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: T_2W 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 cauterization was performed then after 30 minutes rat pups were kept under hypoxia for 30 minutes at 6% O_2. 5h following HI, T_2W images (actively-shielded 9.4T/31cm magnet (Varian/Magnex), 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 mm^3 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 mm^2, 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 was used. Diffusion gradients (G_{diff} = 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 $D_{\perp}$) 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:

	Ipsi EPO vs. Contra EPO	Ispsi NaCl vs. Contra NaCl
Anatomy	Recovery	Abnormal
DTI	No * changes	FA *↓ EC Ipsi FA *↑ SCx Ipsi
MRS	[tNAA] and [Gln]/[Glu] *↓ Ipsi	No * changes
Histo.	GFAP: no glial scar (in 80% of ?) MBP: less altered	GFAP: glial scar MBP: WM injury

Table 1: Summary of the results (*: $p<0.05$; ↑: increase; ↓: decrease)

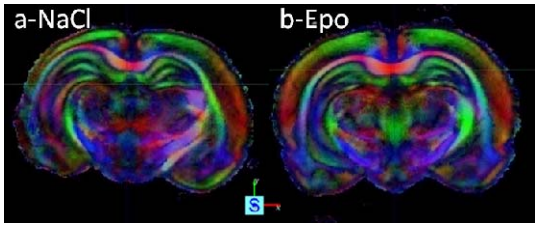

Figure 1: Direction encoded color maps of a typical rat pup brain 22 days following HI, with NaCl (a) and EPO (b) treatment. In term of anatomy, the recovery of the EPO rat is obvious.

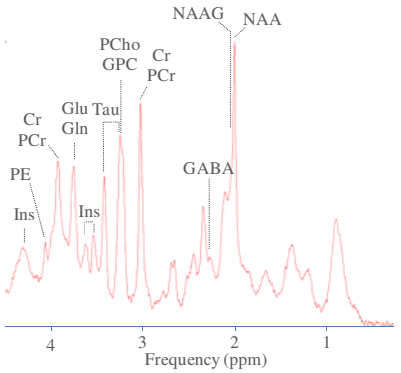

Figure 2: Typical spectrum in the contralateral cortex of an EPO P25 rat pup at 9.4T.

Ex-vivo DTI: Anatomic and DT 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-EPI 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. ipsilateral NaCl and 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 group is so thin (fig. 1, a) that it's difficult to measure spectra in the cortex without outer structure contaminations.

Immunohistochemistry: The effect of EPO is different between male and female. On qualitative assessment, MBP staining appeared less altered in the female of EPO group than in the ones of NaCl group, no obvious differences were observed in males. GFAP-positive staining was seen in ipsilateral cortex of NaCl animal as already reported (glial 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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