Exogenous iron redistribution between brain and spleen after the administration of the $^{57}Fe_3O_4$ ferrofluid into the ventricle of the brain

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ABSTRACT

Iron clearance pathways after the injection of $^{57}Fe_3O_4$-based dextran-stabilized ferrofluid into the brain ventricles were studied by Mössbauer spectroscopy and histologically. The nanoparticles appeared in spleen tissues within 3 h after transcranial injection. We separated and independently estimated concentrations of iron encapsulated in nanoparticles and iron encapsulated in proteins in the all rat organs. It was found that the dextran coated initial nanoparticles of the ferrofluid disintegrated in the brain into separate superparamagnetic nanoparticles within a week after the injection.The nanoparticles completely exited from the brain in a few weeks. The exogenous iron appeared in the spleen in 3 h after the injection and remained in the spleen for more than month. The appearance of additional component in Mössbauer spectra of spleen samples revealed a fundamental difference in the mechanisms of processing of iron nanoparticles in this organ, which was also confirmed by histological examination.

1. Introduction

Magnetic ferrofluids are used today in many industrial, scientific and medical applications including drug delivery, gene therapy, magnetic hyperthermia, contrasting of the magnetic-resonance images (MRI) or 3D magnetic particle imaging (MPI) [1–7]. The ferrofluid containing nanoparticles used for implementation of these procedures might be introduced into a body either by direct injections into the target area or by intravenous injections with further delivery through the blood stream into a pathological area. For the last method, the ferrofluids, based on iron oxide nanoparticles, are considered as the most convenient and promising tool. Their advantage is based both on their magnetic properties and iron biocompatibility in the live body. The human body contains several grams of endogenous iron, mainly in the form of ferritin, hemosiderin or gem-containing proteins, such as the blood hemoglobin. Natural metabolism of iron in a body is the very intensive process. In the case of iron deficiency the additional iron can be obtained by the breakdown of hemoglobin or other iron-containing proteins. In reverse, an excess of the exogenous iron after medical procedures will be transformed to the endogenous iron-containing

proteins, such as ferritin and hemoglobin.

Magnetic nanoparticles based technologies are very promising for the diagnostics and treatment of brain oncological or neurological diseases [7–11]. Drug delivery through the blood–brain barrier (BBB) still is a challenge in the treatment of brain's disorders. Magnetic nanoparticles are considered to be one of the possible delivery systems for passing through the BBB. The delivery method is based on the concentration of a magnetic drug in the blood vessels of the brain by an external magnetic field followed by the transportation of the drug through the barrier by monocytes/macrophage. The possibility of such delivery of the magnetic drugs into the brain was confirmed experimentally by [12]. The alternative strategy consists of invasively bypassing the BBB by drilling a hole in the skull and injecting probes or drugs intracerebrally or intracerebroventricularly [13]. This method has a number of advantages; however, the procedure of opening the skull is very dangerous and can cause long-term consequences [14,15]. The main problem whether there is a mechanism of biodegradation or excretion of iron nanoparticles from the cerebral cavities requires a clear answer for the introduction of the method into clinical practice. In our previous works, we developed an experimental method for an
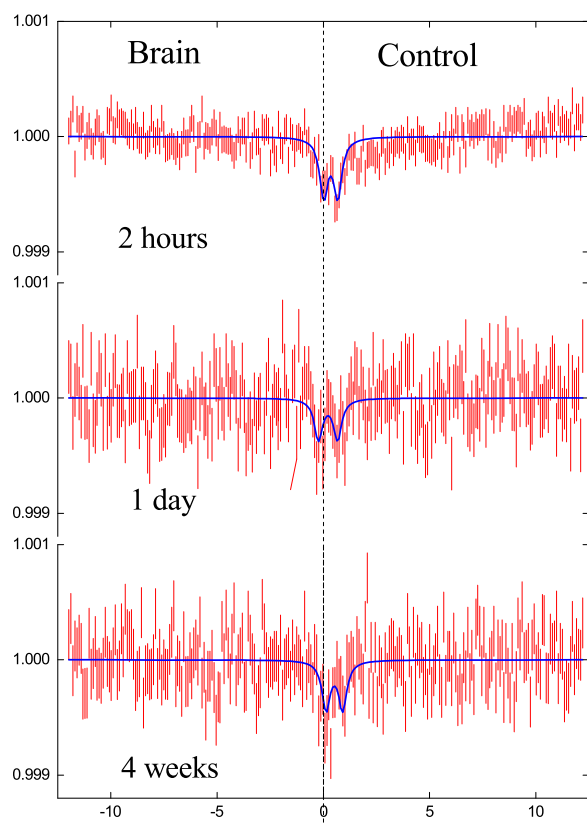

Fig. 1. ^{57}Fe Mössbauer spectra of control rat brain samples at 2 h, 1 day and 4 weeks after transcranial injection collected at 300 K.

evaluation of the distribution, clearance and excretion of magnetic particles in the body, based on joint analysis of the Mössbauer spectra of the same sample collected at various physical conditions [16,17]. According our previous experience, iron oxide nanoparticles after injection into the tail vein of mice accumulated mainly in the liver and spleen with subsequent biodegradation. The first fast stage in biodegradation is destruction of the outer polymer shell, which leads to the decrease of the magneto-dipole interaction between the neighboring superparamagnetic nanoparticles within several hours after intravenous injection of the ferrofluid. As a result, the Mössbauer spectra of the studying liver and spleen lyophilized samples changed from the magnetically split sextet, usually observed in the spectra of the initial dried ferrofluid, to a doublet with intensity increasing over time after injection. The final products of such biodegradation process are so-called iron-containing proteins such as ferritin, hemosiderin and transferrin [18–21]. The Mössbauer spectrum of the iron-containing proteins show in the temperature range of 78–300 K demonstrates a doublet with a practically stable line shape [18,19]. Consequently a presence of iron-containing proteins in studying sample can be easily established from the group of temperature-dependent Mössbauer spectra. Unfortunately, we are not able to determine the exact protein composition in the tissue specimens using our experimental technique. Hereinafter the term "iron-containing proteins" means one of the abovementioned proteins or their mixture.

In this work we further developed this method to study the redistribution of transcranially injected ferrofluid between internal organs. The most challenging part of this study was to collect Mössbauer spectra of the brain tissue specimens having a very low iron concentration. The probability of the Mössbauer effect is proportional to the amount of the ^{57}Fe nucleus (stable isotope) in the sample and the natural iron contains only 2.2% ^{57}Fe. To enhance the sensitivity of the Mössbauer spectroscopy, we synthesized ^{57}Fe-en-

riched dextran nanoparticles.

2. Experiment

2.1. Magnetic nanoparticles

The ^{57}Fe enriched iron oxide ferrofluid was prepared as previously reported in [21–23]. ^{57}Fe$_3$O$_4$ magnetic nanoparticles were synthesized by co-precipitation of the water solutions of iron chlorides FeCl$_2$ and ^{57}FeCl$_3$ in 0.1 M HCl (Fe^{2+}:Fe^{3+}=1:2) in 30% solution of ammonium hydroxide, NH$_4$OH. After incubation at 90 °C, the particles underwent magnetic separation and flushing in 2 M HNO$_3$ followed by their introduction into distilled H$_2$O to form a suspension. The as-produced suspension was supplemented with 70 kDa dextran from Leuconostoc spp. (Sigma, USA). After re-incubation at 80 °C, the particles were triply flushed in dH$_2$O by centrifuging. Then the particles were sorted for the selection of batches of particles with similar dimensions. The final nanoparticles contain 50% of dextran. The enriched to 96% by ^{57}Fe isotope powder of ^{57}Fe$_2$O$_3$ used in this work was obtained from JSC "PA "Electrochemical Plant", Russia. The initial trivalent ^{57}FeCl$_3$ was synthesized by direct solving ^{57}Fe$_2$O$_3$ in HCl. As a result, the as-produced ^{57}Fe$_3$O$_4$ nanoparticles were enriched in the ^{57}Fe isotope up to 66%, which exceeds the ^{57}Fe content in magnetic nanoparticles of natural isotope composition in more than 30 times.

2.2. Rats

Details of the experiment on laboratory animals were described previously [23,24]. The right lateral ventricles of the brain of 17 Wistar rats were injected transcranially with 5 µL of ferrofluid containing 5 mg of magnetic ^{57}Fe$_3$O$_4$ nanoparticles suspended in physiological saline. The injections were performed under chloral hydrate anesthesia. The animal care facility was kept under a natural light/dark cycle. The rats were housed individually and were provided with water and food *ad libitum*. The procedure was performed in compliance with the NIH guide for the care and use of laboratory animals (NIH Publication No. 8023, revised 1996). The rats were euthanized at 2 h, 1, 3, 7, 28, 49 and 84 days after the injection and the organs were extracted. To prevent the oxidation processes the extracted organs were placed in 4% paraformaldehyde solution in phosphate buffer (pH=7.2–7.4). In the control experiment series we used the same protocol and injected transcranially 5 µL of physiological saline.

2.3. Mössbauer spectroscopy

For Mössbauer studies, the lyophilized rat brain and spleen samples were ground to the powder form. The Mössbauer absorption spectra of ^{57}Fe nuclei were measured in transmission geometry using an conventional spectrometer working in the constant acceleration mode. The radioactive source ^{57}Co(Rh) of about 20 mCi activity was used. Isomer shifts were determined relative to the centroid of α-Fe. The samples were measured at the temperatures 78 K and 300 K and in the presence of a magnetic field of 3.4 kOe at 300 K. The analysis of all Mössbauer spectra was carried out in the frame of the many-state relaxation model formalism [26,27]. We estimated concentrations of the exogenous and endogenous iron using the joint analysis method as described in [17].

2.4. Histology

Sampling of the material for histological studies was performed using perfusion as previously reported in [22]. For this purpose the animals were anesthetized with Nembutal (50 mg/kg). Then the spleen was extracted and placed in 4% paraformaldehyde solution in phosphate buffer (pH=7.2–7.4). For histological studies the organs were placed in 20% sucrose solution in phosphate buffer pH=7.2–7.4) at a temperature of 40 °C for 12 h. Then they were dried by filter paper and
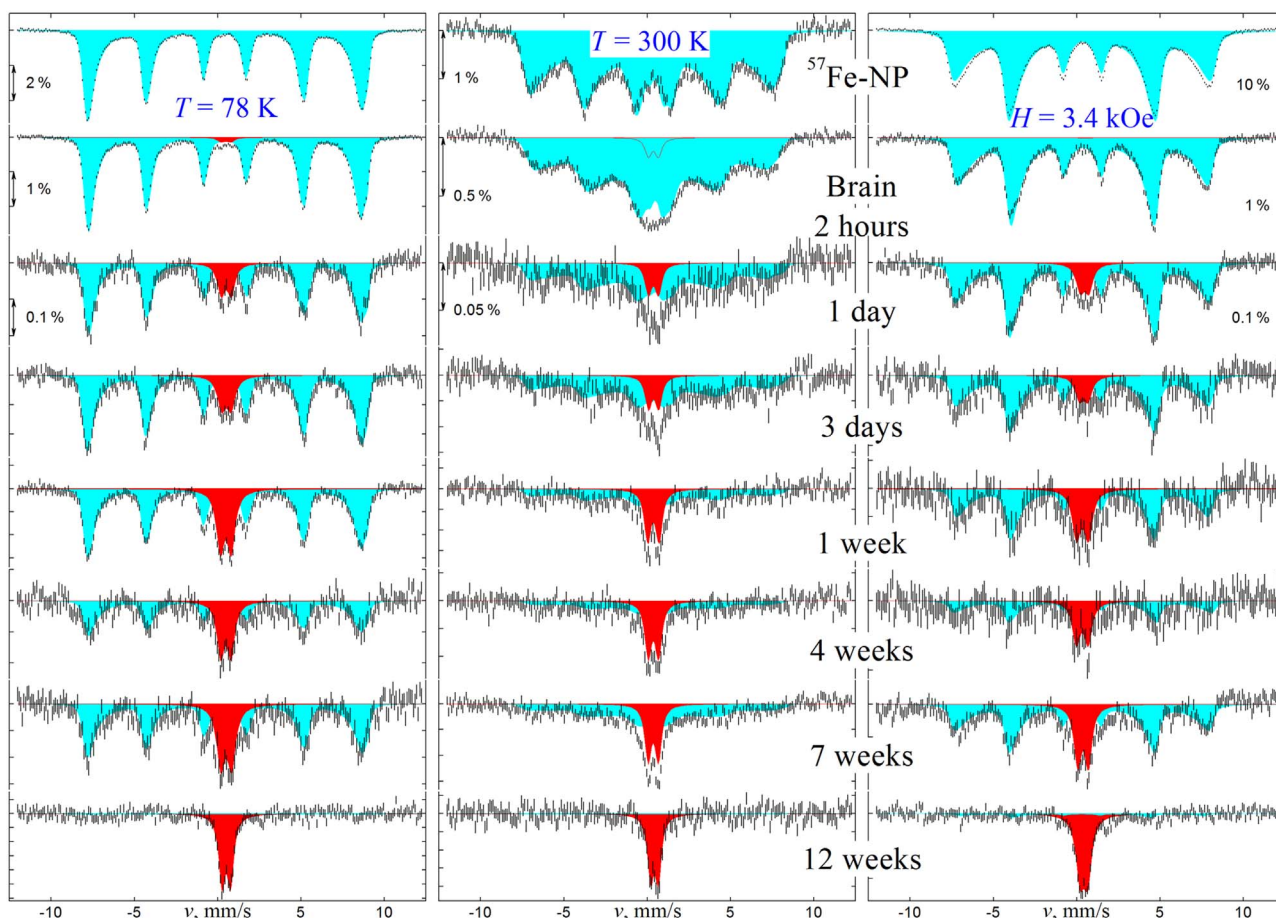
Fig. 2. ^{57}Fe Mössbauer spectra of rat brain samples at 2 h, 1 day, 3 days, 1 week, 4 weeks and 7 weeks after transcranial injection collected at 78 K, 300 K.
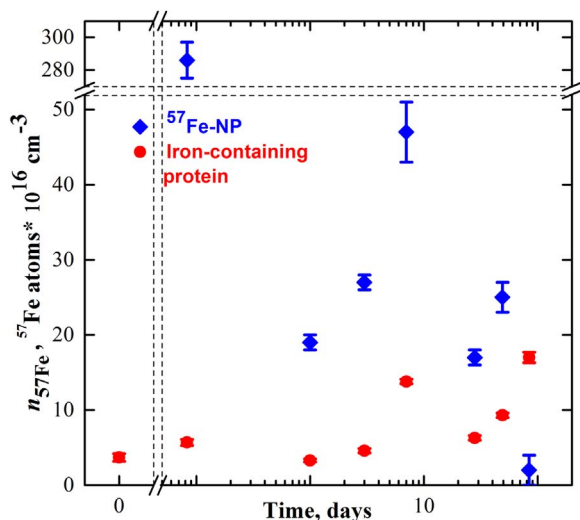

Fig. 3. Concentrations of exogenous and endogenous iron in rat brain samples.

frozen in liquid nitrogen vapor. The slices thick of 18 μm were produced on a cryostat Zeiss Microm HM 505 E at the temperatures 20 °C to 22 °C.

2.5. Stain on ferric iron

Portion of sections was stained by the Perls [28] (the reaction product—Prussian blue). We first prepared staining solution containing 2% aqueous solution of potassium ferrocyanide and 2% solution of hydrochloric acid, which were mixed together in equal proportions immediately before use. Staining was performed as follows: the dye was applied to the slices for 30 min, then the glass slides with slices were put in 70% (30 s), 96% (30 s), and twice in 100% (30 s) alcohol, and after 2 min the glass slides with slices were placed in toluene. Slices were entered in the medium (Bio Optica, Italy) under a coverslip. Stained slices were examined under a light microscope Axioplan 2 (Zeiss, Germany). As a result of the staining clusters of Fe_3O_4 nanoparticles and clusters of hemosiderin in macrophages become dark blue, cell nuclei, basophilic substance and fibrin acquire different shades of red.

2.6. Stain on ferrous iron

Staining of sections was performed for 1 h in a freshly prepared solution of 0.4 g of potassium ferricyanide in 40 mL of an aqueous solution of hydrochloric acid (2.5 mL of hydrochloric acid and 497.5 mL of distilled water). The slices were then washed in 1% acetic acid (1 mL glacial acetic acid and 100 mL of distilled water). After rinsing in distilled water, sections were stained with the dye in Nuclear Fast Red Sodium Salt for 5 min. After rinsing in distilled water, sections were dehydrated in alcohols of increasing concentration: 70°, 96°, and 100°. The sections were then placed for 5 min in toluene and embedded in the medium (Bio Optica, Italy) under the coverslip. As a result of the staining a ferrous iron becomes blue.
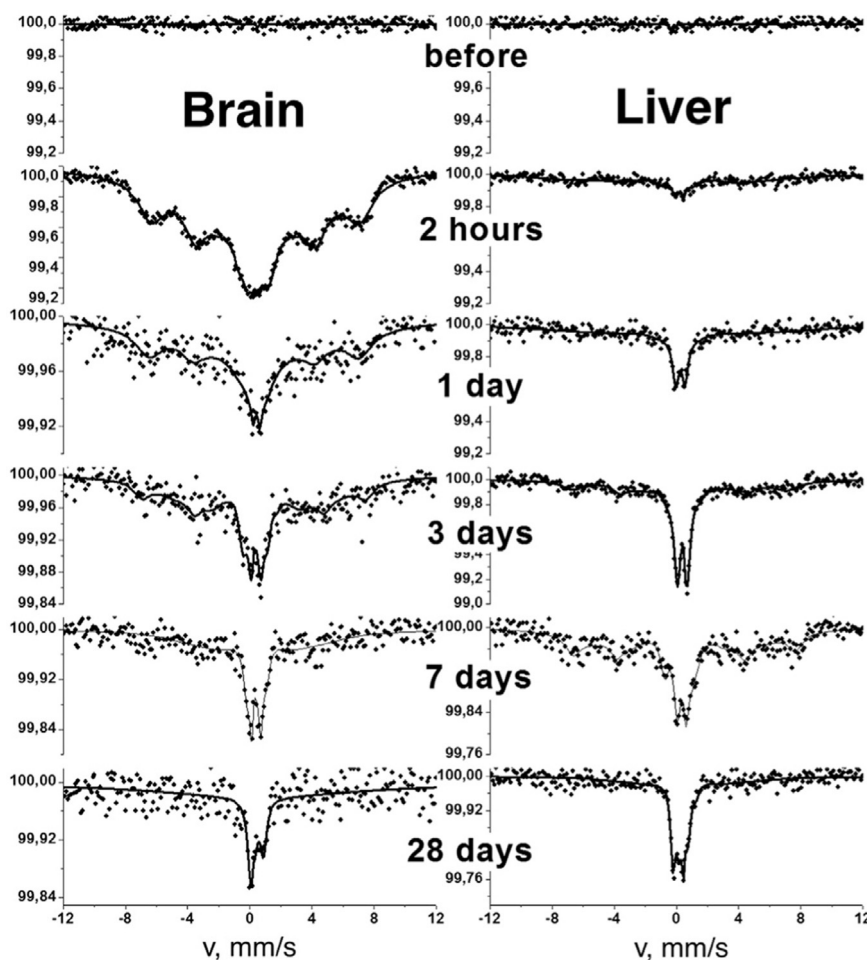
Fig. 4. ^{57}Fe Mössbauer spectra of the rat brain and the rat liver, measured: before injection and 2 h, 1, 3, 7, 28 days after the injection. T=300 K. [24].

3. Results and discussion

3.1. Brain

The control brain samples demonstrated the presence of a single doublet component with isomer shift corresponding to the trivalent iron (Fig. 1), which is the typical picture for the iron-containing protein in the tissues [18,19]. We did not found any principal differences in spectra between control samples at 2 h, 1 day, 7 weeks and others after administration (henceforward "after administration" will be omitted. For example "the 2 h sample" means "the sample of rat euthanized at 2 h after transcranial ferrofluid administration"). A rapid excretion process started immediately after nanoparticle administration into the brain. The rat brain sample at 1 day showed more than ten times intensity decrease the of the hyperfine nanoparticle component in comparison to 2 h sample (Fig. 2). The concentration of iron-containing proteins in 1 day sample seemed to be comparable with the iron concentration in the control sample (Fig. 3). After 1 day takes place a gradual process of reducing the intensity of the nanoparticle magnetic spectral component and the growth of the concentration of iron-containing proteins (the doublet component). Finally, the spectrum of the 12 weeks sample showed almost complete removal of initial nanoparticles from the brain tissue. The iron-containing proteins concentration has increased almost 5-fold compared to the control sample, which is a manifestation of transformation of exogenous iron. A similar result was obtained for histology data of the rat brains, presented in [24], however because of the lower sensitivity of the method used, we did not observe any significant amount of iron in the

samples after 1 week. It should be noted that Mössbauer data showed a certain nonmonotonical behavior. For example, for the 1 week sample, there is an unexpected increase of iron concentration. The most likely reason is that we used for Mössbauer study only half of the brain and another half was saved for histology. And due to the inhomogeneous distribution of the nanoparticles in the brain also observed in histology data [24], the received result may not coincide with the actual iron concentrations in the rat brain. The histology images of the brain tissues did not show any indications of leaks into the peripheral from the injection channels [24].

3.2. Spleen

The exogenous iron appeared in the liver [24] (Fig. 4) and the spleen in several hours after the injection and remained in these organs for several months. The Mössbauer spectrum of 2 h rat spleen sample already demonstrated a six-line component corresponding to initial nanoparticles (Fig. 5). The 300 K Mössbauer spectra of the liver and the spleen samples demonstrated the similar behavior. The concentration of nanoparticles in both organs reached its maximum on the seventh day and then starts decreasing practically to zero after 2 weeks. Together with the usual doublet component of ferric iron-containing proteins in the spleen spectra we observed an additional component with principally different properties. This component has the isomer shift about 0.15 mm/s at 300 K temperature and 0.35 mm/s at 78 K which points to the low spin ferrous iron composition with a large quadruple splitting ~2 mm/s. The most appropriate candidate for this component is hemoglobin [29,30]. Thus, the Mössbauer spectra of the

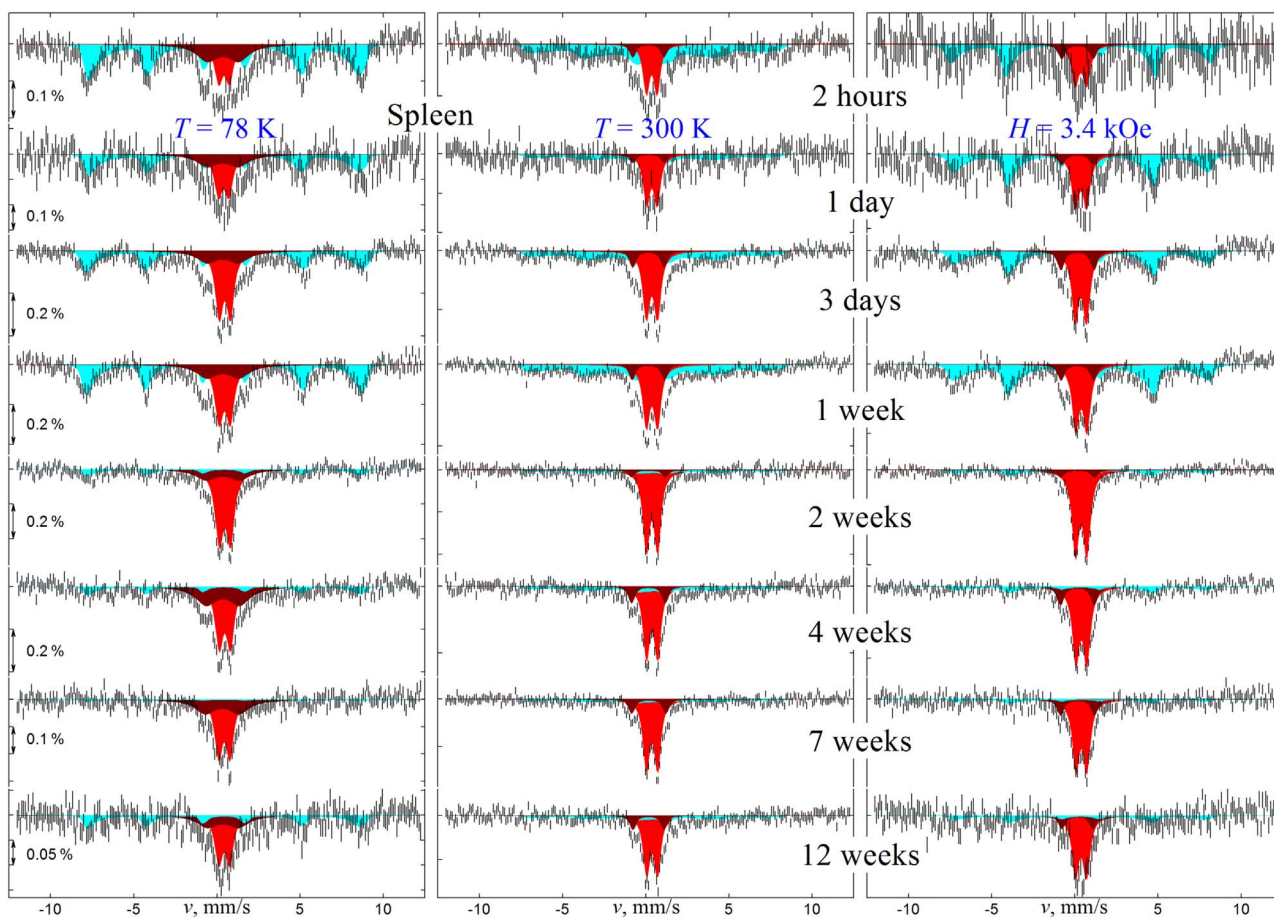**Fig. 5.** ^{57}Fe Mössbauer spectra of rat spleen samples at 2 h, 1day, 3 days, 1 week, 4 weeks and 7 weeks after transcranial injection collected at 78 K, 300 K.
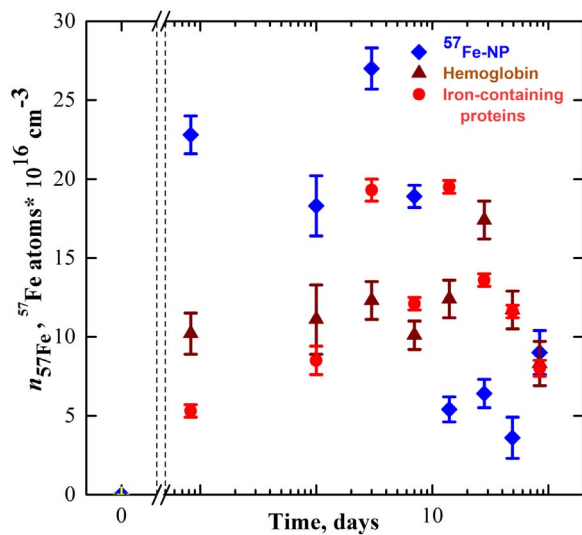

Fig. 6. Concentrations of exogenous and endogenous iron in rat spleen samples at 2 h, 1 day, 3 days, 1 week, 4 weeks and 7 weeks after transcranial injection obtained from Mössbauer spectra.

spleen specimens were analyzed in assumption of the presence of the different components: 1) Nanoparticles (broadened six-line structure transforming at 78 K to usual sextet), 2) iron-containing protein (ferritin, hemosiderin and transferrin) which forms a doublet with low quadruple splitting and 3) hemoglobin (doublet with large quad-

rupole splitting). The spectral area of this component significantly decreases with increasing temperature from 78 K to 300 K, which also indicates to the hemoglobin as a material with a low probability of recoilless absorption of gamma-quanta. The concentration of this component does not undergo significant changes over time after injection (Fig. 6).

According to histology data, iron localized in spleen follicles (Fig. 7). Follicles with iron were detected in all other periods of the experiment. The number of follicles Fe^{2+} is slightly varied during the experiment. The number of follicles with Fe^{3+} iron was maximal at the 3rd, 7th and 14th days of the experiment. The 2 h sample contains more follicles with ferrous iron than follicles with ferric iron. In all other samples any noticeable difference between the number of follicles with Fe^{2+} and Fe^{3+} iron was not observed.

4. Conclusion

We showed that the dextran coated magnetic nanoparticles following their injection into the cerebrospinal liquid of the live brain undergo an intensive transformation into iron-containing proteins, same as the nanoparticles after their injection into the blood [16]. The blood brain barrier does not prevent the transfer of the nanoparticles from the ventricles of the brain to the liver and the spleen. We showed a principal difference in biodegradation processes in the liver and the spleen despite the fact that this both of the organs contain a large amount of the same iron depositing protein and ferritin, in these organs. The spleen samples also contained significant amount of hemoglobin. We were able to separate contribution of these proteins to Mössbauer spectra and estimate concentration of each component. The received results were confirmed by histology data.
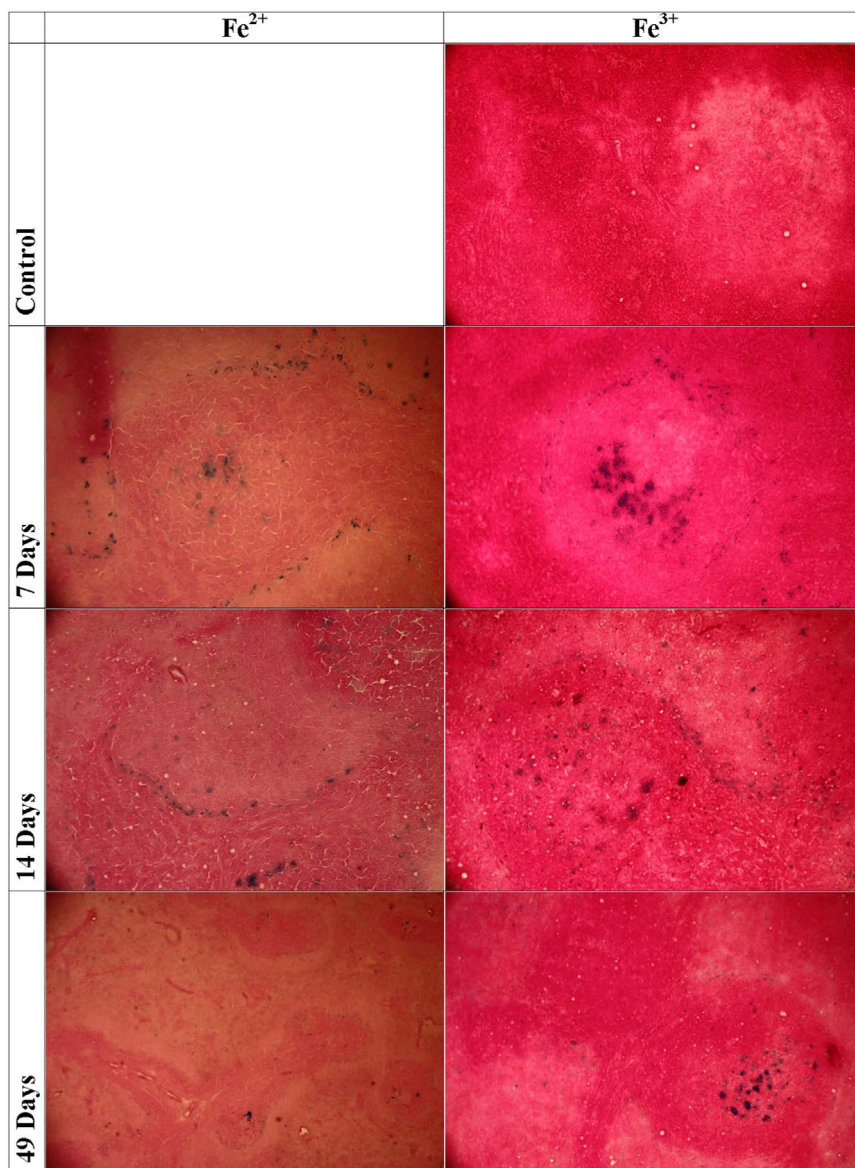
Fig. 7. Cross-sections of the spleen (4 samples) stained on ferrous and ferric iron.

Acknowledgement

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