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Assessment of liver phagocytic activity using EPR spectrometry and imaging

Nicolas Charlier[a], Audrey M. Neyrinck[b], Nelson Beghein[a], Nathalie M. Delzenne[b], Bernard Gallez[a,*]

[a]Biomedical Magnetic Resonance Unit, Louvain Drug Research Institute, Université catholique de Louvain, 1200 Brussels, Belgium
[b]Laboratory of Pharmacokinetics, Metabolism, Nutrition and Toxicology, Louvain Drug Research Institute, Université catholique de Louvain, 1200 Brussels, Belgium

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Abstract

The aim of the present study was to evaluate the usefulness of electron paramagnetic resonance (EPR) spectroscopy and imaging in assessing the phagocytic activity of the liver after administration of India ink. We conducted experiments on livers from control rodents and from rodents in which the Kupffer cell population had been depleted by pretreatment with gadolinium chloride. The EPR signal intensity recorded in liver homogenates was about two times lower in $GdCl_3$ treated rats than in control rats. EPR imaging carried out on precision-cut liver slices indicated a good correlation between the depletion of Kupffer cells and the EPR signal intensity.

Keywords: EPR; ESR; Spectroscopy; Imaging; Ink; Liver

1. Introduction

Electron Paramagnetic Resonance (EPR) spectroscopy is the method of choice for detecting, characterizing and quantifying free radicals in biological systems. The development of low-frequency EPR instruments has made it possible to perform EPR measurements on biological samples and in vivo. Studies that require measurement of the spatial distribution of free radicals within the sample can be performed by EPR imaging (EPRI) using appropriate field gradients [1]. Except for melanin, which is the only endogenous radical that can be detected directly in large biological samples [2], EPRI requires administration of paramagnetic contrast agents, such as nitroxides [3,4], triarylmethyl radicals [5–7], or charcoals [8]. The most important applications of EPRI so far are the measurement of oxygen tension [9,10], pH [11,12] and redox status [13].

Another potential field of application of EPRI is molecular EPRI, using paramagnetic compounds that could be selectively delivered and accumulated in targeted sites. This approach could potentially be achieved using targeted nitroxides [14,15], trityls or nanoparticles. In the present study, we used India inks. India inks are suspensions of carbon black nanoparticles dispersed in aqueous media. These materials have been noted for their oxygen sensitivity and have been used for oximetry purposes in vivo in animals [16,17] Using commercial inks and synthetic inks, based on dispersion of carbon blacks [18] in biocompatible suspending agents [19] these compounds were also tested in the first EPR clinical trials at Dartmouth Medical School [20]. Early after the discovery of the sensitivity of their EPR linewidth to oxygen, India inks were used in liver oximetry [21,22]. Indeed, India ink particles are quickly taken up by Kupffer cells and accumulate rapidly in the liver. In these studies, the authors addressed questions about the modulation of liver oxygenation after various treatments: intoxication by carbon tetrachloride [21] or after reversible interruption of the blood flow [22]. As far as we know, there is no study which has tried to correlate the accumulation of India ink, as measured by EPR, to phagocytic activity. The rationale for this study was that the administration of India ink is currently used in histology to assess the number of macrophages in liver tissue

* Corresponding author. CMFA/REMA, B-1200 Brussels, Belgium. Tel.: +32 2 7647391; fax: +32 2 7647390.
E-mail address: bernard.gallez@uclouvain.be (B. Gallez).

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[23,24]. As a key step for further investigations using in vivo EPR, the aim of the present study was to answer the following questions. (1) Is EPR spectroscopy in liver samples able to detect differences in phagocytic activity? (2) Does EPR imaging of liver slices correlate with standard histology? To answer these questions, we carried out experiments on livers from control rodents and from rodents in which the Kupffer cell population had been depleted by pretreatment with gadolinium chloride ($GdCl_3$) [25].

2. Material and methods

2.1. Treatments

The animal experiments were carried out in compliance with national care regulations. Male Wistar Rats (Janvier, Le Genest-St-Isle, France, 150–200 g body weight) were used in this study. $GdCl_3$ (Sigma-Aldrich, Bornem, Belgium; 30 mg/ml in NaCl 0.9%) was administered intravenously 24 h before ink injection to deplete the Kupffer cell population. It is generally considered that this period allows maximal depletion of the Kupffer cell population [26]. The control rats were injected with saline (intravenous 1 ml/kg) 24 h prior to ink injection. Twenty-four hours after the $GdCl_3$ or saline injection, the rats were deeply anesthetized by intraperitoneal injection of ketamine/xylazine (80 mg and 8 mg/kg, respectively). India ink (Pelikan N°17, Hannover, Germany) diluted 1:9 in saline was injected via the tail vein (1.0 ml/kg; slow infusion over 1 min). The ink was allowed to circulate for 20 min. To avoid any signal being detected from the circulation, the blood was then removed by flushing saline containing heparin (Leo Pharma, Wilrijk, Belgium; 125 U.I./ml) via an 18G needle inserted into the aorta. The flushed blood was allowed to escape by an incision into the right atrium. The process takes about 5 min to flush almost all the blood. The liver was then carefully excised, weighed and divided into two parts for further experiments: one part was homogenized (with half its weight of saline) using a Potter-Elvehjem tissue grinder for further X-band EPR spectrometry; the second part of the liver was placed in 10% formalin for about 48 h to be fixed before being processed for histological examination.

2.2. Histology

After fixing the samples (n=5) in 10% formalin for 48 h, they were embedded in paraffin blocks and cut into histological slices (5-μm thickness). No coloration was needed because the black spots of the macrophages were observed better without coloration. Pictures were taken with an optical microscope (Nikon Alphashot-2 Y52, 100× magnification) and a digital camera, Nikon CoolPix 4500, to evaluate the distribution of macrophages in the liver.

2.3. EPR spectroscopy

Samples of homogenized liver (12 μl) were put into a Teflon capillary tube (inner diameter: 0.65 mm). The Teflon tube was placed in a quartz tube open at both ends before being positioned in the EPR spectrometer cavity. An EMX X-band spectrometer from Bruker (Rheinstetten, Germany) was used. The spectrometer was working at 9.42 GHz and equipped with a variable temperature controller, BVT-3000. Measurements were made at 310°K and at 21% oxygen. The following spectrometer parameters were used: center field: 335.2 mT; sweep width: 3 mT; power: 12.7 mW; modulation frequency: 100 kHz; modulation amplitude: 0.1 mT; conversion time: 20.48 ms; time constant: 20.48 ms; number of points: 512; number of scans: 20.

2.4. Precision-cut liver slices

In an other set of mice, after India ink administration and excision of the liver as described, the different lobes of the

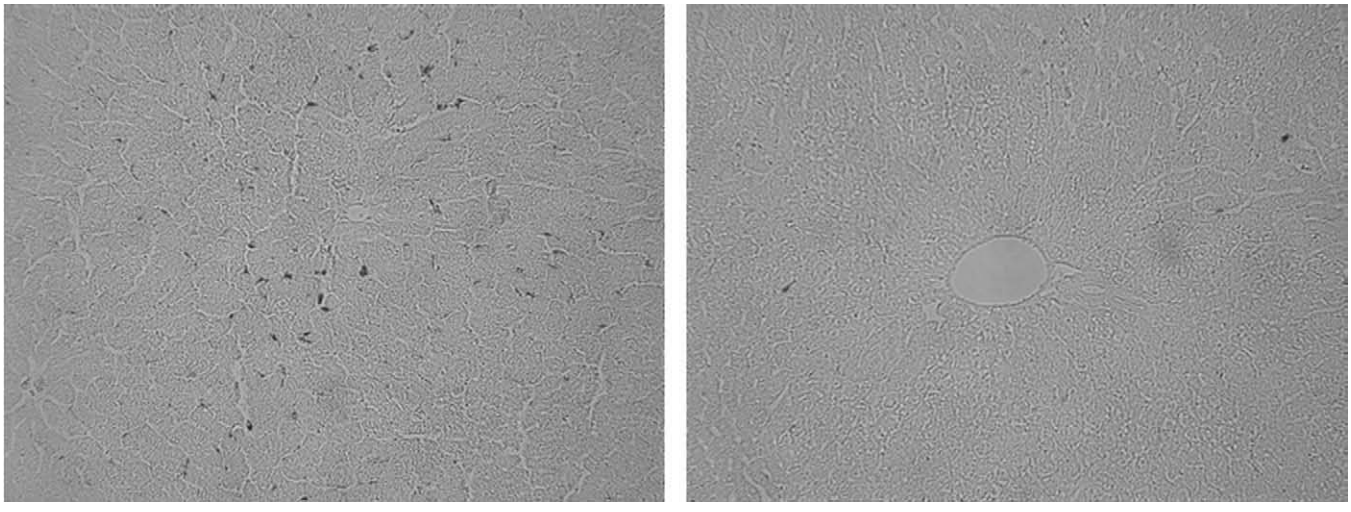

Fig. 1. Histological sections of liver after intravenous administration of India ink. (Left) Control rats. (Right) $GdCl_3$-treated rats. The dark spots correspond to the accumulation of India ink in Kupffer cells. Note the large decrease in the uptake of India ink in the liver of $GdCl_3$-treated rats.

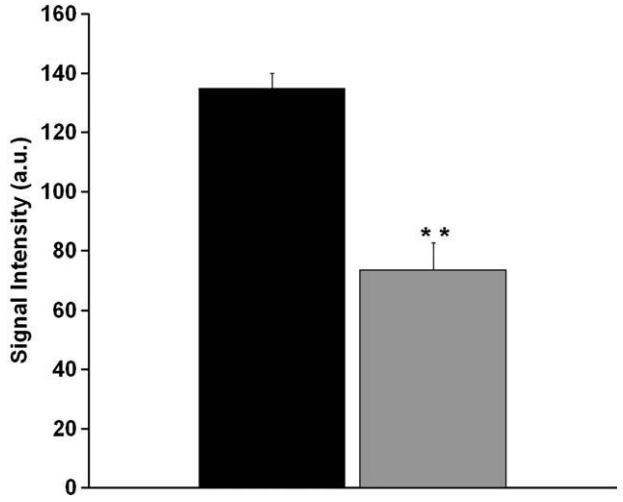

Fig. 2. EPR signal intensities (arbitrary units±S.E.M., n=5) recorded in liver homogenates after administration of India ink. The black column represents the control group; the grey column, the $GdCl_3$-treated group. Note the decrease in signal intensity in the treated group due to decreased phagocytic activity.

liver were separated. Using a hollow cylindrical-shaped headed drill, cylindrical sections were taken through the liver (diameter 8 mm). With the help of a Krumdieck tissue slicer, precision-cut liver slices (PCLS) were prepared in saline (thickness 0.5 mm) as previously described [27]. The PCLS where carefully cut into a rectangular shape (about 3/5 mm dimensions) to fit the EPR tissue sample cavity and stored in saline at −80°C. PCLS were used because of the ability to reproducibly obtain slices of the same thickness.

2.5. EPR imaging

The PCLS where placed in a tissue sample cell (Suprasil from Wilmad LabGlass, 5/10 mm cavity dimension, 0.5 mm deep). The tissue sample cell was then closed using a quartz plate and put into the Super High Q cavity of an Elexsys X-band spectrometer/imager from Bruker. To visualize the differences in India ink content, 2D spatial images were taken of PCLS from control and $GdCl_3$-treated rats. The following spectrometer parameters were used for the reference spectrum: center field: 350.08 mT; sweep width: 3 mT; power: 26 mW; modulation frequency: 100 kHz; modulation amplitude: 0.15 mT; conversion time: 10.24 ms; time constant: 20.48 ms; number of points: 512; scan: 35. Parameters for the 2D images were as follows: sweep width: 21.3 mT; Field of view: 20 mm; gradient: 4 mT/cm; pixel size: 0.5 mm; 31 projections. The sweep width of the image was chosen according to the following formula:

$$\Delta B = GL + \Delta \mathrm{Bo}$$

Where: ΔB is the minimal sweep width of the image to encompass the full probe function ; G is the gradient in Gauss; L is the field of view; and ΔBo is the sweep of the spectrum. ΔB should be at least 110 G or 11 mT.

3. Results and discussion

We first assessed the degree of Kupffer cell depletion after $GdCl_3$ treatment. Histological sections are shown in Fig. 1. On the left (control rats), the dark spots indicate the presence of Kupffer cells in the histological section. On the right ($GdCl_3$-treated rats), we can observe a large decrease in the number of Kupffer cells. These results are consistent with previously published data using similar protocol [23,24].

X-Band EPR spectroscopy was then used to evaluate the influence of the depletion in Kupffer cells on the intensity of the EPR signal recorded from liver homogenates; the results are shown in Fig. 2. The signal intensities (expressed in arbitrary units±S.E.M., n=5) were 134.8±5.2 and 73.4±9.2 for the control rats and $GdCl_3$-treated rats, respectively. This difference was highly significant (P<0.01; Student t test). These results indicate that the EPR signal intensity recorded in the liver after administration of India ink directly reflects the phagocytic activity of the liver. To determine in a more quantitative way, the amount of India ink accumulated in the liver was compared to calibration curves established with liver homogenates doped with India inks. We found out that about 95% of the administered India ink is taken up by the liver 20 min after administration. Due to the large accumula-

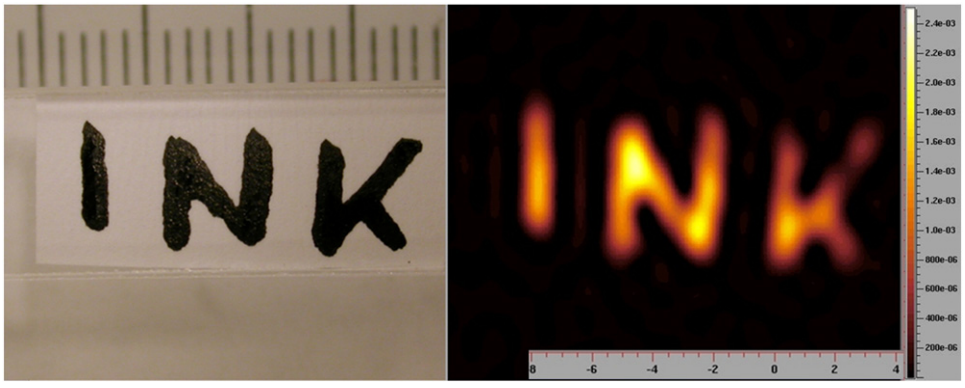

Fig. 3. Writing in ink on paper and its corresponding EPR image (on the right). Space between bars: 1 mm.

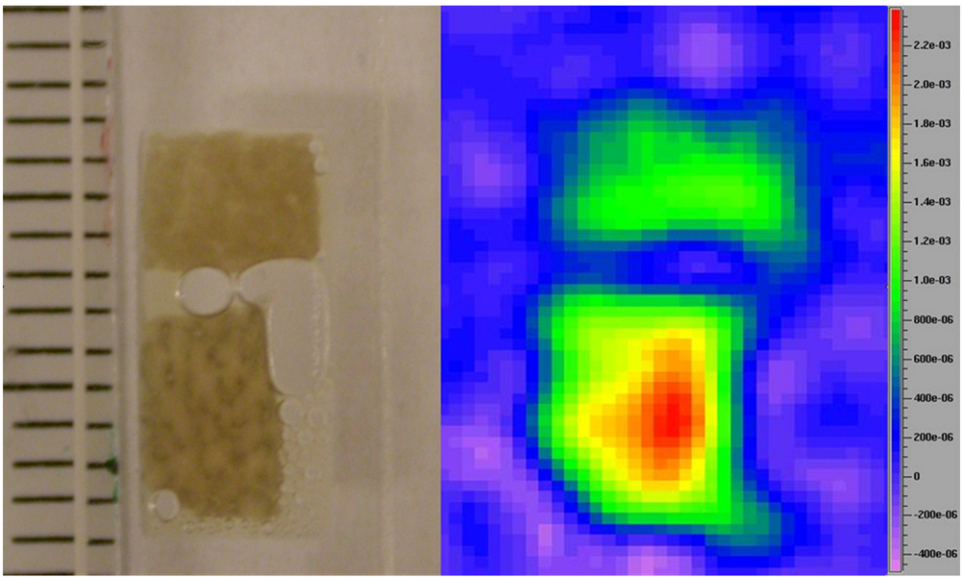

Fig. 4. EPR imaging experiment on PCLS from rats. (Left) Typical samples in the tissue cell cavity. PCLS of $GdCl_3$-treated rats were put horizontally in the upper part of the cavity and PCLS from the control group were placed vertically in the lower part of the cavity. (Right) Corresponding EPR image. Note the decrease in signal intensity in the PCLS from the treated group due to decreased phagocytic activity. Space between bars: 1 mm.

tion of India ink in the liver, the method was sensitive to situations with a decrease in Kupffer cells, as observed after depletion by $GdCl_3$. The 24-h period was selected because this time period allows maximal depletion of the Kupffer cell population, but it is likely that the technique could be applied to see early depletion as observed by Hardonk et al. [26]. However, the technique is probably unable to detect subtle increase in the number of macrophages in the liver. In another set of experiments, we failed to observe an increase in India ink accumulation after lipopolysaccharide treatment (data not shown). This is consistent with the fact that the liver is already saturated in inks in a normal liver, and a further increase in ink concentration in the liver is not detectable.

Finally, we investigated whether EPRI could reflect the accumulation of ink as seen in histology. India ink has not previously been used as a paramagnetic tracer in EPRI. Preliminary experiments carried out with ink writing on paper supported the feasibility of this approach (Fig. 3). The large EPR linewidth (approximately 0.8 mT) of the ink tested in the present study required application of large field gradients to image the samples. In our case, we applied a gradient of 4 mT/cm in order to discriminate two points at a distance of 1 mm from each other. We applied 2D EPRI on PCLS from control and $GdCl_3$-treated rats. Typical samples examined in the tissue cell EPR cavity are presented in Fig. 4 (on the left). PCLS of $GdCl_3$-treated rats were put horizontally in the upper part of the cavity and PCLS of the control group were placed vertically in the lower part of the cavity. A slight difference in color intensity can be observed between the control (darker PCLS) and treated (lighter PCLS) groups. Interestingly, the 2D EPR images (Fig. 4, right) correlate well with the accumulation of India ink related to the phagocytic activity of the liver.

In conclusion, our study indicates that the phagocytic activity of the liver can be assessed by EPR spectroscopy and imaging. For the proof of principle, we performed the study using a 9-GHz spectrometer/imaging system that offers a high sensitivity. The prospect for the future will be to apply this concept in vivo using low frequency EPR spectrometers and imaging systems in order to have a non invasive measurement of this phagocytic activity. The large EPR linewidth of the ink tested in the present study precludes detection in vivo because of the low signal-to-noise ratio. However, it is likely that this approach could be successful in vivo using paramagnetic nanoparticles that have a narrower linewidth, such as charcoals [28,29] or carbon blacks [18]. Another potential application of this concept would be to visualize the recruitment of macrophages in inflamed tissues.

Acknowledgments

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