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Short communication

Graphite furnace atomic absorption spectrophotometry—A novel method to quantify blood volume in experimental models of intracerebral hemorrhage

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ABSTRACT

Intracerebral hemorrhage (ICH) accounts for 10% of all strokes and has a significantly higher mortality than cerebral ischemia. For decades, ICH has been neglected by experimental stroke researchers. Recently, however, clinical trials on acute blood pressure lowering or hyperacute supplementation of coagulation factors in ICH have spurred an interest to also design and improve translational animal models of spontaneous and anticoagulant-associated ICH. Hematoma volume is a substantial outcome parameter of most experimental ICH studies. We present graphite furnace atomic absorption spectrophotometric analysis (AAS) as a suitable method to precisely quantify hematoma volumes in rodent models of ICH.

1. Introduction

Intracerebral hemorrhage (ICH) accounts for 10% of all strokes in western communities (Lovelock et al., 2007) with a considerably poorer prognosis than cerebral ischemia (Adeoye et al., 2008). ICH volume is an independent predictor of functional outcome and mortality (Ruiz-Sandoval et al., 2007). For decades, ICH has been regarded as the most untreatable cause of stroke and neglected by stroke researchers. In parallel with recent clinical trials investigating the effects of aggressive blood pressure lowering and coagulation activation in the acute phase of ICH, experimental research has gained in importance. Common ICH models encompass the collagenase injection model (Rosenberg et al., 1990), which has been adapted to model anticoagulation-associated ICH (Foerch et al., 2008), the autologous blood injection model (Gong et al., 2001) as well as models of intracerebral microhemorrhage (Lauer et al., 2011). Besides functional neurological outcome, hematoma volume is one of the crucial outcome parameters of all experimental ICH studies. Brain imaging, particularly magnetic resonance imaging (MRI), has the disadvantage that it is costly, time-consuming and does not allow a quantification of hematoma volume in the strict sense. The widely used hemoglobin (Hb) assay based on the photometric determination of cyanohemoglobin is a reliable test but meets serious limitations when other dyes are administered in vivo or post mortem as we will demonstrate in this study for Evans blue (EB). EB is widely used to examine the integrity of biological barriers as the blood–brain barrier (BBB) (Belayev et al., 1996). EB is an autofluorescent dye with a broad excitation peak between 470 and 600 nm and an emission peak at 680 nm and therefore interferes with the cyanohemoglobin-based Hb assay which is read at 540 nm. In this study, we show that graphite furnace atomic absorption spectrophotometric analysis (AAS) is a simple and straightforward alternative method to precisely quantify hematoma volume in experimental ICH. This technique measures heme-bound and non-heme iron and is not affected by co-administered dyes.

2. Materials and methods

2.1. Animals

All experiments were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and the ARRIVE guidelines. The study was approved by the local governmental authorities (Regierungspraesidium Darmstadt, Germany). We used 12-week-old male C57BL/6J mice weighing on average 27.3 ± 1.2 g for comparative hematoma volume measurements with graphite furnace AAS and the Hb assay as the currently widely used "gold standard".

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2.2. Standard curve determination

A first set of non-ICH mice ($n = 5$) was used to determine standard curves for ICH volume based on hemoglobin content determination and graphite furnace AAS, respectively. Defined blood volumes (0 μl, 5 μl, 10 μl, 15 μl and 20 μl) were added to homogenized samples of largely blood-free brains derived from transcardially perfused mice. The samples were subjected to the procedures described in detail below. 3 additional mice were used to determine the "naïve" brain iron content (without exogenously added blood). These samples served as a background correction factor which was subtracted from the absolute iron values of the standard curve samples. To verify the precision of the iron detection method, five incremental aliquots of 1, 5, 10, 15, and 20 μl of mouse blood were diluted in 3 ml phosphate buffered saline (PBS) and subjected to the same procedure as brain tissue samples.

2.3. Virtual model of ICH with EB coadministration with known volumes of blood and EB solution

A second set of animals ($n = 3$) was used to create a "virtual model" to ascertain the exactitude of the graphite furnace AAS in quantifying hematoma volume in the lipid–protein matrix of the brain. Brains were taken from natively perfused mice, and 5 μl blood and 0.5 μl of 4% EB were added. The blood volume (5 μl) was chosen as a typical hematoma volume that can be found 24 h after collagenase injection (Lauer et al., 2011), and the EB volume as a typical volume of extravasated 4% EB solution in our models of brain injury (data not shown).

2.4. Determination of hematoma volume in ICH mice

A third set of mice ($n = 3$), were subjected to the collagenase model of ICH. We stereotactically injected collagenase Type VII into the right striatum (0.3125 IU in 0.5 μl, Sigma–Aldrich, Taufkirchen, Germany). The coordinates for the burr hole were 0.0 mm anterior to the bregma and 2.0 mm to the right from the midline, and the needle was inserted 3.0 mm into the depth of the basal ganglia. After 30 min, EB was injected via the i.v. route. Mice were transcardially perfused with 30 ml normal saline (NaCl 0.9%) 2.5 h after collagenase injection and 2 h after administration of EB. Brains were removed and frozen at −20 °C until they were subjected to hematoma volume determination via the Hb assay or AAS, respectively.

2.5. Hemoglobin assay

Hemoglobin (Hb) content was assessed as described previously (Foerch et al., 2008) by photometric evaluation of samples exposed to Drabkins's reagent (Sigma–Aldrich, Taufkirchen, Germany) at 540 nm and calculation of hematoma volumes along a standard curve ranging from 5 to 20 μl.

2.6. Graphite furnace AAS of brain samples

The concentration of total iron was measured by graphite furnace atomic absorption spectrometry with a HGA 700 computer controlled system (Perkin-Elmer 1100B, Perkin-Elmer, Waltham, MA, USA) using a working calibration curve of three composite standards freshly prepared from commercial 10,000 ppm stock solution (Iron Standard Solution, 10 g/L as iron nitric acid, Sigma–Aldrich, Taufkirchen, Germany) by dilution in deionised water containing 0.2% nitric acid (69% Nitric acid, Merck KGaA Darmstadt, Germany). Acidified water retains the iron ions in solution and stabilizes the analyte during the ash stage. Temperature programs were created with up to 12 different steps (from 80 °C up to 2650 °C) in order to reach a maximum analyte sensitivity, ramp, and hold times. Argon (inlet pressure 300–450 kPa) was required to remove matrix vapors, which were formed during thermal pretreatment at an internal flow rate of 300 ml/min, and stopped during automization to obtain interference-free results. Absorption from a hollow cathode Fe lamp was measured at 248.3 nm. A deuterium lamp was used to correct for the background absorption to obtain maximal analyte sensitivity. The characteristic mass for iron was calculated to be 5.0 pg ± 20%. Using peak area measurements, the sampler was programmed to inject 20 μl of prepared calibration samples in quintuples to develop a linear calibration response over 0–30 ng/ml. The maximum limit of detection was 30 ng/ml and the dilution of the brain tissue samples was adapted to the measuring range of the calibration curve, and to the expected iron content and samples were measured sextuply. Iron content of unknown samples was calculated along a standard curve ranging from 5 to 20 μl. The established average mouse brain iron content (3.9 ± 0.3 μg) was subtracted from the measured values to reach a zero intercept in the plot of our linear standard curve.

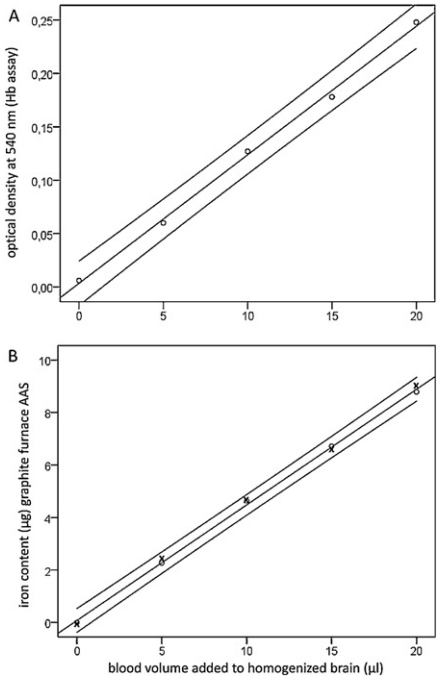

Fig. 1. Standard curves of Hb assay and AAS and validation of AAS by dummy samples with defined blood content. (A) Defined blood volumes (0 μl, 5 μl, 10 μl, 15 μl and 20 μl) were added to samples of largely blood-free homogenized brains of transcardially perfused mice. The samples were subjected to the Hb assay and read at 540 nm. $r^2 = 0.998$. (B) Standard samples were prepared as described above and subjected to graphite furnace AAS. Natively perfused control brains contain 3.9 ± 0.3 μg iron ($n = 3$). This value was subtracted from the values of all samples. To validate the precision of the standard curve based on the subtraction of native brain iron content, five samples with defined blood volumes (0 μl, 5 μl, 10 μl, 15 μl, 20 μl) in 3 ml PBS were measured. $r^2 = 0.999$. The graph depicts an overlay of the standard curve and the dummy samples with defined blood volumes in PBS.

2.7. Evans blue assay

The EB assay was performed as described before (Czech et al., 2009) with photometric evaluation of samples after lipid and protein precipitation with 50% trichloroacetic acid with excitation at 600 nm and emission at 680 nm.

2.8. Statistical analysis

The equations of the standard curves are given as $y = ax + b$. We show the CI for a, Pearson's correlation coefficients (r^2) and the level