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Viscoelastic evaluation of different knee osteoarthritis therapies

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This study deals with the viscoelastic evaluation of the efficacy of two ordinary knee osteoarthritis therapies: injections of exogenous high molecular weight hyaluronic acid and arthrocentesis. The results were also compared with "placebo" treatment. Synovial fluids were extracted from osteoarthritic knee joints of 60 patients before and after the different treatments and were analysed in a rheometer with coaxial cylinders arrangement at 37 °C. Frequency sweep experiments provided the values of shear storage modulus G' and shear loss modulus G'' as function of oscillation frequency. The quantified viscoelasticity gave information about the mechanical performance of synovial fluids and hence the long-term efficacy of osteoarthritis therapies. Moreover, the results were interpreted in terms of macromolecular structure and hyaluronic acid concentration and molecular weight in pathological synovial fluids.

1. Introduction

The performance of human articular joints is strictly connected with the viscoelastic properties of synovial fluid (SF), which determine load transmission, lubrication, wear inhibition and protection of the articular cartilage and soft tissue surfaces from mechanical stresses during joint function [1–3].

The viscoelasticity of SF mainly depends on the concentration, molecular weight and interactions with other molecules, especially proteins, of its major macromolecular component, hyaluronic acid (HA), naturally occurring mucopolysaccharide or glycosaminoglycan consisting of residues of D-glucoronic acid and N-acetyl-D-glucosamine [4, 5].

It has been reported that, because of superoxide ions action and reduced synthesis by synoviocytes, the HA concentration in SF and its degree of polymerization decrease in the course of osteoarthritis, a degenerative disease of the hyaline articular cartilage of synovial joints, which often culminates with the total loss of cartilage [6].

As a consequence of these modifications, the rheological properties of SF from arthrosic joints appear to be very different from those of normal ones [1, 7–9]: the reduction of SF viscoelasticity allows cartilage–cartilage contact which results in an increase of the surface wear.

Intra-articular injections of exogenous HA as sodium salt (Na-hyaluronate) were seen to be effective, in several clinical studies [10–13], in knee osteoarthritis treatment, because this application can normalize SF, reduce pain and improve joint function, decreasing inflammatory processes in the synovial tissue and promoting the reparative process of articular cartilage [14].

It has been proposed that the reported increase in HA concentration and intrinsic viscosity after hyaluronate injections is not due to the persistence of exogenous HA in the knee joint, but rather because the injected HA could stimulate the *in vivo* production of endogenous HA.

This study deals with the rheological evaluation of synovial fluids extracted from osteoarthritic knee joints before and after HA, arthrocentesis and placebo therapies in order to obtain some objective results about the efficacy of this specific treatment.

Moreover, it investigates the suitability of rheological experiments on SF to better characterize joint diseases.

2. Materials

The present rheological study was run in parallel with a clinical study [14]. In the clinical trial 100 patients (minimum age 40 years) were enrolled with a diagnosis of congenital or acquired knee osteoarthritis determined clinically and radiologically at least 6 months before the trial. In this work, the rheological investigation was performed on the synovial fluids extracted from the knee joints of 60 among the abovementioned patients. They were randomly assigned to three different treatment groups with the following schedule:

"Placebo" group: five intra-articular injections of 2 ml of physiological solution (placebo) after arthrocentesis with a 1-week interval between each injection.

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"Arthrocentesis" group: five arthrocentesis (aspiration of knee effusion) with a 1-week interval.
"Hyalgan®" group: five intra-articular injections of exogenous Na-hyaluronate as Hyalgan® 20 mg/2 ml after arthrocentesis with a 1-week interval between each injection.

Arthrocentesis with withdrawal of all fluid was carried out only at the time of first application (initial) and 1 week after the last injection (final) to carry out the rheological measurements on the collected synovial fluids, whose volume was at least 3 ml.

The exogenous HA was injected in the osteoarthritis knee joints as Hyalgan® (Fidia S.p.A., Abano Terme, Italy), whose active ingredient is a specific fraction of HA with a molecular weight between 500 and 730 KDa, a high degree of molecular definition and purity, obtained by extraction from rooster's combs.

3. Methods

The rheological properties of synovial fluids were evaluated on a Bohlin VOR Rheometer (Bohlin Reologi A B, Lund, Sweden) at a controlled temperature of 37 ± 0.1 °C.

The measuring system was the Bohlin "small sample cell" with coaxial cylinders geometry (Couette), which permits the rheological evaluation of low viscosity and low volume (about 1 cm^3) samples.

The outer cylinder (cup) is forced to rotate or oscillate, whereas the stress transferred from the fluid to the inner cylinder (bob) is measured by means of a linear variable displacement transducer (LVDT) system.

The synovial fluids after extraction from the knee joints of the patients, were centrifuged to separate cells and immediately stored at − 80 °C without preservatives. Before the test, the samples were allowed to reach room temperature and then put into the thermostated measuring cell to stabilize at 37 °C.

The non-linear flow properties of pathological SF were evaluated through steady shear measurements to determine the viscosity η as a function of shear rate $\dot{\gamma}$. The tests were carried out at different $\dot{\gamma}$ ranging from 10^{-1} up to 10^2 s^{-1}.

The small-amplitude oscillatory shear experiments allow the measurement of the unsteady response of a sample and hence the determination of its linear viscoelastic properties [15, 16].

The material is subjected to a sinusoidal shear strain:

$$\gamma = \gamma_0 \sin(\omega t)$$

where γ_0 is the shear strain amplitude, ω is the oscillation frequency and t the time.

The mechanical response expressed as shear stress τ of viscoelastic materials is out of phase with the imposed deformation and is given by

$$\tau = G'(\omega)\gamma_0 \sin(\omega t) + G''(\omega)\gamma_0 \cos(\omega t)$$

where $G'(\omega)$ is the shear storage modulus and $G''(\omega)$ is the shear loss modulus.

G' gives information about the elasticity or the energy stored in the fluid during deformation, whereas G'' describes the viscous character or the energy dissipated as heat during flow.

Simultaneous information about the fluid viscous and elastic behaviour as a whole are given by the absolute value of complex shear modulus G^* defined as

$$G^*(\omega) = \sqrt{G'^2 + G''^2}$$

or by the absolute value of complex viscosity η^* defined as

$$\eta^*(\omega) = \frac{\sqrt{G'^2 + G''^2}}{\omega}$$

In order to identify the linear viscoelastic response range of the materials, strain sweeps were performed on selected samples at three different oscillation frequencies (0.1, 1, 10 Hz), with a strain γ_0 varying logarithmically from 0.005 to 0.5.

The viscoelastic properties were measured by means of oscillatory frequency sweeps covering the physiological frequencies of knee movement, ranging from 0.5 Hz (slower knee movements) to 3 Hz (more rapid knee movements).

4. Results and discussion

The rheology of pathological synovial fluids was investigated through steady-state viscosity and small-amplitude oscillatory measurements in order to evaluate the flow behaviour and the related main material functions of this system [17].

The synovial fluid, a dyalisate of blood plasma, is a complex solution of hyaluronic acid, proteins, ions and other biological substances. It performs essentially two functions: biological, as nutritive to articular tissues, and mechanical, working as lubricant and shock-absorber.

The mechanical response of this material may be largely ascribed to the physical characteristics of hyaluronic acid and to its concentration, molecular weight and molecular weight distribution, even if the physical and non-covalent interactions within the molecule itself and with other molecules, especially proteins and ions present in solution with the formation of protein-mucopolysaccharide complexes and ion binded intermolecular associations, could not be neglected [18].

Another important characteristic influencing the rheology of SF, is the HA behaviour as an anionic polyelectrolyte [19], because of the presence of carboxyl groups, which are dissociated in solution. Therefore, in solution, hyaluronic acid behaves as an expanded random coil molecule and occupies a large hydrodynamic volume because of its chain backbone stiffness, the presence of intramolecular hydrogen bonding and the electrostatic repulsion between its charged groups [20–22]. At physiological concentration and pH, HA molecules overlap and interact through physical "entanglements" or temporary crosslinks and interactions with ions and other link proteins due to the polyelectrolyte nature. These

interactions determine the formation of a transient network structure that is responsible for the viscoelasticity of synovial fluid.

The viscous behaviour is prevalent at low deformation rates (rest and walk) reducing wear due to cartilage contacts, whereas elasticity prevails at high shear rates (run) determining a rapid elastic response to mechanical stresses and preventing articular tissues from compressive damage.

4.1. Steady-state viscosity behaviour

The log–log plot of steady shear viscosity η as function of shear rate $\dot{\gamma}$ for a typical pathological SF is represented in Fig. 1. The marked dependence of η or $\dot{\gamma}$ showed by normal as well as pathological SF is typical of shear thinning (pseudoplastic) polymeric solutions, showing a constant value at low shear rates (first Newtonian plateau) η_0, the zero-shear viscosity, and a subsequent decrease that is well described by the "power law" model:

$$\eta = K\dot{\gamma}^{n-1}$$

However, the non-Newtonian viscosity of pathological SF is much lower than normal SF [9], showing a zero-shear viscosity η_0 in the range 0.01–1 Pa s (normal value $\approx$ 10 Pa s), the consistency K of the power-law model varying from 10^{-2} to 10^{-1} Pa s^n (normal value $\approx$ 1 Pa s^n) and the exponent n in the range 0.5 to -0.1 (normal value ≈ -0.5).

Since the presence of intermolecular interactions is a constraint to flow, and hence favours viscous dissipation, the decrease of η at increasing $\dot{\gamma}$ could be explained in terms of the progressive decrease of steady-state entanglement density [23].

At low shear rates, the uncoupling of HA macromolecules due to flow is balanced by the coupling induced by random molecular thermal motions, so the average number of entanglements remains constant with time and hence the viscosity maintains a constant value η_0.

At increasing deformation rates, the imposed deformation determines the uncoupling of macromolecules no longer balanced by the rate of formation of new entanglements by Brownian motions. In other words there is a decrease in non-covalent or physical cross-link density and a consequent viscosity reduction typical of shear thinning behaviour.

In the same Fig. 1 we compared, for a pathological SF, the data of complex $\eta^*(\omega)$ and steady shear viscosity $\eta(\dot{\gamma})$, that, with the exception of a slight departure at high frequencies or shear rates, agree very well following the Cox–Merz rule [24]:

$$\eta(\dot{\gamma}) = \eta^*(\omega)|_{\omega=\dot{\gamma}}$$

However, the evaluation of the true mechanical response of a polymer solution and the identification of its structure are better accomplished by small-amplitude oscillation tests rather than steady-state measurements which may promote distortion of the macromolecules and structure breakdown because of large deformations [25, 26]. Moreover, joint motions, being unsteady and time dependent, are better represented by dynamic measurements.

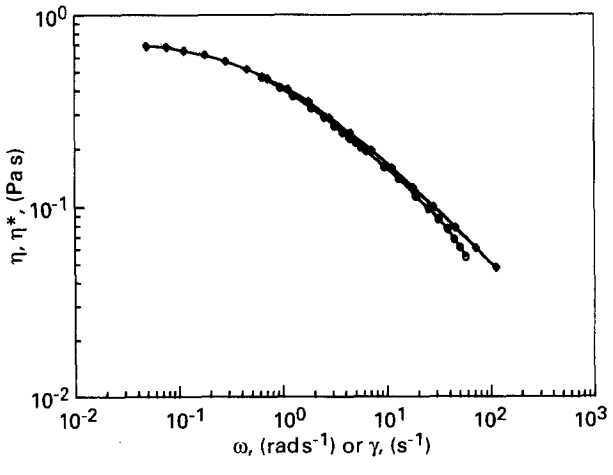

Figure 1 Steady shear viscosity η (–◆–) and complex viscosity η^* (–○–) versus oscillation frequency ω or shear rate, $\dot{\gamma}$, for an osteoarthritic synovial fluid.

4.2. Linear viscoelastic behaviour

Polymer solutions display linear viscoelastic behaviour when the imposed deformation or rate of deformation are sufficiently small in such a way that the mechanical response of the material as stress is directly proportional at any time to the imposed deformation. Under these circumstances, the viscoelastic moduli are independent of strain or strain rate.

Moreover, in linear viscoelasticity, the tension state in the material can be represented only by shear stress, because normal stresses, generally arising in viscoelastic materials, becomes neglectable [16, 23].

The amplitude of the imposed deformation in frequency sweep experiments must be chosen appropriately to ensure that the mechanical response of the material under investigation is within the limits of linear viscoelasticity.

The practical identification of linear viscoelastic behaviour of pathological SF was accomplished through the evaluation of viscoelastic moduli at different strain amplitudes and at constant frequency. Strain sweep experiments at three different oscillation frequencies (0.1, 1, 10 Hz) were performed on selected samples.

The results obtained showed a constant value of G' and G'' over the full range of strain amplitudes allowed by the instrument; for this reason the choice of a strain amplitude of 0.5 for the oscillation tests allowed the investigation of linear viscoelastic behaviour of SF.

The linearity of viscoelastic moduli is exemplified in the plot of normalized complex modulus G^*/G_0^* against strain amplitude γ_0 at 1 Hz frequency (Fig. 2), where G_0^* is the complex modulus extrapolated at zero strain.

The viscoelastic behaviour of normal SF is typical of temporary polymer network solutions, i.e. of solved macromolecular random coils linked by transient junctions: a viscous liquid-like behaviour for slow deformations or low oscillation frequencies and a

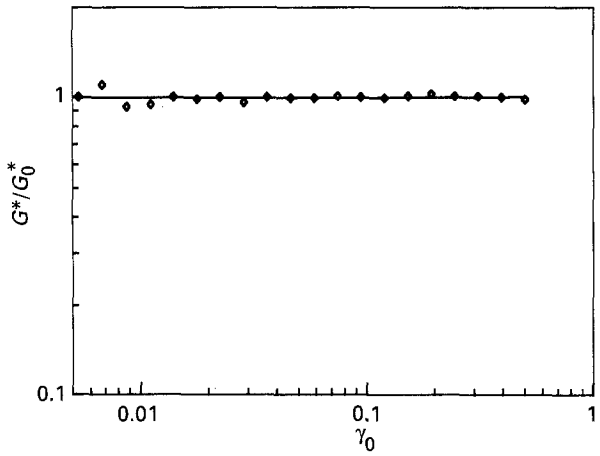

Figure 2 Normalized complex modulus G^*/G_0^* versus strain amplitude γ_0 for an osteoarthritic synovial fluid.

rubbery solid-like behaviour for rapid strains or high frequencies.

The viscous dissipation under strain is generally related to the friction arising from three different interactions:

1. A friction caused by the interactions of single HA molecules with the medium (solvent and other solutes) and by the hydrodynamic interactions among the flow fields of chain segments of single HA molecules. It is proportional to the concentration and the hydrodynamic volume of HA and can be represented by the coil overlap parameter $c[\eta]$, where c is the HA concentration and $[\eta]$ is the intrinsic viscosity (which is generally proportional to M^a, where a is less than unity). This behaviour is typical of dilute polymeric solutions.
2. A friction arising among intermolecular contacts during chain slipping. This contribution to the viscous dissipation depends on the number of intermolecular contacts per molecule and is proportional to the product cM, where M is the molecular weight of the HA polymer. This situation holds for concentrated polymeric solutions with a molecular weight less than a critical value M_c.
3. A friction connected with the formation of entanglements when, in concentrated solutions, the polymer molecular weight exceeds the critical value M_c. In this case, the viscosity is proportional to cM^a, with the exponent a generally found, both theoretically and experimentally, to be equal for most polymers to 3.5.

The elastic component of the mechanical response in these systems can be interpreted in analogy with the rubber elasticity theory, with the difference that the crosslinks are not permanent as in rubber loose networks, but transient, being formed and destroyed continuously because of thermal molecular motions. The chain segments between junctions, under an imposed deformation, are subjected to configurational changes. The intramolecular forces, arising in the deformed chain strands which tend to return to their most probable undisturbed configuration, are responsible for the entropic elasticity of the system. This elasticity is proportional to the instantaneous density of junctions or the number of chain strands per unit volume.

At low strain frequencies and hence for long deformation times, the HA macromolecules have enough time to readjust themselves to their original conformation through the disentanglement of chain segments, one past another. Under these circumstances, the material response is essentially viscous.

On the other hand, when subjected to rapid deformations, the single HA chain strands formed between temporary junctions and without topological obstacles to movement, can adjust very quickly giving rise to the overall elastic response of the material. Frequency sweep measurements allow the evaluation of the mechanical response and of the structure of these solutions.

The viscoelastic behaviour is in fact well represented by the elastic and viscous dynamic moduli as function of oscillation frequency. The qualitative behaviour of G' and G'' for typical normal and pathological synovial fluids is represented in Fig. 3, derived from Balazs [1].

Normal SF present prevalent viscous behaviour at low frequency ($G'' > G'$) and prevalent elastic behaviour at high frequencies ($G' > G''$), the limit between the two regions being represented by the crossover frequency ω_c, which typically moves to higher frequencies as solutions become more dilute and structure disappears [25].

The SF from a young subject has lower ω_c and higher absolute values of G' in all the frequency range and of G'' (with the exception of a small range at higher frequencies) than one from an old subject, showing a reduction in HA concentration or molecular weight with age, even if the overall functionality of SF is substantially not affected. Moreover pathological SF present reduced absolute values of G', G'' and $G'' > G'$ over the whole frequency range under investigation, indicating that there are few interactions among HA molecules and the structure disappears, the rheological properties being now essentially controlled by intramolecular conformational changes affecting hydrodynamic molecular size. In this way, the mechanical response of SF cannot cope with compression and tangential forces arising in everyday life, allowing

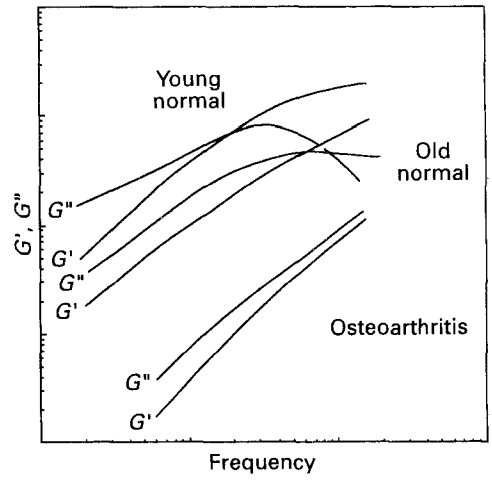

Figure 3 Qualitative frequency sweep plot of shear storage modulus, G', and shear loss modulus, G'', for synovial fluid of young normal, old normal and osteoarthritic knee joints (derived from Balazs E.A. [1]).

cartilage–cartilage contact and increasing wear of the surfaces.

4.3. Rheological properties of pathological synovial fluids after different osteoarthritic therapies

Rheological properties of synovial fluids from osteoarthritic knee joints were evaluated before and after three different therapies, in order to assess their long-term efficacy:

1. Arthrocentesis; withdrawal of inflamed SF.
2. Hyalgan®; injections of exogenous HA.
3. Placebo; injections of physiological solution.

Preliminar measurements were performed on few samples of pathological synovial fluids (not belonging to the above-mentioned groups) before and after storage at − 80 °C to ascertain that the refrigeration had no influence on the viscoelastic properties of these fluids. The experiments were carried out a short time after aspiration from the knee with the samples maintained at room temperature and, on the same samples, ten days after storage at − 80 °C. The results obtained showed that there was no significative modification to the viscoelastic properties of SF after freezing as illustrated by the dynamic curves of one such sample in Fig. 4.

The viscoelastic evaluation of the efficacy of the different therapies was essentially based on the analysis of SF frequency sweeps. Dynamic moduli for SF before treatment were characterized by values of G'' always higher than G', indicating the presence of a dilute system and an overall reduction of rheological properties respect to normal values. After treatment it was possible to note three different conditions, in order of more-structured organization and consequent increasing viscoelasticity of the material:

1. no change or slight reduction in viscoelasticity (Fig. 5a);
2. increase in G' and G'' absolute values, but no crossover frequency (Fig. 5b);
3. increase in G' and G'' absolute values, with the presence of a crossover frequency (Fig. 5c).

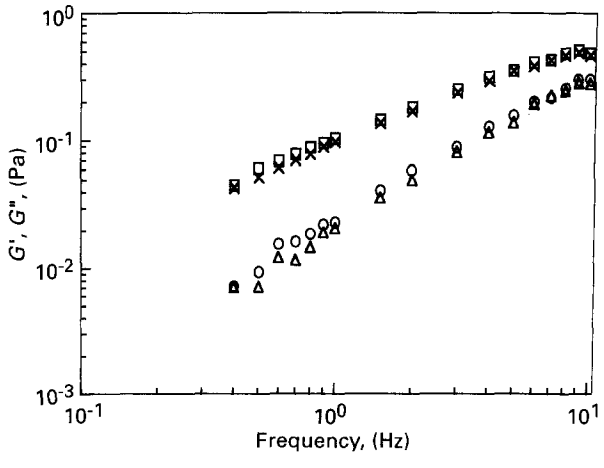

Figure 4 Dynamic moduli G' and G'' of a pathological synovial fluid before and after storage at − 80 °C: –○– G' before; –□– G'' before; –△– G' after; –✳– G'' after.

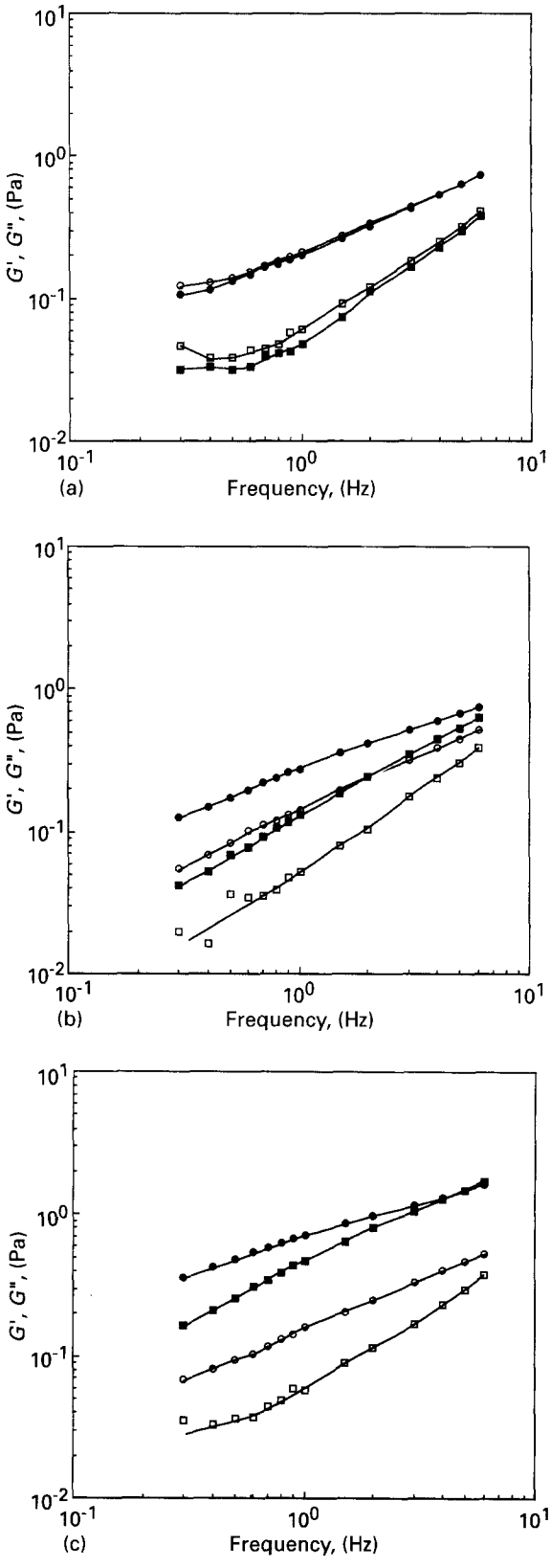

Figure 5 Frequency sweep plots of G' and G'' for three pathological synovial fluids before and after therapy: –□– G' initial; –■– G' final; –○– G'' initial; –●– G'' final.

It is clear, from the above discussions, that increased viscoelasticity leads to better functionality of SF and hence to reduction of cartilage wear and damage. Moreover the fluids before treatment presented very different absolute values of dynamic moduli, suggesting different degrees of inflammation. For this reason it was not possible to deal with absolute values, but it was rather preferred to analyse the variations relative

to initial values, in terms of:

$$\Delta G = \frac{G_{fin} - G_{in}}{G_{in}}$$

where G_{in} and G_{fin} are, respectively, G' or G'' of SF before and after treatment.

The variations ΔG were evaluated at every investigated frequency for the three different therapies. As an illustration we present in Figs 6 and 7, respectively, the plots of the probability function $f(\Delta G)$ and of the distribution function $F(\Delta G)$ versus the variations ΔG in G' and G'' at a given frequency (3 Hz) for a treatment group (Hyalgan). Because of the non-symmetric character of the ΔG distribution, as a measure of central tendency, the median value $\tilde{\Delta}G$ [27] was obtained as solution of the equation:

$$F(\Delta G) = \tfrac{1}{2}$$

where $F(\Delta G)$ is the constructed distribution function of the distributions under consideration.

The $\tilde{\Delta}G$ values for G' and G'' in the range of frequencies investigated are reported in Table I and as histograms in Fig. 8a and 8b.

The medians of G' and G'' for "placebo"-treated SF vary roughly in the range $\pm$ 10%, indicating, as expected, no significant variations with respect to initial conditions.

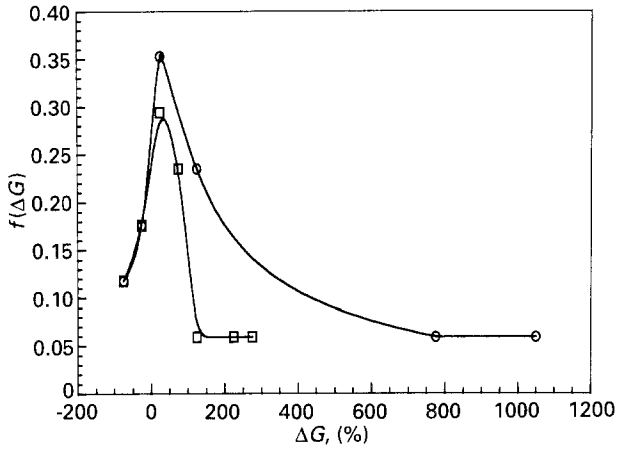

Figure 6 Probability function f versus percentage variation ΔG of dynamic moduli (–○–) G' and (–□–) G'' with respect to initial values for Hyalgan®-treated group at oscillation frequency of 3 Hz.

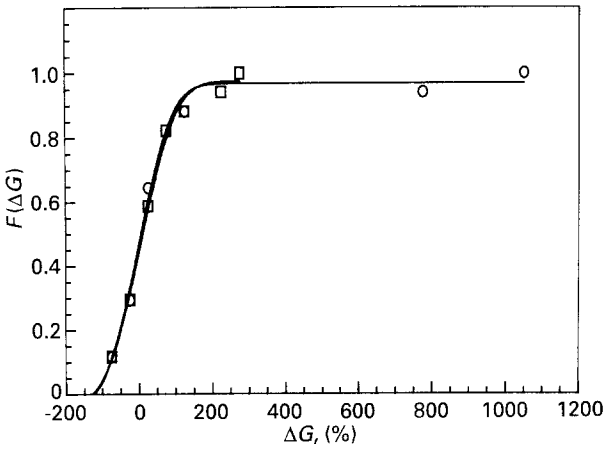

Figure 7 Distribution function F versus ΔG constructed from the probability function f of Fig. 6: –○– G'; –□– G''.

Referring to "arthrocentesis" therapy, there is a significant positive variation in elasticity, whereas an evident decrease in viscous behaviour characterizes almost all the frequency range. This somewhat atypical behaviour could be explained as follows. The viscous dissipation in SF is strongly dependent on HA concentration and molecular weight, but only in a slight manner on HA complexation. On the other hand, the elasticity, being related to HA dynamic interactions, is strongly dependent on the presence of HA/protein complexes [4]. On this basis, we can assume that, after arthrocentesis, a decrease in HA concentration and MW and an increase in concentration of proteins and phospholipids interacting with HA could occur [28, 29]. In other words, there is no stimulation of endogenous HA production by synoviocites and an increase of proteic fraction in SF due to poor synovial barrier [30].

"Hyalgan®" treated SF presents positive variations in both G' and G'', generally higher than 20%, with a maximum of up to 80% for the elastic modulus at 0.6 Hz. From these results, an increase in HA concentration and MW can be deduced.

TABLE I Percentage variation in median values $\tilde{\Delta}G$ for G' and G'' relative to synovial fluids after three different therapies.

Frequency (Hz)	Arthrocentesis		Hyalgan®		Placebo	
	$\tilde{\Delta}(G')$ (%)	$\tilde{\Delta}(G'')$ (%)	$\tilde{\Delta}(G')$ (%)	$\tilde{\Delta}(G'')$ (%)	$\tilde{\Delta}(G')$ (%)	$\tilde{\Delta}(G'')$ (%)
0.4	15.18	– 15.30	41.60	37.79	– 2.99	5.37
0.5	46.02	– 15.64	29.24	28.33	– 8.01	10.22
0.6	63.59	– 9.75	82.09	27.22	– 10.75	11.44
0.7	8.44	– 11.89	29.36	16.25	– 0.07	– 4.03
0.8	19.97	– 6.65	38.12	16.45	27.29	12.96
0.9	12.96	– 14.83	30.97	20.09	– 10.86	– 7.43
1	24.52	– 9.07	20.91	33.81	– 13.62	– 1.46
1.5	26.07	– 7.17	18.96	13.44	4.30	– 0.26
2	28.28	– 5.10	18.97	20.00	– 3.75	2.94
3	5.33	– 5.13	11.79	11.25	– 1.6	4.05
4	0.00	3.73	11.00	9.52	– 0.6	2.23

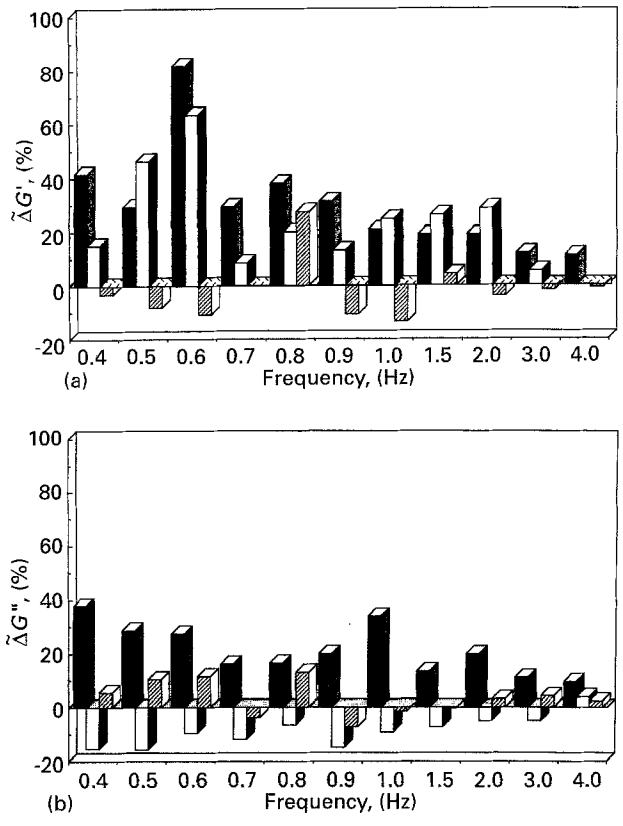

Figure 8 Histograms of percentage variation in median values $\tilde{\Delta}G$ for shear storage modulus G' (a) and shear loss modulus G'' (b) versus oscillation frequencies relative to synovial fluids after three different therapies: ■ Hyalgan; □ Arthrocentesis; ▨ Placebo.

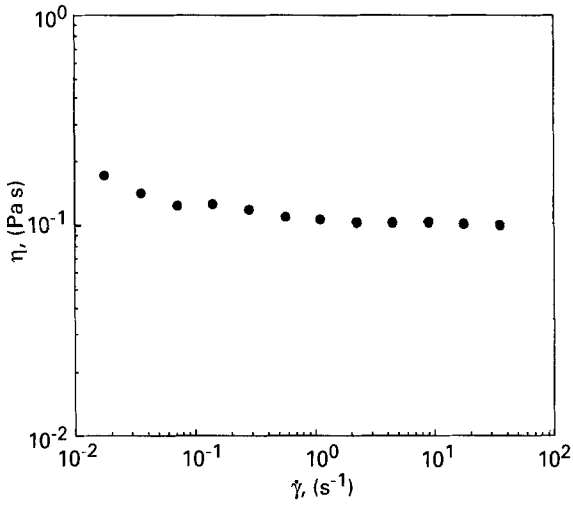

Figure 9 Steady-state viscosity as function of shear rate for Hyalgan®.

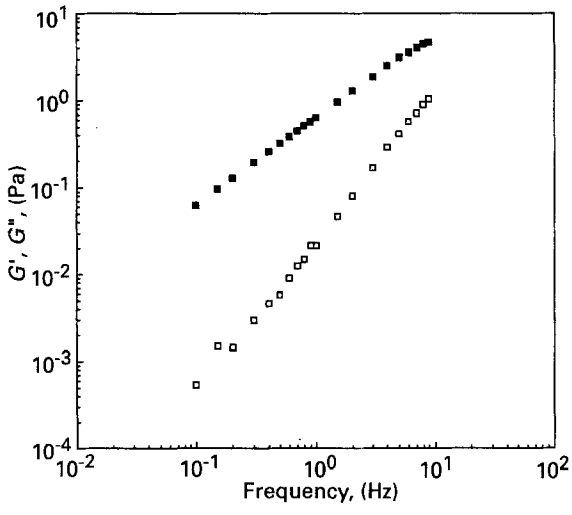

Figure 10 G' (□) and G'' (■) as function of oscillation frequency for Hyalgan®.

Moreover, the hypothesis that exogenous HA, despite its short life in the joint, enhances, as *in vitro* [31], the *in vivo* production of higher molecular weight HA by synoviocites and hence the normalization of SF and joint function, can be also confirmed by rheological analysis. In fact Hyalgan® has an essentially viscous behaviour as represented by the Newtonian character of viscosity (η constant with $\dot{\gamma}$) (Fig. 9) and the prevalence of G'' over G' in oscillation tests (Fig. 10) with absolute values of viscoelastic moduli less than the corresponding values for pathological synovial fluids. It appears from the above considerations, that the emmision of exogenous HA could not account, by itself, for the lasting increase in SF viscoelasticity, but is fundamental to the promotion of new HA synthesis.

5. Conclusions

Rheological analysis of pathological synovial fluids gives a set of useful information for the evaluation of different knee osteoarthritis therapies. This technique allows investigation of the mechanical behaviour and of the macromolecular structure of these complex biological solutions. The quantified viscoelasticity of SF is a measure of its *in vivo* mechanical functionality and can be related to hyaluronic acid concentration and molecular weight.

The increase in dynamic elastic and viscous moduli of exogenous HA-treated SF is a measure of its normalization, confirming the results of clinical studies.

The reduction in viscous dynamic modulus of arthrocentesis-treated SF could be ascribed to HA concentration and MW decrease, whereas the increase in elasticity is proposed to be related to the formation of new interactions of HA with other molecules, especially proteins.

Acknowledgements

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