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Anti-neutrophil antibody enhances the neuroprotective effects of G-CSF by decreasing number of neutrophils in hypoxic ischemic neonatal rat model

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Abstract

Objectives—Neonatal hypoxia ischemia (HI) is an injury that can lead to neurological impairments such as behavioral and learning disabilities. Granulocyte-colony stimulating factor (G-CSF) has been demonstrated to be neuroprotective in ischemic stroke however it has also been shown to induce neutrophilia, ultimately exacerbating neuronal injury. Our hypothesis is that coadministration of anti-neutrophil antibody (Ab) with G-CSF will decrease blood neutrophil counts thereby reducing infarct volume and improving neurological function post HI brain injury.

Methods—Rat pups were subjected to unilateral carotid artery ligation followed by 2.5h of hypoxia. Animals were randomly assigned to five groups: Sham (n=15), Vehicle (HI, n=15), HI with G-CSF treatment (n=15), HI with G-CSF+Ab treatment (n=15), and HI with Ab treatment (n=15). Ab (325μg/kg) was administered intraperitoneally while G-CSF (50μg/kg) was administered subcutaneously 1h post HI followed by daily injections for 3 consecutive days. Animals were euthanized at 96h post HI for blood neutrophil counts and brain infarct volume measurements as well as at 5 weeks for neurological function testing and brain weight measurements. Lung and spleen weights at both time points were further analyzed.

Results—The G-CSF treatment group showed tendencies to reduce infarct volume and improve neurological function while significantly increasing neutrophil counts. On the other hand, the G-CSF+Ab group significantly reduced infarct volume, improved neurological function and

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decreased neutrophil counts. The Ab alone group showed reversal of the neuroprotective effects of the G-CSF+Ab group. No significant differences were found in peripheral organ weights between groups.

Conclusion—Our data suggest that coadministration of G-CSF with Ab not only prevented brain atrophy but also significantly improved neurological function by decreasing blood neutrophil counts. Hence the neuroprotective effects of G-CSF may be further enhanced if neutrophilia is avoided.

Keywords

Granulocyte- colony stimulating factor (G-CSF); Anti-neutrophil antibody (Ab); Hypoxia-ischemia (HI); Neurological function; Neutrophil; Neonatal

Introduction

Hypoxia ischemia (HI) refers to the insufficient blood and oxygen supply to the brain that results in severe brain damage and the development of neurological impairments such as cerebral palsy; cognitive, behavioral, socialization and learning difficulties; seizures and encephalopathy. It is the main cause of mortality and morbidity in infants; affecting two to four of 1000 full-term births and nearly 60% of premature births (Bracewell and Marlow 2002; Ferriero 2004; Vannucci and Vannucci 1997; Volpe 2001). Current clinical treatments available such as anticonvulsants, therapeutic hypothermia, and fluid and electrolyte management, have proven only some degree of success (Koenigsberger 2000; Zanelli, et al. 2009), thus the necessity for alternative strategies to either replace or amplify the current therapeutic protocols.

Granulocyte – colony stimulating factor (G-CSF), a 20-kDa hematopoietic growth factor, stimulates survival, proliferation and development of neuronal stem cells and regulates maturation and survival of neutrophil granulocyte precursors (Roberts 2005; Schneider, et al. 2005; van Raam, et al. 2008). G-CSF has anti-apoptotic (Komine-Kobayashi, et al. 2006; Schabitz, et al. 2003; Schneider, et al. 2005) and anti-inflammatory (Gibson, et al. 2005) effects and has been shown to confer neuroprotection in a number of in vivo studies (Popa-Wagner, et al. 2010; Solaroglu, et al. 2009; Solaroglu, et al. 2006; Yata, et al. 2007). Rats treated with G-CSF tend to have lower infarct volumes, less brain tissue loss and improved long term neurological function (Beck, et al. 2003; Fathali, et al. 2010).

However, G-CSF has been identified as the main component in the generation of neutrophilic granulocytes and is in widespread clinical use for the treatment of neutropenia (Schabitz, et al. 2010). G-CSF in conjunction with HI further increases the upregulation of endothelial cell adhesion molecules which captures circulating neutrophils (Justicia, et al. 2003; Vemuganti, et al. 2004). Neutrophils aggregate into cerebral microvasculature leading to breakdown of blood flow and may worsen brain damage (del Zoppo and Mabuchi 2003; Stoll, et al. 1998). A number of studies have shown, both in adult and neonatal animal models of cerebral ischemia, that neutrophils accumulate within cerebral blood vessels and then extravasate into the brain parenchyma (Barone, et al. 1991; Garcia, et al. 1994; Matsuo, et al. 1994; Shiga, et al. 1991). There is evidence that neutrophils contribute to ischemic

injury in adult and neonatal animals as neutrophil depletion has been reported to be markedly protective (Heinel, et al. 1994; Hudome, et al. 1997; Matsuo, et al. 1994; Shiga, et al. 1991). No study to date has examined whether the neuroprotective effects of combined treatment with G-CSF and anti-neutrophil antibody (Ab) can translate into decreasing infarct volumes and brain tissue loss, associated with improvements in neurological function, or whether there is an additive benefit against systemic organ atrophy.

This study aims to investigate whether coadministration of G-CSF with Ab will amplify G-CSF's neuroprotective effects by reducing neutrophil accumulation in blood vessels and thereby significantly reducing brain atrophy and improving long term neurological function. To test this hypothesis we randomized P10 rat pups into five groups: sham, vehicle, G-CSF, G-CSF+Ab and Ab. Treatments were administered four times: 1h and for 3 consecutive days post HI. Infarct volume and neutrophil counts were measured at 96h post HI; Organ weights and brain atrophy at 5 weeks while neurological function was measured at 2 weeks and 5 weeks post HI.

Materials and Methods

All protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University. The animals were cared for in accordance with the Guidelines of the Committee. Sprague Dawley rat mothers, with litters of 10–12 pups (a mix of male and female pups), were purchased from Harlan Labs (Livermore, CA). A total of 75 P10 unsexed Sprague Dawley rat pups were used: 40 rats were euthanized at the 96h time point and 30 rats were euthanized at 5 weeks. Mortality rate was 5/75, which is 6%. The rat pups were divided into the following groups: sham (n=15), HI + Vehicle (n=15), HI + G-CSF (n=15), HI + G-CSF+ Ab (n=15) and HI + Ab (n=15). In the following experiments, the effects of the Ab alone treatment was not further tested in the long term neurological outcome studies as it did not alter the infarct volume compared to the vehicle group.

Hypoxia Ischemia model

The model that is described herein is the standard neonatal Hypoxia-Ischemia model (Rice-Vannucci Model (Rice, et al. 1981)). Ten days after birth, neonatal rat pups were placed into a temperature-controlled chamber for induction of general anesthesia. The animals were then exposed to 3% isoflurane gas in air for induction of anesthesia, and 1.5% isoflurane in air for maintenance of anesthesia. Throughout the surgical and postoperative period, temperature was controlled with heating blankets and incubators. After induction of anesthesia the neck of the pups was prepared and draped using standard sterile techniques. Next, a small midline neck incision on the anterior neck was made with a No. 11 blade surgical knife (approximately 3–5 mm in length). Using gentle blunt dissection the right carotid artery was isolated and gently separated from surrounding structures. The carotid artery was then ligated with 5-O surgical suture. All bleeding was controlled with gentle pressure and electrocautery as needed. The surgery was performed aseptically and the time taken per surgery was 5–9 min. After the surgical procedure was completed, the rats were allowed to emerge from anesthesia and recover for 1h. Thereafter, they were placed in a 500-ml airtight jar partially submerged in a 37 °C water bath to maintain a constant thermal

environment. A gas mixture of 8% Oxygen and 92% Nitrogen was delivered into the jars through inlet and outlet portals. The rat pups were exposed to this gas mixture for 2h 30min. Thereafter, animals were returned to their mothers.

Treatment method

Rat pups were allowed to rest for 1h on a warm blanket before initiating therapy. Human recombinant G-CSF (50 μg/kg) (Fathali, et al. 2010) (Amgen, Thousand Oaks, USA), Anti-Neutrophil antibody (325 μg/kg) (Friedrich, et al. 2011) (Accurate Chemical, Westbury, NY, USA), G-CSF and Ab together or Phosphate Buffered Solution (PBS)/Saline was administered subcutaneously or intraperitoneally. The first treatment was given at 1h post HI followed by the same treatment once per day for an additional 3 days (a total of four injections).

Infarct Volume Measurements

Animals were deeply anesthetized at 96h post HI for sacrifice. Brains were removed and sectioned into 2 mm slices and immersed into a 2% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC) solution (Fisher scientific, Waltham, MA, USA) at 37 °C for 5 min, followed by a 10% formaldehyde solution. The infarct volume was traced and analyzed by Image J software (NIH).

Blood sample collection

Animals were euthanised at 96h post HI for blood collection. Cardiac puncture was performed and about 500µl of blood was collected into EDTA tubes for neutrophil count measurements. Samples were then sent to Antech Diagnostics for analysis.

Lung Bleeding measurement

Lung bleeding was assessed at 5 weeks post HI. It is graded on a scale from 0 to 3, with 0 indicating no bleeding at all and 3 indicating bleeding in more than ¾ of the lung.

Immunohistochemistry

During deep anesthesia, pups were perfused transcardially with 0.1 M PBS followed by 4% formaldehyde solution (PFA) at 96h post HI. The brains were removed and postfixed (4% PFA, 4°C, 24 hrs), then transferred into a 30% sucrose solution for 2 days. The cryoprotected brains were sectioned at 10 µm thickness with a cryostat (Leica LM3050S) for double fluorescence staining and were observed under OLYMPUS BX51 microscopy.

The cryoprotected sections were washed with 0.1M PBS three times then incubated with blocking solution (10% normal goat serum, 0.1% Triton X-100 in 0.1 M PBS) for 1h at room temperature. Primary antibody, rabbit MPO (1:100, Santa Cruz) was applied (4°C, overnight). Sections were then washed with 0.1 M PBS and incubated for 1h with the secondary antibodiy (anti-rabbit IgG labeled with Alexa Fluor-488, 1:100, Jakson Laboratories Inc) at room temperature. Microphotographs were analyzed with the use of a fluorescent microscope and Magna Fire SP system (Olympus).

Western Blot

Western Blot was performed as described previously (Fathali, et al. 2013; Ostrowski, et al. 2005). Animals were euthanized at 96h post HI. After intracardiac perfusion with cold PBS (pH 7.4) solution, brains were removed and separated into ipsilateral and contralateral cerebrums instantly. Samples were snap-frozen in liquid nitrogen and stored at −80°C immediately until analysis. Whole-cell lysates were obtained by gently homogenizing in RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology, Inc., TX, USA) and further centrifuged at 14,000 g at 4°C for 30 min. The supernatant was used as whole cell protein extract and the protein concentration was measured by using a detergent compatible assay (Bio-Rad, Dc protein assay). Equal amounts of protein (50 μg) were loaded on a 10% SDS-PAGE gel. After being electrophoresed and transferred to a nitrocellulose membrane, the membrane was blocked with 5% non-fat blocking grade milk (Bio-Rad, Hercules, CA, USA) and incubated with the primary antibody overnight at 4°C. The primary antibody used was rabbit MPO (1:1000, Santa Cruz Biotechnology). Nitrocellulose membranes were incubated with secondary antibody (Santa Cruz Biotechnology) for 1h at room temperature. Immunoblots were then probed via ECL Plus chemiluminescence reagent kit (Amersham Bioscience, Arlington Heights, IL) and analyzed using Image J (4.0, Media Cybernetics, Silver Springs, MD).

Neurobehavioral Tests

The following neurobehavioral tests were performed in a blinded setup at 5 weeks post HI. Neurological function was evaluated using modified Garcia, T-maze, foot- fault, rota rod and watermaze.

Briefly, the *Modified Garcia* test (Garcia, et al. 1995) is a sensorimotor assessment system consisting of seven tests with scores of 0 to 3 for each test (with 0 being the worst score and 3 the best; maximum score=21). These seven tests included (1) spontaneous activity, (2) side stroking, (3) vibrissae touch, (4) limb symmetry, (5) climbing, (6) lateral turning, and (7) forelimb walking. Total scores were recorded.

T-maze—Prior to sacrifice at 5 weeks post HI, rats were tested for spontaneous alternation on a T-shaped maze (Matchett, et al. 2007). The T-maze measured 40 (stem) x 46 (arm) x 10 (width) cm. Rats were placed in the stem of the T-maze and allowed to freely explore the two arms of the maze, throughout a 10-trial continuous alternation session. Once an arm was chosen, the rat was placed in the stem of the maze again, and the trial repeated. Absolute numbers of left and right choices were recorded, and the spontaneous alternation rate calculated as the ratio of the alternating choices to the total number of choices.

In the *foot-fault test* rats were placed on a horizontal grid floor (square size 28 x 3 cm, wire diameter 0.4 cm) for 2 min (Barth and Stanfield 1990). Foot-fault was defined as when the animal inaccurately placed a fore- or hindlimb and fell through one of the openings in the grid. The number of foot-faults for each animal was recorded.

Rota Rod test assessed motor impairment using an accelerating rotarod (Columbus Instruments Rotamex, OH, USA). The mean duration (in seconds) on the device was recorded as the average of three rotarod trials (Sayeed, et al. 2007).

Water maze test—evaluated the ability to learn spatial locations and memory (Hartman and Warren 2005). This test requires the rats to find a hidden (submerged) platform in a pool of water using visual cues in the room. The animals participated in both cued and hidden tests. All trials lasted a maximum of 60 sec, at which point the rats were manually guided to the platform if needed. All activities were recorded and the animals' swim paths were measured for quantification of distance, latency, and swimming speed by the Video Tracking System SMART-2000 (San Diego Instruments Inc., CA).

Statistical Analysis

All data were expressed as mean $+/-$ SEM. Statistical differences between two groups were analyzed using the two-sided t-test with unequal variances. Multiple comparisons were statistically analyzed with one-way analysis of variance followed by Tukey multiple-comparison post hoc analysis or Student-Newman-Keuls test on ranks using SigmaPlot 10.0 software. A P value of $p < 0.05$ was considered statistically significant.

Results

Coadministration of G-CSF with Ab reduced infarct volume and neutrophil counts at 96h post HI

The vehicle group showed 31% infarction in the right hemisphere, which was reduced to 19% in the G-CSF+Ab treatment group (figure 1A; $p < 0.004$, 30.957 ± 1.564 vehicle vs 18.746 ± 2.983 G-CSF+Ab). The G-CSF alone group (figure 1A; $p < 0.116$, 30.957 ± 1.564 vehicle vs 21.693 ± 4.692 G-CSF), showed a tendency to reduce infarction but significance was not reached. Finally, the Ab alone treatment group (figure 1A; $p < 0.733$, 30.957 ± 1.564 vehicle vs 30.275 ± 1.034 Ab) had the same infarct percentage as the vehicle group.

Blood neutrophil counts were significantly increased by G-CSF administration when compared to sham (figure 1B; $p < 0.001$, 0.32 ± 0.285 sham vs 0.44 ± 0.36 G-CSF), while G-CSF +Ab and Ab groups had significantly decreased neutrophil counts in the blood when compared to G-CSF and vehicle ($p < 0.001$, 0.02 ± 0.02 G-CSF+Ab vs 0.08 ± 0.05 Ab vs 0.44 ± 0.36 G-CSF vs 0.4 ± 0.27 vehicle).

Brain neutrophil counts were also significantly increased by G-CSF treatment compared to all other groups ($p < 0.05$, 1.8 ± 0.185 G-CSF vs 1 ± 0.06 sham, 1.082 ± 0.09 vehicle, 1d. 097 ± 0.13 G-CSF+Ab) as seen from Western Blot analysis (figure 1C). Immunohistochemical staining showed similar trends to Western Blot as seen from the representative pictures (figure 1D).

Treatment reduced lung injury post HI

The spleen weight in the vehicle group was significantly lower than the sham group at 5 weeks after HI ($p < 0.05$, $0.541 +/- 0.0273$ Vehicle vs. $0.649 +/- 0.0252$ Sham); however there

was no intergroup difference found at 96h and 5 weeks when the spleen weight was normalized to the body weight as the spleen to body weight ratio (figure 2A). There was no significant difference found in the lung weights at 96h and 5 weeks, however the lung to body weight ratio was higher in the vehicle group than the sham group at 5 weeks ($p<0.05$, 0.00840+/−0.000717 Vehicle vs. 0.00637+/−0.000436 Sham) after HI. The increased lung to body weight ratio was reduced by both treatments ($p>0.05$, treatment groups vs. Sham) (figure 2B).

Figure 2C shows that animals in the vehicle group had more lung bleeding compared to sham or treatment groups. The graph represents the severity of bleeding ranked on a score from 0 to 3, with 0 showing no bleeding (as seen in sham) and 3 having more than ¾ of lung bleeding (as seen in vehicle). G-CSF treatment group showed significantly reduced bleeding when compared to vehicle ($p<0.05$, 1±1 G-CSF vs. 3±2.25 vehicle) while G-CSF+Ab group showed a tendency for improvement but significance was not reached.

Combinational treatment of G-CSF with Ab reduced brain atrophy and improved physical development at 5 weeks post HI

HI injury caused severe brain atrophy of the lesioned hemisphere ($p<0.001$, 107.946±0.791 sham vs 64.071±5.061 vehicle, % of ipsi-/contra-lateral brain weight) as seen in figure 3A (arrows represent the injured side). Rats injected with G-CSF+Ab showed significantly less brain tissue atrophy when compared to the vehicle group ($p<0.001$, 76.699±4.545 G-CSF +Ab vs 64.071±5.061 vehicle, % of ipsi-/contra-lateral brain weight).

Figure 3B shows the difference in physical development (body weight) between rats injected with vehicle (PBS), G-CSF and G-CSF+Ab at the completion of the four-day treatment period. At 35 days post HI, vehicle rats (n=8) had gained less weight (n=8) ($p<0.05$, compared to sham, 202.429±13.688 vehicle vs 236.714±12.192 sham, body weight) whereas body weight was augmented by G-CSF and G-CSF+Ab (n=9) treatment ($p<0.05$, compared to vehicle, 222.629±7.053 G-CSF and 230.125±16.355 G-CSF+Ab). Furthermore, animals treated with G-CSF+Ab showed increased weight gain than animals treated with G-CSF only ($p>0.05$).

Combined G-CSF and Ab improved long-term neurological function at 5 weeks post HI

In order to test the effects of G-CSF and G-CSF+Ab treatment on the neurological impairments induced by HI, neurological function was assessed using modified Garcia test, T maze, foot-fault, rota rod and watermaze at 5 weeks post HI. In all behavioral tests, animals in the vehicle group performed significantly worse than sham operated rats ($p<0.05$). In watermaze, G-CSF+Ab treatment group showed significantly improved neurological function while the G-CSF alone group only showed a tendency to improve behavior but significance was not reached (figure 4A). In addition, the combinational treatment group also had significantly improved sensorimotor coordination as assessed by rota rod (figure 4B) and foot-fault tests (figure 4C) ($p<0.05$, compared to vehicle). In the T Maze and modified Garcia test no differences were seen among any of the groups (figure 4D and 4E).

Discussion

In the present study we tested whether G-CSF+Ab combinational therapy could enhance the neuroprotective effects of G-CSF by decreasing neutrophilia and thereby resulting in further prevention of brain atrophy and improved neurological function in a rat model of neonatal HI. We found that G-CSF+Ab treatment was more effective than G-CSF alone treatment in the HI rat pup model. Combinational treatment further improved body weight, reduced brain tissue loss and decreased neurological deficits following HI. These findings provide clinically relevant evidence for the potential development of new therapeutic strategies against HI-induced brain injury in neonates.

The current study focuses on a major bone marrow derived cytokine, G-CSF, and its effects when combined with anti-neutrophil antibody (Ab). G-CSF is known to stimulate the survival, proliferation and differentiation of bone marrow cells including endothelial cells and neutrophilic granulocytes (Broudy, et al. 1994; Hess, et al. 2002; Hess, et al. 2004; Kocher, et al. 2001; Powell, et al. 2005; Takamiya, et al. 2006). It is one of the most studied growth factors in the setting of stroke as it can cross the blood brain barrier (BBB) (Zhao, et al. 2007). Clinically, increasing evidence has shown the therapeutic potential of G-CSF in patients with ischemic stroke (Shyu, et al. 2006; Sprigg, et al. 2006).

As the main hematopoietic growth factor, G-CSF has the ability to recruit new neutrophils from the bone marrow while simultaneously delaying apoptosis of mature neutrophils (Roberts 2005; van Raam, et al. 2008). Hence neutrophil accumulation results, which can exacerbate brain injury through vessel blockage, enhancement of vascular permeability, parenchymal injury by hydrolytic enzyme release, lipid mediator synthesis, and active oxygen species production (Matsuo, et al. 1994). A number of studies have reported that neutrophil depletion can reduce brain injury after stroke (Lees, et al. 2003; Matsuo, et al. 1994; Shimakura, et al. 2000). According to those studies, we believe that the depletion of neutrophils results in the reduced release of chemical mediators in the ischemic area and lessens the damage related to the no reflow phenomenon, thus suppressing post ischemic brain injury. Therefore, it is of particular interest to study the effects of the combinational treatment of G-CSF with Ab on neurological function, brain injury and systemic organ protection. We tested this concept by administering G-CSF alone, Ab alone, and a combination of G-CSF+Ab.

The main outcome of HI is brain damage and permanent tissue loss. While G-CSF reduced infarct percentage by approximately 10% at 96h post HI (no significance reached), the G-CSF+Ab treatment group significantly reduced infarction by about 15% compared to vehicle (figure 1A). We believe that significance was not reached in the G-CSF group due to two reasons: 1) The injury model was too severe: previous studies have reported that G-CSF reduced infarction when administered subcutaneously at a dose of $50\mu g/kg$ (the same dose as used in this study) (Fathali, et al. 2010; Matchett, et al. 2007), but, these studies induced overall infarct volumes of approximately 20%. For this study we have developed a model that yields over 40% infarction when measured at 48h post HI (data not shown) and over 30% at 96h post HI (figure 1A). Therefore, the dose used only showed tendencies but no significance as infarct volume was too severe compared to other studies. 2) G-CSF

upregulates neutrophils (explained below) which contribute to brain injury, hence explaining why G-CSF+Ab group had less infarction compared to the G-CSF group. Similar results were seen at 5 weeks where the percentage of ipsilateral/contralateral hemisphere weight was significantly increased in the combinational treatment group (figure 3A). However, Ab alone treatment group had similar infarct volume percentage compared to vehicle group and therefore was not further tested in the long term neurological outcome studies. At the same time we saw that neutrophil counts were significantly increased in the G-CSF group (as previously shown (Matchett, et al. 2007)) and were reduced in the G-CSF+Ab and Ab alone groups in blood (figure 1B). To further support our hypothesis and the results mentioned above, MPO levels (marker for neutrophils) were measured in the brain using Western Blot and immunohistochemical analysis. The Western Blot data and immunohistochemistry pictures clearly show that G-CSF treatment group significantly increased neutrophil counts in the brain while the coadministration group reversed the neutrophilia to nearly similar levels as the sham group (figure 1C and 1D). Our data show that neutrophil counts were increased both in the peripheral blood and brain after G-CSF treatment and reduced after G-CSF+Ab treatment; hence the results suggest that depleting neutrophils while simultaneously treating with G-CSF confers greater neuroprotection than treatment with G-CSF alone.

One of the major consequences of HI is growth retardation, which can be used as an indicator for general well being (Kim, et al. 2008). From the body weight data in the present study, it is evident that injured animals had lower weight gain compared to sham animals at 35d post HI while G-CSF treatment groups reversed that effect. However, the combinational treatment group further improved weight gain outcome (Figure 3B). This clearly shows that G-CSF+Ab treatment improves physical development during the critical period following brain injury.

At 5 weeks post HI, the absolute spleen weight in the vehicle group was lower than that of the other groups. Along with the reduced spleen weight, there was also reduced body weight. Consequently, there was no significant difference in the spleen to body weight ratio between groups, suggesting the smaller spleen observed in the vehicle group resulted from HI-induced growth retardation, which is consistent with our previous findings (Fathali, et al. 2010). It is known that HI may also cause injury to various peripheral organs such as the kidney, liver, and lungs (Agarwal, et al. 2008). In this study, we examined the lungs and found that vehicle operated rat pups had severe lung bleeding, an observation consistent with the high incidence of pulmonary hemorrhage reported clinically in preterm infants (Lodha, et al. 2011). Although the cause of lung bleeding after HI is not entirely clear, it is possible that hypoxia increases pulmonary vascular permeability (Dehler, et al. 2006) and pulmonary microvascular pressure (Swenson, et al. 2002), resulting in edema and bleeding. Interestingly, G-CSF treatment was found to significantly reduce lung bleeding after HI. Further investigation will be needed to determine the mechanisms of this effect from G-CSF.

The bleeding in the lungs might have led to the increased lung weight as shown by the lung to body ratio (figure 2B). Treatment reduced lung weight as the treated groups show no significant difference from the sham group (p>0.05, treatment groups vs. Sham).

Aside from promoting weight gain, G-CSF+Ab resulted in less brain atrophy and improved motor performance. The combinational treatment group was shown to prevent the long-term loss of brain tissue. The hippocampus and the sensorimotor cortex are critical for regulation of sensorimotor function, learning and memory which are highly affected by HI (Spandou, et al. 2005). As already known, damage to those regions causes severe deterioration in functional performance. According to watermaze, rota rod and foot-fault test, G-CSF treatment group alone showed a tendency to improve functional recovery, however, significance was not reached. On the other hand, the combinational treatment group (G-CSF +Ab) showed significant improvements in neurological function (figure 4), again confirming our hypothesis that administering Ab in conjunction with G-CSF enhances G-CSF's neuroprotective effects even in more severe cases.

In this study we demonstrated that G-CSF+Ab improved body weight, reduced brain tissue damage and improved long term neurological function when assessed at 96h and 5 weeks post HI in the neonatal rat pup. Consistent with previous studies, we demonstrated that HI increases neutrophils in the blood and G-CSF further exacerbates this effect thus contributing to brain damage as no significant neuroprotection was reached in any of the neurobehavioural tests with G-CSF. However, combining G-CSF with Ab resulted in neutrophil depletion and yielded neuroprotection in all of our outcome studies and behavioral tests. Thus, our results suggest that G-CSF+Ab further improved G-CSF's neuroprotective effects by decreasing neutrophil accumulation in vessels.

Overall, the above findings from this study are clinically relevant and provide the foundation for exploring these treatments for clinical translation. Both G-CSF and Ab are attractive candidates for therapeutic treatment as they have minimal side effects such as bone or musculoskeletal pain, anemia, thrombocytopenia and injection site reactions (Hubel and Engert 2003a; Hubel and Engert 2003b). They both showed neuroprotective properties, specifically attenuating long term brain damage and improving long term neurological function. Our results illustrate the synergistic treatment effect of G-CSF with Ab, which was able to further enhance G-CSF's neuroprotective effects and confer greater neuroprotection by depleting neutrophil accumulation.

Acknowledgments

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Highlights

1. G-CSF+Ab treatment was more effective than G-CSF alone treatment after HI.

2. G-CSF+Ab reduced infarct volume.

3. G-CSF+Ab prevented brain atrophy and significantly improved neurological function.

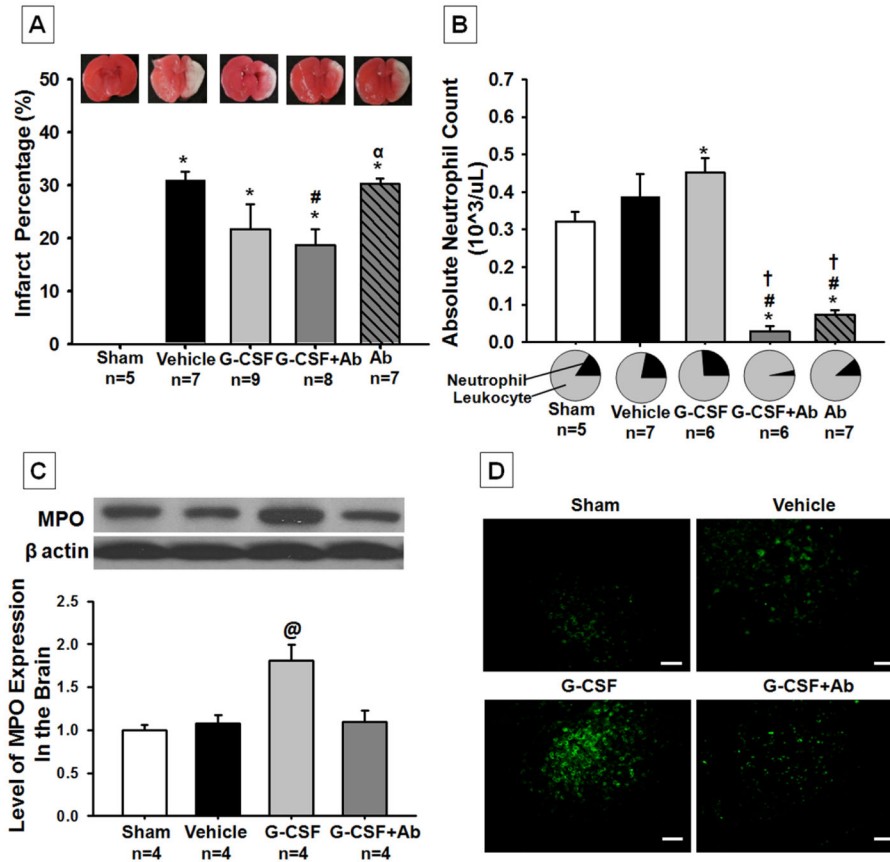4. Ab enhanced G-CSF's neuroprotection by reducing blood neutrophil counts post HI.

Fig. 1. Effects of Treatment on Infarct Volume (A) Blood Neutrophil Counts (B) Brain Neutrophil Counts (C) and Representative Pictures of Infiltrating Neutrophils in Brain (D) at 96h post HI

Postnatal day-10 rats were subjected to HI. Intraperitoneal (IP) treatment with G-CSF, G-CSF+Ab or Ab began at 1h post HI and continued daily for 4 days. **(A)** While the G-CSF treatment group showed a tendency to reduce infarction, the G-CSF+Ab group significantly reduced infarct volume when compared to vehicle (p<0.004, 30.957±1.564 vehicle vs. 18.746±2.983 G-CSF+Ab). **(B)** Absolute neutrophil counts were significantly increased in the G-CSF treatment group when compared to vehicle and were successfully reduced in the combinational treatment (G-CSF+Ab) and Ab alone group (Data represent +/− SEM; *p<0.05 versus sham, #p<0.05 versus vehicle, †p<0.05 versus G-CSF, α p<0.05 versus G-CSF + Ab). **(C)** Western Blot analysis showed that MPO expression (marker for neutrophils) was significantly increased in the G-CSF treatment group when compared to all other groups (Data represent +/− SEM; @p<0.05 versus sham, vehicle and G-CSF+Ab). **(D)** Immunohistochemical staining of MPO (neutrophils) in the brain showed a significant increase in neutrophil counts after G-CSF treatment compared to other groups (White bar in picture represents 100μm).
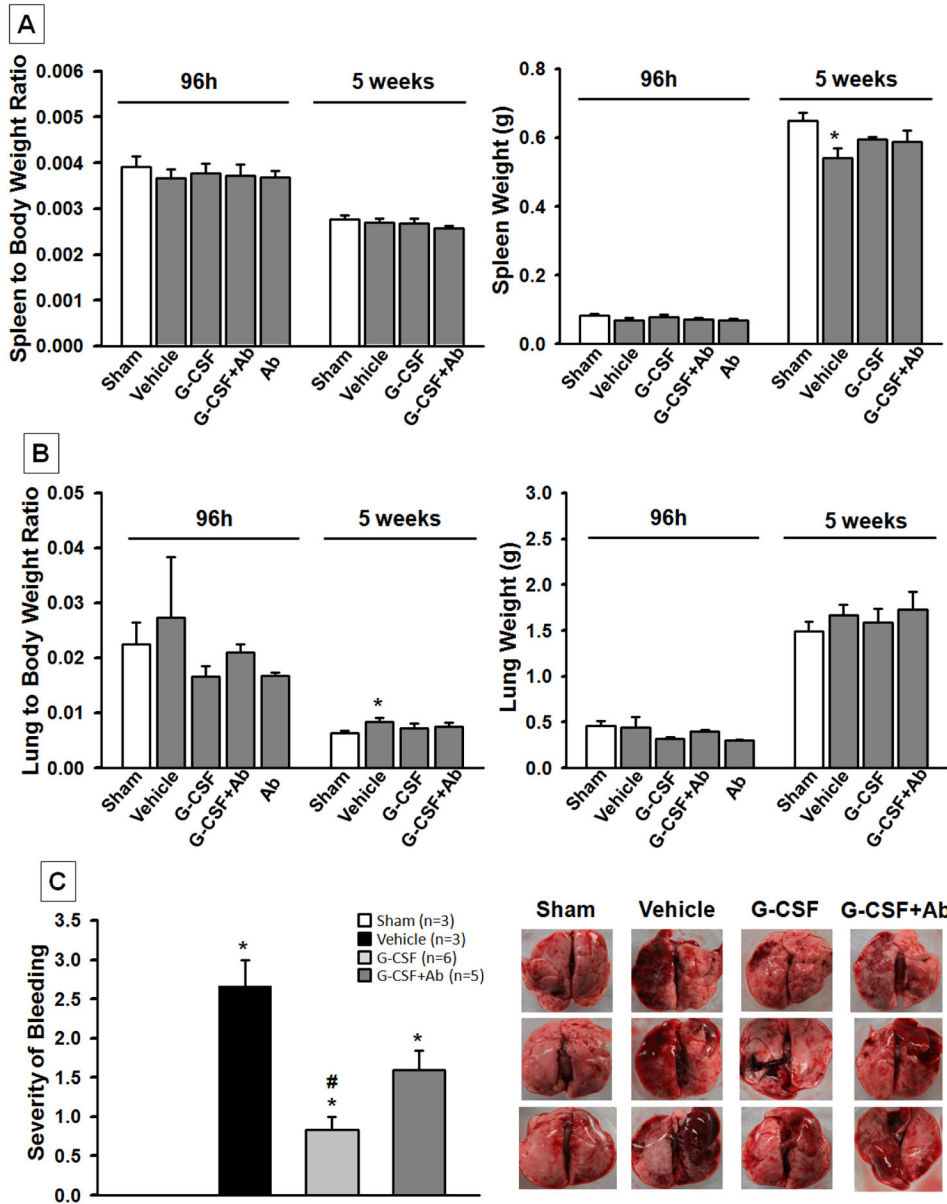
Fig. 2. Effects of Treatment on Organ Weight at 96h and 5 weeks post HI and Lung Bleeding
The absolute spleen weight in the vehicle group was significantly lower than the sham group at 5 weeks post HI. There was no intergroup difference in the spleen to body weight ratio at 96h or 5 weeks **(A)**. There was a significant increase in the lung to body weight ratio in vehicle group at 5 weeks post HI when compared to sham **(B)**. Treatment groups improved lung to body weight ratio (p>0.05 vs. Sham). The graph in part **(C)** shows the severity of lung bleeding at 5 weeks post HI. It is graded on a scale from 0 to 3, with zero meaning no bleeding at all and 3 meaning more than ¾ of lung bleeding. The vehicle group showed severe bleeding compared to sham; while G-CSF significantly reduced lung bleeding compared to vehicle, G-CSF+Ab treatment group also showed tendency to reduce bleeding

but significance was not reached (Data represent +/− SEM; *p<0.05 vs. sham, #p<0.05 vs. vehicle).
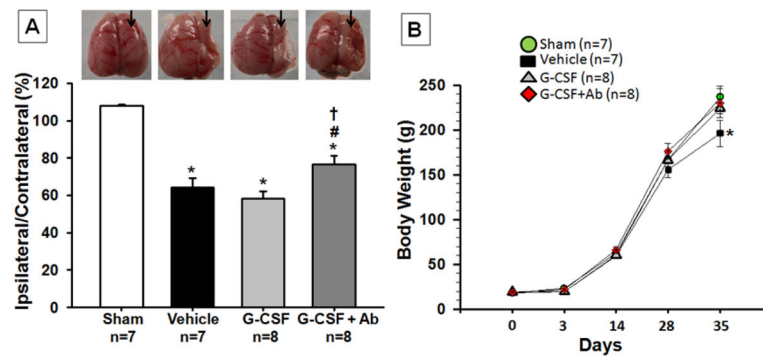
Fig. 3. Effects of Treatment on Brain Atrophy (A) and Body Weight (B) at 5 weeks post HI
(A) Significant loss of right-to-left hemispheric (RH: LH) weight ratio is evident in vehicle rats and significantly improved with G-CSF+Ab treatment at 5 weeks post HI. (Representative pictures shown; Data represent +/− SEM; *p<0.05 versus sham, #p<0.05 versus vehicle, †p<0.05 versus G-CSF). **(B)** At 5 weeks, the vehicle group showed a loss in body weight compared to sham and treatment groups. (Data represent +/− SEM; *p<0.05 vehicle vs. sham, G-CSF and G-CSF+Ab).
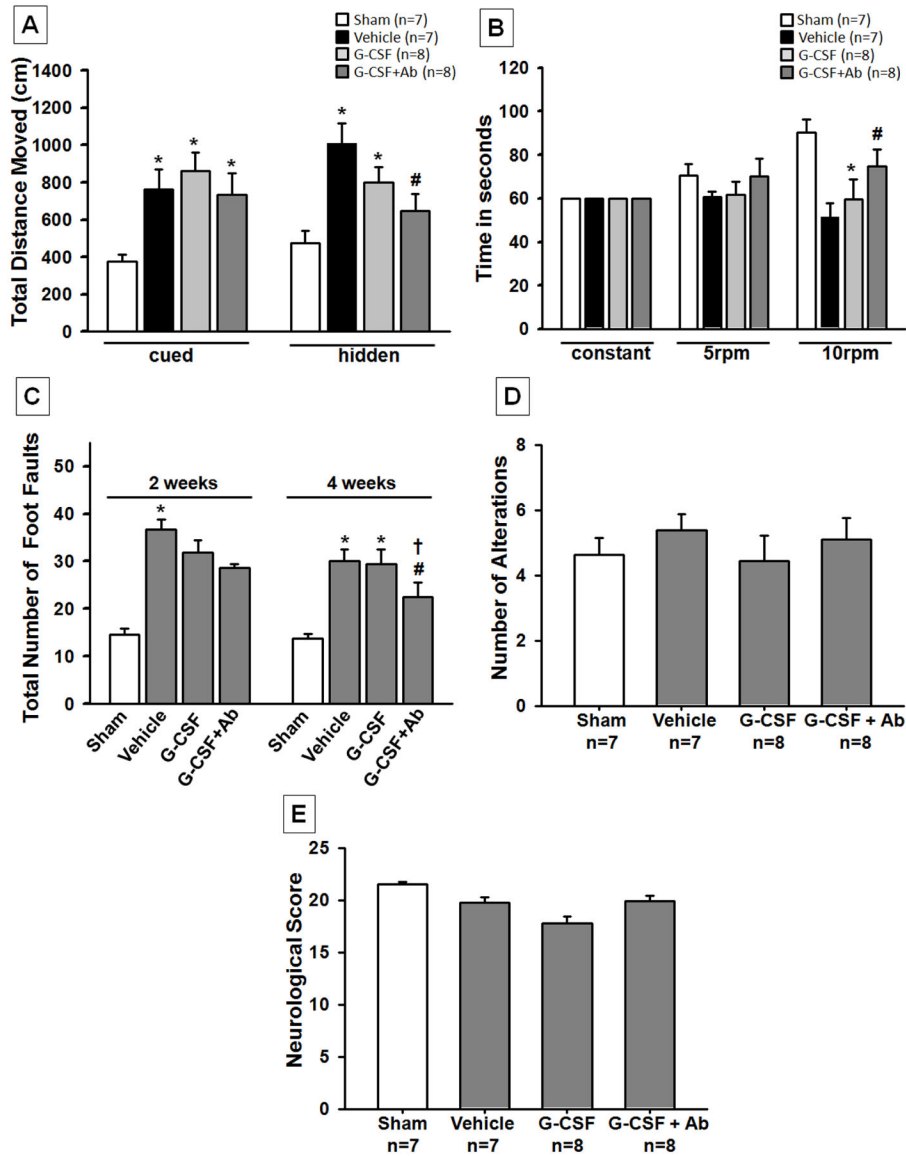
Fig. 4. Functional Outcome post HI

The combinational treatment group (G-CSF+Ab) showed significant improvement in neurological function according to **(A)** Morris Watermaze test, where spatial memory and learning was evaluated at 5 weeks post HI, **(B)** Rota Rod where sensori-motor function was evaluated at 5 weeks and **(C)** Foot fault test, which shows the total number of foot faults at 4 weeks. The results show that ischemia-induced foot slips were significantly reduced by treatment with G-CSF+Ab. T-maze **(D)** and Modified Garcia Test **(E)** did not show any significant improvements among treatment groups although there was a tendency for improvement. (Data represent +/− SEM; *p<0.05 versus sham, #p<0.05 versus vehicle, †p<0.05 versus G-CSF).