Biological, biochemical and biomechanical characterisation of articular cartilage from the porcine, bovine and ovine hip and knee

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Abstract

This study aimed to determine the optimal starting material for the development of an acellular osteochondral graft. Osteochondral tissues from three different species were characterised; pig (6 months), cow (18 months) and two ages of sheep (8 - 12 months and > 4 year old). Tissues from the acetabulum and femoral head of the hip, and the groove, medial and lateral condyles and tibial plateau of the knee were assessed. Histological analysis of each tissue allowed for qualification of cartilage histoarchitecture, glycosaminoglycan (GAG) distribution, assessment of cellularity and cartilage thickness. Collagen and GAG content were quantified and cartilage water content was defined. Following biomechanical testing, the percentage deformation, permeability and equilibrium elastic modulus was determined. Results showed that porcine cartilage had the highest concentration of sulphated proteoglycans and that the condyles and groove of the knee showed higher GAG content than other joint areas. Cartilage from younger tissues (porcine and young ovine) had higher cell content and was thicker, reflecting the effects of age on cartilage structure. Cartilage from older sheep had a much higher elastic modulus and was less permeable than other species.

1   *Introduction*

2   Osteoarthritis (OA) is the most common disorder affecting joints [1]; this degenerative

3   disease causes progressive degradation of articular cartilage, bone and surrounding joint

4   tissues, resulting in pain and a loss of function [2]. In a proportion of patients OA has been

5   reported to originate from an initially minor cartilage defect [3], which, due to the avascular

6   and aneural nature of cartilage, is unable to heal and deteriorates with regular joint loading

7   and articulation [4]. However, OA may also result from changes in bone, soft tissues or

8   secondary to trauma or acute injury. Osteochondral lesions in themselves can cause pain,

9   swelling and mechanical problems, reducing the quality of life for patients [5]. Current

10   orthopaedic treatments for chondral defects, such as lavage and debridement [6, 7] and

11   marrow stimulation [8] have shown variable long term outcomes. Similarly,

12   perichondrial/periosteal grafting, mosaicplasty [9] and autologous chondrocyte implantation

13   [10] may result in donor site morbidity amongst other complications [5, 11]. Due to the

14   limitations of current treatments, chondral lesions present an ideal target for early

15   intervention tissue engineered therapies in the OA disease process. It is proposed here that an

16   acellular, xenogenic, osteochondral biological scaffold may provide the ideal graft for defect

17   repair.

18

19   Articular cartilage has a highly organised, specialised composition and structure, which

20   allows it to function as a low friction, load-bearing surface [12]. The major tissue components

21   are collagen type II, proteoglycans, water and cells. The collagen forms a characteristically

22   oriented fibril network which provides the tensile strength of the tissue [13, 14].

23   Proteoglycans are dispersed throughout this network and bind water in the tissue, allowing it

24   to resist compression [15]. Chondrocytes are responsible for cartilage matrix homeostasis

25   [16].

1

2　The aims of this study were to provide a comprehensive analysis of articular cartilage from

3　four animals as a potential source of acellular xenogeneic biological scaffold, assessing the

4　various matrix components and the resultant mechanical properties of each tissue. Cartilage

5　from the hip and knee joints of pigs, cows and two ages of sheep was assessed. Cellularity,

6　cell distribution, GAG content and distribution, collagen concentration, water content and

7　cartilage thickness was determined and the percentage deformation, permeability and

8　equilibrium elastic modulus of each tissue defined. The characterisation of these different

9　tissues will enable determination the most optimal starting material for the development of an

10　acellular xenogenic osteochondral biological scaffold.

11　## *Materials and Methods*

12　### **Osteochondral samples**

13　Legs of 9 month old pigs, 8 - 12 month old lambs, 4 - 8 year old ewes and femurs of 18

14　month old cows were obtained from a local accredited abattoir within 24-76 hr of slaughter.

15　Extra-skeletal tissues were removed using a scalpel and forceps to expose the joint surfaces.

16　Exposed cartilage was regularly moistened with phosphate buffered saline (PBS [Oxoid]) to

17　maintain hydration. Long bones were held in a specially designed clamp to immobilise them

18　while allowing access to the joint surfaces, acetabula were held in a bench-top vice. Using an

19　electric drill with a corer attachment, osteochondral plugs, 9 mm diameter and 12 mm deep,

20　were collected from the femoral head, patellofemoral groove, medial and lateral condyles and

21　medial and lateral tibial plateau of all species. Bovine acetabular tissue was not available and

22　was collected from sheep and pigs. Plugs were washed in PBS before being stored at -20 °C

23　on PBS moistened filter paper. During dissection, macroscopic observations of the cartilage

24　from each species was made.

1 **Histology**

2 Osteochondral plugs (n=5) were fixed for 48 hours in 10 % (v/v) neutral buffered formalin

3 (NBF) [Bios Europe Ltd], before being decalcified in 12.5 % (w/v) diaminoethane tetraacetic

4 acid disodium salt [Na_2EDTA pH 7: Fisher Scientific] for 4 weeks or until soft enough to be

5 cut with a scalpel. Plugs were bisected before being dehydrated and paraffin wax embedded

6 using an automated process [Lecia TP 1020, Lecia Microsystems]. Sections of 6 μm

7 thickness were cut through the cartilage surface into the bone, encompassing the different

8 cartilage zones and subchondral bone. Haematoxylin and eosin (H&E) [Bios Europe Ltd]

9 staining was used to assess tissue histoarchitecture. From the resultant digital images, Image J

10 software was used to delineate the superficial, middle and deep zones of cartilage (top 15 %,

11 middle 50 % and bottom 35 % of cartilage thickness respectively [17]) and cell shape

12 analysis was performed on 500 $μm^2$ regions from each zone, giving particle count, area and

13 roundness data. Roundness was calculated by the software as follows:

$$roundness = \frac{4 \times area}{\pi \times major\ axis^2}$$

14 with a measurement of 1.0 being perfectly round. One measurement for each zone was

15 performed per image. GAG distribution within the tissue was visualised by staining with

16 alcian blue [Bios Europe Ltd] (pH 2.5) and counterstaining with periodic acid Schiff and

17 Mayers haematoxylin [Sigma]. This stain provided good definition of the cartilage-bone

18 interface, so image software was used to measure the cartilage thickness. Measurements

19 (n=6) were taken from the cartilage surface to the point at which alcian blue staining

20 terminated, the mean of these measurements was calculated for each image.

21 **Biochemical Assays**

22 *Sulphated sugar assay:* Cartilage from each joint area (n=5) was cut away from the

23 subchondral bone and lyophilised to a constant weight before being enzymatically digested

1   using papain solution (5 ml, 50 U.ml^{-1} papain [Sigma] in PBS at pH 6 with 5mM L-cysteine

2   hydrochloride [Sigma] and 5mM Na_2EDTA [Fisher Scientific]) for 36-48 hours at 60 °C.

3   GAG content was quantified following a method adapted from Farndale *et al.* [18]. Briefly,

4   standard concentrations of chondroitin B sulphate [Sigma] were produced, 1,9-

5   dimethylmethylene blue [Sigma] solution (250 µl) was added to each standard and sample

6   (40 µl) in a clear flat bottomed 96-well plate, the plate was agitated for 2 minutes before the

7   absorbance was read on a spectrophotometer [Multiscan Spectrum, Thermo Labsystems] at

8   525 nm. Interpolation of sample absorbance from the standard curve gave the GAG

9   concentration of each digest which was then expressed in µg.mg^{-1} of dry weight of tissue.

10

11   *Hydroxyproline assay:* Cartilage was lyophilised, as above, before being hydrolysed in 6 M

12   hydrochloric acid [HCl: VWR] for 16 hours at 80 °C followed by neutralisation with 6 M

13   sodium hydroxide [NaOH: Fisher]. Hydroxyproline content was quantified following a

14   method adapted from Edwards & O'Brien [19]. Briefly, standard concentrations of trans-4-

15   hydroxy-L-proline [Sigma] were produced, Chloramine-T oxidisation solution [Sigma] (100

16   µl) was added to each standard and sample (50 µl) in a clear flat bottomed 96-well plate, the

17   plate was agitated for 5 minutes. Ehrlich's reagent [Sigma] (100 µl) was then added and the

18   plate was incubated in a water bath at 60°C for 45 minutes before the absorbance was read at

19   570 nm on a spectrophotometer. Interpolation of sample absorbance from the standard curve

20   gave the hyrdoxyproline concentration of each sample. This was then expressed as µg.mg^{-1}

21   dry weight of tissue.

22

23   Water content of samples (n=5) was determined during cartilage lyophilisation. Sample

24   weight was recorded prior to and post dehydration, the ratio of wet weight to dry weight gave

25   the water content of each cartilage sample.

1   Biomechanical Testing

2   *Compressive testing using an indenter:*

3   Methods used have been previously described by Pawaskar *et al*. [20] and Taylor *et al*. [21]

4   to assess biomechanical properties of cartilage attached to bone. Briefly, osteochondral plugs

5   were compressed in a purpose built indentation rig using a 3 mm diameter, hemispherical,

6   stainless steel indenter under a load of 0.8 N. Plugs were submerged in PBS during testing to

7   maintain cartilage hydration. The deformation of cartilage was measured at a sampling

8   frequency of 5 Hz over one hour, after which all samples had reached equilibrium. Following

9   compression, pins were fully rehydrated in PBS before cartilage thickness was measured. A

10   needle indenter was used to penetrate the cartilage, lowering at a rate of 4.5 mm.min^{-1}; the

11   resistance to motion was measured using a 500 N load cell [Instron 3365]. An increase in

12   load was recorded when the needle first contacted the cartilage surface and a second increase

13   when entering the bone, the distance between these two changes in load was taken as the

14   cartilage thickness. Deformation of cartilage was normalised to thickness to give percentage

15   deformation for each pin. The permeability and equilibrium elastic equilibrium modulus of

16   cartilage were derived from the cartilage thickness, deformation and water content using a

17   finite element (FE) method developed by Pawaskar *et al*. [20]. Briefly, an FE model of the

18   cartilage pin and indenter is produced, to which an initial permeability and equilibrium elastic

19   modulus are assigned. The FE model produces a deformation curve which is compared to the

20   experimental deformation curve. The input material properties are altered iteratively until the

21   modelled curve matches as closely as possible with the experimental curve. The accuracy of

22   these modelled properties is assessed by the closeness-of-fit R^2 value for the curves, generally

23   acceptable over 0.6 for biological tissues.

1   **Statistical analysis**

2   Numerical data was analysed using Microsoft Excel. For each set of results the mean and

3   95% confidence limits were calculated. For data expressed as a ratio or percentage, values

4   underwent arcsine transformation before being statistically analysed, means and upper and

5   lower 95% confidence limits were then back transformed prior to presentation. Group means

6   were compared using the T-test following one-way or two-way (cell shape data) analysis of

7   variance (ANOVA). A p-value <0.05 was accepted as significant.

8   *Results*

9   Upon dissection, physical differences were observed between species. The length of the

10   femurs from femoral head to the front of the condyles was ~ 20 cm in both pigs and sheep;

11   however this was double in cows, ~ 40 cm. This size difference was reflected in the size of

12   the joints; the bovine femoral head measured ~ 8 cm from the terres ligament to femoral

13   neck, while the porcine was ~ 4 cm and ovine only ~ 2 cm. This pattern in joint size was seen

14   for both the hip and knee. Cartilage generally appeared white, glossy and healthy with

15   exception of ovine (> 4 yr) cartilage which had a yellowed appearance in most cases. Upon

16   drilling, the bone of porcine and young ovine was notably softer than that of bovine and older

17   ovine samples. Old ovine bone, despite being hard, was also found to be quite brittle when

18   cores were extracted from the joints.

19

20   H&E staining of osteochondral sections from each species showed characteristic chondrocyte

21   organisation (Figure 1), with linear columns of cells in the deep zone perpendicular to the

22   surface. This was less pronounced in porcine and young ovine, where the cell groups showed

23   a more rounded morphology.

24

1   Across most joint regions porcine and young ovine cartilage tended to have higher cellularity

2   than bovine and old ovine cartilage. This trend was observed within the superficial and deep

3   zones, but was less pronounced in the middle zone, where porcine cartilage showed lower

4   cellularity (Figure 2; $p<0.05$, ANOVA). There was no significant difference in the cell area

5   between species or joint region in the superficial or middle zones, cells of the deep zone

6   tended to be larger than those of the middle or superficial zones. Porcine cells were larger

7   than those of other species in the deep zone, significantly so in the acetabulum, femoral head,

8   femoral groove and medial condyle (Figure 3; $p<0.05$, ANOVA). Cells in the superficial and

9   middle cartilage zones in all species and joint regions tended to have a roundness value

10   around 0.75, however porcine cells in the deep zone were less round than those of other

11   species, significantly so in the acetabulum, femoral head, groove and medial condyle (Figure

12   4; $p<0.05$ ANOVA)

13

14   Staining of sections with alcian blue showed the distribution of GAGs within the cartilage

15   tissues. All tissues showed characteristically less staining at the superficial zone becoming

16   more intense through the middle and deep zones, then depleting in the calcified zone with the

17   bone unstained.

18

19   Thickness of cartilage for each species was measured from digital images of the stained

20   sections (Figure 5). Porcine tibial cartilage was significantly thinner than that of the groove

21   and medial condyle, young ovine femoral head cartilage was significantly thinner than that of

22   the medial tibia, no significant difference between joint areas was seen in bovine or old ovine

23   ($p<0.05$ ANOVA), however there was a trend for cartilage of the femoral knee (groove

24   and/or condyles) to be thicker than other joint areas.. Porcine and bovine cartilage was

25   significantly thicker than ovine cartilage in a number of joint regions.

1

2   Following quantitative analysis it was found that porcine cartilage generally had significantly

3   higher GAG content than the other species, and old ovine cartilage generally had the lowest

4   (Figure 6). Across the joint regions a similar pattern was seen to thickness: femoral knee

5   cartilage tended to have higher GAG content. In the pig, cartilage of the groove and both

6   condyles had a significantly higher GAG content than that of the medial side of the tibial

7   plateau, only the medial condyle and groove showed higher concentration than the lateral

8   side. Cartilage from the medial condyle of the older sheep had a significantly higher GAG

9   content than that of the acetabulum, groove and tibia. Cartilage of the bovine lateral condyle

10   had significantly fewer GAGs than the medial tibia (p<0.05 ANOVA).

11

12   The cartilage hydroxyproline content (representative of collagen) was quantified.

13   Hydroxyproline concentration showed an inverse relationship to GAG content (Figure 7)

14   with porcine cartilage having the lowest hydroxyproline concentration in most joint regions

15   whilst having the highest GAG concentration. The opposite was true of cartilage from the

16   older sheep, which had significantly higher hydroxyproline cartilage than other tissues but the

17   lowest GAG. Cartilage of the porcine lateral condyle had significantly lower hydroxyproline

18   content than that of the medial condyle, there were no other significant differences within

19   species (p<0.05 ANOVA).

20

21   Analysis of the water content of cartilage from each species showed that all values fell

22   between 70–82 % (Figure 8). There was no significant difference between any of the species

23   except for bovine, in which cartilage of the tibia had significantly higher water content than

24   the femoral head and medial groove, the lateral condyle also showed higher water content

1   than the femoral head. Across the species only bovine lateral tibial cartilage was significantly

2   higher in water content than old ovine (p<0.05 ANOVA).

3

4   The mechanical properties of cartilage from the different species and joint regions are shown

5   in Figure 9 and Table (1), detailing the percentage deformation of cartilage after one hour, the

6   permeability, elastic equilibrium modulus and thickness of each tissue. Properties could not

7   be determined for ovine groove cartilage as the joints were too small to collect suitable pins.

8

9   There was a trend toward cartilage from the tibias and lateral condyles having higher

10   percentage deformation than cartilage of the hip. Porcine tibial cartilage showed significantly

11   greater percentage deformation than all other joint areas. Bovine femoral head cartilage,

12   medial condyle and medial tibia cartilage had significantly lower percentage deformation

13   than that of the lateral condyle and tibia. Young ovine acetabular cartilage deformed

14   significantly less than that of the femoral head. Cartilage of the old ovine acetabulum

15   deformed significantly less than that of the lateral condyle and tibial plateau. Old ovine

16   cartilage from the acetabulum deformed significantly less than other species. Young ovine

17   femoral head cartilage deformed significantly more than other species. Porcine lateral

18   condyle cartilage deformed significantly less than bovine and old ovine. Bovine medial tibial

19   cartilage deformed significantly less than other species (p<0.05 ANOVA).

20

21   Generally the equilibrium elastic modulus of porcine cartilage was lowest, increasing in

22   bovine, further increasing in young ovine cartilage and old ovine cartilage showed the highest

23   equilibrium elastic modulus in all cases. A trend was seen in all species across joint regions,

24   for cartilage of the tibia to have a lower modulus than areas of the femoral knee and hip.

25

1 Permeability showed an inverse relationship with equilibrium elastic modulus, with ovine

2 being least permeable and porcine being most permeable. Permeability increased in the tibial

3 plateau and was lowest in cartilage of the hip.

4

5 *Discussion*

6 As a starting point the xenogenic source material for decellularisation would be similar to

7 human cartilage in terms of composition, structure and mechanical properties. An additional

8 benefit could be to find a starting material with a higher GAG content than human cartilage,

9 as it is reported that decellularisation leads to a loss of GAGs within acellular scaffolds, and

10 therefore diminished mechanical properties [22-25]. This study looked at the biological,

11 biochemical and biomechanical properties of different osteochondral tissues. Various regions

12 of the porcine, bovine and ovine hip and knee were analysed to determine which would be

13 the best material for development of a decellularisation protocol.

14

15 From initial observations, cartilage of older sheep (>4 years) had a yellowed appearance, as

16 seen in aged, osteoarthritic cartilage [26]. This would make it an inferior substitute for

17 healthy young cartilage in vivo. The joint size has implications for the practicality of a

18 species; more osteochondral samples can be harvested from a large bovine joint than porcine,

19 and even fewer from an ovine joint. The apparent softness of porcine and to some extent the

20 younger ovine bone highlighted the immaturity of these tissues.

21

22 H&E staining showed that cellular orientation was more defined in bovine and old ovine

23 cartilage indicating a fully mature tissue. Cell shape analysis identified that the cells of the

24 deep zones of porcine cartilage were larger and less round than those of the cartilage deep

25 zones of other species, these observations of characteristically hypertrophic chondrocytes

12

1   suggested that the porcine tissues are immature and still undergoing growth. As in humans,

2   the cellularity of cartilage showed a trend of decreasing with age [27], with older ovine (> 4

3   years) and bovine having fewest cells and porcine (6 months) having the most. The difference

4   in cellularity between joint regions in pigs related to the cartilage thickness. Cartilage from

5   the groove had significantly fewer cells than that of the tibia; however, tibial cartilage was

6   significantly thinner than that of the groove. Thicker cartilage has been reported to have

7   lower cell density than thin cartilage [28]. Comparison between cartilage zones indicated that

8   in thick porcine cartilages, there was higher cell density in the superficial and deep zones

9   compared the middle zone. For decellularisation, a starting material with fewer cells would

10   be preferable.

11

12   Cartilage thickness has been reported to increase as body weight increases [28]. This was not

13   necessarily supported in the present study. Although bovine and porcine cartilage were

14   thicker than that of ovine, despite the body mass of a cow being greater, porcine cartilage was

15   thicker. Julkunen *et al*. [29] showed that immature cartilage is thicker than that of mature

16   animals, and that thickness decreases with maturity. This was supported by comparison of the

17   two age groups of sheep, with the less mature ovine cartilage tending to be thicker than that

18   of the older sheep. Cartilage of the femoral condyles and groove showed a trend toward being

19   thicker than that of the hip and tibia in all species, this trend was also seen when comparing

20   the thicknesses for these joint regions in humans [30-32]. Simon *et al.* [33] have shown that

21   cartilage thickness is relative to joint congruence, so in joints with a high level of congruence,

22   such as the hip, cartilage is thinner. Human cartilage was on average thicker than that of any

23   species in this study, Shepherd *et al.* [30] report the thickness of human femoral head

24   cartilage to range between 1.08-2.40 mm where as ovine cartilage was only ~ 0.68 mm in this

25   area, porcine 1.14 mm and bovine 1.53 mm. Cartilage from the human groove ranged

1 between 1.76-2.59 mm, ovine cartilage is again much thinner; 0.86 mm in young sheep and

2 0.63 mm in old sheep. Cartilage from the porcine groove was 2.04 mm and bovine 1.52 mm,

3 within or approaching the range of human cartilage thickness. The relative thickness of

4 human cartilage probably reflects the increased loads placed on human knee cartilage due to

5 the bipedal posture, as discussed by Stockwell [28]. It is likely that a decellularised xenogenic

6 graft with cartilage of a similar thickness to the surrounding host tissue would integrate and

7 function better, therefore porcine and bovine tissues would be preferred.

8

9 Porcine cartilage had the highest GAG content; this was likely due to the young age of the

10 tissue, as the concentration of proteoglycans is reportedly higher in immature cartilage, and

11 decreases with age [34]. Bolton *et al*. [35] suggest this is due to changes in chondrocyte

12 activity; aggrecan and link protein synthesis decreases with maturity. Except for cartilage of

13 the medial condyle, there was no significant difference in the GAG content of bovine and

14 ovine cartilage. Again, cartilage of the groove and condyles showed higher GAG content than

15 other joint regions. The relationship in these joint areas of increased GAG content alongside

16 increased thickness has been observed when tissues undergo increased loading or activity

17 [36]. However, forces acting on the healthy knee are reportedly lower than those of the hip

18 [37]. This trend across the joint regions may again be due to the congruence of the joints,

19 affecting the contact area and therefore stresses. Increased load on a very local scale may

20 cause specific remodelling at that site, to increase thickness and GAG concentration so to

21 decrease contact pressure. There is limited information in the literature regarding

22 concentrations of GAGs per dry weight of human cartilage. Various figures have been

23 reported for cartilage of the femoral head, Vilim *et al*. [38] quote 54.7 and 38.6 mg.g^{-1} GAG

24 per wet weight cartilage from two donors, while Hollander *et al*. [39] reported values ranging

25 between 25 – 40 μg.mg^{-1} wet weight. In this study porcine femoral head cartilage was shown

1   to have 78 ± 30 µg.mg^{-1} (n=5 $\pm$ 95 % CL) GAG per wet weight, bovine cartilage was 68 ± 8

2   µg.mg^{-1}, young ovine was 45 ± 18 µg.mg^{-1} and old ovine was 62 ± 16 µg.mg^{-1}, all higher than

3   values reported for human cartilage. GAG content was reported to be between 5-10 % total

4   cartilage mass, as described for human cartilage by Mow *et al.* [14]. The comparatively high

5   GAG content of cartilage found for all species indicates any could reasonably replace human

6   cartilage, however, porcine having highest GAG concentration would be preferred as loss of

7   GAGs is a limitation in decellularisation processes[22-25].

8

9   Hydroxyproline content showed an inverse relationship to GAG content. Porcine cartilage

10   had the lowest collagen content while old ovine had the highest. Muir *et al.* [40] assessed

11   collagen content of human cartilage and found great variation between individuals and joints.

12   They found no correlation between cartilage collagen content and age. This study only

13   quantified collagen content and did not look qualitatively at collagen fibre alignment,

14   although content was constant collagen orientation may differ.

15

16   On the whole, tissue water content did not vary significantly between species or joint area,

17   only bovine tibial cartilage showed significantly higher water content. The water content of

18   human cartilage varies between 68-85 % [14], the water content of animal tissues is currently

19   within the same range, although, this may differ once cartilage has undergone

20   decellularisation.

21

22   Within a species, percentage deformation of cartilage tended to be higher in the tibia and

23   lower in the hip, this was reflected in the equilibrium elastic modulus derived for each type of

24   tissue, with tibial cartilage showing a lower modulus. Permeability was also higher in the

25   tibia than the hip as the GAG content of these tissues was lower, increased water flow

1   through the tissue would mean that load support from the cartilage fluid phase was less in

2   these tissues, therefore, the tissue deformed more. The relationship between material

3   properties and biological/biochemical composition was most pronounced in the bovine knee;

4   cartilage of the medial condyle and medial tibia was thicker and had higher GAG content

5   than its lateral counterpart, cartilage of the medial side of each joint also showed lower

6   permeability, and stiffer equilibrium elastic modulus (lower percentage deformation).

7

8   Between species, old ovine cartilage was stiffest and porcine was the least stiff. Old ovine

9   was stiffer than young ovine despite young ovine having higher GAG content, bovine was

10   also stiffer than porcine, despite porcine having a higher GAG content. This was likely due to

11   the quality of GAGs within the tissue, mature bovine and ovine cartilage may have more

12   effective proteoglycan structures which are better immobilised in the ECM compared to

13   porcine and young ovine, therefore these mature tissues had lower permeability and stiffer

14   cartilage. Old ovine cartilage was less permeable and stiffer than bovine cartilage, which may

15   be a result of further GAG maturity, or an effect of ovine cartilage being much thinner than

16   bovine. In this study, thinner cartilage tended to have a stiffer equilibrium elastic modulus.

17

18   The equilibrium elastic modulus of bovine lateral condyle cartilage was similar to the

19   equilibrium aggregate modulus quoted for human (0.70 MPa) and bovine (0.89 MPa)

20   previously [14, 31]. Porcine cartilage also had a similar elastic modulus, but was lower; this

21   was possibly as a result of having softer subchondral bone. Old ovine cartilage had a much

22   higher elastic modulus, so may be too stiff to replace human cartilage. Cartilage from all

23   species was less permeable than values reported for human cartilage [14, 31].

24

Conclusion

A limitation of this study was that tissues from animals of different ages were compared; this was due to the restricted availability of animals bred from the food chain for human consumption. Cartilage properties were found to vary greatly between species and joint region. For decellularisation, the optimal starting material should have properties as similar to human cartilage as possible. Porcine cartilage from 6 month old animals was the thickest of all the tissues studied and the GAG content was high, however the cartilage was highly cellular and, did not have a fully defined structure due to the immaturity of the tissue. Conversely bovine cartilage available from 18 month old animals was fully mature and less cellular, the GAG content was lower than porcine cartilage, but in the range reported for human. Bovine cartilage was also relatively thick. Both porcine and bovine cartilage had equilibrium elastic moduli similar to the equilibrium aggregate modulus reported for human cartilage. Ovine cartilage from 8-12 month and >4 year olds was very thin. Young ovine cartilage, like porcine was not fully mature, with a higher cell density and less well defined cellular distribution. Ovine cartilage again had a lower GAG content than porcine.

It has been found that porcine condylar cartilage and cartilage from the bovine groove and condyle would make the most optimal starting materials on which to develop a decellularisation protocol. Additionally, this study has highlighted the affect of species, age and joint region on the physical properties of articular cartilage, allowing a more informed selection of animal model for fundamental studies of cartilage biotribology.

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9

10 *Statement of Interests and declaration for healthcare compliance*

11 E Ingham and J Fisher are academic founders of Tissue Regenix and are shareholders and

12 advisers to Tissue Regenix Group PLC.

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Table 1. Material properties of cartilage from various joint regions of the pig, cow and sheep.

Equilibrium elastic modulus (MPa)	Porcine		Bovine		Ovine (8-12 month)		Ovine (> 4 years)	
	Mean	R^2	Mean	R^2	Mean	R^2	Mean	R^2
Acetabulum	1.30	0.99	-	-	1.69	0.98	4.32	0.91
Femoral head	[a]1.13	0.97	[a]1.59	0.91	1.22	0.94	[a]2.52	0.95
Groove	1.04	0.93	0.86	0.94	-	-	-	-
Medial condyle	[b]0.74	0.63	[b]1.17	0.80	1.31	0.99	[b]2.04	0.89
Lateral condyle	[b]0.66	0.67	[b]0.81	0.81	1.42	0.98	[b]2.02	0.86
Medial tibia	[b]0.88	0.86	[b]0.85	0.73	0.95	0.99	[b]2.02	0.87
Lateral tibia	[b]0.70	0.86	[b]0.81	0.85	1.19	1.00	[b]1.96	0.82

Permeability, k x10^{-16} (m4/Ns)	Porcine		Bovine		Ovine (8-12 month)		Ovine (> 4 years)	
	Mean	R^2	Mean	R^2	Mean	R^2	Mean	R^2
Acetabulum	3.04	0.99	-	-	1.12	0.98	0.37	0.91
Femoral head	5.70	0.97	1.78	0.91	2.99	0.94	0.76	0.95
Groove	4.49	0.93	6.33	0.94	-	-	-	-
Medial condyle	7.18	0.63	4.49	0.80	2.91	0.99	1.64	0.89
Lateral condyle	6.77	0.67	5.45	0.81	2.73	0.98	1.59	0.86
Medial tibia	8.09	0.86	4.52	0.73	3.67	0.99	1.30	0.87
Lateral tibia	10.29	0.86	5.75	0.85	2.87	1.00	1.37	0.82

Thickness (mm)	Porcine		Bovine		Ovine (8-12 month)		Ovine (> 4 years)	
	Mean	95% CL	Mean	95% CL	Mean	95% CL	Mean	95% CL
Acetabulum	1.12	0.15	-	-	0.85	0.12	1.06	0.45
Femoral head	1.22	0.05	1.32	0.13	0.48	0.07	0.52	0.10
Groove	1.70	0.53	1.36	0.10	-	-	-	-
Medial condyle	2.23	0.20	1.28	0.17	0.87	0.12	0.71	0.24
Lateral condyle	2.08	0.23	0.93	0.15	0.80	0.22	0.55	0.18
Medial tibia	0.87	0.15	1.70	0.36	0.76	0.23	0.61	0.24
Lateral tibia	0.82	0.08	1.02	0.21	0.70	0.28	0.59	0.27

Thickness data is expressed as the mean (ovine 8-12 months, acetabulum and groove n=5; all other n=6) ± 95 % confidence limits, Elastic equilibrium modulus and permeability data was modelled on mean deformation data and R^2 values for modelled and experimental deformation curve agreement are presented. [a]From Taylor et al. [21], [b] from McLure et al. [41], all other data from the present study.
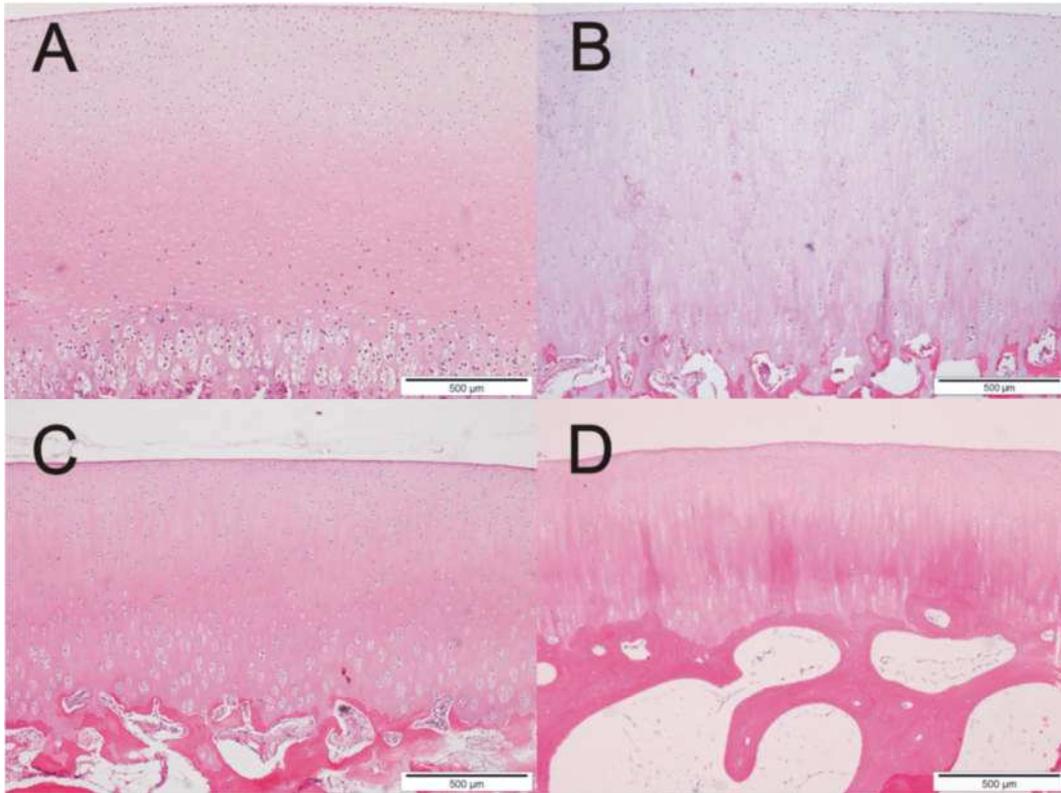
Fig. 1. **H&E staining of osteochondral pins from the medial condyle**. A – pig, B- cow, C

8-12 month old sheep and D – > 4 year old sheep. Size bars are 500 μm.
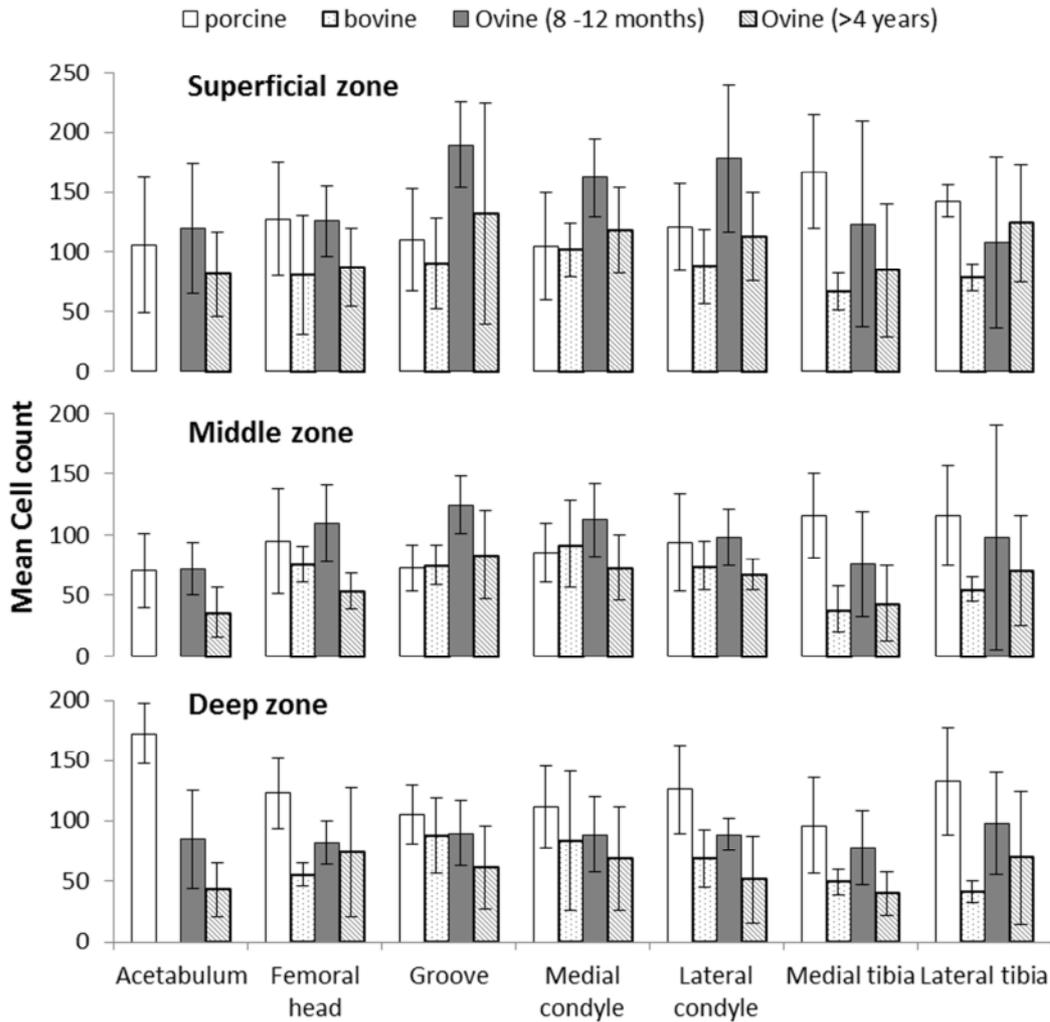
Fig. 2. **Cellularity of superficial, middle and deep zone cartilage from various joint regions of the pig, cow and sheep**. Data is expressed as the mean (n=5) ± 95 % confidence limits. Data was analysed by two-way analysis of variance. This showed that for superficial zone cartilage, there was no significant variation in cell number between joint regions (p= 0.276). The effect of species on the cell number was dependent on the joint region (species x joint region interaction p= 2.74 x 10^{-8}). For the middle zone cartilage, there was no significant variation in cell number between joint regions (p= 0.164) and the effect of species on the cell number was again dependent on the joint region (species x joint region interaction p=0.035). For the deep zone cartilage there was no significant variation in the cell number between joint regions but there was highly significant variation in the cell number between

species ($p = 3.39 \times 10^{-12}$) with no significant interaction between species and joint region ($p = 0.384$). Perusal of the data indicated that this was due to the higher cellularity of the porcine deep zone cartilage compared to the bovine and ovine cartilages.
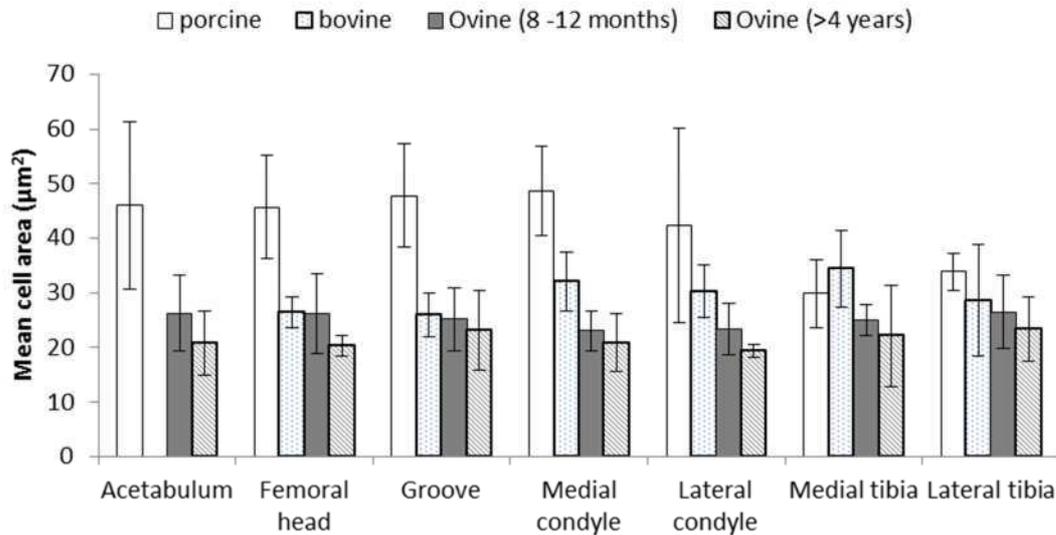
Fig. 3. **Size of chondrocytes in deep zone cartilage from various joint regions of the pig, cow and sheep**. Data is expressed as the mean (n=5) ± 95 % confidence limits. Data was analysed by two-way analysis of variance which revealed no significant variation in the size of the chondrocytes between joint regions ($p = 0.387$). The effect of species on the size of the chondrocytes in the deep zone ($p = 1.13 \times 10^{-26}$) was dependent on joint region (species x joint region $p = 9.47 \times 10^{-5}$). Perusal of the data indicated that, although the chondrocytes in the deep zone of acetabular and femoral joint regions of the porcine cartilage were larger than in the ovine and bovine cartilages, this was not the case for the tibial cartilages.
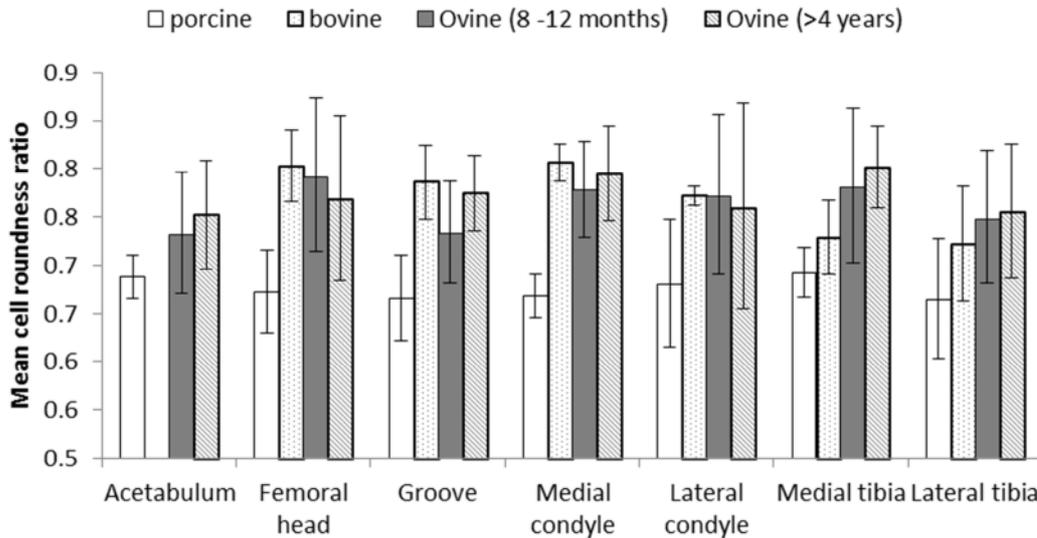
Fig. 4. **Roundness of chondrocytes in deep zone cartilage from various joint regions of the pig, cow and sheep**. Data is expressed as the mean (n=5) ± 95 % confidence limits. Data was analysed by two-way analysis of variance which revealed no significant variation in the roundness of the chondrocytes in the deep zone between joint regions (p=0.1) but a highly significant variation in the roundness of the chondrocytes in the deep zone between species (p=5.38 x 10^{-15}) with no significant interaction between species and joint region (p=0.344). Perusal of the data indicated that this effect was due to the chondrocytes in the deep region of porcine cartilages being less round than the chondrocytes in the deep regions of bovine and ovine cartilages.
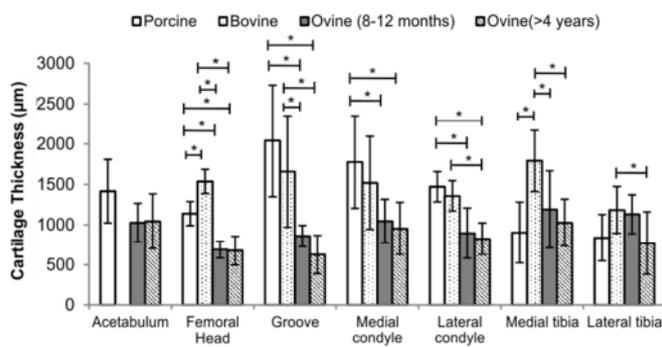
Fig. 5. **Thickness of cartilage from various joint regions of the pig cow and sheep**. Data is expressed as the mean (n=5) ± 95 % confidence limits. Data for a given tissue site was

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analysed by one-way analysis of variance and individual means compared by the T-method .

* indicates p<0.05.
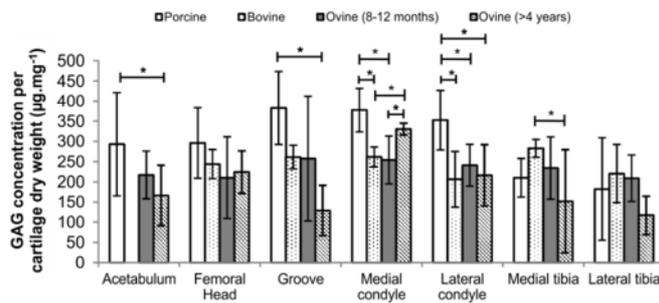
Fig. 6. **GAG content of cartilage from various joint regions of the pig cow and sheep**. Data is expressed as the mean (n=5) ± 95 % confidence limits. Data for a given tissue site was analysed by one-way analysis of variance and individual means compared by the T-method . * indicates p<0.05.
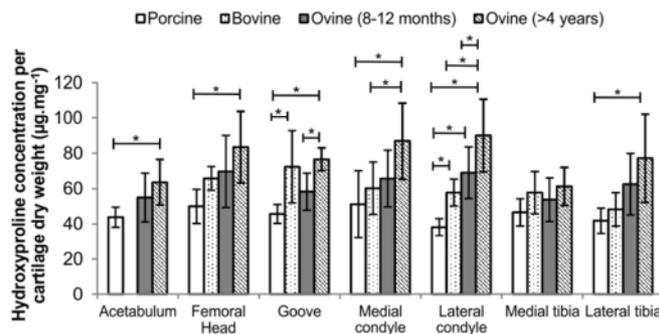
Fig. 7. Hydroxyproline content of cartilage from various joint regions of the pig cow and sheep. Data is expressed as the mean (n=5) ± 95 % confidence limits. Data for a given tissue site was analysed by one-way analysis of variance and individual means compared by the T-method . * indicates p<0.05.
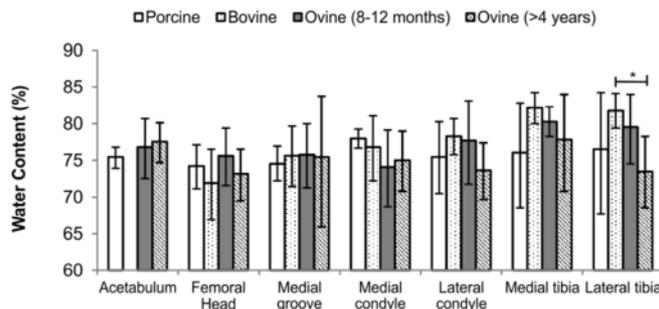
Fig. 8. **Water content of cartilage from various joint regions of the pig cow and sheep**.

Data is expressed as the back transformed mean (n=5) ± 95 % confidence limits. Data for a given tissue site was arcsine transformed then analysed by one-way analysis of variance and individual means compared by the T-method . * indicates $p < 0.05$.
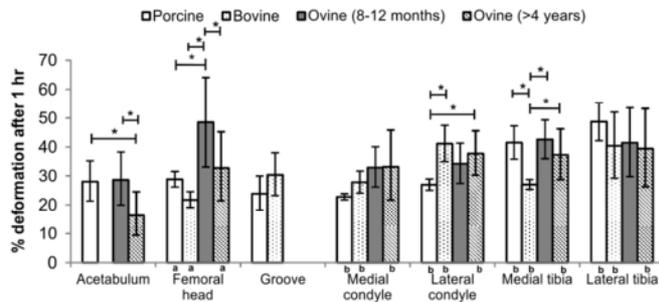
Fig. 9. **Percentage deformation of cartilage from various joint regions of the pig cow and sheep.** Data is expressed as the back transformed mean (ovine 8-12 months, acetabulum and groove n=5; all other n=6) ± 95 % confidence limits. Data for a given tissue site was arcsine transformed then analysed by one-way analysis of variance and individual means compared by the T-method . * indicates $p < 0.05$. [a]From Taylor *et al*. [21], [b] from McLure *et al*. [41], all other data from the present study.