Effects of Magnesium Treatment in a Model of Internal Capsule Lesion in Spontaneously Hypertensive Rats

Clotilde Lecrux, PhD; Christopher McCabe, PhD; Christopher J. Weir, PhD; Lindsay Gallagher, HNC; Jim Mullin, HNC; Omar Touzani, PhD; Keith W. Muir, MD; Kennedy R. Lees, MD; I. Mhairi Macrae, PhD

Background and Purpose—The study aim was to assess the effects of magnesium sulfate (MgSO4) administration on white matter damage in vivo in spontaneously hypertensive rats.

Methods—The left internal capsule was lesioned by a local injection of endothelin-1 (ET-1; 200 pmol) in adult spontaneously hypertensive rats. MgSO4 was administered (300 mg/kg SC) 30 minutes before injection of ET-1, plus 200 mg/kg every hour thereafter for 4 hours. Infarct size was measured by T2-weighted magnetic resonance imaging (day 2) and histology (day 11), and functional recovery was assessed on days 3 and 10 by the cylinder and walking-ladder tests.

Results—ET-1 application induced a small, localized lesion within the internal capsule. Despite reducing blood pressure, MgSO4 did not significantly influence infarct volume (by magnetic resonance imaging: median, 2.1 mm³; interquartile range, 1.3 to 3.8, vs 1.6 mm³ and 1.2 to 2.1, for the vehicle-treated group; by histology: 0.3 mm³ and 0.2 to 0.9 vs 0.3 mm³ and 0.2 to 0.5, respectively). Significant forelimb and hindlimb motor deficits were evident in the vehicle-treated group as late as day 10. These impairments were significantly ameliorated by MgSO4 in both cylinder (left forelimb use, \(P<0.01\) and both-forelimb use, \(P<0.03\) vs vehicle) and walking-ladder (right hindlimb score, \(P<0.02\) vs vehicle) tests.

Conclusions—ET-1–induced internal capsule ischemia in spontaneously hypertensive rats represents a good model of lacunar infarct with small lesion size, minimal adverse effects, and a measurable motor deficit. Despite inducing mild hypotension, MgSO4 did not significantly influence infarct size but reduced motor deficits, supporting its potential utility for the treatment of lacunar infarct.

Key Words: endothelin ■ lacunar infarct ■ magnesium ■ functional recovery ■ white matter
MgSO₄ and MgCl₂ in rodent models of both reversible and permanent middle cerebral artery occlusion, with a reduction in infarct volume of up to 61% (reviewed in Muir⁸). In vitro studies⁹,¹⁰ indicated a potential neuroprotective effect of magnesium on white matter damage, but to date this concept has not been tested in vivo. To assess the effects of magnesium on white matter damage and functional recovery, we established a model of white matter ischemia in the adult rat. The IC was chosen, first because it is one of the larger white matter tracts in the rodent brain and second because Frost et al.⁸ recently reported that ET-induced lesions of the IC result in a measurable sensorimotor deficit in rats. Furthermore, a unilateral section of the pyramidal tract at the brainstem level results in deficits in voluntary motor functions and impairment of both forelimb and hindlimb functions.¹¹⁻¹³

Hypertension represents the most important modifiable risk factor for stroke, including lacunar infarction.¹⁴ In light of the Intravenous Magnesium Efficacy in Stroke trial subgroup analysis demonstrating treatment benefit in patients with higher-than-average BP, the aim of this study was to induce ischemia in the IC of the spontaneously hypertensive rat (SHR) to study the effects of MgSO₄ on lesion size and sensorimotor deficit.

Materials and Methods

Animals

Experiments were performed under license from the UK Home Office and were subject to the Animals (Scientific Procedures) Act of 1986. Adult male SHR (mean ± SD weight, 335 ± 18 g; Charles River) were subjected to unilateral ET-1 injection into the left IC. Animals were randomly allocated to active (n = 15) or control (n = 15) treatment by an independent technician who prepared the injection materials accordingly and provided this in masked form to the researcher. Criteria were set to exclude animals that died before 11 days or that failed to demonstrate infarction within the IC territory. Analysis was thus conducted on a per-protocol basis. Investigators who measured infarct size with magnetic resonance imaging (MRI) and histology and scored behavioral data were blinded to treatment allocation. In a separate group of SHR, blood magnesium concentration and BP were determined after repeated injections of either vehicle (n = 4) or magnesium (n = 4). Magnesium levels were also determined in 2 adult Sprague-Dawley rats. A schematic illustration of the experimental protocol is shown in Figure 1A.

ET-1 Injection Into the IC

Animals were anesthetized with halothane (1.5%) in a mixture of O₂ and N₂O (30%:70%, vol/vol) delivered via face mask. Body temperature was maintained at 37 ± 0.5°C with a heating pad. Animals were placed in a stereotaxic frame, and a craniectomy was made with a temperature-controlled drill for placement of a 30-gauge needle into the left IC (posterior, 1.9 mm to bregma; lateral to midline, 7.0 mm; ventral from brain surface, 0.48 mm; angle, 25°; Figure 1B). The 25° angle prevented the needle track from passing through the cerebral ventricles and motor cortex. After ET-1 injection (200 pmol/μL, 0.05 μL/min, total volume 1 μL), the needle was left in place for 10 minutes. All animals survived the protocol, and no significant adverse effects were recorded. Despite precautions, 1 rat exhibited a cortical lesion and 1 rat displayed a thalamic lesion on MRI. These 2 rats were subsequently excluded from analysis. Final group sizes were 15 in the vehicle-treated group and 13 in the magnesium-treated group.

Magnesium Administration and Dose

The schedule of magnesium injections was determined from previous neuroprotective studies and analysis of magnesium plasma levels after repeated injections. Blood samples were collected via an externalized femoral artery cannula in animals subjected to ET-1 ischemia and repeated subcutaneous injections of magnesium. Plasma magnesium concentration was determined from blood samples collected at various time points after ischemia (15 minutes and every 30 minutes thereafter for 5 hours). The final dosing protocol was as follows: first injection of magnesium (300 mg/kg) 30 minutes before ET-1 with repeated injections of 200 mg/kg every hour thereafter for 4 hours.

T2-Weighted MRI

MRI was carried out 2 days after ET-1 injection on a Bruker Biospec 7-T MRI system. Anesthesia was induced with 5% and maintained with 1.5% halothane (in 30% O₂:70% N₂O, vol/vol). Rats were intubated and mechanically ventilated at 60 beats/min. Heart and respiration rates were monitored throughout the procedure, and body temperature was maintained at 37°C. A rapid-acquisition relaxation enhancement, T2-weighted sequence was used to determine the precise lesion location: rapid-acquisition relaxation enhancement factor 16, repetition time 5086 ms, echo time 70.1 ms with an in-plane resolution of 250 x 250 x 250 μm, and 15 slices. A second T2-weighted image set was acquired throughout the lesion: rapid-acquisition relaxation enhancement factor 16, repetition time 5086 ms, echo time 70.1 ms with an in-plane resolution of 117 x 117 x 500 μm with 25 contiguous slices. Infarct areas were manually delineated from the MRI images with the use of Paravision software and multiplied by the interslice distance to generate infarct volume.

Behavioral Tests

Both cylinder¹⁶ and walking-ladder¹⁷ tests were performed before ischemia to obtain baseline values and then on days 3 and 10 after ischemia. To limit variability, all behavioral tests were performed at the same time (11 AM to 3 PM) by the same investigator.

Cylinder Test

The cylinder test¹⁶ was used to assess forelimb use asymmetry. In brief, the rat is placed inside a perspex cylinder (diameter 20 cm, height 30 cm) for a maximum of 10 minutes or 20 rears. No habitation to the cylinder before the experiment was allowed. All sessions were video-recorded for subsequent analysis. This test was
Walking-Ladder Test

This test was developed to assess fine motor deficits precisely17 and requires rats to cross an elevated horizontal ladder (length, 1 m) of irregularly spaced rungs (diameter, 1 mm). The irregular pattern limits the rat’s ability to learn the rung arrangement. All trials were recorded on video and visualized image by image to determine each paw placement score. Scores were assessed as previously described17: 0=total miss, 1=deep slip, 2=slight slip, 3=re-placement, 4=correction, 5=deep drift, and 6=correct placement. An error was defined as a score of 0, 1, or 2 and a slight error, as a score of 3, 4, or 5. Animals were handled regularly before behavioral testing and trained to cross the ladder before test sessions. Each rat was tested on 5 different irregular rung patterns during each session.

Histology Analysis

Animals were euthanized and transcardially perfused with heparinized saline followed by 4% paraformaldehyde 11 days after ischemia. Brains were processed and embedded in paraffin wax, and 6-μm sections throughout the lesion were stained with Luxol Fast Blue for analysis of white matter damage. Ischemic damage was quantified microscopically on 13 sections throughout the lesion, with infarct volume calculated from infarct areas and the distance between measured sections.

Statistical Analysis

Infarct volumes are expressed as median and interquartile range and were compared between treatment groups by 2-sample t tests. There was no significant difference in plasma magnesium levels between Sprague-Dawley (SD) rats and SHR. ANCOVA after adjusting for baseline values. All behavioral data are presented as mean±SEM.

Results

Effect of MgSO₄ on Plasma Magnesium Level and BP

The dosing regimen maintained mean plasma magnesium levels >1.40 mmol/L18 over the course of the injection schedule (the Table) and induced a modest but significant hypotensive effect in SHR (Figure 2).

ET-1–Induced Ischemic Damage in the IC

ET-1–induced ischemic damage, measured on day 2 by T2-weighted MRI (Figure 3A), was not significantly different between the vehicle (median 1.6 mm³, interquartile range 1.2 to 2.1, n=15) and magnesium-treated (2.1 mm³, 1.3 to 3.8, n=13) groups (Figure 3B). Correct lesion localization in each animal was confirmed by histology on day 11 (Figure 3C). The lesion size as assessed by histology was not significantly different between groups but was significantly decreased compared with day 2 in both vehicle (0.3 mm³, 0.2 to 0.5, P<0.0001) and MgSO₄–treated (0.3 mm³, 0.2 to 0.9, P<0.001) groups (Figure 3B). In both groups, the MRI lesion volume calculated on day 2 was significantly correlated with the histology lesion volume on day 11: vehicle (P<0.01) and magnesium (P<0.001; Figure 3D).

Cylinder Assessment of Forelimb Asymmetry

Before ischemia, forelimb use was ≈50% for “both” forelimbs simultaneously and 25% use for each individual forelimb (left or right forelimb alone) in both vehicle and

Table. Plasma Levels of Mg²⁺ (mmol/L) After Repeated MgSO₄ Injections

<table>
<thead>
<tr>
<th>Time</th>
<th>First Injection (300 mg/kg)</th>
<th>Second Injection (200 mg/kg)</th>
<th>Third Injection (200 mg/kg)</th>
<th>Fourth Injection (200 mg/kg)</th>
<th>Fifth Injection (200 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.54±0.06‡</td>
<td>1.75±0.02‡</td>
<td>1.68±0.06‡</td>
<td>1.99±0.02‡</td>
<td>1.85±0.30‡</td>
</tr>
<tr>
<td>2 Hours</td>
<td>1.29±0.08‡</td>
<td>2.03±0.25‡</td>
<td>2.14±0.12‡</td>
<td>1.61±0.02‡</td>
<td>0.92±0.08*</td>
</tr>
<tr>
<td>6 Hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
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Normal range is 0.66–0.95 mmol/L.36 Statistical differences for each strain compared with baseline: *P<0.05, †P<0.01, ‡P<0.001 (repeated-measures ANOVA, Fisher’s protected least significant difference test). There was no significant difference in plasma magnesium levels between Sprague-Dawley (SD) rats and SHR.
magnesium groups (Figure 4A). ANOVA with repeated measures revealed a significant modification of forelimb use in the vehicle group after the lesion, with a decrease in “both” forelimb placements at day 3 (P<0.02) and day 10 (P<0.003) compared with baseline and an increase of left forelimb use at both time points (P<0.02), whereas there was no modification of forelimb use after ischemia in the magnesium group (Figure 4A). Magnesium treatment provided a significant improvement of forelimb use after ischemia compared with the vehicle group on both the left forelimb (P<0.01) and “both” forelimb (P<0.03) use, as revealed by ANCOVA.

Figure 4B shows the variation in the total number of forelimb placements in the cylinder. IC ischemia induced a decrease in the total number of placements in the vehicle group (~15%, P<0.03), whereas there was no significant improvement of forelimb use after ischemia compared with the vehicle group on both the left forelimb (P<0.01) and “both” forelimb (P<0.03) use, as revealed by ANCOVA.
of MgSO4 on white matter ischemia with these outcome measures.

Specific white matter damage in the IC was induced by ET-1 injection in SHR. Early MRI assessment of infarct location and size was confirmed by late histologic evaluation and behavioral assessment revealing a measurable motor deficit. This model has subsequently been used to test the influence of MgSO4 on white matter ischemia with these outcome measures.

ET-1 is one of the most potent vasoconstrictors, and its local administration produces ischemic injury by a prolonged but reversible reduction of local blood flow. ET-1 has consequently been used as a tool to induce localized focal cerebral ischemia in rats. Models have been developed with ET-1 to induce ischemia within a particular artery territory, (eg, stereotaxic administration adjacent to the middle cerebral artery, and topical administration onto the exposed middle cerebral artery), with the extent of the damage related to the concentration of ET-1 administered. Frost et al subsequently administered ET-1 into the IC of Sprague-Dawley rats to produce a white matter lesion with effects on behavioral deficits on placing and sensorimotor tests, which we adapted for use in the SHR. However, this required the design of a new set of stereotaxic coordinates because SHR have enlarged ventricles, and adverse effects of ET-1 were encountered when the needle track passed through the cerebral ventricles. These new stereotaxic coordinates also avoided any needle track damage to the sensorimotor cortex.

With this approach, ET-1 induced a small but reproducible lesion that was quantified by early T2-weighted MRI. Because we included a vehicle control for the magnesium study but not a sham control, we cannot discount the possibility that mechanical damage, unrelated to ischemia, was responsible for or contributed to the lesion. However, in the study of Frost et al, a surgery control group (1-μL vehicle injection into the IC) showed no histologic damage or behavioral deficits. Therefore, the lesion identified in the current study is more likely a consequence of ET-1–induced ischemia rather than mechanical damage induced by the injection needle or volume.

MRI did not provide sufficient resolution to determine whether the damage was located precisely within the white matter or whether any damage extended into the neighboring gray matter. Therefore, histlogic staining, specific for white matter (Luxol Fast Blue), was used to confirm the localization of the lesion to the IC in both groups. Lesion volumes as assessed by histology were significantly smaller compared with those derived from MRI. This is most likely due to a combination of the resorption of acute brain edema by day 11 and brain shrinkage due to dehydration of the brain during histologic processing.

Our lesion model revealed significant motor deficits of both the forelimb and hindlimb as measured by specific motor tests. The cylinder test showed forelimb use impairments during the 10 days after lesion induction. The walking-ladder test provided evidence of hindlimb placement impairments during this same period.

Although magnesium decreased BP, it did not significantly influence lesion size but produced a beneficial effect on functional outcome on both the forelimbs and the right hindlimb compared with the vehicle group. The significant effect of magnesium was more pronounced in the magnesium group. This difference between groups at day 3 was statistically significant (P < 0.03). At day 10, the right hindlimb score was not different from baseline in the 2 groups. The specificity of the motor deficit induced by left IC ischemia was confirmed by the absence of left-side deficits, on either forelimb or hindlimb placement. The left hindlimb score was increased compared with baseline at day 3 in the vehicle group (P < 0.01) and at day 10 in the magnesium group (P < 0.003) (Figure 5).

Discussion

Specific white matter damage in the IC was induced by ET-1 injection in SHR. Early MRI assessment of infarct location and size was confirmed by late histologic evaluation and behavioral assessment revealing a measurable motor deficit. This model has subsequently been used to test the influence of MgSO4 on white matter ischemia with these outcome measures.
Several studies have demonstrated that magnesium crosses the blood-brain barrier in both animals and humans. Transport from the blood to the cerebrospinal fluid is regulated by active transport that maintains cerebral concentrations higher than those in serum (1.1 mmol/L vs 0.8 mmol/L in the rat). Previous rodent studies have revealed that systemic administration of magnesium leads to an increase in the cerebrospinal concentration of up to 25% for 6 hours after treatment. Even if the cerebrospinal concentration increases only modestly, there is preferential uptake of magnesium by pathologic regions, including those affected by focal ischemia (eg, hippocampus and cortex), resulting in magnesium levels sufficient to inhibit N-methyl-D-aspartate (NMDA) receptors. Furthermore, intracellular free magnesium concentrations may be increased by dissociation from ATP after stroke. Taken together, even small elevations of extracellular magnesium concentration may lead to a protective effect.

The mechanisms involved in white matter damage and functional recovery are still inadequately understood, and therefore, we can only hypothesize regarding the possible mechanisms for the observed magnesium-associated preservation of motor function. Magnesium-induced neuroprotection could include both vascular and neuroglial mechanisms. In spinal cord injury models, magnesium-induced vasodilation may improve blood flow and reduce vaso spasms. Several mechanisms for the vascular effects of magnesium have been proposed, including antioxidant actions, blockade of voltage-dependent calcium channels, release of NO, and inhibition of ET-1 vasoconstriction. However, if the severity or duration of ischemia were reduced by magnesium, one would expect a reduction in infarct volume, and this was not apparent in our data.

Magnesium may provide cellular protection via a number of potential mechanisms. First, protection of oligodendrocytes, the main cell type constituting white matter, may occur via voltage-dependent blockade of ion flux through the glutamateergic NMDA receptor. Oligodendrocytes express several subunits of the NMDA receptor. Therefore, magnesium could contribute to white matter integrity by blocking NMDA receptors involved in excitotoxic damage. Second, magnesium inhibits the sodium–calcium ion exchanger, a major mediator of calcium influx in neurons and therefore excitotoxic damage independent of the early action of glutamate on NMDA receptors. However, this hypothesis requires further investigation to confirm the presence of transient receptor potential melastatin receptors in white matter and the inhibitory effect of magnesium on these receptors in vivo. A beneficial effect of magnesium via an effect on gray matter could also be involved, because axotomy at the level of the IC induces death of nearly 50% of corticospinal neurons within the first week. In the brain, 80% of intracellular magnesium is bound to ATP, and magnesium is an essential cofactor in the activation of ATPases and is consequently involved in energy-dependent pathways and protein synthesis. Because magnesium is involved in ATP regeneration after ischemia, its cerebroprotective action may be attributed to maintaining ATP levels during periods of relative hypoxia. Therefore, the beneficial effect of magnesium could extend to an influence on neuronal survival via a direct effect on the neuronal cell body in the cortex.

In conclusion, stereotaxic injection of ET-1 into the IC of SHR resulted in a small, reproducible white matter lesion and a measurable motor deficit in the contralateral forelimb and hindlimb. In this model of cardiovascular risk, systemic magnesium administration induced modest hypotension with no change in infarct size and an improvement in motor deficit, supporting further research on the potential utility of magnesium for the treatment of lacunar infarct.

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Disclosures
None.

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