Allometric Dose Retranslation Unveiled Substantial Immunological Side Effects of Granulocyte Colony–Stimulating Factor After Stroke

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Background and Purpose—Granulocyte colony–stimulating factor (GCSF) showed robust neuroprotective and neuroregenerative properties after stroke in rodents but failed to meet study end points in patients. Because immunologic side effects of GCSF may have escaped preclinical testing because of nonallometric dose translation, we hypothesized those as possible reasons.

Methods—Stroke was induced in C57BL/6 mice by 45-minute filament middle cerebral artery occlusion. GCSF was administered at 50 and 832.5 μg/kg body weight. Treatment was controlled by vehicle injection, sham surgery, and naive animals. Immune cell counts were assessed in blood, spleen, and brain by multidimensional flow cytometry 1 day after stroke.

Results—High-dose GCSF significantly altered myeloid and T-cell subpopulations in blood and spleen and caused a tremendous increase of monocytes/macrophages infiltrating the ischemic brain.

Conclusions—Dose-dependent immunomodulation superimposes central nervous system-specific effects of GCSF after stroke. Adaption of dose or treatment time may overcome this drawback. (Stroke. 2014;45:623-626.)

Key Words: granulocyte colony-stimulating factor ■ immune system ■ stroke

The peptide hormone granulocyte colony–stimulating factor (GCSF) was the most recent candidate for the treatment of stroke. Successfully tested in numerous rodent studies, it yielded neutral results in a large multicenter randomized clinical trial. Two issues may have jeopardized the translational process, both being potentially relevant for future research. First, preclinical doses cannot be linearly translated to patients. It was hence suggested to apply the body surface area normalization method to convert preclinical into clinical doses. Accordingly, back-calculating the GCSF dose that was used clinically (135 μg/kg)2 implies cumulative (3 days) GCSF dosages of 1665 μg/kg (mice) or 833 μg/kg (rats) for comparable and thus predictable rodent studies. These values were undermatched significantly in most preclinical trials with mean cumulative dosages of 50 μg/kg (mice) and 100 μg/kg (rats),1 illustrating a dose gap between preclinical and clinical application. Second, GCSF as a drug candidate for neurological disorders has been investigated predominantly from a CNS-centric viewpoint, but little is known on its immunologic effects during stroke pathophysiology.4 Because stroke outcome is significantly determined by sterile inflammation and systemic immune imbalance, GCSF as a key regulator of the immune system5 may unforeseeably influence these processes. We therefore decided to investigate whether and how GCSF dose adjustment influences poststroke inflammation in mice.

Materials and Methods

Experimental Stroke and Treatment Regime

Animal procedures were approved by local state authorities (TVV12/11) and performed according to the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines. A total of 72 adult male C57BL/6 mice were assigned randomly to 5 experimental groups: naive (n=3), sham (n=9), middle cerebral artery occlusion (MCAO) (n=20), GCSF low dose (n=20), and GCSF high dose (n=20). Animals were anesthetized with 2% isoflurane in a 30%O2/70%NO mixture, and focal cerebral ischemia was induced by filament occlusion (45 minutes) of the right middle cerebral artery. Mice received intraperitoneal injections of GCSF of either 50 or 832.5 μg/kg body weight distributed to 2 partial doses immediately after reperfusion and 12 hours later. GCSF treatment was controlled by application of 5% glucose in the others groups.

For details on further group allocation and end point measurements, see Methods in the online-only Data Supplement.

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Results

Neurological Outcome and Peripheral Immunomodulation

The number of animals with high ranks (ie, more severe neurological deficit) in the categorical Bederson score was significantly increased by high-dose GCSF, whereas the infarct volume did not differ among MCAO groups (Figure 1A). When analyzing the impact of GCSF on circulating leukocyte populations, reduced numbers of T, B, and natural killer cells were found in all operated mice. Myeloid cells were not altered, but granulocytes (polymorphonuclear cells [PMN]) were significantly increased by high-dose GCSF, whereas the infarct volume did not differ among MCAO groups (Figure 1A). When analyzing the impact of GCSF on circulating leukocyte populations, reduced numbers of T, B, and natural killer cells were found in all operated mice. Myeloid cells were not altered, but granulocytes (polymorphonuclear cells [PMN]) were

Figure 1. Impact of granulocyte colony–stimulating factor (GCSF) on neurological deficit, infarct volume, and peripheral leukocyte distribution 24 hours after middle cerebral artery occlusion (MCAO). A, Bederson score (percentage distribution, animal numbers are indicated in columns) and edema-corrected hemispheric lesion volume (%HLVe). *P<0.05. B, Natural killer (NK) cells, mononuclear (mn) myeloid cells, granulocytes (polymorphonuclear cells [PMN]), B cells, cytotoxic T cells (CD8+), and T-helper cells (Th, CD4+) were assessed in blood and spleen. *P<0.05 naive vs sham/MCAO/GCSF low/high; †P<0.05 naive vs GCSF low/high; ‡P<0.05 naive vs GCSF high. C, Th-cell subpopulations in spleen and blood. D, Serum cytokines. **P<0.01, ***P<0.001 (n=3–8). HD indicates high dose; IL, interleukin; and LD, low dose.

Figure 2. Differentiation of circulating myeloid subpopulations. A, Nongranulocyte CD11b+ cells were distinguished by CD11c/major histocompatibility complex (MHC) II/Ly6C. Q1, CD11c+/MHCII–/Ly6C+ and – cells significantly increased after granulocyte colony–stimulating factor (GCSF) high treatment. Histogram, note the shift toward Ly6C+ in GCSF high group. CD11c+/MHCII– (Q3), and CD11c+/MHCII+ (Q4) did not differ among groups. B, Lymphoid dendritic cell counts in blood and spleen. *P<0.05, **P<0.01, and ***P<0.001 (n=3–6). MCAO indicates middle cerebral artery occlusion.
significantly increased after GCSF treatment (Figure 1B). In the spleen, B cells decreased, whereas PMN increased because of surgery. T cells were significantly reduced after GCSF treatment. Again, myeloid cell counts were not influenced by surgery or treatment (Figure 1B). Next, we analyzed specific T-helper (Th) cell subpopulations revealing a decline of Th1 cells after MCAO that was reversed by high-dose GCSF. Th2 cells were decreased significantly because of surgery, but not further affected by MCAO or GCSF. Th17 cells showed a trend toward decrease after MCAO and increase after high-dose GCSF treatment. Regulatory T-cell counts were unaltered in the spleen (not shown) but significantly elevated in circulation after high-dose GCSF (Figure 1C).

Serum levels of some cytokines were below (interferon-γ, tumor necrosis factor-α; not shown) or along (interleukin-1β; Figure 1D) the detection limit. Circulating interleukin-6 was significantly decreased by GCSF treatment irrespective of the dose. Interleukin-12/23p40 was not affected by GCSF. Determination of serum GCSF revealed a 10-fold difference between the dose groups (Figure 1D). We next differentiated the heterogeneous group of blood mononuclear myeloid cells by CD11c, major histocompatibility complex (MHC) II, and

Figure 3. Brain infiltration of leukocytes 24 hours after middle cerebral artery occlusion (MCAO). A, Infiltration of CD45+ leukocytes, Ly6G+ granulocytes (polymorphonuclear cells [PMN]) and CD3+ T cells. B, Differentiation of CD11b+ myeloid infiltrate by F4/80+ MHCII/ Ly6C. Major histocompatibility complex (MHC) II+/Ly6C+ cells (Q2) and MHCII−/Ly6C+ cells (Q3) were significantly increased by high-dose granulocyte colony-stimulating factor (GCSF). C, CNS gene expression of cytokines and chemokines was unaltered after GCSF treatment. *P<0.05, **P<0.01, and ***P<0.001 (n=3–6). CCL indicates CC chemokine ligand; CXCL, CXC chemokine ligand; IL, interleukin; TGF, transforming growth factor; and TNF, tumor necrosis factor.
Ly6C expression. CD11c+/MHCII−/Ly6C− resident monocytes were significantly reduced after MCAO but restored by high-dose GCSF (Figure 2A, Q1). Circulating CD11b+/CD11c+/MHCII−/Ly6C+ dendritic cell (DC) precursors were doubled by high-dose GCSF (Q1). Blood myeloid (Figure 2A, Q2) and lymphoid (Figure 2B) DCs were not affected by MCAO or GCSF. Splenic CD8+ lymphoid DC were decreased by MCAO but not influenced by GCSF (Figure 2B). Ly6C+ inflammatory monocytes (Q4; MHCII+/CD11c−), including their activated variant (Q3, MHCII+/CD11c−), were also unchanged among experimental groups.

CNS Immunomodulation

MCAO caused a significant leukocyte influx into the ischemic hemisphere. Low-dose GCSF had no significant effect, but high-dose treatment tripled leukocyte counts within the brain (Figure 3A). This increase was not caused by PMN (Figure 3A) or B cells (not shown), but by T cells (Figure 3A) and mononuclear myeloid cells (Figure 3B). The latter population could be differentiated into MHCII+ cells, which primarily comprised macrophages, DC, and activated monocytes (Q2). Besides, MHCII−/Ly6C+ inflammatory monocytes were also increased in the GCSF high group (Q3). Finally, we found no significant effect of both GCSF doses on the gene expression of relevant chemoattractant factors and inflammatory cytokines in the ischemic hemisphere (Figure 3C).

Discussion

Our study indicates a considerable impact of high-dose GCSF treatment on poststroke immune responses, reflecting the GCSF dose given in a recent clinical trial when considering allometric dose retranslaction. Immunomodulation induced by high-dose GCSF was further associated with worse neurological outcome. In contrast, GCSF dosing complying with most preclinical studies did not influence immune responses or functional outcome. We hypothesize that the surprising failure of GCSF in patients might, at least partly, be explained by dose-dependent immunologic side effects. This hypothesis is corroborated by the fact that patients with stroke receiving GCSF presented fever, tachycardia, mononcytosis, and higher C-reactive protein levels. However, our findings are limited to the first 24 hours after stroke, and the relationship between dose-dependent immune alterations and neurological outcome requires confirmation in long-term studies.

Systemic effects of GCSF, such as increased mobilization of regulatory T cells, immature DC, and certain monocyte subpopulations, are in line with previous studies beyond the stroke field and may indicate a conducive dose-dependent gain of peripheral tolerance at larger time scales. During acute stroke, however, it was recently shown that regulatory T cells accumulate within the microvasculature and contribute to brain damage by compromising cerebral perfusion. Thus, the increase of circulating regulatory T cells and the compensation of stroke-induced Th1 cell depression may serve as possible explanations for detrimental effects of high-dose GCSF treatment early after stroke.

Another interesting finding was the distinct increase of monocytes/macrophages infiltrating into the ischemic lesion after high-dose GCSF treatment. We did neither observe shifts in splenic or circulating monocyte counts nor increased expression of chemoattractants that could explain this finding. One could speculate that high doses of GCSF induce functional changes of monocytes or modulate adhesion molecules on circulating cells and the CNS vasculature. It is also uncertain which consequences may arise from increased monocyte/macrophage counts in the ischemic lesion. A recent study in healthy mice indicated primarily beneficial effects, but the situation may turn in comorbid patients.

In conclusion, we unveiled substantial immunomodulatory effects of GCSF at a dose corresponding to that used in a recent clinical trial. Within the first 24 hours after stroke, these changes seem to be detrimental, but long-term consequences are yet unknown. The delayed influence of high-dose GCSF on poststroke immune responses and functional outcome should be entirely understood before terminally amortizing GCSF as stroke treatment candidate. Application of lower doses to target neuroprotection while avoiding adverse immunomodulation or a timed administration (after the peak of thromboinflammation, but timely for regenerative effects) is a possible approach for future research.

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Disclosures

None.

References

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals and group allocation
The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was performed according to the ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). All experiments were approved by local state authorities (protocol number TVV12/11). A total of 72 male mice (C57BL/6, Charles River Laboratories, Sulzfeld, Germany; 12-14 weeks old, body weight 25-30g) were enrolled in the study. Animals were singly housed with free access to food and water and kept on a 12h/12h light-dark cycle. Mice were assigned to the following five experimental groups by balanced randomization drawing lots: naive (n=3), sham-surgery (n=9), MCAO (n=20), GCSF low dose (n=20), GCSF high dose (n=20). Naive were exclusively examined by flow cytometry while the remaining groups were further divided into two subgroups. Group 1: analysis of leukocyte subpopulations in blood, spleen and brain by flow cytometry (sham n=6, MCAO n=6, GCSF low dose n=6-8, GCSF high dose n=6-9). Group 2: gene expression of chemoattractants and inflammatory cytokines in the brain by RT-PCR (sham =3, MCAO n=7, GCSF low dose n=7, GCSF high dose n=7). Animals from the PCR group were also used to measure serum cytokine levels by cytometric bead array (CBA). Another 3 animals were used to determine the infarct volume in the MCAO, GCSF low and GCSF high group, respectively.

Experimental stroke model
Focal cerebral ischemia was induced by transient middle cerebral artery occlusion (tMCAO) as described previously. Briefly, mice were anaesthetized with 2.0% isoflurane in 30%O₂/70%N₂O. Body temperature was maintained at 36.5°C ± 0.5°C by a feedback-controlled heating device. After ligation of the common carotid artery and the external carotid artery a standardized silicon-rubber coated monofilament (60SPPK10; Doccol corporation, Sharon, USA) was introduced in the common carotid artery and advanced to the origin of the middle cerebral artery. The filament was removed after 45 minutes to allow reperfusion. In sham-operated animals the filament was immediately withdrawn after occluding the MCA to avoid ischemia. After surgery mice were kept in heated cages for 2 hours.
Exclusion criteria
Exclusion criteria were defined as follows: i) prolonged operation time > 15min ii) no reperfusion after filament withdrawal iii) excessive bleeding iv) death within 24h after MCAO. Altogether, 8 mice were excluded from the study (n=2 in sham group, n=4 MCAO, n=2 GCSF low dose). Moreover, due to technical problems (coagulated blood samples, cell aggregation compromising valid cell counting, inappropriate organ processing) some blood (naive n=0 / sham n=0 / MCAO n=3 / GCSF low dose n=0 / GCSF high dose n=0), spleen (0/0/1/1/2) and brain samples (0/0/0/1/0) had to be excluded from further analysis.

Drug administration
In the low dose group GCSF administration followed common preclinical application protocols. Mice received 50µg/kg body weight Neupogen (rhG-CSF; Amgen GmbH, Thousand Oaks, USA) intraperitoneally immediately after MCAO, followed by an injection of the vehicle solution (5% glucose) 12 hours later. In the GCSF high dose group the applied dosage was back-calculated from the first day dose of a recent clinical trial by the body surface area normalization method. In the AX200 trial, patients received 135µg GCSF per kg body weight over 72 hours, hereof half dose (± 67.5µg/kg) on day 1. To convert this dose to a dose based on the surface area of mice, it needs to be multiplied by the $K_m$ factor for humans (37) and then divided by the $K_m$ factor for mice (3) (Animal equivalent dose (µg/kg) = human dose (µg/kg) * $K_m$ human/$K_m$ mouse). This calculation resulted in a mouse equivalent dose of $67.5µg/kg * 37/3 = 832.5µg/kg$ body weight in our study. Due to the short plasma-half time of GCSF the dose was distributed to two partial doses and applied immediately after MCAO as well as 12h hours later. The administrations in both treatment groups were controlled by intraperitoneal injections of 5% glucose immediately after stroke and 12h later in the sham-operated and MCAO group.

Functional outcome
Prior to sacrifice (24 hours after surgery) the neurological deficit was assessed by an investigator blinded to the group allocation using a modified Bederson score: 0=no deficit, 1=decreased extension of forepaw, 2=circling, 3=loss of postural reflexes, 4=death.

Tissue sampling
24 hours after MCAO or the respective sham operation mice were sacrificed by CO$_2$ exposition under deep inhalation anesthesia with isoflurane. The thoracic cavity was opened,
the right atrium was punctured and peripheral blood was collected in monovettes containing 2mM EDTA. Subsequently, animals for flow cytometric analysis were transcardially perfused with 50 mL of Hanks Balanced Salt Solution (HBSS), whereas animals for RT-PCR and histology were perfused with phosphate buffered saline (PBS), respectively. For flow cytometry, brains and spleens were harvested and shortly stored in ice-cold HBSS. For gene expression analysis, brain tissue was dissected on ice and ipsi- and contralateral hemispheres were separately dissociated by razor blades. All specimens were directly frozen in liquid nitrogen and then stored at -80°C until further use.

**Processing of peripheral blood**

Absolute leukocyte counts (WBC) were determined by an animal blood cell counter (scil Vet abc, SCIL animal care company GmbH, Viernheim, Germany). Leukocyte subsets were identified and categorized according to their antigen expression using multichannel flow cytometry. For this, 50 µL anticoagulated EDTA-blood was diluted in 50 µL PBS and incubated with a mixture of monoclonal anti-mouse antibodies (of either ‘CD45.2-FITC, Nk-1.1-PE, Ly6G-PerCP-Cy™5.5, CD25-PE-Cy™7, CD3-APC, CD4-APC-efluor®780, CD8a-efluor®450, B220-Horizon™V500’ or ‘CD45.2-FITC, Lineage of CD3-PE/Nk-1.1-PE/CD19-PE/Ly6G-PE, CD4-PerCP-Cy™5.5, CD11c-PE-Cy™7, CD8-APC, Ly6C-APC-Cy™7, MHCII-efluor®450, CD11b-Horizon™V500’) for 20 min at 4°C. Erythrocytes were lysed by incubation with 1x BD FACS Lysing Solution for 10min followed by two washing steps with PBS containing 3% fetal calf serum (PBS/3% FCS). Remaining leukocytes were resuspended in 300µL PBS/3% FCS and temporarily stored at 4°C in the dark until acquisition. For the quantification of circulating cytokine levels, serum (n=6 per group) was separated from coagulated blood samples by centrifugation at 5000 rpm for 5min and stored in aliquots at -80°C. Cytokine secretion levels of IFNγ, TNFα, IL-1β, IL-6, IL-12/23p40 and serum levels of rhG-CSF were analyzed by Cytometric Bead Array (BD Biosciences, Heidelberg, Germany).

**Processing of spleen tissue**

Spleen tissue was mechanically dissected using razor blades and further dissociated through a 100µm metal sieve using glass pestles. The obtained single cell suspension was collected in PBS/3% FCS and centrifuged at 300 g for 10 min. The cell pellet was resuspended in 1 mL Roswell Park Memorial Institute (RPMI) Medium 1640 and once more filtered through a 40 µm cell strainer followed by rinsing with 7 mL RPMI medium. Total cell counts and viability were determined by trypan blue exclusion in a hemocytometer. Likewise, total leukocyte
counts were defined by counting in Turk’s solution. For flow cytometric analysis, 3 x 10E5 leukocytes were primarily incubated with anti-murine CD16/CD32 FC-Receptor blocking reagent for 10 min at 4°C and thereafter labeled with the above mentioned mixture of monoclonal antibodies for additional 20 min at 4°C in the dark. After incubation, cells were washed, resuspended in 300 µL PBS/3% FCS and temporarily stored at 4°C in the dark until acquisition.

T helper subpopulations were analyzed by anti-mouse Th1/Th2/Th17 Phenotyping Kit (BD Biosciences). Therefore, a total of 5 x 10E6 leukocytes per mL RPMI medium were stimulated with 50 ng/mL PMA and 1 µg/mL Ionomycin for 5h at 37°C, 5% CO2 and 95% humidity. Extracellular secretion of cytokines was inhibited by adding 0.67 µL BD GolgiStop™ Protein Transport Inhibitor per mL medium after 1 h incubation time. For intracellular fluorescent staining, cells were harvested, washed twice in RPMI 1640 medium and centrifuged at 300 g for 10 min. Cells were counted in Turk’s solution in a hemocytometer and 10E6 leukocytes were fixed, permeabilized and labeled with provided ‘IFNγ-FITC/IL-17A-PE/CD4-PerCP-Cy™5.5/IL-4-APC’ antibody cocktail and additional CD3-APC-efluor®780 T cell marker according to the manufacturer’s instructions.

**Processing of brain tissue**

Single brain hemispheres were cut into 3 pieces and homogenized through a 100 µm cell strainer by continuous rinsing with 15 mL of cold HBSS. Suspensions were centrifuged for 5 min at 4°C, the supernatant was discarded and the pellet was resuspended in 1 mL digestion buffer (2 U/mL of Liberase TL (Roche, Switzerland) in HBSS with Ca/Mg). Digestion was performed over a period of 1 h at 37°C under continuous rotation. Thereafter, single cell suspensions were sieved through 70 µm cell strainers with 3 mL of washing buffer (HBSS without Ca/Mg containing 10% FCS and 200 U DNAse I (Roche)) followed by 15 mL of DNAse-free washing buffer. Samples were centrifuged at 286 g for 5 min at 18°C. The supernatants were discarded and the pellets were each resuspended in 5 mL of 25% Percoll in HBSS with 3% FCS solution and centrifuged at 521 g for 20 min at 18°C without break. The supernatants and the myelin layer were discarded and the pellets were resuspended in 10 mL of washing buffer. The samples were again centrifuged at 286 g for 5 min at 10°C. Cells were finally resuspended in 100 µL of washing buffer and cell counts as well as viability were determined by trypan blue exclusion in a hemocytometer. For flow cytometric analysis, 2.5 x 10E5 cells were primarily incubated with anti-murine CD16/CD32 FC-Receptor blocking reagent for 10 min at 4°C and subsequently labeled with the following monoclonal anti-
mouse antibodies: CD45.2-FITC, CD3-PE, Ly6G-PerCP-Cy™5.5, CD19-PE-Cy™7, F4/80-Alexa Fluor®647, Ly6C-APC-Cy™7, MHCII-efluor®450, CD11b-Horizon™V500. After incubation for 20 min at 4°C cells were washed, resuspended in 300 µL PBS/3% FCS and stored temporarily at 4°C in the dark until acquisition.

Flow cytometry
All flow cytometric analyses were performed by an investigator blinded to the group allocation using a 3-laser FACS Canto II equipped with FACS Diva software (BD Biosciences). Absolute cell numbers were calculated by multiplication of total leukocyte counts per spleen or WBC count per mL by the percentage of the depicted subpopulations out of the viable leukocyte gate (single cell and life gate as well as CD45 positive cells) respectively. Total counts of brain infiltrating leukocytes were determined by additional Trucount Tube measurement (BD Biosciences) of CD45 labeled brain cell suspensions.

qPCR analysis
Total RNA of 100 mg dissociated brain tissue was extracted by homogenization in 1 mL TRIzol reagent (Life Technologies, Darmstadt, Germany) using an ULTRA-TURRAX® (Ika, Staufen, Germany) and further purified by RNeasy Mini Kit (Qiagen). Single-strand cDNA copies were generated from 5 µg purified RNA using Superscript III Synthesis Kit (Life Technologies) according to the respective manufacturer’s instructions. A cDNA dilution of 1:10 was used as reaction template and mixed with Quantitect Primer assays (CCL2 (QT00167832), CXCL1 (QT00115647), IL-1β (QT01048355), IL-6 (QT00098875), IL-10 (QT00106169), TGFβ (QT00145250), TNFα (QT00104006), GCSF (QT00105140)) and QuantiTect SYBR Green PCR Kit (Qiagen) as suggested by the manufacturer. Quantification of mRNA expression was performed and monitored using an ABI 7900 real-time PCR system (Applied Biosystems, Darmstadt, Germany). Data was analyzed using the relative standard curve method, normalized on the average cycle threshold of the housekeeping genes (YWHAZ (QT00105350), B2M (QT01149547), RPL22 (QT01758120)) and relativized to the gene expression of the sham control.

Infarct volume
For histological analysis, animals were transcardially perfused with PBS followed by 4% formalin solution. Removed brains were vitrified in sucrose solution (30%) and frozen at -
80°C. Afterwards, brains were cut into 20μm thick coronal sections. For infarct volume determination, every 25th section (i.e. every 500μm) between bregma +2.5mm and -3.4mm was stained with cresyl-violet and acquired using a Nikon Coolscan V ED scanner. The volumes of the hemispheres, ventricles and of the ischemic lesion were assessed by ImageJ (National Institute of Health). Finally, the edema-corrected lesion volume (%HLVe) was calculated as described previously.\(^7\)

**Statistics**

Data were presented as mean ± standard deviation. Except for the neurological scoring, statistical differences were determined by one-way analysis of variance (ANOVA). When appropriate, Bonferroni’s post hoc test was applied for the following group comparisons: i) naive vs. sham; ii) sham vs. MCAO; iii) MCAO vs. GCSF low; iv) MCAO vs. GCSF high; v) GCSF low vs. GCSF high. Neurological scores were analyzed using the Fisher's exact test. P-values ≤ 0.05 were considered statistically significant. Statistical analyses were performed by GraphPad Prism (Version 5.03).

**References**


