Osteoarthritis and Cartilage 22 (2014) 1271–1281

Osteoarthritis and Cartilage

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Biomechanical and cellular segmental characterization of human meniscus: building the basis for Tissue Engineering therapies

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ARTICLE INFO

Article history:
Received 29 November 2013
Accepted 5 July 2014

Keywords:
DMA (dynamic mechanical analysis)
Flow cytometry
Histomorphometry
Meniscus
Micro-computed tomography
Tissue engineering

SUMMARY

Objective: To overcome current limitations of Tissue Engineering (TE) strategies, deeper comprehension on meniscus biology is required. This study aims to combine biomechanical segmental analysis of fresh human meniscus tissues and its correlation with architectural and cellular characterization.
Method: Morphologically intact menisci, from 44 live donors were studied after division into three radial segments. Dynamic mechanical analysis (DMA) was performed at physiological-like conditions. Micro-computed tomography (CT) analysis of freeze-dried samples assessed micro-structure. Flow cytometry, histology and histomorphometry were used for cellular study and quantification.
Results: Anterior segments present significantly higher damping properties.
Mid body fresh medial meniscus presents higher values of E' compared to lateral. Cyclic loads influence the viscoelastic behavior of menisci. By increasing the frequency leads to an increase in stiffness. Conversely, with increasing frequencies, the capacity to dissipate energy and damping properties initially decrease and then rise again.
Age and gender directly correlate with higher E' and tan δ. Micro-CT analysis revealed that mean porosity was 55.5 (21.2–89.8)% and 64.7 (47.7–81.8)% for freeze-dried lateral and medial meniscus, respectively. Predominant cells are positive for CD44, CD73, CD90 and CD105, and lack CD31, CD34 and CD45 (present in smaller populations). Histomorphometry revealed that cellularity decreases from vascular zone 1 to zone 3. Anterior segments of lateral and medial meniscus have inferior cellularity as compared to mid body and posterior ones.
Conclusion: Menisci are not uniform structures. Anterior segments have lower cellularity and higher damping. Cyclic loads influence viscoelastic characteristics. Future TE therapies should consider segmental architecture, cellularity and biomechanics of fresh tissue.

Introduction

In recent years, we have been assisting to a turn in the paradigm for treating meniscus-related problems, i.e., trends changed from removal to preservation, repair or replacement[1,2].

Promising results were obtained from meniscal allograft transplantation, but several limitations remain[1]. Acellular scaffolds are currently available for partial meniscus replacement[3,4]. Despite promising outcome, it has been reported early decrease of mechanical properties of the implants[5], resorption or diminished size over time[2,3]. Moreover, the final tissue obtained is different from native fibrocartilage[2,3]. Scaffold's porosity[6] and cellular pre-seeding can affect integration and maturation of meniscus' scaffolds[7]. Highly

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http://dx.doi.org/10.1016/j.joca.2014.07.001
1063-4584/

porous scaffolds have shown to integrate better and mature to a greater extent[8]. The best *in vitro* cell-seeding strategy remains to be established[9], but is known to influence integration and maturation[8].

While clinical studies still refer to acellular scaffold's replacement strategies, most pre-clinical authors favor the respect for the basic triad of Tissue Engineering (TE): combined use of scaffolds with cells and/or bioactive agents besides mechanical stimulus[2,10–12]. Furthermore, cell-laden scaffolds have shown improved mechanical properties when preservation of chondrogenic phenotype and proper Extra cellular matrix (ECM) production[13] were considered.

Maher *et al.*[14] found relevant discrepancy between *in vitro* and *in vivo* models aiming to develop a pre-clinical test platform for functional evaluation of meniscus scaffolds[14]. This problem reinforces the need to gain a deeper knowledge on meniscus biology prior to TE-based products are applied in the clinical setting. A major challenge is related to *in vitro* maintenance of the phenotype of human meniscus' fibrochondrocytes[15] or control the chondrogenic differentiation of these cells[16,17].

No biomechanical data on fresh human menisci is available once most research reports on animal studies[18,19] or human frozen specimens[20–22].

Therefore, further characterization of meniscus tissue is required for development of more effective TE approaches[23].

This study aims to determine segmental biomechanics of fresh human meniscus and further biologic characterization of this tissue.

Our hypothesis is that the meniscus tissue is heterogeneous thus biomechanics, cellularity and architecture presents segmental variation.

Method

Sources and selection of human biological samples

Thirty lateral and fourteen medial menisci from forty-four live human donors were harvested during Total Knee Arthroplasty (TKA) and were randomly distributed for all different parts of the study. The clinical indication for total joint arthroplasty was unicompartmental osteoarthritis (OA). Cases involving trauma or infection were excluded. Ages of TKA patients ranged between from 46 to 70 years. Only morphologically intact menisci [Fig. 1(A)], not torn and without macroscopic signs of degeneration were included. All patients where radiographically evaluated to exclude chondrocalcinosis and only Kellgren–Lawrence (KL) classification up to grade 2 in correspondent harvesting compartment were included. Furthermore, the histological record of the degenerative condition of the tissues was performed. Harvesting was performed with cold blade in aseptic conditions and samples kept in phosphate buffered saline (PBS) solution with 1 wt% of an antibiotic–antimycotic mixture (Invitrogen, Carlsbad, CA, USA) containing 10,000 U/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B as Fungizone® antimycotic in 0.85% saline. All menisci were kept at 4°C and processed within 24 h.

Donors were selected from candidates list for TKA proposed independently of surgeons involved in present study. Donors received informed written consent and study received approval from the Institutional Review Ethical Committee.

Dynamic mechanical analysis (DMA)

The viscoelastic measurements were performed using a TRITEC8000B DMA equipped with compressive mode (unconfined compression test). The measurements were carried out at physiological-like conditions (37°C and pH 7.4). Each fresh meniscus was sectioned into three segments [Fig. 1(B)]: anterior, mid body and posterior. Within the region of interest, samples were cut in cylindrical shapes with about 4 mm diameter and 4 mm thickness using a biopsy punch [Fig. 1(C)], and were stored in PBS solution. Samples were always analyzed immersed in a liquid bath placed in a Teflon® reservoir. Cylinders that were damaged during processing were excluded from the final analysis. A total of 108 cylinders (15 donors) were tested. The geometry of the tested samples was measured, and afterwards, samples were placed between two parallel plates and immersed in the PBS solution. A microtome was used, when needed to achieve parallel surfaces. After equilibration at 37°C, the DMA spectra were obtained during a frequency scan ranging from 0.1 to 10 Hz. During the frequency scans, three cycles were performed under constant strain amplitude (50 μm). A preload of 0.2 N was applied to each sample to ensure that the entire scaffold surface was in contact with the compression plates before testing. The storage modulus (E' – which relates to the stiffness of the material) and loss factor ($\tan\delta$) were determined for each segment corresponding to a given patient and the mean value for the patient's segment (two or three samples depending on the amount of tissue harvested) was considered for final statistical analysis. Loss factor represents the ratio of the amount of energy dissipated by viscous mechanisms relative to energy stored in the elastic component. Thus, $\tan\delta$ provides information about the damping properties of the material. The loss modulus (E''), was calculated from the values of E' and $\tan\delta$: $E'' = E' \times \tan\delta$. Mean age of all evaluated patients was 59 (53.1–64.9), mean height was 161 cm (141.4–180.6), mean weight 74 kg (53.0–95.5), mean body mass index (BMI) was 28.7 (21.8–35.6) and the mean number of viable cylinders obtained from each segment was three.

Micro-computed tomography analysis (Micro-CT)

The frozen and freeze-dried meniscus samples (10 lateral meniscus and 9 medial meniscus) were cut into pieces with 12 ± 2 mm (longest dimension) and scanned with the μ-CT SkyScan 1072 scanner (SkyScan, Kontich, Belgium). The resolution pixel size was between 15.5 μm and 18.8 μm and the same value was used for all pieces within a meniscus. The X-ray source was set at 38–50 keV and 201–248 μA. Projections ($n = 486$) were acquired over a rotation range of 180° with a rotation step of 0.45°. The binary images were then used for the morphometric analysis (CT Analyser, v1.12.0.0, SkyScan), and for the creation of the 3D models (CT Vol Realistic 3D-Visualization, v2.2.1.0, SkyScan). For the meniscus the attenuation coefficient values were used as 0.008577–0.045336. The slices were converted into binary images with a dynamic threshold of 46–255 (gray values).

Isolation of meniscus cells

The explant meniscal tissue was kept in PBS solution (pH 7.4) containing 1% (v/v) antibiotic–antimycotic mixture. Human meniscus cells (HMC's) were isolated from the explants using an enzymatic standard protocol. Briefly, meniscus tissue was separated from fat and vascularized tissue. Then, the samples were washed several times with PBS containing 1% (v/v) antibiotic–antimycotic mixture, and cut into small pieces. Tissue digestion was performed by adding a mixture of 10–20 mL a-MEM culture medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) of an antibiotic–antimycotic mixture and collagenase type II (1:1) from *Clostridium histolyticum* (Sigma–Aldrich, USA). An incubation period of 24 h took place at 37°C in a humidified atmosphere of 5% CO_2. The obtained cell suspension was passed through a cell strainer, and cultured in a T-75 culture flask with a-MEM medium supplemented with 10% (v/v) FBS and 1% (v/v) of an antibiotic–antimycotic mixture, until reaching confluence.

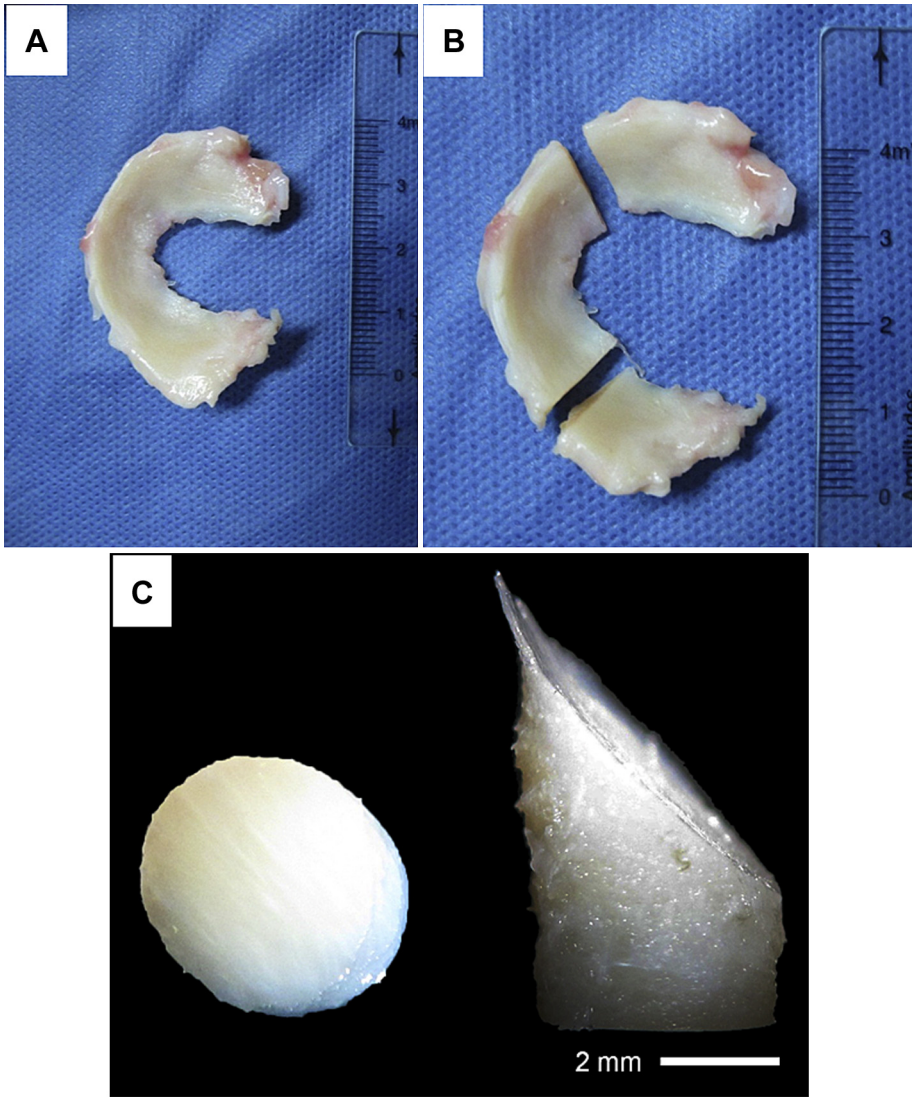

Fig. 1. Lateral meniscus obtained from live donor that undergone a TKA for unicompartmental OA (A), meniscus division in anterior, mid body and posterior segments (B), and harvesting of 4 mm cylinders (C).

Surgical debris ($n = 3$) obtained by biopsy from 16, 18 and 21 years old patients with acute traumatic irreparable lesions (one lateral and two medial menisci) without osteoarthritic changes provided cells to serve as phenotype control.

Fluorescence Activated Cell Sorting (FACS) analysis

Cell samples were isolated and analyzed by flow cytometry ($n = 5$). Cells from different samples, after trypsinization were labeled with fluochrome-conjugated antibodies (CD31-APC, CD34-PE, CD44-PE, CD45-FITC, CD73-PE, CD90-APC, CD105-FITC). Briefly, cells were incubated with the monoclonal antibodies for 20 min and protected from light at room temperature. After this incubation procedure, the samples were washed in PBS by centrifugation and fixed with 1% formaldehyde (v/v). Data were acquired on a FACSCalibur flow cytometer and analyzed using CellQuest software.

Histological and histomorphometric analyses

Anterior, mid body and posterior portions obtained from lateral and medial meniscus of 10 donors were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections with 4 μm were deparaffinized in xylene and rehydrated through decreasing percentage of ethanol and kept in PBS before staining. Sections were stained using hematoxylin and eosin (H&E) and observed in an Axio Imager.Z1m light microscope (Zeiss, Germany). Histomorphometric analysis was performed to determine cellularity among different segments (anterior, mid body, and posterior), zones (1 – vascular, 2 – central, and 3 – avascular) and type of meniscus (lateral and medial) according to International Society of Arthroscopy, Knee Surgery and Orthopedic Sports Medicine (ISAKOS) classification[24]. The WCIF ImageJ software program (USA) was used for facilitating the counting of the cells. A total of 200 photomicrographs (2800 μm × 2100 μm) were analysed per segment of meniscus. The method was independently repeated by three different investigators.

Statistical analysis

The statistical analysis used SPSS software (IBM SPSS Statistics ver.20.0; USA). For the mechanical analysis, the Mann–Whitney *U* test was used. When the categorical variable had more than two non-ordered groups the Kruskal–Wallis test was used instead. As

parametric test, to assess relationship between continuous variables the Pearson correlation was also tested. Independent samples *t* tests were used to study differences in the mean values of the cell counting for zonal, segmental and type of meniscus. Complementary to this test the Levene test was computed in order to conclude if the variances between groups were significant. Significance level considered was $P < 0.05$.

Results

DMA

No statistically significant differences were found between the two subgroups relative to the type of meniscus (medial or lateral) concerning gender, height, weight, BMI or number of cylinders per segment (see also Supplementary data, Figs. S1–S3).

Figure 2 presents the viscoelastic behavior of both menisci in the three different segments.

For lateral meniscus: the E' of anterior segment at 0.1 Hz is 0.46 MPa and increases to 0.51 MPa at 10 Hz. On mid body the E' increases from 0.46 MPa to 0.52 MPa (0.1–10 Hz). The anterior segment has higher damping (tan δ: 0.20 and 0.18 for 0.1 and 10 Hz, respectively) than mid body (0.17–0.16 for 0.1 and 10 Hz, respectively). Concerning posterior segment, E' increases from 0.73 MPa to 0.82 MPa, and the tan δ values are 0.15 and 0.14 at 0.1 and 10 Hz, respectively. In summary, anterior and mid body segments have very similar stiffness' response; posterior segments are stiffer (have the ability to store more energy) and dissipate more energy while the anterior segments have the higher damping properties.

For medial meniscus: E' values range for 0.1–10 Hz are: 0.28 MPa–0.33 MPa, 0.83 MPa–0.93 MPa and 0.59 MPa–0.68 MPa, for anterior, mid body and posterior segments, respectively. Anterior has the higher damping properties (0.23 at 0.1 Hz and 0.20 at 10 Hz) followed by posterior (0.18 at 0.1 Hz and 0.17 at 10 Hz) and mid body (0.17 at 0.1 Hz and 0.15 at 10 Hz) segments.

Table I summarizes the segmental biomechanical analyses for medial and lateral menisci at 1 Hz (frequency which has been described during normal ambulation[25]).

The E' of both menisci (medial and lateral), for all the segments studied, tends to increase when increasing the frequency. Anterior segments present significantly higher tan δ for both menisci (either in separate or combined analysis) comparing to mid body or posterior ones ($P < 0.001$).

Table I
Viscoelastic properties (E, E'' and tan δ) of fresh lateral and medial meniscus studied at 1 Hz (frequency which can be found during normal ambulation)

Meniscus	Segment	E' (MPa) (95% CI)	E'' (MPa) (95% CI)	tan δ (95% CI)
Lateral	Anterior	0.48 (0.01–0.94)	0.06 (0.02–0.09)	0.15 (0.07–0.23)
	Mid body	0.48 (0.25–0.71)	0.05 (0.04–0.07)	0.13 (0.09–0.17)
	Posterior	0.77 (0.59–0.94)	0.09 (0.07–0.11)	0.12 (0.10–0.14)
Medial	Anterior	0.30 (0.12–0.48)	0.05 (0.03–0.06)	0.18 (0.13–0.23)
	Mid body	0.87 (0.24–1.51)	0.10 (0.04–0.16)	0.13 (0.10–0.17)
	Posterior	0.63 (0.00–1.26)	0.09 (0.00–0.18)	0.15 (0.12–0.18)

The medial anterior segments are the less stiff and have the highest damping properties of all studied segments (Fig. 2 and Supplementary data Tables 1s and 2s). The medial mid body has higher E' comparing to lateral ($P < 0.001$).

The results suggest that the cyclic loads influence the viscoelastic behavior: by increasing the frequency leads to an increase of the menisci's stiffness which was evident for all menisci tested (independently of medial/lateral or different segments).

Regarding the E'', lateral and medial menisci present the same behavior: all the menisci possess higher capacity to dissipate energy at lower frequencies (0.1 Hz) and at higher frequencies (6.3 Hz–10 Hz).

Similarly, tan δ is also influenced by the cyclic loads in the same trend as E''.

Significant differences were found respecting gender with higher values of E' and tan δ for males (Mann Whitney's test P value = 0.05 and E' = 0.005 for tan δ).

Age also greatly influences either E' (Pearson cor. = 0.18; $P < 0.001$) and tan δ (Pearson cor. = −0.25; $P < 0.001$); increased stiffness is associated with increasing age. Increased BMI associates with a decrease in E' (Pearson cor. = −0.19; $P = 0.04$) but not on tan δ (Pearson cor. = −0.02; $P = 0.86$).

Micro-CT analysis

The mean porosity was 55.5 (39.3–71.6)% and 64.7 (56.6–72.7)% for lateral and medial meniscus, respectively. By its turn, the mean

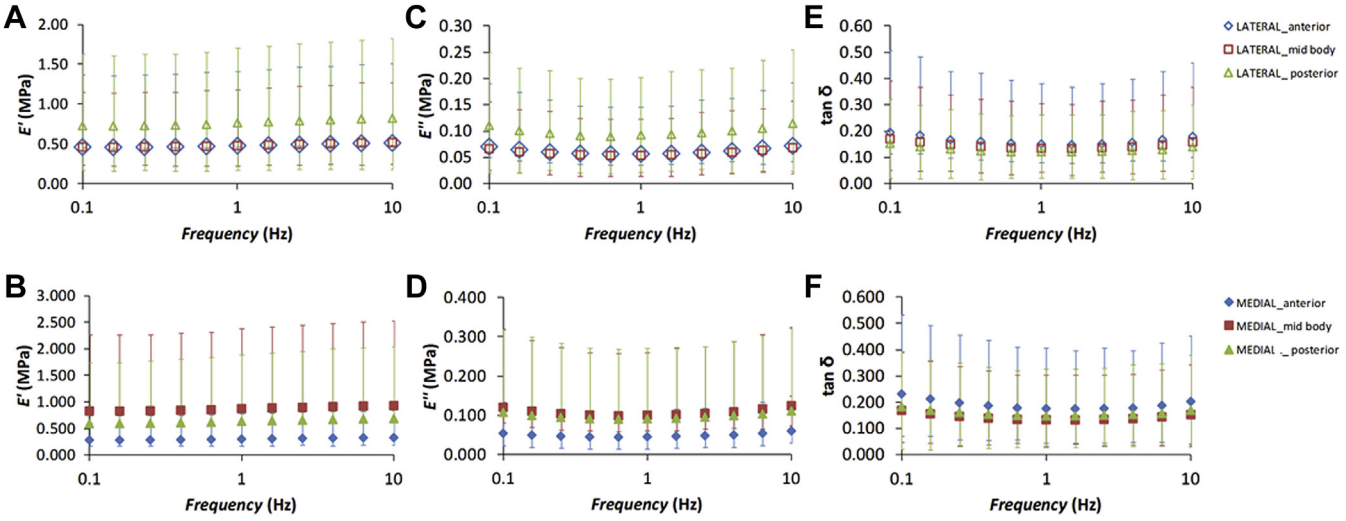

Fig. 2. DMA of fresh lateral and medial meniscus studied at several frequencies (mean with 95% confidence interval (CI), $n = 108$ samples from 15 donors): segmental evaluation of E' values for lateral (A) and medial menisci (B); segmental evaluation of E'' values for lateral (C), and medial (D) menisci; and segmental evaluation of tan δ for lateral (E) and medial (F) menisci. Numeric data are presented on Table 2s of Supplementary data.

Table II
Summary of studies considering meniscus biomechanics

Species	Meniscus' location	Compressive modulus (MPa)				Type of test	Reference
Human	**Dynamic:**					Dynamic	Bursac *et al.*[22]
(frozen menisci)	Medial						
	Anterior	1.7					
	Mid body	0.75					
	Posterior	0.4					
	Lateral						
	Anterior	0.8					
	Mid body	1					
	Posterior	1.2					
	Static:					Static	
	Medial						
	Anterior	0.068					
	Mid body	0.032					
	Posterior	0.02					
	Lateral						
	Anterior	0.039					
	Mid body	0.05					
	Posterior	0.06					
Human	**At equilibrium:**	**3% strain**	**6% strain**	**9% strain**	**12% strain**	Unconfined compression	Chia and Hull[21]
(frozen menisci)	Axial						
	Anterior	0.037	0.052	0.073	0.138		
	Mid body	0.023	0.030	0.046	0.080		
	Posterior	0.025	0.012	0.037	0.033		
	Radial						
	Anterior	0.042	0.069	0.041	0.103		
	Mid body	0.021	0.019	0.028	0.029		
	Posterior	0.034	0.056	0.056	0.097		
	At physiological loading rate:						
	Axial						
	Anterior	0.140	0.276	0.567	1.130		
	Mid body	0.064	0.128	0.302	0.669		
	Posterior	0.041	0.084	0.184	0.356		
	Radial						
	Anterior	0.101	0.201	0.446	0.966		
	Mid body	0.052	0.105	0.240	0.547		
	Posterior	0.078	0.112	0.178	0.301		
Bovine (frozen)	Tibia1 plateau surface	0.42				Compression	Fithian *et al.*[20]
Bovine (frozen)	Central portion of the medial meniscus	0.024				Unconfined compression	Lai and Levenston[32]
Bovine	Posterior	0.393				Confined compression tests	Proctor *et al.*[29]
	Anterior	0.440					
Baboon (frozen)	Superior					Creep and recovery indentation experiments	Sweigart *et al.*[19]
	Anterior	0.17					
	Mid body	0.18					
	Posterior	0.18					
	Inferior						
	Anterior	0.16					
	Mid body	0.18					
	Posterior	0.15					
Bovine (frozen)	Superior						
	Anterior	0.21					
	Mid body	0.14					
	Posterior	0.11					
	Inferior						
	Anterior	0.16					
	Mid body	0.11					
	Posterior	0.13					
Canine (frozen)	Superior						
	Anterior	0.28					
	Mid body	0.22					
	Posterior	0.26					
	Inferior						
	Anterior	0.26					
	Mid body	0.20					
	Posterior	0.19					
Human (frozen)	Superior						
	Anterior	0.15					
	Mid body	0.10					
	Posterior	0.11					
	Inferior						
	Anterior	0.16					
	Mid body	0.11					

(*continued on next page*)

Table II (*continued*)

Species	Meniscus' location	Compressive modulus (MPa)	Type of test	Reference
	Posterior	0.09		
Lapine (frozen)	Superior			
	Anterior	0.50		
	Mid body	0.13		
	Posterior	0.12		
	Inferior			
	Anterior	0.39		
	Mid body	0.17		
	Posterior	0.15		
Porcine (frozen)	Superior			
	Anterior	0.27		
	Mid body	0.17		
	Posterior	0.14		
	Inferior			
	Anterior	0.18		
	Mid body	0.13		
	Posterior	0.13		
Bovine	Inferior; posterior	0.12	Confined compression	Joshi *et al.*[28]
Human	Inferior; posterior	0.22		
Canine	Inferior; posterior	0.15		
Porcine	Inferior; posterior	0.27		
Human	Anterior	0.2	Confined compression	Hacker *et al.*[30]
	Mid body	0.22		
	Posterior	0.28		
Porcine (frozen)	Pericellular matrix:		Atomic force microscopy	Sanchez-Adams *et al.*[31]
	Outer	0.15		
	Middle	0.05		
	Inner	0.03		
	Extracellular matrix:			
	Outer	0.32		
	Middle	0.25		
	Inner	0.07		
Human (frozen menisci)	**Strain = 10%:**	For an analytical model	Confined compression	Seitz *et al.*[33]
	Medial			
	Anterior horn	0.07		
	Pars intermedia	0.06		
	Posterior horn	0.08		
	Lateral			
	Anterior horn	0.09		
	Pars intermedia	0.06		
	Posterior horn	0.08		
	Strain = 15%:			
	Medial			
	Anterior horn	0.06		
	Pars intermedia	0.04		
	Posterior horn	0.05		
	Lateral			
	Anterior horn	0.06		
	Pars intermedia	0.04		
	Posterior horn	0.07		
	Strain = 20%:			
	Medial			
	Anterior horn	0.08		
	Pars intermedia	0.05		
	Posterior horn	0.06		
	Lateral			
	Anterior horn	0.07		
	Pars intermedia	0.05		
	Posterior horn	0.09		

interconnectivity was 26.3 (12.9–39.7)% and 31.7 (17.9–45.5) for lateral and medial meniscus, respectively. The mean trabecule thickness values were defined as 143.4 (114.4–172.5) μm for lateral and 139.2 (110.6–167.9) μm for medial meniscus, while the mean pore size was 152.6 (113.2–192.1) μm for lateral and 189.0 (164.3–213.8) μm for medial meniscus. Figure 3(A)–(C) shows the 3D software-based reconstruction and 2D images of an entire lateral meniscus of left knee. By its turn, Fig. 3(D) shows porosity (%) variation across the menisci from the bottom part (tibial surface) to top part (femoral surface).

Isolation of meniscus cells and FACS analysis

The enzymatic method used for meniscus cells isolation proved to be reliable as it consistently permitted to obtain viable cells. Figure 4(A) shows a typical microscopy image of meniscus cells depicting different morphologies, i.e., rounded and fusiform-like shapes. Different samples including all viable cells isolated from a given meniscus were analyzed ($n = 5$) using flow cytometry [Fig. 4(B)–(E)]. Cell populations were positive for CD105, CD73 and CD90. This population is negative for hematopoietic markers such

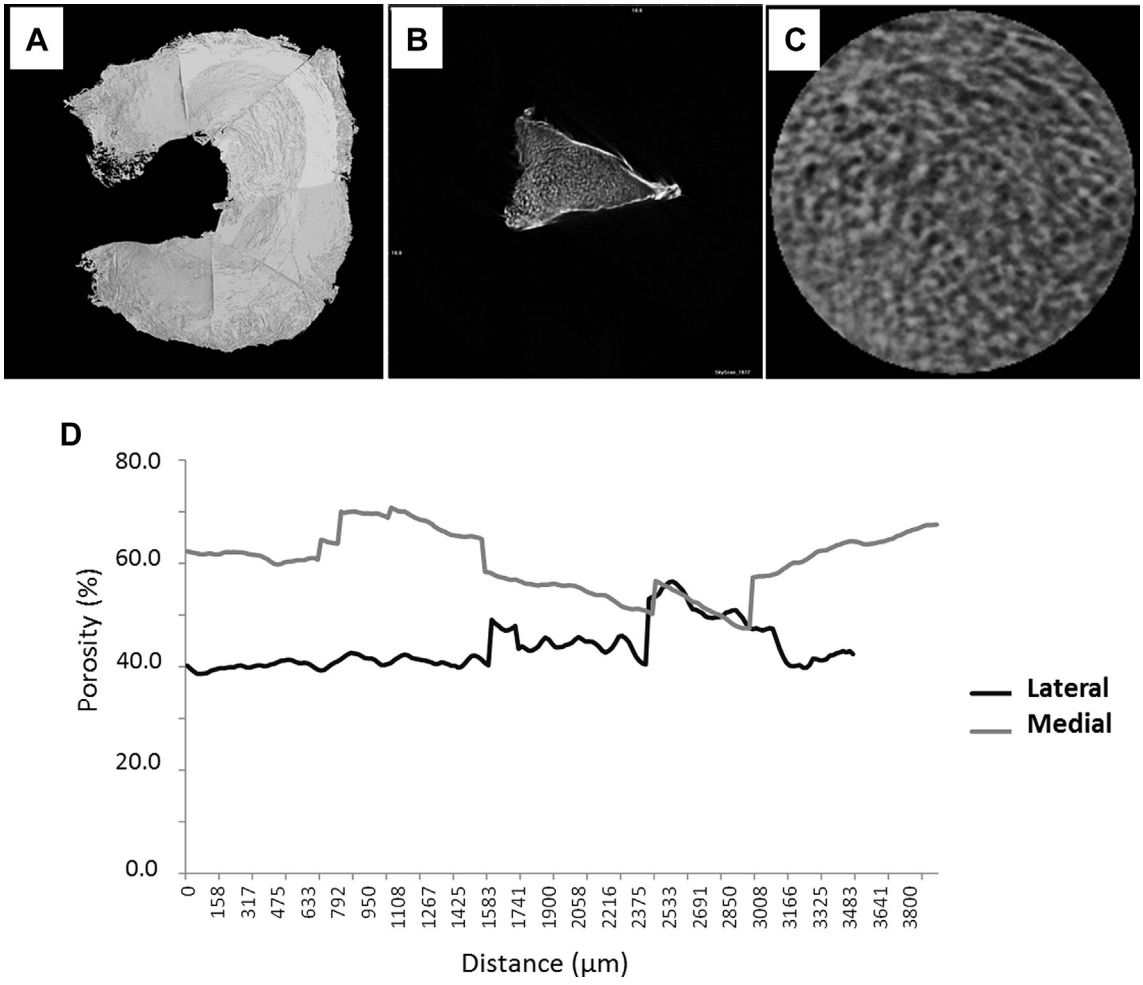

Fig. 3. 3D reconstruction from micro-CT images of freeze-dried lateral meniscus of left knee (A); 2D micro-CT images of a part of freeze-dried medial meniscus of left knee (B, C), change of porosity (%) across the menisci from the bottom part (tibial surface) to top part (femoral surface) (D).

as CD45 and CD34. The population has an expression of less than 3% for CD31 and for CD34. Similar results were registered for the samples of young patients with acute injuries ($n = 3$).

Histology and histomorphometric analysis

Meniscus tissues were divided according to ISAKOS classification[24]. The mean cell number quantification in the different segments of lateral and medial menisci are presented in Table III. It was consistently observed a gradual decrease of cellularity from zone 1 to zone 3, in all segments of both lateral and medial meniscus. Moreover, it was observed a relatively lower cellularity in anterior segments of both (lateral or medial) menisci as compared to mid body or posterior segments.

Discussion

DMA is an adequate tool to characterize the mechanical/viscoelastic properties of biomaterials[26,27].

Herein, it is presented the first biomechanical characterization of fresh human meniscus and the description of different meniscus' segments concerning E', E'' and tan δ (Fig. 2). Results herein presented have shown that anterior segments present significantly higher damping properties. Moreover, the mid body of medial meniscus is significantly stiffer than the lateral.

The results suggest that cyclic loads influence the viscoelastic behavior of the menisci. By increasing the frequency leads to an increase of the stiffness which was evident for all menisci tested independently of segmental variation. However, with ascending frequencies, the capacity to dissipate energy and damping properties (at low frequencies) initially decrease and then rise again at higher frequencies (6 Hz–10 Hz).

Previous studies have reported on the mechanical behavior of menisci[19–22,28–33] (Table II). The values of the compressive properties are quite different from the ones reported in the present study due to variations in the testing method, test location and species studied. Our study was conducted to determine the variation in compressive and viscoelastic properties of fresh human menisci at different topographical locations which could lead to different compressive properties.

In present study the values of E' ranged from 0.28 to 0.83 MPa and the values of E'' ranged 0.06–0.12 MPa. Bursac *et al.*[22] also reported similar values of E' ($\approx$0.4–1.7 MPa) and E'' ($\approx$0.03–0.2 MPa) by performing dynamic unconfined compression. By comparing only the lateral anatomical site, they shown that the E' at the posterior segment was higher than the anterior and mid body segments as in our study. However, the results obtained for the medial meniscus were different once they obtained stiffer menisci for the anterior segment oppositely to our findings. It is demonstrated that the properties of menisci are influenced when

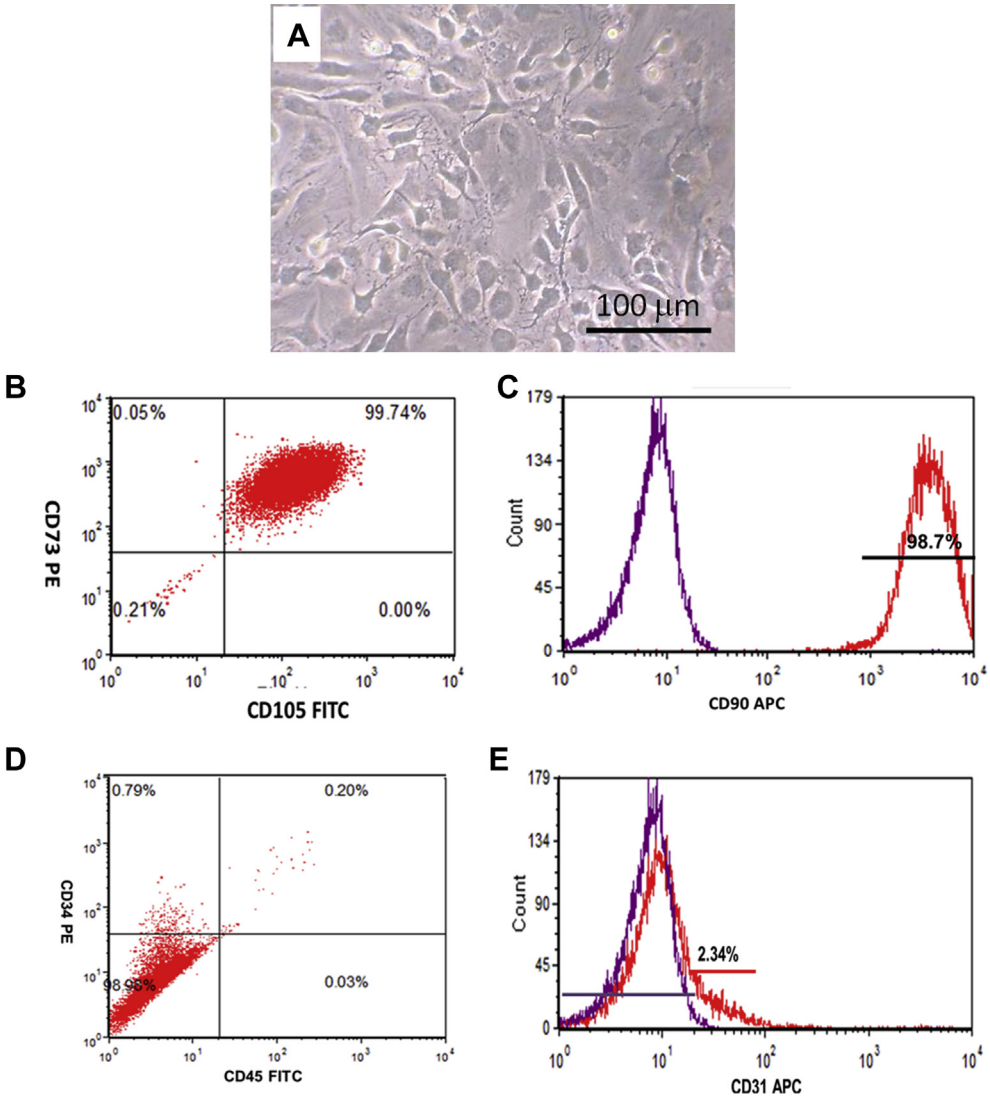

Fig. 4. Microscopy image of the HMC's in culture after isolation using an enzymatic digestion method. It is possible to observe meniscus cells depicting rounded and fusiform-like morphologies (A). The mean value ($n = 5$ patients) of percentage of positive cells for markers CD73, CD90, and CD105, is 97.2 (95.6–98.9)%, and for the marker CD44 the mean percentage value is 96.7 (95.1–98.2)%. The same densities were also analyzed for the expression of CD31, CD34 and CD45, and the obtained mean percentage values are 2.1 (1.8–2.4)%, 3.1 (2.9–3.3)% and 0.2 (0.1–0.2)%, respectively. Besides, two different passages (P2, P7) were analyzed ($n = 2$); and it was found that the analyzed markers and their percentages of expression were maintained.

they are subjected to different loading patterns, thus providing valuable clinically/physiologically-related information.

Several studies performed in different cartilage-tissues[34–39] also observed that viscoelastic properties were frequency-dependent. Taken all together it is clear that the dynamic compressive moduli are region-specific and dependent on the loading frequency not only for human menisci as herein reported but also in different cartilage-tissues. Such information has high relevance and should be considered when evaluating the *in vivo* behavior of this tissue. Moreover, these insights must be combined with the current knowledge of kinematics and contribution from different segments of either menisci in the function of the knee[40–43]. Loading conditions of the joint is fundamental in interpreting the functional properties of the tissues[44].

As limitations, we should consider the variability within the measured parameters related to differences among donors (gender, age, BMI, condition) amplified by the regional and anatomical variability which is expected when dealing with biologic samples. Some variability is also inherent to harvesting and manipulation process; however such source of bias is expected to be homogenously distributed among all the experiment. However, the aim of this study was to study global variations through segments of medial and lateral menisci and significant results could be found.

Lateral meniscus is known to be more mobile and having a higher role in load transmission within the less congruent lateral knee compartment comparing to medial meniscus[45]. The lower mobility of medial meniscus can also influence conversion of axial load into meniscal hoop stresses[45]. The aforementioned might be implicated in the herein presented findings. There are still many gaps in understanding the functional biomechanics of the menisci however this knowledge should be considered in subsequent research. The higher stiffness registered among males in present study requires comprehension of both anatomic and functional differences between genders and should be subject of further investigation.

The increased of dynamic stiffness with increasing age was somewhat expected to some extent and constitutes one of the observations of present study. In our study, it was also found that

Table III
Cell's distribution: mean number of cells per 5.88 × 10^6 μm^2 in the different segments and zones of lateral and medial menisci (including 95% CI; $n = 10$ donors)

Meniscus			Mean (95% CI)
Lateral	Anterior	Zone 1 (vascular)	217.9 (129.6–306.2)
		Zone 2 (central)	130.7 (66.8–194.6)
		Zone 3 (avascular)	62.7 (27.9–97.5)
	Mid body	Zone 1 (vascular)	286.0 (255.4–316.6)
		Zone 2 (central)	119.3 (92.5–146.1)
		Zone 3 (avascular)	74.9 (57.9–91.9)
	Posterior	Zone 1 (vascular)	430.4 (281.2–579.6)
		Zone 2 (central)	427.3 (200.0–654.6)
		Zone 3 (avascular)	241.4 (104.2–378.6)
Medial	Anterior	Zone 1 (vascular)	348.8 (295.3–402.3)
		Zone 2 (central)	106.1 (82.3–129.9)
		Zone 3 (avascular)	63.8 (51.4–76.2)
	Mid body	Zone 1 (vascular)	439.8 (346.6–533.0)
		Zone 2 (central)	181.1 (112.6–249.6)
		Zone 3 (avascular)	72.2 (53.9–90.5)
	Posterior	Zone 1 (vascular)	428.0 (375.5–480.5)
		Zone 2 (central)	64.0 (55.2–72.8)
		Zone 3 (avascular)	45.7 (36.9–54.5)

increased BMI is associated with a decrease in E' but not on tan δ. Such combination of lower E' but higher tan δ could eventually be explained by greater difficulty of the structure to recover when a higher load (correspondent to higher BMI) is systematically applied. Influence of gene expression might also be implicated[46]. No definite conclusion could derive from this observation but it seems a relevant trend requiring subsequent study.

Another limitation of present study is the use of samples from a population over 46 years old and osteoarthritic joints. However, scarce information is provided regarding the source of samples studied in previously reported studies[22] in which little is known about donor's gender, age or physical condition. Furthermore, considering human studies, only frozen samples have been used for biomechanical studies[19,22]. Freezing is known to significantly alter menisci biomechanical features[47] representing an important bias to be considered. The data herein described relates to adult population with registered joint degeneration and no direct extrapolation should be made to different subgroups (e.g., adolescents or young adults). Nevertheless, one can assume that it represents one step closer to understanding physiologic behavior and biologic characteristics of human menisci, and helps establishing minimum requirements for meniscus implants. By providing description of human donors' characteristics and sample status, it is envisioned to provide a solid starting point for further research.

No significant differences were registered between freeze-dried medial and lateral menisci concerning mean porosity, mean interconnectivity, mean pore size or mean trabecule thickness. The obtained values might be used as guides for scaffolds' design aiming meniscus regeneration. The different structural organization and content of any scaffold influences its biomechanical behavior and biocompatibility (including cell's viability)[6,8].

The cell-associated matrix (CAM) of one of the populations of meniscus cells is known to be composed of high amounts of type I and II collagen and low amounts of aggrecan[48]. On the other hand, a second population synthesizes a CAM containing high amounts of type I collagen, low amounts of type II collagen and high amounts of aggrecan. This population is known to be CD44+CD105+CD34−CD31−[48]. A third population, CD34+ (a stem cell marker), has also been described but not associated to significant CAM production[48]. The zone 1 of the meniscus contains more stem cells than zone 3 and these cells are known to play a role in meniscal regeneration[49]. It has also been described that no relevant differences exist in matrix production of mesenchymal stem cells (MSCs) and fibrochondrocytes of osteoarthritic patients as compared to young adults, when cultured in pellet form[50]. The results of present study reinforce the previously reported data. In this study, high expressions of CD73, CD90, and CD105 were registered combined with poor expression of CD31 and CD34. CD45 (marker for hematopoietic stem cells) was only present in an even smaller percentage of cells. This small number of CD45+ hematopoietic cells might play a role on chondrogenic differentiation of MSCs[51]. It has been realized that meniscus-resident MSCs are efficient colony formers, possess strong chondrogenic activity, and share the same set of typical cell-surface markers as bone-marrow-derived MSCs[52]. In addition, it has also been recognized that paracrine signaling by MSCs might play a decisive role in stimulating repair responses[49,52].

No differences in FACS analysis were found between cells isolated from our study group when compared with cells isolated from young patients without OA (controls). Baker *et al.*[53] studied meniscus cells from surgical debris and found significant variability concerning type of cells and amongst donors. Similarly to the present study, these variations did not correlate with patient age or disease condition[53]. It can be hypothesized that cells are the same regardless of age or OA despite admitting possible different activity levels in cell adhesion molecules (CAMs) production or some specific gene expression[46].

One can assume that cells from morphologically intact menisci (harvested from arthritic joints involving mainly the opposite compartment) might be a viable source for tissue-engineered constructs under adequate conditions. It has been demonstrated superior tissue integration and favorable biochemical properties of cell-based regenerated tissues as compared to acellular techniques[54].

Samples were always harvested from the less affected joint compartment from patients with unicompartmental knee arthritis as an attempt to minimize any possible influence of arthritic environment on the studied meniscus. To overcome selection bias, the KL score and indication for Total knee replacement (TKR) were defined prior to selection of donors by orthopedic surgeons not participating in the herein reported research. Donors that had been previously included were latterly randomly selected from institutional waiting list for TKR. It has been recognized that cell density and distribution might have decisive role in further differentiation and maturation of constructs[55]. It has also been reported that the more vascularized region of meniscus contains more stem cells[55]. One of our findings is that anterior segments of both lateral and medial meniscus have relatively lower cellularity (number of cells per area) as compared to mid body and posterior ones. So, despite the known circumferential variation from vascular part to the central and avascular parts there is also a radial variation in cells' distribution. This fact might correlate with different participation of menisci's segments in joint kinematics.

Conclusions

Cyclic loads influence the viscoelastic behavior of menisci. Mid body medial meniscus presents higher storage modulus (compared to lateral). Anterior segments are more prone to dissipate mechanical energy as compared to mid body and posterior ones. Anterior segments also have lower cellularity. Age, gender and BMI can influence biomechanical properties but not the basic type of cells. This study provides the first segmental biomechanical characterization of fresh human menisci, also combining the study of micro-architecture, cell's phenotype and distribution. The results herein presented confirm the initial hypothesis of our study, i.e., segmental patterns of variation can be found in meniscus tissue concerning biomechanical features, architecture and cellularity.

These data should be taken into account during development of cell-laden constructs for future clinical implantation aiming meniscus repair/substitution.

Contributions

Hélder Pereira, Sofia G. Caridade, Ana M. Frias, Joana Silva-Correia, Diana R. Pereira, Ibrahim Fatih Cengiz, and Joaquim Miguel Oliveira were involved in the conception and design of the study, acquisition of data, and analysis and interpretation of data. Hélder Pereira, Ibrahim Fatih Cengiz and Joaquim Miguel Oliveira were also responsible for drafting the article. João F. Mano, João Espregueira-Mendes and Rui Luís Reis were the revision of intellectual content and final approval of the manuscript.

Role of the funding source

The authors thank to the Portuguese Foundation for Science and Technology, FCT through the project OsteoCart (PTDC/CTM-BPC/115977/2009), as well as Doctoral and Post-Doctoral fellowships (SFRH/BD/81356/2011 to D. R. Pereira, SSFRH/BD/64601/2009 to S. G. Caridade, and IF/00423/2012 to J. M. Oliveira). The financial support of MultiScaleHuman project (Contract number: MRTN-CT-2011-289897) in the Marie Curie Actions-Initial Training Networks is also acknowledged.

Competing interests

The authors declare no conflicts of interest.

Acknowledgments

The authors thank the contribution of the clinical panel of Centro Hospitalar Póvoa de Varzim-Vila do Conde for the contribution on the human tissue collection.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2014.07.001.

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