Neuroimaging in former preterm children who received erythropoiesis stimulating agents

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BACKGROUND: In premature children, erythropoiesis-stimulating agents (ESAs) may improve developmental outcome. It is not clear which of the several potential mechanisms are responsible for this improvement. High-resolution MRI and diffusion tensor imaging characterize brain structure and white matter organization, offering possible insight into the long-term effect of ESAs on brain development.

METHODS: MRI scans were performed at 3.5–4 years of age on former preterm infants treated with ESAs or placebo, and on healthy term controls. Mean cortical thickness, surface area, and fractional anisotropy (FA) were compared across study groups, and were correlated with general IQ measures.

RESULTS: Univariate analysis found no significant effect of ESAs on cortical thickness ($P = 0.366$), surface area ($P = 0.940$), or FA ($P = 0.150$); however, there was a greater increase in FA among ESA-treated girls. Group analysis found significant correlations between FA and Full-Scale IQ ($P = 0.044$) and Verbal IQ ($P = 0.036$), although there was no significant relationship between Full-Scale IQ and FA among just the preterm children.

CONCLUSION: ESA treatment may have a preferential effect on white matter development in girls, although factors other than just whole-brain FA are involved in mediating cognitive outcome.

Prematurity affects 15 million infants annually (1). Although survivability has improved in recent decades, premature infants still face major long-term sequelae including cerebral palsy, visual problems, and intellectual disability. Outcome for infants born weighing $<1{,}000$ g is particularly worrisome, with 71% of survivors experiencing some degree of neurodevelopmental impairment (2). Reducing this disease burden is an important public health issue throughout the world.

Recent work suggests that erythropoiesis-stimulating agents (ESAs) such as erythropoietin (Epo) and darbepoetin (Darbe) may provide neuroprotection after premature birth. ESAs are essential for normal brain development and augment a variety of potential neuroprotective mechanisms including promoting neurogenesis and angiogenesis (3,4) and inhibiting apoptotic, excitotoxic, and oxidative injury to neurons and oligodendroglia (5–11). ESAs may improve neurologic outcome of term infants with hypoxic ischemic encephalopathy (12), and appear safe in premature infants as well (13), decreasing major morbidity (14) and possibly improving cognitive outcome at 18–22 months (15), 3.5–4 years (16), and at 10 years (17), although some trials reporting no difference have been published (18,19).

Although accumulating evidence supports a role for ESAs in the management of premature children, it is not clear which of the several different mechanisms of action are responsible for beneficial effects in this clinical population. It would be helpful if dosing decisions or patient selection could target specific neuronal mechanisms. MRI might provide this information. High-resolution MRI and diffusion tensor imaging (DTI) demonstrate significant consequences of premature birth (20–30). Although recent neuroimaging reports suggest that ESAs given in the first few days of life to premature infants reduce brain injury at term (31,32), we are not aware of any studies that characterize neuroimaging at later ages, or those that integrate neuroimaging and cognitive outcome in children treated with ESAs.

Our recent work demonstrated improved cognitive outcome at 3.5–4 years of age in premature children treated with ESAs (16). Here, we report results of DTI and high-resolution MRI in this cohort. Our hypothesis was that ESAs would improve cortical structure and white matter organization reflected by a trend toward normalizing surface area, cortical thickness, and fractional anisotropy (FA). Furthermore, we predicted that these anticipated anatomic effects of ESAs would contribute to better cognitive outcomes.

METHODS

Participants

This investigation is part of a larger ongoing study of developmental follow-up after prematurity being conducted at the University of

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New Mexico. The initial study (NCT 00334737) enrolled preterm infants with 500–1,250 g birth weight at ≤48 h of age. Infants were randomized to one of the three groups: Epo, 400 U/kg, given three times a week; Darbe, 10 μg/kg, given once a week, with sham dosing two other times per week; or placebo, consisting of three sham doses per week. Dosing continued until 35 completed weeks of gestation. Details of methods and developmental outcome at 2 years have been published (15).

Children enrolled in the initial study were eligible for the BRITE (BRain Imaging and Developmental Follow-up of Infants Treated with Erythropoietin; NCT 01207778) follow-up study, performed at the University of Utah and the University of New Mexico. In addition, healthy children previously born term (TC) without hospital complications were enrolled at the New Mexico site. Institutional Review Boards approved the study at both sites.

Developmental Assessments

Developmental assessments were acquired at 3.5–4 years of age using the Wechsler Preschool and Primary Scale of Intelligence-III (WPPSI-III) (33) administered by certified examiners (J.L. and M.S.) masked to the treatment group. The WPPSI-III is standardized and normed for children at 2 years, 6 months through 7 years of age, and is a widely used scale of general cognitive abilities. Demographic measures were collected from parents. Results of developmental assessments at 3.5–4 years have been published (16).

Statistical Analysis

Rather than evaluating a very large number of specific regions with less statistical power, we focused on "global" neuroimaging variables, one each for cortical surface area, cortical thickness, and FA. Univariate analyses of treatment effects were conducted for each global imaging variable, and significant findings were followed up with more detailed analyses. As noted below, site of imaging acquisition and gender were included as covariates in all analyses. Statistical analyses were conducted across all three groups (placebo, ESA, and term controls) for descriptive purposes; our hypotheses of beneficial effects of ESA on the three brain variables were tested through three univariate general linear models. As each hypothesis was pursued through a single analysis, correction for multiple comparisons was not necessary.

MRI Data Acquisition

Structural T1 images were obtained from Magnetization-Prepared Rapid Gradient Echo sequences at both sites using a 3 T MRI scanner, and analyzed using the FreeSurfer data-processing program as previously described (25). FreeSurfer provides separate measures of volume, surface area, and cortical thickness for each anatomic region. In the analyses reported below, we focus on total cortical surface area and mean cortical thickness.

Diffusion images were acquired in New Mexico using a 30-gradient direction coil and 2 mm slice thickness, and at the Utah site using a 24-gradient direction coil with 3.4-mm slice thickness. Data were processed using the FSL software package (www.fmrib.ox.ac.uk/fsl). FA images were calculated and normalized to a template using a nonlinear registration algorithm (fnirt/FSL). A 50-region Johns Hopkins atlas was used to calculate mean FA values over 50 atlas-defined regions (34).

RESULTS

Participants

Seventy-seven participants were followed up as part of the BRITE study (16), of whom 67 provided at least some adequate imaging data sets at 3.5–4 years of age. The 10 scan failures were because of excessive movement. All 21 former preterm Utah subjects and 14 former preterm New Mexico subjects who did not fall asleep naturally were sedated with chloral hydrate. Because initial developmental testing revealed no significant differences between the Epo and Darbe groups, these children were combined into a single ESA-treated group. The final cohort consisted of 11 premature children treated with placebo (UNM male/female = 2/3, Utah male/female = 4/2), 33 premature children treated with ESAs (UNM male/female = 9/7, Utah male/female = 9/8), and 23 term born healthy control children. This was a subset of the same cohort of children who underwent full developmental assessment (reported in Ohls *et al.* (16)).

Table 1. Group characteristics for participants contributing imaging and cognitive data.

	Placebo ($N = 11$)		ESA ($N = 33$)		Term ($N = 23$)		Placebo vs. ESA	ESA vs. term
	Mean	SD	Mean	SD	Mean	SD	*P*	*P*
Age at testing (months)	48.55	3.04	48.91	3.83	45.09	2.13	0.77	<0.001
Gestational age at birth (weeks)	27.88	1.39	27.22	1.69	39.04	1.38	0.25	<0.001
Gender (M/F)	6/5	—	18/15	—	10/13	—	0.99	0.41
Full-Scale IQ	78.73	21.02	92.21	16.17	102.30	13.00	0.03	0.016
Income	3.73	1.74	4.76	2.14	5.00	1.66	0.16	0.64
Maternal education	4.27	1.10	4.82	1.24	5.36	1.36	0.20	0.13
Maternal age	24.09	3.83	27.82	6.68	29.45	7.46	0.09	0.40
Number of family moves	2.73	1.90	1.33	1.32	1.23	1.44	0.01	0.78
Number of children under 6	2.36	1.43	1.55	0.67	1.68	0.78	0.01	0.49
Ethnicity: Hispanic/Anglo	4/7	—	13/20	—	14/9	—	0.86	0.11
Primary language: English/Spanish	11/0	—	28/5	—	20/3	—	—	0.82
Socioeconomic composite (SEC)	−0.12	0.71	−0.02	1.09	0.05	0.23	0.77	0.81
Family stress composite (FSC)	0.99	1.08	−0.10	0.78	−0.20	1.00	0.001	0.65

ESA, erythropoiesis-stimulating agent.
Note: independent samples' *t*-tests and χ^2 analyses compared groups (though the χ^2 involving placebo group vs. ESA could not be calculated for primary language because one entry was zero). Higher SEC indicates greater income and maternal education. Higher FSC corresponds to more family moves, more young children at home, and younger mothers.

Demographic Data

Basic demographic information on each group is provided in Table 1. There were no significant differences between placebo and ESA groups in terms of age at testing, gestational birth age, or gender. Principal components analysis was used to reduce seven demographic variables into a "socioeconomic composite" and a "family stress composite". Higher scores on the socioeconomic composite indicated greater income and education, and higher scores on the family stress composite indicated more family moves, more children in the home, and younger maternal age. The placebo group had higher scores than the ESA groups on family stress composite ($P = 0.018$).

Imaging Data

Global imaging data are summarized in Table 2. Total surface area, mean cortical thickness, and FA are indicated. To reduce the set of 50 FA values for statistical analyses, we used principal components analysis and identified a single factor (termed "PC FA") with an eigenvalue >1, capturing 69.49% of total variance. In Table 2, FA values were expressed as *T*-scores (mean = 50 and SD = 10). Because this was a two-site imaging study, we evaluated whether imaging data were affected by scanner/site; univariate *t*-tests demonstrated that imaging site indeed did have a significant effect on mean cortical thickness ($P < 0.001$) and PC FA ($P < 0.001$), and therefore we covaried site in all statistical analyses.

Group differences in global imaging parameters

Separate univariate general linear model analyses were conducted for each imaging variable. Fixed effects were gender, group (placebo, ESA, and term), and site of image acquisition (NM vs. UT), whereas the two demographic factors served as covariates. Our initial analysis included all three groups. There was no significant effect of group for mean cortical thickness ($F(2, 54) = 1.917$, $P = 0.157$, partial eta squared = 0.066), or cortical surface area ($F(2, 50) = 0.844$, $P = 0.436$, partial eta squared = 0.033). However, there were significant differences in mean PC FA across the three groups ($F(2, 50) = 6.547$, $P = 0.003$, partial eta squared = 0.208), with the term group showing greatest PC FA. In a follow-up analysis, the term group PC FA was significantly greater than that in the ESA-treated preterm group ($F(1, 41) = 8.408$, $P = 0.006$, partial eta squared = 0.170 (Table 2).

Our second set of analyses evaluated the effect of ESAs on just the preterm children (ESA-treated vs. placebo). There was no significant group effect for mean cortical thickness ($F(1, 34) = 0.838$, $P = 0.366$, partial eta squared = 0.024), surface area ($F(1, 34) = 0.006$, $P = 0.940$, partial eta squared = 0.000), or PC FA ($F(1, 35) = 2.171$, $P = 0.150$, partial eta squared = 0.058). However, a gender by group interaction was found ($F(1,35) = 5.738$, $P = 0.022$, partial eta squared = 0.141). Figure 1 shows that ESA treatment increased PC FA in females more than males.

Specific White Matter Tracts in Females

We identified white matter tracts most affected by ESA treatment in females. Partial correlations were obtained between group (placebo vs. ESA) and each tract (averaging left and right hemisphere values for bilateral tracts to reduce the number of correlations examined), controlling for site, socioeconomic composite, and family stress composite. Those tracts showing the greatest increase in PC FA with treatment were as follows: cingulum ($r = 0.687$, $P = 0.003$), anterior corona radiate ($r = 0.628$, $P = 0.009$), superior longitudinal fasciculus ($r = 0.606$, $P = 0.013$), and the inferior fronto-occipital fasciculus ($r = 0.543$, $P = 0.030$). Figure 2 identifies the location of these fiber tracts most affected by ESA treatment in girls.

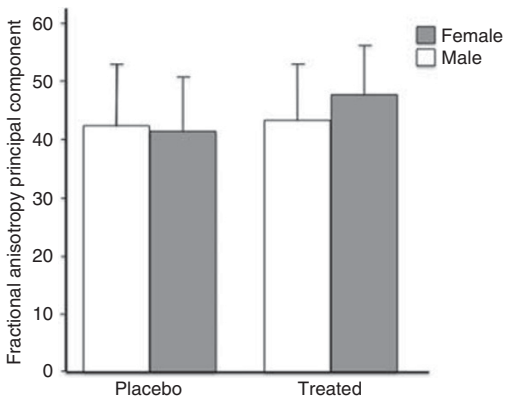

Figure 1. ESA treatment effect on fractional anisotropy by gender. Interaction of gender and group (placebo, treated) on global fractional anisotropy of white matter tracts. Fractional anisotropy principal component is expressed as a *T*-score (mean for entire sample, including term, was 50, SD = 10). ESA, erythropoiesis-stimulating agent.

Table 2. Global neuroimaging variables

	Placebo			ESA			Term		
	N	Mean	SD	*N*	Mean	SD	*N*	Mean	SD
Mean cortical thickness (mm)	10	2.94	0.20	32	2.87	0.22	23	3.01	0.08
Cortical Surface Area (mm^2)	10	15,9930.70	20,085.42	32	16,1542.47	21,385.91	23	16,0055.13	13,614.81
PC FA	11	42.18	9.38	32	45.49	9.62	18	58.80	5.35

ESA, erythropoiesis-stimulating agent.
Note: only high-quality data are reported; some participants did not provide data for each variable. PC FA is the first principal component of fractional anisotropy values for 50 white matter tracts, expressed as a *T*-score (mean = 50, SD = 10). See text for statistical data.

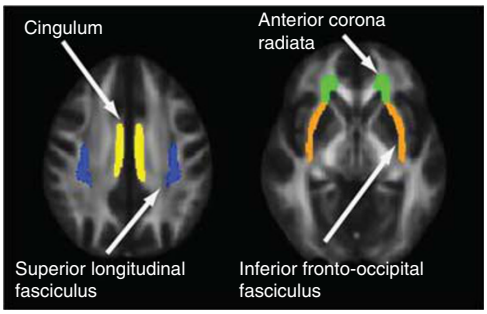

Figure 2. Fiber tracts most affected by ESA treatment in girls. The four fiber tracts most affected by ESA treatment in girls were cingulum, anterior corona radiata, superior longitudinal fasciculus, and inferior fronto-occipital fasciculus. Mean FA was calculated over each of these tracts as identified above. ESA, erythropoiesis-stimulating agent; FA, fractional anisotropy.

Neuroimaging and Cognition

Our prior publications on BRITE participants reported the beneficial effects of ESAs for Full-Scale IQ (FSIQ) (16) (see also **Table 1**). Given the above results regarding the effects of ESAs on PC FA, we examined relationships of PC FA with Full-Scale IQ, and also with the two components of FSIQ—Verbal IQ (VIQ) and Performance IQ (PIQ). All analyses controlled for site, gender, test age, and the two demographic variables. Across all participants in the three groups, the partial correlation of PC FA with FSIQ was $r=0.273$, $P=0.044$; for VIQ, $r=0.284$, $P=0.036$; and for PIQ, $r=0.219$, $P=0.108$. Among just the children born preterm (placebo plus ESA), the partial correlations were as follows: for FSIQ, $r=0.014$, $P=0.933$; for VIQ, $r=0.109$, $P=0.515$; and for PIQ, $r=-0.021$, $P=0.902$). Thus, although across all groups FSIQ and VIQ had a significant relationship with PC FA, there was no relationship among just the preterm children (placebo and ESA), suggesting that, for this group, the beneficial effects of ESAs on IQ were not directly mediated by PC FA.

DISCUSSION

This is the first study to evaluate neuroimaging at 4 years of age in premature children treated with ESAs. We previously found that early ESA treatment improves cognitive outcome. Our current results suggest that ESAs may improve white matter development, albeit only in females. Although greater PC FA was associated with higher IQ scores when all subjects were pooled together, there was no relationship when the preterm group was analyzed separately, suggesting that in preterm children, the beneficial effects of ESAs on IQ are driven by factors other than just a general improvement in white matter structure.

Preclinical work identifies multiple cellular actions of ESAs that may contribute to neuroprotection (for review, see Wu and Gonzalez (35)). Because these mechanisms affect changes in both gray and white matter, a single neuroimaging approach is unlikely to capture the complexity of an *in vivo* response to ESA intervention. Recent animal studies suggest that diffusion tensor imaging and MR spectroscopy may be more sensitive to ESA treatment effects than a general measure of cortical volume (36–39). This is consistent with our findings of no difference in mean cortical thickness or surface area between treatment groups, but with a trend toward increasing FA from untreated former preterm children (lowest), to ESA-treated former preterm children, to normal term-born children (**Table 2**).

In addition to demonstrating a trend toward improving white matter FA in ESA-treated children, we found an unexpected gender effect—ESAs increased FA in females more than that in males. The reason for this gender difference is not clear. This was not because of site because as noted above, our analyses included site, gender, and treatment group. FA is a measure of water diffusion, ranging from 0 to 1, with higher FA values in white matter generally felt to reflect greater axonal density, diameter, or myelination. Thus, FA closer to 1 presumably reflects greater white matter integrity. There is a suggestion in the preclinical literature of a gender effect—Wen *et al.* (40) published a study of stroke in neonatal rats demonstrating that females benefit from ESA treatment more than males do; however, this has not been replicated to our knowledge.

Interestingly, a gender effect has been noted in transfusion studies evaluating liberal vs. restrictive blood transfusion criteria. In a long-term study from the University of Iowa, liberally using blood transfusions in preterm children was associated with adverse neurocognitive outcome and reduced the brain volume, worse in females (41,42). The authors noted that erythropoietin levels were higher in infants receiving fewer blood transfusions, leading to a hypothesis that perhaps liberal use of blood transfusions delays the body's natural erythropoietin response, which reduces the accompanying neuroprotection associated with erythropoietin. Thus, the findings of McCoy *et al.* (41,42) and our data are consistent with what may be a greater female sensitivity to erythropoietin levels in premature infants. Further studies are required to replicate these findings and characterize the mechanism of action for any gender effect.

Recently O'Gorman *et al.* (32) reported an ESA-related increase in FA of several white matter tracts, but without a gender effect. Differences between our results and the results by O'Gorman *et al.* could be related to study design. O'Gorman used high-dose Epo (3,000 IU/kg) given three times over the first 2 days of life, whereas we treated with lower doses for a much longer period of time (through 35 completed weeks of gestation). Also, although our studies had comparable numbers of DTI data sets, we had fewer untreated subjects (32 treated/11 untreated) than those in the O'Gorman study (24 treated/34 untreated). Finally, a major difference was age at imaging. Both studies employed a 3 T MRI scanner, however O'Gorman *et al.* scanned at term, whereas our group performed MRI assessments much later, at 3.5–4 years of age. Longitudinal studies will be required to determine whether there is an age-related gender effect after ESA treatment.

Although we found that ESA treatment improved cognitive outcome and, separately, that it was associated with a trend toward increasing PC FA, the two findings were not statistically related. Thus, our hypothesis of a causal link between treatment, brain, and IQ was not substantiated. The hypothesis was based on an assumption that ESA treatment affects all areas of the brain, which might be expected to improve global FA and thus to increase IQ. This was a reasonable assumption, as a number of authors have already reported that global FA (43,44) and regional FA (45) are related to cognitive outcome in premature children. However, these were not treatment studies, and whether ESA therapy affects the FA/IQ relationship is not known.

After identifying a potential treatment effect in females, an exploratory analysis was performed just in girls that identified four fiber tracts with significant increases in FA related to ESA treatment. The superior longitudinal fasciculus (arcuate fasciculus) was one of these tracts. Others have reported reduced FA in the arcuate fasciculus as a result of prematurity, correlating with language skills (44). This raises the question of whether ESA neuroprotection might preferentially affect specific fiber tracts such as the arcuate fasciculus that might be at greater risk of injury related to prematurity.

Our study has several limitations. Low subject numbers limited our approach to data analysis, as we were unable to evaluate correlations between multiple brain regions and developmental outcome. Also, this was a two-site study, and because we found that scanner differences significantly affected data, all analyses were controlled for site. In addition, we report here results from a single time point. Age-related changes in cortical structure and white matter development have been reported in healthy populations, and recent work suggests that in at least one region, the corpus callosum, diffusion abnormalities present at term are less apparent by 7 years of age. It is not known whether similar accelerated imaging recovery occurs after ESA treatment.

Clearly, adequately powered, longitudinal studies will be necessary to disentangle the effects of age on regional brain development and cognitive outcome after ESA therapy. Our study is a step in this process. We note the importance of controlling for imaging site variables, and identify a potential gender effect of ESA therapy on white matter development. Although we were unable to identify a specific imaging variable associated with improved cognitive outcome, our findings suggest that white matter integrity, perhaps of specific tracts, may be more important than gray matter structure in mediating the ESA treatment effect.

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