EXTENDED REPORT

Analysis of lapine cartilage matrix after radiosynovectomy with holmium-166 ferric hydroxide macroaggregate

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Synovectomy has sometimes been used to treat refractory joint inflammation instead of more conventional treatments such as intra-articular cortisone treatment. Radiosynovectomy provides an attractive alternative to surgical synovectomy and has been in clinical use for 50 years.¹ The objective of radiosynovectomy is to destroy the pannus and inflamed synovium by direct radiation, with the expectation that the regenerated synovium will be free of disease. A radiopharmaceutical agent with an appropriately strong β emission can be administered directly into the joint, where it affects the synovial lining, leaving the adjacent cartilage apparently unaffected.² Radiosynovectomy has been shown to give results at least as good as those of arthroscopic synovectomy.^{3–5} The benefits to patients include increased joint movement and reduced swelling, pain, and effusion.⁵

Although mature cartilage has been considered to be resistant to radioactivity,^{2–7} minor injury to articular cartilage remains a concern of radiosynovectomy. In some studies β radiation has been associated with chondrocyte injury, and radiosynovectomy using phosphorus-32 (^{32}P , β_{max} 1.7 MeV) with injuries of both articular cartilage and the growth plate.^{8–9} A report on the use of gold-198 (^{198}Au , β_{max} 0.96 MeV) indicated that rat articular cartilage is extremely resistant to radiation.⁶

In addition to direct radiation, synovial damage, subsequent joint inflammation, and fibrosis may also contribute to articular cartilage damage after radiosynovectomy. Extensive fibrosis of the subsynovial tissues has been reported after radiosynovectomy with yttrium-90 (^{90}Y , β_{max} 2.3 MeV), dysprosium-165 ferric hydroxide macroaggregate (^{165}Dy]FHMA, β_{max} 1.3 MeV), or ^{32}P .^{10–12} Fibrosis may hamper filtration and resorption of synovial fluid, and lead indirectly to articular cartilage damage because articular cartilage relies

Objective: To study the short and long term effects of radiosynovectomy on articular cartilage in growing and mature rabbits.

Methods: The articular cartilage of the distal femurs of rabbits was examined four days, two months, and one year after radiosynovectomy with holmium-166 ferric hydroxide macroaggregate (^{166}Ho]FHMA). Arthritic changes were evaluated from histological sections by conventional and polarised light microscopy, and glycosaminoglycan measurements using safranin O staining, digital densitometry, and uronic acid determination. Proteoglycan synthesis was studied by metabolic [^{35}S]sulphate labelling followed by autoradiography, and electrophoretic analysis of extracted proteoglycans. Northern analyses were performed to determine the mRNA levels of type II collagen, aggrecan, and Sox9 in cartilage samples.

Results: Radiosynovectomy had no major effect on the histological appearance of articular cartilage in mature rabbits, whereas more fibrillation was seen in ^{166}Ho]FHMA radiosynovectomised knee joints of growing rabbits two months after treatment, but not after one year. Radiosynovectomy did not cause changes in the glycosaminoglycan content of cartilage or in the synthesis or chemical structure of proteoglycans. No radiosynovectomy related changes were seen in the mRNA levels of type II collagen, whereas a transient down regulation of aggrecan and Sox9 mRNA levels was seen in young rabbits two months after ^{166}Ho]FHMA radiosynovectomy.

Conclusions: ^{166}Ho]FHMA radiosynovectomy caused no obvious chondrocyte damage or osteoarthritic changes in mature rabbits, but in growing rabbits some transient radiation induced effects were seen—for example, mild cartilage fibrillation and down regulation of cartilage-specific genes.

entirely on synovial fluid for its nutrition.¹³ Despite fibrosis, less cartilage destruction was reported in knees treated with ^{90}Y than in untreated ones.¹⁰ In other investigations on allergic arthritis, there was no evident sparing of articular cartilage after treatment with radiosynovectomy.^{11–12}

Holmium-166 (^{166}Ho) has excellent physical properties for radiosynovectomy. A reasonably short half life (27 h) decreases the risks associated with eventual leakage of the radioisotope, and strong β radiation energy (β_{max} 1.8 MeV) ensures efficient radiation of the synovium. Its ionising γ radiation (6% 81 keV) increases the total radiation to the body only slightly. Extra-articular leakage of the radioisotope is minimised with the use of ^{166}Ho conjugated with ferric hydroxide macroaggregate particles (^{166}Ho]FHMA).^{14–15}

Despite these advantages, the effects of radiosynovectomy with ^{166}Ho]FHMA on articular cartilage have not been previously investigated. This study aimed at filling this gap in our knowledge and studying the effects of ^{166}Ho]FHMA radiosynovectomy on rabbit articular cartilage using morphometric, biochemical, and molecular biological analyses.

MATERIALS AND METHODS

Animals

This study was performed on 51 New Zealand white rabbits. At the onset of the study 29 animals were growing (mean age 3.7 months, range 106–122 days, weight 2.4 kg, referred to as

Abbreviations: GAGs, glycosaminoglycans; ^{166}Ho]FHMA, holmium-166 ferric hydroxide macroaggregate; IM, intramuscularly; MEM, minimum essential medium; SDS, sodium dodecyl sulphate

young rabbits) and 22 were mature (mean age 1 year 3 months, range 446–519 days, weight 5 kg, referred to as old rabbits). The rabbits were housed in individual stainless steel cages with a floor area of 0.6×0.6 m, and height of 0.45 m. The study protocol was approved by the Animal Care and Use Committee of the University of Helsinki.

Protocols for [¹⁶⁶Ho]FHMA injection and sample preparation

[¹⁶⁶Ho]FHMA was injected into the knee joints through the suprapatellar ligament of 26 young and 20 old rabbits anaesthetised with medetomidine (0.3–0.5 mg/kg body wt intramuscularly (IM); Domitor, Orion Co, Espoo, Finland) and ketamine (20–25 mg/kg body wt IM; Ketalar, Warner Lambert/Parke Davis, Solna, Sweden). One knee of each rabbit was injected with 0.4 ml of radioactive [¹⁶⁶Ho]FHMA (referred to as the [¹⁶⁶Ho]FHMA treated knee), and the contralateral knee with 0.4 ml of non-radioactive [¹⁶⁶Ho]FHMA (referred to as the control knee). [¹⁶⁶Ho]FHMA was prepared under good manufacturing practice conditions (MAP Medical Technologies OY, Tikkakoski, Finland). Another five negative control animals received no injections. Old rabbits were treated with a higher dose of [¹⁶⁶Ho]FHMA owing to their larger body size (on average 121 MBq in old rabbits v 75 MBq in young animals). The animals were killed at periods of four days, two months, and one year after [¹⁶⁶Ho]FHMA injections (6–10 animals in each group).

The hind limbs were removed, skinned, and the femur was fixed onto a ball joint specimen holder. Three cartilage slices were then cut perpendicular to the surface with a high speed machine tool (Dremel, Emerson Electric Co, St Louis, MO, USA) equipped with two cutting discs and a 2 mm spacer. Each sample was continuously cooled with ice cold 0.9% sodium chloride during the harvesting procedure. The first slice was taken from the patellar surface just above the femoral condyles for histology, quantitative autoradiography, and densitometric analyses of glycosaminoglycans (GAGs). The second slice was cut approximately 2 mm proximal to the first one for total sulphate incorporation and uronic acid analyses. The third slice was cut from the superior part of the patellar surface of the femur for electrophoretic analysis of proteoglycans. All the slices were stored in minimum essential medium supplemented with Earle's salts (MEM, Life Technologies, Paisley, Scotland) including 100 U/ml penicillin and 100 µg/ml streptomycin at 4°C until the next day, when they were labelled with [³⁵S]sulphate (50–100 µCi/ml) in Dulbecco's MEM at 37°C in a 5% CO₂ atmosphere. After six hours the labelling medium was replaced by ice cold phosphate buffered saline.

Cartilage histology and assessment of the superficial collagen network

Specimens for histology were fixed in 4% formaldehyde at 4°C for 20 hours followed by decalcification in buffered 10% EDTA and 4% formaldehyde for 12 days at room temperature, dehydration with alcohol, and embedding in Paraplast Plus wax (Lancer Division of Sherwood Medical, Kildare, Ireland).¹⁶ Sections 3 µm thick were cut for safranin O staining, and 5 µm thick sections for haematoxylin and eosin staining, and subsequent histological assessment. The superficial collagen network was assessed by quantitative polarised light microscopy.¹⁷

Digital densitometry of GAGs and thickness measurement of uncalcified cartilage

Densitometric determination of GAG concentration in safranin O stained sections was carried out in the middle of the femoral groove as described earlier.^{16, 17} The thickness of the uncalcified articular cartilage was measured and divided into 12 fractions. The two most superficial fractions represented the superficial, the next five fractions the intermediate, and the deepest five fractions the deep zone. The results were cal-

culated as the mean area integrated optical density proportional to one square micrometre in each zone (OD/µm²).

Uronic acid content

The GAG content of cartilage was calculated as micrograms of uronic acid measured with the *m*-hydroxydiphenyl method per milligram of wet weight.¹⁸

[³⁵S]Sulphate incorporation and autoradiography

For the analysis of total [³⁵S]sulphate incorporation, cartilage specimens were digested overnight at 60°C with 0.05% proteinase K (Roche Boehringer Mannheim, Mannheim, Germany) and 10 mM EDTA in 0.1 M phosphate buffer (pH 7.4). Unincorporated sulphate precursors were removed by PD-10 gel filtration columns (Amersham Pharmacia-Biotech, Uppsala, Sweden), and the radioactivity was measured by liquid scintillation counting (Winspectral, Wallac, Turku, Finland) from PD-10 eluates, and from medium samples using a water soluble scintillation cocktail (Optiphase Hisafe III, Wallac).

For autoradiography, tissue blocks containing articular cartilage and the underlying bone were prepared as described earlier,^{19, 20} with the exception that no background staining was used. Microscopic analysis of the grain area fraction was performed in the middle of the femoral groove using image analysis. Each microscopic view was divided into 10 fields from the cartilage surface to the tidemark. The two most superficial fractions represented the superficial zone, the four consecutive fields the middle zone, and the deepest four the deep zone. The results are presented as the average grain fraction percentage of the total tissue volume.

Electrophoretic analysis of proteoglycan monomers

Cartilage was dissected from the underlying bone, weighed, and extracted with 500 µl of 4 M GuCl in 50 mM sodium acetate (pH 5.8) containing inhibitors of bacterial growth and proteases.²⁰ Extraction was continued at 4°C for 24 hours. Proteoglycans were precipitated in 75% ethanol at 4°C and washed twice in ice cold 70% ethanol. The samples were dissolved in sodium dodecyl sulphate as previously described²¹ for electrophoresis in submerged horizontal 5 mm 1.2% agarose gels. The gels were scanned for densitometry, and exposed to autoradiography films for two weeks. The scanned images of films and gels were analysed by image analysis software (IP-Lab, Scanalytics, Fairfax, VA, USA).²⁰

Northern hybridisations

For extraction of total RNA, both femoral condyles were cut off with a bone cutter, transferred into liquid nitrogen, and kept at -80°C. Before extraction of total RNA using a modification of the guanidium isothiocyanate method, the samples were pulverised under liquid nitrogen.²² For northern analyses, 10 µg aliquots of total RNA were denatured by glyoxal and dimethyl sulphoxide. After electrophoresis on 0.75% agarose gels the RNAs were transferred overnight onto Pall Biodyne membranes as recommended by the supplier (Pall Europe, Portsmouth, UK). The filters were prehybridised and hybridised with cDNA clones for rabbit type II collagen,²³ human aggrecan core protein,²⁴ and Sox9,²⁵ and for the 28S rRNA²⁶ according to a protocol recommended by the supplier. All inserts were labelled with [³²P]deoxycytidine triphosphate using the random priming method (Boehringer Mannheim, Germany). After high stringency washes, the bound radioactivity was detected and quantified on a molecular imager phosphorimager (Bio-Rad, Hercules, CA, USA), and the mRNA signals were corrected for variations in the 28S rRNA levels determined by hybridisation.

Statistical analysis

Differences in parameters between treated and control knees were tested with the non-parametric Wilcoxon matched pairs

Table 1 Number of knees with articular cartilage fibrillation‡

Group	Young rabbits				Old rabbits			
	Four*days (n=8)†	Two months (n=9)	One year (n=9)	Total (n=26)	Four days (n=6)	Two months (n=7)	One year (n=7)	Total (n=20)
Synovectomy	2	3	3	8‡	1	2	0	3
Control	2	0	2	4	0	4	0	4

*Time after [¹⁶⁶Ho]FHMA injections; †number of animals; ‡the data are too few for statistical analysis.

Table 2 Concentration of uronic acid (µg/mg articular cartilage) and total amount of [³⁵S]sulphate incorporated (pmol SO₄/mg cartilage wet wt/h)

Group	Young rabbits			Old rabbits		
	Four days* (n=8)	Two months (n=9)	One year (n=9)	Four days (n=6)	Two months (n=7)	One year (n=7)
Concentration of uronic acid						
Synovectomy	4.780 [0.408]†	NA	7.491 (1.031)	4.317 (0.553)	5.979 (0.520)	8.529 (0.615)
Control	4.804 (0.238)	NA	7.926 (1.045)	4.393 (0.401)	6.033 (0.645)	7.198 (0.666)
Normal controls	4.393‡	NA	8.068	4.116	4.116	6.096
Total Amount of [³⁵ S]sulphate incorporated						
Synovectomy	24.6 [2.2]†	12.4 (2.4)	10.2 (2.2)	10.8 (2.1)	10.7 (1.9)	13.2 (0.5)
Control	21.2 (1.5)	12.7 (2.1)	13.6 (2.3)	7.3 (0.7)	11.0 (0.9)	12.8 (0.3)
Normal controls	15.1‡	8.9	11.3	8.1	8.1	13.1

NA, not analysed.

*Time after [¹⁶⁶Ho]FHMA injections; †mean (SEM); ‡mean values of negative controls.

The numbers of paired observations are in parentheses in the column heads. The two tailed non-parametric Wilcoxon's matched pairs signed ranks test was used for statistical analysis; no significant differences were found between the synovectomy and control groups.

signed rank test because the data did not fulfil the criteria for parametric testing.

RESULTS

Table 1 summarises the results of the histological analyses of rabbit knee joints at four days, two months, and one year after radiosynovectomy. In young rabbits superficial articular cartilage fibrillation was seen more frequently in [¹⁶⁶Ho]FHMA treated joints than in control joints. Vertical clefts disrupting the articular cartilage surface were only seen in old rabbits: in three animals processed four days after radiosynovectomy, and in two animals processed two months after the treatment. These changes occurred equally in [¹⁶⁶Ho]FHMA treated and control knees.

The average thickness of uncalcified cartilage in young rabbits was 388 µm, and in old rabbits 505 µm. No differences in

cartilage thickness were found between [¹⁶⁶Ho]FHMA treated knees and control knees.

The intensity of safranin O staining increased from the superficial zone towards deep articular cartilage (data not shown). Synovectomy with [¹⁶⁶Ho]FHMA did not alter safranin O staining in any part of the articular cartilage. Neither were any differences observed in GAG concentration of the articular cartilage determined by uronic acid analyses for the [¹⁶⁶Ho]FHMA treated and control knees (table 2). Metabolic labelling with [³⁵S]sulphate did not reveal any significant effect of radiosynovectomy on proteoglycan synthesis in the incorporation analysis of tissue samples (table 2) or in autoradiographic analysis of the cartilage zones (table 3, fig 1).

Analysis of the size distribution of proteoglycan monomers by SDS agarose gel electrophoresis showed that the proteoglycans were more homogeneous in size in young rabbits than

Table 3 Autoradiography slides; total grain volume fraction (% of the total tissue volume occupied by grains) in superficial, middle, and deep cartilage layers

Group	Young rabbits			Old rabbits		
	Superficial zone	Intermediate zone	Deep zone	Superficial zone	Intermediate zone	Deep zone
Four days after [¹⁶⁶ Ho]FHMA						
Synovectomy	(n=8) 3.174 [0.994]*	3.059 (0.625)	2.931 (0.612)	(n=6) 1.468 (0.391)	1.429 (0.373)	1.111 (0.248)
Control	2.317 [0.457]	3.270 (0.81)	2.920 (0.628)	0.747 (0.123)	0.825 (0.200)	0.838 (0.259)
Normal controls	5.748†	6.448	5.844	1.614	1.767	1.456
Two months after [¹⁶⁶ Ho]FHMA						
Synovectomy	(n=9) 2.318 [0.537]	2.274 (0.389)	1.968 (0.302)	(n=7) 1.248 (0.159)	1.479 (0.144)	1.220 (0.102)
Control	1.568 [0.265]	1.737 (0.259)	1.591 (0.242)	1.907 (0.356)	1.740 (0.305)	1.306 (0.182)
Normal controls	4.66	4.604	3.829	1.614	1.767	1.456
One year after [¹⁶⁶ Ho]FHMA						
Synovectomy	(n=9) 3.442 [0.249]	3.649 (0.164)	3.577 (0.133)	(n=6) 3.230 (0.227)	3.261 (0.253)	3.215 (0.286)
Control	3.883 [0.860]	4.539 (1.172)	4.690 (1.370)	3.493 (0.253)	3.787 (0.349)	3.265 (0.277)
Normal controls	3.529	3.482	2.790	5.022	5.258	4.639

*Mean (SEM); †mean values of negative controls.

Data represent the mean (SEM) (with number of paired observations in parentheses at the head of the column). The two tailed non-parametric Wilcoxon's matched pairs signed ranks test was used for statistical analysis; no significant differences were found between the synovectomy and control groups.

Figure 1 Sections of articular cartilage at the patellar surface of the femur prepared for autoradiography. In an old rabbit four days after [^{166}Ho]FHMA treatment an equal level of [^{35}S]sulphate incorporation is seen in all cartilage zones of (A) control and (B) [^{166}Ho]FHMA treated samples. S, superficial zone; I, intermediate zone; D, deep zone. Bar =200 μm .

in the old ones (fig 2). No obvious changes induced by [^{166}Ho]FHMA radiosynovectomy were seen in the size distribution.

When calculated for a constant amount of 28S rRNA, the levels for pro $\alpha_1(\text{II})$ collagen mRNA were considerably higher in growing rabbits than in rabbits aged over one year. The pro $\alpha_1(\text{II})$ collagen mRNAs remained unchanged after radiosynovectomy, but reduced levels of aggrecan and Sox9 mRNA were seen in the [^{166}Ho]FHMA treated knees of young rabbits two months after treatment in comparison with the contralateral control knees. At the one year follow up, no differences

were seen in the mRNA levels between the [^{166}Ho]FHMA treated and control knees (fig 3).

DISCUSSION

Histological analysis and assessment of fibrillation indicated that [^{166}Ho]FHMA radiosynovectomy caused no obvious adverse effect on articular cartilage in old rabbits. In young rabbits, however, mild cartilage fibrillation was seen more frequently in the radiosynovectomised knees, but only transiently two months after the treatment. Five old rabbits had vertical clefts in both the radiosynovectomised and control

Figure 2 SDS agarose gel electrophoresis of proteoglycans from lapine cartilage. Proteoglycans were extracted from cartilage samples from the patellar surface of the femur two months and one year after [^{166}Ho]FHMA treatment of young rabbits. Proteoglycans were separated in 1.2% agarose gels and stained with toluidine blue. Afterwards the gels were exposed to autoradiography films. Densitometric analysis of films (A and C) and stained gels (B and D) is shown, as well as representative samples from gels and films. 2M, samples taken two months after [^{166}Ho]FHMA treatment; 1Y, samples taken one year after [^{166}Ho]FHMA treatment.

Figure 3 A summary of changes in the mRNA levels of pro α_1 (II) collagen, aggrecan, and Sox9 after $[^{166}\text{Ho}]$ FHMA treatment. The data are compiled from quantitative analyses of northern hybridisations of young and old rabbits at times shown above the diagrams. *Significance in northern analyses ($p < 0.05$).

knees, probably representing a naturally occurring phenomenon. A benefit of using young animals for long term experiments is that they are less likely to have active degenerative processes at the time of experiment. On the other hand, young animals with their greater reparative abilities might make the experimental results less applicable to adult joints with signs of cartilage degeneration.²⁷ The results of this study suggest

that the cartilage of young rabbits is more sensitive to radioactivity induced metabolic alterations. The results also show that active matrix production in growing rabbits helps to prevent permanent cartilage damage.

None of the biochemical analyses gave any information which might explain the increased frequency of fibrillation in the radiosynovectomised knees of young rabbits. Cartilage

thickness remained unchanged after radiosynovectomy, and there was no decrease in articular cartilage GAG concentration as has been found after surgical synovectomy.^{28–30} Similarly to the present study, no decrease in GAG concentration was detected after ⁹⁰Y radiosynovectomy in dogs.³¹ Biochemical analyses also showed that [¹⁶⁶Ho]FHMA radiosynovectomy had no effect on the average size of extracted proteoglycans. In old rabbits, proteoglycan heterogeneity can be explained by the fact that the normal aging process causes partial core protein cleavage of proteoglycans in tissues, whereas the newly synthesised proteoglycans produced by the same tissue exhibit remarkable homogeneity.³²

Chondrocyte defects in the superficial zone have been reported after ⁹⁰Y radiosynovectomy.⁸ Because the superficial chondrocytes would most likely be the first targets of damage caused by the synovial radioactivity, autoradiographic analysis of [³⁵S]sulphate labelled histological sections was performed. No decrease in autoradiographic grain density could be seen in any cartilage zone after [¹⁶⁶Ho]FHMA radiosynovectomy. Similar results have been reported after surgical synovectomy,²⁸ whereas increased synthesis of proteoglycans has been reported after ⁹⁰Y radiosynovectomy.³¹ Higher rates of proteoglycan synthesis and turnover have been associated with incipient cartilage damage.³³ No such increase was evident after [¹⁶⁶Ho]FHMA radiosynovectomy. On the other hand, decreased levels of aggrecan mRNA were seen in the knee cartilage of young rabbits two months after radiosynovectomy. The significance of this observation remains to be determined, as no corresponding changes were seen in the proteoglycan or GAG content of the tissue at this time.

It has been proposed that synovectomy results in some degree of enzymatic degradation of cartilage, which continues until synovial regeneration is complete.²⁸ As discussed above, increased matrix synthesis has been associated with attempted articular cartilage repair and with early osteoarthritis.^{34–36} In our study no up regulation of mRNAs for Sox9, an activator of the chondrocyte phenotype, nor pro α_1 (II) (for type II collagen) and aggrecan, two key components of cartilage matrix, was detected. Careful tissue preparation and analysis of 28S rRNA to confirm equal loading of samples minimised the effect of RNA derived from subchondral bone on the levels of cartilage-specific mRNAs. A lack of a repair process in the present model after [¹⁶⁶Ho]FHMA radiosynovectomy indirectly suggests that no severe radiation induced damage had occurred in articular cartilage. In contrast, radiosynovectomy in young rabbits resulted in a transient decrease (at two months) in mRNA levels of Sox9 and aggrecan. Earlier studies have shown that Sox9 is a transcriptional activator of both the Col2a1 gene^{37–38} and the aggrecan gene.³⁹ The reason why reduced Sox9 mRNA levels did not result in down regulation of pro α_1 (II) mRNA remains unexplained. As the pro α_1 (II) mRNAs levels remained unchanged, it is unlikely that the reduction in the aggrecan and Sox9 mRNAs reflected irreversible chondrocyte damage. The importance of the decreased levels of aggrecan and Sox9 mRNAs remains open because no chondrocyte damage was apparent in the autoradiographic analysis of cartilage samples. Neither were any adverse effects detected in the chemical composition of articular cartilage.

Our results therefore indicate that intra-articular [¹⁶⁶Ho]FHMA treatment does not cause cartilage damage in adult lapine joints despite the reported radiation damage on synovial tissue.⁴⁰ However, studies showed transient harmful effects of [¹⁶⁶Ho]FHMA treatment on immature rabbit cartilage. A higher rate of cartilage matrix synthesis in immature animals compared with adult animals⁴¹ might make the chondrocytes in young cartilage more susceptible to transient irradiation induced derangement of matrix production. Further studies are needed to determine whether cartilage in rheumatoid or osteoarthritic joints resembles young cartilage and might therefore be more susceptible to degenerative

changes. Although some activation has been seen in cartilage matrix production in a transgenic mouse model for osteoarthritis,⁴¹ the mRNA levels of matrix components were much lower than in growing mice. Although [¹⁶⁶Ho]FHMA has already been used in some clinical patients, further studies are needed to define the effects of [¹⁶⁶Ho]FHMA treatment in the osteoarthritic joint of man.

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