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

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The biochemical factors related to moderation of secondary or delayed damage to the central nervous system (CNS) remain undefined. We have recently demonstrated that the weight- drop induced moderate diffuse axonal injury (mDAI) in rats causes a rapid decline in serum ionized magnesium (Mg^{2+}) and a significant increase in the amount of serum ionized calcium (Ca^{2+}) relative to Mg^{2+} (Ca^{2+}/Mg^{2+}). For three hours, serum Mg^{2+} levels remained significantly depressed at 76% of preinjury values ($p<0.05$), but total serum magnesium remained unchanged (tMg, $p>0.05$). Head trauma resulted in a small decrease of Ca^{2+} (about 10%), but a significant increase in the amount of Ca^{2+}/Mg^{2+} (mean value in control group: in injured group for 3 hours after trauma =4.65 ± 0.012 : 5.69 ± 0.015, $p<0.05$) was observed. In order to further investigate the relationship between Mg^{2+} and brain injury, the effect of Mg^{2+} treatment on posttraumatic histological changes (apoptotic changes) was examined following the weight-drop induced brain injury. At 30 min postinjury, animals treated with $MgSO_4$ (750 μmol/kg) showed significant improvements of apoptotic changes when compared to the control group (54.8 ± 1.7, 51.5 ± 3.2 at 12, 24 h in control group, 24.8 ± 2.6, 20.5 ± 1.4 at 12, 24 h in treated group, $p<0.05$). The early decline in serum Mg^{2+} and the increase in the amount of Ca^{2+}/Mg^{2+} immediately following brain trauma uncovered by these findings suggest that they may be a critical factor in the development of irreversible tissue injury. If this proves to be the case, treatment with $MgSO_4$ may be effective in improving histological findings following experimental traumatic brain injury in rats.

Key Words: Ionized magnesium, ionized calcium, apoptosis, $MgSO_4$

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.[7] 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^{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.[10-15] Magnesium is now widely recognized for its importance in a wide variety of critical cellular processes, including glycolysis,[12,16,17]

oxidative phosphorylation,[12,18,19] cellular respiration,[12,20] and protein synthesis.[12] Furthermore, it is essential to many enzymatic reactions, membrane integrity,[12,13] and ATPase function.[20,21] 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).[5,22-24] It is generally assumed that Ca^{2+} enters the cells through both 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.[22,23,25-27] 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.[24,27,28]

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.[28,29]

Recently, phosphorus magnetic resonance spectroscopy (MRS)[21] 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 now 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).[30] 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
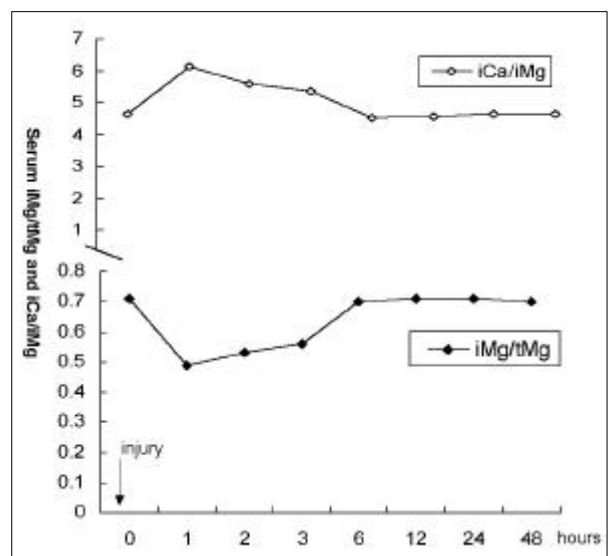

Fig. 1. Diagram demonstrating the ratio of the serum iMg/tMg and iCa/iMg level between the times elapsed after trauma.

the height to 1 m.

A total of 64 adult Sprague-Dawley rats, each weighing 300 to 350 gm, 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 periosteal 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, I 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 $MgSO_4$, (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.[31] 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^{2+} serum levls at 30 min, 1h, 2h, 3h, 6h, 12h, 24, and 48h 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℃. After fixations, all brains were paraffin embedded and cut in 6 μm sections. 6 μ m coronal sections at 3.5 - 3.8 mm posterior to bregma were adhered to poly-L-lysine-coated slides by heating them at 60℃ for 15 - 20 min. After deparaffination and rehydration, the tissue was digested for 15 min in proteinase K (20 μg/ml; DAKO S3020), followed by incubation with 3% H_2O_2 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℃ 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 SBS 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^{2+}, total Mg (tMg), Ca^{2+}, Ca^{2+}/Mg^{2+}, and the percentage of ionized Mg [$(Mg^{2+}/tMg) \times 100$]. Mean values ± SD were compared for statistical significance using Student's t-test and ANOVA with Scheffes' contrast test. A p-value of < 0.05 was considered significant.

RESULTS

Prior to injury, mean serum Mg^{2+} 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).[31] 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 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),[31] which is consistent with previous reports in this model. A subsequent intramuscular injection of MgSO₄ 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)

	Mg^{2+}(mg/dL)	tMg	Mg^{2+}/tMg	Ca^{2+}	Ca^{2+}/Mg^{2+}
0 hr	1.07 ± 0.03	1.51 ± 0.23	0.71	4.98 ± 0.09	4.65
1 hr	0.73 ± 0.01*	1.49 ± 0.16	0.49*	4.48 ± 0.12*	6.14*
2 hr	0.79 ± 0.03*	1.48 ± 0.34	0.53*	4.54 ± 0.11*	5.61*
3 hr	0.84 ± 0.03*	1.49 ± 0.25	0.56*	4.56 ± 0.09*	5.34*
6 hr	0.99 ± 0.04*	1.50 ± 0.12	0.70	4.64 ± 0.12	4.52
12 hr	1.06 ± 0.01	1.51 ± 0.19	0.70	4.76 ± 0.08	4.58
24 hr	1.05 ± 0.03	1.50 ± 0.68	0.70	4.77 ± 0.10	4.65
48 hr	1.06 ± 0.02	1.52 ± 0.12	0.70	4.76 ± 0.14	4.64

Values are means ± SD.
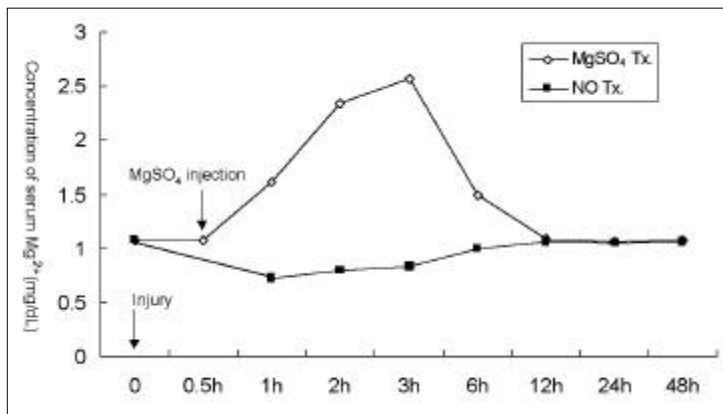*$p < 0.05$, compared with control group.

Fig. 2. Diagram demonstrating serum Mg^{2+} level with and without $MgSO_4$ treatment. About doubling of serum concentrations is shown at 2.5 hours, but return to preinjured level at 11.5 hours.

Table 2. Apoptotic Index (AI) to Elapsed Time after mDAI

	1 hr	2 hr	3 hr	6 hr	12 hr*	24 hr*	48 hr
No Tx[†]	0	0	18.8 ± 1.9	32.9 ± 1.8	54.8 ± 1.7	51.5 ± 3.2	23.5 ± 1.3
Tx	0	0	3.8 ± 2.1	18.9 ± 1.3	24.8 ± 2.6	0.5 ± 1.4	13.5 ± 1.2

*AI decreased by about 45% of no-treated group at 12 - 24 hours after $MgSO_4$ treatment.
Tx, treatment.

We then evaluated the neuroprotective properties of $MgSO_4$ in the posttraumatic treatment paradigm. Administration of an effective dose of $MgSO_4$ (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 Mg^{2+} 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 $MgSO_4$ 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 $MgSO_4$-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 $MgSO_4$ 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 Mg^{2+} plays in calcium transport and accumulation as nature's physiological calcium blocker, alterations in intracellular Mg^{2+} could potentially exacerbate

calcium-mediated neurotoxicity.[32-34] 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.[35,36] 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 autoproteolytic activation 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[37] and increased phospholipid turnover[38] 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,[39] free radical formation,[40] DNA synthesis,[41] the ion ATPase,[12,20,42] platelet aggregation[43] and cerebral vascular tone.[44,45] 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.,[14] 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 brain.

Heath and Vink[31] 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.[33] 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[46] 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.[47] Although further studies are required to resolve this issue, we chose the MgSO₄ based on that reasoning.

Health and Vink[32] 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 pre-eclamptic and eclamptic women.[48] Therefore, we thought that the doubling of serum concentration after single injection (750 μmol/kg, previously published)[32] 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 Mg^{2+} treatment in CNS injury.

REFERENCES

1. Becker DP, Miller JD, Ward JD, Greenberg RP, Young HF, Saka R. The outcome from severe head injury with early diagnosis and intensive management. J Neurosurg 1977;47:491-502.
2. Jennett B, Bond M. Assessment of outcome after severe brain damage: a practical scale. Lancet 1975;1:480-4.
3. McLaurin RL, King LR. Metabolic effects of head injury. In: Handbook of clinical neurology 1976;23:109-28.
4. Ellis EF, WrightKF, Wei EP, Kontos HA. Cyclooxygenase products of arachidonic acid metabolism in cat cerebral cortex after experimental concussive brain injury. J Neurochem 1981;37:892-6.
5. Hayes RL, Jenkins LW, Lyeth BG, Balster RL, Robinson SE, Clifton GL, et al. Pretreatment with phencyclidine, an N-methyl-D-aspartate antagonist, attenuates long-term behavioral deficits in the rat produced by traumatic brain injury. J Neurotrauma 1988;5:259-74.
6. Ikeda Y, Long DM. The molecular basis of brain injury and brain edema: the role of oxygen free radicals. Neurosurgery 1990;27:1-11.
7. Marshall LF. Current head injury research. Curr Opin Neurol Neurosurg 1990;3:4-9.
8. Vink R, Golding EM, Headrick JP. Bioenergetic analysis of oxidative metabolism following traumatic brain injury in rats. J Neurotrauma 1994;11:265-74.
9. Headrick JP, Bendall MR, Faden AI, Vink R. Dissociation of adenosine levels from bioenergetic state in experimental brain trauma: potential role in secondary injury. J Cereb. Blood Flow Merab 1994;14:853-61.
10. McIntosh TK, Vink R, Yamakami I, Faden AI. Magnesium protects against neurological defect after brain injury. Brain Res 1989;482:261-70.
11. Smith DH, Okiyama K, Gennarelli TA, McIntosh TK.

Magnesium and ketamin attenuate cognitive dysfuction following experimental brain injury. Neurosci Lett 1993;157:211-4.

12. Ebel H, Gunther T. Magnesium metabolism: a review. J Clin Chem Clin Biochem 1980;18:257-70.

13. Grubbs RD, Maguire ME. Magnesium as a regulatory cation: criteria and evaluation. Magnesium 1987;6: 113-27.

14. Memon ZI, Altura BT, Benjamin JL, Cracco RQ, Altura BM. Predictive value of serum ionized but not total magnesium levels in head injuries. Scand J Clin Lab Invest 1995;55:671-7.

15. Mauskop A, Altura BT, Cracco RQ, Altura BM. Deficiency in serum ionized magnesium but not total magnesium in patients with migraines. possible role of ICa^{2+}/IMg^{2+} ratio. Headache 1993;33:135-8.

16. Anderson BJ, Marmarou A. Post-traumatic slective stimulation of glycolysis. Brain Res 1992;585:184-9.

17. Garfinkel L, Garfinkel D. Magnesium regulation of the glycolytic pathway and enzymes involved. Magnesium 1985;4:60-72.

18. Abe K, Kogure K, Yamamoto H, Imazawa M, Miyamoto K. Mechanism of arachidonic acid liberation during ischemia in gerbil cerebral cortex. J Neurochem 1987;48:503-9.

19. Fagian MM, Dasilva LP, Vercesi AE. Inhibition of oxidative phosphorylation by Ca^{2+} or Sr^{2+}: a competition with Mg^{2+} for the formation of adenine nucleotide complexes. Biochem Biophys Acta 1996;85:131-7.

20. Jenkins Wt. Yeast mitochondrial F1-ATPase-effects of metal ions. J Cereb Blood Flow Metab 1991;11:1025-30.

21. Headrick JP, Bendall MR, Faden AI. Dissociation of adenosine levels from bioenergetic in experimental brain trauma: potential role in secondary injury. J Cereb Blood Flow Metab 1994;14:853-61.

22. Kudo Y, Ogura A. Glutamate-induced increase in intracellular Ca^{2+} concentration in isolated hippocampal neurones. Br J Pharmacol 1986;89:191-8.

23. Melloni E, Pontremoli S. The calpains. Trends Neurosci 1989;12:438-44.

24. Rothman SM. Synaptic activity mediates death of hypoxic neurons. Science 1983;220:536-7.

25. Tsuda T, Kogure K, Ishii K, Orihara H. Postischemic change of calcium and endogenous antiantagonist in the rat hippocampus studied by proton-induced x-ray emission analysis. Brain Res 1989;484:228-33.

26. Sakamoto N, Kogure K, Kato H, Ohtomo H. Disturbed Ca^{2+} homeostasis in the gerbil hippocampus following brief transient ischemia. Brain Res 1983;364:372-6.

27. Zhang L, Rzigalinski BA, Ellis EF, Satin LS. Reduction of voltage-dependent Mg^{2+} blockade of NMDA current in mechanically injured neurons. Science 1996;274: 1921-3.

28. Linnik MD, Miller JA, Sprinkle-Cavallo J, Mason PJ, Thompson FY, Montgomery LR, et al. Apoptotic DNA fragmentation in the rat cerebral cortex induced by permanent middle cerebral artery occlusion. Mol Brain Res 1995;32:116-24.

29. MacManus JP, Buchan AM, Hill IE, Rasequinha I, Preston E. Global ischemia can cause DNA-fragmentation indicative of apoptosis in rat bran. Neurosci Lett 1993;164:89-92.

30. Marmarou A, Montasser A, Foda MA. A new model of diffuse brain injury in rats. Part 1: pathophysiology and biomechanics. J Neurosurg 1994;80:291-300.

31. Heath DL, Vink R. Blood-free magnesium concentration declines following graded experimental traumatic brain injury. Scand J Clin Lab Invest 1998;58:161-6.

32. Heath DL, Vink R. Improved motor outcome in response to magnesium therapy received up to 24 hours after traumatic diffuse axonal brain injury in rats. J Neurosurg 1999;90:504-9.

33. Zoppi F, Degasperi A, Guagnellini E, Marocchi A, Mindo E, Pazzucconi F, et al. Measurement of ionized magnesium with AVL 188/4 electrolyte analyzer-preliminary analytical and clinical results. Scand J Clin Lab Invest 1996;56:259-74.

34. Iseri LT, French JH. Magnesium: Nature's physiologic calcium blocker. Am Heart J 1984;108:188-93.

35. Ogura A, Miyamoto M, Kudo K. Neuronal death in vitro: parallelism between survivability of hippocampal neurons and sustained elevation of cytosolic Ca^{2+} after exposure to glutamate receptor agonist. Exp Brain Res 1988;73:447-58.

36. Ascher P, Nowak L. The role of divalent cations in the N-methyl-D-aspartate responses of mouse central neurons in culture. J Physiol 1988;339:247-66.

37. Rosner MJ, Becker DP. Experimental brain injury: successful therapy with the weak base, tromethamine. With an overview of CNS acidosis. J Neurosurg 1984;60:961-71.

38. Kontos HA, Wei EP. Superoxide production in experimental brain injury. J Neurosurg 1986;64:803-7.

39. Bara M, Guiet-Bara A. Potassium, magnesium and membranes. review of present and new findings. Magnesium 1984;3:212-25.

40. Gunther T, Hollrieg IV, Vormann J, Bubeck J, Classen HG. Increased lipid peroxidation in rat tissues by magnesium deficiency and vitamine E depletion. Magnes Bull 1994;16:38-43.

41. Rubin H. Specificity of the requirement of magnesium and calcium in the growth and metabolism of chick embryo fibroblast. J Cell Physiol 1977;91:449-58.

42. Hayashi H, Chaudry IH, Clemens MG, Hall MJ, Baue AE. Reoxygenation injury in isolated hepatocytes: effect of extracellular ATP on cation homeostasis. Am J Physiol 1986;63:R573-9.

43. Gawaz M, Ott I, Reininger AJ, Neumann FJ. Effects of magnesium on platelet aggregation and adhesion-magnesium modulates surface expression of glycoproteins on platelets *in vitro* and *ex vivo*. Thromb Haemost 1994; 72:912-8.

44. Altura BT, Altura BM. The role of magnesium in etiology of strokes and cerebrovasospasm. Magnesium 1982;1:277-91.

45. Seelig JM, Wei EP, Kontos HA, Choi SC, Becker DP.

Effect of changes in magnesium ion concentration on cat cerebral arterioles. Am J Physiol 1983;245:22-6.

46. Health DL, Vink R. Neuroprotective effects of $MgSO_4$ and $MgCl_2$ in closed head injury: A comparative phosphorus NMR study. J Neurotrauma 1998;15:183-9.

47. Wallis RA, Panisson KL. Ionic mechanisms of CA1 traumatic neonatal injury. [abstract] J Neurotrauma 1996;13:626.

48. Muir KW, Lees KR. Dose optimization of Intravenous magnesium sulfate after acute stroke. Stroke 1889;29: 918-23.