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Title: USE OF HYDRODYNAMIC FORCES TO ENGINEER CARTILAGINOUS TISSUES RESEMBLING THE NON-UNIFORM STRUCTURE AND FUNCTION OF MENISCUS

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Abstract: The aim of this study was to demonstrate that differences in the local composition of bi-zonal fibrocartilaginous tissues result in different local biomechanical properties in compression and tension. Bovine articular chondrocytes were loaded into hyaluronan-based meshes (HYAFF®-11) and cultured for 4 weeks in mixed flask, a Rotary Cell Culture System (RCCS), or statically. Resulting tissues were assessed histologically, immunohistochemically, by scanning electron microscopy and mechanically in different regions. Local mechanical analyses in compression and tension were performed respectively by indentation-type scanning force microscopy and by tensile tests on punched out concentric rings. Tissues cultured in mixed flask or RCCS displayed an outer region positively stained for versican and type I collagen, and an inner region positively stained for glycosaminoglycans and types I and II collagen. The outer fibrocartilaginous capsule included bundles (up to 2um diameter) of collagen fibers and was stiffer in tension

(up to 3.6-fold higher elastic modulus), whereas the inner region was stiffer in compression (up to 3.8-fold higher elastic modulus). Instead, molecule distribution and mechanical properties were similar in the outer and inner regions of statically grown tissues. In conclusion, exposure of articular chondrocyte-based constructs to hydrodynamic flow generated tissues with locally different composition and mechanical properties, resembling some aspects of the complex structure and function of the outer and inner zones of native meniscus

Response letter

We greatly appreciate the time and effort the Reviewers and Editor have put into evaluating and improving our manuscript. We have copied the Reviewers' questions and comments below and followed them with our responses.

Editor's comments:

In addition to the changes requested by the referees, I would like you to consider whether figures 1 and 3 are really necessary. I like to keep papers concise and I cannot see that these figures add anything to the understanding of the text.

According to the Editor's suggestion, Figures 1 and 3 have been eliminated.

Reviewer #1:

General comments.

Methods. How was the degree of staining of the histological specimens quantified for the purpose of scoring (page 4), or was this done subjectively?

The degree of staining was evaluated subjectively by Donald Salter, Professor of Osteoarticular Pathology at the University of Edinburgh, UK. This has been clarified in the revised text (Page 4, 2nd paragraph)

The measurement and calculation of tensile modulus could be more clearly explained, in particular the dimensions that were recorded during each test (with reference to Fig. 1b, or diagram) and how the measurements at each strain were processed to obtain the data presented in Fig. 6. Also, why were the same tests not performed on native meniscus for direct comparison?

Data presented in Fig. 6 were obtained using standard calculations to derive the elastic modulus of materials based on stress-strain curves. In order to better characterize the dimensions recorded, the description has been revised as follows: *"Changes in dimensions during loading were obtained with a digital image analysis system (Adapted QuickCam® Pro 3000, Logitech Europe S.A., Switzerland and the GNU Image Manipulation Program, www.gimp.org), and were always lower than 10% of the gauge length considered." (Methods, page 6, 1st paragraph).*

The same tensile tests could not be performed on the inner or outer regions of native meniscus: in fact, out of those regions, having a semilunar shape, it would not be possible to punch ring-shaped specimens. This is now discussed in the following sentence: "*Since mechanical tests used in this work are not standard and require specimens of specific geometries, a comparison of the compressive and tensile properties of the engineered constructs with those of the native meniscus could not be established or derived from data in the literature*,, (Discussion, Page 10, 1st paragraph)

Discussion. The authors state that the mechanical properties of the HYAFF scaffold were negligible. Nevertheless, the scaffold must surely influence tissue formation: to what extent does the presence of the scaffold contribute (directly or indirectly) to the differences between the inner and outer regions reported here?

We now clarify that "*Since the mechanical properties of the non-woven mesh scaffold used are negligible, the mechanical functionality of the bi-zonal engineered constructs was mainly due to the differential deposition of extracellular matrix by chondrocytes*." (Discussion, page 9, last paragraph) and discuss that "*The HYAFF®-11 non-woven meshes used in this study were supportive of the formation of bi-zonal tissues. The composition and open structure of the meshes may have contributed respectively to the deposition of GAG in the inner region and to the outgrowth of cells towards the outer capsule.* " (Discussion, page 8, last paragraph)

To what extent might these differences also be accounted for by the increase in cell number and viability when cultured under dynamic as opposed to static conditions? Likewise, is the preferential orientation of the collagen fibers not due simply to the increased rate of growth of the fibrous capsule when cultured under conditions of improved mass transport rather than hydrodynamic shear per se (N.B. the RCCS is considered a low shear environment)?

We now describe that "*In the outer capsule, cells were elongated and at a higher density than in the central region*" (Results, Page 6, last paragraph) and discuss that "*More fundamental studies would be required to determine the role of increased mass transport and/or increased cell density following application of hydrodynamic flow in the development of the bi-zonal tissues*." (Page 9, 1st paragraph)

Specific Comments
Introduction
p2, line 3: delete 'turbulent' as true turbulence is unlikely to exist within the culture vessels (the Reynolds number is not high enough). (See also p9, line 2).
The term has been deleted.

Methods
p3, line 13: replace 'due to' with 'to account for'
The change has been made.

p3, line 14: Sentence 'For each experimental condition...' contradicts foregoing statements about the culture conditions. Was the volume in each case equivalent to 10 ml of complete medium per construct?
Yes, the volume of medium was in each case equivalent to 10 ml per construct

p4, line 9: The constructs were examined blind.
The change has been made.

Results
p6, line 19: corresponding to the space 'occupied by' the original scaffold. Delete 'of'
The change has been made.

p7, line 14: Replace 'anisotropic' with 'aligned'. (The fibers themselves are not anisotropic, rather it is their orientation that gives rise to this property.)
The sentence now reads "*SEM analysis indicated that collagen fibers were randomly oriented in the inner region of all constructs…"* (Results, page 7, 3rd paragraph)

Figure Captions
Should not part of the caption to this figure read: 'Fields in (B,C), (E,F) and (H, I) correspond to a specific area respectively within (A), (D), and (G)'?
We thank the Reviewer for finding this inconsistency. The legend has been revised accordingly.

Reviewer #2:

…How do the cells appear in the two different zones in size and shape? Is the cellularity different? Author should include this aspect in the result section for better characterizing their findings.
We now describe that *"In the outer capsule, cells were elongated and at a higher density than in the central region."* (Results, page 6, last paragraph)

Specific comments:
The title is too long. I would be more schematic, as for example" USE OF HYDRODYNAMIC FORCES TO ENGINEER NON-UNIFORM FIBROCARTILAGINOUS TISSUES"…or "USE OF HYDRODYNAMIC FORCES TO ENGINEER NON-UNIFORM MENISCAL TISSUES"…
We agree with the Reviewer that the title is a bit long, but we would prefer to keep it in the original version, in order to more precisely reflect the message of the study.

Introduction, page 1, third paragraph: reference # 16. Authors should not self-cite with manuscripts which were only submitted, without being accepted.
Reference 16 is now In Press. The reference list has been updated

Materials and methods, page 2 first paragraph: 0.15% type II collagenase does not clearly indicate the amount of enzyme used. An indication of the Units would be more accurate. In fact, 22 hours of digestion seems to be a long time, with respect to the overnight digestion of the common protocols. An indication of the vitality of the cells after the enzymatic isolation should also be indicated.
We now detail that the activity of the enzymes used was varying between 180 to 250U/mg and that cell viability after digestion was greater than 80% (Methods, page 2, last paragraph)

Results, page 6, second paragraph, second line: typo in "characterization".
The word has been corrected

Results, page 7, lines 21-22: "The highest elastic modulus in compression was measured in the inner region of the tissues developed within RCCS (0.138±0.07MPa)". Comparing this sentence with figure 6A, it seems that the highest elastic modulus in compression was measured in the inner region of the tissues developed within Mixed Flask. Please, verify!

According to the correct observation, the sentence has been revised as follows: *"The highest elastic moduli in compression were measured in the inner region of the tissues developed within mixed flasks and RCCS (respectively 0.153±0.07 and 0.138±0.07MPa)."* (Results, page 7, 4th paragraph)

Discussion, page 8, second paragraph: reference # 16. Again, Authors should not self-cite with manuscripts which were only submitted.

Reference 16 is now In Press. The reference list has been updated

Figure captions. Figure 2, last line: "respectively within (A), (B), and (C)." Authors probably meant "respectively within (A), (D), and (G)"

We thank the Reviewer for finding this inconsistency. The legend has been revised accordingly.

Figure captions. Figure 3 is not clear: it seems that static culturing is the only one that allows the formation of the two different structures, while this is not presented throughout the entire manuscript. Please, correct or remove the figure.

The figure has been removed

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Mandatory Author Declaration

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from biomaterials@online.be.

Signed by all authors as follows:

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*** Title Page**

USE OF HYDRODYNAMIC FORCES TO ENGINEER CARTILAGINOUS TISSUES RESEMBLING THE NON-UNIFORM STRUCTURE AND FUNCTION OF MENISCUS

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Running Title: Bi-zonal cartilaginous tissues

* Abstract

SUMMARY

The aim of this study was to demonstrate that differences in the local composition of bi-zonal fibrocartilaginous tissues result in different local biomechanical properties in compression and tension. Bovine articular chondrocytes were loaded into hyaluronan-based meshes (HYAFF®-11) and cultured for 4 weeks in mixed flask, a Rotary Cell Culture System (RCCS), or statically. Resulting tissues were assessed histologically, immunohistochemically, by scanning electron microscopy and mechanically in different regions. Local mechanical analyses in compression and tension were performed respectively by indentation-type scanning force microscopy and by tensile tests on punched out concentric rings. Tissues cultured in mixed flask or RCCS displayed an outer region positively stained for versican and type I collagen, and an inner region positively stained for glycosaminoglycans and types I and II collagen. The outer fibrocartilaginous capsule included bundles (up to 2μm diameter) of collagen fibers and was stiffer in tension (up to 3.6-fold higher elastic modulus), whereas the inner region was stiffer in compression (up to 3.8-fold higher elastic modulus). Instead, molecule distribution and mechanical properties were similar in the outer and inner regions of statically grown tissues. In conclusion, exposure of articular chondrocyte-based constructs to hydrodynamic flow generated tissues with locally different composition and mechanical properties, resembling some aspects of the complex structure and function of the outer and inner zones of native meniscus.

Keywords: bioreactor, shear, cartilage tissue engineering, chondrocyte.

INTRODUCTION

Meniscus is a complex fibrocartilaginous tissue, which is often characterized by distinguishing two main regions: a peripheral vascularized zone, about one third of the radial thickness and mainly exposed to circumferential tensile loads, and an inner avascular zone, covering the remaining two thirds and predominantly exposed to compressive forces. The physiological loads applied to the different regions determine the structure and function of the meniscus tissue [1]. The peripheral region of the meniscus is mainly composed by collagen fibers (predominantly type I) arranged circumferentially in large bundles (50-150μm in diameter), proper to resist to large tensile loads. Radial oriented fibers are interwoven with the circular fibril bundles, presumably acting as a "tie" holding the circumferential fibers together, and providing resistance to longitudinal splitting of the meniscus [2]. The inner region is characterized by a unique network of types I and II collagen, and contains a considerable amount of glycosaminoglycans (GAG) [3, 4], directly contributing to resistance in compression [5]. Several types of meniscal lesions frequently occur following traumatic injuries, and removal of the damaged tissue is known to result in reduced protection of the articular cartilage and early degenerative changes [6-8]. Currently, no viable clinical treatment is available that consistently results in preservation of meniscal size, shape and function, particularly for tears occurring in the avascular zone.

Several studies have indicated that tissue engineering has the potential to yield meniscus substitutes which could, immediately after implantation, protect articular cartilage by distributing and absorbing physiological loads [9-12]. The generation of meniscus grafts with a sufficient level of functionality could be facilitated by the application of defined regimes of physical conditioning in bioreactors, similarly to what has been introduced in cartilage tissue engineering [13, 14]. However, the use of bioreactors in meniscal tissue engineering has been so far rather limited [9] and physical parameters for the generation of functional meniscus tissue have yet to be identified [15].

We previously reported that human articular chondrocytes loaded into hyaluronan meshes (HYAFF®-11) generated fibrocartilaginous tissues containing collagen types specific of both the inner and outer zones of the native meniscus, indicating a possible compatibility of their phenotype with meniscus cells [16]. We also demonstrated that articular chondrocytes, seeded into hyaluronan meshes and cultured within a Rotary Cell Culture System (RCCS), developed bi-zonal tissues, with a clearly distinct pattern of spatial

distribution of GAG and collagen. In particular, tissues displayed an outer capsule containing a higher density of elongated cells, stained intensely for collagen and negatively for GAG, and surrounding a central region containing larger amounts of GAG [17]. This effect was likely due to the eddies and flow-induced shear stresses, since a similar pattern was already found in constructs generated within mixed flasks [18].

In this study, we aimed at assessing whether the bi-zonal pattern of the cartilaginous tissues generated by articular chondrocytes in mixed flasks or RCCS resembles the structure and function of meniscus tissue. In particular, based on the known contribution of GAG and collagen respectively to compressive and tensile stiffness, we hypothesized that the collagen-rich outer region is more resistant to tension, whereas the GAG-rich inner region is more resistant to compression. To investigate the local compressive properties of the engineered tissues, we employed indentation-type scanning force microscopy (IT SFM) with a spherical micrometer sized indenter tip [19]. To assess the local tensile properties, we performed customized tension tests on concentric rings punched out from the inner and the outer regions of the same engineered constructs.

MATERIALS AND METHODS

Cell Isolation and Expansion

Articular cartilage was harvested from the femoropatellar grooves of 6-month-old cows, finely minced and digested by incubation for 22 hours at 37°C in 0.15% type II collagenase (180 to 250 U/mg, Worthington Biochemical Corporation, Lakewood, NJ) (1ml solution per 100mg tissue). Isolated cells (with viability greater than 80%, as assessed by trypan blue stain) were resuspended in *complete medium* composed of Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/L glucose with nonessential amino acids), containing 10% fetal bovine serum, 0.1mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.29 mg/mL L-glutamine. Cells were expanded for one passage in *complete medium* further supplemented with 5 ng/mL of Fibroblast Growth Factor-2 (FGF-2) (R&D Systems, Minneapolis, MN), as previously described [20]. Chondrocytes were detached using 0.05% trypsin / 0.53mM EDTA (GIBCO-BRL, CH) and seeded into hyaluronan-based meshes as described below.

Cell seeding and culture into hyaluronan-based meshes

Expanded articular chondrocytes were seeded into non-woven meshes (5.5mm diameter, 1.2mm thick disks), made of esterified hyaluronan (HYAFF®-11, Fidia Advanced Biopolymers, Abano Terme, Italy) at the density $4x10^6$ cells/scaffold ($3.9x10^7$ cells/cm^3). Briefly, $4x10^6$ cells were resuspended in 28μL of *complete medium* and slowly dispersed over the top surface of the dry meshes with a micropipette. After 2 days, the seeded scaffolds were maintained in 6 well-plates (static culture), or transferred either to a mixed flask, or to a Rotary Cell Culture System (RCCS) as described below.

For mixed flask culture, constructs were threaded onto 31-gauge stainless steel wires that were affixed into a silicone stopper at the top of a glass flask (Bellco Glass Inc., NJ; Model #1967). The volume of culture medium was 80 mL, and a 4 cm magnetic bar was stirred at 50 rpm. For RCCS culture, constructs were suspended within a 3.8 cm diameter, 3.5 cm length polycarbonate vessel [17], rotating at an angular velocity of 16 to 50 rpm, with a total of 70 ml culture medium continuously recirculated through the system at a flow rate of 0.6 ml/min. The angular velocity of the vessel was set in order to maintain constructs in a continual freefall condition [21] and was increased during culture time to account for construct growth. For each experimental condition (i.e., static, mixed flask or RCCS culture), cell-scaffold constructs were cultured for 4 weeks in 10 ml of *complete medium* further supplemented with 10ng/ml TGF-β3, 0.1 mM ascorbic acid 2-phosphate and 10 μg/ml human insulin, in order to enhance chondrogenesis [22, 23], with medium changes twice a week.

A total of 4 experiments were performed using 4 different primary cultures. For each of the 4 experiments, at least 3 specimens per experimental group were assessed histologically as described below. For 2 representative experiments, at least 3 specimens per experimental group were assessed immunohistochemically, mechanically and by scanning electron microscopy as described below.

Histological assays

Tissues were rinsed in PBS, fixed in 4% buffered formalin for 24 h at 4°C, embedded in paraffin, and sectioned to a thickness of 7 μm. Sections were stained with Safranin-O for sulfated GAG or with elastica-van Gieson for collagen.

Immunohistochemistry

Cryostat sections (3 to 5 μm thick) were cut from tissues stored frozen at -70°C. Sections, on Superfrost slides (BDH, Poole, UK), were fixed for 5 minutes in ice cold acetone and then stored at -20°C until required. Immunostaining was performed by standard ABC immunoperoxidase method with a panel of antibodies against collagen type I (COL-1 Mouse, Sigma, Gillingham, UK), type II (CIICI, Developmental Studies Hybridoma Bank, Iowa City, USA), type III (Cat No ab6580, Abcam plc, UK), type VI (Cat No ab6588, Abcam plc, UK), and versican (12c5, DSHB, Iowa, USA). Sections immunostained with anti-type I and type II collagen antibodies were pre-incubated with 1mg/ml hyaluronidase (Sigma type 4-2 hyaluronidase from bovine testes) for 30 minutes at 37°C. Negative control sections with appropriate IgG or non-immune animal serum were run in parallel with the experimental sections to confirm the specificity of any positive staining.

Constructs were examined blind by light microscopy and expression of the investigated molecules was subjectively scored as follows: no matrix positive (-); focal (<50% of matrix positive) staining (-/+); extensive (>50% of matrix positive) but weak staining (+/-); extensive (>50% of matrix positive) and strong staining (+).

Scanning Electron Microscopy (SEM)

Beams of 1-2 mm side were cut out from the different regions of the engineered constructs. Proteoglycans were extracted in 100mM Sorensen's phosphate buffer (pH 7±2) containing 1 mg/ml bovine hyaluronidase (type I-S, Sigma, St Louis, MO, USA) and 1 mg/ml trypsin (type I, Sigma, St Louis, MO, USA) for 3 days at 37°C, with refreshment of the solution every 12 h [24]. Specimens were then fixed with 2.5% glutaraldehyde (in PBS, 2.6 mM NaH2PO4, 3 mM Na2HPO4, 155 mM NaCl, 0.01% NaN3 w/v, pH 7.2) for 2 h. After critical point drying, samples were sputtered with platinum and examined by SEM (Philips XL30 *FEG; environmental scanning EM), operated at 10 kV. Specimens from the outer construct regions were imaged at the external surface.

Indentation-type scanning force microscopy (IT SFM)

Micrometer-scale indentation testing was performed by SFM on the surface of cross-sections (about 2 mm thick) of engineered constructs, cut with a vibratom sectioning system (Oxford Instruments, UK), as previously described [19]. Briefly, a micrometer sized borosilicate glass sphere (SPI Supplies, West Chester, PA) was glued with epoxy resin onto the free end of a rectangular tipless silicon cantilevers

(nominal spring constant of k=0.35N/m, MikroMasch, Estonia) using a three-dimensional microtranslation stage as previously described [25]. The radius of the microsphere employed, measured using a silicon calibration grating (TGT01, MikroMasch, Estonia) according to the Neto and Craig method [26], was 1.6μm. Cantilever spring constant was selected to be compatible with an expected elastic modulus of the sample in the range 0.1-1 MPa [27] and was measured by pressing against a reference calibrated cantilever [28, 29], resulting in a spring constant of 0.24 N/m. The SFM spherical tip was used as a microindenter and cyclic load-displacement curves [30] were performed in the force-mapping mode [31] at a 1 Hz vertical displacement frequency. Each force map corresponds to 16x16 indentation curves taken on a 10x10 μm area. The SFM used (BioScan, Molecular Imaging, Inc., Tempe, AZ) was equipped with a 100μm XY scanner with low-coherence laser diode and the standard fluid cell. Load-displacement curves were recorded at a fixed maximum applied load of ~36 nN, which corresponded to an indentation depth of ~150 nm. Such penetration was always <1% of the sample thickness, hence below Bückle's indentation depth limit. Possible boundary interference was avoided by performing indentations at least 100 μm from the outer specimen edge. Dynamic elastic modulus values were derived from load-displacement data based on the model of Oliver and Pharr [32], applied to the mechanical testing of soft, hydrated biological tissues as described by Stolz et al. [19], using the determined indenter radius and cantilever spring constant.

Tensile mechanical test

Two concentric rings were punched out of the engineered constructs using 5.5mm- and 2mm-diameter biopsy punches, in order to compare the local mechanical properties of the inner and outer regions. The outer ring extended from the outer edge of the construct to the 5.5mm-diameter cut. The inner ring was confined between the 5.5mm- and 2mm-diameter cuts. Rings were fixed with hooks to a MTS Synergie 100 Material Testing System (MTS Systems Corporation, Eden Prairie, MN 55344, USA) and stretched by application of a baseline load (0.05 N). Engineering tensile modulus (based on initial rather than instantaneous dimensions) was determined for a specific volume of each specimen, defined by its initial thickness, width and a gauge length established prior to applying any load using graphite marks (placed 4mm apart for the outer rings and 2mm apart for the inner rings). Modulus in tension was measured by cyclic loading at a strain rate of 24 mm/min and reaching increasing nominal strains (300, 600 and

1200μm), within the elastic region of all constructs [33]. Measurements were repeated three times at each strain. Changes in dimensions during loading were obtained with a digital image analysis system (Adapted QuickCam® Pro 3000, Logitech Europe S.A., Switzerland and the GNU Image Manipulation Program, www.gimp.org), and were always lower than 10% of the gauge length considered. The force applied to the specimens was measured by a 2N built-in load cell. Stress-strain curves obtained were typical of collagenous tissues, with a linear relationship resulting from progressive alignment of the collagen fibers. Elastic modulus was calculated as the slope of the linear region of the stress/strain curves. Comparison between moduli of the inner and outer rings of the different constructs was established after taking into account differences in size and assuming all tissues have the same Poisson's ratio (approximated to 0.5 as for articular cartilage [34]).

Statistical analysis

Data are presented as means ± standard deviations. The normality of the populations to be analyzed was tested by measuring the symmetry (skewness) and the peakedness (Kurtosis) of the population. Means were compared by Student's *t*-tests in case of normal populations or by Mann-Whitney tests in case of failure of normality. Statistical analyses were performed using the Sigma Stat software (SPSS Inc., Version 7.5), with $p<0.05$ as the criteria for statistical significance.

RESULTS

In constructs grown for 4 weeks in static conditions, a region overlapping with the space occupied by the scaffold and radially extending from that for about 0.5mm (Fig. 1A,B) was intensely stained for GAG, whereas the construct core was only faintly stained. Staining intensity for total collagen was rather weak throughout the construct cross section, and almost negative at the construct core (Fig. 1C). In contrast, tissues cultured in the mixed flask or the RCCS displayed a central region, corresponding to the space occupied by the original scaffold, with uniform and intense staining for GAG, and a peripheral capsule (about 0.5 to 0.7 mm thick) negatively or faintly stained for GAG and predominantly stained for total collagen (Fig. 1D-I). In the outer capsule, cells were elongated and at a higher density than in the central region.

Based on these observations, two zones were defined for further structural and functional construct characterization: the *inner region* included the space of the original scaffold (disk shaped zone of 5.5mm diameter and 1.2mm thickness), whereas the *outer region* covered the extracellular matrix growing outside the original scaffold space.

Immunohistochemical characterization of the engineered constructs is summarized in Table 1 and representative fields are presented in Figure 2. In the *inner region* of tissues cultured statically, staining for type III collagen and for versican was negative, whereas focal positive stain was observed for types I, II and VI collagen. The *outer region* was similarly stained, with the exception of the most external tissue layer (about 50 μm thick), which was more positively stained for types I, III and VI collagen, as well as for versican. The distribution of the investigated molecules was similar in tissues generated in mixed flask and RCCS, as follows. In the *inner region*, matrix was stained strongly for type II collagen, weakly for type I and VI collagen, and only scattered areas were stained for versican, whereas type III collagen was not detectable. In the *outer region*, a markedly different pattern was observed: matrix was negative for type II collagen, strongly positive for types I and VI collagen and for versican, and scattered areas were stained for type III collagen.

SEM analysis indicated that collagen fibers were randomly oriented in the inner region of all constructs, as well as in the outer region of statically grown tissues (Fig. 3). Instead, in scattered areas of the outer region of tissues cultured in mixed flask or RCCS, collagen fibers appeared oriented parallel to the construct periphery and were arranged in bundles of 0.3-2μm diameter.

Dynamic elastic moduli in compression in the *inner region* of tissues generated within mixed flask or RCCS were significantly higher (respectively 3.2- and 3.8-fold) than in the *outer region* (Fig. 4A). In contrast, stiffness in compression was not statistically different between the regions of statically grown constructs. The highest elastic moduli in compression were measured in the *inner region* of the tissues developed within mixed flasks and RCCS (respectively 0.15±0.07 and 0.14±0.07MPa).

The measured size of the inner and outer rings punched out of the different constructs are displayed in Table 2. Measurements are reported following trimming of the external capsule from the inner rings, in order to eliminate the contribution of the outer region to the tensile properties of the inner rings. Since the punched outer and inner rings respectively covered the previously defined *outer region* and *inner*

region, the tensile stiffness of the outer and inner rings was considered as representative of the *outer region* and *inner region*, respectively. Elastic moduli in tension in the *outer region* of tissues generated within mixed flask or RCCS were significantly higher (respectively 2.2- and 3.6-fold) than in the *inner region* (Fig. 4B). In contrast, stiffness in tension was not statistically different between the regions of statically grown constructs. The highest elastic modulus in tension was measured in the *outer region* of the tissues developed within RCCS (0.41±0.33 MPa).

DISCUSSION

In this study, we demonstrated that cartilage tissues engineered in the mixed flask or the RCCS, but not those grown statically, reproducibly display a bi-zonal structure, resulting in different local functional properties. The two zones can be clearly distinguished as an inner region, corresponding to the space of the original cell-seeded scaffold, and an outer region, corresponding to the outgrown tissue. The former contained larger amounts of GAG, was positively stained for type II collagen and was stiffer in compression, whereas the latter contained larger amounts of collagen organized in fibril bundles, was more intensely stained for type I, III and VI collagen and was stiffer in tension. Thus, in terms of spatial distribution of specific molecules [4, 17, 35-37] and differential mechanical properties [38-40], these regions appear to resemble some biochemical and biomechanical features of the inner and outer zones of native meniscus.

The bi-zonal structure and function of the engineered cartilage tissues was achieved by culture of articular chondrocytes within HYAFF®-11 meshes in the presence of hydrodynamic flow. The capacity of articular chondrocytes to respond to hydrodynamic conditions by differentially expressing molecules present in the inner and outer regions of native meniscus is a further demonstration of their plasticity [41]. Together with a recent study comparing different chondrogenic cells of human origin [16], our results support articular chondrocytes as a valuable cell source for the engineering of meniscus grafts. The HYAFF®-11 non-woven meshes used in this study were supportive of the formation of bi-zonal tissues. The composition and open structure of the meshes may have contributed respectively to the deposition of GAG in the inner region and to the outgrowth of cells towards the outer capsule. Application of convective flow to engineered tissues within mixed flasks prevented the formation of core

regions containing little extracellular matrix and was associated with the formation of an outer fibrous capsule, consistent with previous studies [18]. Our finding that RCCS culture also resulted in the formation of an outer fibrous capsule, as opposed to a previously described uniform matrix distribution [18] or to no changes in matrix production [9], could be explained by the different cell source used (partially expanded bovine chondrocytes as opposed to respectively primary bovine chondrocytes or rabbit meniscal fibrochondrocytes) and/or by the different culture vessel configuration, which was not designed to minimize turbulent eddies and associated shear stresses [17, 21, 42]. This observation highlights the need to better characterize the hydrodynamic environment within the used RCCS, in order to identify the mechanism of our findings and to possibly extend them towards the engineering of meniscus-shaped grafts. More fundamental studies would be required to determine the role of increased mass transport and/or increased cell density following application of hydrodynamic flow in the development of the bi-zonal tissues.

One important component of this study lies in the assessment of the *local* mechanical properties of the engineered cartilage tissues in compression and tension, using innovative methods. SFM-based mechanical indentation testing by micrometer-sized spheres was previously introduced to measure the bulk elastic behavior of articular cartilage, leading to values similar to those derived by indentation testing at the millimeter-scale [19]. Since the area probed by a sphere with a radius of 1.6μm is estimated to be around 1.5 μm^2, we can exclude that indentation measurements are biased by the test of single collagen fibers (whose diameter is in the range of 40-60 nm). Tensile tests with concentric cartilage rings were developed specifically for this study, since the typical dog-bone shaped geometry would not have allowed to capture local mechanical differences at defined distance from the construct periphery.

Statically grown tissues displayed a similar trend in compression and tension, namely that the inner region was slightly less stiff – although not significantly – than the outer region, likely due to overall lower content of extracellular matrix. Instead, the *inner region* and *outer region* of mixed flask- or RCCS-cultured tissues had opposite trends in compression and tension, suggesting a potential for the resulting composite structure to specifically resist to complex loading modalities. Since the mechanical properties of the non-woven mesh scaffold used are negligible, the mechanical functionality of the bi-zonal engineered constructs was mainly due to the differential deposition of extracellular matrix by

chondrocytes. The higher compressive stiffness in the inner region could be directly related to the presence of GAG [9]. Increased tensile stiffness in the outer region of the bi-zonal tissues was likely due not only to the higher collagen content and presence of specific collagen types, but also to the organization of collagen fibers in bundles parallel to the construct edges. Since mechanical tests used in this work are not standard and require specimens of specific geometries, a comparison of the compressive and tensile properties of the engineered constructs with those of the native meniscus could not be established or derived from data in the literature. However, in general terms, in RCCS-grown tissues the *inner region* had a compressive stiffness in the range of the aggregate modulus calculated for meniscus [43], and the *outer region* had a tensile stiffness about 10- or 1000-fold lower than that in native meniscus measured respectively perpendicular or parallel to the principal fiber direction [3]. As compared to the native meniscus, the reduced tensile properties of the engineered constructs may be related not only to a lower amount of collagen, but also to a more limited organization of collagen fibers in large bundles, oriented parallel to the applied loads. In this regard, it would be important to determine whether the level of mechanical functionality reached by the engineered tissues would be sufficient, upon exposure to loads physiologically applied to the meniscus, to allow further development into a tissue more closely mimicking the complex structure and function of native meniscus [4].

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FIGURE CAPTIONS

Figure 1. Histology of chondrocyte-based engineered tissues. Representative fields of engineered tissues, generated after 4 weeks of culture statically (A, B, C), in the mixed flask (D, E, F) or in the RCCS (G, H, I). Sections were stained for GAG with Safranin O (A, B, D, E, G, H) and for collagen with elastica-van Gieson (C, F, I). Fields in (B,C), (E,F) and (G,H) correspond to a specific area respectively within (A), (D), and (G). Scale bar = 200μm (A, D, G) or 100μm (B, C, E, F, H, I).

Figure 2. Immunohistochemistry of chondrocyte-based engineered tissues. Representative immunohistochemical stain for collagen type I (A,B,C), type II (D,E,F) and versican (G,H,I) of cartilaginous tissues cultured for 4 weeks statically (A,D,G), in the mixed flask (B,E,H) or in the rotary cell culture system (C,F,I). Scale bar = 100μm.

Figure 3. Scanning Electron Microscopy (SEM). SEM from representative fields within the *inner region* (A, C, E) and *outer region* (B, D, F) of constructs cultured statically (A, B), in the mixed flask (C, D) or in the rotary cell culture system (E, F).

Figure 4. Biomechanical results. Elastic modulus in compression (A) and in tension (B) of the *inner region* and *outer region* of constructs cultured statically, in the mixed flask or in the rotary cell culture system (RCCS). * = significantly different from the outer region of the constructs cultured in the same condition.

Table 1. Immunophenotypic characterization of engineered tissues.

	Static		Mixed Flask		RCCS	
	Inner	Outer	Inner	Outer	Inner	Outer
Col I	-/+	+	+/-	+	+/-	+
Col II	-/+	-/+	+	-	+	-
Col III	-	-/+	-	-/+	-	-/+
Col VI	+/-	+	+/-	+	+/-	+
Versican	-	-/+	-/+	+	-/+	+

Expression of collagen types I, II, III, VI and versican was assessed in tissue constructs cultured statically, in mixed flask or in the rotary cell culture system (RCCS). Scores are based on the percentage of positive tissue as: no matrix positive (-); focal (<50% of matrix positive) staining (-/+); extensive (>50% of matrix positive) weak staining (+/-); extensive (>50% of matrix positive) strong staining (+).

Table 2. Size of inner and outer rings generated for tensile tests.

	Static	Mixed Flask	RCCS
Inner Ring Thickness (mm)	1.1±0.15	1.2±0.42	1.28±0.27
Outer Ring Thickness (mm)	1.35±0.07	1.88±0.67	2.13±0.39
Outer construct diameter (mm)	6.74±0.1	6.99±0.24	7.05±0.45

Thickness of inner and outer rings generated for the mechanical test in tension from constructs cultured statically, in mixed flask or in the rotary cell culture system (RCCS). Measurements are reported following trimming of the external capsule from the inner rings, in order to eliminate the contribution of the outer region to the tensile properties of the inner rings.

Figure 1

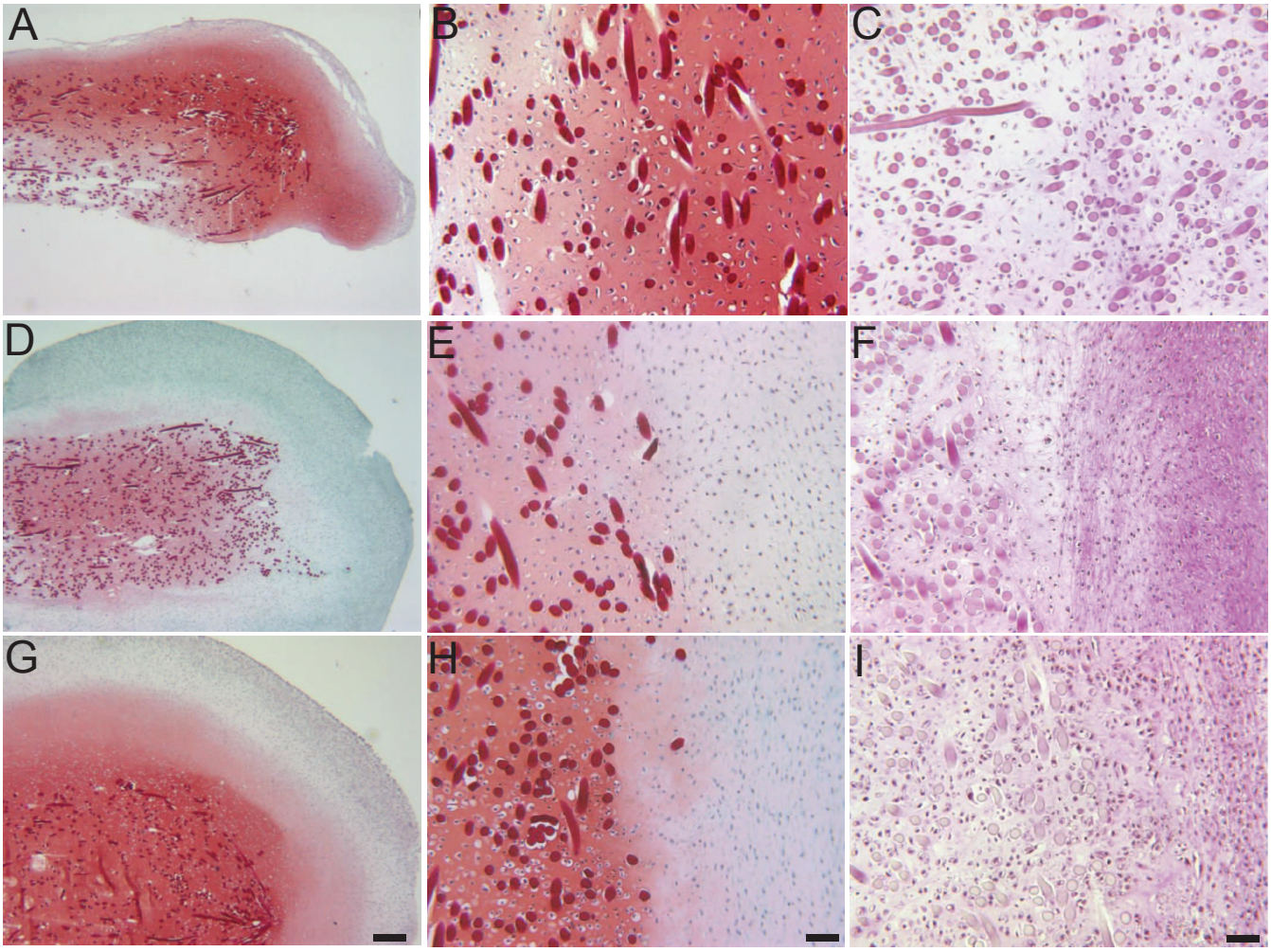

Figure 1
Marsano et al.

Figure 2

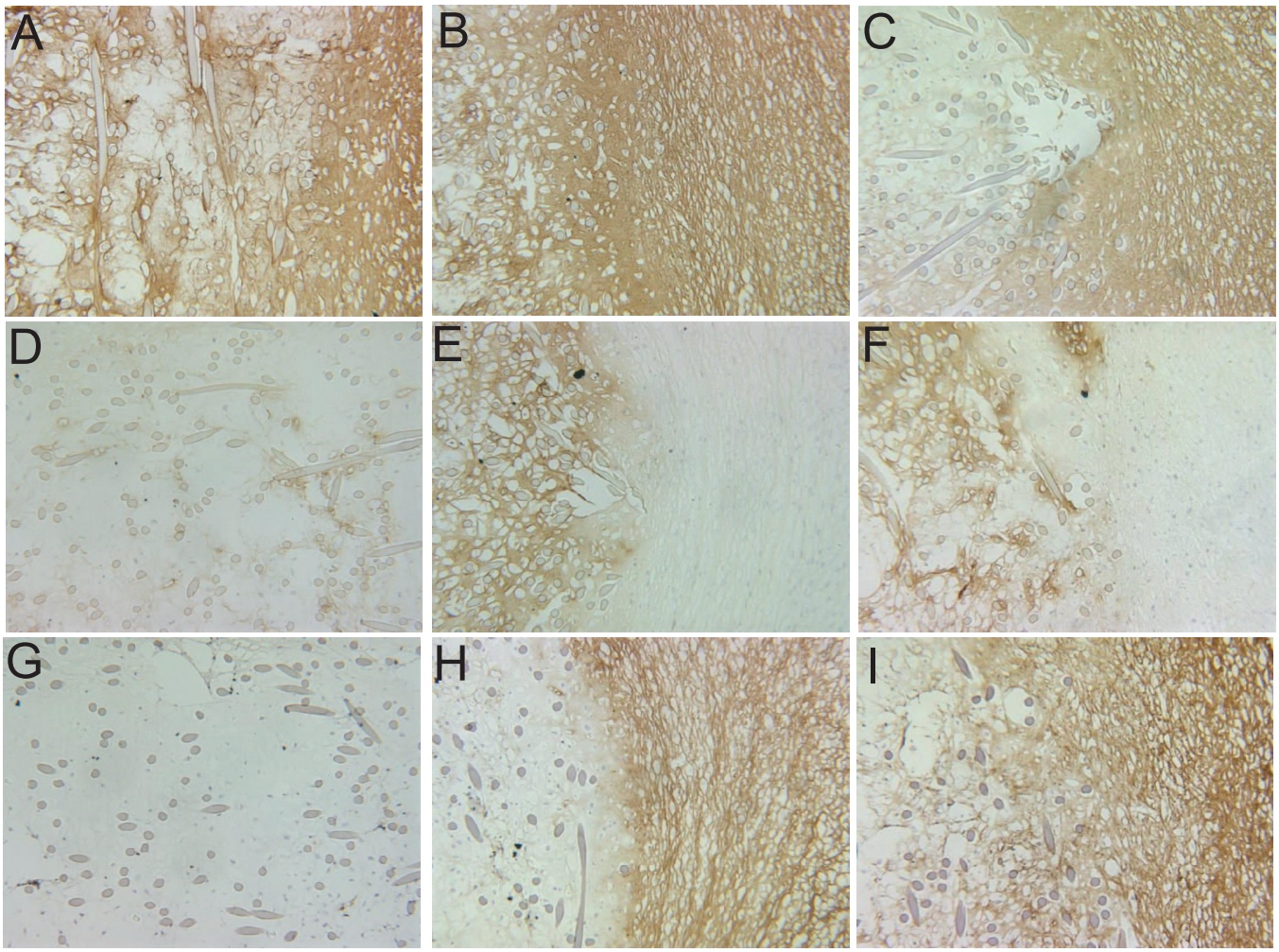

Figure 2

Marsano et al.

Figure 3

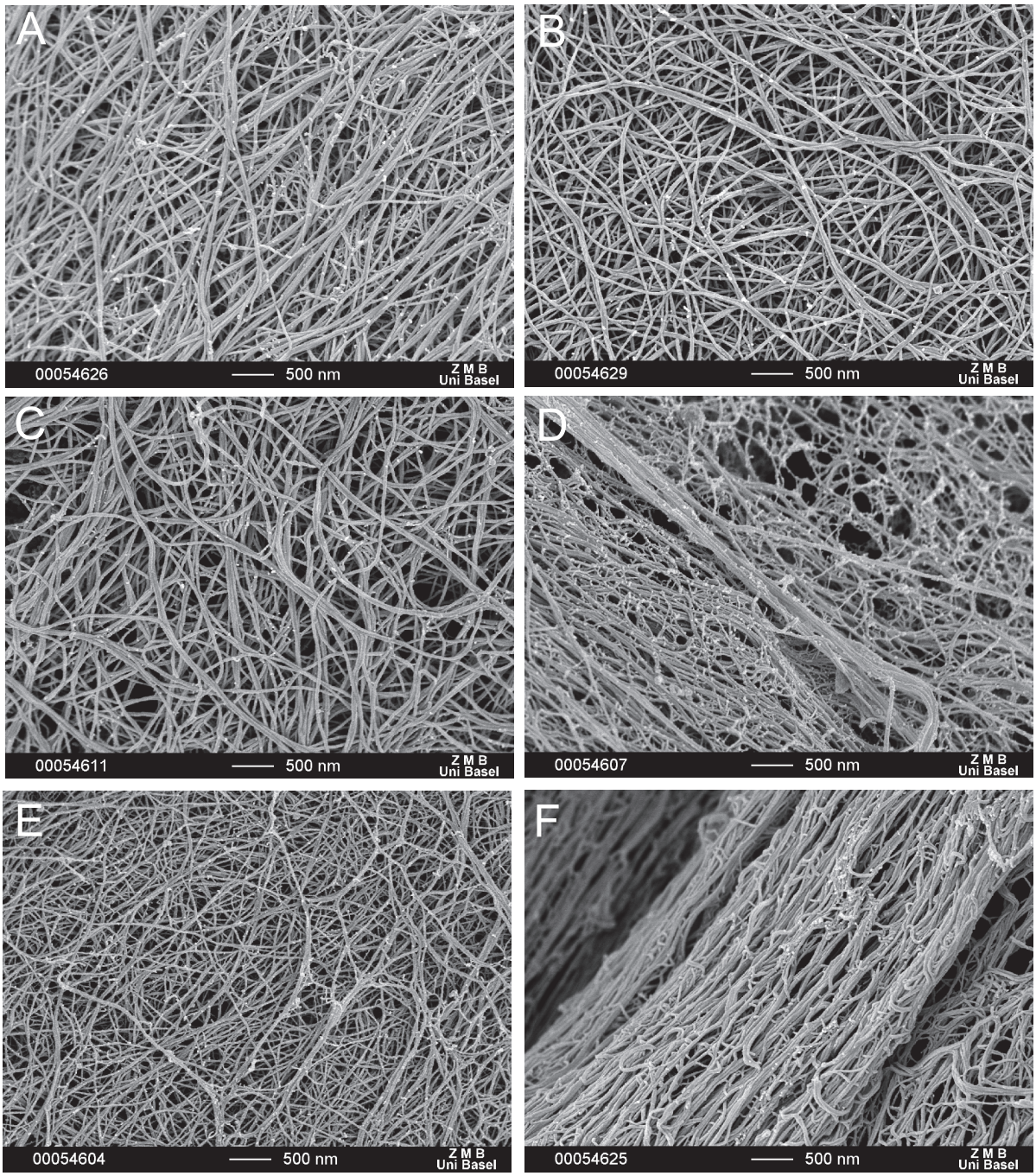

Figure 3

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Figure 4

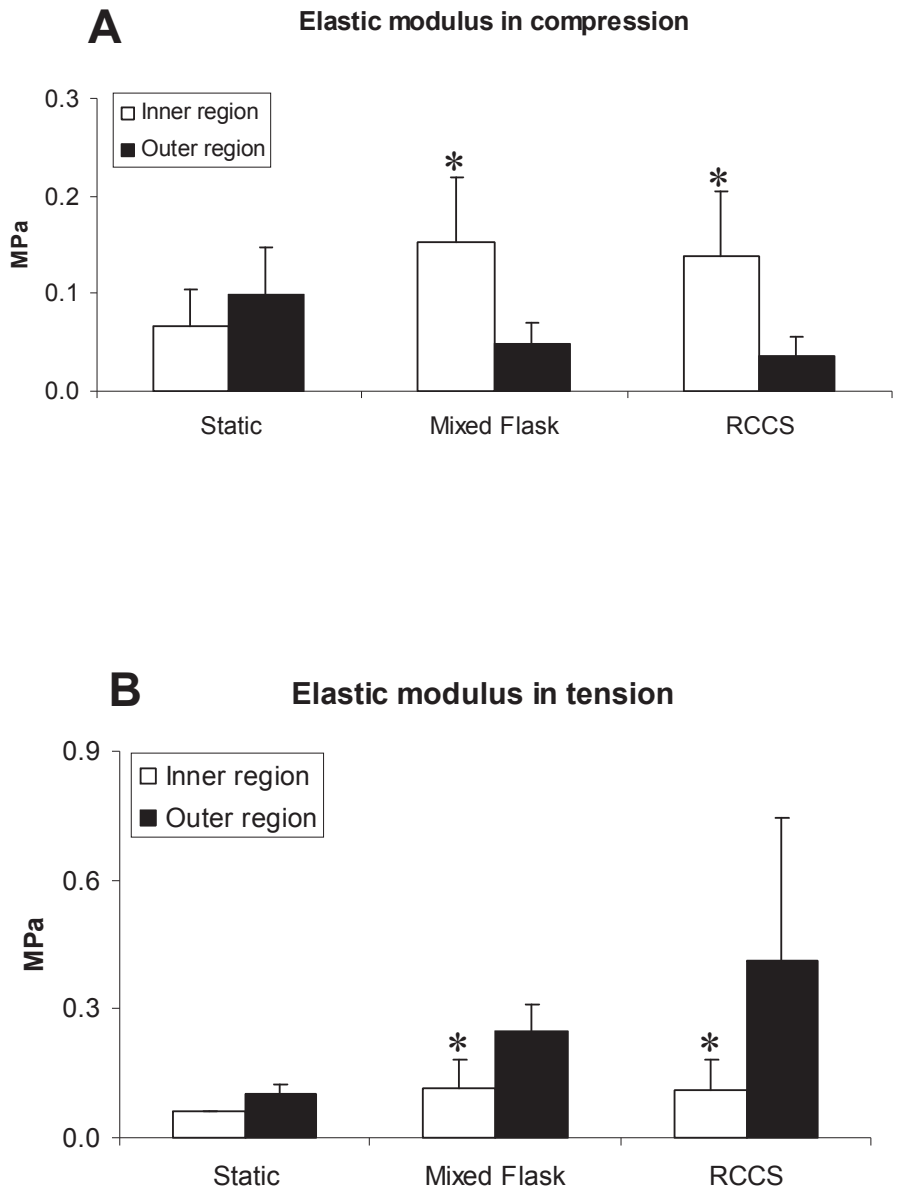

Figure 4

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