Cartilage labelling for mechanical testing in T-peel configuration

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Abstract

Purpose The purpose of this study was to find a suitable method of labelling cartilage samples for the measurement of distraction distances in biomechanical testing.

Methods Samples of bovine cartilage were labelled using five different methods: hydroquinone and silver nitrate (AgNO_3), potassium permanganate (KMnO_4) with sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$), India ink, heat, and laser energy. After the labelling, we analysed the cartilage samples with regard to cytotoxicity by histochemical staining with ethidiumbromide homodimer (EthD-1) and calcein AM. Furthermore, we tested cartilages labelled with India ink and heat in a T-peel test configuration to analyse possible changes in the mechanical behaviour between marked and unlabelled samples.

Results Only the labelling methods with India ink or a heated needle showed acceptable results in the cytotoxicity test with regard to labelling persistence, accuracy, and the influence on consistency and viability of the chondrocytes.

In the biomechanical T-peel configuration, heat-labelled samples collapsed significantly earlier than unlabelled samples.

Conclusion Labelling bovine cartilage samples with India ink in biomechanical testing is a reliable, accurate, inexpensive, and easy-to-perform method. This labelling method influenced neither the biomechanical behaviour nor the viability of the tissue compared to untreated bovine cartilage.

Introduction

Principle biomechanical testing methods of articular cartilage repair described by Ahsan et al. [1] included the push out model [2], the partial overlap model, and the T-peel test [3]. In all three tests, cartilage blocks were cultured in tissue culture, inducing a measurable repair process of opposing cartilage surfaces. In the push out model, a round piece of cartilage was punched out and re-inserted into the same opening, whereas in the partial overlap model two cartilage blocks were positioned in a partial overlap configuration. The T-peel test differed from the other two tests insofar as the cartilage block was incised only halfway. Rupture of the non-incised part of the cartilage block was induced, and the ruptured surfaces were re-joined and measured. Therefore, the T-peel test may be used for simulating two different clinical situations: incisions, for instance, during surgical interventions or ruptured surfaces, such as after accidents. During all these tests, specimen deformity occurs before rupture. This distraction process is best assessed with an optical deformation measurement instrument that measures distances between individual markers on a given specimen. However, this method has never been applied in the context of cartilage repair. Yet, specimen deformation the time point of initial rupture may vary to a large degree, necessitating

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accurate estimations of a deformity [4], which requires the labelling of distances on the cartilage pieces by means of markers.

For histopathological examination, staining methods to provide visible markers depend on the aim of a particular investigation [5]. In order to facilitate the use of this optical deformation measurement system cartilage specimens required visible markers that were neither toxic, nor immediately removable or susceptible of change by liquids, nor altering the cartilage tissue in any way. This optical deformation measurement system required defined markers larger than 100 μm on or within the cartilage specimens for optimal application.

Therefore, we investigated different labelling methods with regard to their accuracy, handling properties, the viability of chondrocytes and the biomechanical impact of the methods on the specimens.

Materials and methods

Bovine cartilage tissue

The entire knee joints of the hind legs of eight to 12-week-old calves were purchased from a local abattoir after approval by a veterinarian. Osteochondral cylinders measuring $20 \times 15 \times 15 \text{ mm}$ were cut from the femoro-patellar joint surface of the femoral part of the knee with an oscillating saw (Stryker Instr.). A master plate divided each cylinder and a microtome (Microm HM440E) into areas sized $12 \times 2.5 \text{ mm}$ measuring 6 mm in thickness (Fig. 1).

Fig. 1 **a** and **b** The pictures and illustrations show the three steps of cartilage block preparation. Osteochondral blocks harvested from the patellofemoral groove of bovine hind legs were cut with a scalpel in horizontal and perpendicular directions. The prepared cartilage region is further dissected by a sledge microtome, which finishes the cartilage tissue for T-peel testing by partially incising the block from one side. **c** The exact geometrical parameters of the prepared cartilage block are given. Parts of the block can be adjusted by clamps, so that the left part of the block can be peeled and measured with a biomechanical testing device

Labelling with Indian ink

To keep the cartilage samples sterile and for better handling, the samples were clamped into a specially designed holding device consisting of two plexiglas layers and one thin (600 μm) aluminum layer for all labelling methods (see Fig. 2a). Black Indian ink was used for labelling by depositing ink pigments into cartilage tissue. Three different ways of depositing were evaluated. To create punctate marks, we punctured cartilage with either a 27 G or a 30 G needle or both that had previously been dipped into black ink. To create longish marks, we cut cartilage surfaces with standard razor blades (GEM) that had been dipped into black ink. Cartilage samples were humidified with PBS-solution.

Labelling with a heated needle or a plane indenter

Moistened cartilage samples were clamped into the holding device. A sewing needle and a plane indenter, made of steel (Fig. 2b), were heated in a gas flame. Different application modes were tested:

1. Labelling with the tip of the needle
2. Labelling with the longitudinal side of the needle
3. Labelling with the tip of the indenter
4. Labelling with the longitudinal side of the indenter (Fig. 2a)

Burn marks were applied at intervals of 2 mm. Labelling with the tip of the needle even allowed intervals of less than 1 mm.

Fig. 2 **a** The picture illustrates the custom-made clamps for mounting the samples into a mechanical testing machine. The clamps are surrounded by a sterile plexiglas bowl to keep the samples irrigated during the T-peel test. **b** Mechanical testing machine for measuring the applied force for T-peeling

Labelling with hydroquinone and silver nitrate (AgNO_3)

A solution of 1.5 ml of 0.1 M phosphate-buffer and 100 mg hydroquinone was mixed. Cartilages were treated in two different ways:

1. Cartilage samples were dipped into hydroquinone solution, labelled with AgNO_3 using a 27 G needle, and washed again in hydroquinone solution
2. Cartilage samples were labelled with AgNO_3 using a 27 G needle and washed in hydroquinone solution

During each procedure, samples were humidified with PBS-solution by keeping them covered in a PBS-filled petri dish.

Labelling with laser

A solid-state laser (Visual 532 s, Carl Zeiss Meditec) was used with a maximum spot size of 50 μm and an irradiation time of 100 ms. Energy levels ranged from 100 mW to 250 mW. After irradiation of untreated cartilages humidified with PBS-solution, the samples were coated with black ink and irradiated again. Humidification was done with PBS solution in a PBS-filled petri dish.

Labelling with potassium permanganate (KMnO_4) and sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$)

A solution of 25 ml of 0.1 M phosphate-buffer and 1 g of $\text{Na}_2\text{S}_2\text{O}_3$ was prepared and divided into two petri dishes. The cartilage sample was washed for five minutes in sodium thiosulphate. KMnO_4 was applied to the cartilage with a

27 G needle. Afterwards, the cartilage sample was washed again in $\text{Na}_2\text{S}_2\text{O}_3$ to remove any remains of KMnO_4 .

Live/dead analysis

To check the viability of chondrocytes, the cartilage samples labelled with ink and burn marks were incubated with the Live/Dead® Viability/Cytotoxicity Assay Kit (L-3224). For this, 10 ml of a 4- μM EthD-1 solution was mixed with 5 μl of 4 mM calcein AM. Cartilage samples were incubated in this solution in a petri dish for 30 minutes at room temperature. Calcein diffuses through cell membranes and fluoresces green under a fluorescence microscope, whereas EthD-1 only penetrates into dead cells and fluoresces red. None of the chemicals used affected the viability of the chondrocytes.

Biomechanical testing

Peeling experiments were conducted to test the stability of cultured 3D-cartilage constructions that are visualised in a stress-strain curve. Different peeling tests are available, such as push out or single lap tests. In our experiment, we used a T-peel test in which a cartilage block was incised only halfway. Rupture of the non-incised part of the cartilage block was induced, and the ruptured surfaces were re-joined and measured. To induce the rupturing process, we designed a special uniaxial clamping tool (Fig. 2a) used in combination with a universal testing machine (Hegewald & Peschke GmbH; Fig. 2b). During the peeling procedure, the cartilage samples were fixed at both thick ends measuring 300 μm and placed in a shallow bowl containing a

Fig. 3 The upper pictures illustrate the T-peel progress of the cartilage block. The numbers 1 to 6 on pictures **a** and **c** refer to the markers on the cartilage sample. It is obvious that the distance between the markers increase during the peeling process. The diagram below shows a typical stress–strain curve depicting the initial bending of the sample until rupture of the cartilage block starts. Only after the rupture, the force needed to peel the cartilage tissue remains the same

physiological saline solution. Peeling was conducted over the entire cartilage sample or at least until collapse. Distraction velocity was chosen at 15 mm/min. The resulting force was measured with a precision of 0.01 N. Additionally, the optical deformation during peeling was measured with a high-resolution camera (Aramis, GOM AG) recording two pictures per second (approximately 120 pictures per run). The distraction between labelling spots could be calculated and thus the distraction of the entire cartilage sample (see Fig. 3).

Results

Acceptable results with regard to labelling persistence, accuracy, influence on consistency and viability of chondrocytes could be achieved with Indian ink and heat labelling.

Labelling with a heated needle or heated indenter

Labelling with the tip of a needle showed only poor visual effects. Furthermore, handling, heating, and labelling were more challenging than in other methods. Using the longitudinal side of a heated needle showed more obvious

visual effects. The best visual effects of heat labelling, however, were achieved with the longitudinal side of the modelling instrument, whereas the tip of the indenter generated burn marks which were too large. The depth of impression was easily controllable by the holding device.

Live/Dead®-analysis was done for specimens treated with a longitudinally applied indenter and a longitudinally applied needle. For the heated indenter, a large area of non-vital chondrocytes was detected measuring between 40 and 200 μm around the impression site sized 300 by 120 μm . Even in less prominent areas, islets of dead chondrocytes were detected. Comparatively little trauma was found in the specimens treated with the heated needle. The impression site itself measured 40 $\times$ 40 μm and was surrounded by an area with non-vital chondrocytes sized 40–80 μm . No remote islets of non-vital chondrocytes were detectable (see Fig. 4a). Figure 5.

Testing these samples for mechanical integrity, the T-peel test showed higher rates of premature collapse than for native samples (see Table 1). Collapsed sites remarkably often included areas of burn marks, showing non-vital chondrocytes at the tearing edge under the microscope.

Fig. 4 Live/Dead®-viability test: red staining indicates dead cells, and green staining viable cells. The area of labeling application is marked by arrows. **a** Heat labelled cartilage with large areas of necrotic cartilage shown in red colour. **b** Ink labelled cartilage with hardly any necrotic cartilage shown in green colour; the black spot visible within the green area is ink

Fig. 5 Overview of the investigations depending on the labelling methods used

Labelling with Indian ink

The application of Indian ink by a needle is easily done. Small, black, puncture marks remain even in small specimens (12 mm × 300 μm), and are not removed by moistening with PBS solution. The Live/Dead®-analysis showed microscopically small traumatised areas (with a maximum of 280 × 200 μm) with only marginally more dead chondrocytes than other non-treated areas (see Fig. 4b). A higher number of dead cells were found after using the razor blade. The traumatised area of this method measured 200 × 600 × 350 μm, and a black straight-line mark remained. The T-peel test on specimens labelled with a needle showed no statistically significant difference to native samples with regard to premature collapse (Table 1).

Labelling with laser

Laser irradiation of untreated cartilage specimens did not show any reproducible results. Even changes of energy levels up to 250 mW and a spot size of 50 μm did not achieve any favourable results. Staining the entire cartilage sample with black ink resulted in a mark on the cartilage surface. The laser beam was augmented by particles of the ink adhering to the cartilage's surface. Different energy levels led to differently sized burn marks ranging from 300 × 540 μm with 250 mW and to 200 × 400 μm with 100 mW.

Table 1 Success rate of T-peel testing depending on cartilage preparation. The set markers by heat labeling weaken the cartilage sample so that premature collapse occurs. Only ink labeling does not alter the mechanical behaviour of the sample

Cartilage preparation/t-peel-test result	No collapse	Premature collapse
Labelled by needle and Indian ink	5 (41.7%)	7 (58.3%)
Labelled by a heated needle	0 (0%)	12 (100%)
Native	9 (50%)	9 (50%)

The depth of the burn marks reached up to 70 μm. Microscopic analysis of the labelled areas showed a large surrounding zone of damage measuring 100 μm and more. Therefore, laser-labelled specimens did neither undergo Live/Dead®-analysis nor T-peel testing.

Labelling with hydroquinone and silver nitrate (AgNO₃)

Markings labelled with hydroquinone and AgNO₃ first showed good clear and accurate marks; however, after the cartilage had been washed in hydroquinone solution, the markers appeared yellowish and blurred. After storage in PBS solution for a few days, cartilage specimens showed loss of elasticity but increased stiffness. Therefore, samples labelled with hydroquinone and AgNO₃ neither underwent Live/Dead®-analysis nor T-peel testing.

Labelling with KMnO₄ and Na₂S₂O₃

After labelling the samples with KMnO₄, marks were immediately washed away, even when KMnO₄ was placed into the specimens by means of a needle. Therefore, this method was not followed-up with further tests.

Discussion

Our investigations revealed a precise procedure for staining (bovine) cartilage specimens. Furthermore, we were able to show that staining with Indian ink does not induce any changes, in either biomechanical behaviour or in the viability of the stained chondrocytes. The reliable marks caused by Indian ink have been described in previous studies on staining entire surfaces of specimens [6]. To our knowledge, no method has been yet described for applying precise, persistent marks measuring less than 200 μm in diameter. This exact marking is necessary to accurately measure distraction distances in biomechanical testing, such as T-peel

tests. The damage to chondrocytes around the marker point may be explained by the pressure caused by puncturing the cartilage with a 27 G needle. Pressure necrosis of chondrocytes in vitro [7] and in vivo [8] has been described in previous studies. In contrast, no evidence has been found in the literature that Indian ink causes necrosis in chondrocytes, either in vitro or in vivo. After the ink staining, the cartilage specimens could be easily kept moist by covering them in PBS solution for several days without losing the marks for further testing, e.g. T-peeling. Nevertheless, the accurate application of ink requires a holding device for the cartilage specimens and a needle no bigger than 27 gauge for a size mark not larger than 200 μm in diameter. In our test series, we observed a short learning curve in acquiring the most efficient and precise technique of applying the marks.

Setting marks with heated instruments is definitely a reliable and easy-to-perform method. The development of comparably large areas of cell necrosis due to heat is an inevitable side effect. Heat in general is a well-known factor in necrosis of chondrocytes not only in vitro [9] but also in vivo [10]. Therefore, this method is not useful in scientific settings that require viable cartilage. We could show that, in a biomechanical T-peel test, this staining method leads to the falsification of results.

Staining with laser was thought to be a precise and easy-to-perform method. Also the application of laser energy is widely used in the clinical treatment of arthroscopically performed chondroplasties. However, no visible marks could be produced, even at high energy levels. We were able to produce small marks after staining the surface with black ink. Microscopy showed a large area of cell damage despite the presence of small visible marks. Necrosis of chondrocytes after the application of laser energy is related to the energy levels used and the duration of laser application [11]. Producing a small visible spot with a laser requires high energy levels and a comparatively long application time so that large areas of cell necrosis around the application area cannot be avoided. Therefore, we also found this method unsuitable for scientific investigations.

Staining cartilage samples with hydroquinone and silver nitrate resulted in precise and well visible marks. But during a time course of 12–24 hours, cartilage samples changed their biomechanical behaviour showing increased stiffness. These samples were moistened as usual with PBS solution to prevent chondrocytes from drying out. Nevertheless, the specimens became unsuitable for biomechanical testing. Rosa et al. [12] described the glycosylated hydroquinone "arbutin" as an effective cryoprotective agent. We could find no previous description in the

literature explaining the results of our investigation. Therefore, we suggest that hydroquinone was responsible for the change in biomechanical behaviour; thus we do not recommend using hydroquinone for treating cartilage samples in biomechanical testing.

Labelling with potassium permanganate and sodium thiosulphate resulted in invisible marks from the beginning. The coloured potassium permanganate was washed away by sodium thiosulphate, so that no reliable marks remained. Thus, we cannot recommend this method for staining cartilage samples either.

Conclusion

In conclusion, small, precise, and persistent marks in cartilage specimens can be reliably produced using Indian ink without any change in chondrocytes or biomechanical behaviour. Thus, we recommend this method of staining for biomechanical testing.

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References

- Ahsan T, Sah RL (1999) Biomechanics of integrative cartilage repair. *Osteoarthritis Cartil* 7(1):29–40
- Reindel ES, Ayroso AM, Chen AC et al (1995) Integrative repair of articular cartilage in vitro: adhesive strength of the interface region. *J Orthop Res* 13(5):751–760
- Englert C, Greiner G, Berner A, Hammer J (2008) T-peel test for the analysis of articular cartilage integration. *Stud Health Tech Informat* 133:95–102
- Fierlbeck J, Hammer J, Englert C, Reuben RL (2006) Biomechanical properties of articular cartilage as a standard for biologically integrated interfaces. *Technol Health Care* 14(6):541–547
- Schmitz N, Laverty S, Kraus VB, Aigner T (2010) Basic methods in histopathology of joint tissues. *Osteoarthritis Cartil* 18(Suppl 3):S113–S116
- Salerno A, Trent R, Jackson PJ, Cook MG (1995) A rapid and safe method to fix India ink on specimen resection margins. *J Clin Pathol* 48:689–692
- Lee JH, Fitzgerald JB, Dimicco MA, Grodzinsky AJ (2005) Mechanical injury of cartilage explants causes specific time-dependent changes in chondrocyte gene expression. *Arthritis Rheum* 52(8):2386–2395
- Spahn G, Wittig R (2003) Biomechanical properties (compressive strength and compressive pressure at break) of hyaline cartilage under axial load. *Zentralbl Chir* 128(1):78–82

9. Voss JR, Lu Y, Edwards RB et al (2006) Effects of thermal energy on chondrocyte viability. *Am J Vet Res* 67(10):1708–1712
10. Lu Y, Edwards RB 3rd, Nho S et al (2002) Lavage solution temperature influences depth of chondrocyte death and surface contouring during thermal chondroplasty with temperature-controlled monopolar radiofrequency energy. *Am J Sports Med* 30(5):667–673
11. Jones N, Sviridov A, Sobol E et al (2001) A prospective randomised study of laser reshaping of cartilage in vivo. *Lasers Med Sci* 16(4):284–290
12. Rosa SC, Gonçalves J, Judas F et al (2009) Assessment of strategies to increase chondrocyte viability in cryopreserved human osteochondral allografts: evaluation of the glycosylated hydroquinone, arbutin. *Osteoarthr Cartil* 17(12):1657–1661