prill *et al.*, 2014), although it has been reported that a loss of viability is not always indicated by a decrease in glucose consumption (Racher *et al.*, 1990). Alkaline phosphatase is produced by osteoblasts and is potentially a good indicator of healthy viable bone.

Methods for the non-invasive assessment of the integrity of the extracellular matrix of osteochondral tissues include the measurement of glycosaminoglycans (GAGs) and matrix metalloproteinases (MMPs) in the organ culture conditioned medium. Previous studies have measured the release of GAGs from organ cultures as an indicator of cartilaginous tissue degradation (Tchetina *et al.*, 2006; Korecki *et al.*, 2007). Both MMP-2 and MMP-9 have been shown to be elevated in the synovial fluid of rheumatoid arthritis patients (Cawston and Young, 2010), which might indicate that these MMPs are potential markers for the degradation of osteochondral tissues.

The longer term aims of our studies are to develop physiological biotribological models of natural cartilage articulations, in order to investigate the impact of novel cartilage substitution therapies on the surrounding and opposing cartilage. Hence, the number of replicate organ cultures will be limited, negating the use of tissue destructive methods for determination of tissue viability during longer term culture. The aim of this study was therefore to investigate the utility of measuring a range of molecular markers in the organ culture conditioned medium as an indicator of tissue viability. We used Triton X-100 to induce cell necrosis by causing cell lysis and camptothecin to induce apoptosis by interfering with topoisomerase I enzymes, in porcine femoral OC organ cultures. We demonstrate that a combination of non-invasive analytical techniques that assess changes in glucose consumption, and release of LDH, alkaline phosphatase (AP), glycosaminoglycans (GAGs) and matrix metalloproteinases (MMPs) into the culture medium can be used to indicate changes in tissue viability, without sacrifice of the tissues.

Materials and Methods

Tissue procurement

Porcine hind legs were obtained from animals 4-6 months old from a local abattoir (J. Penny, Leeds, UK) within 4 h of sacrifice. Under aseptic conditions, all soft tissues were dissected from the femora, which were then clamped using a bench vice. A sterile 9 mm drill was then used to dissect OC plugs from the femoral condyles (Fig. 1a), to a depth of approximately 6 mm, and a sterile 9 mm corer used to release the plugs from the underlying bone. OC plugs were immediately stored in Hank's balanced salt solution (HBSS; Sigma-Aldrich Company Ltd.; product code H8264) and transferred to a sterile class II cabinet. Sterile bone cutters were then used to trim the plugs to a depth of 4-5 mm. Bone marrow was washed out with an overnight heparin (12.5 IU/mL; LEO Pharma, Hurley, UK; product code 009876-04) and antibiotic (200 U/mL penicillin/ 200µg/mL streptomycin [Lonza, Walkersville, MD, USA; product code DE17-603E], 50 µg/mL gentamycin [Sigma-Aldrich Company Ltd., Dorset, UK; product code G1272] and 2.5 µg/mL amphotericin B [Sigma-Aldrich Company Ltd.; product code A2942]) wash in HBSS at 4 °C and subsequent jet washing using phosphate buffered saline (PBS; Lonza; product code BE17-512F) with a Waterflosser Ultra (Waterpik International Inc., Surrey, UK; product code WP-120UK). OC plugs, before and after washing, are illustrated in Fig. 1b and c. OC plugs were washed again using PBS with a Waterflosser Ultra and washed twice for 5 min in HBSS before culture.

Organ culture

Tissues obtained from two animals were used in two separate experiments. OC plugs (*n* = 15; from the same animal) were weighed and 3 replicates were sacrificed for day 0 XTT assay. The remaining 12 were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza; product code BE12-917F) supplemented with L-glutamine (2 mM; Lonza; product code BE17-605E), 100 U/mL penicillin/ 100 µg/mL streptomycin, gentamycin (50 µg/
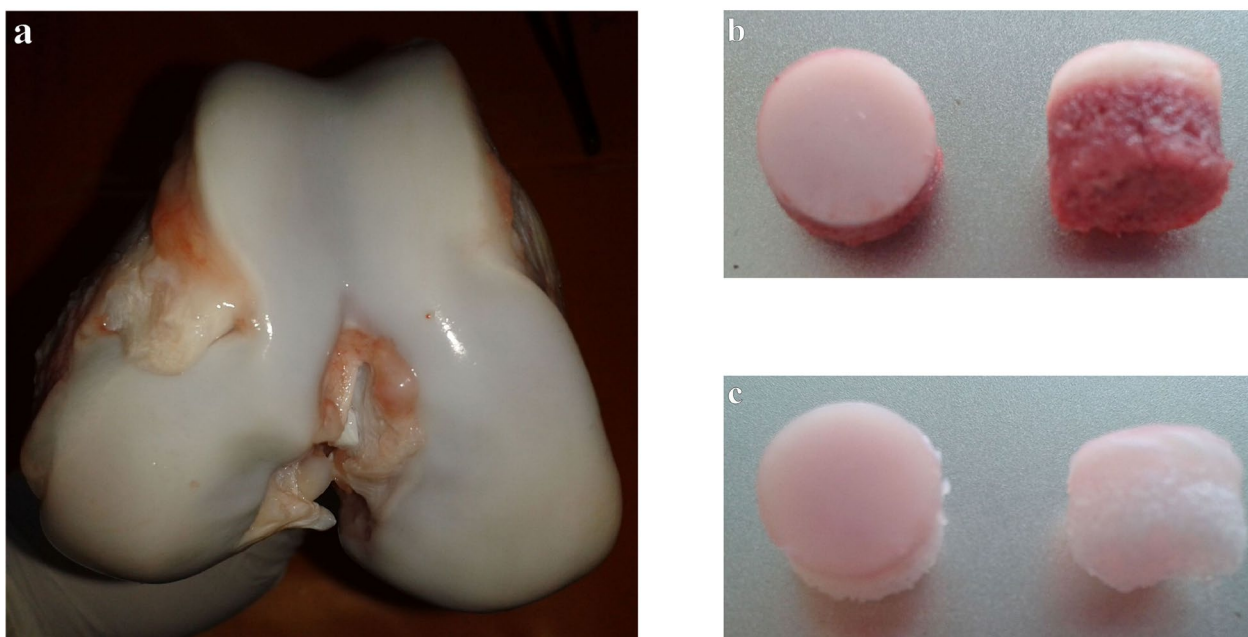
Fig. 1. Images of the osteochondral tissues. Porcine femoral head (**a**) from which 9 mm diameter osteochondral plugs were dissected (**b**). The plugs were washed to remove all blood and marrow (**c**).

mL), amphotericin B (2.5 µg/mL), ascorbic acid (50 µg/mL; Sigma-Aldrich Company Ltd.; product code A8960), dexamethasone (0.1 µM; Sigma-Aldrich Company Ltd.; product code D2915), and ITS™ premix universal culture supplement (0.1 % v/v; Corning B.V. Life Sciences, Amsterdam, The Netherlands; product code 354351) at 37 °C in 5 % (v/v) CO_2 in air. Culture medium was changed and conditioned medium collected at days 1, 3 and 6. At day 6, three OC plugs were sacrificed for XTT assay. Of the remaining nine OC plug cultures, three were spiked with camptothecin (4 µg/mL; Cayman Chemical Company, Ann Arbor, MI, USA; product code 11694), three were spiked with Triton X-100 (0.01 % [v/v]; Sigma-Aldrich Company Ltd.; product code X100), and three were cultured without any additional treatments for the remainder of the culture period. The medium was changed and conditioned medium collected on days 9, 13, 16 and 20. OC plugs cultured with camptothecin ($n = 3$), Triton X-100 ($n = 3$) or culture medium alone ($n = 3$) were sacrificed on day 20 for analysis by XTT assay. The study was repeated, with collection of the conditioned medium using a further $n = 15$ OC plugs from a different animal.

In order to obtain tissue for histological evaluation and analysis of chondrocyte membrane integrity by LIVE/DEAD® cell staining, OC plugs ($n = 15$) were placed into organ culture as described above. OC plugs were harvested at day 0 ($n = 3$) and day 6 ($n = 3$). Of the remaining OC plug cultures ($n = 9$), three were spiked with camptothecin (4 µg/mL), three were spiked with Triton X-100 (0.01 % (v/v)), and three were cultured without treatment for a further 14 days prior to harvest at day 20.

Assay of tissue viability
Tissue viability was analysed using an XTT based *in vitro* toxicology assay kit (Sigma-Aldrich Company Ltd.; product code TOX-2), with some small adaptations to the

manufacturer's instructions. Briefly, pre-weighed tissues (bone or cartilage) were chopped into approximately 2 mm³ pieces using a scalpel and incubated in the XTT solution (1 mL) for 4 h at 37 °C in 5 % (v/v) CO_2 in air and the XTT solution removed and retained. Tetrazolium product was then extracted from the tissues with dimethyl sulfoxide (DMSO; 0.5 mL; VWR Chemicals, Lutterworth, UK; product code 23500.297) for 1 h. XTT and DMSO solutions were then pooled before reading the absorbance of triplicate samples at 450 nm and 690 nm in a 96 well plate (Nunc A/S [Thermo Fisher Scientific], Roskilde, Denmark; product code 269620) using a microplate spectrophotometer (Thermo Scientific, Fisher Scientific, Loughborough, UK; model Multiskan Spectrum) and SkanIt™ RE for MSS 2.1 software (Thermo Software, Thermo Scientific). The absorbances at 690 nm were subtracted from those at 450 nm and XTT content calculated *per* gram of tissue.

LIVE/DEAD cell staining of cartilage tissues
Plasma membrane integrity of the chondrocytes in the cartilage of the OC plugs was visualised using LIVE/DEAD® cell staining (Life Technologies). A cross-sectional slice of the cartilage was cut using a scalpel (< 1mm). LIVE/DEAD® working solution (1 µM calcein-AM and 1 µM ethidium homodimer-1 in PBS) was added to the cartilage slice and incubated at 37 °C in 5 % (v/v) CO_2 in air for 1 h. The cartilage slice was washed three times in PBS before imaging by confocal microscopy (Zeiss LSM510 Meta).

Histological evaluation of cartilage tissues
For histological evaluation, the cartilage was sliced from the bone of the OC plugs using a scalpel. The cartilage samples were fixed in 10 % (v/v) neutral buffered formalin (Atom Scientific) for 48 h and dehydrated through

increasing concentrations of ethanol followed by xylene and embedded in paraffin wax. Serial sections (5 μm) were stained with haematoxylin and eosin (H&E) or safranin O. For safranin O staining, sections were rehydrated, stained with Weigerts` haematoxylin (Atom Scientific) for 3 min, differentiated in 1 % (v/v) acid alcohol, stained with 0.02 % (w/v) aqueous fast green (Sigma) for 5 min and washed briefly in 1 % (v/v) acetic acid before staining in 0.1 % (w/v) safranin O (Acros) for 4 min. Sections were dehydrated and mounted. Images were captured using an upright Carl Zeiss Axio Imager.M2 incorporating an Axio Cam MRc5, which was controlled by Zen Pro software 2012 (Zeiss).

Conditioned medium assays
All conditioned medium samples were analysed from the 20 day untreated control group (C), the group treated with Triton-X 100 (0.01 % [v/v]) from day 6 to 20 (TX), the group treated with camptothecin (4 μg/mL) from day 6 to 20 (CA).

Glucose assay
Conditioned medium was analysed for glucose content using the GlucCell™ glucose monitoring system (Cesco Bioproducts, Atlanta, GA, USA; product code DG1000). Fresh and conditioned culture medium samples (30 μL) were pipetted onto a clean hydrophobic surface in duplicate and measured using glucose test strips (Cesco Bioproducts; product code DGA050) according to the manufacturer's instructions. Average readings were used to calculate total glucose concentration in each sample. The concentrations of glucose in the conditioned medium were subtracted from the concentration of glucose in the fresh medium to determine the amount of glucose used (mg). This was then divided by the tissue weight (g) and again by the number of days in culture to give glucose (mg) utilised *per* gram of tissue *per* day in culture.

Lactate dehydrogenase (LDH) assay
LDH content in conditioned medium was measured using an LDH cytotoxicity assay kit (Cayman Chemical Company; product code 10008882) according to the manufacturer's instructions. Triplicate conditioned medium samples were diluted in assay buffer to bring each within the linear region of the assay. LDH standard was serially diluted in assay buffer (containing fresh culture medium of the same dilution as the samples) to prepare standard LDH solutions in triplicate. Absorbance at 490 nm was measured in a 96 well plate, using a microplate spectrophotometer and SkanIt™ RE for MSS 2.1 software. Linear regression was performed on the absorbances of the standard LDH solutions and the LDH concentrations in the conditioned medium samples were calculated. Average readings were used to calculate the total LDH (mU) in each sample. This was then divided by the tissue weight (g) and by the number of days in culture to give LDH (mU) released *per* gram of tissue *per* day in culture.

Glycosaminoglycan (GAG) assay
Accumulation of sulphated GAGs was measured using a standard dimethylmethylene blue (DMMB) colourimetric

assay (Farndale *et al.*, 1986). Briefly, conditioned medium samples were diluted in assay buffer to bring each within the linear region of the assay. Chondroitin sulphate B standard (Sigma-Aldrich Company Ltd.; product code C3788) was serially diluted in assay buffer (containing fresh medium at the same dilution as the samples) to prepare standard GAG solutions. Diluted samples and standards (40 μL) were mixed with DMMB solution (250 μL) in triplicate in 96 well plates. After 2 min of shaking the plate, absorbance at 525 nm was read using a microplate spectrophotometer and SkanIt™ RE for MSS 2.1 software. Linear regression was performed on the absorbances of the standard GAG solutions and the GAG concentrations in the conditioned medium samples were calculated. Average readings were used to calculate the total amount of GAGs (μg) in each sample. This was then divided by the tissue weight (g) and by the number of days in culture to give GAG (μg) released *per* gram of tissue *per* day in culture.

Alkaline phosphatase (AP) assay
AP content in conditioned medium was measured using the colorimetric SensoLyte® *p*NPP Alkaline Phosphatase Assay Kit (AnaSpec Inc., Fremont, CA, USA; product code 72146) according to the manufacturer's instructions. Triplicate conditioned medium samples were diluted in assay buffer to bring each within the linear region of the assay. AP standard was serially diluted in assay buffer (containing fresh medium at the same dilution as the samples) to prepare standard AP solutions in triplicate. Absorbance at 405 nm was measured using a microplate spectrophotometer and SkanIt™ RE for MSS 2.1 software. Linear regression was performed on the absorbance readings obtained from the standard AP solutions and AP concentrations in the conditioned medium samples were calculated. Average readings were used to calculate total AP (ng) in each sample. This was then divided by the tissue weight (g) and by the number of days in culture to give AP (ng) released *per* gram of tissue *per* day in culture.

Determination of MMP-2 and MMP-9 using gelatin zymography
MMP-2 and MMP-9 activity was assessed in the conditioned medium from 2 replicate samples of the repeat experiment at days 6, 9, 13 and 16 using gelatin zymography (Woessner, 1995). Conditioned medium samples were diluted in zymogram sample buffer (Bio-rad Laboratories Ltd., Hemel Hempstead, UK; product code 161-0764) and electrophoresed under non-reducing conditions on pre-cast 10 % (w/v) SDS-PAGE gelatin zymography gels (Bio-rad Laboratories Ltd.; product code 161-1167). Gels were washed twice in zymogram renaturation buffer (Bio-rad Laboratories Ltd.; product code 161-0765) at 4 °C for 20 min each wash, before wash and overnight incubation at 37 °C in zymogram developing buffer (Bio-rad Laboratories Ltd.; product code 161-0766) containing 1 mM 4-amino-phenylmercuric acetate (APMA; Sigma-Aldrich Company Ltd.; product code A9563). Gels were then stained with SimplyBlue™ Safe Stain (Invitrogen, Life Technologies Ltd., Paisley, UK; product code LC6065) for 3 h and de-stained overnight in distilled water to reveal zones of gelatinase activity.
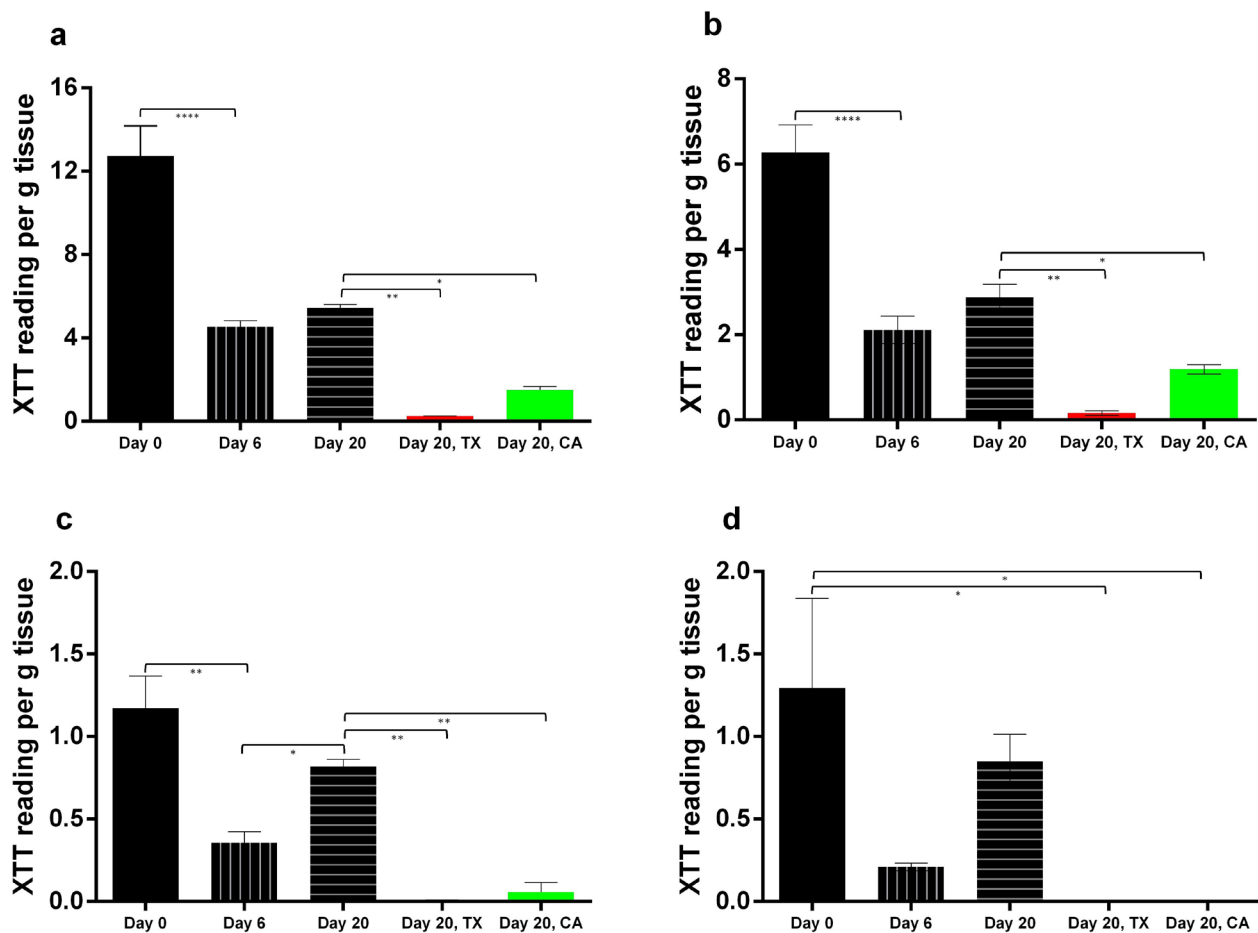
Fig. 2. Viability assessment of osteochondral tissues over 20 days in culture. XTT conversion by cartilage (**a** and **b**) and bone (**c** and **d**) over 20 days in two separate experiments using tissue from two porcine donors (**a** and **c**, tissue from porcine donor 1; **b** and **d**, tissue from porcine donor 2). XTT assays were used to assess the effects of 0.01 % Triton X-100 (TX) and 4 μg/mL camptothecin (CA), added at day 6 of culture, on tissue viability. The data are presented as the mean ($n = 3$) ± standard error. Data were analysed by one way ANOVA and significant differences are indicated by $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

The gels were analysed using an Evolution™ MP Colour camera and Image-Pro® Plus version 5 image analysis software (Media Cybernetics Inc., Rockville, MD, USA).

Statistical analysis

All data were analysed by one-way ANOVA followed by Tukey's multiple comparison test (GraphPad Prism 6, GraphPad Software, San Diego, CA, USA). Results are reported as mean values +/- standard deviation or standard error and considered significant when $p < 0.05$.

Results

Viability of osteochondral tissue organ cultures over 20 days

In the first experiment, XTT assays of tissue viability indicated that the cartilage tissue showed reduced XTT conversion from day 0 to 6 ($p < 0.0001$), but there was no further reduction in the amount of XTT converted by the tissue from day 6 to day 20 in the untreated control cultures (Fig. 2a). Therefore, XTT conversion by the control tissues

remained stable between day 6 and day 20, the period when the test OC plugs were treated with either 0.01 % (v/v) Triton X-100 or 4 μg/mL camptothecin. The XTT assay data at day 20, showed that both Triton X-100 and camptothecin treatment from day 6 resulted in significant loss of XTT conversion by the cartilage tissue ($p < 0.01$ for Triton X-100 and $p < 0.05$ for camptothecin). The repeat study showed the same pattern of results for the cartilage tissue and the same significant differences between XTT conversion data at days 6 and 20, although the actual values for XTT conversion *per* gram of tissue were lower throughout (Fig. 2b).

The bone tissue also showed a significant reduction in XTT conversion from day 0 to 6 in the first study ($p < 0.01$), with a subsequent significant increase to day 20 ($p < 0.05$; Fig. 2c). Therefore, the ability of the bone to convert XTT appeared to recover from day 6 to 20 in the control group. Treatment of the OC plugs with 0.01 % (v/v) Triton X-100 or 4 μg/mL camptothecin, initiated at day 6, caused significant reductions in XTT conversion by the bone compared to the control at day 20 ($p < 0.01$). The repeat study showed a very similar range and pattern
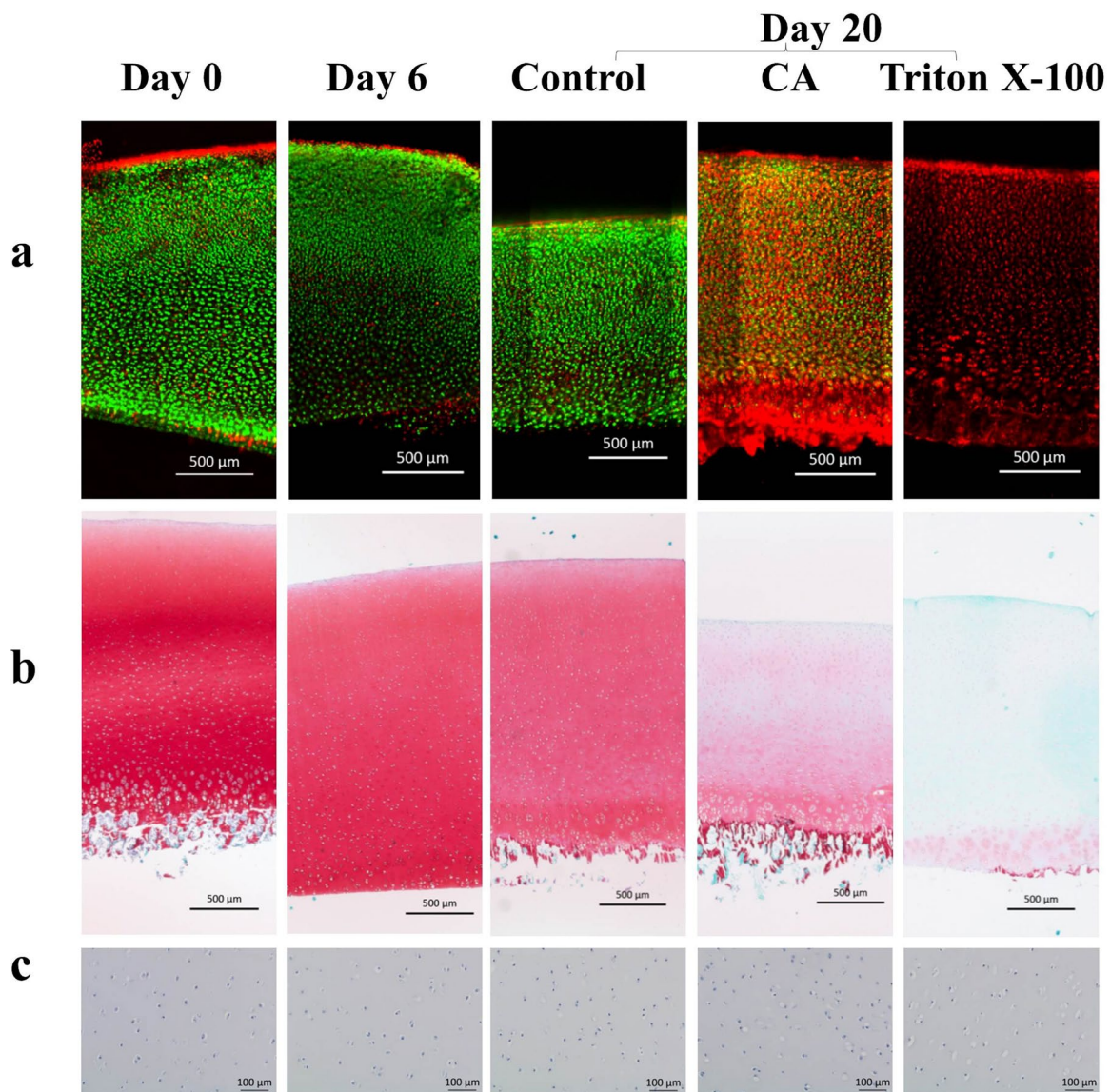
Fig. 3. LIVE/DEAD® cell imaging and histological evaluation of cartilage tissues over 20 days in culture. LIVE/DEAD® stained images of slices of cartilage tissue from OC plug cultures; scale bar 500 µm (**a**); Sections of cartilage tissue from OC plug cultures stained with safranin O; scale bar 500 µm (**b**); High power images of sections of the mid-section of cartilage tissue from OC plug cultures stained with H&E; scale bar 100 µm (**c**). Control; no treatment after 6 days of culture. CA; treated with camptothecin (4 µg/mL) after 6 six days of culture. Triton X-100; treated with 0.01 % (v/v) after 6 days of culture.

of results, although there was greater variation in the data (Fig. 2d). There were no significant changes in the amount of XTT converted between day 0, day 6 and day 20 in the control cultures, but at day 20 the Triton X-100 (0.01 % [v/v]) and camptothecin (4 µg/mL) treated organ cultures showed significantly less XTT conversion compared to day 0 ($p < 0.05$).

LIVE/DEAD® cell staining of cartilage tissues over 20 days of organ culture
The plasma membrane integrity of the chondrocytes in the cartilage of the OC plugs was visualised using LIVE/DEAD® cell staining. Images of the cross-sectional slices are presented in Fig. (3a). At day 0, whilst the majority of

the chondrocytes in the tissue stained green – indicating viable cells, there was a band of dead cells in the cartilage surface zone. At day 6, most of the chondrocytes within the cartilage tissue were viable, again with a band of dead cells in the surface zone. For the control tissue at day 20, LIVE/DEAD® cell staining showed the same pattern, with most of the cells in the tissue remaining viable. This was in contrast to the images of the tissues treated with Triton X-100 or 4 µg/mL camptothecin at day 20. In tissues treated with Triton X-100, there were overall fewer cells and all the remaining cells were stained red, and therefore dead. In tissues treated with camptothecin, whilst there were some chondrocytes that retained membrane integrity throughout the cartilage, most of the cells were dead.
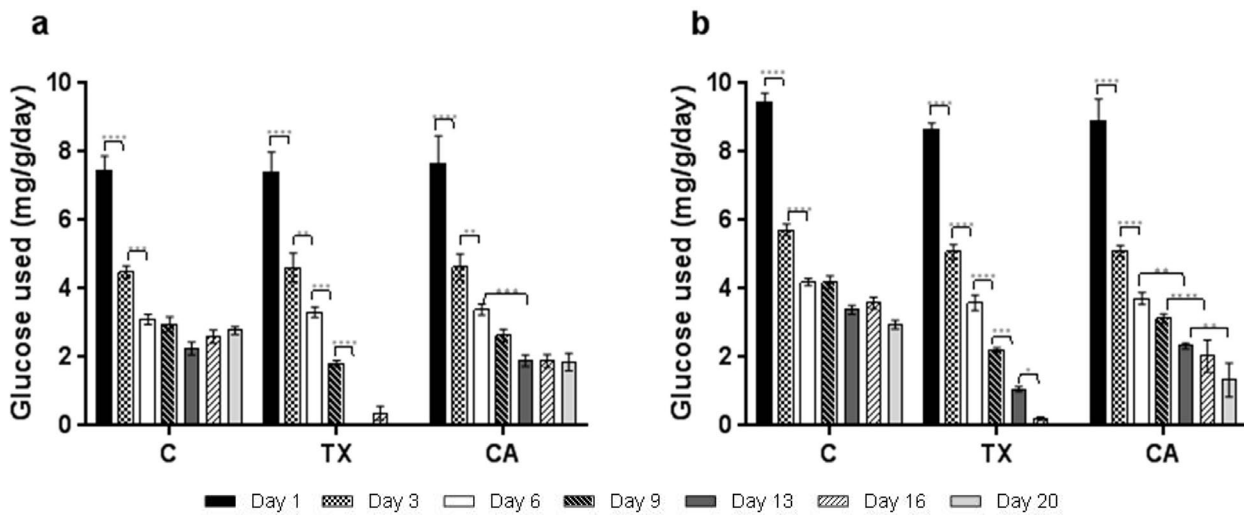
Fig. 4. Glucose consumption of osteochondral tissues over 20 days in culture. Glucose assays were used to assess glucose consumption over 20 days of culture and the effects of 0.01 % Triton X-100 (TX) or 4 µg/mL camptothecin (CA), added at day 6 of culture, compared to untreated controls cultured for the whole culture period of 20 days (C), in two separate studies (**a** and **b**). The data are presented as the mean ($n = 3$) ± standard deviation. Data were analysed by one way ANOVA and significant differences are indicated by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.
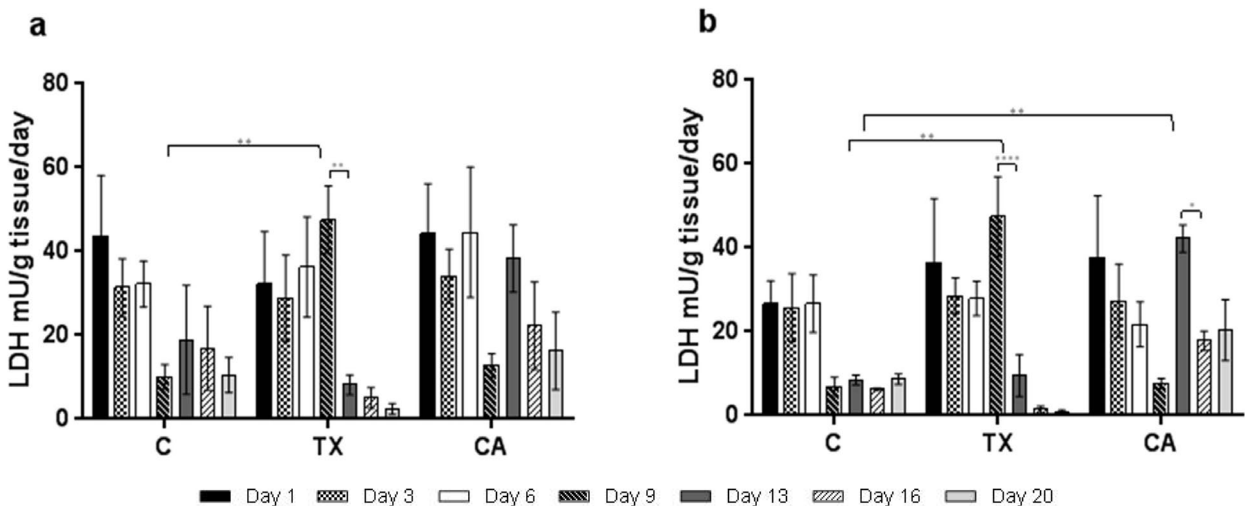

Fig. 5. LDH release by osteochondral tissues over 20 days in culture. LDH assays were used to assess LDH release over 20 days of culture and the effects of 0.01 % Triton X-100 (TX) or 4 µg/mL camptothecin (CA), added at day 6 of culture, compared to untreated controls cultured for the whole culture period of 20 days (C), in two separate studies (**a** and **b**). The data is presented as the mean ($n = 3$) ± standard deviation. Data were analysed by one way ANOVA and significant differences are indicated by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Histological evaluation of the osteochondral tissue cartilage over 20 days of organ culture

Images of sections of the cartilage tissues from the organ cultures stained with safranin O and H&E are shown in Fig. (3b) and (3c) respectively. Images of control tissue sections stained with safranin O revealed strong staining for GAGs at day 0 and day 6 with slight qualitative reduction in staining intensity at day 20. In the cartilage tissue treated with 4 µg/mL camptothecin at day 20, there was an apparent reduction in staining with safranin O compared to the control tissue at day 20, but GAGs were still present in the tissue. In sections of the cartilage tissue treated with Triton X-100 at day 20, there was no positive staining with safranin O indicating a complete loss of GAGs from the tissue. The H&E stained sections

revealed that the cartilage tissue remained structurally intact regardless of the duration of organ culture or mode of treatment. The major difference in the tissues revealed by H&E staining of tissue sections was that in the tissues treated with TritonX-100 at day 20, there were numerous empty lacunae present compared to the control tissue at day 20. This was not a feature of the tissue treated with 4 µg/mL camptothecin at day 20 which was morphologically similar to the control tissue at day 20.

Glucose consumption by osteochondral tissue organ cultures over 20 days

In the control OC cultures there were significant reductions in glucose consumption from day 1 to 3 ($p < 0.0001$) and again from day 3 to 6 ($p < 0.001$), but there were
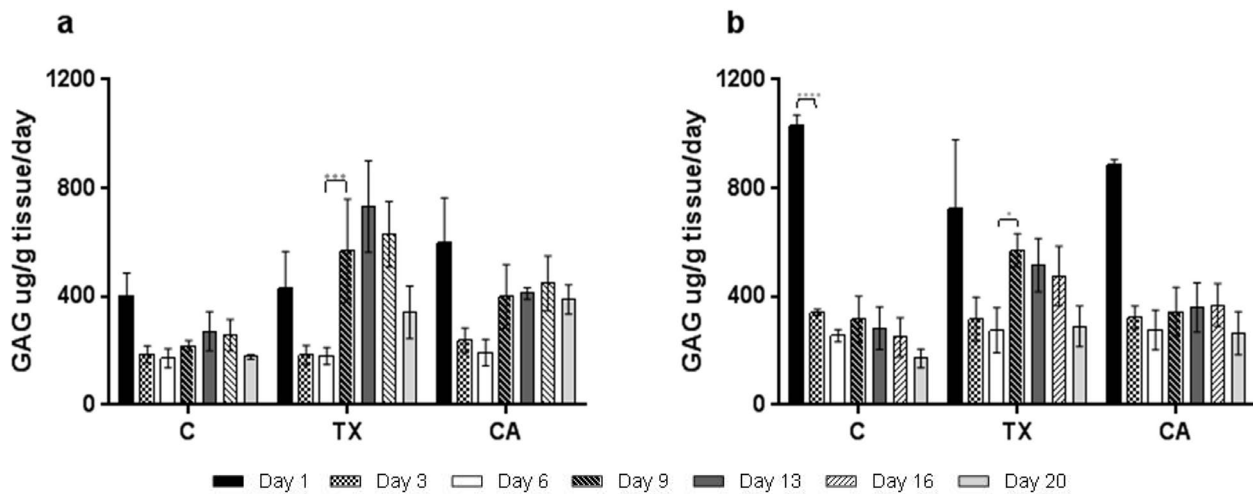
Fig. 6. GAG release by osteochondral tissues over 20 days in culture. GAG assays were used to assess GAG accumulation over 20 days of culture and the effects of 0.01 % Triton X-100 (TX) or 4 µg/mL camptothecin (CA), added at day 6 of culture, compared to untreated controls cultured the whole culture period of 20 days (C), in two separate studies (**a** and **b**). The data are presented as the mean ($n = 3$) ± standard deviation. Data were analysed by one way ANOVA and significant differences are indicated by $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$.
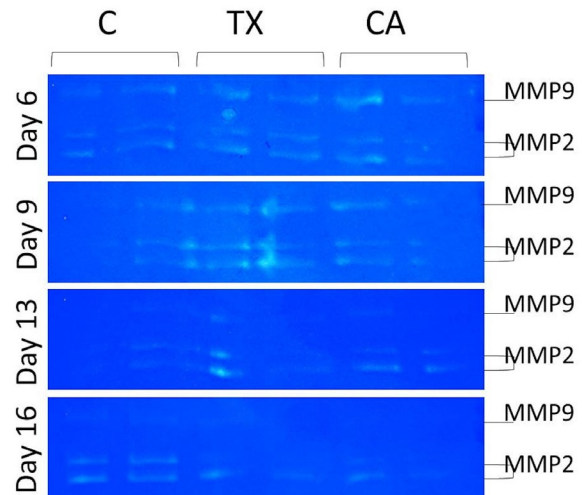

Fig. 7. MMP release by osteochondral tissues over 20 days in culture. MMP-2 (doublet of pro-MMP-2 and active MMP-2) and MMP-9 present in conditioned medium samples collected on days 6, 9, 13 and 16 of a 20 day culture, from 2 samples each of the untreated (C), 0.01 % Triton X-100 (TX) or 4 µg/mL camptothecin (CA) groups. Treatments began after medium collection on day 6 of culture.

no further changes in glucose consumption from day 6 onwards, showing a steady consumption of glucose after an initial settling period. In both studies, there were no significant differences in glucose consumption between any groups before treatments began at day 6 (Fig. 4). Immediately after treatment at day 6, the Triton X-100 treated OC tissues consumed significantly less glucose with a reduction in consumption at each subsequent time point from day 6 until negligible glucose consumption at day 13 (Fig. 4a) and 16 (Fig. 4b) in the first and second study respectively. The camptothecin-treated OC tissues also consumed significantly less glucose after treatment although the reduction in glucose consumption occurred over an extended period of time with consumption reducing over 2 time-points from day 6 to 13 in both studies and further reducing from day 9 to 16 and day 13 to 20 in the second study.

LDH release by osteochondral tissue organ cultures over 20 days

In both studies, there was a trend for higher LDH levels in the first 6 days of culture in all groups, followed by lower, constant levels in control cultures (Fig. 5). There were no significant differences in LDH concentrations in the culture fluids between groups before treatment at day 6. After treatment (day 9), the Triton X-100 treated OC plugs released significantly more LDH than the control OC plugs in both studies ($p < 0.01$ and $p < 0.0001$; Fig. 5a and 5b respectively). This peak in LDH release was short lived, with significantly less LDH released at day 13 compared to day 9 in both studies ($p < 0.01$ and $p < 0.0001$; Fig. 5a and 5b respectively). In the repeat study the camptothecin-treated group released more LDH than the control group on day 13 ($p < 0.001$; Fig 5b), a peak that was short lived, being significantly higher than levels detected later at
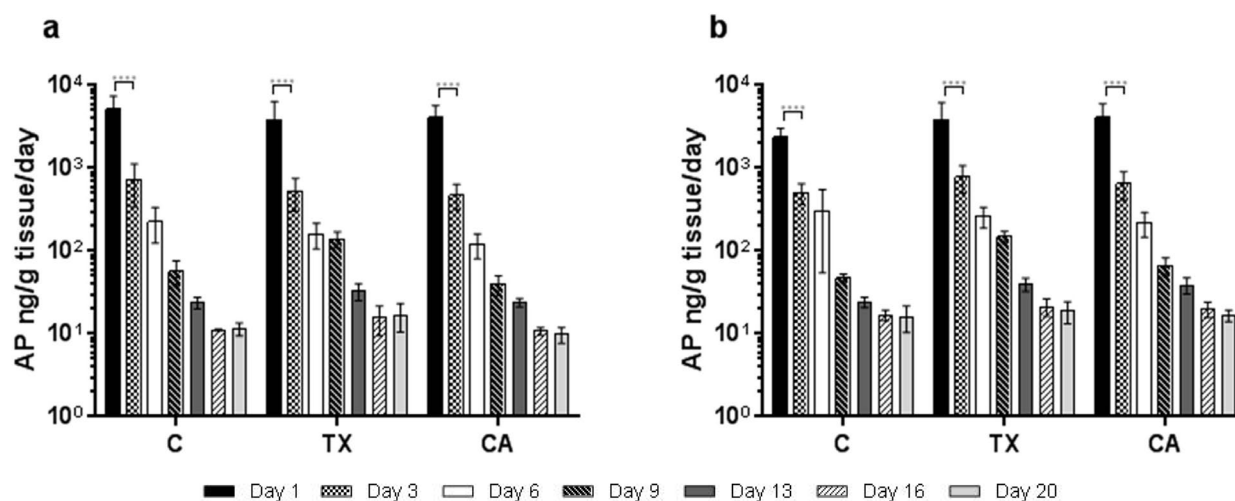
Fig. 8. AP release by osteochondral tissues over 20 days in culture. AP assays were used to assess AP release over 20 days of culture and the effects of 0.01 % Triton X-100 (TX) or 4 μg/mL camptothecin (CA), added at day 6 of culture, compared to untreated controls cultured for the whole culture period of 20 days (C), in two separate studies (**a** and **b**). The data are presented as the mean ($n = 3$) ± standard deviation. Data were analysed by one way ANOVA. No significant differences between the controls and treatment groups were detected.

day 16 ($p < 0.05$). This same pattern of LDH release was observed in the first study although the findings were not statistically significant due to larger variation in the data (Fig. 5a).

GAG release by osteochondral tissue organ cultures over 20 days

In both studies, the levels of GAGs released into the culture medium were relatively high and variable on day 1 in all groups, but then reduced and remained constant in the control group from day 3 onwards (Fig. 6). There were no significant differences in GAG concentrations in the culture fluids between groups before treatment at day 6. In both studies, the Triton X-100 treated OC plugs released significantly higher levels of GAGs immediately after treatment, from day 6 to 9 ($p < 0.001$ and $p < 0.05$; Fig. 6a and 6b respectively). There was no significant effect of camptothecin on GAGs release by the OC plugs.

MMP-2 and MMP-9 release by osteochondral tissue organ cultures over 20 days

MMP-2 was detected in the culture medium as a doublet of pro-MMP-2 and active MMP-2 along with a single MMP-9 band at approximately equal levels in all samples tested at day 6 before any treatments of the OC cultures (Fig. 7). At day 9, following treatment, levels of both MMPs were low in the untreated control OC plug cultures and appeared higher in the camptothecin-treated OC plug cultures, and even higher in the Triton X-100 treated OC plug cultures compared to the controls. MMP-2 was not detected at day 13 in the control samples, but was present at day 16. Levels of MMP-2 reduced in the Triton X-100 samples from day 9 to 16, and more active MMP-2 than pro-MMP-2 was detected in the camptothecin-treated cultures at days 13 and 16. MMP-9 was barely detectable in any of the culture samples at days 13 and 16.

AP release by osteochondral tissue organ cultures over 20 days

In both studies there was an initial high level of AP detected in the culture fluids in all groups, which reduced significantly at day 3 (Fig. 8). From day 3 onwards, there was a general, but not significant, decline in AP concentration released into the culture medium in all OC plug cultures, regardless of treatment. No significant differences were found between treated and control OC plug cultures at any time point.

Discussion

Organ culture is very challenging in three main respects. Firstly, the researcher generally has a limited number of samples due to the practicalities of procuring sufficient tissue. Secondly, maintaining organ cultures *in vitro*, in the appropriate physiological environment, with an adequate oxygen and nutrient exchange system, is often limited. Thirdly, the researcher needs to be able to monitor the viability status of the organ culture, which usually requires harvesting replicate samples at given time intervals for analysis. The aim of this study was to evaluate the use of molecular markers in the conditioned medium of OC cultures as an indicator of the viability of the tissues, negating the need to sacrifice the tissue samples.

In this study, porcine OC tissues were used. This was driven by the longer term aims to develop *in vitro* models that incorporate natural OC tissues for the pre-clinical biotribological testing of novel biomaterial and regenerative therapies for cartilage repair and replacement within a range of diarthrodial joints including the knee, hip and spinal facet joints. The data will however, be of general interest to researchers engaged in organ culture of a range tissues.

Determination of the viability of cells in tissues in organ culture can be complex, since there are two modes of cell death – necrosis and apoptosis. Necrotic cells lose their membrane integrity in an uncontrolled manner, whilst apoptotic cells die through a controlled process that involves cell shrinkage, nuclear fragmentation, and the formation of apoptotic bodies (with no loss of membrane integrity), which are then removed by macrophages *in vivo*. Many assays of cell viability (dye exclusion, intracellular enzyme release) rely upon the necrotic loss of membrane integrity that is not seen with apoptosis. Assays that detect metabolic activity (glucose or oxygen consumption, carbon dioxide production, tetrazolium assays) are also only applicable to necrotic cells or those that have completed apoptosis, and do not detect the early apoptotic cells that are committed to die but are still metabolically active (Browne and Al-Rubeai, 2011). Furthermore, assays that detect apoptotic cells (*e.g.* TUNEL) do not detect necrotic cells or those apoptotic cells that have not yet commenced nuclear fragmentation (Browne and Al-Rubeai, 2011). In this study, a combination of non-invasive assays was used to assess tissue viability and ECM stability alongside the widely used, but tissue sacrificing, XTT assay.

It was found that the OC cultures required a period of up to 6 days to equilibrate to the *in vitro* environment following harvesting. During this initial period of culture, there was a significant reduction in XTT conversion by both cartilage and bone tissues. This was followed by consistent values for XTT conversion by the untreated control OC tissues for up to 20 days of culture, indicating that tissues could be maintained in organ culture. The viability of the cartilage tissue in osteochondral plug organ cultures was also evaluated using LIVE/DEAD® staining. This revealed that at day 0, there was a band of dead cells in the superficial layer of the cartilage and that the majority of the chondrocytes in the middle and lower regions of the cartilage were alive. Exposure to the air and drying out of the cartilage surface during preparation for organ culture most likely accounted for the surface dead zone visible at day zero (Pun *et al.*, 2006). After 6 and 20 days (for the control OC plugs) in culture there was no noticeable change in the LIVE/DEAD® staining pattern of the cartilage tissue indicating that the chondrocytes within the cartilage did not lose membrane integrity in organ culture for up to 20 days. This suggested that the levels of XTT conversion observed in the OC organ cultures at day 0 were elevated due to an initial increase in mitochondrial activity of the chondrocytes following harvest of the OC plugs and placement in organ culture, supporting the need for an equilibration period.

This need for an equilibration period was also confirmed by the maintenance of glucose consumption levels from day 6, indicating no reduction in metabolism in the control cultures after the cultures had equilibrated. The control OC tissues did not show any increase in LDH, AP or GAG release after day 6, although MMP-2 levels did rise towards the end of the culture period. Moreover, the histological appearance of sections of the control tissues stained with H&E and safranin O, at days 0, 6 and 20, indicated that the histo-morphology of the cartilage was maintained with good retention of GAGs. Hence,

it could be inferred that no cell death or breakdown of the ECM occurred between days 6 and 20. In addition to the initial reduction in XTT conversion, the levels of glucose consumption, LDH release and GAG release took 3, 6 and 1 d, respectively, to reach consistent levels. The procedure for harvesting the OC tissues, which involved coring of cylindrical plugs, would have led to mechanical damage to the cells and the ECM at the cut periphery. It is recognised that mechanical damage to healthy cartilage tissue can result in cell death, MMP up-regulation and GAG release (Correro-Shahgaldian *et al.*, 2014). This inevitable damage would have led to some peripheral cell death (LDH release) and attempts by viable cells to remodel the damaged ECM during the initial period of culture which was the most likely explanation for the initially high levels of XTT conversion, glucose consumption, and AP release. Moreover, the collagen network in articular cartilage is under tension and has a characteristic orientation (Jeffrey *et al.*, 1991). The harvesting of OC plugs would have severed the collagen fibres, leading to a loss of tension allowing proteoglycans (GAGs) in the periphery of the tissue to be released into the culture fluid.

This is important when observing patterns of markers detected, as this initial culture period may give rise to erratic data. Allowing a time period for culture equilibration is an accepted technique used in many studies (Jacoby and Jayson, 1976; Fay *et al.*, 2006; Tchetina *et al.*, 2006), and should be routinely implemented during the monitoring of organ cultures. The day 20 XTT results showed that treatment of the OC tissues with both Triton X-100 and camptothecin, resulted in the loss of all bone cell viability and the most of cartilage cell viability, clearly showing that both treatments induced significant cell death within the OC tissues. The day 20 results of the LIVE/DEAD® cell stained cartilage tissue slices that had been treated with Triton X-100 and camptothecin were in agreement with the XTT data.

A reduction in glucose consumption can indicate a drop in metabolism when cell death occurs, except during early apoptosis, although reduction of metabolism would be apparent after completion of apoptosis (Browne and Al-Rubeai, 2011; Prill *et al.*, 2014). The findings of this study are in agreement with this. The inclusion of 0.01 % Triton X-100 from day 6 onwards caused an immediate reduction in XTT conversion and glucose consumption (and therefore metabolism). This was most likely due to the detergent lysing the cells in the cartilage and bone. However, the camptothecin-induced reduction in glucose consumption was delayed, presumably representing a period of early cellular apoptosis during which glucose consumption still occurred as metabolism continued, followed by completion of apoptosis when cell death was complete and glucose consumption reduced.

LDH is present in the cytosol and mitochondria of cells (Brooks *et al.*, 1999). Since necrosis results in the loss of membrane integrity and subsequent release of LDH, the level of LDH in conditioned medium has been used as a marker of cell injury and death in monolayer cell culture (Racher *et al.*, 1990; Wagner *et al.*, 1991; Legrand *et al.*, 1992), and in organ culture of the intervertebral disc (Dudli *et al.*, 2012). A rapid increase in LDH, and subsequent

immediate reduction in the OC tissues treated with Triton X-100, indicated a rapid and uncontrolled lysis of the majority of cells, and release of intracellular enzymes. It might be expected that apoptotic death would not lead to a rapid increase in LDH as chondrocyte apoptotic bodies have been reported to accumulate in the lacunae and interterritorial space (Hashimoto *et al.*, 1998). However, an increase in LDH release was detected in OC cultures treated with camptothecin, after a delay, in this model. Since macrophages are not present in cartilage tissue, it can be hypothesised that the final phagocytic stage of apoptosis was not completed and that the contents (including LDH) of some of the apoptotic bodies were released.

AP is a by-product of osteoblast activity so may indicate active and healthy bone, in addition to possible bone remodelling in the organ culture. However, no effects on AP release were recorded in the treated and untreated OC cultures. Early in the culture, following dissection, AP levels were very high throughout and any small changes in AP would have not been apparent. Later after a period of equilibration, the bone likely settled into a less active state where, even in the control group, AP levels were naturally low.

The levels of GAGs are reduced in cartilage of osteo- and rheumatoid arthritis compared to normal cartilage (Hollander *et al.*, 1991), indicating that GAGs are lost from the tissue as cartilage is degraded. The release of GAGs into conditioned medium has been assessed in organ cultures of full depth articular cartilage (Tchetina *et al.*, 2006) and intervertebral discs (Korecki *et al.*, 2007) to detect/quantify tissue degradation. The breakdown of cartilage and release of GAGs into the conditioned medium was therefore assessed as an indicator of tissue degradation in the present study. The OC tissues treated with Triton X-100, showed an immediate increase in GAG release into the conditioned medium, that might have been due to 1) the release of lyases and hydrolases from the intracellular contents as the cells lost membrane integrity, and 2) release of cell membrane bound GAG that would not necessarily be released from apoptotic bodies or (3) wash-out of proteoglycans from the cut-edge of the ECM as a result of disruption of the non-covalent interactions between aggrecan and long chain hyaluronic acid molecules in the cartilage. Detergents, including Triton X-100 have been reported to deplete tissues of proteoglycans, albeit at concentrations an order of magnitude greater than the 0.01 % used here (Vavken *et al.*, 2009). There was no significant increase in GAG release in the OC tissue cultures treated with camptothecin. In cartilage, apoptotic bodies accumulate and remain in the lacunae and interterritorial space (Hashimoto *et al.*, 1998). With no phagocytic cells present, the apoptotic bodies will eventually break down and release their contents (indicated by LDH results), but enzyme release may not have been adequate for subsequent ECM breakdown. In one of the two studies presented here, there was a non-significant small increase in GAG release after camptothecin treatment, indicating that perhaps slow release of ECM degrading enzymes had begun. The levels of MMP-2 and MMP-9 released into the conditioned medium at day 13 and day 9, respectively, after camptothecin treatment, were higher than those detected in control cultures at the same

points, although not as high as in the OC tissue cultures treated with Triton X-100. Both MMP-2 and MMP-9 have been shown to be expressed by chondrocytes and to play a role in osteoclast resorption during bone degradation (Murphy and Lee, 2005), and both are elevated in rheumatoid arthritic synovial fluid and tissues (Cawston and Young, 2010), suggesting that these MMPs might be expected to play a role in the breakdown of bone and/or cartilage in this model. It is of note that the relative level of active MMP-2 to pro-MMP-2 was higher at days 13 and 16 in the camptothecin-treated cultures, and this agrees with findings that apoptosis can induce the activation of proMMP-2 (Preaux *et al.*, 2002). Overall, however it was difficult to detect overt changes in MMP-9 and MMP-2 using the zymography method in this study. It would have been advantageous to use specific ELISAs, had these been available for the porcine proteins.

The general trends in levels of molecular markers of tissue viability were the same in both study repeats, although the levels of some markers varied. This highlights the need to monitor the day to day changes and general trends in marker levels to predict viability in individual cultures. Glucose consumption and LDH release have been used in combination as markers of tissue viability previously (Racher *et al.*, 1990), and the data presented here support their use to predict loss of tissue viability in OC organ cultures. However, these two markers alone could not be used to indicate whether loss of tissue viability was due to necrosis or apoptosis, because the researcher would not know whether the changes were immediate or delayed. In this study we explored whether the inclusion of assays to assess ECM degradation (GAG release, MMP-2 and MMP-9 release) might indicate whether loss of tissue viability was by necrosis or apoptosis; however, it was possible that the increased levels of GAG release following Triton X-100 treatment, which was not seen with camptothecin-induced apoptosis were due to the detergent effect rather than an effect of the mode of cell death and this requires further investigation.

More recently, lactate monitoring has been used in conjunction with glucose monitoring as a marker of anaerobic metabolism and hypoxia (Boero *et al.*, 2011; Boero *et al.*, 2014). Lactate is potentially a good marker to monitor in addition to those used in the research presented here, as it could be used to show whether oxygen was limiting in larger organ culture models. Other improvements which could be included in future would be to use other relevant markers of ECM degradation in OC organ culture, such as collagenase-cleaved type II collagen (Tchetina *et al.*, 2006). Furthermore, the assays could be adapted to assess levels of different ECM markers for other types of organ and tissue culture.

There have been a number of studies involving organ and tissue culture of different parts of the knee tissues, in which viability was not assessed (Jacoby and Jayson, 1976; Hollander *et al.*, 1991; Fay *et al.*, 2006; Tchetina *et al.*, 2006; Ashwell *et al.*, 2008; Ashwell *et al.*, 2013). The studies utilised minimal sample numbers, and any tissue destructive method of viability assessment was not possible due to restrictive sample numbers. The assessment of soluble markers in the conditioned medium would have

provided important information on tissue viability without the need to increase sample numbers.

In vitro pre-clinical studies of novel therapies and devices are important initial steps in the clinical translation pathway (Anz *et al.*, 2014). Many therapies and devices will not progress beyond this initial phase of development, and therefore efficient pre-clinical test methods are essential. The use of soluble markers to measure organ culture viability enables smaller sample numbers and reduced costs compared to the use of tissue destructive methods.

Conclusions

The data presented showed that both Triton X-100 and camptothecin induced loss of tissue viability in porcine OC organ culture models, but viability was maintained after an initial period of culture equilibration in untreated control cultures. Both glucose consumption and LDH release indicated a loss of viability, immediately following Triton X-100 treatment (necrosis induction), and after a delay following camptothecin treatment (apoptosis induction). The results showed that by using this approach, the viability of OC organ culture models can be quickly and easily monitored. The use of other molecular markers (for example markers of ECM degradation) for differentiation between apoptosis and necrosis requires further investigation.

Acknowledgements

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Discussion with Reviewers

Magali Cucchiarini: The goal of this study is to produce a convenient culture system of osteochondral tissue to easily assess viability based on the analysis of the production of soluble makers. The organ culture model is certainly pertinent as a simple system for future screening of drugs or of other therapeutic candidates. One weakness of the study is the use of non-clinically-/-physiologically relevant molecules (Triton, camptothecin) and it would be necessary to test instead the effects of mediators such as those produced during pathological processes in the cartilage (inflammatory cytokines).

Authors: We agree entirely with this comment. We chose to use Triton X-100 and camptothecin to validate the approach as "positive controls" for cell death by necrosis and apoptosis. Now that the approach to utilise molecular markers in the conditioned medium as an indicator of loss of tissue viability has been established for the osteochondral organ cultures, it will be necessary to test more clinically relevant interventions, which for our research interests will include biomechanical loading and the impact of novel cartilage substitution therapies.

Benjamin Gantenbein: Why do the authors think the GAG release into the media was still increasing although they treated the cells with Triton X-100 or camptothecin after 6 days and beyond? Why was the glucose consumption in the case of camptothecin not behaving the same way as with the treatment of Triton X-100 detergent?

Authors: The increase in GAG release into the culture medium in camptothecin and Triton X-100 treated cultures compared to control cultures beyond day 6 of culture might have been due to breakdown of aggrecan due to release/activation of aggrecanases as observed for MMP-2. The camptothecin treated osteochondral plug cultures did indeed maintain higher levels of glucose consumption than the Triton X-100 treated cultures after day 6 which can be explained by the findings, from the LIVE/DEAD® cell imaging of cartilage slices which showed that at day 20, there were still viable cells in the camptothecin treated cultures which were not found in the Triton X-100 treated cultures.