Magnesium and the blood-brain barrier *in vitro*: effects on permeability and mg transport

Valentina Romeo, Alessandra Cazzaniga, Jeanette A.M. Maier

Dipartimento di Scienze Biomediche e Cliniche L. Sacco, Università di Milano, Via GB Grassi 74, 20157 Milano, Italy

Correspondence: Jeanette A.M. Maier, Dipartimento di Scienze Biomediche e Cliniche L. Sacco, Università di Milano, Via GB Grassi 74, 20157 Milano, Italy.
<jeanette.maier@unimi.it>

Abstract. The blood-brain barrier (BBB) tightly regulates the homeostasis of the central nervous system, and its dysfunction has been described in several neurological disorders. Since magnesium exerts a protective effect in the brain, we assessed whether supraphysiological concentrations of different magnesium salts modulate the permeability and magnesium transport in *in vitro* models of rat and human BBB. Among various formulations tested, magnesium pidolate was the most efficient in reducing the permeability and in enhancing magnesium transport through the barrier. We then compared magnesium pidolate and magnesium sulfate, a widely used salt in experimental models and in clinical practice. Magnesium pidolate performs better than sulfate also in preventing lipopolysaccharide-induced damage to *in vitro* generated BBB. We conclude that magnesium pidolate emerges as an interesting alternative to sulfate to protect BBB and maintain correct intracerebral concentrations of magnesium.

Key words: magnesium, blood-brain barrier, lipopolysaccharide

Introduction

The blood-brain barrier (BBB) represents the crucial interface between the peripheral circulation and the tightly regulated environment of the central nervous system (CNS) [1]. It is constituted by tightly adherent endothelial cells, which line the brain vessels and limit paracellular and transcellular movement of solutes, and astrocytes, which modulate endothelial phenotype [2, 3].

The BBB protects neurons from noxious factors present in the systemic circulation and maintains the highly regulated brain internal milieu, which is a requisite for proper synaptic and neuronal functioning [2, 3]. Therefore, it is not surprising that BBB dysfunction has been reported to have a pathogenic role in neurological disorders such as Alzheimer disease, Parkinson disease, and multiple sclerosis [3].

Several molecules are known to modulate BBB permeability. For example, bacterial lipopolysaccharides (LPS) and inflammatory cytokines, such as Tumor Necrosis Factor (TNF)α, increase BBB permeability [4], while elevated concentrations of Magnesium (Mg) reduces it [5]. *In vivo* Magnesium sulfate ($MgSO_4$) prevents oxLDL-induced BBB permeability without affecting myogenic tone [6]. In addition, $MgSO_4$ is useful in preventing BBB injury in trauma and eclampsia [7, 8].

Interestingly, also chronic stress has been reported to exert an effect on BBB permeability. In a recent paper it was reported that, in mice, chronic social stress alters the morphology of the brain blood vessels and promotes BBB leakiness,

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thus allowing the passage into the brain of inflammatory cytokines that lead to depression-like behaviors [9].

Along with the permeability of the BBB, also the content of Mg in the brain may represent an important parameter to be evaluated. Recent findings highlight a correlation between low levels of serum Mg and acute or chronic neuronal diseases [10] as well as stress and anxiety [11, 12]. Even though Mg is important for cerebral function [10], the mechanisms underlying Mg transport across the BBB remain elusive [10].

The development of *in vitro* models of BBB has yielded important insights into our understanding of the pathophysiology of the BBB. These models have progressed from simple monocultures of endothelial cells to more complex coculture systems in which endothelial cells are grown on porous cell culture membranes and cocultured with astrocytes [13].

In the present study, we took advantage of recently developed *in vitro* models of BBB to compare the ability of different Mg to modulate BBB function. In particular, we performed our experiments on rat BBB to offer insights into *in vivo* studies carried out in rodents. We also used human BBB, which is an interesting model to test neuroprotective agents, because Mg is widely used in clinical practice.

Materials and methods

Chemicals

Magnesium Chloride ($MgCl_2$), Magnesium Threonate (MgThre), Magnesium Pidolate (MgPid), Magnesium Sulfate ($MgSO_4$), pidolic acid, and lipopolysaccharide (LPS) (Sigma Aldrich, St. Louis, Missouri, USA) were dissolved in deionized water, filtered by 0.22 μm filter (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and added to the culture media to reach up to 10 mmol/L final concentration. These high concentrations did not exert any cytotoxicity in our experimental model (data not shown), as previously shown [14].

In vitro models of Blood-Brain Barrier (BBB)

Human endothelial cells (HECs) from the umbilical vein (American Type Culture Collection) were cultured in M199 containing 10% dyalised fetal bovine serum (FBS), 1 mmol/L glutamine, Endothelial Cell Growth Supplement (ECGS, 150 μg/mL), 1 mmol/L sodium pyruvate, and heparin (5 units/mL) [15]. Rat Brain Microvascular Endothelial Cells (RBMEC) (Innoprot, Derio, Bizkaia, Spain) were cultured in Endothelial Cell medium (ECM) supplemented with 5% dyalised FBS and ECGS. Rat cortical Astrocytes (RCA) (Innoprot, Derio, Bizkaia, Spain) and Human Astrocytes (HA) (ScienCell Carlsbad, CA, USA) were cultured in Astrocyte medium (AM) containing 5% dyalised FBS and 1% of Astrocyte Growth Supplement. Different cocultures were developed i) RBMEC/RCA and ii) HECs/HA [13] and defined rat BBB and human BBB, respectively. The BBB *in vitro* models were established using the Transwell system (Corning, Corning NY, USA) with a 0.4 μm pore size. RCAs or HA (35,000/cm^2) were seeded on the underside of the insert precoated with Poly-L-lysine (100 μg/mL). Once they reached confluence, HECs or RBMECs were seeded on the upper side of the membranes (60,000/cm^2) precoated with fibronectin (50 μg/mL). BBB was validated by measuring the transmonolayer electrical resistance (TEER) of the endothelial monolayer at various time points using an EndOhm (World Precision Instruments, Friedberg, Germany). The experiments were repeated 3 times, each sample being replicated 3 times. Results were expressed in çxcm^2 [16] and are presented as the mean ± S.E.M. values.

Evaluation of permeability

The permeability of *in vitro* BBB models was measured using bovine serum albumin-fluorescein isothiocyanate conjugate (FITC-BSA) (Sigma, Saint Louis, Missouri, USA) [5]. Briefly, FITC-BSA (1 mg/mL) was added to the upper chamber and, after different times, it was measured in the lower chamber using the Promega Glomax Multi detection System at excitation/emission wavelength of 495 nm/519 nm. All the experiments were repeated 3 times, each sample being replicated 3 times. Results are shown as % of the control exposed to 1 mmol/L Mg and presented as the mean ± S.E.M. values.

Evaluation of magnesium transport through the BBB

Mg levels in the lower chamber were measured using the fluorescent dye based on diaza-18-

crown-6-hydroxyquinoline (DCHQ5) (kindly donated by Prof. S. Iotti, University of Bologna) as described [17]. Fluorescence intensities were acquired at 510 nm. Mg concentrations were obtained by the interpolation of their fluorescence with the standard curve performed using known concentrations of $MgSO_4$. All the experiments were repeated 3 times, each sample being replicated 3 times. Results are expressed as % of control and presented as the mean ± S.E.M. values.

Statistical analysis

Statistical significance was determined using Student's t test and set as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

Measurement of Transmonolayer Electrical Resistance in human and rat BBB

To set up the system, we measured the electrical resistance of the rat and human BBB *in vitro* at various time points to individuate when the BBB has reached the maximal resistance. *Figure 1A* shows that within 3 days human BBB reaches 435 çXcm^2 and this value is maintained in the following days. Rat BBB reaches the maximal resistance (433 çXcm^2) after 4 days (*figure 1B*). Therefore, all the experiments were performed after 4 days of coculture.

BBB permeability and Mg transport upon exposure to high concentrations of $MgSO_4$

A second set of experiments was performed to individuate the concentration of Mg salts to use in further studies. For this purpose, $MgSO_4$ was utilized, because it was previously used *in vivo* and *in vitro* [5, 7]. Human BBB was exposed to 1, 2.5, 5, or 10 mmol/L $MgSO_4$ and BBB permeability measured by adding FITC-BSA to the upper chamber and evaluating its accumulation in the lower chamber 6 hours later by fluorimetry. *Figure 2A* shows that BBB permeability decreases with the increase of Mg concentration. Maximal effect was observed with 5 mmol/L $MgSO_4$. We then investigated Mg crossing through the BBB. By measuring Mg concentration using a DCHQ5 fluorescent probe [17], we found that Mg accumulated in the lower chamber in a dose-dependent manner (*figure 2B*). Similar results were obtained on rat BBB (Data not shown). Considering that 1 mmol/L is the physiological concentration of Mg in extracellular fluids, we selected 5 mmol/L as the right concentration to test the effect of different organic and inorganic Mg salts on the BBB.

The effects of various Mg salts on a rat *in vitro* model of BBB

We compared the effect of 5 mmol/L $MgSO_4$, $MgCl_2$, MgPid, and MgThre on rat BBB permeability and found that, while all the salts reduce BBB permeability, MgPid and MgThre are the

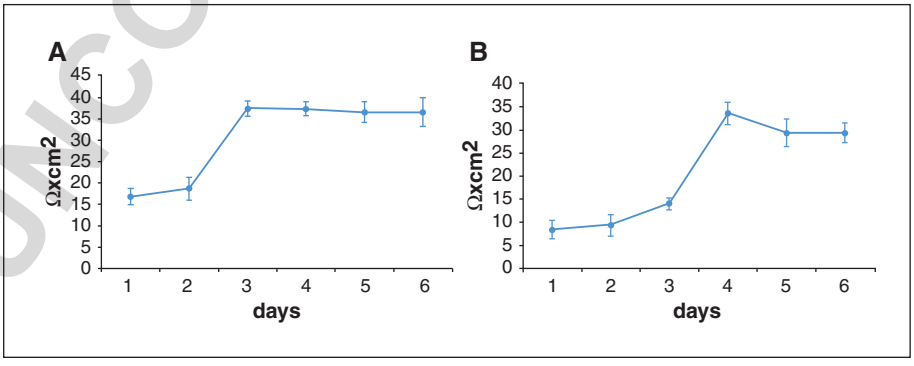

Figure 1. Measurement of TEER in human and rat BBB. TEER measurements were performed every 24 hours (h) for 6 days on human **(A)** and rat **(B)** BBB as described in the methods.

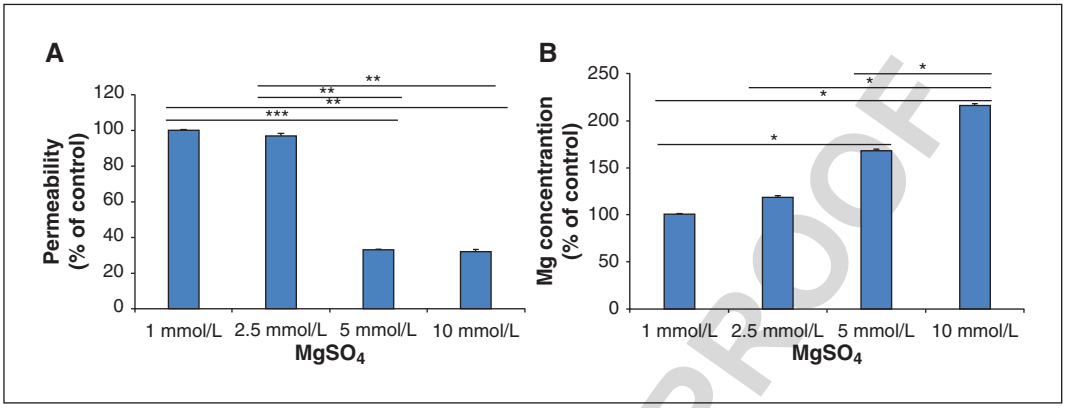

Figure 2. Decreased BBB permeability and increased Mg transport in the presence of high concentrations of $MgSO_4$. Human BBB was exposed to media containing 1, 2.5, 5, or 10 mmol/L $MgSO_4$. 1 mmol/L $MgSO_4$ was used as the control. After 6 hours of treatment, FITC-BSA transit from the apical to the basal chambers was measured by fluorimetry **(A)** and Mg accumulated in the lower chamber was quantified using the DCHQ5 fluorescent probe **(B)**. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

best in inhibiting it (*figure 3A*). We then compared $MgSO_4$, $MgCl_2$, MgPid, and MgThre (5 mmol/L) for their ability to impact Mg transport through the BBB. For this purpose, BBB were exposed to the different salts for 1 and 6 hours and the fluorescent probe DCHQ5 was used to measure how much Mg accumulated in the lower chamber [17]. *Figure 3B* shows that Mg

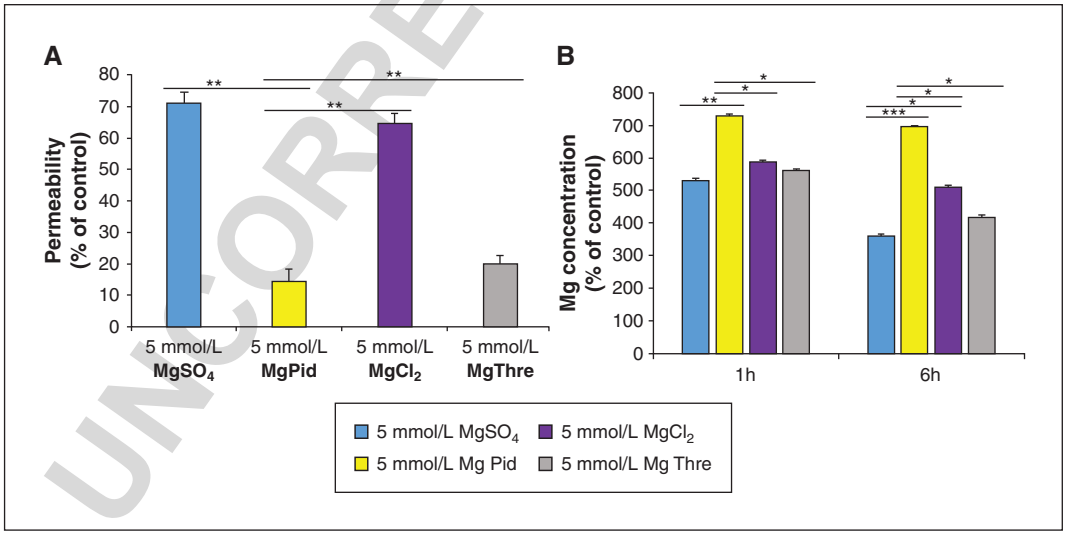

Figure 3. The effects of $MgSO_4$, $MgCl_2$, MgPid, and MgThre on rat BBB. Rat BBB was exposed to $MgSO_4$, $MgCl_2$, MgPid, and MgThre (5 mmol/L) for 6 h. BBB permeability was evaluated after adding FITC-BSA as described. Data are expressed as fold increase *versus* the control in 1 mmol/L $MgSO_4$. **(A)**. Quantification of level Mg accumulated in the lower chamber was performed after 1 and 6 h of incubation with the different Mg forms **(B)**. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

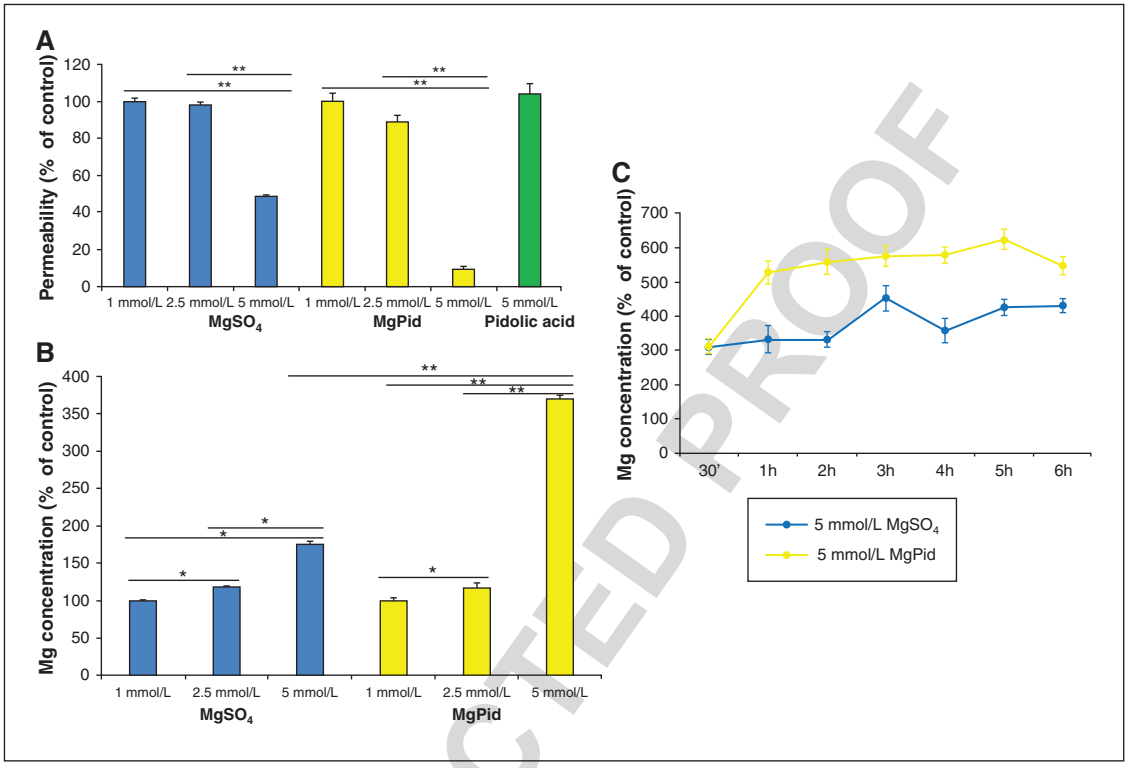

Figure 4. A comparison into the effects of $MgSO_4$ and MgPid on rat BBB. Rat BBB was exposed to different concentrations of $MgSO_4$ and MgPid. Pidolic Acid (5 mmol/L) was used as a control. BBB permeability was evaluated after adding FITC-BSA **(A)**. Mg transport was evaluated after 2 h of Mg salts exposure in a dose-dependent manner **(B)** and in kinetics **(C)** using the DCHQ5 fluorescent probe. $^*P < 0.05$, $^{**}P < 0.01$.

crosses the BBB more efficiently when we use MgPid in respect to the other Mg formulations. We then compared the effects of $MgSO_4$ and MgPid, which have a similar dissociation constant [14].

A comparison into the effects of $MgSO_4$ and MgPid on a rat *in vitro* model of BBB

$MgSO_4$ and MgPid (2.5 and 5 mmol/L) were utilized to measure rat BBB permeability and compared to the control (1 mmol/L $MgSO_4$). By measuring FITC-BSA after 6 hours of treatment, we demonstrate that MgPid is more efficient than $MgSO_4$ in reducing BBB permeability (*figure 4A*). We then tested the transport of Mg in a dose and time-dependent fashion in rat BBB exposed to $MgSO_4$ or MgPid for 2 hours. While at a concentration of 2.5 mmol/L no differences were detected between MgPid and $MgSO_4$, more Mg accumulated in the lower chamber when we used 5 mmol/L MgPid in respect to $MgSO_4$ (*figure 4B*). This difference was detected at all the time points tested (*figure 4C*).

It is well known that lipopolysaccharide (LPS), major endotoxin found in Gram-negative bacteria with strong pro-inflammatory effects, elevates the permeability of BBB [18]. We therefore asked whether high concentrations of Mg salts might prevent or reduce the effects of LPS. Initially, we pretreated BBB with $MgSO_4$ or MgPid (5 mmol/L) for 16 hours. Successively, we added LPS (1 µmol/L). We then measured the amounts of FITC-BSA in the lower chamber and found that both $MgSO_4$ and MgPid prevented LPS-induced increase of permeability (*figure 5*).

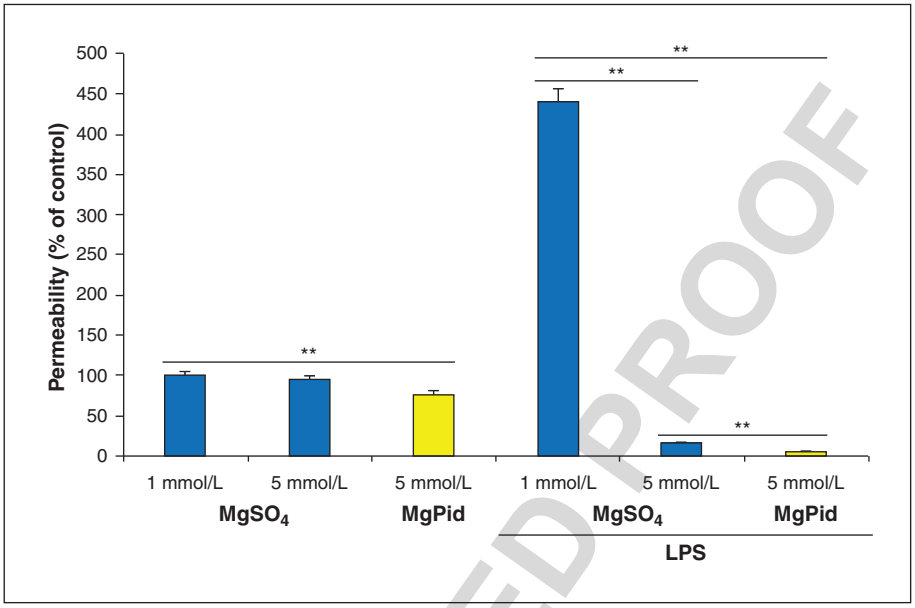

Figure 5. The inhibitory effect of $MgSO_4$ and MgPid on LPS permeabilizing effect in rat BBB. 5 mmol/L of $MgSO_4$ or MgPid were added to culture medium for 16 h before adding LPS (1 µmol/L). 1 mmol/L $MgSO_4$ was used as control. The amounts of FITC-BSA in the lower chamber were measured by fluorometry. $^{**}P < 0.01$.

The effects of various Mg salts on a human *in vitro* model of BBB

We then compared the effect of 5 mmol/L $MgSO_4$, $MgCl_2$, MgPid, and MgThre on human BBB permeability. MgPid is the best in reducing BBB permeability (*figure 6A*). These results highlight a different sensitivity of rat *versus* human BBB, since MgThre is less efficient than MgPid and no significant reduction of BBB permeability was exerted by $MgCl_2$. Next, we compared $MgSO_4$, $MgCl_2$, MgPid, MgThre (5 mmol/L) for their ability to cross the BBB after 1 and 6 hours of incubation. Similarly to the rat model, more Mg crosses the BBB and accumulates in the lower chamber when BBB is exposed to MgPid (*figure 6B*).

Discussion

Dysfunction of BBB has been described in several neurological disorders, including ischemic stroke, inherited and neurodegenerative diseases [3, 19]. Moreover, BBB hyperpermeability was detected in stressed rodents [9, 20, 21], and Mg deficiency in the brain has been associated with BBB disruption [10]. It is therefore interesting to individuate agents that exert a protective role in the BBB and prevent its impairment in response to various challenges. Evidence has been provided about a protective role of Mg on the BBB *in vivo* [7, 22], and a recent paper has highlighted that 10 mmol/L $MgSO_4$ reduce the permeability in an *in vitro* model of human BBB [5]. Such an effect could be the result of the calcium antagonistic effect exerted by Mg on endothelial actin cytoskeleton, which leads to reduced formation of intercellular gap formation [23], thus inhibiting the paracellular movement of solutes through the tight junctions.

In this study we compared the effect of 5 mmol/L of different Mg salts in *in vitro* models of rat and human BBB. While all the salts decreased BBB permeability, MgPid and MgThre were the most efficient in the rat model. In the human model, MgPid was more efficient than MgThre, $MgSO_4$. $MgCl_2$ did not exert any significant

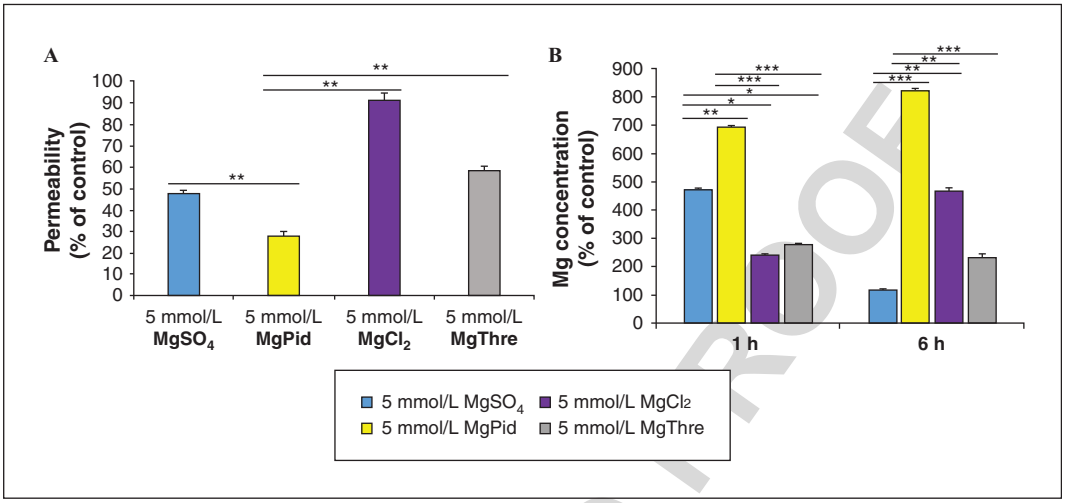

Figure 6. The effects of $MgSO_4$, $MgCl_2$, MgPid, and MgThre on human BBB. Human BBB was exposed to $MgSO_4$, $MgCl_2$, MgPid, and MgThre (5 mmol/L) for 6 h. BBB permeability was evaluated after adding FITC-BSA. Data are expressed as fold increase *versus* the control in 1 mmol/L $MgSO_4$ **(A)**. Quantification of level Mg accumulated in the lower chamber was performed after 1 and 6 h of incubation with the different forms of Mg **(B)**. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

effect. These results indicate that differences in the response obtained in rodents and humans may exist, and therefore caution should be used in interpreting experimental data from animal models and translating them to humans.

We further compared $MgSO_4$, an inorganic salt largely used *in vivo* and *in vitro* [5, 8], and MgPid on rat BBB model. MgPid reduced the permeability of the BBB to FITC-BSA more than $MgSO_4$ both in a time- and -dose-dependent fashion. Moreover, after challenge with LPS, widely used to generate systemic inflammation, MgPid was more efficient than $MgSO_4$ in preserving BBB permeability. These results might have clinical implications in all those conditions characterized by BBB dysfunction. We hypothesize that high concentrations of Mg prevent endothelial tight junction disruption by LPS probably preventing the activation of cyclooxygenase, since indomethacin, which targets the enzyme, prevents LPS-induced BBB increased permeability [24]. Accordingly, high extracellular Mg inhibits cyclooxygenase activity in endothelial cells [25].

Another aspect evaluated in the study was the transport of Mg through the BBB. Mg concentration in the brain is important for neural activity. Indeed, because of its anti-inflammatory function, its inhibitory control over the activation of N-methyl-D-aspartate receptors, and its central role in regulating fundamental cellular function [10], Mg was reported to be neuroprotective. In rats, $MgSO_4$ was found to cross the intact BBB and enter the central nervous system. This transport was proportional to magnesemia [26]. In humans a modest but significant increase in cerebrospinal fluid concentrations was reported after systemic administration of $MgSO_4$ [27].

Of interest, Mg homeostasis has been found to be dysregulated in various neurological disorders. The brain of patients with Alzheimer and Parkinson diseases contains lower concentrations of Mg compared to age-matched healthy controls [3], and reduced cytosolic Mg has been found in the occipital lobes of patients with migraine and cluster headache [28].

In hypomagnesemic individuals Mg supplementation attenuates anxiety and stress symptoms [11]. Similarly, Mg-deficient mice exhibit an anxiety-related behavior, which is due, in part, to the increase in the response of hypothalamic pituitary adrenal axis, a central substrate of the stress response system [12].

It is therefore relevant that both in the rat and in the human model, more Mg crosses the BBB when MgPid is used. On these bases, it is feasible to hypothesize that MgPid might be useful not only to prevent the increase of BBB permeability but also to increase or restore intracerebral Mg concentrations.

Disclosures

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