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Magnetic Tissue Engineering for Voice Rehabilitation – First Steps in a Promising Field

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Abstract. *Background/Aim: The voice is one of the most important instruments of communication between humans. It is the product of intact and well-working vocal folds. A defect of these structures causes dysphonia, associated with a clear reduction of quality of life. Tissue engineering of the vocal folds utilizing magnetic cell levitation after nanoparticle loading might be a technique to overcome this challenging problem. Materials and Methods: Vocal fold fibroblasts (VFFs) were isolated from rabbit larynges and cultured. For magnetization, cells were incubated with superparamagnetic iron oxide nanoparticles (SPION) and the loading efficiency was determined by Prussian blue staining. Biocompatibility was analyzed in flow cytometry by staining with annexin V-fluorescein isothiocyanate propidium iodide, 1,1',3,3,3',3'-hexamethylindodicarbo-cyanine iodide [DiIC$_1$(5)] and propidium idodide- Triton X-100 to monitor phosphatidylserine exposure, plasma membrane integrity, mitochondrial membrane potential and DNA degradation. Results: Isolated VFFs can be successfully loaded with SPION, and optimal iron loading associated with minimized cytotoxicity represents a balancing act in magnetic tissue engineering. Conclusion: Our data are a firm basis for the next steps of investigations. Magnetic tissue engineering using magnetic nanoparticle-loaded cells which form three-dimensional structures in a magnetic field will be a promising approach in the future.*

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Dysphonia is associated with a clear reduction of quality of life (1). Essentially, there are two different reasons for voice disorders: Functional dysphonia means irregular use and interplay of intact normal anatomic structures, whereas organic dysphonia is caused by *e.g.* tissue defects, scarring, and palsy (2). Tissue defects after tumor surgery particularly lead to severe problems for patients who are then faced with a communication barrier with their doctor and family, in addition to their severe disease. Therefore, the potential of tissue engineering of the vocal folds is becoming of ever greater interest.

There are many different approaches to work in the field of tissue engineering of vocal folds (3). One course is the use of scaffolds which are colonized with appropriate cells. After publication of the successful transplantation of a decellularized trachea scaffold (4), this method is now being investigated for vocal fold tissue engineering (5). Others turned their attention to the special structure of the extracellular matrix vocal folds, which is highly important for the biomechanical properties of these structures, and therefore examined the effect of alignment and coating of electrospun fiber constructs on vocal fold fibroblasts (VFFs) (6). Hirano and his group demonstrated the therapeutic effect of basic fibroblast growth factor in vocal fold scarring in an animal model (7) and in humans (8), and consequently went on to test a collagen/gelatin sponge scaffold with sustained release of this important substance (9). There are also tissue engineering methods without the use of scaffolds: Long *et al.* developed a model resembling vocal fold epithelium and *lamina propria* using adipose-derived stem cells in fibrin (10). These bilayered constructs with epithelial and mesenchymal cell phenotypes in a stratified geometry exhibit indentation modulus, microstructure, and vibration similar to that of human vocal fold covers (11).

In our work, we introduced a new method for tissue engineering of vocal folds: magnetic tissue engineering (MTE). This method magnetizes cells in order to arrange them in a magnetic field using superparamagnetic iron oxide nanoparticles (SPIONs). Nanoparticles have already found their way into medicine (12). SPIONs have been especially used for drug delivery (13-18) or as contrast agents for magnetic resonance imaging (19). Three-dimensional tissue cultures based on the principle of magnetic cell levitation to overcome gravitational forces have already been established (20). Since VFFs play a central role in development and retention of the vocal fold, we used rabbit VFF (r-VFF) for our experiments. The structure of the vocal fold and particularly the *lamina propria* of rabbits and humans are very similar (21). The association and biocompatibility of SPIONs was analyzed *ex vivo* in isolated r-VFF in this study with the future aim of performing MTE with such magnetized r-VFF.

Materials and Methods

Materials. Formalin (CH_2O), ethanol (C_2H_5OH), dinatrium hydrogenphosphate (Na_2HPO), sodium citrate (trinatrium citrate-dihydrate $C_6H_5Na_3O_7•2H_2O$), potassium hexacyanoferrate ($K_4[Fe(CN)_6]$), and hydrochloric acid (HCl) were provided by Carl Roth (Karlsruhe, Germany). Lauric acid, Triton X-100, propidium iodide (PI), and non-essential amino acid solution (NEAA) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), L-glutamine, penicillin/streptomycin, phosphate-buffered saline (PBS), Hoechst 33342, fluorescein isothiocyanate (FITC)-labelled annexin V and hexamethylindodicarbocyanine iodide dye $DiIC_1(5)$ were supplied by Life Technologies (Darmstadt, Germany). Amphotericin B was purchased from Biochrom (Berlin, Germany). Trypsin was provided by PAN-Biotech (Aidenbach, Germany). Ringer's solution was supplied by DeltaSelect (Rimbach, Germany). Water used in all experiments was of double-distilled quality.

SPIONs. The SPIONs were synthesized and characterized by the Section of Experimental Oncology and Nanomedicine (SEON), Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital Erlangen, as described before (13): After alkaline precipitation (22), the SPIONs were coated with lauric acid and filtered through a 0.22 μm CME syringe filter (Carl Roth) for sterile application. Characterization was performed using transmission electron microscopy (Philips CM30 TWIN/STEM; Philips Analytical, Eindhoven, the Netherlands), diffuse reflectance infrared spectroscopy (Excalibur FTS 3100 Spectrometer; Varian, Walnut Creek, CA, USA), dynamic light scattering and zeta potential analysis (Zetasizer ZLS 380; Nicomp, Port Richey, FL, USA).

Primary cell culture. Rabbit vocal fold fibroblasts (r-VFF) were isolated and cultured according to the common method of extraction of animal VFFs as described elsewhere (23). Larynges were harvested *post mortem* from New Zealand White rabbits (Charles River, Sulzfeld, Germany). All animals were used for another study by SEON, which was approved by the Animal Research Committee of the responsible authority (Government of Mittelfranken), and no animal was killed in order to excise the larynx. Under an OPMI 1 dissecting microscope (Carl Zeiss, Oberkochen, Germany) the *lamina propria* of the vocal fold was excised and cut into small pieces. Specimens were cultured in 6-well plates with 2 ml DMEM enriched with 10% FCS, L-glutamine (5 mM), 1% amphotericin B, 1% penicillin/ streptomycin, 1x NEAA at 37˚C in 5% CO_2-humidified atmosphere. By morphological criteria such as spindle shape or cell size, fibroblast colonies were identified within 14 days. After reaching 80 to 90% confluence r-VFFs were trypsinized and passaged every two to three days.

Prussian blue staining. Before each experiment, cells were counted using MUSE Cell Analyzer and Muse™ Count & Viability Reagent (Merck, Darmstadt, Germany). r-VFFs were grown in 24-well plates ($5×10^3$ cells per well) on coverslips; after 24 h of incubation, SPIONs were added. After another 48 h, cells were fixed with 4% formalin for 10 min at 4˚C and washed twice with PBS for further staining. For visualization of intracellular iron after incubation of r-VFFs with SPION, cells were stained with Prussian blue. For this procedure, potassium hexacyanoferrate (2%) was mixed with hydrochloric acid (2%) in 1:1 proportion and 250 μl were added onto the coverslips and incubated for 30 min at room temperature. Finally, cells were washed twice with PBS and finally with water. Nuclei were stained with Hoechst 33342. After mounting on microscope slides, images were recorded with a Zeiss Axio Observer Z1 fluorescent microscope (Carl Zeiss).

Flow cytometry. Flow cytometry was performed employing a Gallios cytofluorometer™ (Beckman Coulter, Fullerton, CA, USA). Excitation for FITC and PI was at 488 nm, FITC fluorescence was recorded on an FL-1 sensor [525/38 nm bandpass (BP)], and PI fluorescence on an FL-3 sensor (620/30 nm BP); $DiIC_1(5)$ was excited at 638 nm and recorded on an FL-6 sensor (675/20 nm BP); Hoechst 33342 was excited at 405 nm and recorded on an FL-9 sensor (430/40 nm BP). Electronic compensation was used to eliminate any fluorescence bleed-through. All data were analyzed with Kaluza™ software (Beckman Coulter).

A total of $1.5×10^5$-VFF were seeded into CELLSTAR cell culture flasks with 25 cm^2 growth area (Greiner BioOne, Frickenhausen, Germany). After 24 h, SPIONs were added to a final concentration of 0, 20, 40, 60, 80 μg Fe/cm^2 cell-culture flask area. Cells were incubated another 48 h before harvesting, then cells were washed with PBS and trypsinized. The activity of trypsin was stopped by PBS containing 10% FCS. Media supernatant, washing solution and cell suspension were collected and centrifuged for 5 min at 300 ×g. Supernatants were discarded and the cell pellets were resuspended in PBS.

Four-color cell-death staining was performed as described by Munoz *et al*. (24). Briefly, 50 μl of the cell suspension were stained with 250 μl of a freshly prepared mixture of 20 μg/ml PI, 5.1 μg/ml $DiIC_1(5)$, 1 μg/ml Hoechst 33342 and 0.5 μg/ml annexin V-FITC in Ringer's solution, for 30 min at 4˚C.

For the analysis of DNA degradation, 200 μl aliquots of the cell suspensions were fixed by adding 3 ml of 70% (v/v) ice-cold ethanol and stored at −20˚C for further processing. The cells were then centrifuged (5 min at 1000 × *g* and 4˚C), the supernatant was removed and the cells were washed with PBS once. Then the cells were resuspended in 0.5 ml PBS and 0.5 ml DNA extraction buffer [192 ml of 0.2 M Na_2HPO, 8 ml 0.1% Triton X-100 (v/v), pH 7.8] was added and cells were incubated for 5 min at room temperature. Cells were then centrifuged (5 min at 1,000 × *g* and 4˚C), the

supernatant was removed and cells were resuspended in 1 ml DNA staining solution [1 mg/ml sodium citrate, 0.1% Triton X-100 (v/v), and 50 μg/ml PI in water] and incubated for 30 min in the dark (25). DNA content was analyzed *via* flow cytometry. In order to identify single cells, forward and side scatter was used; further, pulse processing was used to exclude cell doublets and PI was measured with 620/30 nm BP. By using markers set within the analysis program, the percentage of cells in each cell-cycle phase (G_0/G_1, S phase, G_2/M) can be analyzed.

Results

Internalization/attachment of SPIONs by r-VFFs. The association of SPIONs with the r-VFFs was analyzed with Prussian blue staining (blue). r-VFFs were seeded and treated with different concentrations of SPIONs (20, 40, 60, 80 μg/cm^2). After 48 h, Prussian blue (SPIONs) and Hoechst 33342 (nuclei) staining was performed. Mock-treated cells served as control.

Microscopic analysis revealed a dose-dependent association of SPIONs with r-VFFs. Regarding the question of whether the nanoparticles are internalized into or attached onto the cells, it was not possible to make a final decision based on fluorescence microscopy only. Nevertheless, we observed a clear perinuclear distribution and accumulation of SPIONs, which gives us very good reason to suppose cell internalization is more likely than surface attachment alone (Figure 1).

r-VFF viability after treatment with SPIONs. We used an established multiparameter method (24) to detect cell death in flow cytometry employing the markers annexin V-FITC, PI, $DiIC_1(5)$ and Hoechst 33342 (Figure 2A). Annexin V binds to phosphatidylserine, which is exposed by cells undergoing apoptosis. The non-permeable DNA-intercalating dye PI was used for the analysis of plasma membrane integrity. Annexin V-negative/PI-negative cells were considered viable, annexin V-positive/PI-negative cells apoptotic, and PI-positive cells necrotic. Additionally, the mitochondrial membrane potential was analyzed using the cationic dye $DiIC_1(5)$, which primarily accumulates in mitochondria with active membrane potential. Hoechst 33342 served as nuclear staining, which in this setting enabled us to clearly discriminate between SPION agglomerates (no staining) and cells (nuclear staining).

Loading cells with SPION for 48 h resulted in a dose-dependent increase in the number of dying and dead cells as reflected by loss of plasma membrane integrity (PI positivity) and loss of mitochondrial membrane potential [$DiIC_1(5)$ negativity] (Figure 2B and C). As indicated by the annexin V/PI staining, the majority of the cells exhibited a necrotic phenotype (Figure 2B).

In line with these findings, the analysis of cellular DNA content by PI-Triton X-100 staining revealed a dose-dependent increase of subG_1 DNA, indicating DNA degradation of cells which had been treated with SPIONs (Figure 2D).

In summary, loading of cells with SPIONs in an attempt to avoid cytotoxicity represents a balancing act in magnetic tissue engineering. Based on our results, we would recommend a concentration of about 40 μg/cm^2 SPIONs as a compromise between loading and toxicity for further experiments.

Discussion

Voice disorders have variable causes and therefore treatments. Especially scars and defects of the vocal folds are a problem for a successful or even satisfying therapy. Due to these difficulties, one tries to overcome these problems by vocal fold tissue engineering. One aim of this technique is the formation of a *de novo* cellular structure, which *e.g.* can substitute a violated, defective tissue after (tumor) surgery in form and function. Another application is the construction of reliable and valid models for investigating biological and biomechanical properties of the vocal fold *in vitro*.

There are two main obstacles in tissue engineering of vocal folds: The first is the highly complex architecture of such folds (26). The vocal fold is composed of the *lamina propria*, sandwiched between epithelium and vocal muscle. The *lamina propria* can also be divided into three parts: the stratum superficiale (Reinke-Space), *stratum intermedium* and *stratum profundum* (27). Hirano's Body-Cover Model has proven its value in facilitating our understanding of the difficult biomechanics of such architecture (28). The second obstacle is the biological environment, which needs to resemble the physiological situation as closely as possible. For this reason, Gaston *et al*., for example, developed a special bioreactor to examine the response of VFFs to a vibratory stimulus and found a significant increase in their proliferation compared to mesenchymal stromal cells (29).

A basic problem of tissue engineering is that conventional cell culture means two-dimensional cell growth, which is generally associated with different cell function and behavior compared to the three-dimensional tissue, which therefore limits its informative value (30). To overcome this challenge of three-dimensional cell culture, different methods have been described. 3D-Printing is an interesting technique which is believed to have great potential in regenerative medicine (31). It is already in use for the goal of bio-fabricating artificial blood vessels (32), but has not yet found its way into tissue engineering of vocal folds. Seeding of cells onto artificial scaffolds is one way to go as already mentioned and this line should be continued: New materials are being tested and new approaches are investigated (33). In the case of vocal fold tissue engineering it would be particularly desirable to disclaim scaffolds, because the vocal fold has very special vibration properties which are required for vocalization, and of course a scaffold, if not resorbed (34), has to meet this demand.

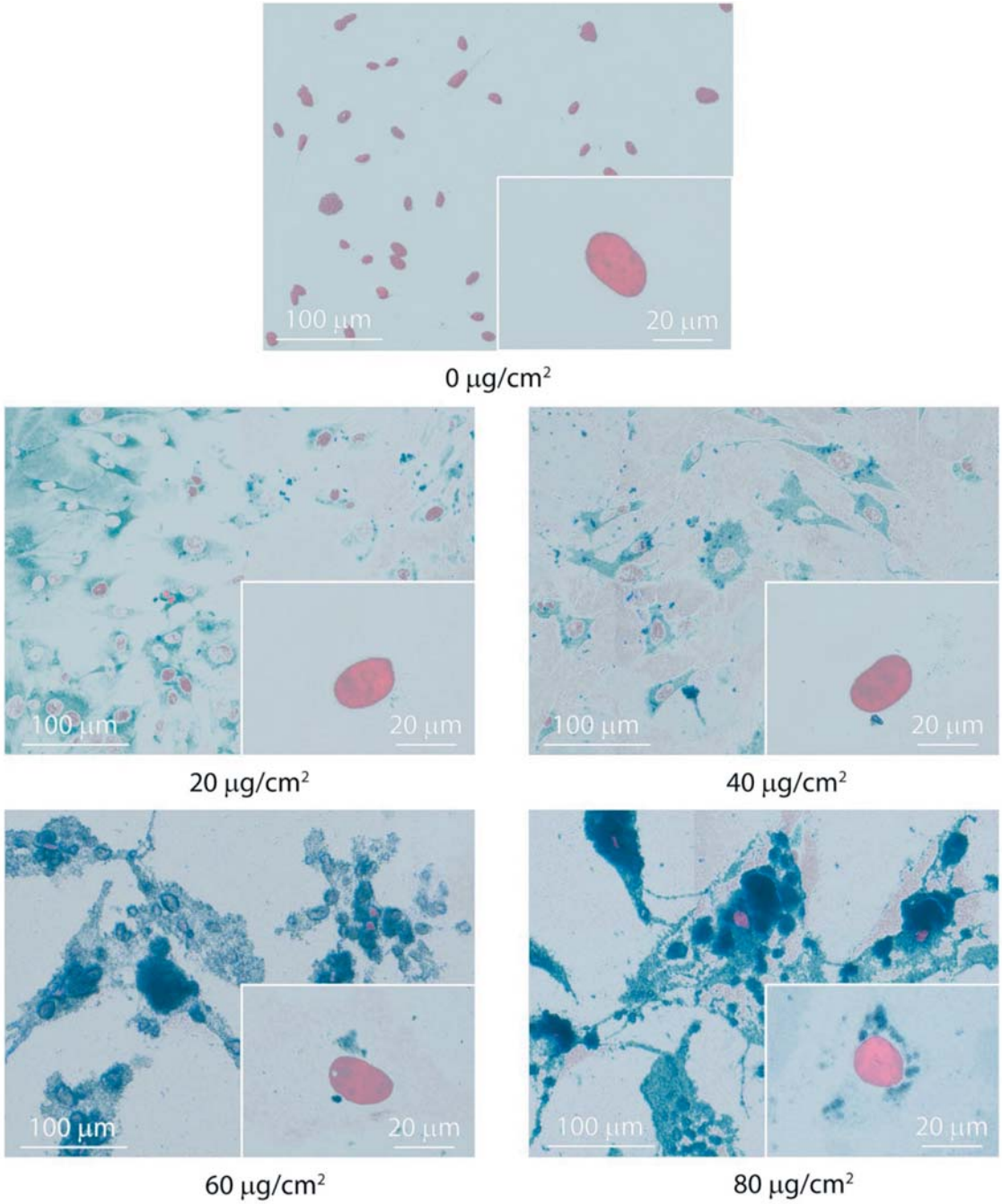

Figure 1. *Internalization/attachment of superparamagnetic ironoxide nanoparticles (SPIONs) by rabbit vocal fold fibroblasts (r-VFFs). Prussian blue and Hoechst 33342 staining of r-VFFs treated with SPIONs at different concentrations in transmission microscopy. Due to the perinuclear localization of the SPIONs, internalization into the cells is assumed. For high SPION concentrations, additional accumulation on the cell surface is assumed. Blue: Prussian blue staining; red: Hoechst 33342 staining.*

Magnetic tissue engineering is a new and innovative technique, not in need of scaffolds or other auxiliary framework for cell seeding, and seems to have great potential in tissue engineering of vocal folds. The method was first reported by Souza *et al*. in 2010 and "… does not require a specific medium, engineered scaffolds, matrices or moulded gels" (20). It was applied using different cell types (cell lines, primary cells, stem cells), different cell lines

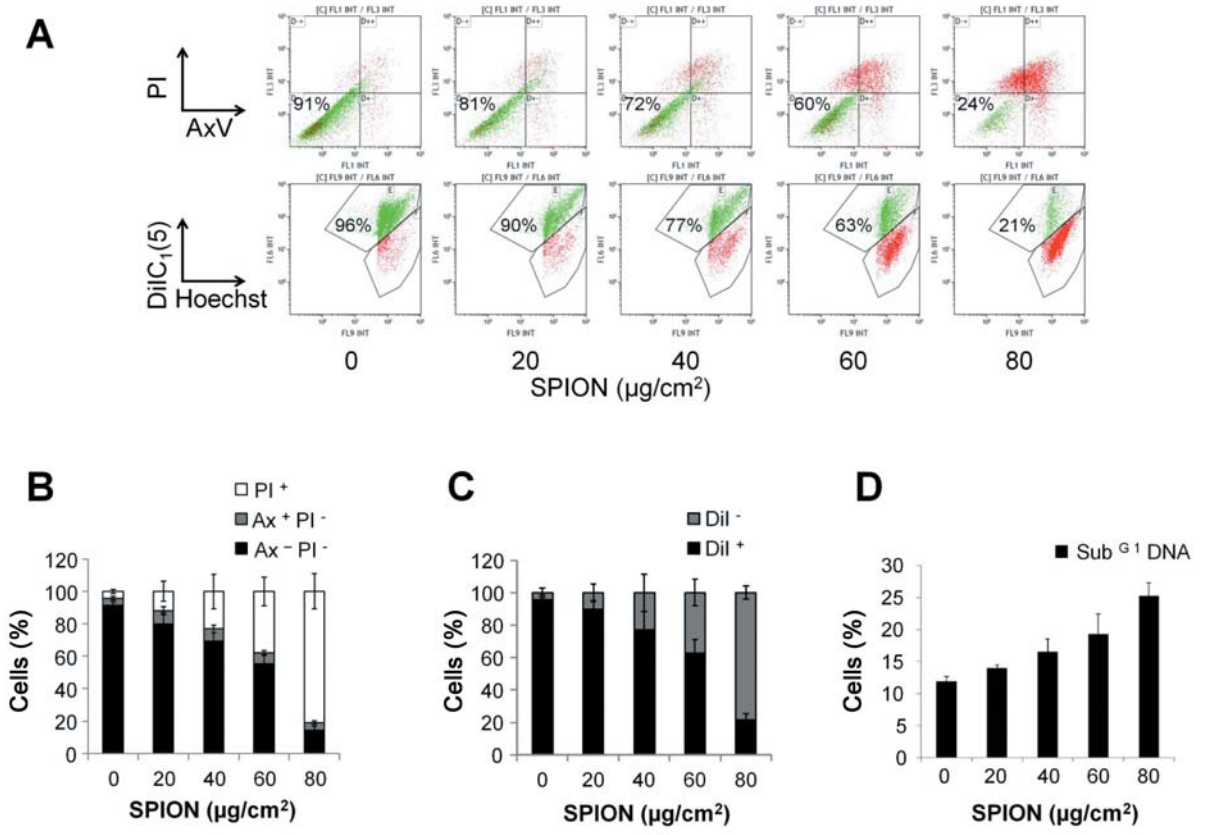

Figure 2. *Cell viability after internalization/attachment of superparamagnetic iron oxide nanoparticles (SPIONs) by rabbit vocal fold fibroblasts (r-VFFs). A: Raw data files of flow cytometric multiparameter cell-death staining. Cells were analyzed using annexin V-fluorescein isothiocyanate/propidium iodide (AxV/PI) staining (upper row), and with $DiIC_1(5)$/Hoechst33342 staining (lower row). Viable cells are depicted in green, dying/dead cells are depicted in red. B: Qualitative analysis of data shown in (A). AxV staining reflects phosphatidylserine exposure and PI staining loss of plasma membrane integrity. Thus, AxV^-/PI^- cells were considered viable, AxV^+/PI^- cells apoptotic, and PI^+ cells necrotic. C: 1,1',3,3,3',3'-Hexamethylindodicarbo-cyanine iodide (Dil) staining shows the state of the mitochondrial membrane potential. DiI^+ cells were regarded as viable, whereas DiI^- cells were undergoing cell death. D: PI-Triton X-100 staining reflects the DNA content of cells. The dose-dependent increase of subG1 DNA reflects DNA degradation after incubation with SPIONs. Data are the mean values of duplicates of three independent experiments with standard deviations.*

(pulmonary fibroblasts, hepatocytes, *etc.*) of different origins (mouse, rat, pig, human) and then published in Nature Protocols in 2013 (35). Magnetic tissue engineering has already been successfully used for a three-dimensional co-culture model, which will also be necessary for producing vocal folds (36).

Magnetic nanoparticles are a substantial part of nanomedicine and are used in many fields, *e.g.* cancer therapy (37) and imaging (38). But like any new technology, nanotechnology has associated risks as well as potential applicatory and for this reason, nanotoxicology is a subject of great interest (39). Nanoparticles are being tested with all kinds of procedures, *e.g.* real-time cell analysis (40), and in regard to various aspects, *e.g.* the immune system (41). The whole field is in a state of flux and new systems are continually being developed to improve biocompatibility (42).

Consequently, our first step was to analyze the effect of our nanoparticles, which have been proven in drug delivery (13), on r-VFFs. To examine the association of SPIONs to r-VFFs we used Prussian blue staining, which is a "special stain" for iron (43) and has already proven its value in the detection and localization of SPIONs (44). Due to the clear perinuclear distribution of SPIONs, we hypothesized internalization of the nanoparticles into the cells (45). We determined SPION toxicity to be dose-dependent, and DNA degradation was triggered due to cell death. Loading of cells with 20 µg/cm^2 induced hardly any toxicity, whereas loading with 80 µg/cm^2 caused a significant amount of cell death. On this basis, we prefer a middle course and use of a SPION concentration of 40 µg/cm^2. All in all, the loading of cells with enough SPIONs for satisfying magnetization and simultaneously avoiding cytotoxicity represents a balancing act in magnetic tissue engineering.

This investigation is the basis of future magnetic tissue engineering of vocal folds. As far as we are aware, it is the first to report the effect and biocompatibility of SPIONs on r-VFFs.

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