High Dose Magnesium Sulfate Exposure Induces Apoptotic Cell Death in the Developing Neonatal Mouse Brain

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Key Words

Magnesium sulfate • Apoptosis • Caspase-3 • Pre-eclampsia • Preterm labor • Neurodevelopment

Abstract

Background: Magnesium sulfate (MgSO₄) is often used as a treatment for pre-eclampsia/eclampsia and preterm labor, resulting in the exposure of a significant number of neonates to this drug despite a lack of evidence suggesting that it is safe, or effective as a tocolytic. While there is evidence that MgSO₄ may be neuroprotective in perinatal brain injury, recent reviews have suggested that the effects are dependent upon dose, and that higher doses may actually increase neonatal morbidity and mortality. There is a lack of evidence investigating the neurotoxic effects of neonatal magnesium (Mg) exposure on the developing brain, specifically in terms of neurodevelopmental apoptosis, a cell-killing phenomenon known to be potentiated by other drugs with mechanisms of action at Mg-binding sites (i.e. NMDA receptor antagonists such as MK-801, ketamine, and PCP). **Objective:** To investigate the effects of Mg exposure on the neonatal mouse brain at different postnatal ages to determine whether MgSO₄ treatment causes significant cell death in the developing mouse brain. **Methods:** C57Bl/6 mice were treated with four doses of MgSO₄ (250 mg/kg) on postnatal days 3

(P3), 7 (P7) or 14 (P14). Caspase-3 immunohistochemistry, cupric silver staining, and electron microscopy techniques were used to examine Mg-treated brains for neurotoxic effects. **Results:** Qualitative evaluation using cupric silver staining revealed widespread damage throughout the brain in P7 animals. Results of electron microscopy confirmed that the cell death process was apoptotic in nature. Quantitative evaluation of damage to the cortex, caudate-putamen, hippocampus, thalamus, and cerebellum showed that Mg treatment caused significant brain damage in animals treated on P3 and P7, but not P14. **Conclusions:** Administration of high doses of Mg may be detrimental to the fetal brain, particularly if exposure occurs during critical periods of neurodevelopment.

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Introduction

Magnesium sulfate (MgSO₄) has been used in clinical obstetrics for over 70 years to treat pre-eclampsia/eclampsia and preterm labor, conditions that complicate approximately 3 and 12.4%, respectively, of pregnancies in the US each year [1]. Currently, MgSO₄ is the standard of care for preventing and treating eclamptic seizures, and although its effectiveness as a tocolytic is controver-

sial, it is estimated that over 150,000 women in the US receive tocolytic therapy at or before 34 weeks of gestation each year, with MgSO_4 used as a first line of treatment in the majority of cases [2–4]. In recent years, MgSO_4 has been studied extensively as a potential neuroprotectant to prevent or improve outcomes of brain injury resulting from perinatal hypoxia/ischemia such as encephalopathy, intraventricular hemorrhage, periventricular leucomalacia and cerebral palsy [5–13]. However, it remains controversial whether MgSO_4 is safe [14, 15] or effective as a tocolytic [16–18], or provides neuroprotection for premature at-risk neonates [19–26]. Recent reviews evaluating the use of MgSO_4 for neuroprotection of the fetus or as a tocolytic in women at risk for preterm birth concluded that neuroprotective effects have not been established and MgSO_4 is ineffective at delaying or preventing preterm birth, and may actually increase neonatal morbidity and mortality [27–29].

Magnesium (Mg) is second only to potassium in abundance as a cation in the human body, and it is an important factor in many cellular functions, including energy production, synaptic neurotransmission, and intracellular signaling. The basis for its use as a tocolytic is due to the observed effects of Mg in reducing myometrial contractility through extra- and intracellular mechanisms of action [30–33]. Its most widely recognized and well studied role in the brain is in relation to the NMDA glutamate receptor ion channel where it performs a physiological blocking action that prevents ion flow through the channel under conditions of normal membrane polarization. Mg is therefore regarded as a modulator of neuronal excitability, and when administered as an exogenous agent it is presumed to suppress neuronal activity.

It has been shown that various drugs that inhibit neuronal activity, including NMDA antagonists, trigger widespread apoptotic neurodegeneration in the developing brain and cause long-term neurobehavioral deficits [34–37]. The window of vulnerability to these agents coincides with the developmental period of synaptogenesis, also known as the brain growth spurt period, which in mice and rats occurs primarily from days 0 to 14 postnatally, but in humans extends from approximately mid-gestation to several years after birth [38]. In rodents, this critical period of neurodevelopment during postnatal days 0–14 roughly corresponds to the third trimester of human development [39]. The literature pertaining to exposure of the immature human brain to MgSO_4 has focused almost exclusively on whether it is therapeutically beneficial as an antenatal neuroprotectant for premature infants at risk of brain damage due to hypoxic/ischemic

encephalopathy, intraventricular hemorrhage, periventricular leucomalacia and cerebral palsy. Neonates born with high serum Mg levels clear Mg very slowly, and therefore are exposed to Mg levels beyond the upper limit of the normal range for a prolonged period of time [40].

There are no studies addressing whether exposure of the normal developing rodent brain to high concentrations of Mg might trigger neurodevelopmental apoptosis. Therefore, in the present study we administered a high-dosage MgSO_4 treatment regimen to infant mice at three stages of development and determined whether this caused an abnormal increase in neuroapoptosis in the developing brain.

Materials and Methods

Animal Procedures and Drug Dosing

All animal care procedures were conducted in accordance with guidelines developed by the National Academy of Science and approved by the Washington University School of Medicine Animal Studies Committee. C57BL6 mice were used in all experiments (Harlan, Ind., USA). To control for litter variability, a litter matching approach was used. That is, to evaluate any given experimental condition, control and experimental pups were taken from the same litters, so that each experimental condition had its own group of littermate controls. From prior studies it is known that peak sensitivity to drug-induced developmental neuroapoptosis occurs in the postnatal day 3 (P3) to P7 period, but some neuronal groups remain sensitive until at least P14. Therefore, we administered MgSO_4 or saline to infant mice at P3, P7, or P14 and evaluated the brains 8–24 h later by histological methods that allow detection and quantification of apoptotic neurons. It has been reported [41] that MgSO_4 is sometimes administered to human obstetrics patients in doses that may exceed 50 g in a 24-hour period, which is in the range of 1 g/kg. To approximate this dosing regimen, we administered MgSO_4 by intraperitoneal injection to infant mice at a dose of 250 mg/kg at time zero and at 1, 2 and 3 h, for a total dose of 1 g/kg. Pups were excluded from the study if they appeared malnourished or if at the beginning of the experiment their stomach was not full of milk (as visualized through the relatively transparent abdominal wall). In addition to histological studies, separate groups of animals were used to monitor Mg blood levels on P7 and P14 following administration of MgSO_4 by the above-described dosing regimen.

Histology

Three histological methods were employed: DeOlmos cupric silver staining, activated caspase-3 (AC3) immunohistochemistry, and electron microscopy.

DeOlmos Silver Stain. This method faithfully stains neurons that are dead or dying, but it is not useful for distinguishing between apoptotic and nonapoptotic forms of cell death. Silver is taken up and retained by dying neurons, even after they have deteriorated into multiple small fragments. We used this stain for

screening purposes to assess the pattern of cell death induced by MgSO_4 compared to the pattern that occurs spontaneously as a natural phenomenon in control animals. A total of eight coronal sections sampled from the olfactory bulb to the lower brain stem were used for this purpose. For this study, P7 mice ($n = 4$) treated with MgSO_4 or saline were deeply anesthetized and perfused with fixative (4% paraformaldehyde in tris buffer) 24 h following the first dose of MgSO_4 . This late time interval was used because it allows enough time for all neurons that die as a result of treatment to become impregnated with silver. It is ideal for detecting the pattern of cell death but not for counting dying neurons because at 24 h many of the dead neurons have disintegrated into small fragments that cannot be identified and counted as an intact cell body. Following perfusion fixation, the brains were removed from the skull and postfixed in the perfusate solution for 2 days, embedded in agar and cut on a vibratome into 70- μm -thick sagittal sections, and then stained by the method of DeOlmos et al. [42].

AC3 Immunohistochemical Staining. This is an excellent method for detecting neurons that are in early stages of apoptotic cell death. This method is selective for apoptosis in that in animal models in which both excitotoxic and apoptotic cell death occur, it only stains the neurons undergoing apoptosis [43]. Neurons in early stages of apoptosis have abundant AC3 distributed throughout their cell body and dendritic tree and they have not yet begun to decompose. Therefore, the entire neuron, including its dendritic arbor, is visualized by this method, making it the method of choice for counting the dying neurons. The ideal time interval for visualizing the majority of dying neurons by the AC3 method is 8 h following the first dose of MgSO_4 . Therefore, at 8 h animals treated with MgSO_4 or saline ($n = 8$) were anesthetized, perfused with fixative and their brains cut into serial sagittal vibratome sections. The sections were washed in 0.01 M PBS, quenched for 10 min in a solution of methanol containing 3% hydrogen peroxide, then incubated for 1 h in blocking solution (2% BSA/0.2% milk/0.1% triton X-100 in PBS), followed by incubation overnight in rabbit anti-active caspase-3 antiserum (DI75, Cell Signaling Technology, Beverly, Mass., USA) diluted 1:1,000 in blocking solution. Following incubation with DI75 primary antibody, the sections were incubated for 1 h in secondary antibody (goat anti-rabbit 1:200 in blocking solution), then reacted in the dark with ABC reagents (standard Vectastain ABC Elite Kit, Vector Labs, Burlingame, Calif., USA), followed by incubation for 15–20 min in VIP peroxidase substrate (Vector Labs). The sections were then mounted on gel-coated glass slides, dehydrated in graded alcohols, and cleared in xylene for evaluation by light microscopy. AC3-positive cell counts were conducted in five brain regions (cortex, caudate/putamen, thalamus, hippocampus, and cerebellum).

Electron Microscopy. We also examined the dying neurons by electron microscopy (EM) to confirm that they have ultrastructural and pathomorphological characteristics of neurons undergoing apoptosis. For EM analysis, P7 animals ($n = 2$) were treated with MgSO_4 . At 8 h post-treatment, the animals were anesthetized and the brains perfusion-fixed as described above except that the fixative solution was 1.5% glutaraldehyde and 1% paraformaldehyde in pyrophosphate buffer (pH = 7.4). Following postfixation for 2 days in the perfusate solution, the brains were sliced into 100- μm -thick slabs, osmicated overnight (1% osmium tetroxide), dehydrated in graded ethanols, cleared in toluene, and

embedded flat in araldite. Thin sections (1 μm) were cut at selected rostrocaudal levels of the brain using glass knives (1/2 inch wide) and an MT-2B Sorval ultratome. Areas of special interest from a given block were then trimmed to a smaller size. Ultrathin sections were cut and suspended over a formvar coated slot grid (1 $\times$ 2 mm opening), stained with uranyl acetate and lead citrate and viewed in a JEOL 100CX transmission electron microscope. Areas showing AC3-positive cells were scanned for neurons showing various stages of neurodegeneration to evaluate whether ultrastructural changes were consistent with those seen in apoptotic cell death.

Quantitative Cell Counts

AC3 immunohistochemistry is a more sensitive method than the De Olmos silver stain for identifying apoptotic neuronal death in the developing brain, and identifies an early time point in the cell death process, when the neuron is still intact. This allows for visualization of the entire cell, including the nucleus, cell body, and dendritic tree, and a more precise identification of dying neurons for quantitative counts.

Separate groups of 16 pups ($n = 8$), randomly chosen from at least 4 litters, were treated with MgSO_4 or saline for histological evaluation of neuroapoptosis at ages P3, P7, and P14. At 8 h post-treatment, the animals were anesthetized, perfused with fixative and their brains cut into serial sagittal vibratome sections (70 μm). Every 6th sagittal section was chosen for evaluation using unbiased, systematic random sampling according to stereological principles [44, 45]. This permitted sampling 6 to 10 sections from half of each brain depending on the area of interest and age. These sections were imaged and quantitatively evaluated with Stereo Investigator 7.0 (MicroBrightField, Inc., Colchester, Vt., USA) using Windows XP Pro operating system installed on a Dell PC connected to a Prior Optiscan motorized stage (ES103 XYZ system, Prior Scientific Inc., Rockland, Mass., USA) mounted on a Nikon Labophot-2 microscope. Stereo Investigator software was used to trace the boundaries of the brain regions of interest (cerebral cortex, hippocampus, caudate-putamen, thalamus, and cerebellum). The program was then used to measure, mark, and count neurons, and to calculate the area of each region.

To be included in the cell count, profiles needed to demonstrate a distinct soma with visible dendritic processes, or if no processes were evident, only those with a soma diameter $\geq 8 \mu\text{m}$ (at 20 $\times$) were counted. The preliminary population estimator function of Stereo Investigator was used to mark and count each profile to ensure that no profile would be missed or counted twice. To obtain an estimate of the total number of AC3-positive profiles in the brain region of interest, regional counts from each section were summed and then divided by the total area counted. The density of AC3-positive neurons in each brain region for a given treatment condition was calculated using the following formula: (total number of positive cells/total area in $\mu\text{m}^2 \times 10^6$)/70 $\mu\text{m} \times 1,000$ = total number of profiles per mm^3 . The counts were performed by an experienced histopathologist (C.C.) blind to the treatment condition.

Magnesium Levels

For determination of Mg levels, animals were treated ($n = 6$ –8) with the same dosing regimen as in the histological studies. Levels were obtained in separate groups of P7 and P14 animals. After receiving the last dose of MgSO_4 or saline, animals were

decapitated at either 30 or 90 min (4.5 and 6 h after receiving the first dose) and the trunk blood was immediately collected in a Microtainer serum separator tube (Becton Dickinson and Company, Franklin Lakes, N.J., USA). The samples were then centrifuged at 3,000 rpm for 30 min and analyzed on an Eastman-Kodak DT-60 analyzer (Eastman – Kodak, Rochester, N.Y., USA). The 4.5-hour time point was chosen to approximate peak blood levels. Mg is tightly regulated by the kidney and hypermagnesemia augments renal elimination, therefore, 6-hour values were obtained to confirm that levels were decreasing when treatment was terminated.

Imaging

Photomicrographs of the various histological techniques were obtained using a Qimage digital camera attached to a Nikon Labophot-2 microscope. Digital montages were created and processed with Surveyor 7.0 software (Objective Imaging, London, UK).

Statistical Analysis

Statistical analyses were conducted using Prism 4 (GraphPad Software Inc., San Diego, Calif., USA) installed on a Mac OS X operating system. Analysis of variance (ANOVA) models were used to analyze the data. All data are shown as Mean $\pm$ SEM, with a probability value for significance of $p < 0.05$. A priori planned comparisons were conducted where appropriate, with Bonferroni correction for multiple comparisons (probability value for significance of $p < 0.01$).

Results

Behavioral Observations

The MgSO_4 -treated animals began to demonstrate evidence of drug effect approximately 5–10 min after the initial dose. Although tone was difficult to assess, spontaneous movement in the treated animals was decreased compared to controls. Righting reflex remained intact but sluggish and all animals showed a robust response to tail pinch. The drug-induced effects waned by approximately 45–50 min and the effects did not seem to be additive with subsequent dosing. Treated animals showed no evidence of cardiorespiratory impairment compared to saline controls; observation of the pups was conducted every 30 min during the duration of the experiment, and it was noted at all observation points that their skin was pink with no discoloration, and they were not having any respiratory difficulty. The animals remained with their mother throughout the experiment and it was also noted that they were able to nurse without difficulty, and that their stomachs contained milk for the duration of the experiment. At the time of sacrifice (8 h), no differences in behavior were identified between the control and treated animals.

Fig. 1. Results of cupric silver staining show neurodegeneration 24 h after exposure to MgSO_4 . Shown here are coronal hemisections of P7 mouse brains treated with saline or Mg. The largest amount of damage occurred in superficial and deep cortical layers (shown above), subiculum, caudate-putamen, hippocampus, and thalamus (shown above). These results provided the basis for the selection of brain regions to be evaluated using AC3 immunohistochemistry.

Neurodegenerative Effects

De Olmos cupric silver staining identified a large number of neurons that were degenerating 24 h after exposure to MgSO_4 . Although this method does not distinguish between apoptotic and non-apoptotic cell death, the neurons were in an advanced stage of degeneration, shrunken, and often subdivided into several small fragments, making it difficult to identify individual neurons. While the observed degeneration was widespread, specific brain regions and specific populations of neurons were affected. The largest amount of damage occurred in superficial and deep cortical layers, caudate-putamen, hippocampus, and thalamus (fig. 1). These results pro-

Fig. 2. Composite photomicrograph illustrating the difference in magnitude and pattern of neuroapoptosis as detected by AC3 immunohistochemistry between a P7 saline- and Mg-treated brain overall (top), and in each region of interest (bottom).

vided the basis for the selection of brain regions to be evaluated using AC3 immunohistochemistry.

Activated Caspase-3 Immunohistochemistry

AC3 positivity was noted to occur in the same population of neurons identified as undergoing degeneration with the De Olmos technique (fig. 2). Sagittal sections

demonstrated similar AC3 damage in the areas noted in the transverse sections as well as periventricular/aqueductal areas of the lateral and third ventricles and in the cerebellum (fig. 3).

Because differences in volume could confound the results for the quantitative cell counts/densities, ANOVAs were conducted to determine whether there were signif-

Fig. 3. Photomicrographs of the cerebellum of a P7 brain demonstrating the difference in AC3-positivity in an animal treated with Mg vs. an animal treated with saline.

Table 1. Mg levels (mean mEq/dl $\pm$ SEM) according to age group measured at two different time points

Time point	Treatment groups			
	P7		P14	
	saline	MgSO ₄	saline	MgSO ₄
30 min	2.10 $\pm$ 0.07	11.80 $\pm$ 1.32	1.81 $\pm$ 0.09	10.51 $\pm$ 1.14
90 min	2.36 $\pm$ 0.13	8.37 $\pm$ 1.45	1.96 $\pm$ 0.16	7.69 $\pm$ 1.27

ificant differences in calculated brain volume between treatment groups for each age group. The results of 2×5 (treatment by region) ANOVAs for the P3, P7, and P14 data showed that there were no significant differences between treatment groups in brain volume that could confound the cell density results ($p = 0.50, 0.48, 0.36$, respectively). Separate 2×5 mixed-model ANOVAs (between treatment groups $\times$ within brain regions) were conducted for each age group. If a main effect of Mg treatment was found, a priori planned comparisons were conducted between groups for each brain region.

Mg treatment at P3 caused significant brain damage compared to normal apoptosis observed in saline controls [$F(1,14) = 11.73$; $p = 0.004$]. MgSO₄-treated animals had increased cell death in the cortex, caudate-putamen, hippocampus, thalamus, and cerebellum compared to

saline controls [$F(1,14) = 11.62, 5.73, 12.24, 5.64, 10.95$, respectively; fig. 4a].

The results were similar for P7 animals; MgSO₄-treated animals displayed a greater number of neurons that were positive for AC3 compared to saline-treated controls [$F(1,14) = 11.78$; $p = 0.004$]. This treatment effect was found in the cortex, caudate-putamen, hippocampus, thalamus, and cerebellum [$F(1,14) = 16.50, 7.21, 11.84, 10.55, 9.82$ respectively; fig. 4b].

There was no overall significant main effect of treatment for P14 animals compared to saline controls [$F(1,14) = 0.11$; $p = 0.74$; fig 4c].

Magnesium Levels

As previously described, blood Mg levels were obtained 30 min after the last dose of MgSO₄ to approximate peak levels, and 2 h after the last dose to confirm appropriate renal elimination. Mean ($\pm$ SEM) Mg levels are presented in table 1. In order to exclude any age-related differences in blood Mg levels, factorial ANOVAs were conducted to determine whether there were significant differences between treatment groups for each age group. The results of two 2×2 (treatment by time) ANOVAs for the P7 and P14 animals showed that there was no significant difference between groups in blood Mg levels ($p > 0.05$).

Although these levels are undoubtedly higher than would be expected in clinical obstetrics, levels as high as 4–8 mg/dl are considered therapeutic. Also, because it has been found that fetal Mg levels are higher than maternal levels, even in untreated fetal-neonates [46], and that high serum levels persist due to slow clearance [40], it can be expected that human fetal neonates exposed to prolonged use could have serum Mg levels at or above the upper limit of the therapeutic range. Typically, it is not until levels are greater than 15 mg/dl that muscle paralysis and respiratory failure can occur [47].

Electron Microscopy

Apoptosis was originally defined using ultrastructural criteria, with EM histological evaluation as the standard method for determining whether the observed neurodegeneration is an apoptotic process [48]. EM was therefore performed to confirm ultrastructurally that the neurodegeneration was apoptotic in nature. Neurons from selected regions, identified as having dying neurons with the other histological techniques, were examined. Degenerating neurons were condensed and had clumped chromatin with fragmentation of the nuclear membrane, verifying apoptotic degeneration (fig. 5).

Fig. 4. Results of the quantification of cell death in P3, P7, and P14 animals according to brain region (mean $\pm$ SEM; $n = 8$). **a** In animals treated at P3, statistical results showed an overall effect of MgSO₄ treatment, with every brain region showing an increase in cell death compared to saline controls. **b** At P7 there was also a significant overall effect of MgSO₄ treatment, with increased

apoptosis observed in every brain region. **c** In P14 animals there was no significant overall treatment effect. CTX = Cortex, CP = caudate/putamen, HC = hippocampus, TH = thalamus, CER = cerebellum. Probability values for individual planned comparisons between groups for each brain region are listed below each set of bars.

Fig. 5. Results of EM evaluation of caspase-3-positive neurons in a Mg-treated animal show ultrastructural neurodegenerative changes consistent with apoptotic cell death. This degenerating neuron shows an advanced stage of apoptosis, with condensation of cytoplasm (white arrows), clumped nuclear chromatin (white asterisk), swollen mitochondria (white arrowhead) and the absence of a neuronal membrane with mixing of nucleoplasm and cytoplasm.

Discussion

In this study, we have administered MgSO₄ to infant mice at a postnatal developmental age corresponding to a critical period of synaptogenesis in the human fetal-neonate, and determined that at P3 and P7 this triggered a significant increase in the rate of neuroapoptosis in several regions of the developing brain. It is well established that various NMDA antagonist drugs trigger developmental neuroapoptosis in the infant rodent brain, and that peak sensitivity to this effect is in the P3 to P7 period [49]. Mg is known to inhibit neuronal excitability by several mechanisms, most notably by blocking ion flow through the NMDA receptor channel. Thus, although other mechanisms may play a role, it seems likely that the neuroapoptogenic action demonstrated for Mg in the present study could be mediated by its action at NMDA receptors.

For many years, Mg has been a mainstay in the management of pre-eclampsia/eclampsia and preterm labor, and more recently has been under evaluation as a neuroprotectant in conditions such as perinatal asphyxia, hypoxic/ischemic encephalopathy, intraventricular hemorrhage and periventricular leukomalacia. In view of these many indications for which Mg is being used or considered for use without clear-cut evidence for its efficacy or safety, several multicenter clinical trials have recently

been undertaken and/or are in the planning stages. In a study referred to as the Magnesium and Neurological Endpoints Trial (MagNET), 165 fetuses exposed either to Mg or to another tocolytic were evaluated for adverse outcomes. This trial was terminated after 15 months because of 10 deaths in the Mg-exposed group versus 1 death in the group not exposed to Mg [50, 51]. A more detailed analysis of the data from this study [41] revealed that infants having the highest blood levels of ionized Mg at birth had a significantly higher rate of intraventricular hemorrhage, and those exposed to a total Mg dose of 50 g or more had an increased risk of lenticulostriate vasculopathy (a rare mineralizing lesion in the thalamus and basal ganglia). In the Australian Collaborative Trial of Magnesium Sulphate (ACTO MgSO₄), Crowther et al. [52] evaluated the outcome of 1062 premature infants born at ≤ 30 weeks gestation after receiving treatment with either MgSO₄ (4 g loading dose followed by intravenous infusion of 1 g/h for up to 24 h) or saline. For the primary outcomes of total mortality, cerebral palsy, and combined death or cerebral palsy no differences reached statistical significance. However, there was a statistically significant reduction in substantial motor dysfunction among survivors in the MgSO₄ group and the combined outcome of death or substantial motor dysfunction. Marret et al. [53] recently reported data from a French study (PREMAG trial) in which mothers at <33 wks gestation expected to deliver within 24 h were given either 4 g MgSO₄ or saline. There were 688 neonates evaluated for the primary endpoints of cerebral white matter injury or death with no differences between these outcomes reaching statistical significance. In a subsequent study evaluating the same patients at 2 years of age for secondary outcomes of death, motor dysfunction, cognitive dysfunction, and cerebral palsy both individually and in combination (eight total outcomes), three outcomes did reach statistical significance – the combination of (1) death and motor dysfunction, (2) death and motor and

cognitive dysfunction, and (3) death and cerebral palsy and cognitive dysfunction [54].

The above findings suggest that doses of Mg in a very high range (50 g or more, which for a small woman would be 1 g/kg) may be associated with increased mortality and gross CNS injury, whereas at substantially lower doses, Mg may be associated with a slight improvement in acute neurological outcome. The dose of MgSO₄ used in the present study (1 g/kg administered over a 4-hour period) is comparable to the high doses in the MagNET study that were associated with gross CNS lesions and a high mortality rate. It is difficult to interpret human risk based on mouse data, because it is not possible with any precision to establish cross-species dose equivalencies. However, it is generally recognized, due to metabolic and other factors, that it usually requires a much higher drug dosage to produce a toxic effect in mice than in humans. Moreover, in our mouse study we examined the brains histologically for subtle neuropathological changes (apoptotic cell death), whereas the only outcome measures evaluated in the human studies were gross neuropathology or death. The dose threshold, in any species, for causing gross neuropathology or death is likely to be much higher than for causing subtle cell death by apoptosis, and the mechanisms underlying apoptotic cell death are different from those causing gross neuropathology or death. Therefore, even if lower doses reduce the incidence of death or cerebral palsy at 2 years of age, it is still possible that these same doses might trigger neuroapoptosis and subsequent neurocognitive disturbances in infants exposed to Mg but without any obvious neurologic disabilities at birth. At a minimum, long-term follow-up studies designed to detect neurocognitive disturbances in humans (particularly in exposed children that demonstrate grossly normal neurological development) would be required to help clarify this issue.

References

- Martin JN, Thigpen BD, Moore RC, Rose CH, Cushman J, May W: Stroke and severe preeclampsia and eclampsia: a paradigm shift focusing on systolic blood pressure. *Obstet Gynecol* 2005;105:246–254.
- Mittendorf R, Pryde P, Khoshnood B, Lee KS: If tocolytic magnesium sulfate is associated with excess total pediatric mortality, what is its impact? *Obstet Gynecol* 1998;92:308–311.
- Grimes DA, Nanda K: Magnesium sulfate tocolysis: time to quit. *Obstet Gynecol* 2006;108:986–989.
- Sibai BM: Magnesium sulfate is the ideal anticonvulsant in preeclampsia-eclampsia. *Am J Obstet Gynecol* 1990;163:1141–1145.
- Gathwala G, Khera A, Singh I: Magnesium therapy in birth asphyxia. *Indian J Pediatr* 2006;73:209–212.
- Ichiba H, Yokoi T, Tamai H, Ueda T, Kim TJ, Yamano T: Neurodevelopmental outcome of infants with birth asphyxia treated with magnesium sulfate. *Pediatr Int* 2006;48:70–75.
- Perlman JM: Intervention strategies for neonatal hypoxic-ischemic cerebral injury. *Clin Ther* 2006;9:1353–1365.

- 8 Wiswell TE, Graziani LJ, Kornhauser MS, Stanley C, Metron DA, McKee L, Spitzer AR: Effects of hypocarbia on the development of cystic periventricular leukomalacia in premature infants treated with high-frequency jet ventilation. *Pediatrics* 1996;98:918–924.
- 9 Nelson KB, Grether JK: Can magnesium sulfate reduce the risk of cerebral palsy in very low birthweight infants? *Pediatrics* 1995;95:263–269.
- 10 Schendel DE, Berg CJ, Yeargin-Allsopp M, Boyle CA, Decoufle P: Prenatal magnesium sulfate exposure and the risk for cerebral palsy or mental retardation among very low-birth weight children aged 3 to 5 years. *JAMA* 1996;276:1805–1810.
- 11 Kuban KC, Leviton A, Pagano M, Fenton T, Strassfeld R, Wolff M: Maternal toxemia is associated with reduced incidence of germinal matrix hemorrhage in premature babies. *J Child Neurol* 1992;7:70–76.
- 12 Perlman JM, Rollins NK, Evan D: Neonatal stroke: Clinical characteristics and cerebral blood flow velocity measurements. *Pediatr Neurol* 1994;11:281–284.
- 13 FineSmith RB, Roche K, Yellin PB, Walsh KK, Shen C, Zeglis M, Kahn A, Fish I: Effect of magnesium sulfate on the development of cystic periventricular leukomalacia in preterm infants. *Am J Perinatol* 1997;14:303–307.
- 14 Nassar AH, Usta IM: Magnesium sulfate compared with nifedipine for acute tocolysis of preterm labor: a randomized controlled trial. *Obstet Gynecol* 2007;110:1170–1171.
- 15 Nassar AH, Salti I, Makarem NN, Usta IM: Marked hypocalcemia after tocolytic magnesium sulphate therapy. *Am J Perinatol* 2007;24:481–482.
- 16 Cox SM, Sherman ML, Leveno KJ: Randomized investigation of magnesium sulfate for prevention of preterm birth. *Am J Obstet Gynecol* 1990;265:6633–6637.
- 17 Crowther CA, Hiller JE, Doyle LW: Magnesium sulphate for preventing preterm birth in threatened preterm labor. *Cochrane Database Syst Rev* 2002;4:CD001060.
- 18 Lyell DJ, Pullen K, Campbell L, Ching S, Druzin ML, Chikara U, Burrs D, Caughey AB, El-Sayed YY: Magnesium sulfate compared with nifedipine for acute tocolysis of preterm labor: a randomized controlled trial. *Obstet Gynecol* 2007;110:61–67.
- 19 Doyle LW, Crowther CA, Middleton P, Marret S: Magnesium sulphate for women at risk of preterm birth for neuroprotection of the fetus. *Cochrane Database Syst Rev* 2007;CD004661.
- 20 Leviton A, Paneth N, Susser M, Reuss ML, Allred EN, Kuban K, Sanocka U, Hegyi T, Hiatt M, Shahrivar F, Van Marter LJ: Maternal receipt of magnesium sulfate does not seem to reduce the risk of neonatal white matter damage. *Pediatrics* 1997;99:E2.
- 21 Marret S, Doyle LW, Crowther CA, Middleton P: Antenatal magnesium sulphate neuroprotection in the preterm infant. *Semin Fetal Neonatal Med* 2007;12:311–317.
- 22 Paneth N, Jetton J, Pinto-Martin J, Susser M: Magnesium sulfate in labor and risk of neonatal brain lesions and cerebral palsy in low birth weight infants. The Neonatal Brain Hemorrhage Study Analysis Group. *Pediatrics* 1997;99:E1.
- 23 Canterino JC, Verma UL, Visintainer PF, Figueroa R, Klein SA, Tejani NA: Maternal magnesium sulfate and the development of neonatal periventricular leukomalacia and intraventricular hemorrhage. *Obstet Gynecol* 1999;99:396–402.
- 24 Kimberlin DF, Hauth JC, Goldenberg RL, Bottoms SF, Iams JD, Mercer B, MacPherson C, Thurnau GR: The effect of maternal magnesium sulfate treatment on neonatal morbidity in < or = 1,000-g infants. *Am J Perinatol* 1998;15:635–641.
- 25 Weintraub Z, Solovechick M, Reichman B, Rotschild A, Waisman D, Davkin O, Lusky A, Bental Y: Effect of maternal tocolysis on the incidence of severe periventricular/intraventricular haemorrhage in very low birthweights infants. *Arch Dis Child Fetal Neonatal Ed* 2001;85:F13–F17.
- 26 Boyle CA, Yeargin-Allsopp M, Schendel DE, Holmgren P, Oakley GP: Tocolytic magnesium sulfate exposure and risk of cerebral palsy among children with birth weights less than 1,750 grams. *Am J Epidemiol* 2000;152:120–124.
- 27 Doyle LW, Crowther CA, Middleton P, Marret S: Magnesium sulphate for women at risk of preterm birth for neuroprotection of the fetus. *Cochrane Database Syst Rev* 2007;CD004661.
- 28 Crowther CA, Hiller JE, Doyle LW: Magnesium sulphate for preventing preterm birth in threatened preterm labor. *Cochrane Database Syst Rev* 2002;CD001060.
- 29 Mittendorf R, Pryde PG: A review of the role of magnesium sulphate in preterm labour. *BJOG* 2005;112:84–88.
- 30 Fomin VP, Gibbs SG, Vanam R, Morimiya A, Hurd WW: Effect of magnesium sulfate on contractile force and intracellular calcium concentration in pregnant human myometrium. *Am J Obstet Gynecol* 2006;194:1384–1390.
- 31 Cunze T, Rath W, Osmers R, Martin M, Warneke G, Kuhn W: Magnesium and calcium concentration in the pregnant and non-pregnant myometrium. *Int J Gynaecol Obstet* 1995;48:9–13.
- 32 Lemanciewicz A, Laudanska H, Laudanski T, Karpiuk A, Batra S: Permeability of fetal membranes to calcium and magnesium: possible role in preterm labour. *Hum Reprod* 2000;15:2018–2022.
- 33 Mizuki J, Taska K, Masumoto N, Kasahara K, Miyake A, Tanizawa O: Magnesium sulfate inhibits oxytocin-induced calcium mobilization in human puerperal myometrial cells: possible involvement of intracellular free magnesium concentration. *Am J Obstet Gynecol* 1993;169:134–139.
- 34 Fredriksson A, Archer T, Alm H, Gordh T, Eriksson P: Neurofunctional deficits and potentiated apoptosis by neonatal NMDA antagonist administration. *Behav Brain Res* 2004;153:367–376.
- 35 Fredriksson A, Archer T: Neurobehavioural deficits associated with apoptotic neurodegeneration and vulnerability for ADHD. *Neurotox Res* 2004;6:435–456.
- 36 Fredriksson A, Ponten E, Gordh T, Eriksson P: Neonatal exposure of a combination of N-methyl-D-aspartate and gamma-aminobutyric acid type A receptor anesthetic agents potentiates apoptotic neurodegeneration and persistent behavioral deficits. *Anesthesiology* 2007;107:427–436.
- 37 Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF: Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci* 2003;23:876–882.
- 38 Dobbins J, Sands J: Comparative aspects of the brain growth spurt. *Early Hum Dev* 1979;3:79–83.
- 39 Rice D, Barone S: Critical periods of vulnerability for the developing nervous system: evidence from human and animal models. *Environ Health Perspect* 2000;108S3:535–544.
- 40 Mittendorf R, Stratford R, Khoshnood B, Lee KS, Pryde P: Persistence of elevated serum magnesium levels in the neonate. *Am J Obstet Gynecol* 2001;184:S50.
- 41 Mittendorf R, Dammann O, Lee KS: Brain lesions in newborns exposed to high-dose magnesium sulfate during preterm labor. *J Perinatol* 2006;26:57–63.
- 42 DeOlmos J, Ebbesson S, Heimer L: Silver methods for the impregnation of degenerating axoplasm; in Heimer L, Robards N (eds): *Neuroanatomical Tract-Tracing Methods*. New York, Plenum, 1990, pp 117–168.
- 43 Young C, Tenkova T, Dikranian K, Olney JW: Excitotoxic versus apoptotic mechanisms of neuronal cell death in perinatal hypoxia/ischemia. *Curr Mol Med* 2004;4:77–85.
- 44 Gundersen HJ, Bagger P, Bendtsen TF, et al: The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS* 1988;96:857–881.
- 45 West MJ, Slomianka L, Gundersen HJ: Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat Rec* 1991;231:482–497.

- 46 Pryde PG, Borg MJ, Mittendorf R, Elin RJ: Cord-blood ionized magnesium (iMg) levels exceed maternal levels in both untreated and tocolytic magnesium sulfate treated preterm neonates. *Am J Obstet Gynecol* 1999;180: S106.
- 47 Witlin AG: Prevention and treatment of eclamptic convulsions. *Clin Obstet Gynecol* 1999;42:507–518.
- 48 Dikranian K, Ishimaru MJ, Tenkova T, Labruyere J, Qin YQ, Ikonomidou C, Olney JW: Apoptosis in the in vivo mammalian fore-brain. *Neurobiol Dis* 2001;8:359–379.
- 49 Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovskya V, Turski L, Olney JW: Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* 1999;283:70–74.
- 50 Mittendorf R, Covert R, Boman J, Khoshnood B, Lee KS, Siegler M: Is tocolytic magnesium sulphate associated with increased total paediatric mortality. *Lancet* 1997;350:1517–1518.
- 51 Mittendorf R, Dambrosia, Pryde PG, Lee KS, Gianopoulos JG, Besinger RE, Tomich PG: Association between the use of antenatal magnesium sulfate in preterm labor and adverse health outcomes in infants. *Am J Obstet Gynecol* 2002;186:1111–1118.
- 52 Crowther CA, Hiller JE, Doyle LW, Haslam RR: Effect of magnesium sulfate given for neuroprotection before preterm birth: a randomized controlled trial. *JAMA* 2003;290: 2669–2676.
- 53 Marret S, Marpeau L, Zupan-Simunek V, Eurin D, Leveque C, Hellot MF, Benichou J: Magnesium sulphate given before very-preterm birth to protect infant brain: the randomized controlled PREMAG trial. *BJOG* 2007;114:310–318.
- 54 Marret S, Marpeau L, Follet-Bouhamad C, Cambonie G, Astruc D, Delaporte B, Bruel H, Guillois D, Pinquier D, Zupan-Simunek V, Bénichou J: Effect of magnesium sulphate on mortality and neurologic morbidity of the very-preterm newborn with two year neurological outcome: results of the prospective PREMAG trial. *Gynécologie Obstétrique & Fertilité* 2008;36:278–288.

Erratum

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