Introduction

Joint fluid therapy with intra-articular injection of hyaluronic acid (HA) is widely used for the treatment of osteoarthritis (OA) in knee joints. Initially, the concept of joint fluid therapy was to restore the viscoelastic properties of the synovial fluid since the HA in OA patients has a lower molecular weight and lower concentration than in healthy patients. However, the mechanism of action is not entirely clear since HA has been shown to have several biological actions (see, for example, the reviews) that could explain the long-lasting positive effects that HA injections have with patients suffering from OA.

A number of different HA products for OA treatment are available, including unmodified HA solutions, solutions of modified HA, and cross-linked HA gels. The intra-articular duration of unmodified HA has been measured in a rabbit model. The half-life of unmodified HA solutions is less than 1 day and only slightly dependent on the molecular weight. As the main reason for modifying HA is the desire to prolong the residence time in the joint, it is important to determine to what extent this is achieved with different manufacturing methods. The use of different HA raw materials and different cross-linking techniques will influence the properties of final products. It is not yet known to what extent such differences may affect the elimination rate. Only one study (poster abstract) has been identified in the literature, where the elimination of a product composed of modified HA (hylan G-F 20) has been studied in the rabbit model. The half-life of the modified HA solution as well as of the gel component of this product was increased compared to that of unmodified HA. Using the NASHA™ manufacturing technique, particles of a cross-linked HA gel are produced. The elimination kinetics of this gel from the knee joint has been studied in human volunteers. A prolonged half-life was observed compared to the known kinetics of

Abstract

Objective: The aim of the present study was to investigate the intra-articular duration of Durolane™ in a rabbit model to allow comparison between Durolane™ residence time and data reported for other hyaluronic acid products as well as native hyaluronic acid. Design: 14C-labeled Durolane™ was manufactured by labeling the cross-linker used for stabilization. A single injection of approximately 0.3 mL 14C-labeled Durolane™ was administered intra-articularly in both knee joints of male New Zealand White rabbits. At days 1, 2, 3, 7, 28, 60, 96, and 120 after injection, the knee joints of 4 animals were collected, and the radioactivity of the remaining gel was measured. The obtained data were fitted by exponential models to calculate the half-life of the gel. Two additional rabbits were used for histology of the joint 127 days after the injection. Results: The elimination of 14C-labeled Durolane™ followed first-order kinetics with an apparent half-life of approximately 32 days. Histology showed no morphological changes in the knee joints. Conclusions: This study shows that Durolane™ has a half-life of 32 days in the rabbit knee joint, which is much longer compared to literature data on hyaluronic acid and other modified hyaluronic acid products.

Keywords
osteoarthritis, hyaluronic acid, intra-articular, duration, Durolane™
Materials and Methods

Test Article Description

\[^{14}\text{C}\]-labeled Durolane™ was manufactured using a pilot scale process. The \[^{14}\text{C}\]-labeling was introduced with the 1,4-butane-diol diglycidyl ether (BDDE) cross-linker used for stabilization during the NASHA™ manufacturing process. The labeled BDDE was manufactured by Analyscentrum (Stockholm, Sweden). The resulting \[^{14}\text{C}\]-labeled Durolane™ gel had a specific activity of 0.86 dpm/µg.

A solution of \[^{14}\text{C}\]-labeled modified HA was prepared by heat degradation of \[^{14}\text{C}\]-labeled Durolane™ followed by filtration through a 0.22-µm filter. This solution (referred to as the \[^{14}\text{C}\]-labeled modified HA solution) had a specific activity of 0.22 dpm/µg.

Animal Management and Intra-articular Injection

The animal experiments were approved by the Regional Animal Ethics Committee of Uppsala, Sweden (no. C168/7). All animals were allowed an acclimatization period of 14 days prior to the commencement of the experiment. New Zealand White male rabbits (Hasslösa, Lidköping, Sweden), with a body weight of 3.0 to 3.5 kg, were used in the study. The animals were caged individually and fed a commercial rabbit diet K-1 from Lactamin AB (Stockholm, Sweden). They were provided with food once daily and had free access to water. The animals were checked daily for change in food intake, activity, and other things as signs of change in general health status. The animals were weighed at arrival, after conditioning, at study start, on a regular basis during the study, and at termination.

Prior to the study, a number of euthanized rabbits were used for development and evaluation of the injection technique and the dissection procedure. To visualize the gel, it was colored by using green food coloring. In order to ensure that the depth of the injection was correct, plastic spacers of varying lengths were fitted on the needle and tested. The knee was fixed in an upright position during the insertion of the needle. After injection, the joints were opened to evaluate the distribution of the colored gel inside the joint. It was concluded that a spacer length of 33 mm was optimal for proper deposition of the gel. In addition, approximately 5 cm needed to be dissected to ensure that there was no loss of joint tissue or joint fluid (for details, see below).

The animals in the study were anesthetized by intramuscular injection of ketamin hydrochloride, 50 mg/mL, 35 mg/kg body weight, and xylazine hydrochloride, 20 mg/mL, 5 mg/kg body weight. The injection sites were shaved and disinfected prior to the intra-articular injection. A 22-gauge needle fitted with the plastic spacer (33 mm) was inserted into the joint via a cranial approach through the patellar ligament to a depth of 11 mm. Once the needle was in the joint, a single injection of approximately 0.3 mL \[^{14}\text{C}\]-labeled Durolane™ or 0.3 mL of the \[^{14}\text{C}\]-labeled modified HA solution was slowly injected (approximately 10 seconds) intra-articularly into both knee joints of the rabbit. The syringes were weighed before and after injection to determine the exact amount injected.

Duration Study

Thirty-two animals were injected with \[^{14}\text{C}\]-labeled Durolane™ and divided into groups of 4 animals. At days 1, 2, 3, 7, 28, 60, 96, and 120 after injection, the respective groups of animals were euthanized by an intravenous injection of pentobarbital, 100 mg/mL, and the knee joints were collected and weighed. The knee joint was dissected by removing the surrounding muscles without opening the joint capsule. The femoral bone was cut approximately 2.5 cm in the proximal direction and the tibial and fibular bone approximately 2.5 cm in the distal direction of the joint space. The resulting 5 cm contained articular cartilage, joint capsule, synovial membrane, and synovial fluid. Three additional animals were injected with the \[^{14}\text{C}\]-labeled modified HA solution, euthanized at day 7, and dissected as described above.

The individual knee joints were homogenized in a mixer together with approximately 35 mL water, and the resulting slurry was allowed to dry in a vacuum desiccator with phosphorus pentoxide as drying agent. Measurements of the radioactivity were performed at Active Biotech Research AB (Lund, Sweden). The radioactivity of the dried knee joint homogenates was measured in triplicate by combustion in an oxidizer (model 307, Packard Instrument Co., Downers Grove, IL, USA). The \[^{14}\text{C}\]-labeled carbon dioxide generated during the combustion was trapped, and the radioactivity was subsequently measured by liquid scintillation. The mean value of the triplicate measurements was used for further calculations. The accuracy of the preparation procedure was studied by injecting \[^{14}\text{C}\]-labeled Durolane™ in 6 joints from euthanized rabbits followed by sample preparation and measurements as described above.
The actual amount of $^{14}$C-labeled Durolane™ in the knees was calculated from the measured activity and plotted as percentage of the initial specific activity of the gel at the time of injection. The obtained data were fitted by exponential models.

**Histology**

Two rabbits were injected with $^{14}$C-labeled Durolane™ intra-articularly, and the animals were euthanized by an intravenous injection of pentobarbital 127 days after the injection. The knee joints were fixated in 4% buffered formaldehyde solution. After washing in 70% ethanol overnight, the joints were decalcified in Parengy (Bie & Berntsen A/S, Herlev, Denmark) for 20 days. Every second day, the Parengy solution was changed. At day 20, the bone was soft, and all samples were cut, with use of a razor blade, in 2 halves by midline sagittal sectioning. These preparations were then dehydrated overnight and embedded in paraffin. The samples were further sectioned at 4 µm and dried at 37 °C overnight. The dewaxed and rehydrated sections were then stained with hematoxylin/eosin in a Leica ST 4040 autostainer (Leica Microsystems GmbH, Nussloch, Germany). Finally, the hematoxylin/eosin–stained sections were evaluated using a light microscope (DMR XE, Leitz, Wetzlar, Germany).

**Statistical Methods**

The average of the triplicate radioactivity measurements on each joint was used for further calculations. The relative standard deviation (RSD) of the triplicate measurements was calculated.

The average and standard deviation ($s$) of the data were calculated for each group (time point) of animals. Two data points outside 2 standard deviations from the average were regarded as outliers and removed from further analyses. The variability within time points was expressed as the standard error of the mean: \( SEM = s/\sqrt{n} \), where \( n = 8 \) \((n = 7 \) for 2 groups with outliers).

The kinetics of the change in percentage remaining gel (y) over time (T) was evaluated by fitting 1-term (\( y = ae^{bT} \)) and 2-term (\( y = ae^{cT} + ce^{dT} \)) exponential models in the Curve Fitting Toolbox in Matlab (version 7.4, The MathWorks Inc., Natick, MA, USA) using the trust region algorithm. Confidence bounds (C) for the fitted coefficients (b) were given by \( C = b \pm t(S) \), where \( t \) depends on the confidence level (95%) and was computed using the inverse of the Student t cumulative distribution function. \( S \) is a vector of the diagonal elements of the estimated covariance matrix of the coefficient estimates.

**Results**

All rabbits remained healthy during the study and tolerated the intra-articular injection without any adverse reactions. The body weights showed normal development. No visual signs of inflammatory reaction were seen during autopsy at any of the time points after injection.

**Duration Study**

The measured radioactivity of the knee joints is presented in Figure 1 as the percentage of the initial radioactivity of the gel at the time of injection for each joint. At the end of the study, the percentage remaining gel observed was 0.5% to 39.6%. The variation within time points varied between SEM 4% (time points 1, 7, and 96 days) and 9% (time points 3 and 28 days), that is, without any trend over time. The average RSD for the triplicate radioactivity analyses was 5.8%, showing that the joint preparations were homogeneous and that the analysis method had good precision. The accuracy of the preparation procedure was studied by injecting gel in joints from euthanized rabbits, resulting in an average recovery of 97% (data not shown).

The elimination of $^{14}$C-labeled Durolane™ was found to follow first-order kinetics with an apparent half-life of approximately 32 days; with a 95% confidence interval, the half-life is within the range of 24 to 46 days. Two-term exponential models did not give realistic results due to the combination of few time points and relatively large variation in the data. One of the 6 joints injected with the $^{14}$C-labeled modified HA solution showed a remaining activity of 0.7% after 7 days. The other 5 joints did not show a detectable activity.
Histology

The histopathology study was made to investigate if any histological findings could be seen in the joint that could be related to the treatment. The 2 investigated rabbit knee joints showed no morphological changes. No inflammatory cells could be seen in the synovial membranes or in the joint cavities. Furthermore, no changes were observed in bones or cartilage of the knee joint (Fig. 2).

Discussion

Duration Study

The percentage remaining radioactivity was on an average 89% for the day 1 animals, showing that almost all of the injected gel remained in the joints after 1 day. The percentage remaining radioactivity decreased for the subsequent groups due to the elimination that was observed to follow first-order kinetics. Some variation in the data at each time point was observed; for example, in the day 3 group, 5 of the joints had a remaining activity above 90%, while 2 joints (from 2 different animals) had close to 40% remaining activity only. In the day 120 group, the remaining activity varied between 0.5% and 40%, with 5 joints below 5% and 3 above 32%. However, this variation is in the range of what can be expected, considering that each data point represents an individual animal. The variation observed adds an uncertainty to the fit of the data, and this is manifested by the 95% confidence interval of the half-life, which is 24 to 46 days. The knee joints that had been injected with the 14C-labeled modified HA solution showed virtually no remaining activity after 7 days, indicating that the activity observed in the duration study is associated with Durolane™ gel particles rather than degradation products.

Even if the mechanism of action is not entirely clear, it has been shown that HA reduces pain associated with OA.13-19 Assuming that it is the presence of the HA formulation in the synovial fluid that is the reason for the effect, a longer residence time would be beneficial since the positive effects of exogenously added HA are extended during a longer time and fewer injections are needed. The number of intra-articular injections should preferably be kept at a minimum due to the fact that each injection is associated with a risk of adverse events. Fewer injections will also be more cost effective, and it is naturally more convenient for the patient.

This study shows that the half-life of Durolane™ is much longer than literature data on the half-life of unmodified HA, which is less than 1 day.7 The radioactivity remaining in the joint most likely represents gel as the 14C-labeled modified HA solution was found to be eliminated within 7 days. Support also comes from the Lindqvist et al.12 study, where soluble HA trapped in the gel was found to be rapidly eliminated. As the ether bond between linker and HA is difficult to cleave, bound linker is unlikely to dissociate from the HA chains. Therefore, remaining radioactivity does not represent cross-linker alone. The half-life found for Durolane™ will thus reflect the rate of degradation of the gel and the subsequent elimination of the liberated HA from the synovial fluid.

The half-life found in the present study is consistent with earlier findings in humans reported by Lindqvist et al.12 However, it is somewhat more difficult to interpret the data from the human study since end group–labeled HA was used. The end group labeling with 131I makes the data more reflective on the molecular weight distribution of HA in the product and could potentially explain why 3 half-lives (1.5 hours, 1.5 days, and 4 weeks) were found for Durolane™ in the human study. The terminal half-life in the Lindqvist et al.12 study is, however, close to the 32-day half-life of Durolane™ in the present study. The 2 shorter half-lives found in the human study were explained by low molecular weight fragments and high molecular weight HA trapped in the gel structure. As no early time points were evaluated in the present study, a half-life associated with unbound HA was not detected.

The duration of Durolane™ can also be compared to the duration of hylan G-F 20, another modified HA product for OA treatment. Hylan G-F 20 is a mixture of a gel and a fluid component, where 90% w/v of the HA comes from hylan A fluid and 10% w/v from hylan B gel. The present study and the study of hylan G-F 20 by Larsen et al.10 are quite similar: both studies investigated the half-life of the gel in rabbits by radiolabeling the cross-linker even though the cross-linker and the radioisotopes differ. Larsen et al.10
dissected out synovial fluid and joint tissues separately, but both studies report only the total recovery of radiolabeled material from the knee joint. Therefore, the results from the 2 studies can be regarded as comparable. The half-life of the Durolane™ gel found in the present study is 32 days, which is clearly longer than the 8.8 days reported by Larsen et al.10 for the gel component of hylan G-F 20. However, the half-life for the hylan G-F 20 product, consisting of both hylan A and hylan B, is considerably shorter than 8.8 days since one must take into account that the majority of the HA in hylan G-F 20 (90%) is in the form of the hylan A fluid, which has a half-life of only 1.5 days. A possible explanation for this longer half-life of the Durolane™ gel compared to the hylan B gel of hylan G-F 20 could be that Durolane™ is more resistant to free-radical degradation than hylan G-F 20 (K. Edsman, H. Lärkner, and J. Näström, unpublished data).

### Histology

The histology shows no morphological changes in the knee joints of the investigated rabbits injected with 14C-labeled Durolane™, indicating that the injected gel product was well tolerated.

### Conclusion

A single injection of Durolane™ has a half-life in the rabbit knee joint of 32 days, which is similar to the half-life found in humans. This study demonstrates that Durolane™ has a much longer residence time in the knee joint compared to literature data on HA and other modified HA products.

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### Declaration of Conflicting Interests

At the time of the study, authors K.E., R.H., H.L., L.I.N., A.K., Å.W., Å.H.K., and J.N. were all employed by Q-Med AB Sweden.

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