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NEGATIVE RESULT

Certain types of iron oxide nanoparticles are not suited to passively target inflammatory cells that infiltrate the brain in response to stroke

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Intravenous administration of iron oxide nanoparticles during the acute stage of experimental stroke can produce signal intensity changes in the ischemic region. This has been attributed, albeit controversially, to the infiltration of iron-laden blood-borne macrophages. The properties of nanoparticles that render them most suitable for phagocytosis is a matter of debate, as is the most relevant timepoint for administration. Both of these questions are examined in the present study. Imaging experiments were performed in mice with 30 minutes of middle cerebral artery occlusion (MCAO). Iron oxide nanoparticles with different charges and sizes were used, and mice received 300 μmol Fe/kg intravenously: either superparamagnetic iron oxide nanoparticles (SPIOs), ultrasmall SPIOs, or very small SPIOs. The particles were administered 7 days before MCAO, at the time of reperfusion, or 72 hours after MCAO. Interestingly, there was no observable signal change in the ischemic brains that could be attributed to iron. Furthermore, no Prussian blue-positive cells were found in the brains or blood leukocytes, despite intense staining in the livers and spleens. This implies that the nanoparticles selected for this study are not phagocytosed by blood-borne leukocytes and do not enter the ischemic mouse brain.

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INTRODUCTION

There is a profound inflammatory response to stroke that includes recruitment of blood-borne leukocytes to the brain. Because one of the roles of monocytes on their maturation is phagocytosis, it is generally believed that this subpopulation of leukocytes can take up contrast agents from the blood stream and bring them to the ischemic brain. For magnetic resonance imaging (MRI), superparamagnetic iron oxide nanoparticles (SPIOs) are widely used, reviewed in (refs 1–3). In general, they consist of an iron oxide core that can be encased in a variety of metabolizable monomers or polymers; the most common coating used is polysaccharides. The coating influences overall hydrodynamic diameter and surface charge, which in turn determines the pharmacokinetic properties of the particles such as blood half-life, uptake, and elimination.[4,5] These properties are extremely important when attempting to use these agents to target blood-borne monocytes. Superparamagnetic iron oxide nanoparticles are generally grouped according to size. Micron-sized particles of iron oxides are the largest (0.7 to 3 μm), followed by SPIOs (60 to 200 nm), ultrasmall SPIOs (USPIOs, 10 to 60 nm), and very small SPIOs (VSOPs, $<$10 nm). The superparamagnetism of iron oxide nanoparticles means they exhibit high transversal relaxivity and are thus generally used to produce signal loss in T_2- and T_2*-weighted images, although some particles exhibit significant longitudinal relaxivity and can produce hyperintensity in T_1-weighted images under certain conditions.

USPIOs are the most commonly used nanoparticles to attempt to image the monocyte response to stroke. This is generally because their small size affords them some degree of protection from uptake by the mononuclear phagocyte, or reticuloendothelial, system; i.e., the macrophages in the lymph nodes, spleen, and liver (Kupffer cells).[6] Therefore, their blood half-life is slightly longer than that of SPIOs. The first preclinical study administered USPIOs 5 hours after permanent middle cerebral artery occlusion (MCAO) in the rat and observed signal loss within the first 2 days in T_2-weighted images.[7] Prussian blue (iron stain)-positive cells were detected in the lesion boundary at 7 days. However, the same group was only able to observe a delayed (48 to 72 hours) hyperintense signal change in the ischemic hemisphere on T_1-weighted images when the same strategy was used in a transient model of MCAO,[8] which is more consistent with the known timecourse of monocyte infiltration to the ischemic brain.[9,10]

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These studies served as a launching platform for this technique and clinical studies began to emerge that showed a more heterogenous pattern of USPIO signal enhancement in stroke patients. When USPIOs were administered 6 days after symptom onset, a strong T_2* signal decrease was observed on account of the blood-pool effect of the iron, which paralleled an increase in T_1 contrast that was attributed to parenchymal accumulation.[11,12] Earlier USPIO administration (between 24 to 96 hours of symptom onset) resulted in T_1 enhancement in only one-third of patients.[13] This highlighted the need for further preclinical investigation with particular focus on delaying the timepoint of USPIO administration. However, studies that used this approach were less promising. The first preclinical study administered USPIOs for up to 3 days after transient MCAO in mice and no signal changes were observed in any of the images.[14] Comparable results were obtained by another group that administered USPIOs between 3 and 6 days after transient MCAO in rats.[15]

Despite exhibiting a poor half-life when compared with USPIOs, SPIOs consistently exhibit much higher uptake by different type of macrophages *in vitro*.[16,17] Therefore, if their administration is timed correctly, they could have a better chance for uptake *in vivo*. This was tested by a group that administered SPIOs continuously (between 3 and 14 days) after transient MCAO in the rat.[18] Focal regions of signal loss were observed in T_2*-weighted images between 3 and 4 days after MCAO in the lesion boundary and ventricles that correlated with Prussian blue-positive cells. Another group observed signal loss in the ischemic boundary in T_2*-weighted images when SPIOs were administered 7 days before MCAO.[19] Therefore, the goals of the present study were, first, to investigate the most suitable type of iron oxide nanoparticle for imaging blood-borne macrophages. We used USPIOs and SPIOs in accordance with the literature, as well as VSOPs. This is the first time VSOPs have been used to attempt to track blood-borne monocytes in experimental stroke. In the second instance, we aimed to identify the most relevant timepoint for contrast agent administration, which is also lacking in the literature. For this purpose, the three different nanoparticle formulations were infused either at 7 days before MCAO, at the time of reperfusion, or 72 hours after MCAO.

MATERIALS AND METHODS

Experimental Design

All experiments were approved by the Landesamt für Gesundtheit und Soziales under the license numbers G 0200/07 and A 0045/11, and performed in accordance with the German Animal Welfare Act. Animals were housed in a temperature (22 °C ± 2 °C), humidity (55% ± 10%), and light (12/12 hours light/dark cycle) controlled environment and given *ad libitum* access to food and water.

To assess the potential of MRI to detect iron-containing macrophages in the brain, 42 male C57 BL6 N mice (8 weeks of age, Charles River, Sulzfeld, Germany) received 30 minutes of MCAO ($n = 33$) or sham surgery ($n = 9$). Animals randomly received one of three different types of iron oxide nanoparticles (300 μmol Fe/kg): SPIOs, USPIOs, or VSOPs coupled to the fluorescent dye Dy-682 (Dyomics, Jena, Germany), at one of three different timepoints with respect to MCAO: 7 days before, at reperfusion, or 72 hours after MCAO. Magnetic resonance imaging was performed daily for up to 5 days after MCAO. At the conclusion of the MRI experiments, terminal blood samples were taken to harvest the blood leukocyte fraction and livers, spleens, and brains were harvested for histological analysis.

To determine blood iron content and leukocyte uptake of the nanoparticles closer to the time of injection in our specific model, 12 additional mice underwent MCAO and randomly received one of the three different types of iron oxide nanoparticles (300 μmol Fe/kg, $n = 4$, each) at 72 hours after MCAO. Blood samples were collected just before and at approximately 2, 5, 15, 30, and 45 minutes after injection and mice were subsequently euthanized. Iron content was measured in the blood samples and organs (heart, lungs, kidney, and portions of the liver and spleen) with magnetic particle spectroscopy (MPS), and an additional blood sample was taken before euthanasia to harvest the blood leukocyte fraction.

To confirm that blood-borne macrophages are indeed capable of migrating to the ischemic brain, additional experiments were performed in bone marrow chimeras (see Supplementary Methods and Results).

MCAO Procedure

Animals weighed between 19 and 27 g (average 24.1 ± 1.4 g) at the time of the MCAO. Anesthesia was achieved using isoflurane in a 70:30 nitrous oxide:oxygen mixture, and core body temperature was maintained at 37.2 ± 0.8 °C using an automated heat blanket with temperature feedback (Harvard Apparatus, Hugo Sachs Elektronik, March-Hugstetten, Germany). Transient occlusion of the MCA was performed using a modified intraluminal filament technique. Mice were placed in a supine position and the neck was shaved and cleaned. A midline incision was made in the neck, and the left sternomastoid muscle was retracted to expose the left carotid artery (CA). A silk suture was tied around the CA below the bifurcation into the internal and external carotid arteries, respectively. A loose suture was placed around the external carotid artery and secured externally. Another loose suture was placed on the internal carotid artery and a microclip was placed directly above it. A small incision was made in the CA and a 20-mm-long filament coated with 5 mm of silicone to a final diameter of 190 μm (Doccol Corporation, Redlands, CA, USA) was inserted into the incision. The loose internal carotid artery suture was tightened around the filament, the microclip released, and the filament advanced up the internal carotid artery until resistance was felt (~9 mm). The animals were recovered and the filament was left in place for 30 minutes. Reperfusion was induced by withdrawing the filament and ligating the CA around the incision site. All other threads were removed, muscles and glands guided back into place, and the incision sutured. Xylocain gel was applied to the sutured wound before recovery. Animals received 1.5 mL of physiological saline subcutaneously daily until weight stabilized and were provided with wet diet to facilitate eating.

Magnetic Resonance Imaging Measurements

Anesthesia was also achieved using isoflurane. Magnetic resonance imaging experiments were conducted on a 7 T Pharmascan 70/16 (Bruker BioSpin MRI GmbH, Ettlingen, Germany) equipped with actively shielded gradient coils (300 mT/m, rise time 80 μs). Radio frequency transmission and reception were achieved with a 20-mm (inner diameter) quadrature mouse head volume resonator (RAPID Biomedical, Würzburg, Germany). During scanning, body temperature and respiration rate were monitored with an MRI compatible system (Small Animal Instruments, Stony Brook, NY, USA).

The entire imaging protocol was selected to be completed within 30 minutes (FOV: 25 × 25 mm^2, matrix: 256 × 256, 12 contiguous 0.6-mm-thick slices). It consisted of a spin-echo multislice multiecho T_2 sequence (TR/TE: 3000/10.5 ms, 16 echoes, 9 minutes), a fast low-angle shot T_1-weighted sequence (TR/TE: 150/3.5 ms, flip angle 30°, 4 minutes), a rapid acquisition with relaxation enhancement T_1-weighted sequence (TR/TE: 800/13.2 ms, 2 averages, rapid acquisition with relaxation enhancement factor 2, 3 minutes), and a fast low-angle shot T_2*-weighted sequence (TR/TE: 1000/7.2 ms, flip angle 30°, 4 minutes) that was not strongly T_2*-weighted to avoid strong endogenous susceptibility effects.

Contrast Agents

One of three different types of iron oxide nanoparticles (for details regarding nanoparticle synthesis and characterization, see Supplementary Methods) were administered (300 μmol Fe/kg) intravenously at one of three timepoints: 7 days before MCAO, at reperfusion, or at 3 days after MCAO. Polyethylene glycol-coated SPIOs with a net positive charge (87 nm diameter, $R_1 = 11$, $R_2 = 295$ (mmol/L s^{-1}) at 1.4 T in water), polyacrylic acid-coated USPIOs with a net negative charge (17 nm diameter, $R_1 = 17$, $R_2 = 56$ (mmol/L s^{-1}) at 1.4 T in water), and citrate-coated VSOPs coupled to the fluorescent dye Dy-682 (9 nm diameter, $R_1 = 14$, $R_2 = 33$ (mmol/L s^{-1}) at 1.4 T in water) were adjusted for osmolarity for *in vivo* use with 15% D-mannitol (Sigma-Aldrich, Taufkirchen, Germany). The dye concentration in the VSOP-treated group resulted in an overall dose of 9 μmol Dye/kg to the mice. In the case of the animals that received contrast agents at 3 days after MCAO, the intravenous infusion was performed while the animals were inside the magnet, thus providing pre and post contrast images. Additionally, before the iron infusion, these animals received a bolus of 0.5 mmol/kg of Gadolinium diethylenetriaminepentaacetic acid (Magnevist, Bayer HealthCare Pharmaceuticals, Berlin,

Germany) to assess blood–brain barrier integrity with pre and post contrast T_1-weighted images.

MR-Image Analysis

Quantitative T_2 maps were fitted on a voxelwise basis using a monoexponential decay function in Paravision version 4 software (Bruker BioSpin). Subsequently, all data were exported to ImageJ version 1.44p freeware (National Institutes of Health, Bethesda, MD, USA). An unblinded observer used the Sync Windows Analyze Tool to draw two circular (1.2 mm diameter) regions of interest (ROIs) on the slice image that was located 0.14 mm from bregma:[20] intact and ischemic striatum. T_2 (ms) was measured in these ROIs from the T_2 maps and data were expressed as a ratio of the intact to ischemic hemisphere. An additional large rectangular ROI (24×4 mm) was also drawn above the brain on the T_1- and T_2*-weighted images. Subsequently, signal-to-noise ratios in the striatum were calculated by dividing the signal intensity of each ROI by the standard deviation of the noise in the large rectangular ROI. These data were also expressed as a ratio of the intact to ischemic hemisphere.

Magnetic Particle Spectroscopy

Quantification of nanoparticle iron content in blood samples from 12 MCAO mice was determined immediately after nanoparticle injection using a commercial MPS (Bruker BioSpin MRI GmbH).[21] Primarily, this device is dedicated to quantify the performance of magnetic nanoparticles as tracers for a novel imaging modality called magnetic particle imaging.[22] Magnetic particle spectroscopy detects specifically the nonlinear magnetic response of magnetic nanoparticles exposed to an oscillating magnetic field. Biological tissue and paramagnetic blood iron do not contribute to the MPS signal.

Blood samples (between 50 and 100 μL) were collected in ethylenediaminetetraacetic acid-coated capillaries before and at approximately 2, 5, 15, 30, and 45 minutes after injection (72 hours after MCAO) of the different nanoparticle formulations. After blood sampling, animals were euthanized with an overdose of anesthetic and perfused transcardially with physiological saline. The heart, lungs, kidneys, livers, and spleens were extracted and weighed, and the livers and spleens were divided for MPS and histology. Blood samples and organs (up to 180 μL) were collected in polymerase chain reaction compatible fast reaction tubes (Applied Biosystems, Darmstadt, Germany) and placed into the pick-up coil of the MPS system. The magnetic response of the samples to an AC magnetic field of a frequency f_0 of 25 kHz and an amplitude B_{excit} of 25 mT was recorded for 10 s. From the time-dependent signal, the harmonics spectrum of magnetic moments is gained by Fourier transformation showing prominent amplitudes at odd multiples of f_0 evoked by the magnetic nanoparticles. The third harmonic A_3 of the spectrum was taken as a measure of the absolute nanoparticle iron content. By referring to a reference sample (5 μL of the stock nanoparticle suspension) of known iron content measured under the same conditions, the total amount of iron in each sample was determined. From the magnetic moment sensitivity of the device (approximately 5×10^{-12} A/m^2) and the measured A_3 amplitude of the reference, we estimated individual detection limits for each of the three different iron oxide nanoparticles, which are determined by the particle size: 1 ng of iron for SPIOs, 30 ng of iron for USPIOs, and 50 ng of iron for VSOPs.

Blood Leukocyte Harvest

Terminal blood samples were collected from all mice and processed to retrieve the leukocyte fraction. The samples were adjusted to equal volumes (5 mL) with 1 mol/L phosphate-buffered saline and embedded on 2.5 mL of Histopaque-1083 (Sigma-Aldrich). They were subsequently centrifuged for 30 minutes at 850 *g* at room temperature. The leukocyte-containing phase was removed from the gradient, washed with RPMI medium (also containing 10% fetal calf serum, 1% penicillin and streptomycin, and 1% glutamate), and centrifuged at 120 r.p.m. at 4 °C for 8 minutes. The cells were washed in phosphate-buffered saline, fixed for 20 minutes at room temperature in 4% paraformaldehyde, washed, and placed on slides.

Histology

Mice that underwent MRI for up to 5 days after MCAO were euthanized with intraperitoneal chloral hydrate (4% in water), perfused with physiological saline, and the brains, livers, and spleens were snap frozen in -40 °C methylbutane, sectioned to 20 μm using a cryostat (Leica Microsystems, Wetzlar, Germany) and stored at -80 °C. Brains and pieces of the livers and spleens from the 12 additional animals used to determine blood iron content were also treated in this fashion. Sections from each organ, as well as the slides containing the blood leukocytes, were stained with Prussian blue to detect iron using the Accustain Iron Kit (Sigma-Aldrich). Slides were placed in equal parts of 4% potassium ferrocyanide ($K_4Fe(CN)_6$) and 1.2 mmol/L of HCl for 10 minutes at room temperature. They were subsequently rinsed in deionized water and counterstained in 1% pararosaniline hydrochloride for 4 minutes. The slides were rinsed again, rapidly dehydrated in 70, 80, 96, and 100% alcohol, placed in xylene, and subsequently coverslipped with Vitro Clud (Langenbrink, Emmendingen, Germany).

RESULTS

Overall mortality within the primary study was low (1/42), two mice were euthanized prematurely because of excessive weight-loss in accordance with our animal care guidelines and one animal was excluded on the basis that no MCAO was observed in the T_2-weighted images. Group assignments and drop outs are presented in Table 1. Only one of the additional 12 mice that were used for blood nanoparticles iron content analysis by MPS was euthanized prematurely and another died after particle injection.

MR-Image Analysis

Repetitive MRI of mice for up to 5 days after MCAO did not reveal any focal regions of hypointensity in T_2- or T_2*-weighted images, or hyperintensity in T_1-weighted images, that could be attributed to any of the three used iron oxide nanoparticles (Figure 1). In the animals that received nanoparticles at 72 hours after MCAO (Figure 1, bottom panel), the infusion was performed while the mice were inside the MRI so that the blood-pool effect could be observed with pre (72 hours) and post contrast (72.5 hours) images. An overall signal decrease, which was particularly prominent in large blood vessels, was observed in T_2*-weighted images after infusion of VSOPs, SPIOs, and USPIOs (Figure 1, bottom row). However, this effect was no longer present the following day (96 hours after MCAO, Figure 1, bottom row) and no additional hypointensities were observed at any other timepoint. Blood–brain barrier breakdown was also evident in the animals

Table 1. Experimental groups and hemispheric lesion volume

Groups	n[a]	n[b]	*Reason*	*Hemispheric lesion volume (72 hours)*
VSOPs 7 days before MCAO	4	4		14% ± 12
VSOPs at reperfusion	4	4		20% ± 16
VSOPs 72 hours after MCAO	3	3		29% ± 11
SPIOs 7 days before MCAO	4	4		31% ± 13
SPIOs at reperfusion	4	3	euthanized	33% ± 12
SPIOs 72 hours after MCAO	3	2	excluded, no MCAO	33% ± 9
USPIOs 7 days before MCAO	4	4		36% ± 16
USPIOs at reperfusion	4	2	1 died, 1 euthanized	43% ± 14
USPIOs 72 hours after MCAO	3	3		22% ± 16
Sham animals (1/group)	9	9		—

MCAO, middle cerebral artery occlusion; SPIOs, superparamagnetic iron oxide nanoparticles; USPIOs, ultrasmall SPIOs; VSOPs, very small SOPIs. [a]Initial group sizes. [b]Final group sizes.

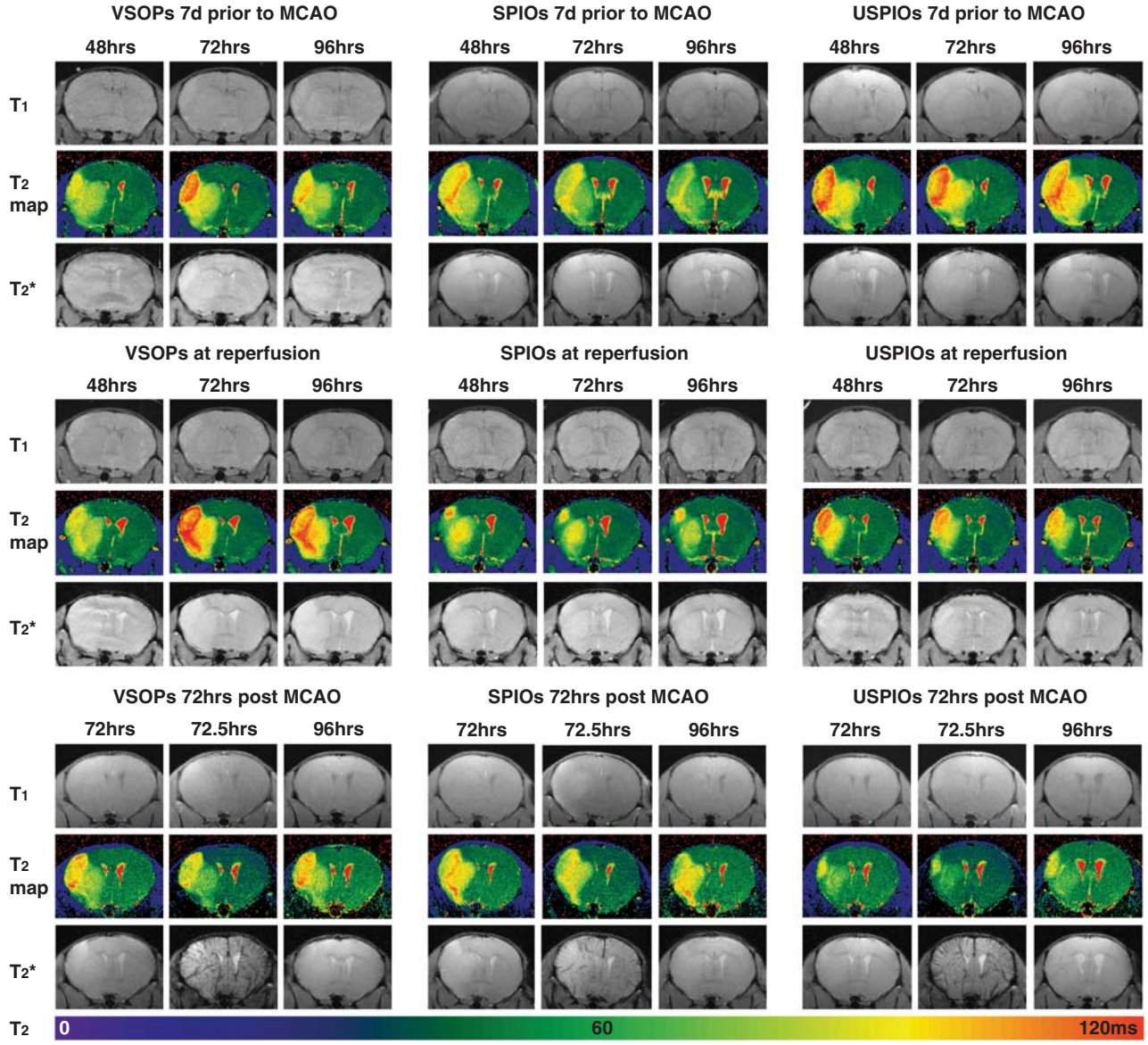

Figure 1. Images from animals treated with intravenous contrast agents at different timepoints with respect to middle cerebral artery occlusion (MCAO). A timecourse of T_1-weighted images, T_2 maps, and T_2*-weighted images from a representative animal in each of the treatment groups (left panel—VSOPs, middle panel—SPIOs, and right panel—USPIOs) after MCAO. The top panel contains animals that received iron oxides 7 days before MCAO, the middle panel: at the time of reperfusion, and the bottom panel: at 72 hours after MCAO. T_2 values (ms) are indicated in the color scale bar below the images. Note the blood-pool effect of the iron oxides after infusion (72.5 hours) in the T_2*-weighted images in the bottom most row. SPIOs, superparamagnetic iron oxide nanoparticles; USPIOs, ultrasmall SPIOs; VSOPs, very small SPIOs.

that received iron at 3 days after MCAO as the ischemic region became hyperintense in T_1-weighted images after Gadolinium diethylenetriaminepentaacetic acid infusion (Figure 1, bottom panel).

Semiquantification of the signal-to-noise ratio in T_1- and T_2*-weighted images, and T_2 (ms), was performed in the intact and ischemic striatum to try and elucidate subtle changes in signal. Subsequent values were expressed as a ratio of the intact to ischemic hemisphere; thus, a value of 1 would indicate that there is no difference in signal-to-noise ratio, or T_2, between hemispheres. Both T_1 and T_2* ratios in animals treated with VSOPs at 7 days before MCAO or at the time of reperfusion remained unchanged (around 1) at all measured timepoints after MCAO (Figure 2A and C). However, the T_2 ratios of these animals were $\sim$0.6 within the first 48 hours of MCAO (Figure 2B), which is not surprising given that T_2 values are increased in the ischemic territory because of vasogenic edema. As edema resolves T_2 values will decline slightly, which is visualized by the gradual increase in T_2 ratios over time in the animals treated with VSOPs at 72 hours after MCAO (Figure 2B). In these same animals, no changes in T_2 or T_2* ratios were observed directly after injection of the iron (72 to 72.5 hours; Figure 2B and C). However, a decrease in the T_1 ratio was observed at this timepoint because of the Gadolinium diethylenetriaminepentaacetic acid injection (Figure 2A).

Similar results were obtained from the animals treated with SPIOs and USPIOs (Figure 3 A–C and D–F, respectively). No changes in T_2* ratios were observed over time in either SPIO (Figure 3C) or USPIO (Figure 3F) treated animals when nanoparticles were infused at 7 days before MCAO, at reperfusion, or at

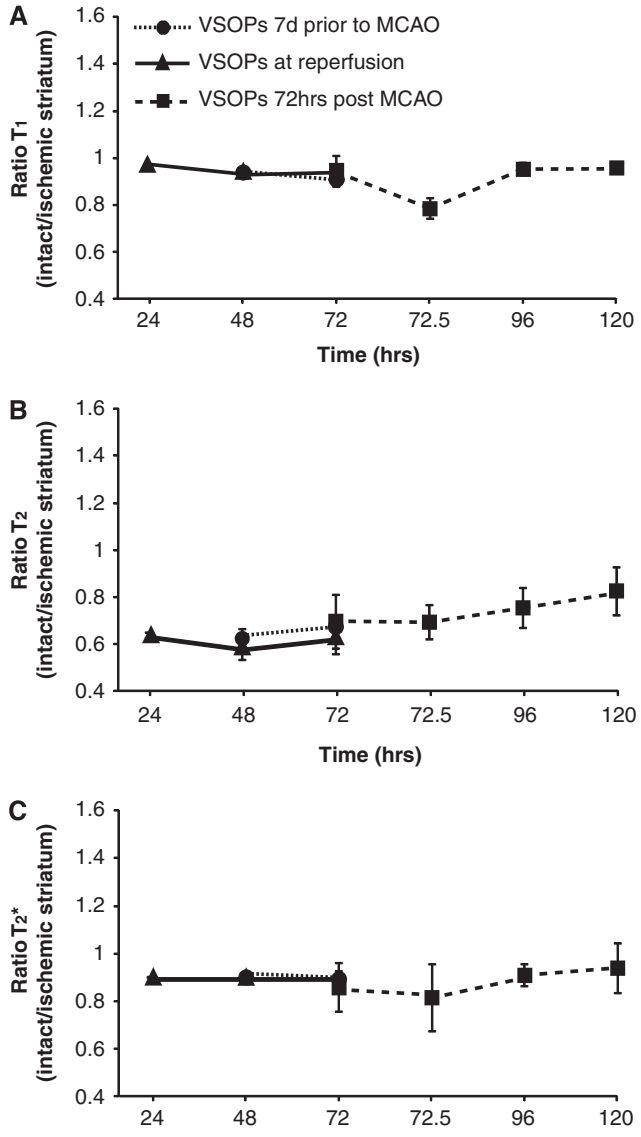

Figure 2. Signal intensity changes in the images over time in VSOP-treated animals. Semiquantitative analysis (means ± s.d.) of the signal-to-noise ratio in the striatum, expressed as a ratio of the intact to ischemic hemisphere in T_1 (**A**) and T_2*-weighted images (**C**), for all VSOP-treated groups (circles with dotted line—VSOPs at 7 days before MCAO ($n = 4$), triangles with solid line—VSOPs at reperfusion ($n = 4$), and squares with dashed line—VSOPs at 72 hours after MCAO ($n = 3$). T_2 values in the striatum are expressed as a ratio of the intact to ischemic hemisphere (**B**). Note the decrease in the T_1 ratio after Gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) administration (72.5 hours) in the animals that received VSOPs at 72 hours after MCAO. MCAO, middle cerebral artery occlusion; VSOPs, very small superparamagnetic iron oxide nanoparticles.

72 hours after MCAO. This was also the case for T_1 ratios. However, there was a decrease in the T_1 ratio at 72.5 hours after Gadolinium diethylenetriaminepentaacetic acid injection in the animals treated with SPIOs at 72 hours after MCAO (Figure 3A). This effect was not observed in the animals treated with USPIOs at 72 hours after MCAO (Figure 3D). Animals in this group did not exhibit such severe lesions, as is indicated by the lower T_2 values and thus T_2 ratio of only 0.8 (Figure 3E). T_2 ratios of the other USPIO, and SPIO-treated groups, were comparable (beginning ~0.6 and increasing to 0.8 by 120 hours after MCAO).

Magnetic Particle Spectroscopy

Magnetic particle spectroscopy revealed that the concentration of magnetic nanoparticles in the blood was negligible before injection. However, the nanoparticle iron concentration increased dramatically in all three groups within the first 2 minutes of the infusion (between 100 and 200 ng Fe/mg of blood) (Figure 4). This response began to decline as early as 5 minutes after injection and was nearly at baseline levels by 45 minutes, and this pattern was similar for all three nanoparticle formulations.

Hearts, lungs, and kidneys also contained a negligible amount of nanoparticle iron. However, the portions of the spleens and livers (respectively) from all three groups contained high amounts of nanoparticle iron: VSOPs (362 ± 56 and 197 ± 82 ng Fe/mg of tissue), UPSIOs (223 ± 76 and 172 ± 53 ng Fe/mg of tissue), and SPIOs (41 ± 3 and 58 ± 21 ng Fe/mg of tissue). When this was corrected for total organ weight, assuming homogenous distribution, the liver contained ten times more nanoparticle iron than the spleen in VSOP- and USPIO-treated animals, and 30 times more in the SPIO-treated animals. Overall particle concentration, in general, was lower in the SPIO-treated group.

Histology

Three brain sections (1.42, 0.14, and − 1.7 mm from bregma) from each of the animals were examined for the presence of Prussian blue-positive cells. No Prussian blue-positive cells were observed in any of the examined sections (Figure 5). However, the livers and spleens of all animals in the study were highly positive for iron. Iron in the spleen was not always homogenously distributed and appeared to be both intracellular and extracellular. Prussian blue staining was always observed inside the cells of the liver. The blood leukocyte fraction was also examined and none of the slides contained any Prussian blue-positive cells.

DISCUSSION

The present study administered three different types of iron oxide nanoparticles, each with a different size and coating, at three different timepoints with respect to MCAO. Interestingly, no regions of circumscribed signal change that could be attributed to iron were observed in T_2*-, T_2-, or T_1-weighted images under any combination of conditions. A detailed examination of the corresponding tissue sections was unable to identify any Prussian blue-positive cells in the ischemic brains, although the livers and spleens from all groups, at all timepoints, contained high amounts of iron. We were also unable to find any Prussian blue-positive cells among the blood leukocytes that were harvested from terminal blood samples. Repetitive blood sample analysis in another group of ischemic animals revealed that all three particle types, irrespective of differences in formulations, rapidly disappeared from the blood within 45 minutes and at this time were only detectable in the livers and spleens and not the other organs or blood leukocytes. Taken together, these results suggest limited application for the use of these type of iron oxide nanoparticles to image the peripheral macrophage response to transient MCAO. We would like to make the controversial suggestion that the available literature actually supports our findings. Because negative results are excluded from the literature, researchers are under pressure to place a positive spin on their results. This means that even though the research community is aware that this technique has certain limitations, many publications still promote iron oxides as a useful tool to image blood-borne monocytes. With this article, we hope to bring this issue forward for discussion.

The notion that USPIO-induced signal change in MR images is because of the infiltration of monocytes that have phagocytosed contrast agents from the blood has been losing momentum over the last few years. The landmark studies in rat and mouse[7,23] were followed by results that suggested some of the observed signal

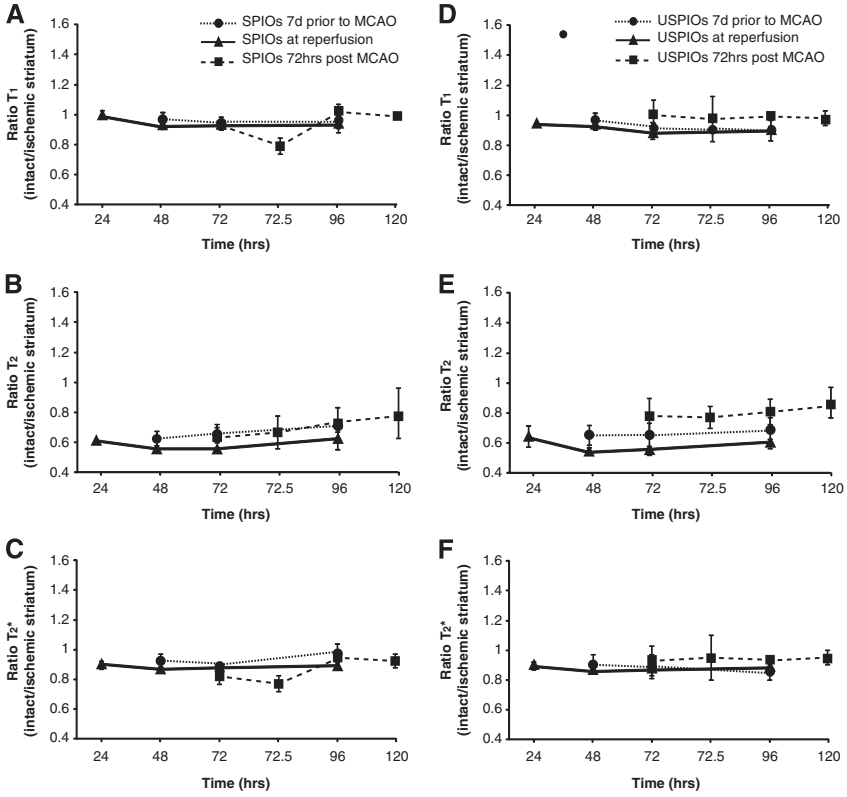

Figure 3. Signal intensity changes in the images over time in SPIO- and USPIO-treated animals. Semiquantitative analysis (means ± s.d.) of the signal-to-noise ratio in the striatum, expressed as a ratio of the intact to ischemic hemisphere in T_1- (**A**) and T_2*-weighted images (**C**) for SPIO-treated groups (circles with dotted line—SPIOs at 7 days before MCAO ($n = 4$), triangles with solid line—SPIOs at reperfusion ($n = 3$), and squares with dashed line—SPIOs at 72 hours after MCAO ($n = 2$). T_2 values in the striatum are expressed as a ratio of the intact to ischemic hemisphere (**B**). Similar results are presented for USPIO-treated animals in the right panel (**D**–**F**). Note the decrease in the T_1 ratio after Gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) administration (72.5 hours) in the animals that received SPIOs at 3 days after MCAO. MCAO, middle cerebral artery occlusion; SPIOs, superparamagnetic iron oxide nanoparticles; USPIOs, ultrasmall SPIOs; VSOPs, very small SOPIs.

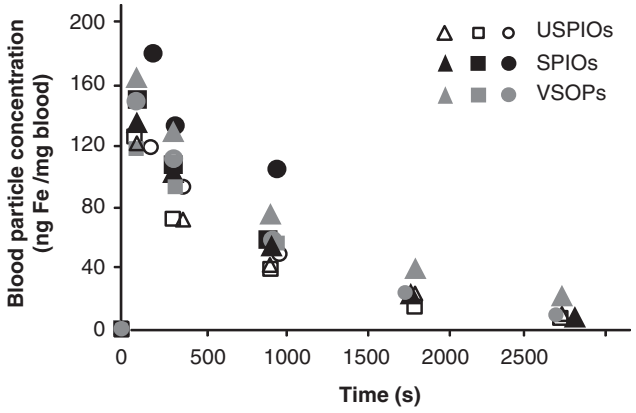

Figure 4. Nanoparticle iron concentration (ng Fe/mg blood) in the blood as measured by magnetic particle spectroscopy before (0 second) and up to 45 minutes (2700 seconds) after injection in animals treated with VSOPs (gray symbols), USPIOs (empty symbols), and SPIOs (black symbols). Note: each symbol corresponds to a different animal ($n = 3$ per group). SPIOs, superparamagnetic iron oxide nanoparticles; USPIOs, ultrasmall SPIOs; VSOPs, very small SOPIs.

change may be because of passive entry of the USPIOs into the brain and/or accumulation in the cerebral spinal fluid and interstitial space[24] and experiments using transient models were never as promising.[8,14,15] The results of the present study using a transient model are in agreement, with the notable exception that we did not observe a delayed hyperintensity develop in T_1-weighted images that was reported by one group.[8] While clinical studies have also reported T_1 increases when USPIOs were administered 6 days after symptom onset,[11,12] it is still not clear if this represents accumulation of iron-containing cells. The T_1 effect can overcome the susceptibility effect when iron concentration is low[25,26] or when the iron is extracellular as opposed to clustered intracellularly.[27,28] Therefore, it is possible that the T_1 hyperintensity could reflect low amounts of iron in the interstitial space.[23,29]

In contrast to USPIOs, very little work has been done with SPIOs on account of their short half-lives. One group attempted to overcome the restrictions imposed by poor circulation times by administering SPIOs on a daily basis, and were able to observe signal change in the ischemic brain;[18] unfortunately, we only administered SPIOs once per animal. Another group has suggested that administration of SPIOs 7 days before MCAO allows these particles to accumulate in the bone marrow progenitor cells; thus, any contrast observed in the brain after stroke would be from cells that originated in the bone marrow and not because of passive entry of contrast into the brain.[19] The preloading strategy was not successful in our hands, although it is not clear if SPIOs are capable of accumulating in the bone marrow. This property has generally been attributed to USPIOs on account of their half-life, and even USPIOs show relatively little bone marrow accumulation beyond 24 hours in rodents.[30,31] The SPIOs

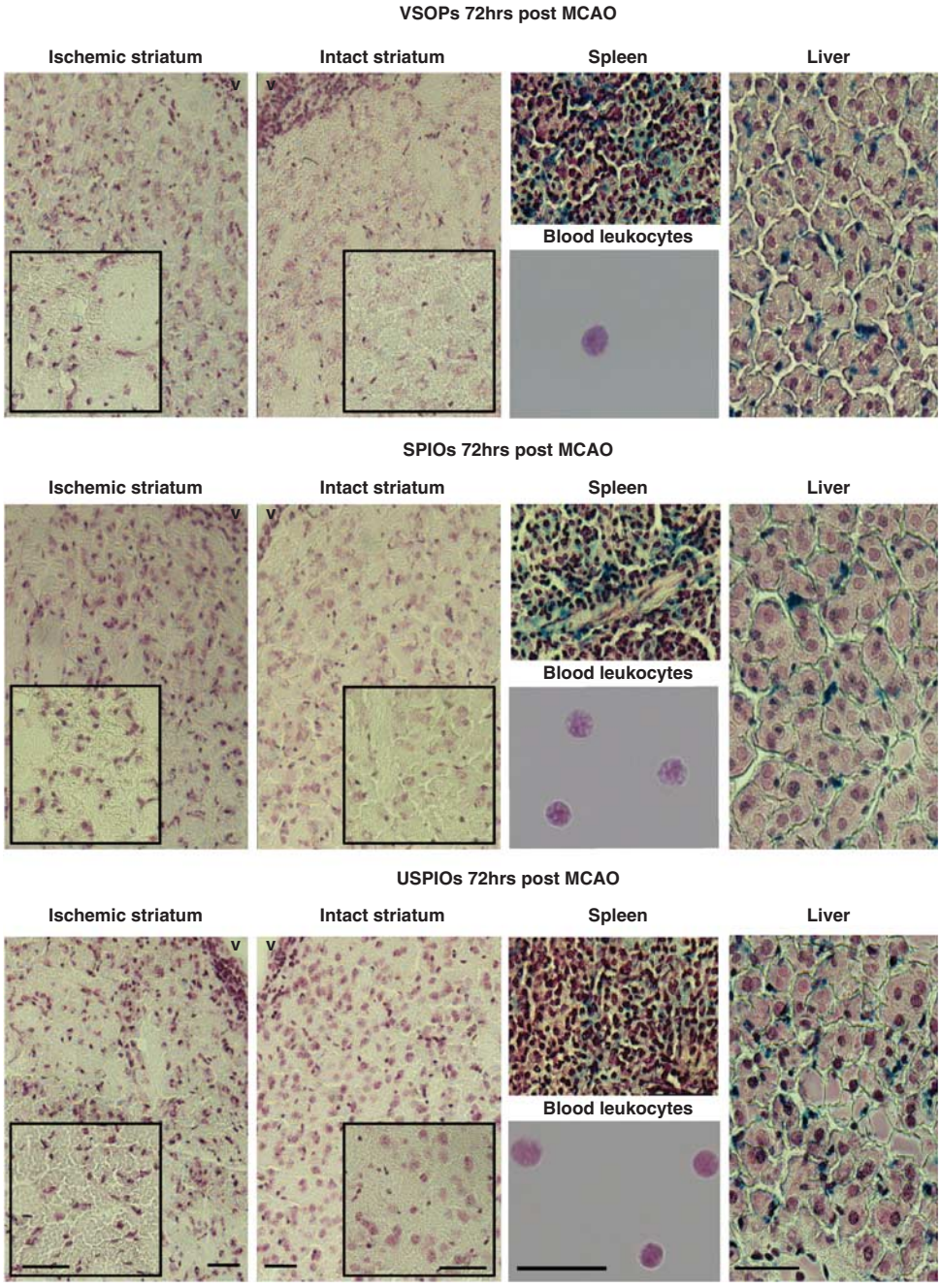

Figure 5. Prussian blue staining of the tissues and leukocyte fractions. Prussian blue sections from a representative animal treated with VSOPs (top row), SPIOs (middle row), and USPIOs (bottom row) at 72 hours after MCAO. The ischemic and intact striatum images contain higher magnification insets. Prussian blue-positive cells were only found in the spleens and livers. Note: scale bars in the leukocyte images correspond to 16 μm, all other scale bars correspond to 50 μm. The letter v in the top corner of the intact and ischemic striatum denotes the ventricles as an anatomical reference. MCAO, middle cerebral artery occlusion; SPIOs, superparamagnetic iron oxide nanoparticles; USPIOs, ultrasmall SPIOs; VSOPs, very small SOPIs.

used in the present study would be more suitable than most other formulations for this purpose, because they were coated with polyethylene glycol to increase circulation time. As we did not measure the bone marrow, we cannot exclude the possibility that the SPIOs accumulated there.

The fact that none of the particles appear to be suited to track blood-borne monocytes is not entirely surprising if we consider their historical development. The first-generation SPIOs (Endorem, Guerbet, Paris, France, Feridex, Amag Pharma, Lexington, MA, USA; coated in dextran with a neutral charge) were developed for iron replacement therapy. Subsequent formulations were coated with carboxy-dextran and possessed a negative charge (SH U 555 A/Resovist, Bayer Schering, Berlin, Germany) because this reduced side effects of administration. Both formulations are sterically stabilized, which means they are not stable for long *in vivo*.[6] Early pharmokinetic studies in rodents revealed that SPIO half-life was extremely short (less than 10 minutes) as nearly 90% are absorbed by Kupffer cells and macrophages in the spleen;[32] even coating them with polyethylene glycol, as is the case in the present study, does not

extend the half-life much beyond this. Thus, the properties of SPIOs make them unattractive as a candidate to target monocytes. Even though USPIOs exhibit slightly longer circulation times (~80 minutes in rats),[33] this is still not very long when trying to observe a process that lasts for several days and involves a constant turnover of cells. What was also not well considered when we began infusing these particles into experimental stroke models was the fact that blood-borne monocytes may not necessarily behave in the same way after their maturation into macrophages as the macrophages in the liver and spleen. It is well known that USPIO uptake *in vitro* by monocytic cells is relatively poor.[16,17,24,34] Thus, it is not surprising that uptake may also be poor *in vivo*, although few groups have actually examined this in their models. Blood leukocytes harvested from animals with MCAO directly after USPIO infusion (6 hours), or 24 hours later, were Prussian blue negative.[24] The results of the present study are in agreement with this.

It is necessary to point out that there are several limitations with the present study. The first is that we did not include statistical analysis of our results on account of the small group sizes, which makes it difficult to make definitive conclusions. However, as the results were emerging as overwhelmingly negative, we could not justify the continued use of resources and animals. The second is that our experiments were conducted solely in mice, which is unfortunate as we cannot directly compare our findings with those that were obtained in rats. The third important limitation was that we chose to focus our attention on nanoparticle formulations that were consistent with those used in the published literature (namely, USPIOs and SPIOs). As was previously mentioned, the half-lives of these formulations are not conducive for labeling blood-borne leukocytes. While the same appears to be true for the VSOPs used in the present study, it is possible that similar particles with longer circulation times, such monocrystalline iron oxide nanocompound,[35] or cross-linked iron oxide nanoparticles, could result in uptake by blood-borne leukocytes.

In conclusion, certain formulations of iron oxide nanoparticles, at least the ones used in the present study, appear to have a limited application as agents to label blood-borne monocytes of bone marrow origin in a mouse model of transient focal ischemia.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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