

The effect of hyaluronic acid and phospholipid based lubricants on friction within a human cartilage damage model

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Abstract

The lubricating abilities of different formulations of high molecular weight hyaluronic acid (HA), dipalmitoyl phosphatidylcholine (DPCC) and mixtures of both HA and DPCC were assessed in an in vitro model. Levels of start-up friction were determined using an osteoarthritis (OA) damaged human cartilage model set within a specially designed friction rig. To examine the long term benefits of HA, the extent of penetration of HA into cartilage tissue was investigated using fluorescently labelled HA and confocal microscopy. It was found that in this model, all formulations of HA and the majority of DPCC lubricants reduced friction (HA 5 and 10 mg ml⁻¹, DPCC 200 mg ml⁻¹ reductions of 51.9%, 46.7% and 46.5% respectively), compared to a Ringers solution control. Lubrication was found not to be concentration dependant for HA formulations, but concentration was key for DPCC lubrication (100 mg ml⁻¹ reduced friction by only 15.9%). By combining HA and DPCC (HA/DPCC; 5 mg ml⁻¹/100 mg ml⁻¹ and 10 mg ml⁻¹/200 mg ml⁻¹), a further improvement was noted (69.5% and 61.9%, respectively) as the mean levels of friction were reduced by up to a further 17% than the most effective individual formulation (HA 5 mg ml⁻¹). Penetration of HA into bovine cartilage by up to 300 µm from the surface was observed over a 48 h period. It was observed that HA specifically targeted the chondrocytes as it was primarily found within the lacunae surrounding the cells.

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1. Introduction

Osteoarthritis (OA) is a degenerative and debilitating disorder of diarthrodial joints affecting approximately 70% of 70 year olds and a total of 8.5 million people in the UK [1]. It is associated with progressive damage of articular cartilage leading to considerable pain resulting in loss of mobility and the requirement of continuous healthcare for patients [1]. There is evidence that OA is associated with systemic features such as genetic inheritance, oestrogen deficiency and high bone mineral density levels. In addition, biomechanical factors have been implicated in the development of the disease, for example weakness of the muscles surrounding the joint, obesity, loss of lubrication and joint laxity. Some of these biomechanical

factors may occur due to the normal aging process and as a community-based survey has shown, the incidence of OA increases by 2–10 fold from the ages of 30–65, and increases further after the age of 65 [2] indicating that OA is age related.

Treatment options aim to return the affected joint to an acceptable level of mobility. The mainstay of current treatment is providing pain relief to try and delay the eventual need for total joint replacement (TJR). However, the demand for TJRs by younger patients has increased, and current prostheses have limited life-spans leading to the need for revision surgery after 10–25 years. Hence there is a need for new, effective treatments for the younger OA patients.

The natural joint is lubricated by synovial fluid (SF), which is highly viscous, enabling it to cushion and lubricate the joint. Hyaluronic acid (HA) is a major component of SF and articular cartilage and plays an important role in

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lubrication of the cartilage surface in addition to helping maintain the structural resistance of cartilage to compressive forces. The high molecular weight of SF HA has been suggested to be important in its lubricating ability [3,4]. In OA affected joints, HA has a reduced molecular weight [5]. Whether this is a consequence or a cause of OA is uncertain.

Within recent years, a new form of therapy, viscosupplementation, has been introduced to treat OA of the knee. It involves the replenishment of the SF via intra-articular injections of a therapeutic HA lubricant. It has been shown that the molecular weight of HA is crucial and the range required to illicit a beneficial response is between 500 and 3800 kDa [3,4]. HA viscosupplementation of SF is believed to restore viscoelasticity and the lubricating ability to SF and therefore assist in protecting the cartilage surface [4]. In addition to these properties of HA, it has been suggested that it may have other beneficial effects. A study by Mensitieri et al. [6] showed that supplementation with high molecular weight HA enhanced the rheological properties of SF for a period longer than the injected HA's known half-life. This may suggest prolonged effects of HA within the joint. This has been supported by other studies showing that HA can induce stimulation of HA synthesis by synovial cells and chondrocytes, stimulate chondrocyte growth and inhibit cartilage degradation [3,7–9].

Studies conducted over the past five years have indicated that HA based therapeutic intra-articular injections over a 5 week injection course have benefits for up to 6 months. These benefits include pain relief as effective as nonsteroidal anti-inflammatory drugs and increased function of the joint [10–13]. The use of these therapies is believed to be mainly beneficial in the early and moderate stages of OA whilst there is remaining cartilage cover of the articulating surfaces. However, despite this evidence, this form of therapy is still not universally accepted as a treatment for OA [14,15] and as such is not widely used.

There are three approved HA based therapeutic lubricants commercially available in the US specifically for the treatment of OA of the knee. These three lubricants are: Hyalgan[®] (Sanofi-Syntheolabo Inc.) and Supartz[®] (Smith & Nephew Inc.) which are naturally extracted and purified forms of sodium hyaluronate and Synvisc[®] (Wyeth-Ayerst Pharmaceuticals) which is a derivative of hyaluronan, hylan-G-F20.

It has long been suggested that phospholipids are responsible for the boundary lubrication of articular cartilage surface [16]. This property has been specifically attributed to a particular phospholipid, dipalmitoyl phosphatidylcholine (DPPC), which has been demonstrated to reduce friction on articulating surfaces under boundary lubricating conditions [16–18]. In a recent *in vivo* study, it was shown that the addition of DPPC liposomes to high molecular weight HA showed greater lubricating ability and better protection of the cartilage tissue than HA alone [19]. These studies suggest the importance of lipids in the lubrication of articular cartilage and have highlighted the

potential of DPPC as a beneficial additive to HA based therapeutic lubricants.

The aims of this investigation were to determine the ability of HA, DPPC and mixtures of HA and DPPC to reduce start-up friction in an OA damaged human cartilage model, since the highest values of friction occur at the start up of motion after prolonged loading. In addition, the extent of penetration by HA into the cartilage tissue was examined using fluorescently labelled HA and confocal microscopy.

2. Methods

2.1. Cartilage samples

Tibial plateaus and femoral heads were obtained from patients undergoing total joint replacement surgery under the care of Mr. M.H Stone, Consultant Orthopaedic Surgeon, Leeds General Infirmary. The samples were collected with full consent from the patients and with local ethical approval. Cartilage pins (5 mm diameter) with a complete covering of cartilage were removed from the femoral heads using a specially designed pin cutter. The tibial plateau, once collected, was trimmed of excess tissue and bone. In order to remove residual fat and create uniform samples, both the plateau and pins were treated in chloroform:methanol (2:1) solution using three 10 min washes in 250 ml of solution and rinsed in Ringers solution between each wash.

2.2. Grading of cartilage samples

The tibial plateau was divided into sections approximately 10 mm × 5 mm in size (i.e. an area large enough for a sliding friction test) using a grid. The damage was assessed according to of the guidelines of the International Cartilage Repair Society (ICRS) cartilage injury evaluation package [20]. The damage was graded according to the depth of cartilage removed and any resultant fibrillation. Grades 0–4 were used in which 0 represented healthy cartilage and 4 represented cartilage that had been worn to the bone. Grades 1 and 2 were areas of low damage and grades 3 and 4 were areas of high damage. It was found that the majority of areas available for testing (i.e. flat regions), had high levels of damage. Therefore effective comparisons of lubricant formulations could only be made using highly damaged cartilage samples (Grades 3 and 4) using this patient group.

2.3. Start-up friction measurements

2.3.1. Apparatus

In order to obtain the start up of friction values from articulating cartilage, a pin on plate friction rig was developed specifically for this study and designed to accommodate large human tibial plateaus. The friction rig is shown in Fig. 1.

2.3.2. Method

A putty mould (using Labrosil-70; Dreve-Dentamid GMBH, Umma, Germany) was made of the tibial plateaus. This was to help create a flat test surface and secure the plateau during testing. The putty mould was secured to a metal plate using an adhesive. The plate, with the putty mould and the tibial plateau attached, was placed into the metal tray and secured using a series of clamps to prevent any movement of the sample during measurements. The tray was secured to the sliding platform and positioned to allow full contact between the cartilage pin and the test area on the plateau. The cartilage pin was fixed in a holder ensuring that the pin sample protruded 5 mm from the holder with the cartilage surface exposed. The cartilage pin was held securely perpendicularly to the plate allowing the cartilage surfaces to come into contact, preventing any

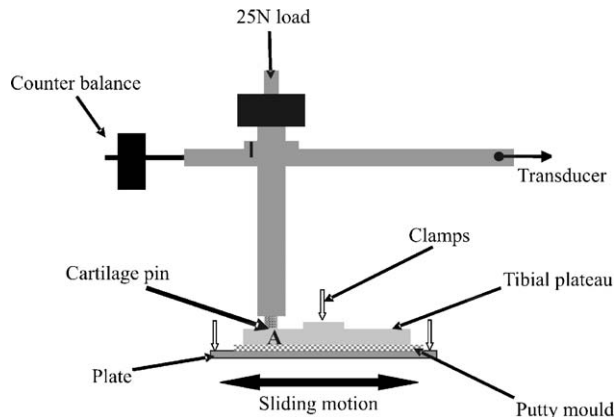


Fig. 1. Schematic diagram of the friction rig—sample section. A—The area of contact between cartilage pin and plate. The sample section was connected to the drive via a crank shaft which allowed controlled movement of a sliding platform. Fixed to the sliding platform was the sample tray. The pin holder was suspended above the sample via a bridge and kept level using a counter balance. The load was rested on top of the pin holder during tests. The transducer detected vibrations produced by any sliding friction which were then transformed into an electrical signal, amplified and read as a voltage using an oscilloscope.

movement of the pin or the holder which could create false readings. Once in position, a 25 Newton load was placed directly onto the cartilage pin holder therefore applying a pressure of 1.3 MPa across the pin's surface area. This arrangement was left for a period of 10 min. Once the 10 min period had elapsed the rig was run at a sliding motion of 4 mm s^{-1} . The friction measurements were read as peak voltage readings from an oscilloscope after approximately 2 s. The conditions for determination of the start-up friction have been validated in previous studies [21,22].

A standard linear relationship between mass and voltage was established prior to any testing. By using the equation derived from the straight line graph ($y = mx + c$) it was possible to determine the friction coefficient from the voltage measurements taken during tests using the equation:

$$\mu = \left[\frac{y - c}{m} \right] / F,$$

where μ is the coefficient of friction, y the voltage, c the X -axis intercept, m the gradient and F the load (25 N).

2.4. Formulation of lubricants

2.4.1. HA lubricant

Arthrase[®] and a sample of dry HA was kindly donated by DePuy International (UK). Arthrase[®] is a 10 mg ml^{-1} gel of bio-synthetically produced HA with an approximate molecular weight of 3 million Da, supplied in 5 ml syringes.

2.4.2. Preparation of DPPC liposomes

Concentrations of 100 and 200 mg ml^{-1} of DPPC (Lipoid, Germany) were made using the following protocol. DPPC powder was weighed to appropriate amounts and placed in a 50 ml, sterile, plastic conical tube with a screw top closure. The DPPC was dissolved in ethanol (30 ml). The solution was transferred to a pre sterilised 500 ml round bottom flask. Using a rotary evaporator with a 56°C water bath, the excess ethanol was reduced to yield a thin and even white coating on the inner surface of the flask. The DPPC was rehydrated by adding the appropriate amount of phosphate buffered saline (PBS) solution. The flask was heated at 56°C until the DPPC was fully removed from the inner surface of the flask and a white fluid suspension of liposomes was formed. Once hydration was

complete, the liposomes were kept at 56°C prior to any addition of HA or stored at 4°C .

2.4.3. Addition of HA to DPPC liposomes

To produce the mixture of HA and DPPC at a concentration of $5 \text{ mg ml}^{-1}/100 \text{ mg ml}^{-1}$, the 10 mg ml^{-1} solution of HA (Arthrase[®]) was added to 200 mg ml^{-1} of DPPC. The HA solution was incubated at 56°C for 5 min to equilibrate and then an equal volume of the HA solution was carefully added to the prepared DPPC suspension. For the higher concentrations (10 and 200 mg ml^{-1}) a corresponding amount of dry HA was added to a 200 mg ml^{-1} DPPC suspension. The mixtures were placed into a 56°C water bath for 15 s followed by vortex mixing until no agglomerations were observed in the suspension. At the end of the process, all mixtures were stored at 4°C .

2.4.4. Lubricant formulations and test procedure

The different lubricant formulations of HA and DPPC tested for their effect on the start-up friction coefficient are listed in Table 1. Individual tests for each lubricant were carried out using a flat area of the tibial plateau with a matching cartilage pin. Each lubricant was tested on six different areas using different cartilage samples. Ringers solution was used as a control test lubricant to determine if any change in friction occurred over time. Ringers solution was used as the control lubricant since it has previously been demonstrated that there is no significant difference in the start-up friction in experiments set up under boundary lubrication conditions between Ringers solution and SF [21]. When operating under boundary lubrication conditions at start up of motion, the viscosity of the lubricant has been shown to have a limited effect. Under these conditions friction is determined by the intrinsic biphasic lubrication properties of the cartilage and the presence of additional boundary lubricants [22].

2.4.5. Start-up friction test protocol

The area for testing on the tibial plateau was selected. A suitable cartilage pin (removed from a femoral head) was selected to match the test area to provide complete contact between the two cartilage surfaces. The cartilage samples were soaked in a Ringers solution at 4°C for a period of 24 h. The cartilage samples were placed into the friction rig and secured. Fresh Ringers solution was added (approximately 40 ml) and five repeat measurements were made. The cartilage samples were removed and approximately 1 ml of test lubricant was added to each cartilage surface. The samples were placed into a container and left to soak at 4°C for a period of 24 h. Approximately 5 ml of Ringers solution was added to the base of the container to prevent the samples from drying out. The cartilage samples were placed back into the friction rig after 24 h and a further five repeat measurements were made.

For every test, the mean coefficient of friction (μ) with the test lubricant was compared with the mean μ of the primary test in Ringers. A total sample size of 6 was used for each lubricant and each test comprised of five repeats.

Variations in the base-line friction levels between cartilage samples meant that direct comparisons between each sample would have been inappropriate. This test procedure allowed the comparison between the control tests in Ringers solution to the secondary test in lubricant. The percentage change in friction caused by the lubricant was then determined. This enabled comparison of the percentage change in friction between different lubricants.

2.4.6. Analysis of data

The mean coefficient of friction, $\pm 95\%$ confidence, for each sample tested was calculated. For each replicate test within each lubricant group, the percentage of friction was calculated. This data was arc sin transformed and then analysed by one-way analysis of variance carried out using Microsoft Excel. Any significant difference between lubricant groups was determined using the T-method (for multiple comparisons amongst groups of means) on the transformed data. The means and upper and lower confidence limits were back transformed to percentages for presentation purposes.

Table 1
Test lubricant formulations

Lubricant	Formulation
A (control)	Ringers solution
B	HA 5 mg ml ⁻¹
C	HA 10 mg ml ⁻¹
D	DPPC 100 mg ml ⁻¹
E	DPPC 200 mg ml ⁻¹
F	HA/DPPC 5 mg ml ⁻¹ / 100 mg ml ⁻¹
G	HA/DPPC 10 mg ml ⁻¹ / 200 mg ml ⁻¹

2.5. Incubation of cartilage with fluorescein-labelled HA

Small samples of healthy bovine cartilage, of approximately 5 mm diameter and 2 mm thickness, were removed from a fresh bovine knee joint using a biopsy punch. The cartilage samples were placed surface-up onto a glass slide and fluorescein-labelled HA (FL-HA; 0.1 ml; kindly donated by Onyx Scientific, UK) was carefully applied to the cartilage surface using a Pasteur pipette. The slides, with the cartilage, were placed into a plastic four slide histology container and then covered with foil to protect the FL-HA from any light source. The samples were incubated at 4 °C for 48 h, transferred to a cryostat where they were embedded in Cryo-M-Bed medium. During the embedding process, the samples were placed on their y-axis to allow a cross-sectional cut through the cartilage surfaces. The sections were cut into 5 µm thick sections using a cryostat. This procedure was carried out using four samples taken from different cartilage specimens.

2.6. Confocal imaging

The cryosections obtained were examined using a LSM510 META confocal microscope using an argon laser at 488 nm. Transmission phase contrast and fluorescence images were acquired and digitally recorded. Fluorescent images were overlaid onto the corresponding phase contrast images allowing the visualisation of FL-HA with reference to the cartilage structure and specifically the location relating to the chondrocytes. Images were captured at 230 and 600 times magnification.

3. Results

3.1. Friction coefficient measurements

The results for the start-up friction coefficients before and after treatment with the lubricants are shown in Fig. 2A–G.

There were no significant reductions in friction found with any of the samples tested with Ringers solution after 24 h compared to Ringers at time zero ($p > 0.05$; Fig. 2A). However, there was a significant increase in friction found with the repeat measurements in Ringers for sample 4. Four out of the six samples tested with HA (5 mg ml⁻¹; Fig. 2B) showed significant reductions ($p < 0.05$) in friction, only samples 1 and 3 showed no significant differences between the Ringers and the 5 mg ml⁻¹ HA lubricant ($p > 0.05$). The largest reduction was seen for sample 2, with which there was a reduction of 0.158. There was a significant difference in friction found between the Ringers

solution and the 10 mg ml⁻¹ HA lubricants in all of the individual samples tested ($p < 0.05$; Fig. 2C). The largest reduction recorded was 0.146 with sample 4.

There was only a significant difference found between the Ringers solution and 100 mg ml⁻¹ DPPC lubricant with samples 2 and 3 ($p < 0.05$; Fig. 2D). These samples showed a mean reduction in friction coefficient of 0.05. There was a significant difference found between Ringers solution and 200 mg ml⁻¹ DPPC lubricant in all samples tested, except sample 5 ($p < 0.05$; Fig. 2E). The largest reduction in friction was 0.278, sample 6. There was a significant reduction ($p < 0.05$) in friction found in all samples tested with HA/DPPC (5 mg ml⁻¹/100 mg ml⁻¹ formulation) when compared to Ringers (Fig. 2F). The largest reduction in friction was 0.176, sample 6. There was a significant reduction in friction found with all samples tested with HA/DPPC (10 mg ml⁻¹/200 mg ml⁻¹ formulation) when compared to Ringers except sample 2 ($p < 0.05$; Fig. 2G). The largest reduction in friction was 0.336, sample 4.

The percentage reduction in friction in the presence of each test lubricant is compared in Fig. 3. The majority of lubricants tested reduced friction levels by at least 45% when compared to the Ringers control, the only exception being the 100 mg ml⁻¹ formulation of DPPC. This lubricant only reduced the level of friction by a mean of 15.9%. Statistical analysis showed that all of the lubricants, with the exception of the 100 mg ml⁻¹ DPPC formulation, to be significantly different to the Ringers control ($p < 0.05$). In addition, when the lubricants were compared with the 100 mg ml⁻¹ DPPC formulation only the two mixtures of HA and DPPC (HA 5 mg/DPPC 100 mg and HA 10 mg/DPPC 200 mg) showed a significant difference ($p < 0.05$). These two formulations caused the largest reduction in friction of 69.5% and 61.9%, respectively. The remaining DPPC formulation (200 mg ml⁻¹) produced a reduction in friction of 46.5% which was comparable to the two HA formulations, 5 and 10 mg ml⁻¹ (51.9% and 46.7%, respectively). The Ringers control showed a small reduction in friction of only 4.6%.

3.2. Confocal imaging

The extent of HA penetration was determined by measuring the distance that fluorescence progressed into the cartilage. This was calculated by using cells or cell groups as visual markers in the field of view displayed on a computer monitor using LSM 5 Image Browser. The results are shown in Figs. 4–6.

A layer of FL-HA was observed on the surface of the cartilage, Fig. 4. This was apparent as a uniform fluorescent green line, approximately 20 µm thick, lining the surface of the cartilage. Chondrocytes appeared silver in colour (as a result of the phase contrast imaging) and appeared to have a normal, healthy morphology. At 230 × magnification, it could not be determined if HA had penetrated into the cartilage any further than the superficial layer.

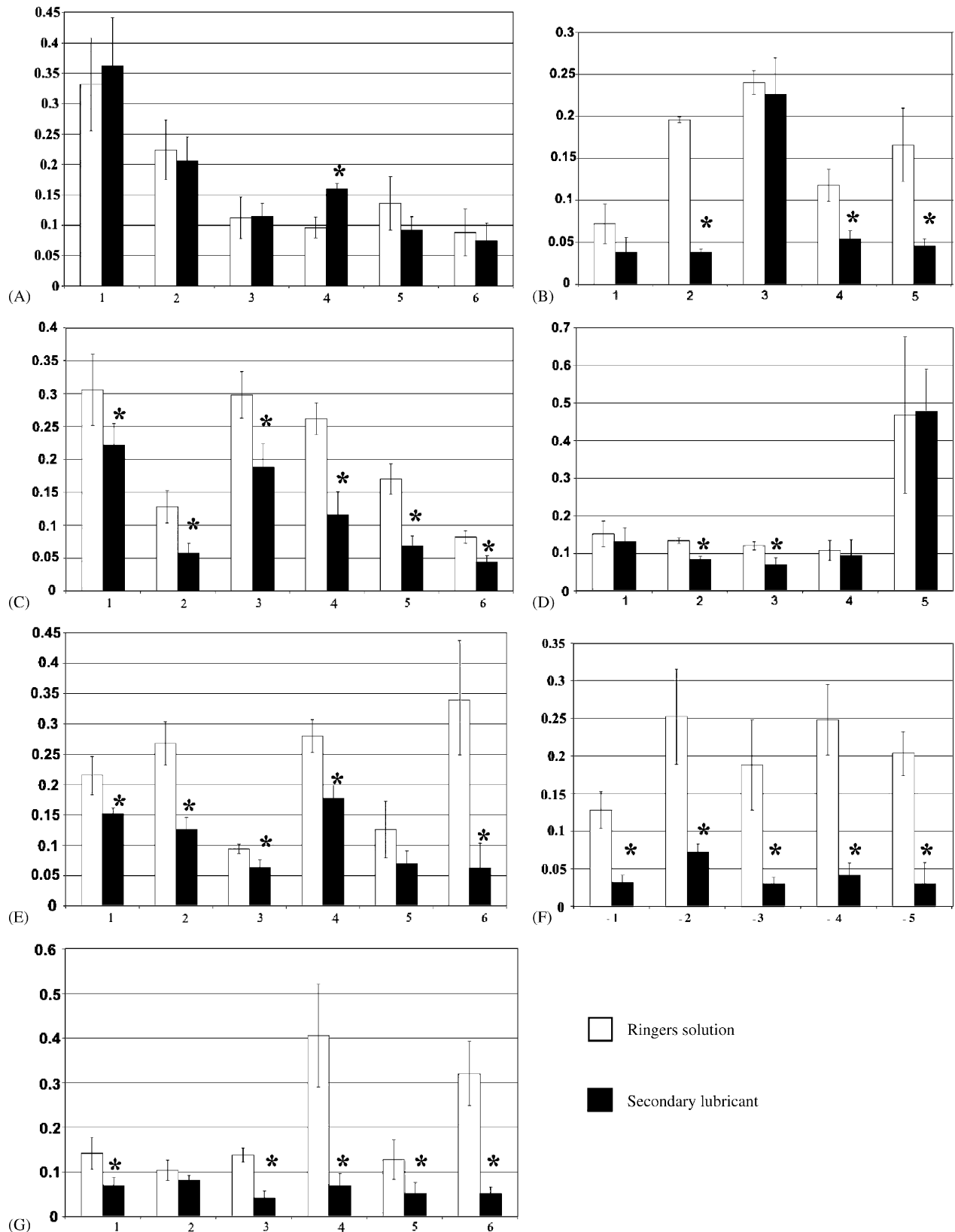


Fig. 2. Mean coefficients of friction of individual samples measured in Ringers solution followed by measurements in a secondary lubricant. The data represents the mean ($n = 5$) of each individual test with 95% confidence limits. Data was analysed by one way ANOVA and calculation of the minimum significant difference by the T-method. * $p < 0.05$ (ANOVA). Secondary Lubricant: (A) the control, ringers solution, (B) a formulation of 5 mg ml⁻¹ of HA, (C) a formulation of 10 mg ml⁻¹ of HA, (D) a formulation of 100 mg ml⁻² of DPPC, (E) a formulation of 200 mg ml⁻¹ of DPPC, (F) a formulation of 5 mg ml⁻¹ of HA with 100 mg ml⁻¹ of DPPC, (G) a formulation of 10 mg ml⁻¹ of HA with 200 mg ml⁻¹ of DPPC.

Images captured at a higher magnification ($\times 600$) are shown in Fig. 5. The 20 μm layer of FL-HA on the cartilage surface was clearly visible. The penetration of the HA was

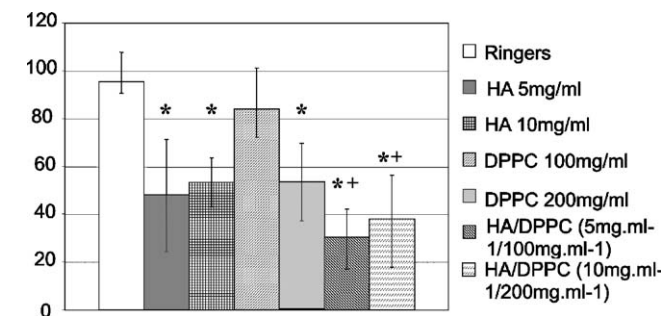


Fig. 3. Mean percentage reduction of friction for all lubricants tested on human cartilage surfaces. The data represents the mean ($n = 6$) percentage reduction in friction for each lubricant with upper and lower 95% confidence limits determined following arc sin transformation. Data was analysed by one-way ANOVA of arc sin transformed data, calculation of the minimum significant difference by the T-method. Data was back-transformed for presentation. * $p < 0.05$ compared to Ringers control (ANOVA). + $p < 0.05$ compared to DPPC 100 mg ml^{-1} (ANOVA).

more apparent in these images. The green fluorescent colour was observed throughout the superficial layer and penetrated into the intermediate layer of the cartilage (over a distance of 100 μm). The FL-HA was not evenly distributed throughout the tissue and appeared to be concentrated in the chondrocyte lacunae (Fig. 5B).

To show the extent of penetration of FL-HA into the cartilage, a series of images were taken progressing from the cartilage surface into the intermediate layer. The images shown in Fig. 6 were achieved through selecting a prominent chondrocyte or group of chondrocytes and using them as a marker point to determine the location of the next image. Throughout this technique, a series of three images were captured beginning at the cartilage surface and ending in the intermediate layer. The 20 μm surface layer of FL-HA can be seen again in Fig. 6. These images show that the FL-HA had penetrated 302 μm into the cartilage; as measured between chondrocyte markers in the series of images. The chondrocytes were used as markers as their lacunae contained concentrated levels of FL-HA compared to the surrounding matrix which made them easily identifiable.

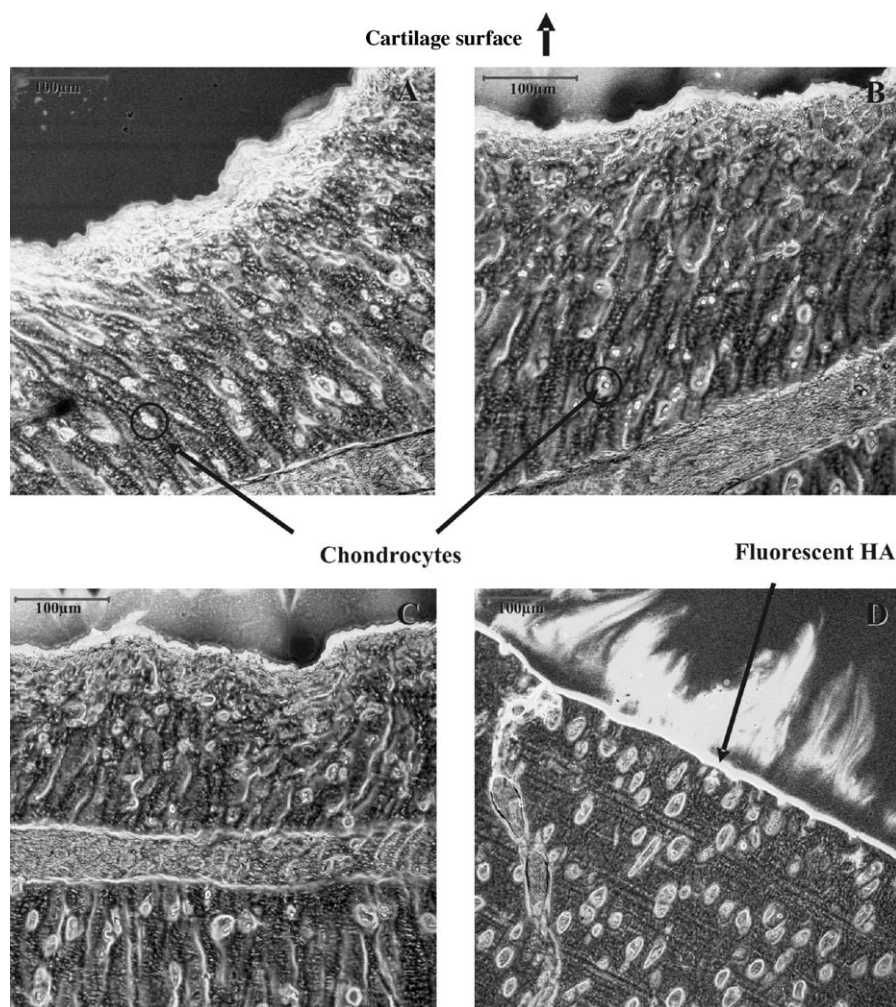


Fig. 4. Confocal and phase contrast overlay images of the cartilage surface at $\times 230$ magnification. Images A–D show the surface of bovine articular cartilage coated with FL-HA with phase contrast images overlaid onto confocal images obtained using an argon laser. Each image (A–D) was taken from a different specimen.

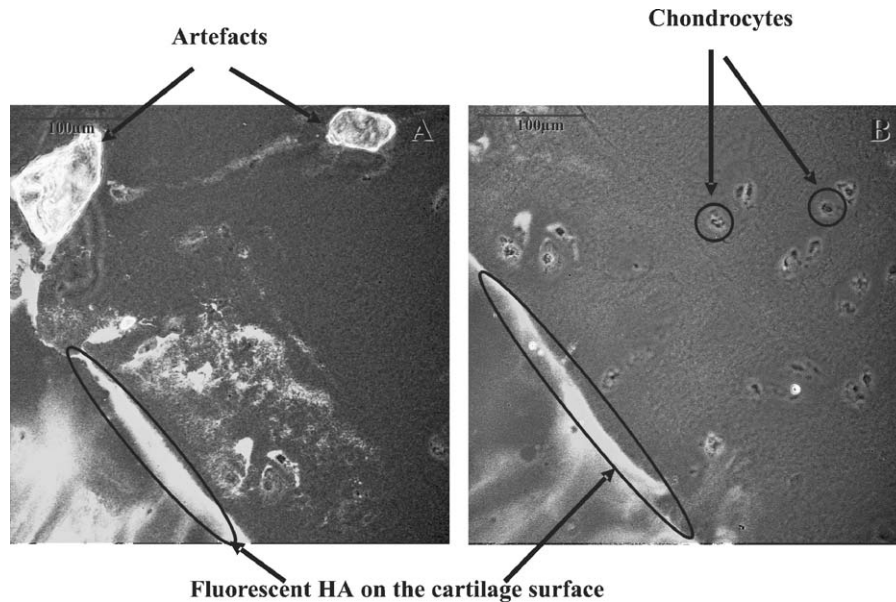


Fig. 5. Confocal overlay images of the cartilage surface at $\times 600$ magnification. Images A and B show the surface of bovine articular cartilage coated with FL-HA with phase contrast images overlaid with confocal images acquired using an argon laser. The images were taken from two different specimens.

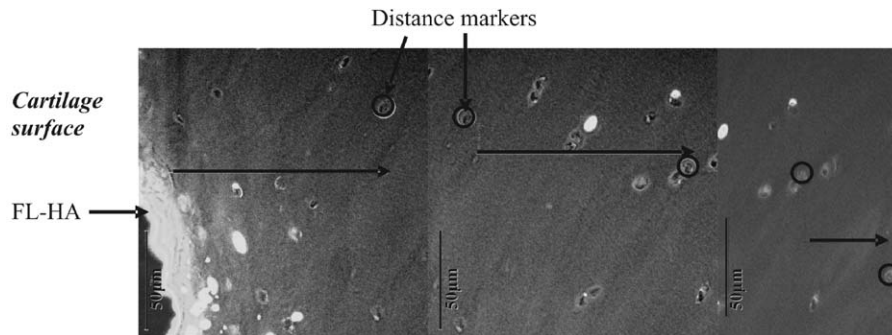


Fig. 6. A series of confocal overlay images showing the penetration of FL-HA into the cartilage surface ($\times 230$ magnification).

4. Discussion

This study initially aimed to test the effects of various therapeutic lubricant formulations on the start up friction of human cartilage with different levels of damage. The damage levels of cartilage used, however, were limited to grades 3 and 4, which were severely damaged (i.e. areas where damage has at least penetrated through over 50% of the cartilage surface). Due to the limitations of the supply of cartilage, it was not possible to evaluate the efficacy of the lubricants in normal healthy or intermediate OA diseased conditions. It would be important to carry out such experiments in the future to determine the effectiveness of different formulations of lubricants at different stages of OA in order to assess the value of introducing therapeutic lubricants at earlier stages of OA.

The start up friction of the cartilage was determined and not the sliding dynamic friction value. Start up friction was chosen since the highest values of friction occur at the start up of motion after prolonged loading. It was not possible

to also measure sliding friction, due to the limitations of the rig used, as it was only capable of determining the friction between two flat surfaces. As the tibial cartilage samples obtained were curved and uneven in nature, the areas available for testing were small and could only support the measurement of start up friction. The procedure for measuring the start up friction level was designed in an attempt to minimise the effect of variations between samples. This was achieved through chloroform:methanol washes of the samples, repeat testing and by initial testing in Ringers followed by lubricant testing with every individual sample. The coefficient of friction results showed the variation between each sample. The comparison of the test in the lubricant to the initial test in Ringers overcame this variation and enabled the effects of each lubricant formulation to be compared.

The study showed that HA and DPPC (200 mg ml^{-1}) individually reduced the friction level of human articular cartilage. However, the lower concentration of DPPC (100 mg ml^{-1}) did not significantly reduce friction levels.

Furthermore, the combined lubricant formulations ($5\text{ mg ml}^{-1}/100\text{ mg ml}^{-1}$ and $10\text{ mg ml}^{-1}/200\text{ mg ml}^{-1}$ of HA/DPPC) achieved a greater mean reduction in friction when compared to any other formulation. Previous studies have indicated that HA alone could lubricate damaged cartilage surfaces [23,24] and that DPPC was an effective boundary lubricant of articulating surfaces [16,17]. The extent to which the combined formulations of HA and DPPC would reduce friction has only been examined recently in a study by Kawano et al. [19] who investigated the effect in an OA model in rabbit stifles. HA showed a reduction in damage and friction in the model but the friction levels were still significantly different to the control. Stifles injected with the mixture of HA and DPPC did not differ in friction coefficient level or histologically when compared to the control. This led to the conclusion by Kawano, that the addition of DPPC to HA would improve intra-articular therapy. The effects of the HA/DPPC mixture in Kawano's study support the data presented here.

HA has been reported to reduce friction [23,24], at both low and high concentration (5 and 10 mg ml^{-1}). In addition, it has been reported that the viscosity of HA has an effect on lubrication and therefore the reduction in friction [24]. In this study, changes in concentrations were tested and these changes altered the viscosity of the HA. However, there was no significant difference between the two formulations of HA (5 and 10 mg ml^{-1}) used and it was, in fact, the lower concentration of HA that produced the greater mean reduction in friction. The concentration of HA may not be a crucial factor in lubricating ability, when operating under boundary lubrication conditions at start up of motion. As stated previously, under these conditions friction is determined by the intrinsic biphasic lubrication properties of the cartilage and the presence of additional boundary lubricants [22].

In addition to the individual HA lubricant formulations, the high concentration of DPPC (200 mg ml^{-1}) alone also significantly reduced friction level. The reduction in friction was equal to both HA formulations and there was no significant difference between any of these three formulations. The lubricating role of surface active phospholipids (SAPLs) and more specifically DPPC has been widely considered [17,25]. The results presented here support the contention that DPPC may provide lubrication on damaged cartilage. The capacity of DPPC to lower the friction was concentration dependant. This has been suggested in some studies examining the lubricating abilities of lipids where it was found that there was a minimum concentration of lipid required to produce a similar low level of friction compared to that of healthy SF [26].

In a previous study by Hills [27], it was found that commercially available lipids, samples obtained from sheep SF, and a mixture of DPPC with HA were effective lubricants. All lubricants reduced friction and the addition of HA to DPPC was found to be beneficial. From the results found in this study, it was suggested that lipids and

HA were important molecules in lubrication of articular cartilage, but that they worked together in synergy due to the increase in lubricating ability observed. Hills attributed this synergy to HA's ability as a "wetting" agent to reduce surface energy and its ability to act as a carrier for lipids to aid in their absorption to the cartilage surface but dismissed HA as a lubricant in its own right. However, it was observed in this study that HA was able to reduce friction on its own, suggesting adequate lubricating properties.

A study conducted by Hills et al. [28], using enzyme treatment of cartilage and SF, found that damage to phospholipids in both the cartilage and SF resulted in a loss of lubrication but that damage to HA had no effect. However, a study by Higaki et al. [17] found that both HA and DPPC, could lubricate cartilage and supported the suggestion by Hills [27] that lipids may lubricate as a multilayer on the cartilage surface. A more recent study by Kumar et al. [29] used enzymes to damage cartilage. It was found that hyaluronidase treatment did not increase the friction levels of cartilage lubricated by a saline solution, but chondroitinase ABC and alkaline protease treatment did increase the friction. However, when chondroitinase ABC and alkaline protease treated cartilage were lubricated with HA formulas, there was a reduction in friction observed.

The results from this study showed that when HA was applied to cartilage it coated the surface and, after 48 h, small amounts of the HA penetrated into the cartilage and targeted chondrocytes. This was demonstrated by the fluorescence associated with lacunae containing chondrocytes in the superficial zone near the cartilage surface and up to $300\text{ }\mu\text{m}$ into the mid zone. Since the experiments were carried out on cut specimens, it was possible that some of the FL-HA penetrated into the tissue through the cut edges. However, there was no obvious fluorescence layer associated with the cut edges, and the intensity of fluorescence appeared to follow a diminishing gradient from the surface into the tissue. The penetration of HA observed, was limited and appeared to exclusively target the chondrocytes. The reason for the apparent targeting of the lacunae is not known. However, it has been suggested that HA binds to the CD44 receptors found on the cell membrane [30]. The importance of this is that if HA is going to have a positive biological effect in damaged cartilage it would have to interact with the cells and in this study evidence has been provided that this may occur.

The observed penetration of HA into cartilage and association with chondrocytes in this study may further support the claims that HA can have long term therapeutic benefits. This is supported by the suggestion that HA can stimulate chondrocytes to up-regulate their synthesis of new HA molecules [7] and can reduce the production and activity of proinflammatory mediators and MMPs [7]. However, any resultant activity from this interaction was not investigated in this study and further work would need to be carried out to investigate this process and to further the assumptions made in previous studies.

5. Conclusions

The application of HA and DPPC onto damaged human cartilage, resulted in improved lubrication between the cartilage surfaces. The effectiveness of HA was not dependant on concentration, however concentration had an effect on the ability of DPPC to lubricate. Furthermore, the combination of DPPC with HA provided an increase in its ability to lubricate. The reason for this effect is not fully understood. In addition to improved lubrication, there may be further benefits in this combination which have not been highlighted in this study.

FL-HA formed a covering over the cartilage surface and over a period of time, some HA penetrated into the cartilage layers and specifically targeted the chondrocytes in the superficial and mid zones. This penetration reached up to approximately 300 μm from the cartilage surface. The mode of this targeting and the role HA plays is not known although there are suggestions of direct interactions with the chondrocytes through receptor sites (CD44) to promote the synthesis of new HA molecules.

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