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Brief report
Cell labelling with superparamagnetic iron oxide has no effect on chondrocyte behaviour

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Summary

Background: Tissue engineering and regenerative medicine are two rapidly advancing fields of research offering potential for effective treatment of cartilage lesions. Today, chondrocytes are the cell type of choice for use in cartilage repair approaches such as autologous chondrocyte implantation. To verify the safety and efficacy of such approaches it is necessary to determine the fate of these transplanted cells. One way of doing this is prelabelling cells before implantation and tracking them using imaging techniques. The use of superparamagnetic iron oxide (SPIO) for tracking of cells with magnetic resonance imaging (MRI) is ideal for this purpose. It is non-radioactive, does not require viral transfection and is already approved for clinical use as a contrast agent.

Objective: The purpose of this study was to assess the effect of SPIO labelling on adult human chondrocyte behaviour.

Methods: Cells were culture expanded and dedifferentiated for two passages and then labelled with SPIO. Effect on cell proliferation was tested. Furthermore, cells were cultured for 21 days in alginate beads in redifferentiation medium. Following this period, cells were analysed for expression of cartilage-related genes, proteoglycan production and collagen protein expression.

Results: SPIO labelling did not significantly affect any of these parameters relative to unlabelled controls. We also demonstrated SPIO retention within the cells for the full duration of the experiment.

Conclusions: This paper demonstrates for the first time the effects of SPIO labelling on chondrocyte behaviour, illustrating its potential for *in vivo* tracking of implanted chondrocytes.

Key words: Superparamagnetic iron oxide, SPIO, Cartilage, Chondrocyte, Magnetic resonance imaging, Cell tracking, Cell labelling.

Introduction

In recent years, implantation of marrow derived stromal cells (MSCs) and chondrocytes into joints for the treatment of cartilage-related disorders has become increasingly common. Given the risks involved in cell based therapies, much research is currently focused on tracking of cells following implantation. This ability to track cells following implantation is not the only consideration to be taken into account when choosing imaging methods. It is also important to be able to visualise the defect and surrounding tissue to assess repair longitudinally. For both of these purposes magnetic resonance imaging (MRI) is at the fore of clinical potential. The use of superparamagnetic iron oxide (SPIO) particles for this purpose has proved promising in recent years. In this approach, small particles of iron oxide are introduced into cells and can be visualised as a hypointense signal by MRI[1–8] both *in vitro* and *in vivo*. While the general consensus is that there are no major effects of labelling on MSCs, some minor differences in chondrogenic and osteogenic differentiation have been observed[6,9,10]. Interestingly, no research has been reported on the effects of SPIO labelling on the redifferentiation capacity of dedifferentiated chondrocytes following *in vitro* expansion. The purpose of this research was to determine the efficacy and effect of labelling *in vitro* expanded chondrocytes on cell viability, proliferation and redifferentiation capacity.

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Materials and methods

CELL CULTURE

Chondrocytes were harvested from full-thickness articular cartilage from knees of humans undergoing knee replacement surgery by digestion in pronase (2 mg/ml, Sigma, St. Louis, MO, USA) for 2 h followed by overnight collagenase B (1.5 mg/ml, Roche Diagnostics). Chondrocytes were subsequently seeded at a density of 7500 cells/cm^2 in T175s and cultured for two passages, prior to labelling.

CELL LABELLING

Cells were labelled with Endorem (Guerbet S.A., Paris, France) and Lipofectamine 2000 (Invitrogen, Breda, The Netherlands) as previously described[11]. Briefly, 20 μl of both Endorem and Lipofectamine were mixed

separately with 500 μl of Opti-MEM (Invitrogen). They were then combined and left for 20 min. The culture medium was replaced by Opti-MEM and 3.25, 9.7, 29.3, 87.9 or 263.7 μl (equivalent to 2, 6.25, 18.75, 56.25, or 168.75 μg iron content) of the SPIO–Lipofectamine suspension was added drop-wise totalling 1 ml of suspension per well of a six-well plate containing 200,000 cells per well. Subsequently, the cells were incubated at 37°C/ 5%CO_2 for 24 h and then rinsed in Phosphate Buffered Saline (PBS). Four conditions, Control, Labelled, SPIO alone and Lipofectamine alone were used and were subsequently cultured under the same conditions.

ALAMAR BLUE ASSAY

The Alamar Blue assay (Invitrogen) was used as a measure of cell viability according to the manufacturer's instructions. Briefly, 70,000 cells were added per well of a 12 well plate and left overnight. They were then labelled overnight according to the above protocol. This was followed by two PBS washes and medium was added for 4 h. After this period Alamar Blue was added at a 1:10 concentration to the medium present. 4 h later 100 μl of this medium-Alamar mix was placed in a 96 well plate, read at 570 and 600 nm and analysed according to the provided calculations.

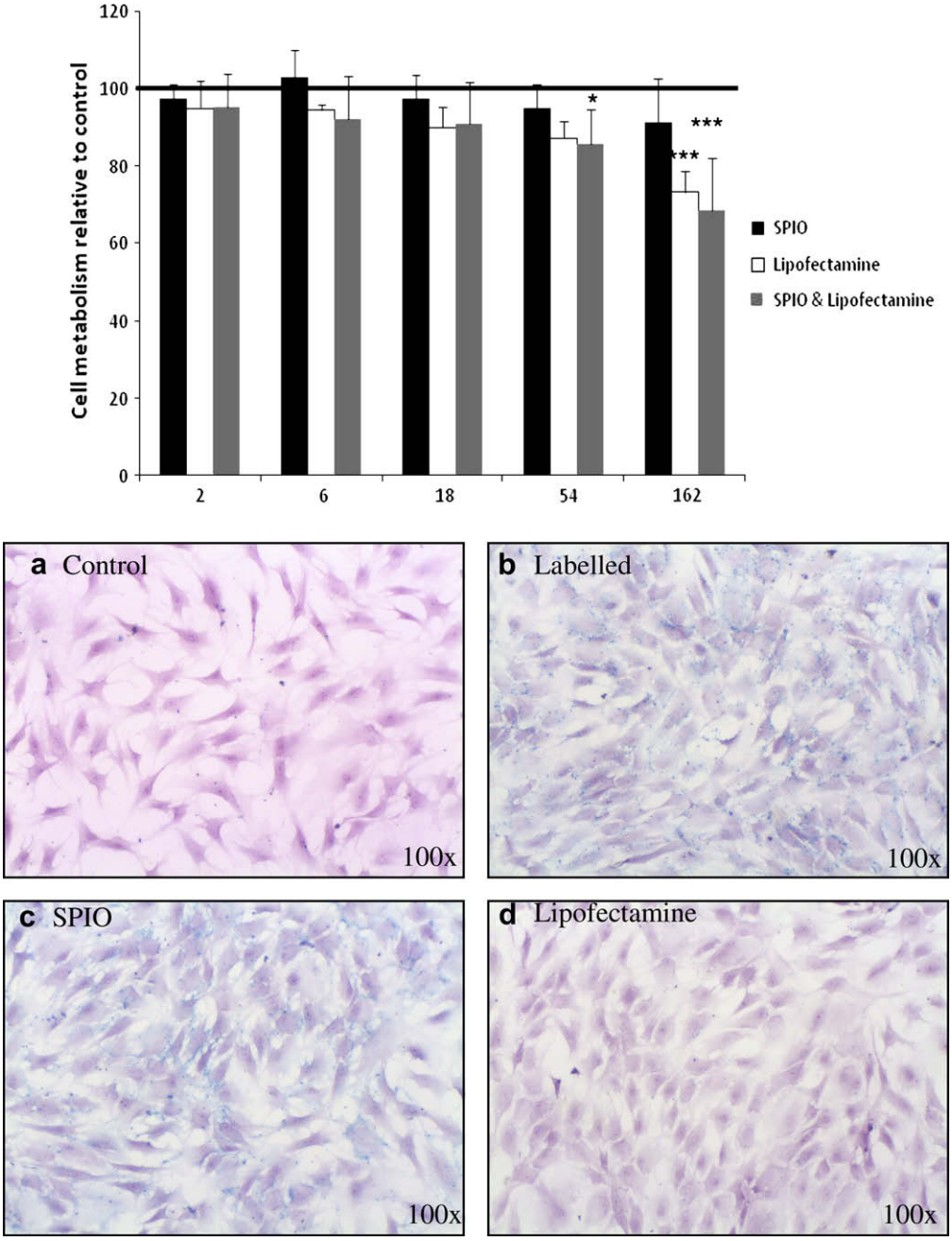

Fig. 1. Chondrocytes are labelled overnight by SPIO–Lipofectamine. Effects of varying concentrations of SPIO labelling was assessed by Alamar Blue cytotoxicity assay. Metabolic activity of cells is expressed as a percentage of unlabelled control cells. SPIO labelling had no effect on cell viability until the highest dose of 168 μg of SPIO per well. This detrimental effect was likely caused by the Lipofectamine. Duplicate samples were tested for four donors (two-way ANOVA $*P < 0.05$, $***P < 0.001$ significantly different from control). Figure 1(b) illustrates effective labelling of chondrocytes at a dose of 18 μg of iron per well.

CULTURE OF CHONDROCYTES IN ALGINATE

Chondrocytes were trypsinised, rinsed, resuspended in 1.2% (w/v) alginate (Keltone LV) in 0.9% NaCl (Sigma) at a concentration of 4×10^6 cells/ml, and beads were made as described previously[12]. Transferred to six-well plates (Corning BV, Schiphol-Rijk, The Netherlands), beads were cultured in 75 μl/bead serum-free chondrogenic induction medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with transforming growth factor (TGF)-β2 10 ng/ml (R&D Systems, UK), IGF-1 10 ng/ml (Sigma, Missouri, USA), L-ascorbic acid 2-phosphate 25 μg/ml (Sigma), and Insulin transferrin and selinium (ITS+) (diluted 1:100; BD Biosciences, Bedford, MA), 50 μg/ml gentamycin and 1.5 μg/ml fungizone and cultured for 21 days. On day 21 beads were harvested for RNA extraction, Glycosaminoglycan (GAG) and DNA assays and for histology.

BIOCHEMICAL ASSAYS

Alginate beads were digested overnight at 56°C in papain buffer (200 μg/ml papain in 50 mM Ethylene Diamintetraacetate (EDTA) and 5 mM L-cystein). GAG amount in the digest was quantified using dimethylmethylene blue (DMB) assay[13]. Interference from alginate in the assay was prevented by performing the test at pH 1.5 as has been described previously[14]. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-DMB. The amount of GAG was monitored by spectrophotometer, using the ratio A_{530}:A_{590} with chondroitin sulfate C (Sigma) as a standard. The amount of DNA was determined using ethidium bromide with calf thymus DNA (Sigma) as a standard.

IMMUNOCYTOCHEMISTRY FOR COLLAGEN TYPE II

To prepare cytospins, three alginate beads cultured for 21 days were dissolved in 55 mM sodium citrate acid. After fixation in cold acetone, the cytospins were incubated with a monoclonal antibody against type II collagen (1:100, II-II6B3; Developmental Studies Hybridoma Bank) at room temperature for 1 h. This was followed by incubation with link and label from the link-label kit (BioGenex, San Ramon, CA, USA) each for 30 min. Freshly prepared neo-fuchsin substrate was used to achieve staining. The number of cells positive for collagen type II per 100 cells was scored in four fields of sight per specimen.

GENE EXPRESSION ANALYSES

Primer and probe oligonucleotide sequences for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), COL X, SOX 9, COL II, and Aggrecan (AGC) assays are published by Mandl *et al.*[15], Uitterlinden *et al.*[16] and de Mos *et al.*[17], respectively. All Taqman assays were performed using triplicate biological samples per donor using Taqman® Universal Polymerase chain reaction (PCR) Master Mix (Applied Biosystems, #4324020) as described previously.

PREPARATION OF BONE PLUGS FOR MRI

Eight millimeter bovine osteochondral plugs were prepared and a 4 mm chondral defect was punched from the centre of each one. Into these defects cells prepared in alginate gel were added. Gels contained 1000, 10,000 or 100,000 labelled cells or 10,000 unlabelled cells as a control. MR imaging was performed on a clinical 3.0-T MRI scanner (General Electric Medical Systems, Milwaukee, WI, USA) with the standard imaging gradient set and specialized signal reception coils for high resolution scanning. For *in vitro* samples, a custom-designed 1.0-cm-diameter surface coil (Flick Engineering Solutions B.V, Winterswijk, The Netherlands) was used for signal reception. After image-localisation scans, a three-dimensional (3-D) rf-spoiled gradient recalled (SPGR) sequence was selected with the imaging volume positioned parallel to the bottom of the well containing the bone plugs. Imaging parameters were as follows: repetition time (TR) = 48 ms, echo time (TE) = 5.0 ms, flip angle = 8°, read-out bandwidth = 8.1 kHz, field-of-view (FOV) = 1.7×1.7 cm^2, matrix size = 192×160, number of excitations (NEX) = 1.0, scan time = 11 min 30 s. Section thickness was set to 100 μm and 86 slices encoded sections. These parameters yielded a voxel size of $88 \times 106 \times 100$ μm^3.

DATA ANALYSIS

Relative gene expression among experimental groups was analysed by one-way ANOVA. A *P*-value <0.05 was considered to indicate statistically significant difference. Cells were cultured from four patients separately and carried out in triplicate for all genes analysed. Results for GAG, DNA and number of collagen II positive cells were also analysed by one-way ANOVA from four donors.

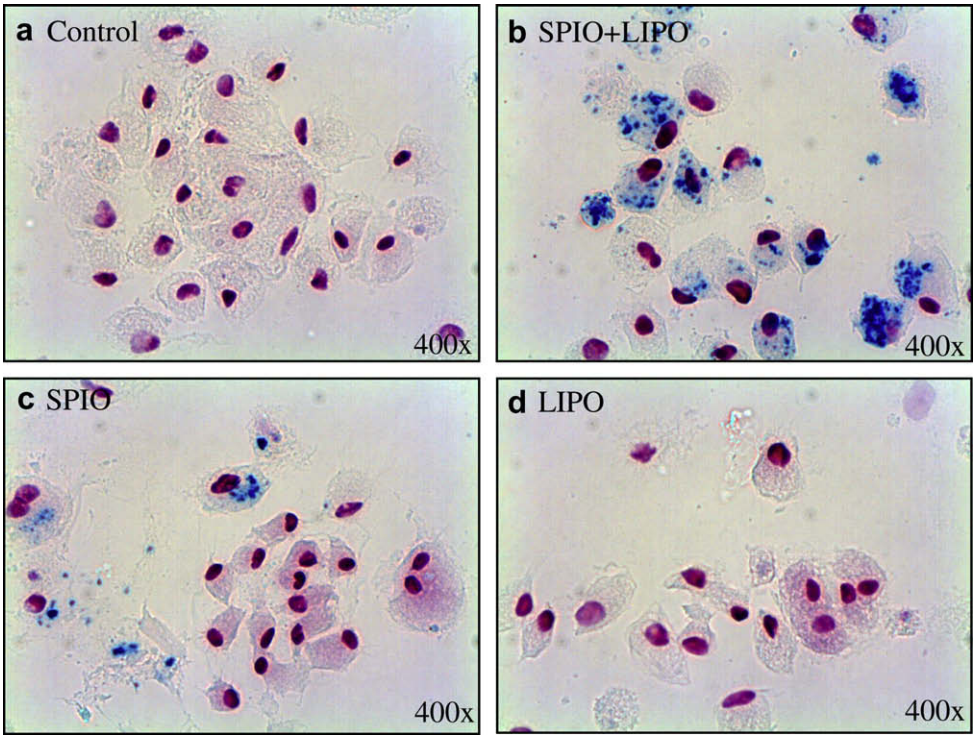

Fig. 2. SPIO is retained intracellularly for 21 days in alginate. Following 21 days culture in alginate beads in the presence of chondrogenic redifferentiation medium, beads were dissolved and prepared as cytospins for histological staining for iron. Figure 2 demonstrates the presence of iron particles in discrete intracellular clusters as evidenced by Prussian blue staining for iron. Some iron positive cells were also observed in the SPIO only condition. Although this was not quantified, there were much fewer positive cells compared to the labelled condition.

Results

SPIO LABELLING HAS NO EFFECT ON CHONDROCYTE VIABILITY

Labelling of chondrocytes at a range of doses had no effect on cell viability as assessed by an Alamar Blue cytotoxicity assay until a slight effect was observed at a dose of 54 μg SPIO per well in the combined SPIO/Lipofectamine condition and in the Lipofectamine only condition at 168 μg [Fig. 1(a)]. This was likely due to the large amount of Lipofectamine present which is known to be cytotoxic at high doses[18]. Based on previous experiments to assess labelling efficiency and these findings, a dose of 18 μg SPIO per well was used for subsequent experiments. Labelling overnight at this concentration led to a very high labelling efficiency as can be seen in the Prussian blue staining [Fig. 1(b)]. There was negligible iron uptake in the SPIO only condition.

LABELLED CHONDROCYTES RETAIN SPIO FOR UP TO 3 WEEKS IN ALGINATE

To demonstrate the retention of iron particles after 21 days in alginate beads, Prussian blue staining was carried out. In unlabelled controls, no positive staining was observed [Fig. 2(a)]. In the labelled condition, iron particles were retained intracellularly for 3 weeks in alginate beads [Fig. 2(b)]. Interestingly, some cells cultured with SPIO alone and no transfection agent also contained iron [Fig. 2(c)]. This was much fewer cells than in the labelled condition, though the pattern of staining was similar. In both SPIO containing conditions iron seemed to be located in discrete intracellular organelles/vacuoles.

REALTIME QUANTITATIVE RT-PCR AND GAG/DNA ANALYSIS OF CHONDROCYTE REDIFFERENTIATION

To assess chondrocyte redifferentiation mRNA expression levels of four chondrogenic markers were assessed

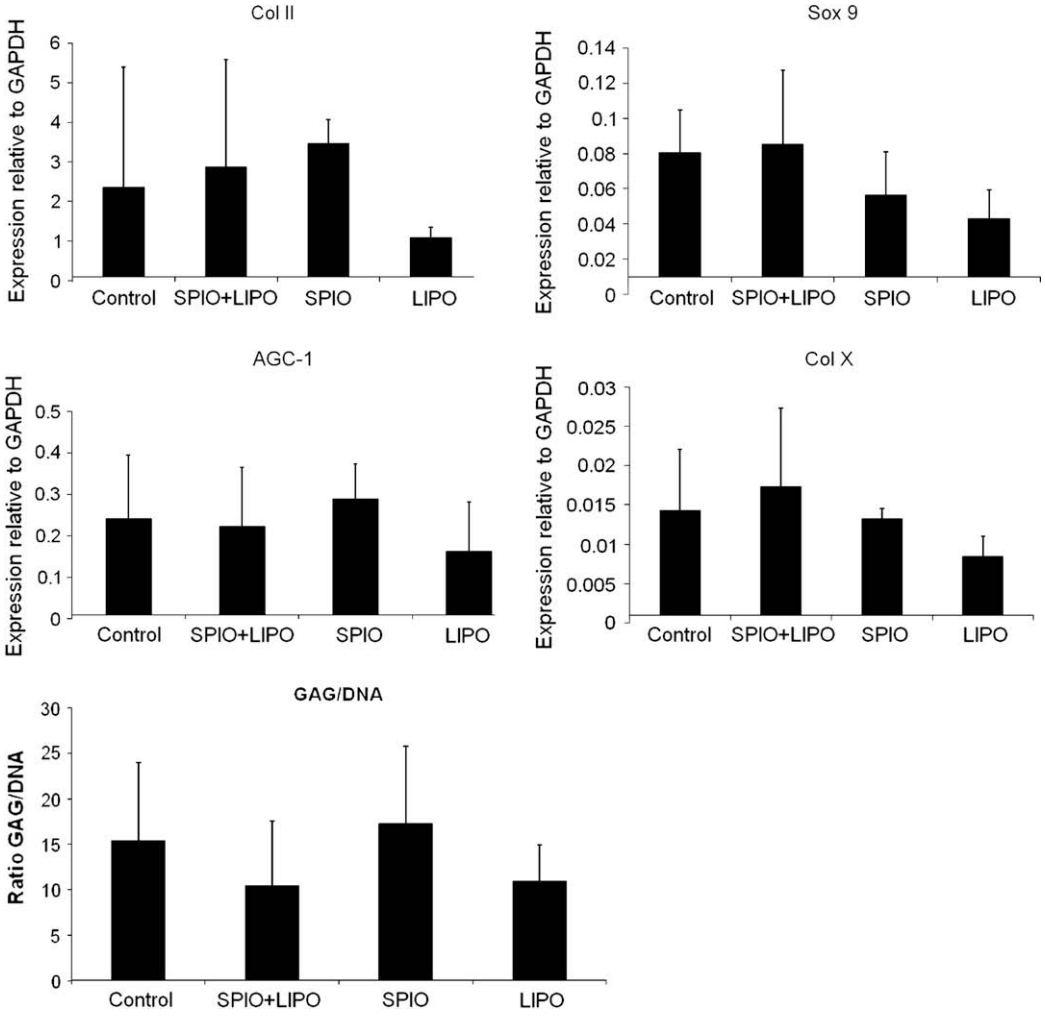

Fig. 3. SPIO labelling does not affect expression levels of common chondrogenic genes or GAG production. Realtime RT-PCR analysis was performed with triplicate samples for each of four donors. Gene expression levels of collagen type II, Sox 9, collagen type X and aggrecan were not significantly different between the labelled condition and unlabelled controls or either of the labelling controls (one-way ANOVA, $n = 4$). All genes expression levels are relative to GAPDH expression. Total DNA per bead and GAG per bead were assayed after 21 days in culture. As can be seen in Fig. 3, GAG production per DNA was not significantly altered by iron oxide labelling. Total DNA levels were also not significantly different from unlabelled controls.

and compared to control levels following 21 days in alginate with chondrogenic redifferentiation medium. For all four genes analyses, collagen type II, Sox 9, collagen type X and aggrecan, no significant difference was observed between control samples and any treatment condition. While a trend towards lower gene expression was observed in the Lipofectamine condition, this did not reach statistical significance ($n = 4$, $P = 0.6187$, 0.2574, 0.4672 and 0.7217, respectively for the four genes Fig. 3). There was no effect of any of the treatment conditions on the amounts of DNA present in the alginate beads ($n = 4$, $P = 0.9145$, data not shown). GAG production was also not significantly affected by cell labelling ($n = 4$, $P = 0.581$), though a slight decrease in GAG production was observed in both the labelled and Lipofectamine conditions indicating a possible effect of Lipofectamine on cell behaviour.

COLLAGEN TYPE II PROTEIN EXPRESSION

As collagen type II protein expression is the definitive indicator of the chondrogenic phenotype, immunocytochemistry was performed (Fig. 4). While there was some inter donor variation in the amount of positive cells, there was no significant difference between any of the conditions ($n = 4$, $P = 0.9491$).

TRACKABILITY OF LABELLED CELLS IN A CARTILAGE DEFECT

As a proof of principle different concentrations of labelled cells were placed in an *ex vivo* cartilage defect model to determine the ability to track cells and differentiate from surrounding cartilage (Fig. 5). At 1000 labelled cells in a 4 mm defect no cells were detectable above background. However, at a concentration of 10,000 and 100,000 cells hypointense signals were observed in the defect clearly distinct from the surrounding cartilage. No signal was observed from 10,000 unlabelled cells.

Discussion

This paper for the first time examines the effects of SPIO labelling on human chondrocyte behaviour/performance, a vital aspect to treatment of cartilage damage by the Autologous chondrocyte implantation (ACI) technique. To assess the effects of SPIO labelling on redifferentiation capacity of chondrocytes, a standard alginate bead experiment was performed. Chondrocytes were cultured in 3-D in the presence of TGF β for 21 days. Redifferentiation was confirmed by the expression of cartilage specific genes, proteoglycan production and collagen type II protein expression. None of these criteria were significantly altered by transfection of the cells with SPIO particles or SPIO or transfection agent alone. Throughout the culture period, SPIO was retained intracellularly as evidenced by Prussian blue staining.

The ability to safely track implanted cells is extremely important in cell therapies both to exclude migration of cells from the implantation site and to potentially monitor long-term cell survival. The use of SPIO for this purpose is therefore ideal[3]. SPIO labelled cells can be tracked for up to 7 weeks post labelling[10] and have been shown in several recent studies to have no or only minor effects on cell viability and differentiation capacity *in vitro*. The effects of label on implanted cells will have to be investigated further. The added benefit of using SPIO is that MRI techniques for visualisation of cartilage lesions are vastly improved enabling clinicians to monitor the repair of a defect long after the label has been metabolised[19].

The use of Lipofectamine as transfection agent is not ideal as it is currently not approved for clinical use. Interestingly, some iron was present in cells cultured with SPIO alone and appeared to be localised in a similar pattern to the labelled condition. This effect could potentially lead to the labelling of chondrocytes without a transfection agent. This has recently been demonstrated with the SPIO preparation called Resovist/Ferucarbotran[20]. Other possibilities will also be investigated for future work, including the use of electroporation and protamine sulfate to introduce SPIO into cells. Both of these methods have been shown to be successful in the labelling of other cell types[6,21–23].

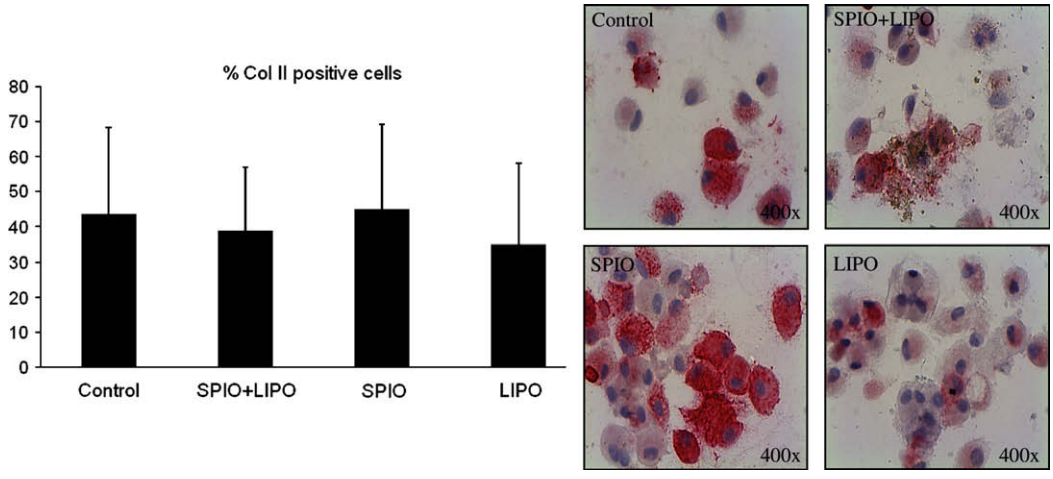

Fig. 4. Collagen type II production is unaffected by SPIO labelling in alginate culture. As definitive evidence of the redifferentiation of dedifferentiated chondrocytes, collagen type II immunocyochemistry was carried out. Approximately 40% of cell stained positively for collagen II with no significant difference in the number of positive cells when labelled with SPIO (one-way ANOVA, $n = 4$). Perceived differences in collagen staining between conditions are simply as a result of cellular distribution on the slides. As can be seen in Fig. 4(a) there was no overall difference in relative number of positive cells.

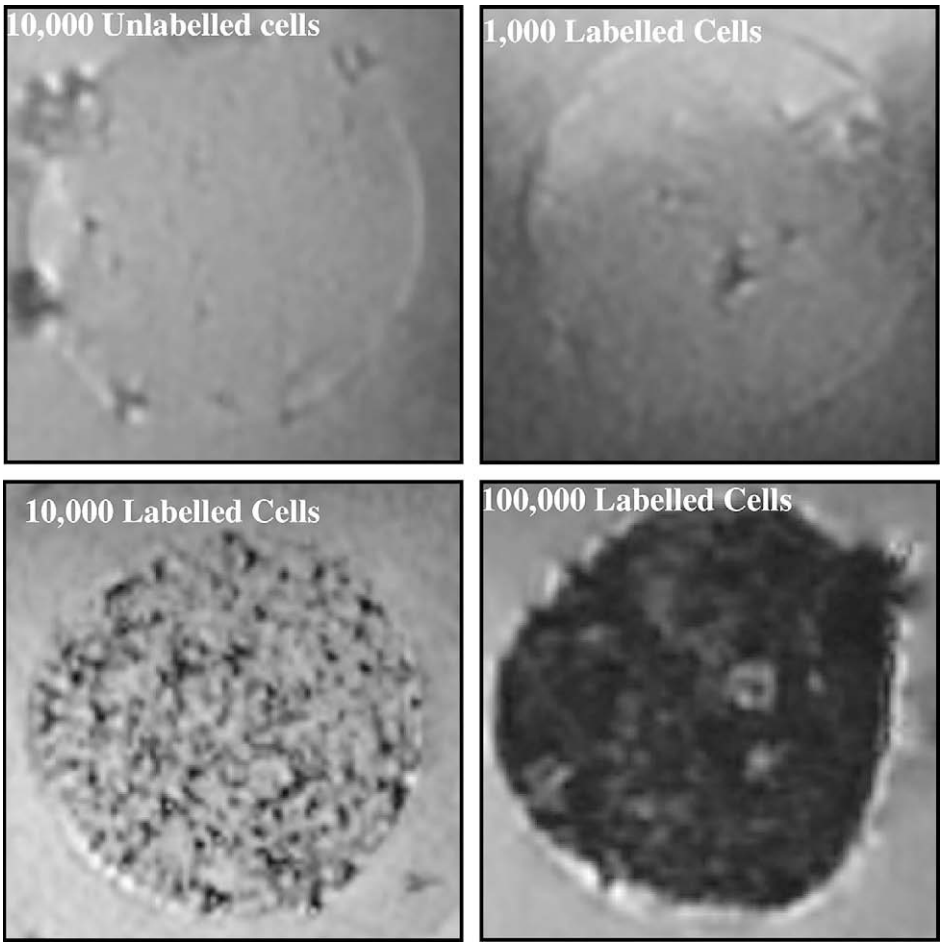

Fig. 5. Cell tracking in *ex vivo* cartilage defect model. MRI pictures of osteochondral plugs with a cartilage defect seeded with cells demonstrates the ability to track as few as 10,000 labelled cells in a gel seeded in a cartilage defect created *ex vivo*. Labelled cells were detectable surrounded by cartilage tissue at the periphery of the images. 10,000 Unlabelled cells served as control and were undetectable. The black signal represents labelled cells surrounded by white cartilage.

In conclusion, this research found no effects of SPIO labelling of chondrocyte redifferentiation as evidenced by gene and protein expression of common cartilage specific markers and proteoglycan production. Iron particles were retained intracellularly for 3 weeks apparently within discrete compartments. This report illustrates the clinical potential of such a labelling method in the treatment of joint related injuries/diseases by cell therapy.

Conflict of interest

The authors have no conflicts of interest.

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