Magnesium dietary manipulation and recovery of function following controlled cortical damage in the rat

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Abstract. Previous research has shown that dietary magnesium (Mg2+) deficiency prior to injury worsens recovery of function and that systemic administration of Mg2+ pre or post-injury significantly improves functional recovery. The purpose of the present study was to determine if manipulations in dietary Mg2+ would alter functional recovery following unilateral cortical injuries. Two weeks prior to injury, rats were placed on a customized diet enriched with Mg2+, deficient in Mg2+, or on a standard Mg2+ diet. Rats were then prepared with unilateral cortical contusion injuries (CCI) of the sensorimotor cortex. Two days following CCI, rats were tested on a battery of sensorimotor (vibrissae-forelimb placing and bilateral tactile adhesive removal tests), as well as the acquisition of reference memory in the Morris water maze. Serum analysis for Mg2+ prior to injury showed a diet-dependent modulation in levels. The Mg2+-enriched diet showed significantly higher levels of serum Mg2+ compared to the normal diet and the Mg2+-deficient diet showed significantly lower levels compared to the Mg2+-normal diet. On the placing and tactile removal tests Mg2+ deficiency significantly worsened recovery compared to the Mg2+-enriched and Mg2+-normal diet conditions. There were no statistically significant differences between the Mg2+-normal and Mg2+-enriched diets on the sensorimotor tests. On the acquisition of reference memory there were no significant difference between diet conditions; however, the Mg2+-deficient diet showed a trend toward impaired performance compared to the other diet conditions. The Mg2+-deficient diet resulted in a larger lesion cavity compared to the other diet conditions. These findings suggest that dietary Mg2+ modulates recovery of function.

Key words: diet, behavior, magnesium, neuroprotection, sensorimotor, TBI, recovery of function

It is well established that Mg2+ is essential for maintaining normal cellular functions such as glycolysis [1], maintaining membrane structure and function [2], protein synthesis and DNA replication [3, 4]. Mg2+ also plays a vital role in the pathophysiological events that occur following injury to the central nervous system (CNS). One of these events is the disruption of normal Mg2+ homeostasis, which has been shown to be very detrimental. Vink and colleagues were the first to identify the disruption of Mg2+ homeostasis following CNS injury [5-7]. These studies have shown that fluid percussion injury (FPI) produced a rapid and severe decline in intra- and extracellular Mg2+ levels, which correlated significantly with the severity of the behavioral deficits observed following injury [5-8]. Heath and Vink have also shown that after severe impact-acceleration injury intracellular levels of free Mg2+ decline for four days post-injury and reach pre-injury levels again by the sixth day [9].

Mg2+ pharmacotherapy has been found to be an effective treatment in models of ischemia, FPI and cortical lesions. For example, it has been shown that administration of magnesium chloride (MgCl2)
following focal cortical injuries significantly improved behavioral outcome and reduced the amount of lesion induced tissue damage [10]. It has also been shown that MgCl₂ facilitated behavioral recovery following lesions that produce chronic impairments. MgCl₂ induced recovery of forelimb placing following large cortical injuries that produced chronic impairments in untreated animals [11, 12]. Similarly, previous research has found that rats treated with daily injections of Mg²⁺ prior to an electrolytic lesion of the sensorimotor cortex (SMC) exhibited improved recovery of function when compared to those treated with saline [13]. Administration of magnesium sulphate (MgSO₄) or MgCl₂ has also been shown to improve functional outcome following FPI and diffuse axonal injury [5, 9, 14-19].

Given the fact that Mg²⁺ is a vital nutrient it might be expected that manipulation of dietary Mg²⁺ levels would have an impact on recovery of function following brain injury. A hallmark study by McIntosh and colleagues administered a Mg²⁺-deficient diet to rats for 14 days prior to FPI [5]. It was found that this diet reduced brain Mg²⁺ levels by 15% and resulted in a 53% mortality in the Mg²⁺-deficient group. It was also found that this diet significantly impaired the functional neuroscore assessment for 4 weeks following the injury. A Mg²⁺-deficient diet has also been shown to exacerbate alcohol-induced stroke fatalities [20]. Furthermore, several recent studies have shown that Mg²⁺ deficiency impairs fear conditioning. In these studies, a Mg²⁺-deficient diet (2-3 weeks) resulted in significant memory deficits in both contextual and cued conditioning tests; as well as increasing N-methyl-D-aspartate (NMDA) hyperfunction [21, 22]. This hyperfunctioning of the NMDA receptor should result in worsened behavioral outcome following TBI. Although, it has been shown that Mg²⁺ deficiency prior to injury worsened behavioral outcome it has yet to be determined how generalizable this effect is. For example, does it disrupt multiple behavioral systems (sensorimotor, motor or cognitive) and what is the neuropathological effect? Furthermore, can a Mg²⁺-enriched diet improve functional outcome when fed prior to injury?

The purpose of the present experiment was to examine the effect of dietary Mg²⁺ manipulation on recovery of function. Rats were placed on one of three different diets (Mg²⁺-normal, Mg²⁺-enriched, or Mg²⁺-deficient) for 2 weeks prior to receiving cortical injuries. Behavioral testing was conducted to assess sensorimotor and cognitive performance. This study will aid in the understanding of the relationship between Mg²⁺ dietary manipulations and behavioral outcome following brain injury.

### Materials and methods

#### Subjects

Forty male Sprague-Dawley rats (weighing 275-350 g) were used as subjects. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee. Rats were maintained on a standard 12-h light/dark cycle with food and water available *ad libitum*.

#### Diet Manipulation

Experimental diets were purchased from Harlan Teklad (Madison, WI). The Mg²⁺-deficient diet (TD02373) was formulated with 0.0 g/kg of MgO. The Mg²⁺-normal diet (TD04253) was formulated with 1.02 g/kg of MgO. The Mg²⁺-enriched diet (TD02372) was formulated with 9.95 g/kg of MgO. All rats were placed onto their formulated diets 2 weeks prior to injury and were allowed to feed and drink *ad libitum*. All behavioral testing and anatomical analyses were conducted without knowledge of the diet assignment. Following surgery all animals were placed back onto normal rodent diets.

#### Serum Mg²⁺ analysis

Immediately prior to injury, blood was collected from the tail vein to be used for the determination of serum Mg²⁺ levels. Samples were frozen and shipped to a clinical laboratory for analysis using the ACE® magnesium reagent and NExCT™ clinical chemistry system (Schiapparelli Biosystems, Inc., Fairfield, NJ, USA).

#### Surgery

The surgical procedure was performed using aseptic procedures and conditions. Animals were anesthetized with a cocktail of ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and then prepared for surgery [23]. When the animal became unresponsive (no ocular or pedal reflexes) it was shaved and scrubbed with 70% alcohol followed by betadine and placed in a stereotaxic device. A midline scalp incision was made in the skin and underlying fascia. A circular craniotomy (5.0 mm) was then performed with a Dremel hand drill and a specially designed drill bit that prevented damaging the meninges or cortical tissue. The craniotomy was unilateral and centered over the cortical region containing the sensorimotor cortex (-0.5 mm posterior and 3.0 mm lateral to bregma). The contusion injury was produced using a sterile, stainless steel impactor tip (3.0 mm diameter) attached to
a piston activated with compressed air. The impactor tip was positioned above the cortex and upon activation (2.75 m/s), made contact with the cortex for 0.5 s, resulting in a 2.5 mm compression of the cortex. It should be noted that the impactor tip did not actually penetrate the cortex, or the meninges, but only momentarily compressed the tissue. Following the contusion, the incision was closed with nylon suture material. The animals were maintained on a heating pad (37°C) until they showed locomotive behavior and were then returned to their home cage. Slain animals were prepped for surgery, anesthetized, placed into the stereotaxic device, given a midline scalp incision and craniotomy, sutured and allowed to recover.

Behavioral analysis

**Vibrissae-forelimb placing**

This test measures sensorimotor dysfunction following cortical injury and has been described in detail [24]. Each forelimb was independently tested for placing reactions by touching the vibrissae to a Plexiglas surface. Testing was conducted on post-operative days 2, 4, 6, 10, 14, 21, 28, 35, and 42. In uninjured animals, vibrissae stimulation elicits a placing response. This response was observed when the animal placed its forelimb on the surface after vibrissae contact. The rats received 10 trials for each forelimb per test day.

**Bilateral tactile adhesive removal test**

This test was used in order to examine sensorimotor deficits following injury [24, 25]. The rats were removed from their cages and gently held while a small adhesive patch (121.5 mm²) was applied to the radial aspect of each forelimb. The order of attachment varied between trials. The rat was then returned to its cage and the order and latency of removal was recorded. Each rat received two trials per test day and was tested on post-operative days 2, 4, 6, 10, 14, 21, and 28. The trial ended when the rat removed both stimuli or at the end of two minutes.

**Cognitive assessment**

The Morris water maze was used to assess cognitive functioning [25]. A blue fiberglass tank, 1.5 m in diameter and 76 cm deep, was filled with water to a depth of 32 cm. A 10 cm² Plexiglas platform was submerged 1.0 cm below the surface of the water. A San Diego Instruments video recording system with SMART tracking software was used to track and record the movement of each rat as it traveled over the dark background. Starting positions were randomly selected in the pool for the reference memory task. Trials measuring reference memory began on post-operative day 11 and continued for 4 days. The submerged platform was always located in the center of quadrant 2, halfway between the wall and the center of the tank. Rats were placed into the water at one of four randomly chosen starting points. Rats completed 4 trials on each test day, being released at each of the starting points. All trials ended when the rat reached the platform or after 90 seconds had elapsed. If the rat did not reach the platform within 90 seconds, it was guided to the platform and remained there for 10 seconds. Swim latency was recorded for each trial.

**Histology**

At 45 days post-injury, rats were anesthetized with Nembutal, (100 mg/kg, i.p.) and transcardially perfused with 0.9% phosphate buffered saline, followed by 4% paraformaldehyde. The brains were carefully extracted from the cranium, post-fixed in 4% paraformaldehyde and then cryopreserved in 30% sucrose for 3 days prior to sectioning. The brain was sectioned frozen on a sliding microtome through the extent of the injury cavity. Coronal slices (40 μm thick) were collected in a cryopreservative solution for storage. The extent of the lesion was analyzed with an Olympus microscope (BX-51) and an Olympus 13.5 megapixel digital camera (DP-70). Images of the sections throughout the extent of the injury coordinates were captured using the digital capturing system and area measures of the lesioned tissue were determined using ImageTool software. The Cavalieri method was used to calculate the volumes of the ipsilateral cortex and the contralateral cortex [26]. The number of sections and the section thickness (40 μm) were multiplied by the mean area of the lesion cavity (calculated at five stereotaxic coordinates surrounding the lesion: 2.20, 1.20, 0.20, -1.20, -2.20 relative to bregma) [27]. The extent of cortical injury was measured by calculating the percent reduction in the ipsilateral cortex compared to the contralateral cortex using the formula (1-(ipsi/contra) x 100) [24].

**Statistical Analysis**

Statistical evaluations were performed with SPSS v15.0 to determine if the Mg²⁺ dietary condition modulated behavioral recovery following injury. Data was analyzed for each behavioral test using analysis of variance (ANOVA) tests following procedures for general linear models with options for repeated measures when appropriate. Huyn-Feldt (HF) probabilities were used when assessing the
repeated measures factor. Post-hoc analyses were performed with Tukey’s HSD tests.

Results

Initial behavioral analyses indicated that there were no significant differences in performance between the animals that received sham procedures and any of the dietary manipulations. Analysis of forelimb placing data with a repeated measures ANOVA of the sham-Mg2+-normal (n = 4) sham-Mg2+-enriched (n = 5) and sham-Mg2+-deficient (n = 4) revealed no significant differences for group [F(2,10) = 0.61, p > 0.56], day [F(1.37,13.69) = 0.78, p > 0.43], or the group x day interaction [F(2.74,13.69) = 0.77, p > 0.52]. The same was also true for the tactile removal data. The effects for group [F(2,10) = 0.03, p > 0.97] and the group x day interaction [F(6.48,83.28) = 0.62, p > 0.72] were non-significant; however, the effect of day was significant [F(3,24,32.38) = 0.64, p < 0.001]. Comparison of swim latencies on the acquisition of reference memory revealed no significant differences for group [F(2,10) = 1.29, p > 0.32] and the group x day interaction [F(5,45,27.26) = 0.64, p > 0.68] were not significant; however, the effect of day was significant day [F(2,73,27.26) = 24.59, p < 0.001]. Thus, the groups were combined to create a single sham-control group. Thus, all subsequent analyses were conducted with the following groups: Mg2+-enriched (n = 9), Mg2+-deficient (n = 9), Mg2+-normal (n = 9), and sham (n = 13).

Serum Mg2+ analysis

Serum Mg2+ analysis was analyzed in a one-way ANOVA including group (Mg2+-normal, Mg2+-enriched, Mg2+-deficient) prior to injury. Two weeks of dietary manipulation of Mg2+ showed a diet-dependent change in serum levels; the main effect for serum Mg2+ was statistically significant, [F(2,27) = 31.05, p < 0.001] (figure 1). Post-hoc comparisons with Tukey’s HSD tests were conducted to determine significant differences within the group factor. Comparison of the Mg2+-normal and Mg2+-enriched diets showed a significant elevation in Mg2+ levels in the enriched group 14 [HSD (16) = 1.10, p < 0.001]. Mg2+-deficient diet showed a significant decrease in Mg2+ level compared to the Mg2+-normal diet [HSD (16) = 1.08, p < 0.001]. Thus, pre-surgical dietary manipulation of Mg2+ resulted in significant alterations of Mg2+ level prior to injury.

Figure 1. The effects of 2 weeks of dietary manipulation of Mg2+ on serum Mg2+ levels prior to injury. The Mg2+-deficient diet significantly reduced Mg2+ serum levels compared to the Mg2+-normal diet (^ = p < 0.001). The Mg2+-enriched diet significantly increased serum Mg2+ levels compared to the Mg2+-normal diet (# = p < 0.001).

Vibrissae-forelimb placing test

The percentage of unsuccessful placing attempts was analyzed in a repeated measures ANOVA including group (Mg2+-normal, Mg2+-enriched, Mg2+-deficient, or Sham) and post-injury test session as the repeated measure. Following injury, the rats became more efficient in placing their contralateral forelimbs on successive trials; the main effect for day was statistically significant, [F(3,21,115.81) = 34.52, p < 0.001]. Unilateral contusions produced significant impairments in forelimb placing; the main effect of group was statistically significant, [F(3,36) = 78.74, p < 0.001] (figure 2). There was a significant difference in the rate of recovery; the group x day interaction was significant, [F(9.65,115.81) = 7.50, p < 0.001]. Post-hoc comparisons were conducted to determine differences within the group factor. Comparison of the Mg2+-normal and Mg2+-enriched diets showed no significant differences in performance on any test day. The Mg2+-deficient diet showed worse behavioral outcome compared to the Mg2+-normal diet on days 14 [HSD (16) = 28.89, p < 0.01], 21 [HSD (16) = 42.22, p < 0.02], 28 [HSD (16) = 76.78, p < 0.001], 35 [HSD (16) = 66.67, p < 0.001] and 42 [HSD (16) = 66.67, p < 0.001].
Bilateral tactile adhesive removal test

The latencies to remove the tactile stimuli were analyzed in a repeated measures ANOVA, including group (Mg²⁺-normal, Mg²⁺-enriched, Mg²⁺-deficient, or Sham) and post-injury test session as the repeated measure. Following injury, the rats became more efficient in removing the contralateral stimuli on their forelimbs on successive trials; the main effect for day was statistically significant, \([F(2.94,105.99) = 57.28, p < 0.001]\). Unilateral contusions produced significant impairments in stimuli removal; the main effect of group was statistically significant, \([F(3,36) = 11.50, p < 0.001]\) (figure 3). There was a significant difference in the rate of recovery; the group x day interaction was significant, \([F(8.83,105.99) = 2.33, p < 0.02]\). Post-hoc comparisons were conducted to determine differences within the group factor. Comparison of the Mg²⁺-normal and Mg²⁺-enriched diets showed no significant differences in performance on any test day \((p > 0.05)\). The Mg²⁺-deficient diet showed worse behavioral outcome compared to the Mg²⁺-normal diet on days 6 \([HSD (16) = 27.72, p < 0.05]\), 10 \([HSD (16) = 39.11, p < 0.007]\), 14 \([HSD (16) = 45.61, p < 0.01]\), and 21 \([HSD (16) = 33.33, p < 0.04]\). The Mg²⁺-increased diet was not significantly different from the Mg²⁺-normal diet.

Reference memory

The swim latencies to find the hidden platform in the MWM was analyzed in a repeated measures ANOVA including group (Mg²⁺-normal, Mg²⁺-enriched, Mg²⁺-deficient, or Sham) and post-injury test session as the repeated measure. Following injury, the rats became more efficient at finding the platform on successive days; the main effect for day was statistically significant, \([F(3.00,108.00) = 34.46, p < 0.001]\). Unilateral contusions produced significant impairments in overall performance; the main effect of group was statistically significant, \([F(3,36) = 5.14, p < 0.005]\) (figure 4). However, there was not a significant difference in the rate of recovery; the group x day interaction was not significant, \([F(9.00,108.00) = 0.60, p > 0.80]\). Post-hoc comparisons demonstrated that the performance on the task between the sham group and the Mg²⁺-normal group was not significant on any of the 4 test days \((p > 0.05)\). This was also the case between the Mg²⁺-normal group and either the Mg²⁺-enriched, or -deficient groups \((p > 0.05)\). The Mg²⁺-deficient diet showed worse behavioral outcome compared to the sham group on days 11 \([HSD (18) = 15.74, p < 0.009]\) and 14 \([HSD (18) = 22.75, p < 0.008]\); whereas, the
The Mg²⁺-enriched group was significant different from the sham group on days 11 [HSD (18) = 20.71, p < 0.001], 13 [HSD (18) = 19.97, p < 0.03], and 14 [HSD (18) = 22.23, p < 0.009].

Lesion analysis

The percent reduction of the injured cortex compared to the non-injured cortex was analyzed in a one-way ANOVA including group (Mg²⁺-normal, Mg²⁺-deficient, or Sham) as the factor in the analysis. There were significant differences in lesion size between groups, the analysis of remaining tissue surrounding the lesion cavity was significant, [F(3,39) = 21.43, p > 0.001] (Figure 5). Post-hoc comparisons with Tukey’s LSD test were conducted to determine significant differences within the group factor. Comparison of the Mg²⁺-normal and Mg²⁺-deficient diets showed a significant increase in lesion severity in the deficient group [HSD (16) = 10.90, p < 0.01]. There was a strong trend toward a reduction in lesion severity in the Mg²⁺-enriched group compared to the Mg²⁺-normal group [HSD (16) = 6.47, p > 0.09].

Discussion

The purpose of the present study was to examine the effect of dietary Mg²⁺ manipulations on recovery of function. After 2 weeks of diet manipulation a serum analysis was conducted at the time of cortical injury and showed that the experimental diets significantly modulated the level of circulating serum Mg²⁺. A strong diet-dependent effect was observed. Rats fed the Mg²⁺-normal diet were found to have on average 2.0 mEq/L of serum Mg²⁺; whereas, the Mg²⁺-deficient group had 0.9 mEq/L and the Mg²⁺-enriched had 3.1 mEq/L of serum Mg²⁺. Thus, the diet manipulations significantly altered serum Mg²⁺ levels and allowed us to determine if these dietary manipulations had any effect on recovery of function following cortical injuries.

It was found that manipulation of dietary Mg²⁺ did have significant effects on recovery of function. Rats fed a 2 week diet deficient in Mg²⁺ were significantly worse on the bilateral tactile adhesive removal and vibrissae-forelimb placing tests compared to injured rats feed a standard laboratory diet. The recovery curve of the Mg²⁺-deficient group showed that very
little recovery of function occurred following the cortical lesions. In fact, on day 42 the average degree of impairment in this group was still at 80%, compared to 10% in the Mg²⁺-normal diet. Likewise, the recovery curve for the Mg²⁺-deficient group on the bilateral tactile removal test also showed severe impairments up to 28 days following cortical injury. Interestingly, during the assessment of reference memory performance it was found that the Mg²⁺-deficient diet was significantly worse compared to the non-injured, sham group, comparatively, the deficient diet was significantly worse compared to the non-injured, sham group, and subsequent behavioral impairments to the same extent as the Mg²⁺-deficient group. However, there were no statistically significant differences between the Mg²⁺-enriched and Mg²⁺-normal groups. This was unexpected, especially given the high serum levels of Mg²⁺ in the enriched group. It is possible that the levels of Mg²⁺ were not high enough to facilitate the recovery of function seen with these behaviors following systemic post-injury administrations [5, 28-30]. Given that serum levels of Mg²⁺ do not correlate well with brain tissue levels, this may also have contributed to the diminished effect in the Mg²⁺-enriched diet. It may also be the case that the pre-injury enriched diet might not offset the injury-induced Mg²⁺ decline and subsequent behavioral impairments to the same degree as post-injury systemic administrations [28-30]. The Mg²⁺-enriched diet appears to have improved performance in the reference memory task to about the same extent as in the Mg²⁺-deficient group. At first this seems paradoxical, however, we have recently shown that daily administration of MgCl₂ impaired the acquisition of reference memory in the MWM [29]. In that study, un-injured rats were given daily injections of 1 or 2 mmol/kg of MgCl₂ 30 minutes prior to their running in the MWM [29]. The timing of these administrations contrasts drastically to the present study, diet enrichment was discontinued at the time of injury (11 days prior to MWM testing) and the rats were placed back on the standard laboratory diet, compared to daily systemic injections prior to the MWM. Unfortunately, we do not have a serum analysis for Mg²⁺ at this time point; however, at the time of injury the levels were extremely high in the enriched diet group, it then must be inferred that the Mg²⁺ levels were high enough to interfere with maze learning. This data suggests that caution is needed with Mg²⁺ supplementation and learning/cognitive based tests. However, this effect only occurred in the injured animals, there were no significant impairments in learning in the Mg²⁺-enriched sham group.

The histological analysis revealed a diet-dependent effect on the percent reduction in the injured cortex, compared to the un-injured, contralateral cortex. The Mg²⁺-deficient diet showed a significantly increased reduction in the injured cortex (29.5%) compared to the contralateral cortex. This group showed the greatest extent of injury compared to all other injured groups. The Mg²⁺-normal group had a 21% reduction in cortical volume. The Mg²⁺-enriched group had the smallest lesions with a 13% reduction in cortical volume. Thus, not only do Mg²⁺ diet manipulations modulate recovery of function following cortical injuries but they also differentially affect lesion size.

The results of the Mg²⁺-deficient diet from the present study are in agreement with those reported by McIntosh and colleagues [5]. The neuroscore is a battery of 5 different tests that measure various reflexive motor and balance tasks and was shown to be significantly impaired in the Mg²⁺-deficient rats following FPI [5]. In the present study, we found that following CCI the deficient diet significantly impaired recovery of function on 2 different sensorimotor tests and on the acquisition of a reference memory task in the MWM. The biggest difference between these 2 studies is in post-injury mortality. Following FPI, a 50% mortality was observed; whereas, following CCI we found no mortality. Given the differences between FPI and CCI (FPI usually shows greater mortality compared to CCI), this result is not surprising.

Conclusion

The results of this study have demonstrated that manipulating dietary Mg²⁺ levels prior to injury had dramatic effects on recovery of function and extent of injury. A Mg²⁺-deficient diet significantly reduced the serum levels of Mg²⁺ and significantly exacerbated sensorimotor and cognitive deficits following cortical injuries. The Mg²⁺-enriched diet increased the serum level of Mg²⁺ prior to injury but did not
significantly improve sensorimotor performance compared to the \( \text{Mg}^{2+} \) control diet; however, cognitive performance in the MWM was impaired. In general, the results of this study suggest that dietary status (especially concerning \( \text{Mg}^{2+} \)) is an important factor in recovery of function following injury and warrants more experimental consideration.

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References

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