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Effect of articular cartilage proteoglycan depletion on high frequency ultrasound backscatter

B. Pellaumail*, A. Watrin†, D. Loeuille†, P. Netter†, G. Berger*, P. Laugier* and A. Saïed*

**Laboratoire d'Imagerie Paramétrique, Université Paris VI—CNRS UMR 7623, Faculté de Médecine Broussais—Hôtel-Dieu, 15, rue de l'Ecole de Médecine 75006 Paris, France*

†Laboratoire de Physiopathologie et Pharmacologie articulaire UMR 7561 CNRS—UHP, Faculté de Médecine Avenue de la Forêt de Haye BP 184, 54505 Vandoeuvre les Nancy, France

Summary

Objective: To study the effect of variations of articular cartilage proteoglycans (PG) on high-frequency ultrasound backscatter.

Design: The study was performed on patellar cartilages of immature and mature rats (N=36). The variation of PG content was induced by enzyme digestion. Control and treated cartilages were explored *in vitro* using a 55 MHz scanning acoustic microscopy, then assessed by histology for the fibrillar collagen organization analysis. The variations of proteoglycan and collagen content were evaluated. Thickness measurements performed on both B-scan images and histologic sections were compared. Ultrasonic radio-frequency signals reflected by the cartilage surface and backscattered from its internal matrix were processed to estimate the integrated reflection coefficient (IRC) and apparent integrated backscatter (AIB).

Results: Although hyaluronidase treatment of immature and mature cartilages removed approximately 50% of the proteoglycans, the echogenicity level of ultrasound images of degraded cartilages was similar to that of controls. IRC and AIB parameters did not significantly vary. Histologic sections of degraded cartilage displayed no change in collagen fiber organization. The thickness mean values measured by ultrasound in PG-depleted groups were significantly higher than in controls, whereas no significant difference in thickness was detected by histological measurement. The increase in cartilage thickness may potentially be explained by a decrease of speed of sound in PG-depleted cartilages that is more likely subsequent to an increase of water content.

Conclusion: Current results indicate that PG depletion has no significant effect on high frequency ultrasound backscattered from rat patellar cartilage. Ultrasound may provide information about variations of PG content via speed of sound measurement. © 2002 OsteoArthritis Research Society International. Published by Elsevier Science Ltd. All rights reserved.

Key words: Cartilage, Proteoglycans, Depletion, Maturation, High-frequency ultrasonography, Tissue characterization.

Introduction

Degeneration of articular cartilage is one of the predominant characteristics of osteoarthritis (OA) which is the most common joint disease in humans. The articular cartilage contains relatively few cells embedded in a large extracellular matrix rich in hydrophilic proteoglycans (PG), collagen fibers and other matrix constituents. The different components of the tissue are interrelated and contribute to its structural integrity. The content of PG and collagen, the collagen fiber characteristics and organization vary in the tissue[1].

During maturation or under pathological processes such as OA, the cartilage thickness and structure undergo progressive changes. The structural modifications involve mainly the PG and collagen content and the organization of the cross-linked collagen fibrillar network. During the cartilage maturation the fiber organization changes, the collagen content increases whereas the PG content and cellularity decrease[2]. Under OA disease process, the PG content is reduced, the cells and collagen fibers are highly altered and the collagen network is disrupted[3].

Radiography (X-ray) is routinely used to confirm the diagnosis of OA disease, but, is unable to directly visualize the cartilage tissue. Conventional magnetic resonance imaging (MRI) and ultrasonography allow a direct evaluation of the patellar cartilage. However, because of their low resolution, these techniques permit the detection of only late-stage lesions of the disease[4].

The development of a high resolution imaging technique may be useful for the non-invasive detection and quantitative assessment of early stages of the cartilage alterations. Our group has shown in previous studies performed on rat patellar cartilage that 50-MHz quantitative ultrasonography allows detection of early cartilage lesions due to a chemically induced OA[5,6]. Also, the technique was sensitive to changes related to cartilage maturation[7]. In particular, we have reported that ultrasonic backscatter from the cartilage matrix increased during the progression of OA, but, decreased with rat aging. We have, then, hypothesized that the backscatter decrease involved during maturation was

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Address correspondence to: Amena Saïed, Laboratoire d'Imagerie Paramétrique CNRS UMR 7623, 15, rue de l'Ecole de Médecine 75006 Paris, France. Tel: (33) 1 44-41-49-68; Fax: (33) 1 46-33-56-73; E-mail: Amena.Saied@lip.bhdc.jussieu.fr

more probably related to changes in the anisotropy of the cartilage extracellular matrix that result from changes in collagen fiber orientation. However, during maturation or OA, changes of both PG content and collagen network organization occur. The precise contributions of each of the constituents (proteoglycans and collagen network organization) in ultrasonic backscatter have not been elucidated yet and remains an open question.

Few studies reported the influence of extracellular matrix proteoglycans on ultrasonic propagation properties of articular cartilage. Agemura *et al*. have reported that removal of proteoglycans from bovine cartilage apparently had some effect on attenuation and speed of sound measured at 100 MHz, but, no clear trend has emerged from the results obtained from the few samples studied[8]. Toyras *et al.*[9] have shown that speed of sound measured at 22 MHz in PG-depleted bovine cartilage was significantly lower than the mean velocity in control cartilage.

The current study aims at obtaining a deeper insight into the influence of proteoglycan changes on ultrasound (US) backscatter. PGs of patellar cartilage taken from immature and mature rats were selectively depleted with a specific enzyme[10] so as to evaluate the impact of proteoglycan depletion in two cartilage groups of different biochemical composition and collagen network organization.

Degraded as well as control cartilage samples were explored *in vitro* using a 55-MHz three-dimensional (3-D) backscatter microscope. Structural cartilage changes were assessed using quantitative acoustic parameters, histologic images and biochemical evaluation of PG and collagen content.

Materials and methods

ANIMAL SAMPLES

Institutional guidelines and legal requirements for laboratory procedures and use of animals were adhered to at all times.

The study included a total of 36 male Wistar rats which were divided into groups of immature rats (N=18, 150 g in weight, 5-week-old) and mature rats (N=18, 300 g in weight, 10-week-old). Within each group of age, eight rats served for ultrasonic exploration then histologic analysis, and 10 rats for biochemical evaluation. The rats were anesthetized and killed by cervical dislocation. Both patellae of each animal were excised. The right patella was degraded whereas the left one served as a control.

PG digestion was accomplished with 1 ml of 0.1 M sodium acetate in saline solution which contained 1000 U/ml of hyaluronidase from ovine testes (2950 U/mg, pH=5.11)[11]. The patellae were incubated at 37°C for 6 h. Then they were washed in 30 ml of solution of 0.1 M sodium acetate and immersed for 2 min in protease inhibitor solution. The control patellae were immersed in a saline solution (0.9%) at 37°C for 6 h.

ULTRASONIC MEASUREMENTS

The ultrasound study included 16 degraded patellae (eight immature and eight mature) and 16 controls (eight immature and eight mature). The samples (approximately 5 mm in sagittal direction by 2 mm in transverse direction) were mounted on a special holder and placed in degassed saline (0.9%) at room temperature for US data acquisition.

Acquisition system

A-mode pulse-echo radio-frequency (rf) data were acquired using a 3-D backscatter microscope which has previously been described in detail[5–7]. The system operated with a polymer 125 MHz resonant frequency focused transducer (Toray, Japan). At focus (7.5 mm), the spectrum of the US signal was centered at 55 MHz and the −6 dB axial and lateral resolutions were 20 μm and 65 μm, respectively. The rf signal backscattered from the tissue was digitized at 400 MHz (8 bits). The patellae were positioned at the focal zone in order that the cartilage was centered on the focus, with the cartilage surface oriented perpendicularly to the probe axis. For 3-D acquisition of signals backscattered from the entire cartilage and subchondral bone, the transducer scanned the sample in two perpendicular directions (40 μm increment step). At each transducer position the rf signal was time averaged to improve the signal to noise ratio and transferred to the computer for the processing of B-mode images.

Estimation of acoustical parameters

Ultrasonic radio frequency signals reflected by the cartilage surface and backscattered from its internal matrix were processed in the frequency domain to estimate two parameters, the integrated reflection coefficient (IRC) and apparent integrated backscatter (AIB) that are device- and operator-independent. The parameters were measured using a standard substitution method, which has been extensively described in previous papers[1,7].

Integrated reflection coefficient. The IRC is a quantitative index of the acoustic energy level reflected from the surface of the cartilage. This parameter is primarily determined by the composition and micro-architecture of the superficial layer, and the surface roughness.

For each patella, 20 A-lines corresponding to a region of interest (ROI) of 80 μm in the transverse direction by 400 μm in the sagittal direction were selected in the central region of the cartilage. Within this ROI, the signal reflected by the cartilage surface (52.5 ns in duration corresponding to 42 μm in depth) was gated and the reflection coefficient compensated for the apparatus transfer function was estimated in decibels (dB) as a function of frequency. The IRC was calculated over the 25–70 MHz frequency range.

Apparent integrated backscatter. The AIB is a quantitative index of the level of acoustic energy backscattered from the cartilage internal structure. This parameter provides quantitative information on the characteristics (concentration, size, orientation, e.g. anisotropy and acoustic impedance mismatch) of sub-resolution scatterers of the explored tissue.

At the central part of each patella, 150 echographic A-scans obtained from a rectangular region of interest of 240 μm in the transverse direction by 1 mm in the sagittal direction were selected. The signal backscattered from a volume of 84 μm in thickness, located at 42 μm beneath the cartilage surface was gated. The apparent backscatter normalized by the apparatus transfer function was

estimated in dB as a function of frequency. Then, the AIB was calculated over the frequency range from 30 to 80 MHz.

Estimation of cartilage thickness

Ultrasonic measurements of the cartilage thickness has been described previously in detail[7]. For each patella, the mean thickness was obtained from a ROI (1 mm in the sagittal direction and 80 μm in the transverse direction) by averaging the values measured on two adjacent sagittal B-scan images of the central region of the patella. The average thickness in μm was obtained taking into account a speed of sound of 1600 m/s[8].

HISTOLOGICAL ANALYSIS

Tissue preparation

The patellae were fixed in 12% neutral buffered formalin solution for 72 h, decalcified (EDTA) for 7–10 days, then dehydrated and embedded in paraffin. Sagittal contiguous sections (5- to 10-μm-thick) were performed in the central part of the patellae corresponding to the US acquisitions. Then, the serial sections were stained with Toluidine blue to estimate the PG content, and with Picrosirius red to estimate the collagen content. The collagen fiber orientation was determined by polarized light microscopy[14].

Histologic measurement of cartilage thickness

The measurement method of histological thickness has previously been described[7]. The mean thickness of site-matched regions identical to the volume examined with ultrasound was estimated on corresponding images of histologic sections stained with Toluidin blue. These sections were selected in such a way that the cartilage thickness was the largest, indicating that they are located in the central part of the patella in the transverse direction. To estimate a mean 'histologic' thickness over the same volume of cartilage approximately, measurements were averaged over three non-adjacent histologic sections (in sagittal plane) separated by approximately 40 μm.

BIOCHEMICAL ANALYSIS

The sulfated glycosaminoglycan and hydroxyproline were quantified for the evaluation of the PG and collagen contents, respectively in the remaining 20 controls (10 immature and 10 mature) and 20 degraded patellae (10 immature and 10 mature). The values of these parameters were expressed in μg per mg of dry cartilage[12,13].

STATISTICS

Because of the small number of samples, a distribution-free statistical method was used to test the difference in thickness, IRC, AIB, PG and collagen content between groups. The medians of degraded cartilage parameters were compared to those of their contralateral controls using the Wilcoxon signed-rank test, with a level of significance α=0.05. The Wilcoxon rank-sum test was used to evaluate the significance of the parameter variations of non-paired groups, e.g. immature and mature controls.

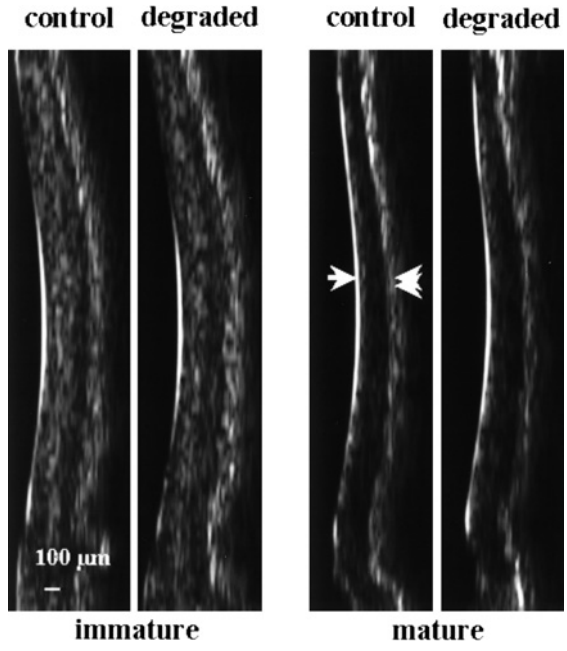

Fig. 1. 50 MHz B-mode images of patellar cartilages in the sagittal plane of an immature and a mature rat. Comparison between control and degraded cartilages of each rat. The arrow and double arrow indicate the cartilage surface and cartilage/bone interface, respectively.

Results

QUALITATIVE ANALYSIS

Ultrasound images

Figure 1 displays high-resolution B-mode images of degraded and control patellar cartilages of immature and mature rats. The image features and echostructure of control cartilages were extensively described by our group in previous articles[6,7].

Two hyperechoic bands delimited the cartilage surface and the cartilage-calcified cartilage/bone interface. For immature cartilage, the first two-thirds of the internal structure appeared hyperechoic. This echogenicity decreased with maturation[7]. After hyaluronidase treatment, no change was detected in the feature characteristics of B-scan images of degraded immature and mature cartilages as compared to controls. In particular, for immature groups, the backscatter level and pattern of the matrix of degraded cartilage were similar to those of control controls.

Histologic images

Images of histologic sections of rat patellar cartilage have been described in details in previous articles[5–7]. Only sections of a mature degraded cartilage and its contralateral control stained with Toluidin blue and Picrosirius red (viewed with polarized light) are shown in Fig. 2.

For control cartilages, the Toluidin blue staining of the extracellular matrix appeared almost homogeneous without zonal variations of proteoglycans with cartilage depth. Section stained with Picrosirius red showed connective tissue in polarized light and revealed the anisotropic structure of cartilage. Briefly, the tissue is composed of layers characterized by collagen fibers of different density, size

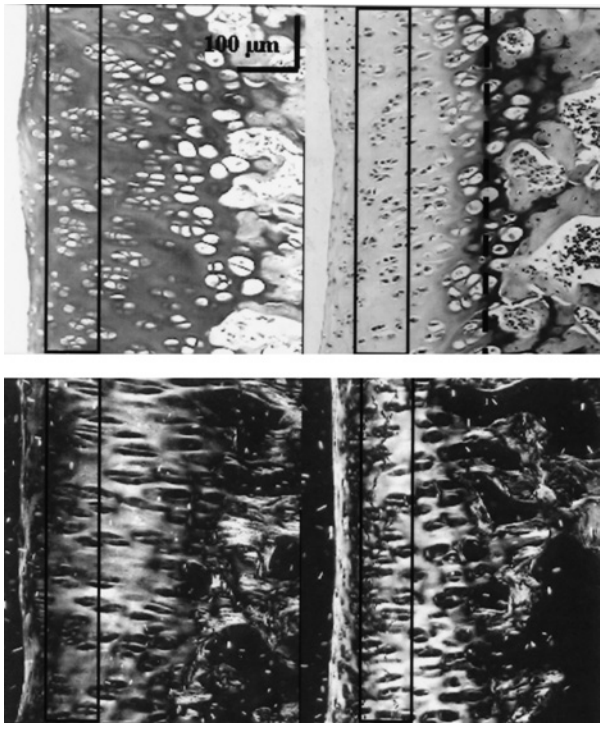

Fig. 2. Histologic section images of patellar cartilages of a mature rat stained with Toluidin blue (top) and Picrosirius red, viewed with polarized light (down). Comparison between control (left) and degraded (right) cartilages. The rectangles (84 µm in width) delimit the extent of the region of measurement of apparent integrated backscatter. On the image of degraded cartilage stained with Toluidin blue, the dotted line indicates the hyaluronidase penetration front.

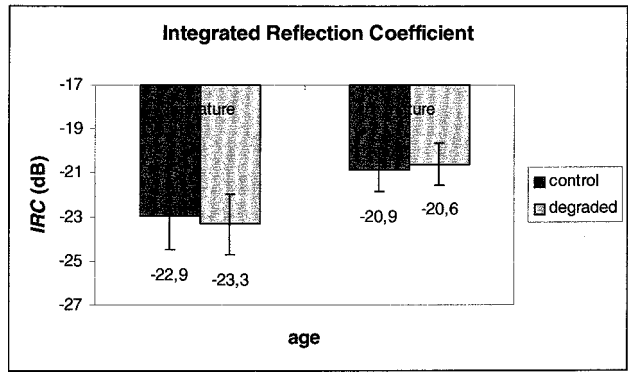

Fig. 3. Mean values of integrated reflection coefficient (IRC) of control and PG-depleted cartilages within immature and mature rat groups. The error bars represent (±) the standard deviation of the IRC over each group.

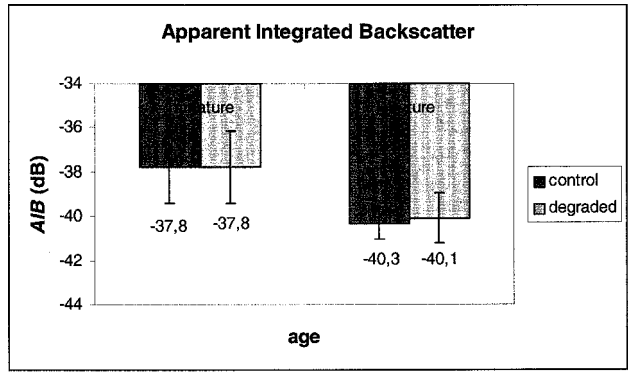

Fig. 4. Mean values of apparent integrated backscatter (AIB) of control and PG-depleted cartilages within immature and mature rat groups. The error bars represent (±) the standard deviation of the AIB over each group.

and orientation. In mature cartilage, the radial zone (with collagen fiber direction orthogonal to articular surface) appeared green.

No morphological alterations were observed on Toluidin blue images of degraded immature and mature cartilages. The cell density and size were not modified. The extracellular matrix was mostly faded, indicating a depletion of an important part of the PGs. An enzyme penetration front was observed at the beginning of the hypertrophic cell zone.

Histologic section visualized with polarized light displayed an organized collagen fibrillar network having an intense orange color mainly in the radial zone. The change in the staining intensity involved almost all the cartilage. However, the organization of collagen fibers within the different layers was not altered by the hyaluronidase digestion.

QUANTITATIVE FINDINGS

Acoustic parameters

The reproducibility of IRC and AIB measurements was reported earlier and found to be 3.5% and 1%, respectively[6].

The mean values of IRC and AIB and their standard deviations were calculated in control and PG depleted cartilage groups and plotted as a function of animal age in Figs 3 and 4, respectively.

The group average IRC values varied between −20.6±1.0 dB and −23.3±1.4 dB. No significant difference was found between mean values of control and degraded cartilages within immature and mature groups (P>0.05). However, as described in a previous study[7], the significant difference in IRC mean value (P<0.05) of controls confirmed that maturation has an effect on cartilage surface.

The mean values of AIB calculated over each group varied between −37.8±1.7 dB and −40.3±0.7 dB. No significant difference was found between control and degraded cartilage within immature and mature groups (P>0.05). However, the group average AIB values, estimated in control groups showed a decline due to the cartilage maturation process (P<0.05)[7].

Cartilage thickness

The reproducibility of thickness ultrasonic measurement assessed in a previous work was 1.3%[6]. For both ultrasonic and histologic measurements, the mean cartilage thickness and the standard deviation were calculated over the eight animals of each group and plotted as a function of rat age in Figs 5 and 6, respectively. A 5% and 11% significant increase in mean values of ultrasonic thickness was detected in depleted immature and mature cartilage groups, respectively, as compared with controls (P<0.05). However, thickness mean values obtained by histological measurements did not exhibit a significant change between

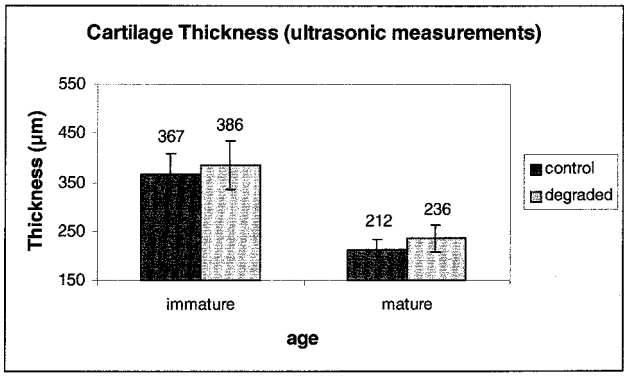

Fig. 5. Mean values of cartilage thickness (ultrasonic measurements) of control and PG-depleted cartilages within immature and mature rat groups. The error bars represent (±) the standard deviation of the thickness over each group.

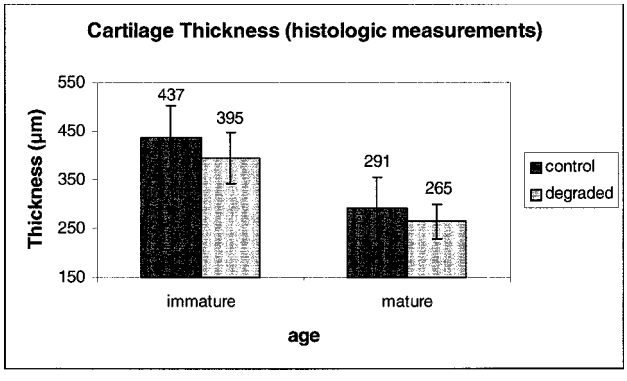

Fig. 6. Mean values of cartilage thickness (histologic measurements) of control and PG-depleted cartilages within immature and mature rat groups. The error bars represent (±) the standard deviation of the thickness over each group.

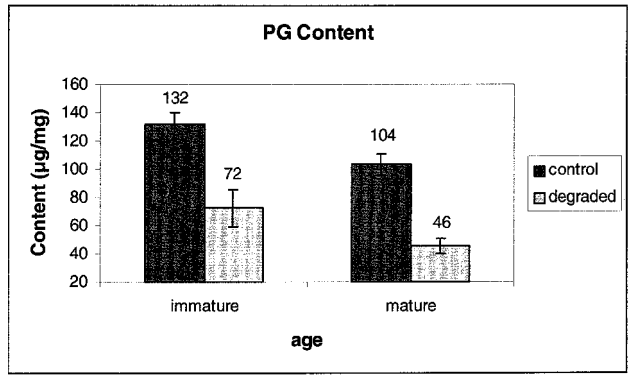

Fig. 7. Mean values of proteoglycan (PG) content of control and PG-depleted cartilages within immature and mature rat groups. The error bars represent (±) the standard deviation of the content over each group.

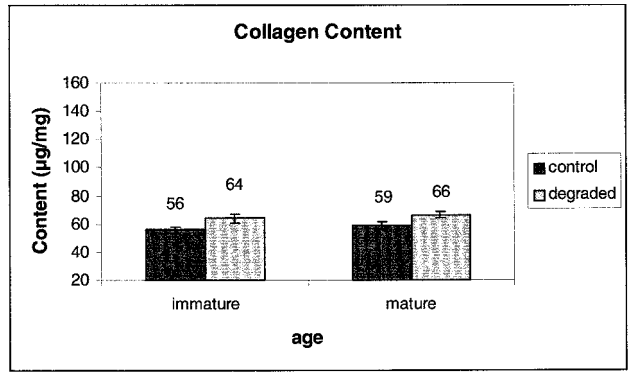

Fig. 8. Mean values of collagen content of control and PG-depleted cartilages within immature and mature rat groups. The error bars represent (±) the standard deviation of the content over each group.

degraded and control cartilages within immature and mature groups (P>0.05). Both ultrasonic and histological thickness mean values of control and degraded groups displayed a decline with age due to cartilage maturation (P< 0.05)[7].

Biochemical data

Figures 7 and 8 reported the effect of hyaluronidase treatment on PG and collagen contents (mean±S.D.) in degraded and control cartilage groups. Within both immature and mature groups, hyaluronidase digestion induced a statistically significant decrease of 45% and 56% (P<0.05) in PG content. The significant decrease (P<0.05) in PG content detected with age in control groups was related to maturation process as has been discussed previously by this group[7].

With regards to collagen content in both immature and mature groups, mean values of degraded cartilage were 14% and 12% higher (P<0.05), respectively, than mean values of controls.

Discussion

The purpose of the current work was to evaluate the influence of proteoglycan content variation on ultrasound backscatter from articular cartilage. Ultrasonic backscatter properties were measured in control and PG depleted cartilages. PG enzyme digestion was performed in immature and mature cartilage groups in order to study the effect of changes in this extracellular matrix component in samples of different biochemical composition and micro-architecture.

The current study reports, for the first time, comparison between results obtained from high resolution imaging and local quantitative characterization of both control and degraded cartilages with histological and biochemical analysis of the samples.

As we have previously described[7], current results have shown that PG content and apparent integrated backscatter mean value displayed similar variation trends between 5 and 10 weeks of rat age. Cartilage maturation was characterized by a significant decrease of 21% in PG content and 6% in mean value of AIB.

After hyaluronidase treatment of immature and mature cartilage groups, biochemical results indicated that approximately 50% of proteoglycans was removed in PG-depleted samples. Images of histologic sections of degraded cartilage viewed with polarized light displayed no significant change in collagen fiber organization but were lighter than those of controls. The absence of significant changes in echographic images and quantitative IRC and AIB parameters of PG-depleted samples as compared with controls indicated that PG depletion has no significant

effect on high-frequency ultrasound backscattered from rat patellar cartilage.

Similar conclusions were reached in an earlier preliminary study on the effect of papain PG-digestion of rat patellar cartilage[14]. We have shown that the first stage of papain digestion involving only PG depletion resulted in no change in 50 MHz B-scan images. However, when the enzyme induced a marked PG depletion followed by slight alterations of the collagen fibers of the superficial layer, echographic images showed a discontinuous and less echoic cartilage surface whereas the cartilage thickness and echogenicity of its internal structure were not changed. An extensive alteration of the collagen network led to an important remodeling of the cartilage that was displayed by marked changes in the echogenicity of B-scan images.

In view of current results and those reported in a recently published article on rat patellar cartilage maturation[7], we demonstrate that changes in high-frequency ultrasound backscatter are related to changes in the extracellular matrix collagen and most likely in its fibrillar network organization.

In addition to the decrease in PG content, biochemical data showed a 13% significant increase in collagen content between control and degraded cartilages within both immature and mature groups. It has been reported that hyaluronidase releases and eliminates selectively proteoglycans[10] and, thus, does not attack collagen. Hence, the increase in collagen mass observed in our biochemical values is, probably, a result of measurement bias that is caused by a decrease of cartilage mass value due to the important loss of proteoglycans.

Histological sections of degraded cartilages viewed with polarized light displayed an increase in light intensity and wavelength in comparison to sections of controls. Enhanced brightness of the birefringence of collagen matrix may result from PG release that yields to the attachment of a larger number of Picrosirius red dye molecules to free cationic charges on the surface of collagen molecules. Regarding the pattern of polarization colors of Picrosirius red-stained collagen, it has been reported that many factors such as change in collagen type, thickening, tight packing and well alignment of collagen fibers play a role in polarization colors of longer wavelength[15]. Since hyaluronidase treatment does, presumably, not denaturalize collagen, one can hypothesize that in PG-depleted cartilages, the collagen fibers are thicker, tightly packed and better aligned than in untreated cartilage and, thus, are more easily depicted. This phenomenon may be responsible for the increase in light wavelength (from green to orange).

Concerning cartilage thickness assessment, we found that for PG-depleted cartilage groups, the thickness mean values measured by ultrasound were significantly higher in comparison with controls, whereas no significant difference in thickness was detected between depleted and control groups by histological measurement. In our ultrasound investigation of cartilage thickness, a constant speed of sound was assumed for both control and PG-depleted specimens. However, previous investigations performed at 22 MHz and 30 MHz have shown that experimentally-induced cartilage PG depletion decreased the speed of sound by 2% to 13%[9,16]. Speed of ultrasonic wave appeared to be sensitive to matrix PG loss and more likely to the subsequent increase of water content of the tissue. Indeed, it has been reported that cartilage treated by hyaluronidase showed an increase in water content[17]. An actual decrease of speed of sound in PG-depleted cartilages may potentially explain the observed apparent increase of cartilage thickness.

In summary, the data of the present study provide significant insights into the relationship between biochemical compositions of rat patellar cartilage and ultrasound backscatter parameters. Our findings demonstrate that proteoglycans do not directly contribute to changes in ultrasound backscattering properties and confirm our previous observation on the major role played by collagen fiber orientation in changes in apparent backscatter occurring during cartilage maturation process or OA experimental models. High resolution ultrasound could be used as a non-destructive means to provide valuable and local information on collagen fibrillar network modifications of joint cartilage related diseases and associated treatments. This potential tool may be very helpful for the quantitative evaluation of pharmacological treatment effects and, in particular, allow non-invasive investigations on small animal models. High-frequency measurements of other acoustic properties such as speed of sound and the frequency-dependent attenuation in immature and mature rat cartilage will be conducted in our laboratory in order to study the influence of the gradient in depth of each cartilage constituent on local ultrasonic parameters. Our ultimate goal is to extend this technique to the clinical field.

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