Apoptotic Change in Response to Magnesium Therapy after Moderate Diffuse Axonal Injury in Rats

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INTRODUCTION

Diffuse axonal injury (DAI) occurs in nearly half of all severe cases of clinical traumatic brain injury. Assessment of the extent of brain damage and prediction of outcome are relatively difficult in the early stages. 1-3 Although it was once believed that DAI occurred as a direct result of tensile forces of the initial trauma, it is now recognized to be a delayed process of progressive neurochemical changes which lead to disconnection. 3-7 Although aggressive strategies regarding diagnosis and treatment of head injury based on control of intracranial pressure and cerebral blood flow are being advocated, the degree of morbidity and mortality continues to remain rather high. 3 This implies that known biochemical indicators may not be sensitive enough to forecast the degree of head injury or its response to treatment. It has been known for sometime that bioenergetic brain changes are a direct reflection of the cellular injury process. Much attention has been focused on the relationship between bioenergetic ion changes and the ultimate neurological outcome. 8-11 Several previous studies have demonstrated that a decrease in bioenergetic brain status occurs after trauma. This is associated with declines in both brain total and free magnesium concentration (tMg, Mg\textsuperscript{2+}). Moreover, alleviation of the magnesium decline by pharmacological means tends to improve the posttraumatic bioenergetic state, as well as the subsequent neurological outcome. Interventions that exacerbate magnesium deficits also exacerbate posttraumatic neurological dysfunction. 12-15 Magnesium is now widely recognized for its importance in a wide variety of critical cellular processes, including glycolysis.

Key Words: Ionized magnesium, ionized calcium, apoptosis, MgSO\textsubscript{4}
oxidative phosphorylation, cellular respiration, and protein synthesis. Furthermore, it is essential to many enzymatic reactions, membrane integrity, and ATPase function. Therefore, a change in magnesium homeostasis may affect some or all of these functions.

An increase in calcium flux has been proposed to be a common final pathway leading to cell death subsequent to both ischemic and traumatic injuries to the central nervous system (CNS). It is generally assumed that Ca\(^{2+}\) enters the cells through voltage-dependent calcium channels following membrane depolarization due to energy failure, and through N-Methyl-D-Aspartate channels (NMDA) operated by glutamate and aspartate. Consequently, the neuronal cell may lapse into cell death subsequent to a large Ca\(^{2+}\) accumulation. Furthermore, Mg\(^{2+}\) may also inhibit excitatory amino acid neurotransmitter (glutamate) release from presynaptic terminals by means of a blockade of voltage-gated calcium channels.

In many cases, intranucleosomal DNA fragmentation has been described, supporting the hypothesis that DNA fragmentation may be a component integral to the apoptotic cell death process in neurons. TUNEL staining and subsequent quantitative analysis provide a tool to localize DNA fragmentation within the cortex of brain architecture.

Recently, phosphorus magnetic resonance spectroscopy (MRS) has been revealed as especially useful for characterizing temporal changes in Mg\(^{2+}\) concentration after traumatic brain injury. While the post-traumatic changes in brain magnesium homeostasis have been acceptably characterized, few studies have provided an accurate description of the nature of free magnesium homeostasis outside of the brain. We hypothesized that a change in Mg\(^{2+}\) and Ca\(^{2+}\) serum concentrations could also provide valuable information related to post-traumatic histological changes following traumatic brain injury and could perhaps be of prognostic significance. Moreover, such a technique would be more practical and cost-effective in the clinical setting than the expensive MRS techniques currently used in all determinations of free magnesium homeostasis after experimental brain injury.

Our study was fueled by three main objectives: first, to determine whether head trauma results in changes in serum Mg\(^{2+}\) and Ca\(^{2+}\) concentration early after brain injury; second, to determine correlation of these changes in relation to divalent cation levels to the degree of apoptotic change; and third, to determine whether magnesium treatment improves apoptotic change.

**MATERIALS AND METHODS**

**Injury device and induction of head trauma**

We used the trauma device introduced by Marmarou and colleagues (Fig. 1). The trauma device consisted of a column of brass weights which fell freely onto a metallic helmet fixed with dental acrylic to the skull vertex of a rat. To ensure high acceleration upon impact, the head was lightly supported to allow for rapid displacement after impact. Each brass weight weighed 50 grams, and they were threaded so that they could be connected to deliver a falling weight ranging from 50 to 500 gm. We previously reported that 450 gm - 2 m impact was considered to be the upper limit for producing severe head trauma. In order to induce a moderate head injury, the energy delivery was reduced to 50% by lowering

![Diagram demonstrating the ratio of the serum iMg/TMg and iCa/iMg level between the times elapsed after trauma.](image_url)
the height to 1 m.
A total of 64 adult Sprague-Dawley rats, each weighing 300 to 350 g, were anesthetized with intraperitoneal injections of Nembutal (pentobarbital, 35 mg/kg). The animals were allowed to breathe spontaneously without tracheal intubation. A midline scalp incision was performed followed by peristomal elevation, which exposes the central area of the skull vault between the coronal and lambdoid sutures. The helmet was crafted from a stainless-steel disc, 1 cm in diameter. After drying the exposed area of the skull vault, the helmet was firmly fixed using dental acrylic. The weight was allowed to drop freely onto the disc from the predetermined height through a Plexiglas tube. Rebound impact was prevented simply by sliding the foam bed containing the animal away from the tube immediately following the initial impact. Rats which died on impact and those suffering skull fractures were eliminated from this study.

**Treatment protocol**

Animals (n=64) were randomly assigned to two groups to receive an intramuscular bolus of MgSO<sub>4</sub> (750 μmol/kg treated group, n=32) and equal volume saline (control group, n=32) at 30 min after induction of mDAI. These doses and single-injection protocol were chosen based on previously published experimental work. Arterial blood samples (0.5 ml) were collected directly into a therumo 1 ml syringe for to determine the normal level prior to injury in both groups and the rats were randomly divided into eight subgroups for checking the Mg<sup>2+</sup> serum levels at 30 min, 1 h, 2 h, 3 h, 6 h, 12 h, 24, and 48 h after injury. Immediately after collection, blood was analyzed using a NOVA biomedical Ultra M3 free magnesium analyzer (NOVA Biomedical Instruments Walthan, MA, USA). The control solution contained a standard concentration of ionized magnesium (0.50-0.64 mmol/L), ionized calcium (1.02-1.18 mmol/L), sodium (129.5-137.5 mmol/L), potassium (4.20-4.60 mmol/L) and pH (7.418-7.468) and was used twice daily to verify instrument performance.

**Histological staining (TUNEL stain)**

The brains were removed from the skulls and immersed in fixative for 24 hr at 4°C. After fixation, all brains were paraffin embedded and cut in 6 μm sections. 6 μm coronal sections at 3.5-3.8 mm posterior to bregma were adhered to poly-L-lysine-coated slides by heating them at 60°C for 15-20 min. After deparaffinization and rehydration, the tissue was digested for 15 min in proteinase K (20 μg/ml; DAKO S3020), followed by incubation with 3% H<sub>2</sub>O<sub>2</sub> solution to prevent activation of endogenous peroxidase. The reaction was terminated with tap water, and the tissue was treated with phosphate buffered saline (PBS) for at least 5 min. Sections were incubated at 37°C in PBS for 1 hr in a humid chamber with a labeling solution containing TdT (0.3 U/ml; Boehringer Mannheim, Indianapolis, IN). After washing with PBS for 5 min, the tissue was counterstained with hematoxylin and mounted in a crystal medium.

Measured values of each animal were pooled from the three slides in order to determine the total number of cells detected by the TUNEL stain and, in adjacent sections, for H & E. An average total cell count was calculated from the three slides at each time point. The data was analyzed as the fraction of the total number of cells detected by H & E which stained positively for TUNEL (AI).

**Analysis and statistical methods**

Mean values were calculated for serum Mg<sup>2+</sup>, total Mg (tMg), Ca<sup>2+</sup>, Ca<sup>2+</sup>/Mg<sup>2+</sup>, and the percentage of ionized Mg [(Mg<sup>2+</sup>/tMg) × 100]. Mean values ± SD were compared for statistical significance using Student’s t-test and ANOVA with Scheffe’s contrast test. A p-value of < 0.05 was considered significant.

**RESULTS**

Prior to injury, mean serum Mg<sup>2+</sup> concentration across all groups was 1.07 ± 0.04 mg/dL (0.44 ± 0.05 mM). This value is slightly lower than those previously published by Health and Vink (0.47 ± 0.04 mM). Consistent with previous results,
moderate diffuse axonal injury resulted in a significant decline ($p<0.05$) from the preinjury level of 1.07 ± 0.04 mg/dL to 0.73 ± 0.01 mg/dL within 1 hour after trauma. Thereafter, the serum ionized magnesium remained significantly depressed ($p<0.05$) with a mean value of 0.84 ± 0.04 mg/dL recorded over the 3 hours following injury. By 12 hours posttrauma, the serum ionized magnesium concentration had returned to preinjury levels.

In contrast to Mg$^{2+}$ concentration, examination of tMg failed to distinguish any significant difference between preinjury and postinjury state. The tMg concentration within 3 hours after injury had declined by only 2%. At the same time however, a decline in the amount of serum Mg$^{2+}$ relative to tMg (Mg$^{2+}$/tMg) represents about 18%. These relative changes indicate that small adjustments in tMg may reflect large changes in Mg$^{2+}$ concentration. After 12 hours, Mg$^{2+}$ and tMg concentration had both returned to preinjury values (Table 1, Fig 1). These studies reveal that acute head injury is associated with graded deficits in serum Mg$^{2+}$, but not in serum tMg. This indicates that there is a predictive value of ionized serum observed in head injuries, but this is not true of total magnesium levels in head injuries.

Diffuse axonal injury was also connected to early deficits in Ca$^{2+}$, which resulted in about 10% depression of Ca$^{2+}$ at 1 hour after injury. Mean Ca$^{2+}$ concentration within 3 hours after injury had declined by 9%. However, a significant rise in the amount of Ca$^{2+}$ relative to Mg$^{2+}$ (Ca$^{2+}$/Mg$^{2+}$ ratio) was observed at 1 hour (about 1.3 times preinjury level; $p<0.05$), remaining significantly increased over the following 3 hours, with a mean value of 5.68 (122% of preinjury level) recorded. This was followed at 6 hours after injury by a return to preinjury levels (Table 1, Fig 1). Although the ionized calcium decreased early on, the ionized magnesium decreased more than the ionized calcium. These results allow one to deduce that the greater the injury, the greater the Ca$^{2+}$/Mg$^{2+}$ ratio. The Ca$^{2+}$/Mg$^{2+}$ ratio is more reliable than the Ca$^{2+}$ concentration.

The mean arterial blood pressure in all animals had returned to approximately preinjury levels within 15 min. of the injury (103 ± 4 mmHg), which is consistent with previous reports in this model. A subsequent intramuscular injection of MgSO$_4$ never produced more than an insignificant 5% change to this value.

A reduction in Mg$^{2+}$ concentration in tissues may contribute to secondary injury through this mechanism. However, none of the other serum analytes measured, including sodium (145–148 mmol/L), potassium (4.2–4.4 mmol/L), and hydrogen ions (pH range=7.39–7.47), demonstrated any significant abnormalities when compared to controls. Baseline blood glucose concentration and those obtained 24 to 40 hours later did not differ. Although blood sugar was slightly increased at 3 hours posttrauma, it was not considered statistically significant ($p>0.05$).

Table 1. Serum Ionized Magnesium (Mg$^{2+}$), Calcium (Ca$^{2+}$), Total Magnesium (tMg), and Ratio of Mg$^{2+}$/tMg, Ca$^{2+}$/Mg$^{2+}$ after Moderate Diffuse Axonal Injury (mDAI)

<table>
<thead>
<tr>
<th></th>
<th>Mg$^{2+}$(mg/dL)</th>
<th>tMg</th>
<th>Mg$^{2+}$/tMg</th>
<th>Ca$^{2+}$</th>
<th>Ca$^{2+}$/Mg$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>1.07 ± 0.03</td>
<td>1.51 ± 0.23</td>
<td>0.71</td>
<td>4.98 ± 0.09</td>
<td>6.65</td>
</tr>
<tr>
<td>1 hr</td>
<td>0.79 ± 0.03</td>
<td>1.49 ± 0.16</td>
<td>0.49*</td>
<td>4.48 ± 0.12*</td>
<td>6.14*</td>
</tr>
<tr>
<td>2 hr</td>
<td>0.79 ± 0.03*</td>
<td>1.48 ± 0.34</td>
<td>0.53*</td>
<td>4.54 ± 0.11*</td>
<td>5.61*</td>
</tr>
<tr>
<td>3 hr</td>
<td>0.84 ± 0.03*</td>
<td>1.49 ± 0.25</td>
<td>0.56*</td>
<td>4.56 ± 0.09*</td>
<td>5.34*</td>
</tr>
<tr>
<td>6 hr</td>
<td>0.99 ± 0.04*</td>
<td>1.50 ± 0.12</td>
<td>0.70</td>
<td>4.64 ± 0.12</td>
<td>4.52</td>
</tr>
<tr>
<td>12 hr</td>
<td>1.06 ± 0.01</td>
<td>1.51 ± 0.19</td>
<td>0.70</td>
<td>4.76 ± 0.08</td>
<td>4.58</td>
</tr>
<tr>
<td>24 hr</td>
<td>1.05 ± 0.03</td>
<td>1.50 ± 0.68</td>
<td>0.70</td>
<td>4.77 ± 0.10</td>
<td>4.65</td>
</tr>
<tr>
<td>48 hr</td>
<td>1.06 ± 0.02</td>
<td>1.52 ± 0.12</td>
<td>0.70</td>
<td>4.76 ± 0.14</td>
<td>4.64</td>
</tr>
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Values are means ± SD.

*p<0.05, compared with control group.
We then evaluated the neuroprotective properties of MgSO₄ in the posttraumatic treatment paradigm. Administration of an effective dose of MgSO₄ (750 μmol/kg, single injection) was initiated 30 min. after mDAI. The time-curve of the magnesium concentration in the rat serum increased rapidly from 1.07 ± 0.03 mg/dL (base level) to a maximum value of 2.57 ± 0.03 mg/dL at 2.5 hours after injection (Fig. 2). Subsequently, the Mg²⁺ level slowly dropped. Physiological levels were reached 11.5 hours after application.

Following impact-acceleration-induced trauma, the control animals demonstrated a significant histological increase in apoptotic changes as assessed by TUNEL staining. By 2 hours posttrauma, the animals showed no apoptotic changes, but at 3 hours after injury, these control animals showed AI of 18.8 ± 1.9 and demonstrated a significant increase in AI at 12 hours and 24 hours (54.8 ± 1.7, 51.5 ± 3.2, p<0.05) followed by a decrease of AI at 48 hours (23 ± 1.3) after injury (Table 2).

In contrast, animals treated with MgSO₄ at 30 min. post-injury had a 3-hour AI of 3.8 ± 2.1, which was significantly better than the AI recorded in the injured control rats (p<0.05). Moreover, these MgSO₄-treated animals demonstrated a significant improvement in AI over the assessment period of 24 hours (24.8 ± 2.6 at 12 hours, 20.5 ± 1.4 at 24 hours) when compared to the control animals (54.8 ± 1.7 at 12 hours, 51.5 ± 3.2 at 24 hours, p<0.05) after injury. Thereafter, the apoptotic index level declined at 48 hours after injury and no statistically significant difference remained between the treated group (23.5 ± 1.3) and the control group (13.5 ± 1.2) at that time (p>0.05, Table 2).

**DISCUSSION**

In this study, traumatic brain injury has been shown to result in a decrease in serum ionized magnesium concentration associated with the histological development of apoptotic change. Moreover, treatment with MgSO₄ at 30 min following drop-weight traumatic brain injury significantly improved histological apoptotic change for a variety of reasons. Due to the important regulatory role that Mg²⁺ plays in calcium transport and accumulation as nature’s physiological calcium blocker, alterations in intracellular Mg²⁺ could potentially exacerbate...
calcium-mediated neurotoxicity. In regards to the electrophysiological function of Mg$^{2+}$, extensive evidence has been reported that N-Methyl-D-Aspartate channels (NMDA), which are permeable to Ca$^{2+}$, Na$^+$, and K$^+$, are blocked by Mg$^{2+}$ in a voltage-dependent manner. Indeed, recent in vitro studies have show a reduction in the voltage dependent magnesium block of NMDA channels following neuronal injury. This reduction in the magnesium block appeared to be linked to a decreased sensitivity of the NMDA channel to magnesium, possibly due to reduced magnesium levels or to a change in the channel structure. Furthermore, Mg$^{2+}$ may also inhibit excitatory amino acid neurotransmitter (glutamate) release from presynaptic terminals due to a blockade of voltage-gated calcium channels. Subsequently, an abnormal elevation of internal Ca$^{2+}$ results in the autophosphorylization of calpain and the activation of phospholipase A2, which results in the breaking down of both the structural protein lining the lipid bilayer and the bilayer itself.

Others have reported altered glycolytic rates and increased phospholipid turnover following brain trauma. These metabolic alterations may be due to the modified Mg$^{2+}$ status of cells, following trauma. Additionally, magnesium also affects membrane structure and permeability, free radical formation, DNA synthesis, the ion ATPase, platelet aggregation and cerebral vascular tone. Recently, evidence has been presented which states that early ischemia after traumatic brain injury may be an important factor in the determination of neurological outcome. These known deficits in regional cerebral blood flow following early after head trauma may occur as a result of a deficit in Mg$^{2+}$ concentration. Memon et al. reported that the deficit in Mg$^{2+}$ in peripheral blood in patients subjected to head trauma may result in spasms of cerebral blood vessels in different regions of the brain. Hein and Vink reported that blood-free magnesium concentration may also provide valuable information regarding post-traumatic magnesium homeostasis following traumatic brain injury in the form of free magnesium homeostasis outside of the brain and correlating with posttraumatic motor deficits. It is not known, however, to what extent an increase in plasma Mg$^{2+}$ is reflected by increases in Mg$^{2+}$ levels at other sites, such as the walls of CNS vessels or the interstitial fluid of brain parenchyma.

We have studied serum tMg, Mg$^{2+}$, Ca$^{2+}$, and apoptotic change in rats treated with or without MgSO$_4$ following acute mDAI. Our studies have shown that acute mDAI is associated with early deficits in Mg$^{2+}$ (at 1 hour, $p<0.05$), but the examination of tMg has failed to illustrate any significant difference between the elapsed times after trauma ($p>0.05$). Although the mechanism of action is unclear, we theorized it may be due to the intact plasma membrane, which is relatively impermeable to divalent cations such as Mg$^{2+}$ during anoxia or hypoxia, becoming increasingly permeable to these ions after reoxygenation. Our results suggest that brain lesions promote deficits in Mg$^{2+}$, which may be a critical initial factor in the development of irreversible tissue damage.

In the present study, we found that there was also an early drop in the serum levels of Ca$^{2+}$ following acute head injury, but the early fall in the percentage of ionized magnesium in the serum was greater than that of ionized calcium. We thought that the Ca$^{2+}$/Mg$^{2+}$ ratio may be more important than Ca$^{2+}$. The rise in Ca$^{2+}$/Mg$^{2+}$ ratio observed in our study was significant between hours one and three post-injury. The rise in the Ca$^{2+}$/Mg$^{2+}$ ratio, particularly in the head injury cases, would cause an increased plasma Ca$^{2+}$ gradient across cerebral vascular smooth muscle cell membranes. This would result in an elevation in cellular free Ca$^{2+}$, which may, in turn, result in the spasm of cerebral blood vessels in different regions of the brain.

Serum hypotonicity of a mild degree is a common finding during the first 3-5 days after head injury; this is thought to be due to primary water retention in excess of salt retention. This water retention is thought to be due either to the release of antidiuretic hormone (ADH) or to inappropriate ADH secretion after head trauma. However, during the initial 8 hr when the Mg$^{2+}$ was measured in our subjects, none of the electrolytes (except Ca$^{2+}$) or biochemical analytes showed any abnormalities. If hemodilution (serum hypotonicity) had been responsible for our reduced Mg$^{2+}$ values, then other analytes should have been altered along with the tMg level.
Hypomagnesaemia could be resultant of an acid-base imbalance which caused shifts in pH levels. However, the blood H+ ion concentration in our study was not different from the time course after trauma (pH range = 7.39–7.47).

Heath and Vink reported that both MgSO4 and MgCl2 salts equally improved outcome within 1 week post-trauma, but MgSO4 may act more rapidly than MgCl2 to restore free magnesium concentration in the brain, and subsequently, improve neurological outcome. Although the reasons for this apparent difference are unknown, a recent paper demonstrating that CA1 injury is dependent upon the presence of extracellular chloride suggests that the chloride form of the salt may be less desirable than other salts, despite its advantage in terms of crossing lipid membranes. Although further studies are required to resolve this issue, we chose the MgSO4 based on that reasoning.

Health and Vink reported that, following administration of the MgSO4 dose given 30 minutes after trauma, intramuscular administration of MgSO4 at 12 hour intervals given over the 1 week posttraumatic assessment did not improve neurological motor outcome in rats when compared with single bolus (30 minutes) treated animals. Serum concentrations of 1.49 mmol/L and above have been neuroprotective in preclinical models of focal cerebral ischemia, and the doubling of serum concentration is known to be efficacious in the prophylaxis and treatment of seizures in pro- eclamptic and eclamptic women. Therefore, we thought that the doubling of serum concentration after single injection (750 μmol/kg, previously published) would be a reasonable goal for the optimization of dosing in the absence of any useful clinical markers in small pilot trials.

The present study indicates that although the decline in blood-free magnesium concentration seemed to follow a temporal pattern, histological evaluation revealed the preventive effects of early MgSO4 treatment against neuronal injury in the brain (AI was deceased about 2 to 5 times compared to control group between 6 h and 24 h after trauma). These observations support the hypothesis that reductions in brain magnesium after trauma may play a role in the pathophysiology of traumatic brain injury.

Our findings provide evidence for divalent cation changes in blood early after traumatic brain injury, which could be of considerable practical diagnostic value in the assessment of the severity of head injury, making estimations of prognosis in such patients more reliable. Use of this biochemical test could serve as a logical basis for monitoring the early reaction of head injuries to therapeutic intervention. Adequate trials in humans have not yet been reported, although the clinical utility of magnesium, e.g. in obstetrics (eclampsia), is proven. Although treatment with MgSO4 improves serum ionized magnesium concentration and has been associated with significant improvement in histological apoptotic changes, future studies will address the mechanism of action of Mg2+ treatment in CNS injury.

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