Research Article

Intraventricular haemorrhage in preterm infants: investigating the pathways involved in astrogliosis to improve neurodevelopmental outcomes

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Abstract

Aims Preterm, low birthweight infants have an increased risk of developing intraventricular haemorrhage (IVH). Substantial haemorrhage can cause blood accumulation in the cerebral lateral ventricles, blocking cerebrospinal fluid (CSF) reabsorption. It can also stimulate migration of neural stem cells (NSCs) and inflammatory molecules into the CSF. This project aimed to generate data to therapeutically support and enhance the endogenous repair mechanisms of the infant brain post injury.

Methods Research at the Regenerative Medicine Laboratory has previously demonstrated that the accumulation of CSF in the lateral ventricles of the brain following IVH can redirect NSC differentiation towards astrocytes. In this study, human neural foetal progenitor cells (hfNPCs) were challenged with the CSF from the lateral ventricles of a baby post IVH (IVH-CSF; samples were collected from one patient at two different time points during therapy progression). hfNPCs were also challenged with IVH-CSF plus pharmacological inhibitors of potentially involved astrogliosis pathways. We screened for two distinct pathways, Janus kinase (JAK) and c-Jun N-terminal kinase (JNK), using their inhibitors (JAK inhibitor I and bosentan/SP600125, respectively). The role of glutamate in astrogliosis was also examined using the drugs ceftriaxone and riluzole. The differentiation profile of the hfNPCs was studied by immunochemistry (S100β in astrocytes, Tuj1 in neurons).

Results Treating hfNPCs with IVH-CSF resulted in a non-significant increase in nuclear and cytoplasmic S100β expression. Exposure of hfNPCs to IVH-CSF and pharmacological inhibitors can impede astrogliosis, although this was not statistically significant.

Conclusions These findings indicate that astrogliosis may reflect neuropathological responses in the resolution of IVH, which are responsible for the compromised neuronal development observed in these patients.

Introduction

Forty per cent of preterm infants (below 1000 g at birth) will develop intraventricular haemorrhage (IVH).[1] Consequently, blood accumulation in cerebral lateral ventricles can block cerebro-spinal fluid (CSF) reabsorption, leading to hydrocephalus. Hydrocephalus stimulates the migration of neural stem cells (NSCs) and inflammatory molecules into CSF.[2] CSF is routinely drained in babies with IVH to prevent a build-up in the cerebral lateral ventricles. Previous research from the Regenerative Medicine Laboratory showed that when neural progenitor cells are treated with CSF samples derived from infants affected by IVH (IVH-CSF), the presence of inflammatory markers, such as IL-6, IL-10, TNF-α, in the IVH-CSF redirects NSC differentiation towards reactive astrocytes. This is detrimental because severe astrogliosis together with glial scar formation may account for compromised neurodevelopment.[3] Furthermore, preterm infants with moderate-to-severe IVH (Grade 3–4) are at higher risk of developing cerebral palsy and severe learning difficulties.[4] Currently, treatment for premature infants suffering from IVH injury is supportive management, such as compressive head wrappings and CSF drainage, which carries its own risks. There is no available treatment to repair injury or improve neurodevelopmental outcomes.[5]

The aim of this study was to investigate whether IVH-CSF stimulates astrogliosis in human neural foetal progenitor cells (hfNPCs) in vitro and whether the response varies depending on whether cells are exposed to early CSF (collected 24 days post IVH) or late CSF (collected 132 days post IVH). Moreover, we hypothesised that blockade of two signalling pathways involved in astrogliosis, Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) and c-Jun N-terminal kinase (JNK), using their inhibitors (JAK inhibitor I and bosentan/SP600125, respectively) would decrease the differentiation of progenitor cells into astrocytes and, hence, reduce astrocyte reactivity.[6,7] Since IVH causes neuronal excitotoxicity by downregulation of glutamate transporters, which leads to neurodegeneration, we also investigated the involvement of glutamate in astrogliosis using the glutamate uptake-enhancer drugs ceftriaxone and riluzole.[8,9]

Methods

The method of hfNPC collection adhered to the recommendations set out by the Polkinghorne Committee (1989) and the UK Department of Health guidelines (1995). Ethical approval was in place for use of hfNPCs from the South Wales Initiative for Fetal Tissue (SWIFT) Research tissue bank in Cardiff (UK). The CSF samples in this study were excess samples of CSF fluid drained from the baby post-IVH as part of routine sampling, which was approved by parental informed consent.

hfNPCs, derived from foetal cortical tissue of terminated pregnancies from SWIFT donors in Cardiff, were treated with Accutase (Biolegend, San Diego, CA, USA). The cells were then grown to neurospheres

for 2 weeks in a growth supporting medium composed of Dulbecco's Modified Eagle Medium (DMEM) and F12 (3:1 DMEM to F12); GlutaMax (2%; ThermoFisher Scientific, Loughborough, UK), penicillin/streptomycin (1%; Sigma-Aldrich, Dorset, UK), B27 (2%; Life Technologies), the mitogenic growth factors human epidermal growth factor (EGF; 20ng/ml) and fibroblast growth factor (FGF; 20 ng/ml; Peprotech), and heparin (5µg/ml; Sigma-Aldrich). This growth medium was replenished twice a week and the cells were stored in a humidified incubator with a controlled environment of temperature of 37°C with 5% CO_2.

The single hfNPCs were harvested and seeded in a 96-well plate at 15,000 cells per well in 50 µl differentiating medium, which was composed of DMEM and F12 (DMEM to F12 at a ratio of 3:1); GlutaMax (2%), N2 (1%; ThermoFisher Scientific) and penicillin/streptomycin (1%; Sigma-Aldrich). CSF was collected 24 days ('early') and 132 days ('late') after IVH from one preterm infant.

After 7 days in the differentiation medium, hfNPCs were treated as follows: control; early CSF; late CSF; early CSF+drug; late CSF+drug; drug. Samples were challenged with CSF at a concentration of 20% . The concentration of experimental drugs used were based on published literature **(Table 1)**.[6-9] Each condition was run in triplicate.

Table 1. Details of the experimental drugs used to treat the hfNPCs.

Drug	Manufacturer	Concentration
JAK inhibitor I	Sigma-Aldrich	10 µM and 50 µM
Bosentan	Sigma-Aldrich	15 µM
SP600125	Sigma-Aldrich	10 mM
Ceftriaxone	Tocris	100 µM
Riluzole	Sigma	100 µM

After 7 days of differentiation, cells were fixed then fluorescent dye immunostained with primary antibodies **(Table 2)** for Tuj1 (neurons) and S100β (astrocytes) and counterstained with Hoechst stain (nuclei). Wells were imaged using automated high-content fluorescent microscopy using an IN Cell Analyzer (Cytiva, Marlborough, MA, USA) to quantify nuclear and cytoplasmic intensity in cells positive for S100β (green stain) and Tuj1 (red stain). The average nuclear and cytoplasmic fluorescent intensity values from each experimental condition was calculated to determine S100β or Tuj1 expression.

Table 2. Details of the antibodies used for immunochemical analysis of experiment.

Antibody	Species	Manufacturer
Primary antibodies		
S100β	Rabbit	Dako[a]
Tuj1	Mouse	Biolegend
Secondary antibodies		
Alexa Fluor 488	Rabbit	Invitrogen
Alexa Fluor 568	Mouse	Invitrogen
Nuclear staining		
Hoechst		Sigma-Aldrich

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Statistical analysis Using the GraphPad Prism statistical analysis programme (GraphPad Software, La Jolla, CA, USA), paired-sample *t* tests and one-way ANOVA were run to assess the significance of differences in nuclear or cytoplasmic expression of S100β or Tuj1 under the different conditions. A result of $p<0.05$ was considered statistically significant. The ratio of the difference between the mean of the two-sample sets and the variation between the sample sets are presented (t(2)); an increasing t(2) value from 0 indicates how the findings becomes progressively dissimilar from the null hypothesis, which could prove that IVH-CSF does have an effect on astrogliosis. The F-value evaluates the ratio of between-group variance and within-group variance (in this case each group is a different drug tested). The higher the F-value, the greater the variance between the means of S100β expression between each individual drug, more than the variance between the samples within each drug.

To analyse the effect of drug treatments, post hoc comparisons using Dunnett's test were conducted to assess the significance of differences between conditions.

Results

Nuclear and cytoplasmic expression of S100β was higher following treatment with early or late CSF when compared with control; however, the differences did not reach significance **(Figure 1)**.

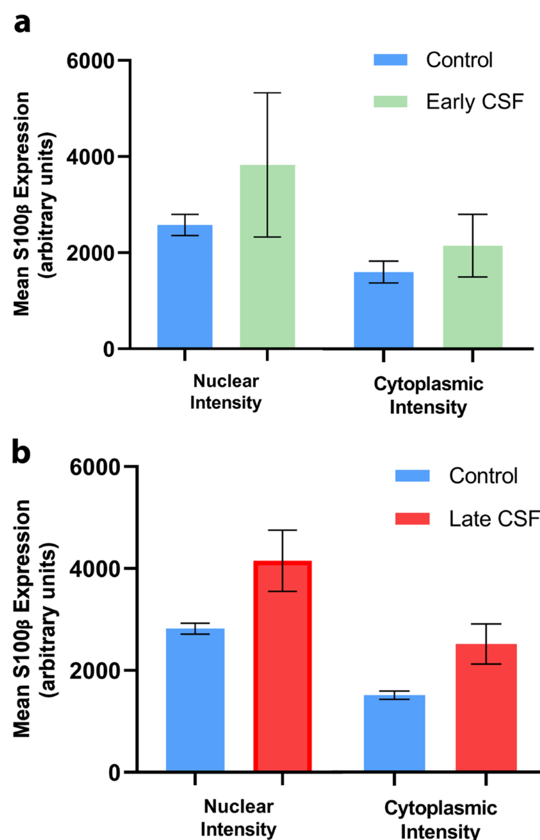

Figure 1. Nuclear and cytoplasmic S100β expression by astrocytes after hfNPCs were left to differentiate for 7 days with or without early or late CSF (20% concentration). Values are presented as mean±SD arbitrary units. **(a)** Evaluation of nuclear (paired-samples *t* test: t(2)=1.689, *p*=0.2332; *n*=3) and cytoplasmic (t(2)=1.230, *p*=0.3439; *n*=3) S100β expression in differentiated cells treated with early CSF. **(b)** Evaluation of nuclear (paired-samples *t* test: t(2)=3.273, *p*=0.0820; *n*=3) and cytoplasmic (t(2)=3.273, *p*=0.0820; *n*=3) S100β expression in differentiated cells treated with late CSF.

Treatment with JAK inhibitor I partially restored the increase in nuclear and cytoplasmic expression of S100β following exposure to early and late CSF; however, this did not reach statistical significance. Treatment with SP600125 resulted in loss of cell viability due to technical issues and so these results were excluded from the statistical analysis. Bosentan treatment non-significantly increased nuclear and cytoplasmic expression of S100β with early CSF and non-significantly decreased nuclear and cytoplasmic expression of S100β with late CSF treatment **(Figure 2)**.

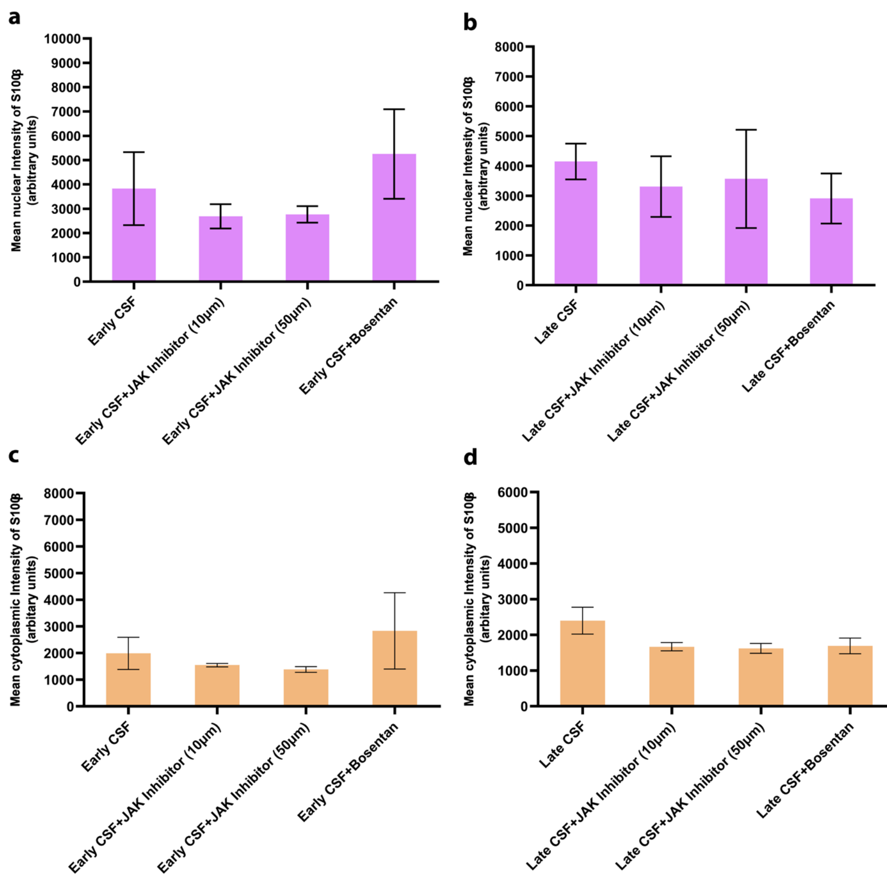

Figure 2. Nuclear and cytoplasmic S100β expression by astrocytes after hfNPCs were left to differentiate for 7 days with early or late CSF (20%), either alone or with the indicated drugs. Values are presented as mean±SD arbitrary units. **(a)** Evaluation of nuclear (one-way ANOVA: F(3,6)=2.401, *p*=0.2610; *n*=3) S100β expression in cells treated with early CSF and drugs. **(b)** Evaluation of nuclear (one-way ANOVA: F(3,6)=0.5438, *p*=0.6031; *n*=3) S100β expression in cells treated with late CSF and drugs. **(c)** Evaluation of cytoplasmic (one-way ANOVA: F(3,6)=1.672, *p*=0.3235; *n*=3) S100β expression in cells treated with early CSF and drugs. **(d)** Evaluation of cytoplasmic (one-way ANOVA: F(3,6)=6.344, *p*=0.1085; *n*=3) S100β expression in cells treated with late CSF showed no significant difference. Post hoc comparisons using Dunnett's test revealed no significant differences between conditions.

Tuj1 is a cytoskeletal protein expressed around the nucleus, but the IN Cell Analyzer segments the cell in such a way that there is an overlap in the staining. Hence the IN Cell Analyzer interprets this as a nuclear component. The perinuclear expression of Tuj1 decreased following treatment with early and late CSF with ceftriaxone **(Figure 3)** and riluzole **(Figure 4)**. Cytoplasmic expression of Tuj1 decreased with late CSF and ceftriaxone treatment; however, outcomes were not significant. Cytoplasmic expression of Tuj1 increased with early and late CSF with riluzole; however, the results were not significant.

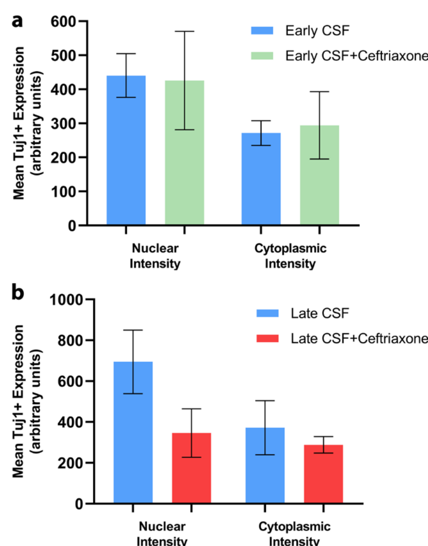

Figure 3. Nuclear and cytoplasmic Tuj1 expression by neurons after hfNPCs were left to differentiate for 7 days in early CSF (20%) alone or in early CSF plus ceftriaxone. Values are presented as mean±SD arbitrary units. **(a)** Evaluation of nuclear (paired-samples *t* test: t(2)=0.1879, *p*=0.8683; *n*=3) and cytoplasmic (t(2)=0.5845, *p*=0.618; *n*=3) Tuj1 expression in cells treated with early CSF and ceftriaxone. **(b)** Evaluation of nuclear (paired-samples *t* test: t(2)=2.578, *p*=0.1233; *n*=3) and cytoplasmic (t(2)=0.8461, *p*=0.4866; *n*=3) Tuj1 expression in differentiated cells treated with late CSF and ceftriaxone.

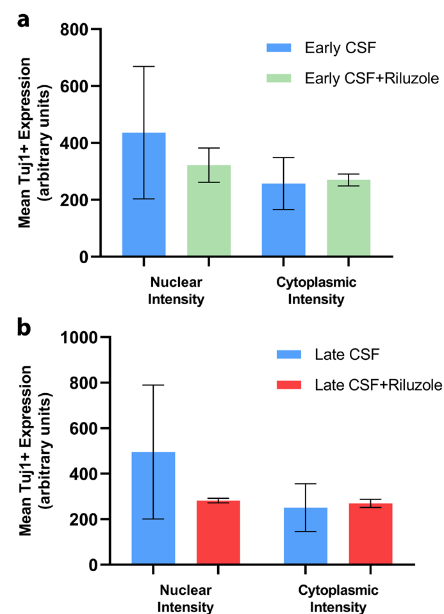

Figure 4. Nuclear and cytoplasmic Tuj1 expression by neurons after hfNPCs were left to differentiate for 7 days in early CSF alone or in early CSF plus riluzole. Values are presented as mean±SD arbitrary units. **(a)** Evaluation of nuclear (paired-samples *t* test: t(2)=0.7580, *p*=0.5276; *n*=3) and cytoplasmic (t(2)=0.1981, *p*=0.8613; *n*=3) Tuj1 expression in cells treated with early CSF and riluzole. **(b)** Evaluation of nuclear (paired-samples *t* test: t(2)=1.223, *p*=0.3459; *n*=3) and cytoplasmic (t(2)=0.2963, *p*=0.7949; *n*=3) Tuj1 expression in cells treated with late CSF and riluzole.

Discussion

Treating hfNPCs with IVH-CSF showed an increase in S100β expression. Although not significant, this increase could be explained by inflammatory cytokines, such as IL-6, IL-8 and TNF-α, which are present in IVH-CSF, directing differentiation of hfNPCs into reactive astrocytes.

Furthermore, the (non-significant) decrease in nuclear and cytoplasmic S100β expression observed with JAK inhibitor I treatment shows how JAK/STAT3 pathway inhibition might be able to impede astrogliosis following IVH. The inhibition of the JNK pathway with bosentan should have caused a decrease in S100β expression, but instead the outcome of the experiment showed an increase of S100β expression. Although this shows that JNK pathway inhibition can control astrogliosis, the unexpected outcome could be attributed to technical errors that exist from data being derived from the first experimental plate.

The results reported here were derived from one experiment. The statistical analysis shows that although trends could be identified, the results were not statistically significant. Hence, further experiments are required to determine whether the treatments had robust effects on astrogliosis. Future experiments should include more independent replicates to obtain significant findings. This study also highlighted the different effects of early and late IVH-CSF on astrocyte reactivity. Future investigations should study the composition of molecules in the CSF collected at the two different time points from infants with IVH. To increase the validity of the results, more CSF samples could be collected at different time points from different preterm infants with IVH.

Conclusion Preterm infants suffering from IVH can experience future neurodevelopmental complications, such as cerebral palsy. Hence, the underlying aim is to generate data to therapeutically support and enhance endogenous repair mechanisms of infant brain post injury. The observed findings of S100β expression suggest that IVH-CSF leads to astrogliosis, which could be rescued by JNK/STAT3 pathway inhibition. Future investigations should focus on modifying drug concentrations to confirm their ability to control astrogliosis.

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