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Full Length Article

Effect of hyaluronic acid-based viscosupplementation on cartilage material properties

Emilie Patois [a,d,*], Marie Gaumet [a,b], Isabelle Badoud [c], Ivo Dellavia [d], Patrick Ammann [c,e]

[a] TRB Chemedica International SA, Route des Jeunes 33 bis, 1227 Carouge, Geneva, Switzerland
[b] Route de Saint-Saphorin 8B, 1112 Echichens, Switzerland
[c] Division of Bone Diseases, Department of Medicine, University Hospitals Geneva, Geneva, Switzerland
[d] TRB Chemedica SA, Chemin Saint-Marc 3, 1896 Vouvry, Switzerland
[e] CMIS, Clinique La Colline, Hirslanden, Avenue de la Roseraie 76A, 1205 Geneva, Geneva, Switzerland

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ABSTRACT

Viscosupplementation by intra-articular injections of hyaluronic acid (HA) is used to treat symptomatic osteoarthritis. Exogenous HA remains in the joint cavity for a short period of time (days) while claimed pain relief period lasts over months. There is a clear lack of understanding of viscosupplementation mechanism of action. Here, we hypothesize that HA penetrates the cartilage and contributes to the restoration of its mechanical quality.

Confocal microscopy and bio-indentation were used to confirm HA penetration into cartilage and modulation of cartilage quality. Bio-indentation was performed on rat distal femurs incubated overnight in HA solutions, using phosphate buffered saline (PBS) as control. For this proof-of-concept evaluation, measurements of elastic modulus (MPa) and of maximal force (μN) were recorded before and immediately after exposure with HA, as well as after an additional washout with PBS. Cartilage thickness at the site of indentation was evaluated by contrast enhanced computed tomography with an ionic contrast agent. Indentation depths were located in the upper part of hyaline cartilage. Ostenil®, commercial product containing 1 % HA, induced a decrease in indentation depth in the range of forces influencing the whole cartilage thickness, together with an increase of the elastic modulus. Then, bio-indentation and size distribution of HA chains via SEC-SLS were assessed for a range of commercially available products. The results showed higher modulation of cartilage quality in the presence of 0.25–1 MDa HA chains.

The present *in vitro* study suggests that HA modulates cartilage quality and might thus explain the long-term beneficial effect of viscosupplementation.

1. Introduction

Osteoarthritis (OA) is the most frequent chronic musculoskeletal disorder and the leading cause of disability in the elderly [1,2]. Appeared in the mid-1990s, viscosupplementation remains commonly administered to treat symptomatic OA, with renewed interest and studies showing some benefits [3–7], despite a lack of convincing evidence and only conditional recommendations for its use in published guidelines [1,2,8]. Viscosupplementation consists in intra-articular injection of hyaluronic acid (HA) based solution to promote joint health and cartilage protection, as first suggested in 1967 by Balazs et al. who proposed to influence healing and regeneration of the cartilage and soft tissues of the joint in case of acute and chronic inflammation and in most degenerative processes of the joint [9]. To date, the corner stone of viscosupplementation is to restore viscosity of the synovial fluid and to promote viscoelastic properties of the joint fluid, by injecting exogenous HA of high molecular weight [10]. HA has already been widely characterized [11–17]. Nowadays, there is a preference toward HA obtained by fermentation, which allows to produce HA with a mean molecular weight (MW) of up to 4×10^6 Da, whereas endogenous HA of the knee synovium is of $6–7 \times 10^6$ Da.

A large effort has been placed to prolong residence time of HA in the joint, leading to a variety of approved products based on linear or crosslinked HA, with MW of $0.25 – 90 \times 10^6$ Da, where crosslinking is

* Corresponding author.
E-mail addresses: Emilie.patois@trbchemedica.com (E. Patois), Marie.gaumet@live.com (M. Gaumet), Isabelle.Badoud@unige.ch (I. Badoud), Labo.RechercheDeveloppement@trbchemedica.com (I. Dellavia), padocos@hotmail.com (P. Ammann).

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used to artificially increase the MW of HA chains. Some viscosupplementation products also include polyols such as mannitol to protect HA from oxidative degradation [18,19]. Unfortunately, residence time of injected HA in synovial fluid of treated joint remains particularly short, from 10 h to a few days, in comparison to claimed pain relief of up to several months [10]. Whereas the rapid and early reduction of pain after intra-articular injection can be related to an improvement of the joint rheology and reduction of inflammation [2], the need for better understanding of long-acting effect on pain and mobility of such treatment persists. Normal healthy tissues are almost always associated with high MW HA, with mean MW higher than 2 MDa in young human cartilage [11,16] and HA content of human articular cartilage of about 500–2500 μg/g [11].

In this study, we investigate viscosupplementation as exogenous supply of high MW HA to the cartilage level. To this end, we have developed an *in vitro* assay based on a bio-indentation method which allows to characterize cartilage properties. HA is a natural compound of the cartilage and its presence in cartilage is early reduced during the onset of OA [16]. We hypothesize that HA injected into the joint cavity penetrates the cartilage and modulates its quality. The osteochondral unit being responsible for the optimal distribution of load during movements and axial compression of the joint, there is an interest in detecting any modulation in the mechanical properties of the tissue. Here we present results obtained during proof-of-concept investigation performed with a linear HA based product and for a group of commercially available products with HA MW ranging from 0.5 to 90 MDa, among which ones a crosslinked HA. We suspected that depending on the size of HA chains, the potential to be integrated into the cartilage could differ. Size distribution measurement of investigated products confirms our hypothesis and sheds light on the importance of 0.25–1 MDa chains to restore cartilage quality.

2. Materials and methods

2.1. Experimental protocol

All experimental designs and procedures were approved by the Animal Ethics Committee of the University of Geneva, Faculty of Medicine, authorization number 31.1.1055/3043/2.

In the first series of experiences, we investigated the penetration of Ostenil® within the cartilage and evaluated the repercussion on cartilage quality as evaluated by cyclic bio-indentation. Femurs were harvested from three 11-month-old rats. Before and after an overnight incubation in 1 % non-chemically modified medium molecular weight HA solution (Ostenil®, TRB Chemedica, Switzerland), we measured alteration of cartilage quality using cyclic bio-indentation. Cartilage thicknesses were evaluated by contrast enhanced computed tomography (Hexabrix®; Guerbet, Roissy, France) at the site of indentation. This allowed an estimation of cartilage depth submitted to deformation depending on the indentation depth performed. This estimation was performed using $d = \sqrt{R \times h}$ where d is the cartilage depth, R is the ray of indentation sphere and h the indentation depth.

In a second series of experiments performed after the proof-of-concept, we compared different HA based solutions using monotonic bio-indentation. *In vitro* tests were performed on distal femurs harvested from 18 adult rats aged 11 months. The femurs were incubated overnight in five different HA based solutions, with mean MW ranging from 0.5 to 90 million Daltons (MDa) : a) Hyalgan® (Fidia farmaceutici, Italy), b) Durolane® (Bioventus LLC, USA), c) Sinovial® (IBSA farmaceutici, Italy), d) Ostenil® (TRB Chemedica, Switzerland) and e) Euflexxa® (Bio-Technology General, Israel). Among the products, four are based on linear HA and Durolane® is based on a crosslinked HA. Controls were incubated in phosphate buffered saline (PBS). Bio-indentation measurements were performed on the condyle, before and after incubation as well as after overnight PBS washout. Indentation depths were located in the upper part of the hyaline cartilage. Cartilage thickness was evaluated by contrast enhanced computed tomography with an ionic contrast agent (Hexabrix®) at the site of indentation.

2.2. Bio-indentation

All samples were prepared according to the following procedure. The distal part of the femur was cut in the transversal plan and was then glued with a fine layer of wax into the center of a Æ 35 mm Petri dish or onto a trapezoidal bloc, which was positioned in such way that the indented zone was always perpendicular to the axis of the indenter. The samples were fully immersed in PBS during the whole test to mimic as closely as possible the physiological conditions. Three indentation zones (medial condyle) according to joint mechanical loading pattern related to rat ambulation were assessed. Zone 1 is submitted to permanent loading during mobilization and rest, Zone 2 to loading during movement with large amplitude while zone 3 is only loaded by the contact with the patella (Fig. 1).

Cyclic indentation. The bio-indentation experiments were performed using Anton Paar Bioindenter™ equipped with spherical ruby indenter (R = 500 μm). Cyclic indentations were done with maximum load of 8 mN and starting load of 0.05 mN. The maximum load was reached progressively during 15 successive cycles. In each cycle, the maximal force (Fmax) was quadratically incremented in respect to the previous cycle; in each cycle, the unloading was done down to 20% of the Fmax of the given cycle. The loading and unloading times as well as the hold period at Fmax for each cycle were set to 10. seconds. Five cyclic indentations spaced by 200 μm were performed in each zone on the rat's femoral cartilage. Data were fitted to a Hertz contact model using Anton Paar software to calculate elastic modulus (MPa) and

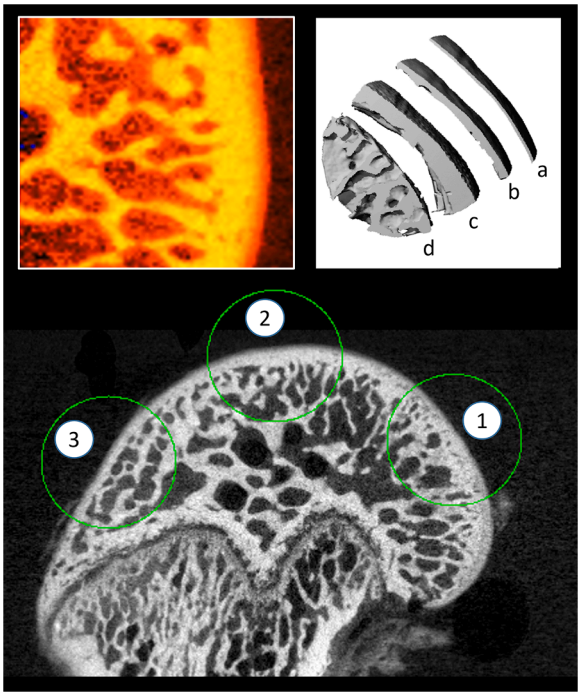

Fig. 1. Cartilage morphology and indention zone. Cartilage morphology assessed by EPIC-μCT (phase-contrast micro-computed tomography using Hexabrix® as contrast agent). Femoral articular cartilage including hyaline (a) and calcified cartilage (b) was segmented by manual contour line. Subchondral compact (c) and trabecular (d) bone were also analyzed on the same scan at the site of indentation. Three different regions of the articular surface were submitted to bio-indentation. Zone 1 corresponds to a part of osteochondral unit submitted to permanent loading during mobilization and rest, Zone 2 to loading during movement with large amplitude and Zone 3 is only loaded by the contact with the patella.

maximal force (μN).

Monotonic indentation. The bio-indentation experiments were performed using Bioindenter Piuma (Optics11, Netherlands). Within each region of interest (ROI), a set of 5 successive indentations were performed at 150 μm-distant location. A spherical indenter with a 50 μm diameter was used. The maximum indentation depth of 5 μm corresponded to 15 % of hyaline cartilage thickness. The protocol consisted in a loading period of 10 s, followed by a hold period of 2 s and finally an unloading period of 10 s. Data were fitted to a Hertz contact model using Optics11 Life software to calculate elastic modulus (MPa) and maximal force (μN).

2.3. Virtual histology of the distal femur

Cartilage morphology was assessed by phase-contrast micro-computed tomography (EPIC-μCT) using Hexabrix® as contrast agent, after all the other investigations. The distal part of the femur including bone metaphyseal and epiphyseal compartments as well as articular cartilage was incubated in a 40 % Hexabrix® solution, as used in studies establishing good correlation between μCT and histology [18]. Scans were performed using a μCT 40 (Scanco Medical, Bassersdorf, Switzerland) at a voxel size of 6 μm. The resulting transversal cross-sectional slices were then re-sliced horizontally using the Scanco Medical software to generate a series of sagittal sections. Femoral articular cartilage including hyaline and calcified cartilage was semi-automatically segmented by manual contour line. Based on an appropriate threshold, according to the histogram of the X-ray attenuation, analysis of the cartilage was performed at the site of indentation (Fig. 1).

2.4. Cartilage penetration assessed by confocal microscopy

A sample of rhodamine-HA, HA labeled with rhodamine 110, was prepared by conducting an esterification reaction in anhydrous organic solvent, dimethylformamide starting from 1.9 MDa HA (#PHI2524, HTL, France). A solution of rhodamine-HA at 2 % was prepared in order to get a similar viscosity level as for a non-labeled 1 % HA solution. Femur samples were immersed in 2 % rhodamine-HA solution and then washed with PBS. Penetration of rhodamine-HA within cartilage was assessed by confocal microscopy. Pictures of stained samples were taken with a Confocal Zeiss LSM710 using 20X objective and aperture setting of 1.0. Stacked images were then 3D visualized with IMARIS software (Bitplane/Oxford Instruments), which also allowed to visualize orthogonal views.

2.5. Hyaluronic acid molecular weight distribution by SEC-SLS

Size-Exclusion Chromatography Static Light Scattering (SEC-SLS) of five HA based solutions obtained by dilution and filtration of commercial products, previously evaluated for their performance in bio-indentation, was achieved using OMNISEC system (Malvern Panalytical, Malvern, UK). SEC-SLS conditions used: combination of three SEC columns to separate polymer molecules based on their hydrodynamic radius, isocratic flow rate of 0.5 mL/min, PBS as mobile phase, column temperature maintained at 40 °C, dn/dc of 0.15 mL/g. After 10x dilution into PBS using very gentle overnight agitation, samples were passed through 200 nm nylon syringe filters and stored at 20 °C in autosampler prior to analysis. SEC-SLS chromatography allowed to measure molecular weights moments Mn and Mw and polydispersity index (PDI), where PDI = Mw / Mn. Reported values of mean MW for tested commercial products, based on available documentation, are: a) Hyalgan® 0.5–0.73 MDa, b) Durolane® 90 MDa, c) Sinovial® 0.8–1.2 MDa, d) Ostenil® 1.6 MDa and e) Euflexxa® 2.4–3.6 MDa.

2.6. Statistical analysis

Mean values were calculated over 3 measurements and compared using an ANOVA. For bio-indentation, measurement set of five indentations were carried out in each of the three sites. Paired T-test were evaluated using the mean of the five indentations in each site. Paired T-test analysis was carried out using SPSS Advanced Statistics software and an ANOVA was used to evaluate inter-group variation. For EPIC-μCT, parameters were analyzed by ANOVA. The level of significance was set to $p < 0.05$. All results were expressed as means ± standard error of the mean (SEM).

3. Results

3.1. Cartilage penetration assessed by confocal microscopy

The penetration of hyaluronic acid within cartilage was demonstrated by confocal microscopy using a 2 % solution of HA labeled with rhodamine 110 (Fig. 2). IMARIS Software allowed visualization of 3D stacked image (Fig. 2a), which provided a picture of the femur surface. In addition, orthogonal views to the condyle surface obtained with IMARIS software showed that labeled HA had penetrated about 30 μm into the cartilage (Fig. 2b to d).

3.2. Modulation of cartilage quality after overnight exposure to hyaluronic acid based solution Ostenil® (proof-of-concept)

The thickness of the hyaline cartilage covering the distal femur of the rat is 0.037±0.005 mm and that of the mineralized cartilage is 0.115 ±0.008 mm as assessed by EPIC-μCT. An indentation of 5 μm has an area of influence of 50 μm and an indentation of 20 μm one of 100 μm. Thus, all the indentation forces used in this protocol are characterized by areas of influence localized in the cartilage (hyaline and mineralized).

During preliminary evaluation, cartilage sample were exposed overnight to HA based solution or to PBS control prior measurement of cartilage properties via cyclic indentation from 0.05 mM to 8 mM. Increasing the indentation force led to deeper indentation into the sample (Fig. 3a) and higher elastic moduli measurements (Fig. 3b). In comparison with PBS control, HA exposure led to smaller indentation depth over the whole range of applied forces with significant difference for forces higher than 3.35 mN (Fig. 3a). As regards elastic modulus, HA exposure led to higher modulus measurements over the whole range of applied forces with significant difference for forces higher than 1.5 mM (Fig. 3b). Ostenil® exposure induced a mean decrease in indentation depth of 8% and a mean increase in elastic modulus of 24 %. The force requested to induce a given deformation depth is larger after Ostenil® incubation. At a given cartilage depth, the elastic modulus is higher after Ostenil® incubation (Fig. 3).

3.3. Comparison of hyaluronic acid-based products on intrinsic cartilage quality

The five investigated HA-based products significantly increased cartilage quality compared to control solution (Tables 1 and 2, Fig. 4, Supplementary data).

Bio-indentation experiments were performed using Bioindenter Piuma (Optics11, Netherlands). All HA-based products significantly increased cartilage elastic modulus compared with the control solution, except for one product. This effect was maintained after the second incubation in PBS (washout period) for four out of five products, containing linear and crosslinked HA. No significant effect was observed for Euflexxa® after washout. Changes in indentation values measured after washout for samples incubated with HA solutions and stability of values observed for the control group exposed to PBS confirmed the effectiveness of the washout period.

The effects on indentation force display the same trend with higher

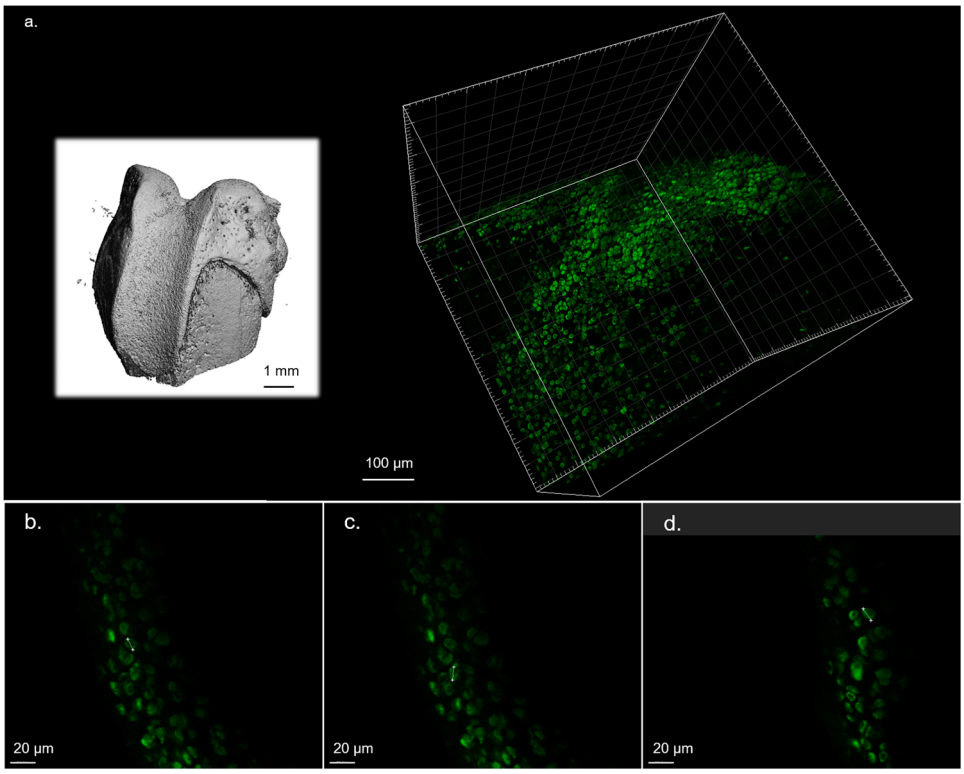

Fig. 2. Evidence of hyaluronic acid penetration into cartilage. Visualization of HA labeled with rhodamine 110 on the femur surface of rat condyle via confocal microscopy (Zeiss LSM710) using 20X objective and aperture setting of 1.0: (a) 3D visualization of stack images and (b to d) orthogonal views to the condyle surface, obtained with IMARIS software (Bitplane/Oxford Instruments).

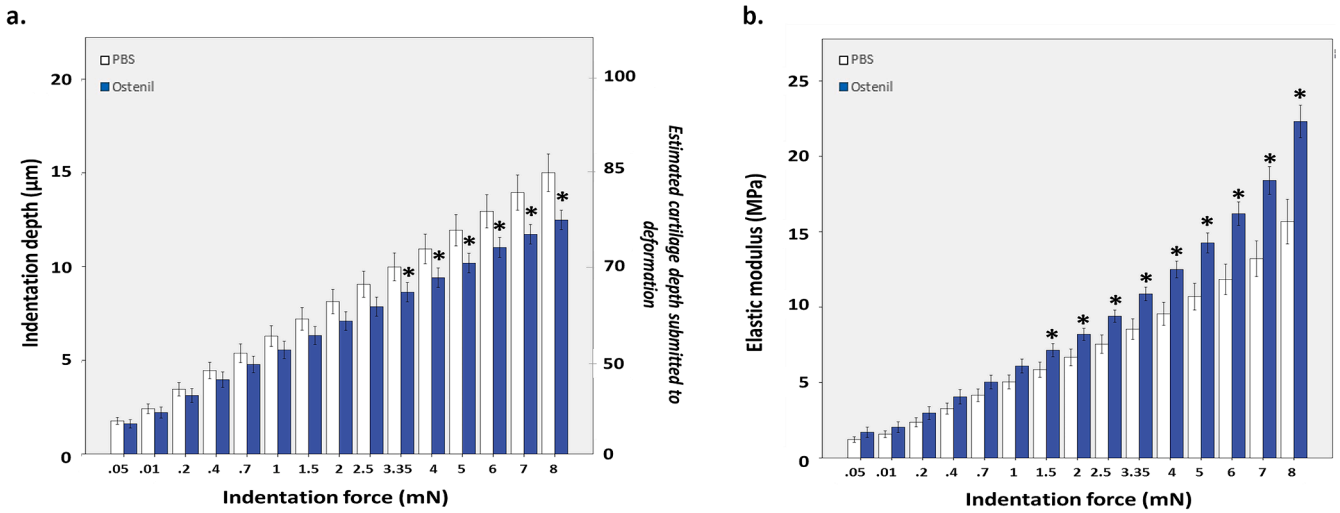

Fig. 3. Proof-of-concept demonstrating a physicochemical effect of HA on cartilage material level properties, as a result of mean over the three investigated zones. Cyclic bio-indentation experiments were performed using Anton Paar BioindenterTM equipped with spherical ruby indenter ($R = 500$ µm). Ostenil® induced (a) a decrease in indentation depth, together with (b) an increase of the elastic modulus.

variability due to the technique.

The positive effect on elastic modulus was observed in all three investigated regions. Among linear HA-based solutions, the formulation with the highest MW (Euflexxa®) was least effective (+2.57 %) compared with products containing mid-range MW HA (+21.59 % in case of Ostenil®). The maximal effect was observed in presence of the linear HA with the lowest MW, *i.e.* Hyalgan®. We therefore propose that the effect on cartilage quality of linear HA-based products is likely to be related with the mean MW of the chains. The crosslinked HA (Durolane®) also significantly improved cartilage quality. Furthermore, these effects were maintained after an incubation in PBS (washout procedure) in most of the cases, indicating that the investigated HA might penetrate and remain within the cartilage.

3.4. Hyaluronic acid mean molecular weight and polydispersity analysis

SEC-SLS chromatography provided insight on molecular weight distributions, number average (Mn), weight average (Mw) molecular weight moments and related polydispersity index (PDI), measured for filtered HA based solutions obtained from commercial products diluted

Table 1
Elastic modulus (MPa) measured by bio-indentation before and after exposure to various products or to PBS control.

		Before exposure	20 hrs exposure	Δ% after exposure	24 hrs washout	Δ% after washout
Zone 1	PBS	3.49 ± 0.34	3.42 ± 0.40	−2.24 ± 3.33	3.51 ± 0.30	+0.91 ± 2.87
	Hyalgan®	3.21 ± 0.15	3.98 ± 0.23*	+24.21 ± 5.61*	3.87 ± 0.03*	+20.98 ± 4.82*
	Durolane®	3.38 ± 0.25	4.18 ± 0.28**	+24.09 ± 2.10**	3.75 ± 0.35	+10.66 ± 2.61
	Sinovial®	3.18 ± 0.22	3.81 ± 0.17*	+20.07 ± 4.95*	3.69 ± 0.24*	+16.04 ± 3.78*
	Ostenil®	3.14 ± 0.15	3.60 ± 0.17**	+14.85 ± 0.07**	3.50 ± 0.32	+11.12 ± 5.16
	Euflexxa®	3.26 ± 0.29	3.39 ± 0.29*	+4.04 ± 0.61*	3.25 ± 0.33	−0.44 ± 3.86
Zone 2	PBS	2.67 ± 0.20	2.62 ± 0.19	−1.71 ± 4.50	2.56 ± 0.20*	−4.51 ± 0.78*
	Hyalgan®	2.18 ± 0.09	3.00 ± 0.11**	+37.90 ± 3.09**	2.95 ± 0.29	+35.44 ± 12.36
	Durolane®	2.56 ± 0.14	2.97 ± 0.24	+15.76 ± 4.28	2.80 ± 0.21	+8.89 ± 2.67
	Sinovial®	2.47 ± 0.09	3.07 ± 0.19*	+23.99 ± 3.45*	2.92 ± 0.06*	+18.09 ± 2.66*
	Ostenil®	2.28 ± 0.05	2.86 ± 0.03*	+25.39 ± 3.31*	2.62 ± 0.06**	+14.74 ± 0.51**
	Euflexxa®	2.30 ± 0.19	2.32 ± 0.18	+0.97 ± 0.87	2.40 ± 0.23	+4.22 ± 1.16
Zone 3	PBS	1.46 ± 0.10	1.44 ± 0.03	−0.35 ± 5.31	1.36 ± 0.00	−6.14 ± 6.26
	Hyalgan®	1.21 ± 0.10	1.52 ± 0.09*	+26.03 ± 5.32*	1.35 ± 0.11*	+11.04 ± 0.99*
	Durolane®	1.22 ± 0.08	1.37 ± 0.06	+12.85 ± 4.59	1.40 ± 0.06	+16.08 ± 9.35
	Sinovial®	1.24 ± 0.07	1.52 ± 0.16	+21.69 ± 7.12	1.46 ± 0.11	+17.47 ± 5.51
	Ostenil®	1.32 ± 0.13	1.63 ± 0.07*	+24.54 ± 6.92*	1.55 ± 0.19	+16.46 ± 5.80
	Euflexxa®	1.32 ± 0.23	1.37 ± 0.32	+2.69 ± 5.86	1.21 ± 0.21	−7.83 ± 4.57

Significant differences are based on paired T-test comparison with measures made before exposure (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$); values are means ± SEM.

Table 2
Maximal force (μN) measured by bio-indentation before and after exposure to various products or to PBS control.

		Before exposure	20 hrs exposure	Δ% after exposure	24 hrs washout	Δ% after washout
Zone 1	PBS	915.77 ± 75.80	782.49 ± 119.45	−15.52 ± 6.98	908.85 ± 38.99	−0.02 ± 4.52
	Hyalgan®	774.84 ± 79.18	830.27 ± 71.91*	+7.47 ± 1.80*	808.87 ± 88.57	+4.41 ± 4.08
	Durolane®	795.58 ± 65.65	912.22 ± 35.96	+15.43 ± 4.93	856.10 ± 74.02	+7.47 ± 1.46
	Sinovial®	757.01 ± 49.39	776.29 ± 28.63	+2.96 ± 3.20	739.01 ± 24.97	−1.46 ± 7.59
	Ostenil®	720.29 ± 30.09	727.11 ± 19.19	+1.22 ± 4.18	719.98 ± 27.19	+0.59 ± 7.60
	Euflexxa®	709.04 ± 37.61	757.35 ± 77.25	+6.58 ± 7.30	786.03 ± 127.11	+9.53 ± 12.59
Zone 2	PBS	727.80 ± 17.80	726.69 ± 29.12	−0.08 ± 4.18	649.91 ± 55.37	−10.96 ± 5.33
	Hyalgan®	628.43 ± 51.42	692.96 ± 56.47**	+10.27 ± 0.54**	700.75 ± 96.10	+10.56 ± 6.09
	Durolane®	703.68 ± 66.72	726.83 ± 127.25	+2.22 ± 12.55	716.09 ± 105.93	+1.47 ± 10.80
	Sinovial®	596.22 ± 18.80	687.75 ± 93.84	+15.88 ± 16.80	514.09 ± 61.24	−13.52 ± 10.65
	Ostenil®	574.94 ± 30.09	620.62 ± 5.77	+8.45 ± 4.86	591.56 ± 6.63	+3.43 ± 5.23
	Euflexxa®	616.84 ± 148.85	641.52 ± 23.03	+21.09 ± 36.04	624.28 ± 75.21	+11.40 ± 22.23
Zone 3	PBS	359.94 ± 77.04	356.45 ± 83.34	−0.08 ± 10.63	419.43 ± 65.29	+24.96 ± 24.88
	Hyalgan®	373.77 ± 90.13	378.69 ± 46.20	+13.36 ± 29.63	336.39 ± 76.47	−1.65 ± 32.06
	Durolane®	407.69 ± 34.93	438.47 ± 78.23	+5.96 ± 9.33	423.51 ± 67.73	+7.73 ± 23.81
	Sinovial®	326.87 ± 35.34	354.18 ± 66.30	+15.92 ± 34.78	361.27 ± 33.14	+15.74 ± 23.99
	Ostenil®	310.21 ± 29.03	419.02 ± 11.39	+38.10 ± 16.24	413.91 ± 10.19	+35.35 ± 10.45
	Euflexxa®	383.16 ± 109.41	442.50 ± 115.59*	+17.33 ± 4.61*	366.95 ± 68.12	+1.49 ± 11.37

Significant differences are based on paired T-test comparison with measures made before exposure (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$); values are means ± SEM.

in PBS buffer (Table 3 and Fig. 5).

Polymer chains from 250 kDA to 7.5 MDa were measured depending on the product. Linear HA-based products showed monomodal size distributions for a) Hyalgan®, c) Sinovial® and d) Ostenil® with MW of 0.5 MDa, 1.0 MDa and 1.3 MDA and PDI of 1.46, 1.33 and 1.33, respectively and one bimodal size distribution with MW of 3.0 MDa and PDI of 1.39 in the case of e) Euflexxa®. Mean MW measured were close to reference of mean molecular weight, with similar SEC-SLS values for Ostenil® and Euflexxa® as already reported [19]. In the case of the crosslinked product b) Durolane®, the measured molecular weight distribution of the diluted and filtered solution was mainly composed of 0.25–1 MDa chains resulting in measured MW different from mean molecular weight available for the products.

Measuring molecular weight distributions allowed us to differentiate between products based on population of chains shorter than 1 MDa. Indeed, among the four commercial products based on linear HA investigated in the study, three showed a significant content of chains shorter than 1 MDa, with Hyalgan®, Sinovial® and Ostenil® showing 92 %, 61 % and 39 % of measured chains of 0.25–1 MDa respectively. The smallest population of chains below 1 MDa was measured for Euflexxa®. As regards Durolane® with reported MW of 90 MDa, chains measured for the solution obtained from such a high MW product were the smallest ones with MW of 200 kDA and no chains bigger than 1 MDa were detected in contrast with the four other products. In addition, our results show that the calculated PDI, defined as the ratio of molecular weight moments Mw over Mn, is not correlated with width of size distribution, commonly referred to as polydispersity.

4. Discussion

The present study demonstrates that high MW HA improves cartilage mechanical properties by penetrating the cartilage. The results underline that molecular weight of HA influence its penetration. Interestingly, this beneficial effect was observed even after the HA was eliminated from the incubation medium, which might explain the long-term efficacy observed in OA patients.

Evaluation of the intrinsic quality of cartilage using bio-indentation has the advantage of providing insight on cartilage proficiency of alteration. This *ex vivo* approach allows to measure a physicochemical effect in the absence of any cellular activities. We used two technologies: the cyclic measurement allows to appreciate the elastic modulus at different depths of the cartilage and thus to distinguish changes within

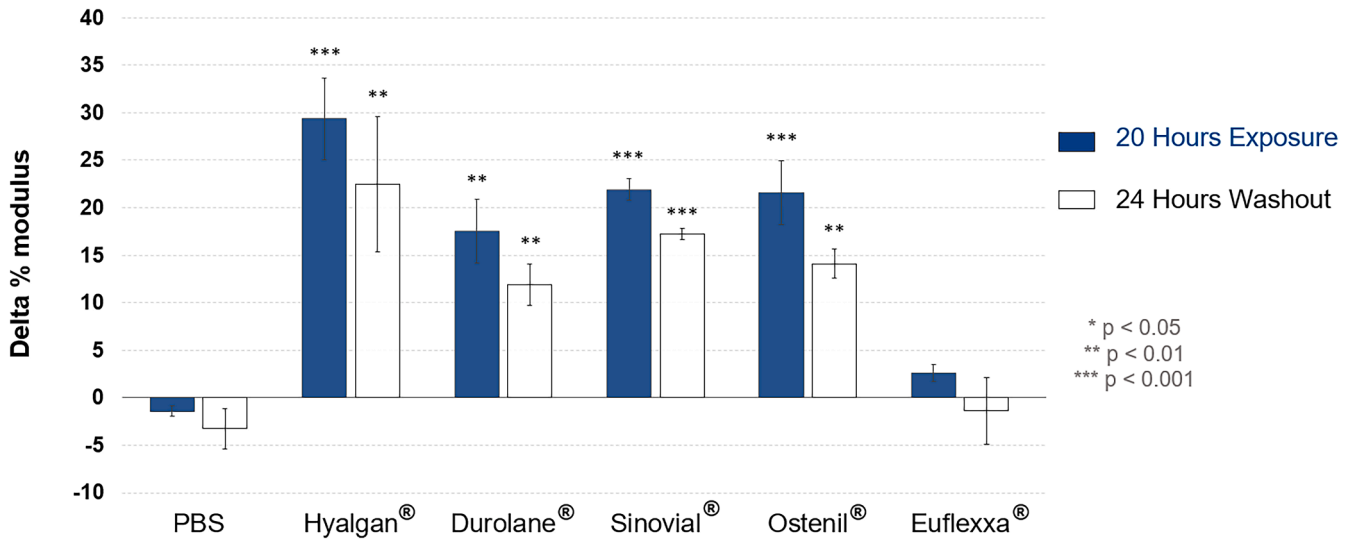

Fig. 4. Effect of hyaluronic acid-based products on intrinsic cartilage quality, as a result of mean over the three investigated zones. (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Table 3
Mean molecular weight of commercial products based on available information, number average (Mn) and weight average (Mw) molecular weight moments measured for 10x diluted in PBS and 0.2 μm filtered solutions, calculated polydispersity index (PDI) and percentage of chains smaller than or bigger than 1 MDa.

Product	Ref. MW (MDa)	Mn, Mw and PDI determined by SEC-SLS			% of chains below 1MDa	% of chains above 1MDa
Hyalgan®	0.5–0.73	Mn = 3.67.10^{e}5Da	Mw = 5.27.10^{e}5Da	PDI = 1.43	92	8
Durolane® (CL)	90	Mn =1.35.10^{e}5Da	Mw= 2.08.10^{e}5Da	PDI = 1.54	100	0
Sinovial®	0.8–1.2	Mn =7.09.10^{e}5Da	Mw = 9.67.10^{e}5Da	PDI = 1.36	61	39
Ostenil®	1.6	Mn = 1.00.10^{e}6Da	Mw = 1.33.10^{e}6Da	PDI = 1.33	39	61
Euflexxa®	2.4–3.6	Mn = 2.16.10^{e}6Da	Mw = 3.00.10^{e}6Da	PDI = 1.39	8	92

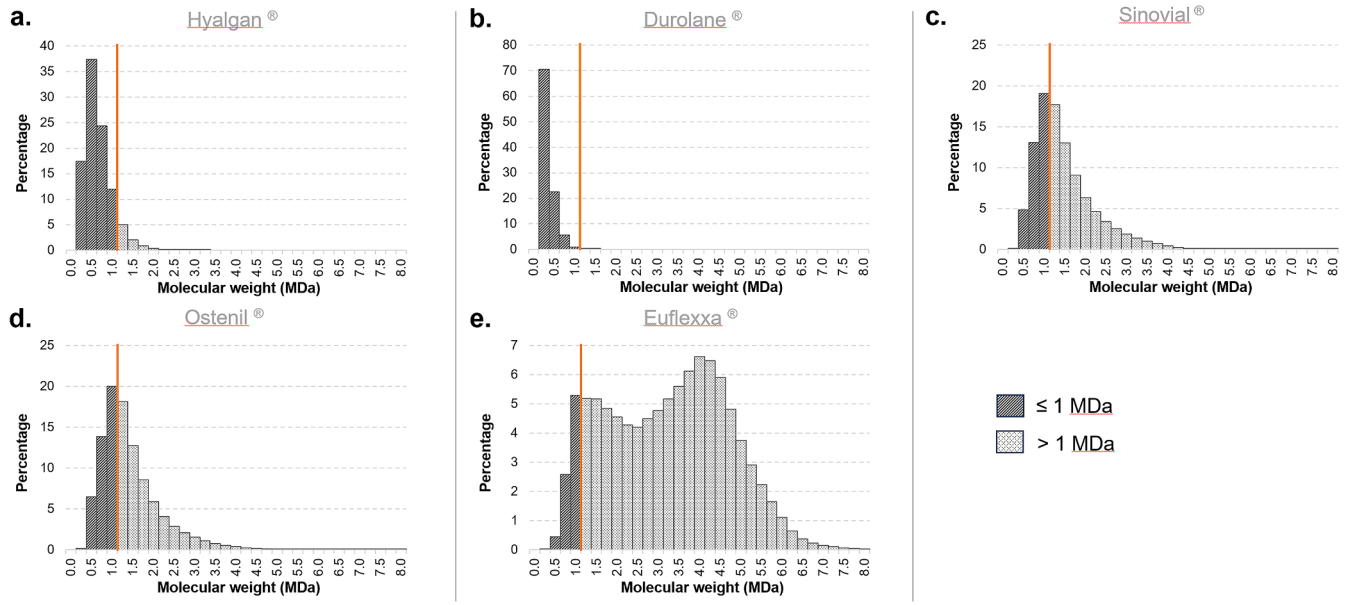

Fig. 5. Molecular weight distributions measured for 10x diluted in PBS and 0.2 μm filtered solutions obtained from a) Hyalgan®, b) Durolane®, c) Sinovial®, d) Ostenil® and e) Euflexxa®. Orange mark indicates 1 MDa. For an easier understanding of size distribution differences, scale was adapted for each substance.

the hyaline and mineralized cartilage. The monotonic approach enables us to assess cartilage quality at a given depth. The latter has the advantage of being faster and less expensive for systematic research. It is interesting to note that indentation characterization has also been used in recent proposals of *in-situ* crosslinked HA based biomaterial to heal damaged cartilage applying load of 0.25 N for 15 min [20–22], differing from fine-tuned forces used to characterize soft hyaline cartilage layers investigated here. Cartilage virtual histology evaluated by contrast enhanced computed tomography using ionic contrast agent such as Hexabrix® allows to assess modification of the subchondral trabecular and cortical bone, and of hyaline or mineralized cartilage at the site of indentation. The major advantage of this technology is to be

non-invasive, allowing investigation at the same localization of histology and mechanics.

It is globally agreed that the decrease in osteoarthritis pain after intra-articular injection of HA is due to improved rheology of the joint and to an anti-inflammatory effect [11]. Since HA completely disappears from intra-articular fluid after a week [23], these mechanisms cannot explain the long-term effect of intra-articular HA injections on pain over >6 months.

Here, we hypothesize that HA injected during viscosupplementation can penetrate the cartilage and contribute to the restoration of its mechanical quality. Confocal microscopy analysis showed that HA labeled with rhodamine 110 penetrates up to 30 μm into the cartilage after washout. A marked concentration of HA was observed near the compartments where the chondrocytes were located. As this study is performed on an *in-vitro* material and chondrocytes are necrotic, it is possible that HA could be accumulated in the cavity after chondrocytes necrosis. To our knowledge this is the first time that an investigation provides evidence of the penetration into non damaged cartilage of high MW HA. It is important to further investigate the effect of high MW HA on cartilage and to avoid the supply of pro-inflammatory HA chains [11]. Our findings are in-line with penetration obtained for oligosaccharides HA into damaged cartilage samples [20–22]. Our result is particularly of interest since HA is visualized after washout, which is in agreement with the rapid clearance of exogenous HA injected in the joint and could explain an improvement in the quality of the cartilage after incorporation in the cartilage tissue on long-term.

It is of interest to note that conditions used in this study allowed to characterize the effect on elastic modulus of HA exposure immediately after exposure as well as after washout of HA. In preliminary experiments, we extended the washout period to 48 h and obtained similar indentations values as after first 24 h washout period, suggesting the absence of additional elimination of HA between 24 h and 48 h. Based on this preliminary study indicating that the maximal washout could be obtained with overnight PBS washout, we chose the conditions of this study.

The measurement of elastic modulus at equilibrium, as intrinsic quality of cartilage, by cyclic bio-indentation systematically shows an increase in elastic modulus and a decrease in indentation depth for a given force. Among the variety of mechanical properties which can be investigated for soft tissues, such as Young's modulus, shear, complex, poison's ratio, yield strength, and ultimate strength [24], elastic modulus at equilibrium measured by indentation is less commonly used than Young's modulus (the measure of elasticity in tension or compression) and relies on complex mathematical model to retrieve mechanical properties. Theoretical understanding of cartilage tissues has been highly studied within the past decades [25–28] and remains a field of concern, not only in the field of tissue bioengineering.

Interestingly and without under-estimating the limitation of the mathematical models used to retrieve elastic modulus measurements [25,29], our results show that indentation is useful to characterize deformation underwent by soft tissues at small length scales. The ability to differentiate between viscosupplementation products effect underlines the interest in indentation method and its use to further characterize soft tissues. Both setups used in this study successfully fulfilled our aim to characterize soft tissues. Virtual histology confirms the absence of anatomic differences of cartilage after HA exposure and underlines the relevance of our results. This observation is a proof-of-concept of the effect of intra-articular HA injection in the modulation of cartilage quality. This information finally explains why, despite the clearance of the exogenous HA, the pain-relieving effect persists. Our findings suggest that the quality-modifying effect on cartilage of HA is physicochemical since the tested tissue is free of any cellular activity in our model. In addition, the results provide a better understanding on why the injection of hyaluronic acid may be ineffective when cartilage is destroyed, as for severe knee OA.

Complementary to the penetration evidence, SEC-SLS measurements provided an additional understanding on the differences between investigated products. We could conclude on a correlation between the cartilage quality improvement and the presence of chains of 0.25 - 1 MDa. Given the radius of gyration of about 200 nm of a HA molecule of 3 MDa [14], it can be expected that the coil-coil configuration of high MW chains prevents them from penetrating cartilage as the size of cartilage pores is of 7 to 10 nm [30,31]. In addition, mean MW, which is commonly characterize HA used in viscosupplementation products, appears as a limited factor to describe the size distribution of the chains.

We thus demonstrate that the effect on the quality of cartilage tissue depends on MW of the HA chains. Hyalgan®, the tested product with the lowest mean MW, demonstrates the best effects on the elastic modulus, while Euflexxa®, with the highest mean MW, has no effect. Products with an average MW of about 1 MDa to 1.3 MDa (Sinovial®, Ostenil®) have the same effect on the quality of cartilage. This observation underlines the limiting effect of high MW HA on its propensity to migrate into the cartilage. In the case of Durolane®, observed cartilage quality improvement can also be explained by the presence of 0.25 to 1 MDa HA chains. Surprisingly, chains measured for the solution obtained from Durolane®, were the smallest ones with MW of 200 kDA, suggesting that only relatively small fragments of chains had got out of a highly crosslinked network. The detection of chains upto 6 MDa for other solutions confirm that the absence of bigger chains in the case of the solution derived from Durolane® is related to the sample and not to detectability. In the case of such crosslinked product, for which a long half-time has been measured [32], it is envisioned that a progressive degradation may occur *in vivo* providing a regular release of medium or short HA chains, thus ensuring long-term efficacy of the product on the quality of the cartilage. Our results show the importance of chains of 0.25 to 1 MDa. It is of interest to note that *in vitro* assays have shown chondroprotective effect of 500–730 kDa HA [33,34]. The recently proposed categorization of viscosupplementation products based on MW as follows : low MW for MW <1MDa, medium MW for MW from 1 MDa to 3 MDa and high MW for MW > 3 MDa [15], independently of crosslinking, appears as a better defined and more appropriate classification than oligosaccharides *vs* high molecular weight and hyper-high molecular weight HA previously used [11,35]. Such classification has already been suggested in earlier studies [36,37], and mainly differs from classification proposed elsewhere by the absence of distinction between high MW and ultra-high MW, where ultra-high MW is used for MW greater than 10 MDa [38]. With the development of techniques such as SEC-SLS or more recently solid-state nanopore sensor [39], we can expect better characterization of HA distributions and new opportunities to understand viscosupplementation mechanisms of action.

Overall, these results suggest a good integration of HA into the cartilage tissue. In addition, if long-term pain relief can be caused by HA penetration into cartilage, such a long-term pain relief effect will depend on the degradation level of cartilage when patients are treated. This requires an adequate injection timing, whose need for further investigation has already been stressed in studies investigating *in vivo* the effect of viscosupplementation at the menisci level with emphasis on the restoration of mechanical properties [40], as well as in some clinical studies. Clinical studies also support the safety and benefits of multiple injections [7].

Since biophysical properties of the chondrocyte microenvironment play a causal role in OA pathogenesis [41], we can expect that the modification of the quality of the cartilage induced by hyaluronic acid could modulate the activity of chondrocytes towards a more anabolic function. Physicochemical alterations as reported in our study might be sufficient to modify the environment of chondrocytes and promote anabolic mechanisms without any pharmacological action.

5. Conclusion

Viscosupplementation has now been used for more than three decades in the case of osteoarthritis to relieve pain, however with a poor

understanding of its mechanism of action. After some controversial period, interest in such treatment is renewed but uncertainty persists since exogenous HA does only remain in the joint cavity for a short period of time (days) compared to claimed relief period (months).

Here, we investigate cartilage quality thanks to a bio-indentation technique. After some proof-of-concept results showing i) HA penetration at the level of cartilage tissue and ii) effect on cartilage quality illustrated by significant decrease of indentation depth and increase of elastic modulus using Ostenil®, we investigated the effect of commercially available products showing a variety of size distributions. This sheds light on the importance of chains of 0.25–1 MDa and confirms that viscosupplementation products can induce cartilage quality alteration, as initially hypothesized by Balazs et al.

Disclosure

EP, MG and ID are or were employees of TRB Chemedica at the time of this study. The authors report no other conflict of interest in this work.

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CRediT authorship contribution statement

Emilie Patois: Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Marie Gaumet:** Investigation, Funding acquisition, Conceptualization. **Isabelle Badoud:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Ivo Dellavia:** Methodology, Formal analysis. **Patrick Ammann:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

EP, MG and ID are or were employees of TRB Chemedica at the time of this study. The authors report no other conflict of interest in this work.

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Supplementary materials

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