Research article

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Improved cartilage integration and interfacial strength after enzymatic treatment in a cartilage transplantation model

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Received: 18 Mar 2004 Revisions requested: 4 May 2004 Revisions received: 30 May 2004 Accepted: 23 Jun 2004 Published: 6 Aug 2004

Arthritis Res Ther 2004, **6**:R469-R476 (DOI 10.1186/ar1216)

Abstract

The objective of the present study was to investigate whether treatment of articular cartilage with hyaluronidase and collagenase enhances histological and mechanical integration of a cartilage graft into a defect. Discs of 3 mm diameter were taken from 8-mm diameter bovine cartilage explants. Both discs and annulus were either treated for 24 hours with 0.1% hyaluronidase followed by 24 hours with 10 U/ml collagenase or left untreated (controls). Discs and annulus were reassembled and implanted subcutaneously in nude mice for 5 weeks. Integration of disc with surrounding cartilage was assessed histologically and tested biomechanically by performing a push-out test. After 5 weeks a significant increase in viable cell counts was seen in wound edges of the enzyme-treated group as compared with controls. Furthermore, matrix integration (expressed as a percentage of the total interface length that was connected; mean ± standard error) was 83 ± 15% in the treated samples versus 44 ± 40% in the untreated controls. In the enzyme-treated group only, picro-Sirius Red staining revealed collagen crossing the interface perpendicular to the wound surface. Immunohistochemical analyses demonstrated that the interface tissue contained cartilage-specific collagen type II. Collagen type I was found only in a small region of fibrous tissue at the level of the superficial layer, and collagen type III was completely absent in both groups. A significant difference in interfacial strength was found using the push-out test: 1.32 ± 0.15 MPa in the enzyme-treated group versus 0.84 ± 0.14 MPa in the untreated controls. The study shows that enzyme treatment of cartilage wounds increases histological integration and improves biomechanical bonding strength. Enzymatic treatment may represent a promising addition to current techniques for articular cartilage repair.

Keywords: cartilage integration, cartilage repair, enzyme, push-out test

Introduction

Localized articular cartilage defects are a major problem for orthopaedic surgeons. Because cartilage has poor ability to heal because of lack of intrinsic repair capacity [1-3], chondral defects do not heal and may increase the risk for early osteoarthritis. A number of different treatment techniques, such as subchondral penetration [4-6], osteochondral transplantation and mosaïcplasty [7-9], perichondrium covering of the defect [10,11] and autologous chondrocyte transplantation [12,13], as well as various enzymatic treatment techniques [14-17], have been tried in either clinical or laboratory settings in an attempt to restore the articular surface. Until now none of these techniques has resulted in long-term, durable and a predictable repair of the articular cartilage. Many researchers focus on the production, or local induction, of hyaline-like cartilage; however, these techniques are generally not directly aimed at local integration with the surrounding healthy cartilage. Variable and suboptimal wound healing and integration may be a cause of potential failure of otherwise promising techniques.

Injury to cartilage results in the formation of an acellular and thus metabolically inactive zone adjacent to the wound interface [18-20], thereby prohibiting significant matrix deposition at the wound interface area and subsequently limiting integration. Ideally, the biochemical composition of the integrative matrix should equal that of native cartilage, with high contents of collagen type II and proteoglycans, and low amounts of collagen types I and III. Furthermore, the biomechanical properties of the interfacial tissue should be within the range of native cartilage in order to prevent excessive strain [21] and mechanical failure.

We previously showed that enzymatic treatment with hyaluronidase and collagenase increased cell density at the wound edges of cartilage explants after 2 weeks of *in vitro* culture [22]. This treatment method could improve cartilage integration in chondral defects and potentially could confer benefit in clinical applications. In the present study we used enzymatic treatment with hyaluronidase and collagenase, and tested how this would affect wound healing and cartilage integration in terms of matrix composition and biomechanical properties. Specifically, we applied a combination hyaluronidase and collagenase treatment on both sides of a cartilage explant, and tested the effect of this treatment on cell viability at the wound edge, production of collagens types I, II and III, collagen fibre orientation, and biomechanical bonding strength.

Methods

Articular cartilage samples were harvested from the metacarpo-phalangeal joints of calves aged 6–12 months. Full-thickness cartilage explants of 8 mm diameter and with a thickness of 0.9–1.2 mm were prepared using a dermal biopsy punch and scalpel. The explants were then randomly divided into two groups. From the centre of the explants, 3-mm cores were punched out, using a custom built device to ensure punching in the exact middle of the explant. Group 1 ($n = 12$) specimens (both outer ring and inner core) were incubated for 24 hours in 0.1% hyaluronidase type I-S from bovine testes (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) followed by 24 hours in 10 U/ml highly purified collagenase VII (Sigma-Aldrich Chemie BV), both in Dulbecco's modified eagle's medium/Hams' F12 with 2% foetal calf serum. Specimens from group 2 (controls; $n = 12$) were incubated in Dulbecco's modified eagle's medium/Ham's F12 culture medium (Gibco, Grand Island, NY, USA) supplemented with 2% foetal calf serum at 37°C for 48 hours (controls). The choices of enzymes, enzyme concentrations and treatment times were based on the findings from our previous *in vitro* study [22]. After 48 hours the samples were washed three times for 10 min in culture medium, and the 3-mm inner cores were reimplanted in their accompanying 8-mm outer rings. Constructs were then implanted in four subcutaneous pockets on the backs of six nude mice (BALB-C nu/nu; Harlan, Horst, The Netherlands), for which approval was obtained from the local animal ethical committee (DEC no.126-01-01). Each mouse carried two enzyme-treated constructs (group 1) and two control constructs (group 2). After 5 weeks the mice were killed by cervical dislocation and constructs were harvested.

Histology

From each mouse, one control and one enzyme-treated construct were processed for histology. Constructs were divided into two halves. One half was fixed in 4% phosphate-buffered formalin and embedded in paraffin, and the other half was frozen in liquid nitrogen and stored at -80°C for later cryosection preparation. Sections (6 μm) were cut using a standard microtome (paraffin) or cryomicrotome and mounted on Starfrost® slides (Knittel, Braunschweig, Germany). Paraffin sections were haematoxylin and eosin stained as well as immunostained for collagen type II. Cryosections were used for thionin (proteoglycan) stain, picro-Sirius Red stain and immunohistochemical stains for procollagen type I, collagen type I and collagen type III.

Evaluation of chondrocyte viability

The number of vital chondrocytes was counted in surface, middle and deep zones in both wounded and unwounded areas using haematoxylin and eosin coloured slides at 400× magnification using a 50 × 50 μm boxed grid. Nuclear and cytoplasmic changes, as described by Kim and coworkers [23], were analyzed to assess cell viability/death. Only cells with visible nuclei were evaluated. Values for wounded cartilage were scored from a 150-μm broad band on both sides of the lesion, and the values for both sides of the interface were then averaged to obtain one value per interface. Furthermore, the cartilage was divided into superficial (100 μm from the surface down), deep (150 μm from the bottom up) and middle (between superficial and deep) zones, as previously described [22]. For comparison, values from unwounded tissue were obtained from the middle part of the outer ring, which is more then 1 mm from the wound edge. Chondrocyte densities are represented as vital cells/mm^2.

For each explant the amount of viable chondrocytes was calculated from the values obtained from two to four sections. Subsequently, the averages for the control and enzyme-treated groups were calculated and used for statistical evaluation.

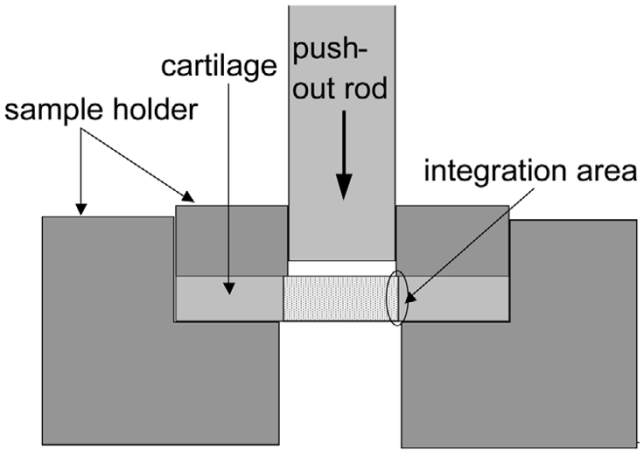
Evaluation of integration

Cryosections were fixed in acetone and stained with 0.04% thionin in 0.01 M sodium acetate for 5 min. For each sample we assessed the percentage of total interface length that had a matrix–matrix connection using a microscope with a 50 μm square grid. A clear distinction could be made between parts with a matrix connection and parts of the cartilage touching each other but without a clearly connected matrix, which were scored as parts with a gap. Interface integration percentages were obtained from measurements of two to four different sections from each sample, resulting in one average value for each interface.

Picro-Sirius Red stain

Cryosections were fixed in acetone and stained with 0.1% Sirius Red F3BA (Direct Red 80; Fluka Chemie, Zwijndrecht, The Netherlands) in a saturated picric acid solution for 1 hour. Brief washing in 0.1% acetic acid was followed by rapid dehydration in 100% alcohol (three changes for 3

Figure 1



Schematic representation of the push-out setup. Displacement transducer and load cell are connected to the push-out rod.

min each), after which a xylene bath (two changes for 5 min each) was used to prepare the slides for mounting with Entellan (Merck, Darmstadt, Germany). Slides were analyzed using a polarized light microscope (Dialux 20; Leitz, Wetzlar, Germany) to evaluate fibre orientation in the interface area. The relative sign of birefringence was determined using the analyzer filter. For semiquantitative analyses, samples from both groups were classified as follows: 0 = no fibres crossing; 1 = occasional fibre crossing; and 2 = many fibres crossing.

Collagen immunostaining

Cryosections were fixed in acetone for procollagen type I, and collagen types I and III staining. For collagen type II staining paraffin sections were deparaffinized using xylene and rehydrated through a graded series of ethanol, after which they were incubated with 0.2% pronase for 30 min to retain antigenicity. Treatment with 1% hyaluronidase (Sigma-Aldrich Chemie BV) was used to unmask the epitopes. Nonspecific binding was blocked using 10% normal goat serum (CLB, Amsterdam, The Netherlands) followed by incubation with the respective antibody for 2 hours. Antibodies used were M38 and II-6B3 (both 1:100; Developmental Studies Hybridoma Bank) for procollagen type I and collagen type II, respectively; ab6308 (mouse monoclonal IgG antibody, 1:500; Abcam Ltd, Cambridge, UK) for collagen type I; and ab6310 (mouse monoclonal IgG antibody, 1:500, Abcam Ltd) for collagen type III. All primary antibodies were previously complexed with goat Fab fragment against mouse conjugated with alkaline phosphatase (GAMAP, 1:400; Immunotech, Marseilles, France) at 4°C overnight. After coupling, 0.1% normal mouse serum was used for 2 hours before usage to capture the unbound GAMAP, after which the antibody solution was used on the slides. Sections were subsequently incubated for 30 min with alkaline phosphatase anti-alkaline phosphatase (APAAP, 1:100 for procollagen I and collagen II, 1:75 for collagen types I and III; Dakopatts, Copenhagen, Denmark). New Fuchsine substrate (Chroma, Kongen, Germany) was used for colour development and haematoxylin for counterstaining, after which slides were mounted using Vectamount (Vecto Laboratories Inc., Burlingame, CA, USA). Negative controls were subjected to the same protocol with omission of the primary antibody.

Mechanical testing

After harvesting of the constructs, the surrounding fibrotic tissue was carefully removed. From each of the six mice, one control and one enzyme-treated construct were frozen using liquid nitrogen and stored in airtight tubes at -80°C for later mechanical testing. Immediately before testing constructs were slowly thawed in airtight tubes. Thickness of the sample was measured to an accuracy of 50 μm using calipers. Constructs were then mounted in a specially designed push-out setup (Fig. 1) on a materials testing machine (LRX; Lloyd Instruments, Fareham, UK) equipped with a 500 N load cell. Push-out tests were performed by leading the push-out rod on top of the 3 mm inner core through the specimen at 10 μm/s. During the test constructs were kept moist by adding a few drops of phosphate-buffered saline on top before starting the test, which on average took 4–5 min. During the test both displacement and load were monitored at a sample frequency of 18 Hz and the output of these values was read out and stored on a desktop computer. For each specimen the peak load-to-failure (maximum observed load) was used to calculate the interface stress-to-failure (maximum load normalized to interface area) as a representative marker of interfacial strength. Furthermore, we also performed push-through tests of intact cartilage for comparison ($n = 4$) and push-out tests of constructs immediately after reassembly of core and annulus ($n = 8$) to determine the friction component of our setup; all this was done in a manner similar to that described above.

Statistical analysis

Values shown are mean ± standard deviation unless otherwise specified. Statistical analyses for both viable cell count and mechanical testing were done using Student's t-test for independent samples. Matrix integration scores and results from polarized light microscopy were analyzed using the Mann–Whitney U test. $P \leq 0.05$ was considered statistically significant.

Results

Histology

Cell counts in the integration area revealed significantly more vital cells near the wound edges in the enzyme-treated group than in the untreated group in all three layers (Table 1), with the largest increase in the superficial layer.

Table 1

Effect of enzymatic treatment on cell viability in cartilage wound edges 5 weeks after subcutaneous implantation in nude mice

Zone	Unwounded	Not enzyme treated	Enzyme treated
Surface	1440 ± 175	1151 ± 133	2316 ± 209*
Middle	787 ± 160	866 ± 27	1097 ± 59*
Deep	646 ± 75	589 ± 16	960 ± 45*

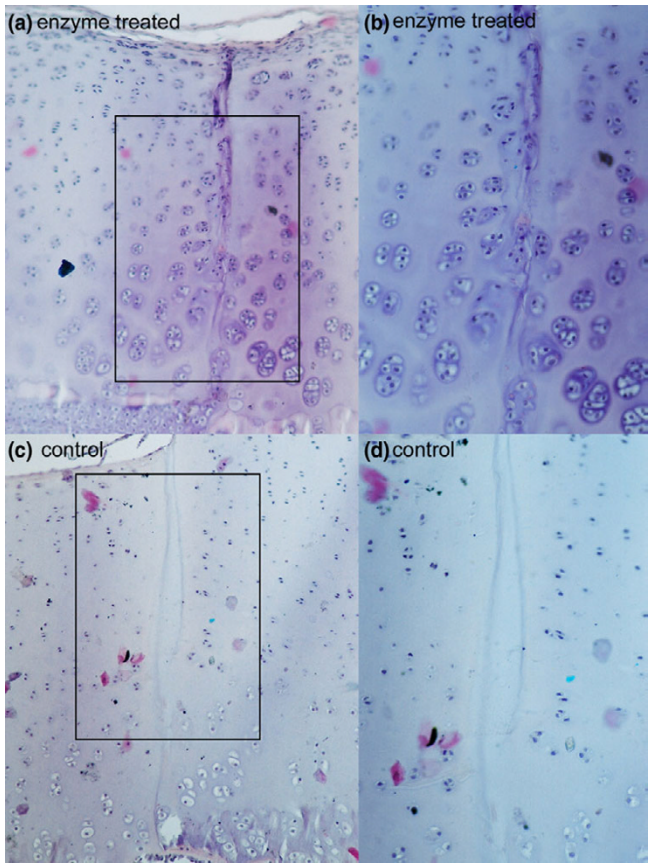
Cartilage was treated with hyaluronidase and collagenase or left untreated and implanted subcutaneously into nude mice for 5 weeks. The number of vital cells were counted in a 150-mm broad band along both sides of the wound edges, as well as in unwounded control areas in surface, middle and deep zones. *$P < 0.05$ versus unwounded areas and non-enzyme-treated wound areas.

Many cells were located in the interface region of the enzyme-treated group (Fig. 2a,2b), but despite the apparently normal average vital cell count in the 150-μm broad band in the untreated control samples, the tissue in the interface region was almost acellular (Fig. 2c,2d). Measurement of matrix integration on thionin stained sections (Fig. 3) revealed an average matrix–matrix connection percentage of 83 ± 15% of wound interface length in the enzyme-treated constructs, as compared with 44 ± 40% in the untreated group ($P < 0.05$), with variability between sections of the same interface typically being less then 15%.

To assess the quality of the newly formed interface matrix we evaluated which types of collagen were present in this new tissue. Immunohistochemical staining revealed the presence of limited amounts of (pro-)collagen type I in the interfaces, which was limited to the area of ingrowth of fibrous tissue from the top surface (Fig. 4a; four out of 10 interfaces in the treated group and three out of 10 interfaces in the control group). Typically, this ingrowth was around 10% of the interface length, with Fig. 4a showing the worst case. Furthermore, an abundance of cartilage-specific collagen type II was found in all interfacial matrices (Fig. 4b), whereas no collagen type III was found in any of the interface areas (Fig. 4c). No clear differences in immunohistochemical staining were observed between the two groups.

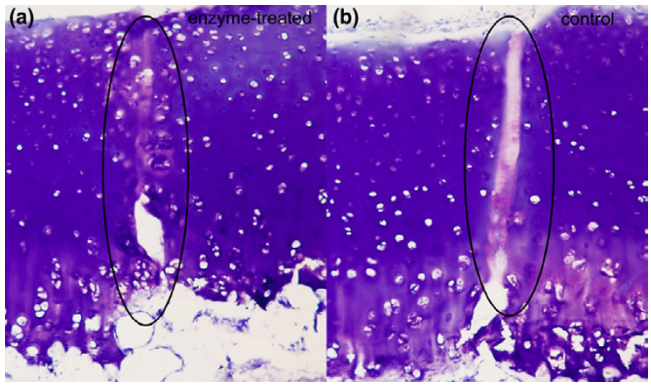
Polarized light microscopy of picro-Sirius Red stained sections indicated that collagen fibres in the wound interface were mainly directed perpendicular to the interface. Many fibres were seen crossing the interface in three out of five treated samples and in none of the control samples. Occasional fibre crossing was observed in two out of five treated samples and in three out of five control samples; in two out of five control samples no fibre crossing was observed. Most of the perpendicularly running fibres in the untreated control group protruded only into the interface (Fig. 5). Statistical analyses showed a significant difference between groups ($P < 0.05$).

Figure 2



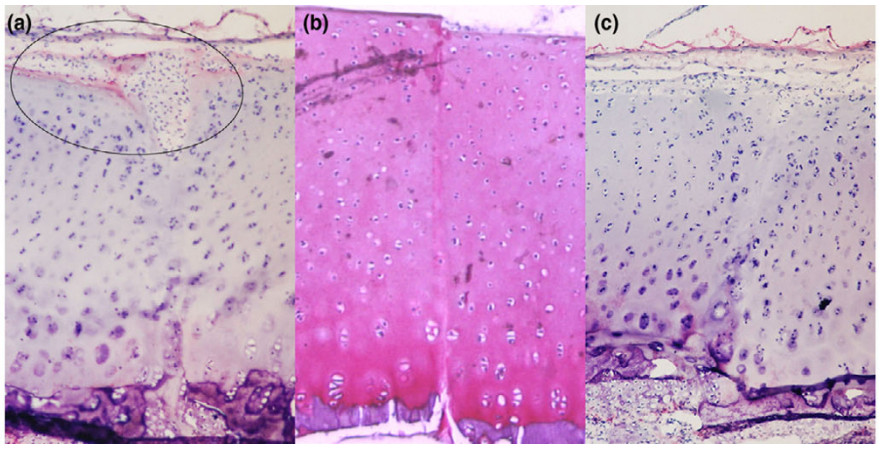
HE-stained sections of enzyme-treated and untreated control constructs 5 weeks after implantation. **(a)** Enzyme treated construct that shows good integration, with cells located in the interfacial region [**(b)** enlargement]. **(c)** Untreated construct that shows a poor integration, with no cells present in the interfacial tissue [**(d)** enlargement]. Magnifications: panels a and c 100×; panels b and d 200×.

Figure 3



Thionin stained sections 5 weeks after implantation in nude mice. **(a)** Enzyme treated and **(b)** untreated control section. Note the clear difference in thionin staining of the interfacial tissue between the enzyme-treated and control section. Interfaces are encircled.

Figure 4



Immunohistochemical stainings for collagens present in the interfacial area of enzyme-treated constructs. **(a)** Collagen type I, with light staining (in red) in the area of fibrous ingrowth (circled). **(b)** Collagen type II, showing medium intensity staining (in red) in the entire matrix of the interfacial area. **(c)** Collagen type III; staining (in red) only present in the surrounding capsule.

Mechanical testing

Mechanical assessment of the cartilage interface between inner core and outer ring by push-out test revealed that the interface connection was stronger in the treated group; the enzyme-treated group exhibited a 58% increase in stress-to-failure over the untreated controls (1.32 ± 0.15 MPa versus 0.84 ± 0.14 MPa). Average force–displacement curves, including standard errors, are shown in Fig. 6.

Furthermore, the push-through strength of intact articular cartilage was 8.8 ± 0.52 MPa, with failure occurring in an annular manner, as with the integrated constructs. Push-out tests performed immediately after reinsertion of the core into the annulus revealed a maximum friction stress of 22.2 ± 9.4 kPa, which is only 1.7–2.6% of the stress measured in the integrated constructs.

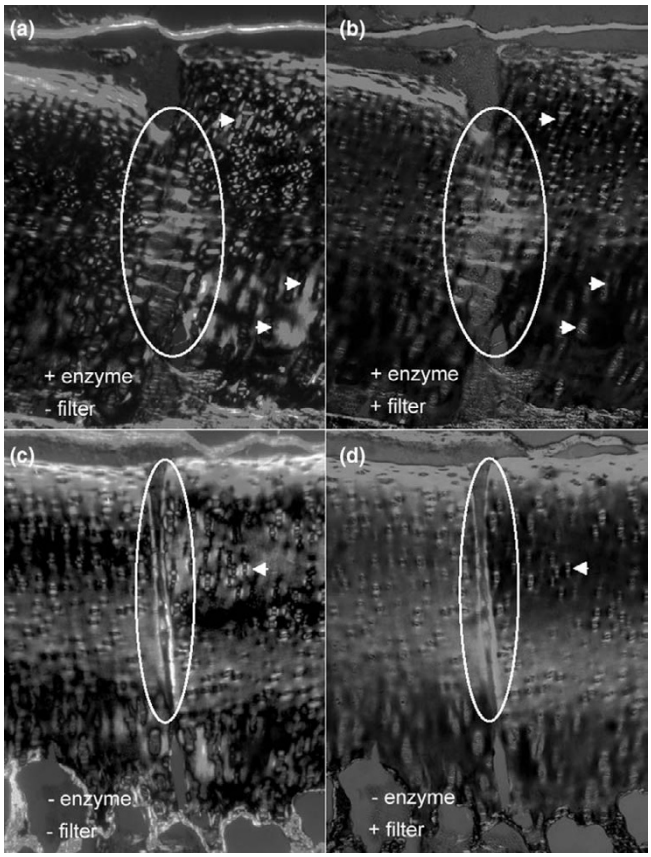
Discussion

In the present study we found an improvement in histological and biomechanical integration of articular cartilage after treatment with a combination of hyaluronidase and collagenase, a protocol that was previously shown to increase chondrocyte densities in wound edges *in vitro* [22]. Our setup of a 3-mm disc placed in an annulus provides a reasonable representation of the *in vivo* situation, in which cartilage is transplanted into a defect with wound edges perpendicular to the surface. Because an *in vitro* culture system might not provide the optimal environment for tissue growth and repair [24], we decided to perform our experiments in the well established nude mouse model [25,26], creating an environment in which there is an ample supply of nutrients.

In this setup, cellularity in nontreated wound edges reached the levels of unwounded cartilage, and cellularity of unwounded cartilage was increased to a level similar to that before implantation, which is in contrast to results from our previous *in vitro* study [22]. We believe that this is due to the nutrient-rich *in vivo* environment. However, in this model we confirmed [22] that the enzymatic treatment protocol enhanced the number of cells near the wound edges as compared with nontreatment, and resulted in better histological integration, as assessed by the percentage of matrix connection in the interfacial area. Furthermore, the repair tissue exhibited collagen fibres crossing the wound edges, and the matrix in both experimental groups exhibited cartilage specific collagen type II, limited (pro-)collagen type I and no collagen type III. This improved integration following enzymatic treatment was further supported by push-out tests, which are similar to tests described by others [24,27].

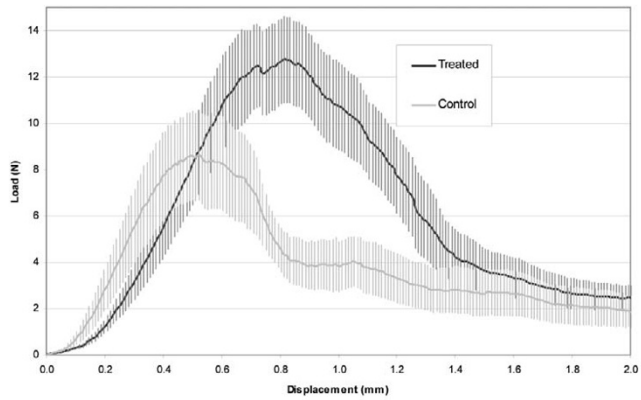
Although enzymatic treatment significantly increased mechanical strength to 1.32 MPa, the interfacial strength was still almost sevenfold less than the 8.8 MPa intrinsic failure strength values observed for intact cartilage. It should be appreciated that the fairly simple normalization to interface area is a rather crude method because the interface stress is not uniformly distributed. Therefore, tests using different sizes or shapes of specimens cannot readily be compared. Because the average thickness of our samples was 1.14 ± 0.28 mm for the treated group and 1.14 ± 0.21 mm for controls, and no correlation could be found between sample thickness and failure strength, we may compare strength values within the present study.

Figure 5



Picro-Sirius Red stained section of the interface regions (circled). **(a, b)** Enzyme treated group, well integrated. **(c, d)** Untreated control group, not integrated. In panels a and c show crossed polarizing filters without analyzer filter; fibres run in parallel and perpendicular directions relative to the interface. Note the squares around individual chondrocytes, signifying pericellular collagen shell (arrowheads). In panels b and d the same field of view is shown as in panels a and c, but this time with the analyzer filter in place, revealing only those fibres that run in a perpendicular direction relative to the interface(circled), pointed out by the fact that the pericellular fibres that run in the parallel direction have disappeared (arrowheads), as well as the lightening up of the superficial cartilage layer. Clearly visible are the fibres crossing through the interface area, thus connecting both pieces of cartilage (panel b) and fibres along the wound edge projecting into the interface area (panel d). Original magnification: 25×.

Figure 6



Average force-displacement curves of push-out tests with standard error bars for untreated ($n = 5$) and enzyme-treated ($n = 6$) curves, respectively. Failure strength in the enzyme-treated group was significantly higher (+58%). The failure of the curve to return to zero can be explained by friction between pushed-out core and sample holder.

Our findings indicate a relation between interfacial strength and cellular activity at the interface. This confirms the results reported by DiMicco and coworkers [28], who used fetal, calf and adult bovine cartilage; after 14 days of culture those investigators found the highest failure stress in calf cartilage at 77 kPa in a single lap shear test. However, Reindel and coworkers [29] found an interface strength of 34 kPa after 3 weeks of culture, and showed that integrative strength was highly dependent on the use of fetal bovine serum in culture, which can influence cellular activity. Dependence of integration on active cell processes is also demonstrated by lack of adhesive strength when combining two lyophilized explant blocks [30]. In an 8-week bioreactor culture of tissue engineered cartilage core constructs with surrounding native cartilage, Obradovic and coworkers [24] found better mechanical integration of very young (5 days) constructs (254 kPa) as compared with more mature constructs (5 weeks; approximating 150 kPa). Peretti and coworkers [26] also used lyophilized explants, which were seeded with chondrocytes and then held together using fibrin glue and placed subcutaneously in nude mice for up to 6 weeks. Tensile testing showed a clear increase of failure strength to 77 kPa, which is 10 times higher than unseeded control explants, with failure always occurring at the interface between new tissue and devitalized matrix. Because cellular activity is clearly an important factor in integration, we should appreciate that in most studies young bovine cartilage is used, which is more cellular than human cartilage. In a previous study, however, we did see similar effects of enzymatic treatment on cell density in human adult articular cartilage [22]. It can be anticipated that, because of the lower cell numbers, the overall repair process might be slower than in the present study but can still be stimulated using enzyme treatment.

Our findings suggest that enzymatic treatment may be a promising technique with which to improve cartilage integration, in addition to currently developing clinical and experimental articular cartilage repair techniques. The cell counts along the wound edge in the control group were comparable to those of native tissue. However, a close look at the histological pictures (Fig. 2c,2d) shows that a thin acellular band is still visible. In the enzyme-treated group cell counts were even higher than those in native cartilage, and histology did not reveal a large acellular band, as seen in the controls (Fig. 2a,2b), thus fulfilling one of the prerequisites for integration, namely the presence of active

chondrocytes close to the lesion site. The high cellularity at the wound edge observed in the present study probably resulted in the increased collagen fibre deposition across the wound gap of adjacent cartilage surfaces, as shown in the picro-Sirius Red slides (Fig. 5). Normally, cross-gap deposition of collagen between native and repair tissue is insufficient in the reparative process that occurs after full-thickness defects [2,31,32]. The observed cross-gap deposition of collagen in the present study coincides with increased interfacial strength, as shown previously in integration experiments using fetal, calf and adult bovine cartilage explants [28]. Those studies showed a correlation between increased adhesive strength and an increased hydroxyproline incorporation in the interface area. Furthermore, inhibition of collagen cross-link formation by β-aminopropionitrile resulted in almost complete loss of integrative repair.

The explanation for the success of the enzymatic treatment technique may be found by examining wound healing in vascularized tissues. In nonvascularized articular cartilage, proteolytic enzyme activity is either lacking or insufficient to degrade and remove the observed acellular band in the wounded areas, as occurs with debris and necrotic tissue in vascularized tissues. Application of enzymes may remove this layer, uncovering an activated area of chondrocytes that are capable of integration. Another possible underlying mechanism of this enzymatic treatment may be the partial degradation of extracellular matrix surrounding the wound edge chondrocytes, which frees chondrocytes from the tight extracellular matrix in which they were entrapped. Because chondrocytes have been shown to have the ability to migrate [33], this may enable them to move to the wound edge in need of repair. A third possible mechanism of the enzymatic degradation of wound edge extracellular matrix may be the stimulation of local chondrocyte proliferation, which can be seen by looking at the cell clusters in the histological images (e.g. Fig. 2). Although we did observe cell division, the exact mechanism by which the enzymatic treatment exerts its effects is still unclear. A more detailed mechanistic study is needed to further elucidate this.

In the present study we demonstrated the potential of hyaluronidase and collagenase treatment in a screening '*in vivo*' environment. Animal experiments with actual articular cartilage defects are needed to determine the value of our findings. Further studies must be undertaken to optimize the enzymatic treatment protocol (e.g. shorter treatment duration) and learn more about the mechanisms involved, such as cell migration to the wound area and matrix deposition, and to improve mechanical interface strength further to the level of intact cartilage, which is still almost an order of magnitude higher. Therefore, longer term studies are required to judge the success of different integration enhancing techniques against the mechanical strength of intact cartilage, and to develop protocols that may become clinically applicable, which in our view could be a valuable addition to existing repair strategies.

Conclusion

The present study shows that enzymatic treatment of cartilage wounds increases histological integration and improves biomechanical bonding strength. Enzymatic treatment may represent a promising addition to current techniques for articular cartilage repair.

Competing interests

None declared.

Acknowledgements

Monoclonal antibodies II-6B3 and M38 were obtained from the Developmental Studies Hybridoma Bank, which is maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA and the Department of Biological Sciences, University of Iowa, Iowa City, Iowa, USA under contract N01-HD-6-2915 from the National Institute of Child Health and Human Development. The authors thank Nicole Kops for immunohistochemistry, Inge van Rensen for her work on cell counting, and Corrina de Ridder for her help in the nude mice experiments. Further thanks go to the company T. Boer & Zn., Nieuwerkerk a/d IJssel, The Netherlands, in particular Ton Looijen, for their kind supply of bovine joints.

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