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Ferumoxides–protamine sulfate is more effective than ferucarbotran for cell labeling: implications for clinically applicable cell tracking using MRI

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The use of superparamagnetic iron oxide (SPIO) for labeling cells holds great promise for clinically applicable cell tracking using magnetic resonance imaging. For clinical application, an effectively and specifically labeled cell preparation is highly desired (i.e. a large amount of intracellular iron and a negligible amount of extracellular iron). In this study we performed a direct comparison of two SPIO labeling strategies that have both been reported as efficient and clinically translatable approaches. These approaches are cell labeling using ferumoxides–protamine complexes or ferucarabotran particles. Cell labeling was performed on primary human bone marrow stromal cells (hBMSCs) and chondrocytes. For both cell types ferumoxides–protamine resulted in a higher percentage of labeled cells, a higher total iron load, a larger amount of intracellular iron and a lower amount of extracellular iron aggregates, compared with ferucarbotran. Consequently, hBMSC and chondrocyte labeling with ferumoxides–protamine is more effective and results in more specific cell labeling than ferucarbotran. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: cell tracking; SPIO labeling; MRI; iron oxide labeling; ferumoxides; protamine sulfate; MSC; chondrocyte

1. Introduction

Cell tracking is a necessary tool for determining the efficacy and safety of cell-based regenerative therapies. Cell labeling using superparamagnetic iron oxide (SPIO) particles allows subsequent *in vivo* cell tracking using magnetic resonance imaging (MRI) and has already entered the clinical arena (1–3). Ferumoxides and ferucarbotran are two SPIO preparations that are clinically used as MRI contrast agents and have both been frequently described for the purpose of cell labeling and tracking using MRI. Both SPIOs have comparable particle sizes (60–150 nm), but whereas ferumoxides particles have a dextran coating, ferucarbotran particles are carboxydextran coated (4). These additional carboxyl groups associated with ferucarbotran lead to a higher affinity to the cell membrane (5). This difference in coating appears to be the main reason why efficient labeling of non-phagocytic cells with ferumoxides requires additional use of a transfection agent, while adequate labeling using ferucarbotran can be accomplished without the need of an additional agent (4,5). Several tranfection agents are being used to facilitate cellular incorporation of ferumoxides (5,6). Amongst them, protamine sulfate is the most interesting from a clinical perspective since it is already FDA approved as a heparin antidote (7). Combining ferucarbotran with a transfection agent has shown controversial results. Some groups reported effective cell labeling (8), but others showed ferucarbotran–transfection agent complexes to coat cell surfaces instead of being incorporated into cells (9).

When applying SPIO-labeled cells either pre-clinically or clinically, an effectively and specifically labeled preparation of cells is highly desired (i.e. a high intracellular iron load and a negligible amount of extracellular iron). Effective labeling is essential to ensure MRI sensitivity (10). Moreover it will likely positively influence the duration of MRI traceability, since the endocytosed SPIO particles are known to dilute upon cell division (11). Extracellular iron, on the other hand, could potentially be endocytosed by host cells, or generate MRI signal voids on its own. Although for both ferumoxides–protamine complexes and ferucarbotran, comparable labeling efficiencies up to

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approximately 100% have been reported regarding human bone marrow stromal cell (hBMSC) labeling, both methods are hampered by the occurrence of extracellular iron nanoparticle aggregates (5,7,12–16).

No publications are known comparing ferumoxides–protamine sulfate complexes with ferucarbotran labeling directly on the same cell-types. This study aims to compare both methods based on accepted, clinically translatable protocols found in current literature. Outcome measures were labeling efficiency, total iron load (TIL) and intra- and extracellular iron load. We performed our experiments on chondrocytes and hBMSCs, the two cell types most commonly used in cell based cartilage repair.

2. Results

2.1. Labeling efficiency and total iron load per cell

SPIO labeling using ferumoxides–protamine or ferucarbotran resulted in histologically clearly visible label uptake in both cell types (Fig. 1). Blue-stained SPIO particles were seen in the cytoplasm of cells, mostly around the nuclei. The percentage of labeled cells was slightly but significantly higher for ferumoxides–protamine compared with ferucarbotran for hBMSCs (96.1 ± 4.5 vs $89.2 \pm 8.0\%$; $p = 0.008$) and chondrocytes (99.8 ± 0.3 vs $97.2 \pm 1.8\%$; $p < 0.001$). Ferumoxides–protamine labeled cells also showed a higher TIL for hBMSCs (89.6 ± 14.9 vs 51.3 ± 4.4 pg/cell) and chondrocytes (75.9 ± 9.5 vs 28.1 ± 3.0 pg/cell) compared with ferucarbotran, as measured by inductively coupled plasma–optical emission spectrometry (ICP-OES). No apparent effects of either labeling procedure were seen in terms of changes in cell morphology or viability.

2.2. Evaluation of intra- and extracellular iron

For further evaluation of the distribution of TIL between the intra- and extracellular compartment we scored all samples using a four-point grading system (Table 1). Figure 2A shows representative examples of all different grades of intracellular iron. Ferumoxides–protamine complexes resulted in a markedly higher intracellular iron load compared with ferucarbotran for both cell types (Fig. 2B). In ferumoxides–protamine labeled hBMSCs $64.2 \pm 26.1\%$ of cells had an intermediate to high intracellular iron load compared with $21.9 \pm 22.0\%$ of ferucarbotran labeled hBMSCs ($p < 0.001$). In ferumoxides–protamine labeled chondrocytes $96.6 \pm 2.2\%$ of cells showed an intermediate to large amount of intracellular iron vs $45.6 \pm 8.4\%$ in the ferucarbotran labeled cells ($p < 0.001$).

The amount of extracellular iron was evaluated using a comparable four-point grading system (Table 1). Figure 2C shows representative examples of all different grades of extracellular iron. Ferumoxides–protamine labeling of cells resulted in less extracellular iron compared with ferucarbotran in both cell types (Fig. 2D). In ferumoxides–protamine labeled hBMSCs $19.3 \pm 13.7\%$ of fields of view showed an intermediate to large amount of extracellular iron, compared with $47.6 \pm 19.1\%$ in the ferucarbotran labeled conditions ($p = 0.001$). When labeling chondrocytes a comparable difference was observed. In $20.2 \pm 11.1\%$ of fields of view an intermediate to large amount of extracellular iron was observed in the ferumoxides–protamine labeled condition, compared with $57.2 \pm 10.2\%$ in the ferucarbotran labeled cells ($p < 0.001$). Extracellular iron presented as aggregates varying in size from barely visible to occasionally found large aggregates up to the equivalent of 50 cell diameters. These aggregates consisted of SPIO, most likely complexed to cells (debris) and extracellular matrix. The largest aggregates were found in the condition where hBMSCs were labeled using ferumoxides–protamine. Especially for hBMSCs, a large donor variation was observed, possibly due to extracellular matrix formation.

3. Discussion

In this study we performed a direct comparison of two SPIO labeling approaches using ferumoxides–protamine complexes or

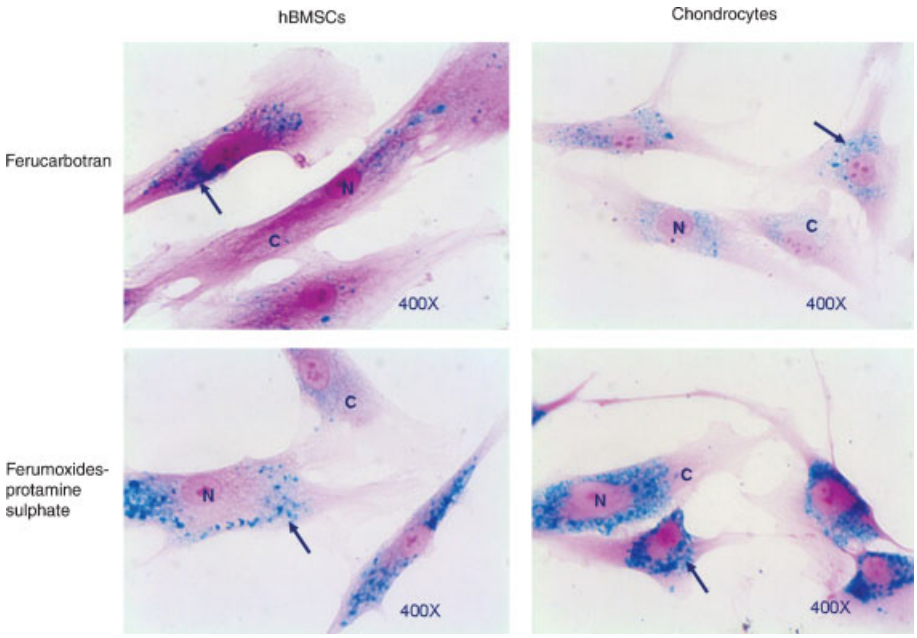

Figure 1. Perl's iron stain of SPIO labeled hBMSCs (left) and chondrocytes (right). Blue stained SPIO particles (arrows) are present in the cytoplasm (C) around the nuclei (N) in all conditions.

Table 1. Description of four-point grading score used for evaluation of intra- and extracellular iron

Iron load	Appearance intracellular compartment	Appearance extracellular compartment
Absent	No blue granules	No aggregates
Low	1–20 blue granules	1–5 aggregates $<$ 1 cell size
Intermediate	21–100 blue granules	$>$5 aggregates $<$ 1 cell size or 1–5 aggregates 1–10 cell sizes
High	$>$100 blue granules or granules no longer separately distinguishable	$>$5 aggregates 1–10 cell sizes or aggregate(s) $>$ 10 cell sizes

ferucarbotran on two primary cell types (hBMSCs and chondrocytes). Both labeling methods show great promise for being translated to a clinical setting, since only clinically applicable compounds are used. Ferucarbotran has an extra advantage, since no transfection agent is needed in order to label non-phagocytic cells.

We found differences in quantity and quality of cell labeling between both approaches. For both cell types ferumoxides–protamine resulted in a higher percentage of labeled cells and a higher TIL compared with ferucarbotran. In addition, ferumoxides–protamine complexes resulted in a higher amount of intracellular iron together with a lower amount of extracellular iron aggregates in both cell-types.

No uniform protocol is described for the two labeling methods. However, for each of the separate strategies, published protocols from different groups are quite similar. We therefore assumed that the described protocols for either ferumoxides–protamine or ferucarbotran represented already optimized protocols. Based on these protocols we developed protocols which offered the best possibility to relate our experiments to these previous reports, taking into account our main objective to compare both techniques. In order to adhere to these objectives some apparent

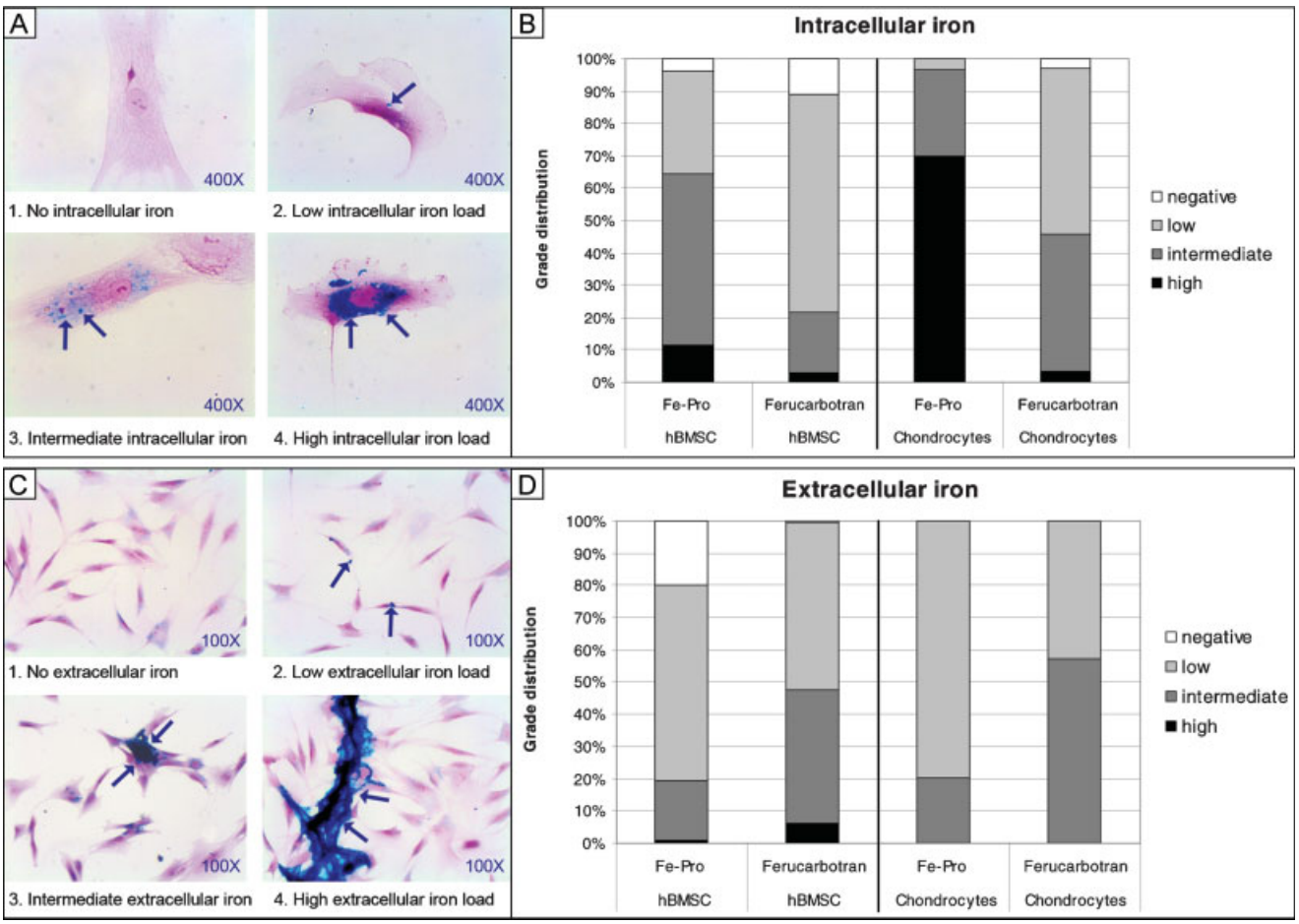

Figure 2. Perl's iron stain of representative examples of all four different intra- and extracellular iron grades (A, C) and intra- and extracellular iron grade distribution (B, D) of hBMSCs and chondrocytes. Cells were labeled using ferumoxides-protamine (Fe-Pro) or ferucarbotran. Ferumoxides-protamine resulted in a higher percentage of cells showing a high to intermediate amount of intracellular iron particles (B) and a lower percentage of fields of view showing a high to intermediate amount of extracellular iron aggregates (D). Arrows: intra- and extracellular blue stained SPIO particles.

discrepancies in protocol occurred, regarding SPIO and serum concentration and the use of a transfection agent. These differences and their potential impact on the obtained result are discussed in greater detail below.

3.1. SPIO concentration

In the ferumoxides–protamine conditions a SPIO concentration of 50 μg/ml was used, while for ferucarbotran a concentration of 100 μg/ml was applied. Both ferumoxides and ferucarbotran are known to be colloidal suspensions containing particles ranging from 60 to 150 nm (4). These particles do not settle out because the energy of Brownian motion largely exceeds gravity (4). By complexing ferumoxides to protamine sulfate, larger particles are formed up to approximately 2000–2500 nm, depending on the ferumoxides–protamine ratio used (17). These larger particles are more susceptible to gravity and are prone to settle out. We consider total added dose of ferumoxides–protamine complexes to be a more determining factor for labeling efficiency and TIL per cell compared to concentration of SPIO particles. We decided to maintain the final concentrations of ferumoxides and ferucarbotran as previously described (50 and 100 μg/ml respectively) while ensuring an identical SPIO dose (100 μg/well of a six-well plate).

3.2. Serum concentration

In our labeling protocols using ferumoxides–protamine or ferucarbotran a serum concentration of 5 or 10% was administered respectively. Influences of serum proteins upon SPIO aggregation and cell internalisation have been reported previously (5,18). Cell labeling in a serum free environment proved not to be preferable for either strategy, resulting in an undesirable amount of extracellular SPIO aggregates (data not shown). We decided to maintain serum concentrations as reported in literature, considering our aim to relate our results to previous reports. Although the total amount of serum proteins and SPIO particles was kept similar for both methods, we cannot rule out the possibility that the difference in serum concentration influenced our results.

3.3. Transfection agent

In order to facilitate cellular incorporation ferumoxides particles were complexed to protamine sulfate, while ferucarbotran was used without a transfection agent. In additional experiments we added protamine sulfate to ferucarbotran, which resulted in a dramatic increase in extracellular SPIO aggregates coating the cell membranes (data not shown). This observation has been reported before when complexing ferucarbotran to a transfection agent (9) and we considered this an unfavorable alternative. As mentioned before, a major advantage of ferucarbotran would be that addition of a transfection agent would not be necessary for effective labeling of non-phagocytic cells.

The increased size of the ferumoxides–protamine complexes could be a reason for the finding that they are taken up more effectively than ferucarbotran. Various groups reported that cellular uptake in transfection experiments is positively influenced by increasing particle size of transfection complexes (19,20). These articles describe the fact that larger particles can sediment faster onto cells and that larger particles have a bigger payload. With increasing size, the surface of particles increases to the second power and the volume to the third power. Consequently, larger particles can deliver more transfection material at a similar cell surface occupation.

Several studies describe the use of ferumoxides–protamine complexes or ferucarbotran for labeling of hBMSCs (4,5,7,12–14,21). In our study both ferumoxides–protamine and ferucarbotran

Table 2. Overview of literature reporting hBMSC labeling using ferumoxides–protamine sulfate or ferucarbotran

Reference	SPIO-TA + concentration SPIO	Labeling efficiency	Cellular iron load (pg/cell)	Extracellular SPIO aggregates
(21) Arbab *et al.* (2004)	Ferumoxides–protamine 50 μg/ml	± 100%	10.9 ± 1.9	NM
(12) Arbab *et al.* (2005)	Ferumoxides–protamine 50 μg/ml	± 100%	NM	Describes heparin wash to remove extracellular aggregates
(7) Pawelczyk *et al.* (2006)	Ferumoxides–protamine 50 μg/ml	± 100%	44.7 ± 0.3	Describes heparin wash to remove extracellular aggregates
(14) Omidkhoda *et al.* (2007)	Ferumoxides–protamine 25–125 μg/ml	71–87%	Absolute value NM	Minimal extracellular aggregates observed
(13) Pawelczyk *et al.* (2008)	Ferumoxides–protamine 50 μg/ml	± 100%	34.8 ± 0.3	Describes heparin wash to remove extracellular aggregates
(16) Janic *et al.* (2009)	Ferumoxides–protamine 100 μg/ml for 15 min, then 50 μg/ml	NM	19.1	Describes adjusted incubation protocol to prevent extracellular aggregates
(4) Hsiao *et al.* (2007)	Ferucarbotran 100 μg/ml	± 100%	23.4	NM
(5) Mailander *et al.* (2008)	Ferucarbotran 250 μg/ml	NM	± 43. After FACS for live cell fraction ± 9	Extracellular aggregates indirectly quantified

Comparable labeling efficiencies and cellular iron loads have been reported for hBMSCs labeling using ferumoxides–protamine sulfate and ferucarbotran. The majority of articles mention the occurrence of extracellular iron aggregates. Mailander *et al.* (5) indirectly quantified this amount of extracellular iron. TA, transfection agent; NM, not mentioned.

resulted in comparable labeling efficiencies to those previously reported and a slightly higher TIL per cell. A possible explanation for this higher TIL could be the fact that we used a different method for our iron content measurements. Differences between methods to determine iron concentration in cells have been reported before (22), and ICP-OES is known to be a very sensitive element detection method (23). Overall we consider our results similar to those previously reported, making it possible to relate our findings to the articles listed in Table 2.

Various modifications of labeling protocols using ferumoxides have been reported in order to decrease the occurrence of extracellular iron deposits, like the use of heparin washes, additional incubation time or a different timing of complex formation (7,12,13,16). We did not include these adjustments in our main experiments, in order to better compare both techniques. In additional experiments, where we included heparin washes (10 U/ml) and additional time to endocytose ferumoxides–protamine complexes, the amount of extracellular iron appeared to decrease during microscopic evaluation (data not shown). This observation was made for both cell types, making ferumoxides–protamine even more favorable over ferucarbotran.

We did not observe increased cell death or changes in morphology between labeled cells vs unlabeled controls for both cell-types. Cell labeling using both SPIOs has been shown not to influence several cell behavior characteristics (4,5,7,12,14,21,24). Labeling chondrocytes using ferumoxides–protamine did result in a distinct higher amount of intracellular iron compared with hBMSC labeling. Possible influences of this vast amount of intracellular iron need to be further investigated before proceeding to any *in vivo* or clinical experiments.

4. Conclusions

We have shown ferumoxides–protamine sulfate to be a more effective and specific way of SPIO labeling compared with ferucarbotran for both primary hBMSCs and human chondrocytes.

5. Experimental

5.1. Cell culture

hBMSCs and human chondrocytes were isolated and cultured using previously described procedures (25,26). hBMSCs were isolated from heparinized fermoral-shaft marrow aspirate of patients undergoing total hip arthroplasty (after informed consent; MEC-2004-142). Chondrocytes were isolated from articular cartilage obtained from patients undergoing total knee replacement surgery (after approval by the local ethical committee; MEC-2004-322). All isolated cells were cultured in DMEM containing 10% FCS, 50 μg/ml gentamicin and 1.5 μg/ml fungizone. Fresh medium was applied every 3–4 days. Cells were trypsinized at subconfluency and subsequently passaged. Cells from the third to the sixth passage, both freshly collected and cryopreserved, were used for labeling experiments.

5.2. Cell labeling

Cells were labeled using ferumoxides (Endorem®, Guerbet S.A., Paris, France) and protamine sulfate (LEO Pharma N.V., Wilrijk, Belgium) complexes or ferucarbotran (Resovist®, Bayer Schering Pharma AG, Berlin, Germany). Labeling protocols used for both SPIOs were based on protocols described in literature.

5.2.1. Ferumoxides– protamine sulfate: based on literature (7,12–14,16,21)

Both hBMSCs and chondrocytes were grown until 80–90% confluency in six-well plates. At that time, existing medium was removed and 1 ml of fresh DMEM containing 10% FCS was added per well. Protamine sulfate was prepared as a fresh stock solution of 1 mg/ml in distilled water. Ferumoxides was diluted in serum-free DMEM to a final concentration of 100 μg/ml. Protamine sulfate was added to the ferumoxides solution to a final concentration of 5 μg/ml. After 3–5 min of intermittent shaking by hand at room temperature, the ferumoxides–protamine solution was added to the freshly applied medium on the cells in a 1:1 v/v ratio (final dose 100 μg of iron per well of a six-well plate).

5.2.2. Ferucarbotran: based on literature (4,5)

Both hBMSCs and chondrocytes were seeded in six-well plates at a density of 50 000 cells per cm^2 on day one. After 24 h, existing medium was discarded. Subsequently, 1 ml of fresh medium was applied and ferucarbotran was added to the media at a final concentration of 100 μg/ml (final dose 100 μg iron per well of a six-well plate).

5.3. Labeling efficiency

Medium was removed after 24 h of incubation with either ferumoxides–protamine complexes or ferucarbotran. Cells were washed twice using PBS, trypsinized and replated in six-well plates (seeding density 10 000 cells/cm^2). Replated cells were fixed within one day and stained using Perl's iron stain (Klinipath BVBA, Duiven, The Netherlands) according to the manufacturers protocol. All samples were evaluated using light microscopy. Labeling efficiency was based on manually counting stained cells. Cells were considered positive if blue granules were present within cell boundaries. A minimum of 100 randomly selected cells was counted per sample at 400× magnification.

5.4. Total iron load per cell

TIL was measured in samples containing trypsinized cells using ICP-OES. Cell pellets of unlabeled and labeled cells were dried for 72 h at 60°C. Then they were digested in 40 μl of a 3:1 v/v mixture of ultra-pure perchloric acid (EM Science, Gibbstown, NJ, USA) and ultra-pure nitric acid (JT Baker, Deventer, The Netherlands) at 60 °C for 24 h. To the digested substance 4 ml MiliQ was added and emission was measured at 259 nm with a Perkin Elmer Optical Emission Optima 4300 DV Spectrometer. The amount of iron per sample was determined by calibration to a standard curve, which was generated using a commercially available ICP-OES standard iron solution (Merck, Schiphol, The Netherlands) in a range of 0–50 μg/ml.

5.5. Evaluation of intra- and extracellular iron

To further discriminate between intra- and extracellular iron load, a grading score was developed. In this score, quantity of both intra- and extracellular iron was graded on a four-point scale

(Table 1). All samples were stained using Perl's iron stain and evaluated using light microscopy. Amount of intracellular iron was based on number of intracellular blue granules. A minimum of 100 randomly selected cells per sample was manually evaluated for intracellular iron according to this score at 400× magnification. Extracellular iron was quantified based on number and size of extracellular SPIO aggregates. Analysis of extracellular iron was based on 40 randomly selected fields of view at 100× magnification in order to get a representative evaluation of the total well. Iron particles were considered extracellular if they were not localized within cell boundaries. Iron within the boundaries of dead cells was not considered extracellular. All samples were evaluated independently by two blinded observers.

5.6. Data analysis

All experiments were performed with triplicate samples for each of the three different hBMSC and chondrocyte donors, except for TIL measurements, which were performed with triplicate samples for one hBMSC and one chondrocyte donor. Independent scores of both observers were compared and proved to be similar. For further calculations all data from both observers regarding intra- and extracellular iron was accumulated per sample. Data is shown in terms of percentage distribution of different grades of intra- and extracellular iron of all evaluated samples. Labeling efficiencies and percentages of different grades were compared using a mixed model ANOVA, in which treatment was considered a fixed factor and the three different donors a random factor. A p value < 0.05 was considered statistically significant.

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