Multimodal NMR assessment of Erythropoietin as a neuroprotective agent following Hypoxia-Ischemia on P3 pup rat brain

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Introduction:
The 3-day old rat (P3) shares some similarities in terms of cortical neuronal, glial and oligodendroglial development to the very preterm infant around 24-28 weeks of gestation. Animal models of periventricular leukomalacia (PVL) which is the most important cerebral alteration as a consequence of a premature birth can be achieved by Hypoxia-Ischemia (HI) as well as inflammation. Erythropoietin (EPO) has been shown efficient in inflammatory models of PVL [1] but has not been used in a very immature HI model. Here we investigated the neuroprotective effect of EPO in a model of neonatal HI injury in the P3 rat pup using high-field multimodal NMR techniques: T2W imaging, Diffusion Tensor Imaging (DTI) and Magnetic Resonance Spectroscopy (MRS) as well as immunohistochemistry.

Materials and Methods:
Animal preparation: P3 Wistar pups underwent moderate HI injury under isoflurane anesthesia. Right carotid artery catheterization was performed then after 30 minutes rat pups were kept under hypoxia for 30 minutes at 6% O2. Sh following HI, T2W images (actively shielded 9.4T/31cm magnet (Varian/Magnetx), 12-cm gradient coils (400mT/m, 120μs), quadrature transceive 17-mm surface RF coil) were performed to detect presence of injury. Injured pups were randomized into NaCl group: injected intraperitoneal with NaCl 0.9% (n=6) and EPO group (n=6) injected with intraperitoneal EPO 10U/g body weight/day during the first week after HI and 5U/g BW 3×/week until P25.

MRS: To study the effects of this chronic treatment, at P25, spectra acquisitions on a voxel of interest of 1.5×1.5×2.5 mm³ both within the cortical lesion (ipsilateral) and the contralateral cortical area were performed using an ultra-short echo time (TE/TR = 2.7/4000 ms) SPECIAL spectroscopy method [2]. 35 to 70 series of FIDs (12 averages each) were acquired, individually corrected for frequency drift, summed together and corrected for residual eddy current effects using the reference water signal. Proton spectra were analyzed with LCMODEL [3].

Ex-vivo DTI experiments were performed with a transceive 25-mm birdcage RF coil. Spin Echo sequence (FOV = 20 × 20 mm², matrix size = 128 × 64, in-plane pixel size = 156 μm, 20 slices of 0.8 mm thickness, 6 averages and TE/TR = 30/5000 ms) with addition of the Stejskal-Tanner diffusion gradients were used. Diffusion gradients (G_x= 22 G/cm, δ = 3 ms and Δ = 20 ms, b-value = 1659 s.mm⁻²) were applied along Dual diffusion gradient sampling scheme [4]. Diffusivity values (ADC, D⊥ and D∥) as well as FA were assessed in the genu of corpus callosum (GCC), external capsule (EC) and the superficial layer of sensorimotor cortex (SCx).

Immunohistochemistry: Anti-Glial fibrillary acid protein (anti-GFAP) staining was used to observe the formation of gliotic scar. Anti-Myelin basic protein antibody (anti-MBP) was used to determine the white matter injury.

Statistical analysis: a Wilcoxon and a Mann-Whitney test were used to compare statistically values between ipsilateral and contralateral side of the same group and between two groups respectively.

Results and Discussion:

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<th>Ipsi EPO vs.</th>
<th>Ipsi NaCl vs.</th>
<th>Ex-vivo DTI</th>
<th>Ex-vivo MRS</th>
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<td></td>
<td>Contra EPO</td>
<td>Contra NaCl</td>
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<td>Anatomy</td>
<td>Recovery</td>
<td>Abnormal</td>
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<tr>
<td>DTI</td>
<td>No * changes</td>
<td>FA ↑ EC Ipsi</td>
<td>FA ↑ SCx Ipsi</td>
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<td>MRS</td>
<td>[tNAA] and [Gln/Glu] ↓ Ipsi</td>
<td>No * changes</td>
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<td>Hist.</td>
<td>GFAP: no glial scar (in 80% of ? ) MBP: less altered</td>
<td>GFAP: glial scar</td>
<td>MBP: WM injury</td>
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Table 1: Summary of the results (*: p<0.05; ↑: increase; ↓: decrease)

Ex-vivo DTI: Anatomical and DTI images showed an anatomical recovery in the EPO group (fig. 1). FA values were found significantly lower in the corpus callosum of the NaCl group compared with EPO (0.66 ± 0.02 vs. 0.74 ± 0.03 respectively, p = 0.002). Indeed, in the NaCl group, FA values in the external capsule were significantly decreased in the ipsilateral side compared with contralateral (0.31 ± 0.02 vs. 0.39 ± 0.03 respectively, p = 0.031) whereas there were no differences in the same region for EPO group (0.39 ± 0.05 vs. 0.42 ± 0.06 respectively, p = 0.56). These results, correlated with MBP staining, provide evidence for protective effects of EPO in white matter (GCC and EC). In the sensorimotor cortex, FA values were found upper in the ipsilateral side compared with contralateral for the NaCl group (0.34 ± 0.04 vs. 0.29 ± 0.02 respectively, p = 0.031). According to GFAP staining, glial scar in the NaCl ipsilateral cortex could explain this increase [5]. In-vivo segmented DT-EPRI results at 14.1T on three rats were consistent with ex-vivo analysis and suggest a comprehensive multimodal in-vivo MR investigation of EPO following HI is feasible at ultra-high magnetic field.

MRS: in the EPO group MRS results showed significant decreases in the ipsilateral cortex compared with contralateral of [tNAA] (7.71 ± 0.68 vs. 8.70 ± 0.80 mM/g respectively, p = 0.031) and [Gln/Glu] (0.29 ± 0.06 vs. 0.42 ± 0.09 respectively, p = 0.031) (typical spectrum in fig. 2). [tNAA] decrease provides evidence for persisting neuronal damage in the ipsilateral cortex of EPO treated animals. The decrease in [Gln/Glu] is consistent with either impairment in glutamate neurotransmission or in glial function through e.g. impairment in glutamate synthesis. For MRS data analysis, there were no other significant differences (i.e. ipsilateral NaCl vs. contralateral NaCl, ipsilateral EPO vs. contralateral EPO vs. contralateral NaCl). In the NaCl group, the absence of significant differences in the metabolite concentrations between ipsilateral and contralateral cortex could be related to partial volume effect: due to abnormal development the ipsilateral cortex of NaCl animal as already reported (gliotic scar) [6]. 80% of EPO treated females presented no gliotic scar, with very few GFAP-positive cells present in the ipsilateral parietal cortex. Once again, this is true for females but not for males.

Conclusion:
On the P3 HI model, EPO appears able to reduce tissue loss (cell death) and white matter injuries but the area of ischemia retains compromised metabolism consistent with incomplete recovery from EPO. Multimodal NMR gives a new insight in the neuroprotective effect of EPO which could be highly relevant for neuroprotective strategies in preterm human neonates.


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