

# Lactoferrine supplementation following Hypoxia-Ischemia in the immature rat brain: macro-, micro-structural and metabolic assessment of the neuroprotective effect using multimodal MR

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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. The two main causes of brain lesion in the early preterm are ischemia and/or inflammation. As such, animal models of preterm brain injury can be achieved by Hypoxia-Ischemia (HI) [1]. Lactoferrin (Lf) is an iron-binding glycoprotein secreted in milk that has an antioxidant activity. In rodent after oral administration, Lf is rapidly transferred from the intestine into various organs including the brain. Lf is also synthesized in brains of both human suffering from Alzheimer's dementia and mouse model of Parkinson's disease, indicating that Lf potentially plays a role in neurodegenerative diseases. Lf has shown a neuroprotective effect in rat brain following prenatal exposure to glucocorticoids [2]. The aim of this work was to assess the neuroprotective effect of Lf in the very immature HI model. For this purpose a multimodal NMR protocol was performed at 9.4T: T<sub>2</sub>W imaging, MRS, DTI and Phase imaging.

**Materials and Methods:** At birth (P0), wistar rat pups were divided in 3 groups: one received Lactoferrin-enriched food (1 g/kg/day – HI-Lf group – n=6) *ad libitum* during lactation and the second had access *ad libitum* to a diet isocaloric to the Lf (HI-Iso group – n=6). The third group represents control rats fed with isocaloric diet (Sham group – n=3). P3 pups from HI-Lf and HI-Iso groups underwent moderate HI injury. Right carotid artery cauterization was performed followed by hypoxia for 30 minutes at 6% O<sub>2</sub>. The Sham group did not undergo any surgery and hypoxia. All MR experiments were performed on an actively-shielded 9.4T/31cm magnet (Varian/Magnex) equipped with 12-cm gradient coils (400mT/m, 120μs) with a quadrature transceive 20-mm surface RF coil. 5h following HI, Fast Spin Echo T<sub>2</sub>W images (TE/TR = 80/4000 ms; FOV = 15×15 mm<sup>2</sup>; matrix size = 256×256, 15 slices of 0.8 mm thick and 8 averages) were performed to detect presence of injury and to quantify the volume of the lesion. To study the effects of Lf, at P25, Fast Spin Echo T<sub>2</sub>W images were acquired (same parameter as P3 except the FOV = 25×25 mm<sup>2</sup>) for volumetric measurements and to position the MRS Volume Of Interest (VOI). Volumetric measurements were performed using BrainVisa/Anatomist [3]: percentage of injured cortex and percentage of cortical loss were calculated at P3 and P25, respectively. After automatic FASTMAP [4] shimming, spectra acquisition on a VOI of 1.5×1.5×2.5 mm<sup>3</sup> within the cortical lesion (ipsilateral for HI-Lf and HI-Iso groups) or the corresponding cortical area (Sham group) was performed using an ultra-short echo time (TE/TR = 2.7/4000 ms) SPECIAL spectroscopy method [5]. 26 series of FIDs (16 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 [6]. DTI acquisition was performed using a semi-adiabatic double spin-echo sequence [7] with the following parameters: Icosahedral 21 directions diffusion gradient sampling scheme ( $b = 1000 \text{ s.mm}^{-2}$ ), FOV = 23 × 15 mm<sup>2</sup>, matrix size = 128 × 64 zero-filled to 256 × 168, 8 slices of 0.8 mm thickness in the axial plane, 6 averages with TE/TR = 42/2000 ms. Diffusivity values (ADC, D<sub>||</sub> and D<sub>⊥</sub>) as well as fractional anisotropy (FA) were derived from the tensor and averaged in ROIs manually delimited in the external capsule (EC), internal capsule (IC), hippocampus (Hp) and cortex (Cx). Gradient-echo MR images (TE/TR = 16/900 ms; FOV = 23×23 mm<sup>2</sup>, Matrix size = 256 × 128 and 18 slices of 0.4 mm thickness and 8 repetitions) were acquired for phase imaging. To remove the effect of large-scale phase shifts across the phase images, ascribed to large-scale B<sub>0</sub> inhomogeneities, a 2D Gaussian high-pass filter with a kernel size of 65 voxels and a width of 5 voxels was applied to all images [8]. A Mann-Whitney test was used to compare statistically values between the different groups.

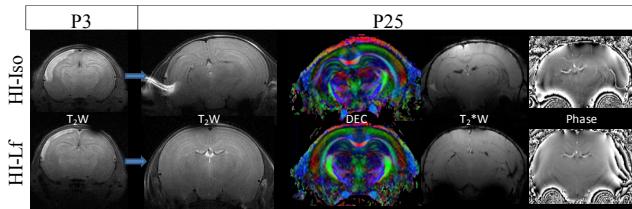
## Results:

**Severity of the injury (Fig. 1):** at P3, 51% and 61% of the pups were injured in the HI-Lf and HI-Iso group, respectively. From P3 to P25, 14% and 40% of the injured pups died in the HI-Lf and HI-Iso group, respectively. At P3 the percentage of injured cortex was equal to 4.9±3.6% vs. 15.0±7.1% (P=0.02) and at P25 the percentage of cortical loss was equal to 4.6±4.8% vs. 16.7±11.9% (P=0.09) for HI-Lf and HI-Iso, respectively.

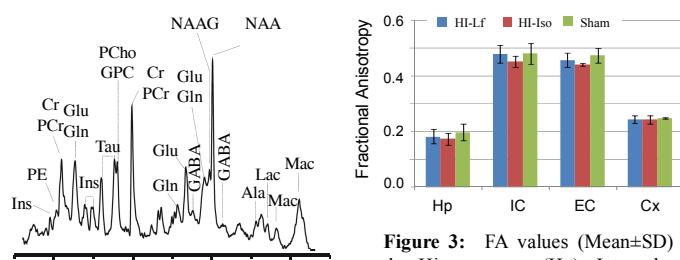
**MRS (Fig. 2):** very good spectral quality was achieved in the current study, as judged from water linewidth, obtained with FASTMAP ranging from 8 Hz to 10Hz. Due to very thin cortical structure in the rat pup brain, MRS was performed on a very small volume of 12 μl placed on the cortex. On the overall study SNR was equal in average to 20 ± 3. Such consistent data was subjected to spectral analysis and absolute quantification by LCModel provided the concentration of 17 metabolites defined as the “neurochemical profile”. Comparing HI-Iso ipsilateral and Sham corresponding cortices, significant decrease of [Asp], [NAA+NAAG] and [Glu+Gln] was observed. In the HI-Lf cortex, only [Glu+Gln] was found significantly lower than in the Sham cortex. Finally, [Tau] was significantly increased in the cortex of the HI-Iso group compared to HI-Lf.

**DTI (Figs. 1 and 3):** In IC and EC, FA values tend to be higher in the HI-Lf group than in the HI-Iso group and comparable to the one of Sham group. In Hp and Cx, HI-Iso group exhibited a tendency to reduced diffusivity values compared to Sham group (data not shown).

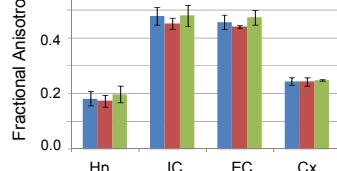
**Phase imaging (Fig. 1):** The Phase contrast showed different contrast in the external capsule of the HI-Lf and HI-Iso groups.



**Figure 1:** Typical evolution of pup rat brain from P3 to P25 for HI-Iso and HI-Lf groups. T<sub>2</sub>W images at P3: in hypersignal the lesion induced by the HI insult larger for the HI-Iso rat. At P25, T<sub>2</sub>W images (the cortical loss is larger for the HI-Iso group than for the HI-Lf group), Direction encoded color (DEC) maps show alteration of the white matter in the external capsule of the HI-Iso rat. T<sub>2</sub>\*W images used for Phase contrast: it appears more difficult to conclude even if the phase contrast seems different between the two rats.



**Figure 2:** Typical spectrum in the ipsilateral cortex of an HI-Lf P25 rat pup at 9.4T.



**Figure 3:** FA values (Mean±SD) in the Hippocampus (Hp), Internal and external capsule (IC and EC) and Cortex (Cx) of the HI-Lf, HI-Iso and Sham groups.

**Discussion and conclusion:** This study shows a characterization of the P3 HI model 22 days following insult by using multimodal NMR techniques: MRI, MRS, DTI and Phase imaging. Using multimodal NMR is very useful to characterize such a model with a compromised metabolism, cortical loss as well as white matter damage 22-d following HI on the P3 pup rat brain. As a result, these techniques allow an accurate assessment of the effects of a neuroprotective treatment such as Lf. Supplemented in the food, the Lf during the lactation seems to have a neuroprotective effect: the number of rats injured, the percentage of injured cortex at P3 as well as the percentage of cortical loss at P25 is reduced in the HI-Lf group. At P25, the metabolism of Lactoferrine supplemented rats is almost normalized and WM integrity seems also almost recovered, whereas it's not the case for the Isocaloric supplemented rats. To conclude, Lactoferrine seems to have a neuroprotective effect following HI in the P3 pup rat but the number of rats needs to be increased to confirm this effect. This result could be of high interest in the clinical field of preterm's brain neuroprotection.

**References:** [1] Kumral A. Neonatology 2007; [2] van de Looij Y. ISMRM 2010. [3] Rivière D. Neuroimage 2003; [4] Gruetter R. MRM 2000; [5] Mlynárik V. MRM 2006; [6] Provencher SW. MRM 1999; [7] van de Looij Y. MRM 2010; [8] Marques JP. Neuroimage 2009;

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