A New Caco-2 Cell Model of in Vitro Intestinal Barrier: Application for the Evaluation of Magnesium Salts Absorption

Ján KYSELOVIČ[1], Nikola CHOMANICOVÁ[2], Adriana ADAMIČKOVÁ[1], Simona VALÁŠKOVÁ[2], Barbara ŠALINGOVÁ[1], Andrea GAŽOVÁ[3]

[1]5th Department of Internal Medicine, Faculty of Medicine, Comenius University Bratislava, Slovakia, [2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Comenius University Bratislava, Slovakia, [3]Institute of Pharmacology and Clinical Pharmacology, Faculty of Medicine, Comenius University Bratislava, Slovakia

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Summary

Experimental data concerning the bioavailability of the different Mg-salts in human organism is inconsistent. Mg-absorption reported by clinical studies largely varies depending on the method used for evaluation. The aim of this study was to evaluate the bioavailability and accessibility of magnesium bound in different Mg-salt compounds, using an *in vitro* model of intestinal cell barrier. The study included a variety of inorganic (oxide, sulphate, chloride, carbonate) and organic salts (lactate, citrate, pidolate). Caco-2 cells were cultivated in a complete culture medium with different magnesium salts treatments in ascending concentrations. The viability and quantity of cells was analysed by FACS. Mg-absorption was analysed by a direct colorimetric assay, measured by spectrometry. T-test identified a significant decrease in cell count treatment with mg-lactate compared with citrate. Mg-pidolate showed a significantly higher cell viability compared with Mg-citrate, Mg-lactate and Mg-chloride. Even though the difference was not significant, we showed that an increase in Mg^{2+} salt concentration progressively decreased the cell count and the viability and the effect was universal for all the used Mg-salt treatments. Mg-citrate, chloride, and sulphate showed a significantly lower absorption compared to Mg-carbonate, pidolate and oxide. Our *in vitro* monolayer model of human intestinal transport showed that viability and quantity of cell decreased with increasing Mg-concentration. We admit that our experiment model may have some limitations in accurately describing an *in vivo* Mg^{2+} absorption. Moreover, it is also necessary to assess the relevance of our data *in vivo* and especially in clinical practice.

Key words

Magnesium salts • Magnesium absorption • Caco-2 cell line

Corresponding author

Andrea Gažová, Faculty of Medicine, Comenius University Bratislava, Institute of Pharmacology and Clinical Pharmacology, Špitálska 24, 813 72 Bratislava, Slovakia. E-mail: andrea.gazova@fmed.uniba.sk, ORCID 0000-0002-4515-5693.

Introduction

Magnesium supplementation utilises different types of magnesium salts, e.g. oxide, chloride, gluconate or lactate (Ranade *et al.* 2001). The bioavailability of elementary magnesium may vary in each of these individual compounds and is still the subject of experimental work and clinical studies. Clinical trials have investigated multiple magnesium compounds in order to determine the most suitable for magnesium supplementation by comparison their respective bioavailability (Kappeler *et al.* 2017, Bøhmer *et al.* 1990, Gegenheimer *et al.* 1994, Schuette *et al.* 1994, Lindberg *et al.* 1990, Firoz *et al.* 2001, Muehlbauer *et al.* 1991, Walker *et al.* 2003). Clinical trials have generally tried to analyse magnesium bioavailability and specifically the compare organic and inorganic magnesium salt sources, (Ranade *et al.* 2001, Wolf *et al.* 2003, Kappeler *et al.* 2017, Schuette *et al.* 1994) focusing primarily on evaluation of urinary excretion or serum levels. The published results suggest that Mg supplementation with magnesium organic compounds such as magnesium citrate and magnesium aspartate might be more efficient compared to the inorganic magnesium oxide. However,

these results are difficult to compare due to differences in the study design and different analysed parameters. The analysis of magnesium absorption is further complicated by endogenous magnesium levels, strictly regulated in several physiological systems of the human body. For example, magnesium concentrations are strictly regulated in human serum, which makes one of the easiest accessible human material unusable for magnesium bioavailability analysis (Brannan *et al.* 1976).

Methods

Cultivation Caco-2 cells

Caco-2 cell line with a homogenous standard phenotype was used for all the reported experiments. Caco-2 cells, supplied by the ECACC passage 25–40th, were cultivated in complete culture medium consisting of DMEM (Dulbecco´s modified eagle´s medium-low glucose, Sigma-Aldrich), 10 % FBS (Fetal bovine serum, Sigma-Aldrich), 5 % Penicillin/Streptomycin solution (Sigma-Aldrich) at 37 °C with 5 % CO2/95 % air atmosphere. The complete culture medium was replaced every 2 days until the cells reached 60 % confluence, when the cultures were passaged. The intestinal cell barrier was formed as previously reported in Natoli M (*et al.* 2011) and Thongon and Chamniansawat (2019) (Brannan *et al.* 1976, Ranade *et al.* 2001).

Corning® HTS Transwell®-24 well permeable supports with HTS Transwell-pore polycarbonate membrane were used. At 100 % confluence, the cells formed a homogeneous polarized cell monolayer. After the intestinal cell barrier was formed, different magnesium salts diluted in cultivation media were added into the upper compartment of the cultivation well in a stepwise manner to establish an ascending concentration of the specific magnesium salt treatments 0.8 mM, 1.5 mM, 2 mM, 2.5mM, 5mM, and 8 mM). Three sets of experiments with different incubation times for magnesium salt treatments (15 min, 2 h, 24 h) were prepared. The absorption of magnesium by Caco-2 cell monolayer was measured in basolateral medium by Xylidyl Blue colorimetric assay. The measured absorbance at the specific concentration was plotted into the graphs.

The analysis of cell viability and cell count of Caco-2 cells

Cell counts (number of cells per ml after trypsinization) and cell viability (percentage of propidium iodide negative cells) was analysed by flow cytometry by MACSQuant Analyze (Miltenyi Biotec, Germany). Individual cell samples were released from the bottom of the Petri dish with 3 ml of a 0.25 % trypsin/EDTA solution. Trypsin was stopped after 3 min of incubation by dilution in a complete culture medium and cells centrifuged for 10 min, 300G. The pellet was washed in PBS, centrifuged again for 10 min, 300G and resuspended in AutoMACS running buffer (Miltenyi Biotec, Germany). Cell count was measured.

Caco-2 cell samples were further diluted (1 x 10^6 cells/ml) for viability analysis. 5 µl of propidium iodide (Miltenyi Biotec, Germany) was added for viability assessment just prior to measurement. At the beginning of the analysis, conflict cases representing cell clumps or impurities were filtered out by gating. The obtained results were finally evaluated in the MACSQuant program.

Xylidyl blue method for measurement of Mg^{2+} concentration in cell cultivation media

The colorimetric estimation of Mg^{2+} in cultivation using the MAGNESIUM Xylidyl Blue Monoreagent, from Spectrum Diagnostics. Magnesium ions form a coloured chelate complex when reacting with xylidyl blue in alkaline solution, the intensity of the colour is proportional to the magnesium concentration. The spectrometry was prepared in window of the wavelength 400 – 700 nm to identified maximum and minimum absorbance Mg^{2+} cation in cultivation medium. Extrapolated data was stated for maximum and minimum of spectrophotometry curves at concentration 0.8 mM, 1.5 mM, 2 mM, 2.5 mM, 5 mM, 8 mM, of magnesium lactate, sulphate, citrate, chloride, carbonate, oxide, and pidolate. The absorbance values in maximum and minimum was used to prepare the calibration curve for the specific magnesium salts.

Statistical analysis

Statistical analysis of the experimental data was conducted via GraphPad Prism 9.0.0 (A P value ≤ 0.05 was considered as significantly relevant).

Results

1. Viability and cell count of Caco-2 cells under different magnesium salts treatments

The cell count of Caco-2 cells after cultivation

Caco-2 cells were cultivated for 24 h in

a complete cultivation medium with different magnesium salt treatments – citrate, lactate, sulphate, chloride, pidolate, oxide and carbonate in 0.8 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM, 5 mM and 8 mM concentrations. The viability and cell count were analysed by flow cytometry (Miltenyi Biotec, MACSQuant® Analyzer). No significant differences (one-way ANOVA, multiple comparison) were observed in cell number and viability between different magnesium salt treatments with increasing concentrations (Fig. 1). However, a paired T-Test (Table S1, Table S2, Fig. S1) showed a significant decrease of cell count for magnesium lactate compared to magnesium citrate (p=0.0459).

The viability Caco-2 cells after cultivation

We showed significant differences in cell viability between individual magnesium salt treatments. Paired T-Test (Table S3) identified a significantly higher viability of Caco-2 cells (89.98 %±1.608) under magnesium pidolate treatment compared to magnesium citrate (80.39±7.468), magnesium lactate (75.03±12.06) and magnesium chloride (70.35 %± 31.15) treatment, respectively (pidolate vs citrate p=0.0286, vs lactate p=0.0327, vs chloride p=0.0063) (Fig. 2).

The increased concentration of Mg^{2+} salts – lactate, chloride, and oxide progressively decreased the viability however the effect was considered insignificant. Magnesium oxide treatment (at 8 mM) was the only treatment significantly decreasing cell viability compared with the baseline (Table 2). Pearson correlation p-value identified significant difference between magnesium salts treatments (Fig. S2, Table S4).

2. Magnesium absorbance

The consumption of Mg^{2+} cations by cells was estimated through colorimetric analysis of the cultivation media, in order to assess bioavailability and accessibility of magnesium from different magnesium salts.

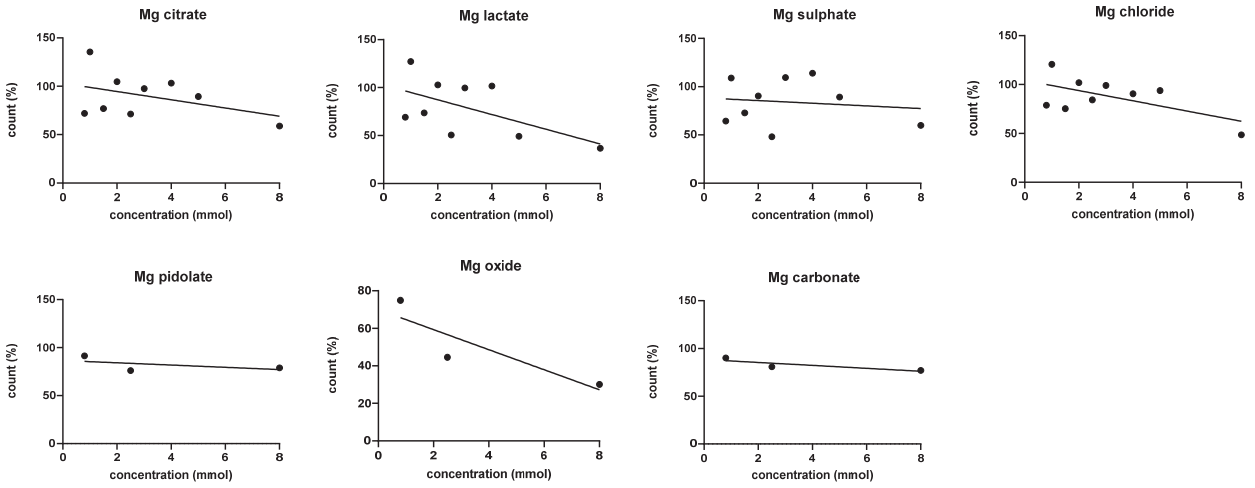

Fig. 1. The effect of different magnesium salt treatments on Caco-2 cell count. Graphic representation of average values from three independent experiments measured by flow cytometry.

Table 1. Cell counts as a percentage of untreated after 24h of individual magnesium treatments.

	citrate	lactate	sulphate	chloride	pidolate	oxide	carbonate
c = 2.5 mM	71.27 %	50.50 %	48.04 %	84.29 %	76.01 %	44.59 %	80.86 %
c = 8 mM	58.93 %	36.66 %	59.91 %	48.54 %	78.94 %	30.07 %	77.03 %

Table 2. Cell viability as a percentage of untreated after 24h of individual magnesium treatments.

	citrate	lactate	sulphate	chloride	pidolate	oxide	carbonate
c = 2.5 mM	99.35	89.60	97.23	90.52	99.07	92.81	92.68
c = 8 mM	97.40	80.56	97.90	81.89	101.74	26.69	89.76

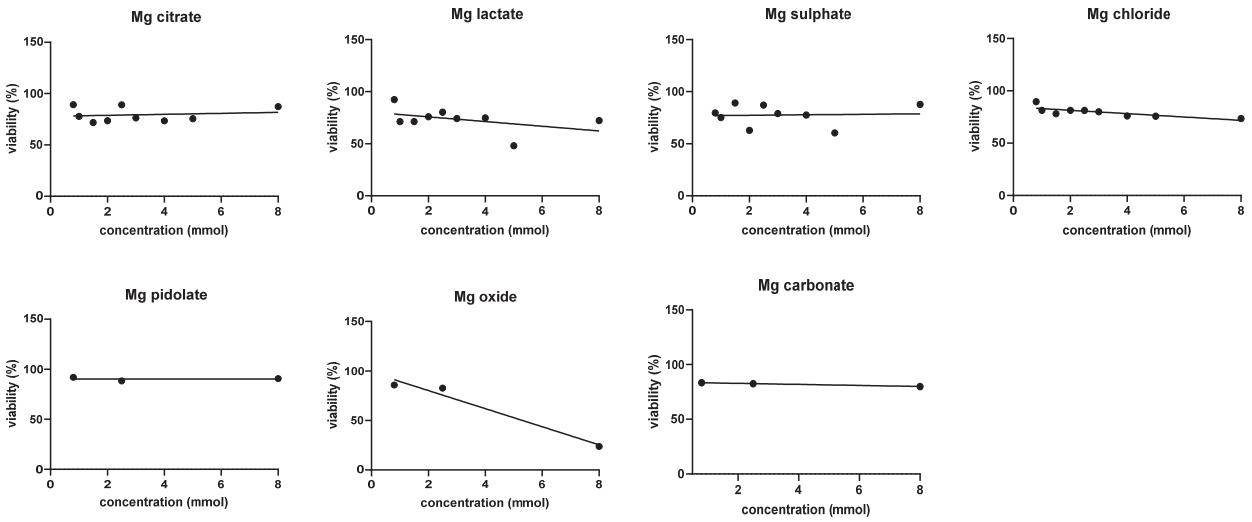

Fig. 2. The effect of different magnesium salt treatments on Caco-2 cell viability. Graphic representation of average values from 3 independent experiments measured by flow cytometry.

As the magnesium absorbance for each individual magnesium salt treatment was measured at the same equimolar concentration of Mg^{2+}, it was possible to compare the amount of the absorbed Mg^{2+} in all the tested treatments under the same experimental conditions. The measured data as well as the calculation of magnesium absorption at 0.8 mM, 2.5 mM and 8 mM concentrations after 15 minutes' incubation is shown in Fig. 3. The statistical analysis showed significant differences between the absorption of magnesium pidolate, oxide and carbonate compared with other salts (Table S5).

The data concerning magnesium absorption after 2 hours' magnesium salt treatments at 0.8 mM, 2.5 mM and 8 mM concentrations are shown in Fig. 4. The statistical analysis showed significant differences between the absorption of magnesium pidolate, oxide and carbonate compared with other salts (Table S6).

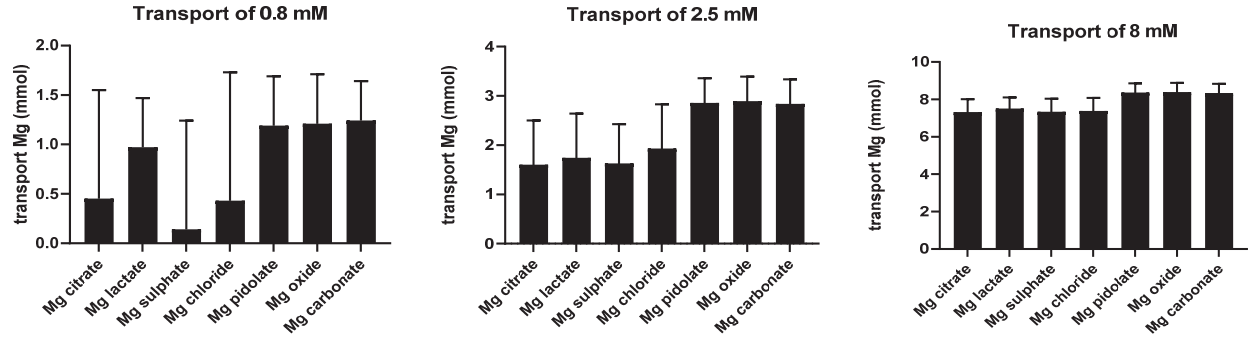

Fig. 3. The absorption of Mg^{2+} after 15minute magnesium salt treatments.

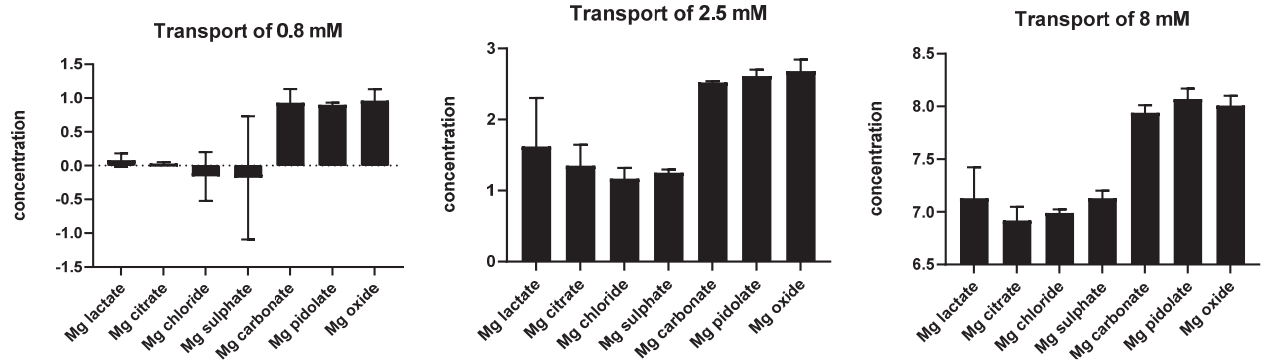

Fig. 4. The absorption of Mg^{2+} after 2 hours magnesium salt treatments.

Discussion

Magnesium deficiency in human body has various clinical manifestations. Currently, magnesium deficiency is frequently diagnosed in pathological conditions like cardiovascular diseases or diabetes mellitus (Workinger *et al.* 2018). Therefore, it is of most importance to prevent magnesium deficiency through enriched magnesium diet or supplementation with magnesium dietary products.

Magnesium supplementation utilises different types of magnesium salts, e.g. oxide, chloride, gluconate or lactate (Ranade *et al.* 2001). The bioavailability of elementary magnesium may vary in each of these individual compounds and is still the subject of experimental work and clinical studies. Clinical trials have investigated multiple magnesium compounds in order to determine the most suitable for magnesium supplementation by comparison their respective bioavailability (Kappeler *et al.* 2017, Bøhmer *et al.* 1990, Gegenheimer *et al.* 1994, Schuette *et al.* 1994, Lindberg *et al.* 1990, Firoz *et al.* 2001, Muehlbauer *et al.* 1991, Walker *et al.* 2003). Clinical trials have generally tried to analyse magnesium bioavailability and specifically the compare organic and inorganic magnesium salt sources, (Ranade *et al.* 2001, Wolf *et al.* 2003, Kappeler *et al.* 2017, Schuette *et al.* 1994) focusing primarily on evaluation of urinary excretion or serum levels. The published results suggest that Mg supplementation with magnesium organic compounds such as magnesium citrate and magnesium aspartate might be more efficient compared to the inorganic magnesium oxide. However, these results are difficult to compare due to differences in the study design and different analysed parameters. The analysis of magnesium absorption is further complicated by endogenous magnesium levels, strictly regulated in several physiological systems of the human body. For example, magnesium concentrations are strictly regulated in human serum, which makes one of the easiest accessible human material unusable for magnesium bioavailability analysis (Brannan *et al.* 1976).

The magnesium absorption takes place, unlike with other minerals, along the entire length of the gastrointestinal tract (Workinger *et al.* 2018, Schuchardt *et al.* 2017). Magnesium uptake is mediated by 2 distinct transport systems – active and passive. The homeostasis depends on the intestinal absorption, bone and soft tissue deposition and renal function (Thongon and Chamniansawat, 2019). Even though the essential mechanisms of magnesium absorption and transport have been previously described, the results and conclusions of many experimental works concerning magnesium absorption remain contradictory (Wolf *et al.* 2003). In vitro model may offer the possibility to better control experimental conditions and understand the discrepancies in the absorption of the various magnesium salts. While the intestine is responsible for the magnesium absorption, the intestinal barrier experimental model (a monolayer from human Caco-2 cell line) has been used and validated for testing magnesium absorption and transport (Natoli *et al.* 2011, Thongon and Chamniansawat 2019). Interestingly, Thongon in his 2019 paper studied the role of purinergic P2Y receptors in the regulation of Mg^{2+} absorption in normal and omeprazole-treated intestinal epithelium-like Caco-2 monolayers. (Thongon *et al.* 2011). Caco-2 monolayers have then been used as a model for studying the regulation of intestinal Mg^{2+} absorption (Thongon and Krishnamra 2012, Ekmekcioglu *et al.* 2000, Thongon and Krishnamra 2011, Xu *et al.* 2013). Caco-2 cells are a widely accepted in vitro intestinal transport model for study of metabolism and toxicity (Natoli *et al.* 2011, Thongon and Chamniansawat, 2019, Thongon and Krishnamra 2011). In prior studies, this model has also been used to assess the effects different pharmaceutical treatments on the absorption of magnesium (Thongon and Chamniansawat 2019, Thongon and Krishnamra 2011) or to analyse magnesium bioavailability from magnesium-fortified spirulina (Perrine Planes *et al.* 2002).

The present study evaluated the bioavailability of different Mg salts using the Caco-2 cell monolayer as an in vitro model for intestinal nutrient bioavailability study. We have tested for the first time the biological effect of inorganic and organic magnesium salts on the quantity and viability Caco-2 cells. Caco-2 cells were cultivated in a complete culture medium with different magnesium salt treatments (magnesium citrate, lactate, sulphate, chloride, pidolate, oxide and carbonate) in increasing concentrations. The quantity and viability of Caco-2 cells decreased with an increase magnesium salt concentration. Magnesium citrate, sulphate and pidolate treatments showed the lowest negative effects on the viability of the cell culture and cell count. On the other hand, magnesium oxide at 8 mM decreased cell count and viability by more than 70 %.

Magnesium is a divalent cation, which plays a critical role the mineral's absorption (Schuette *et al.* 1994, Lindberg *et al.* 1990). At lower magnesium

concentrations, a transcellular and saturable transport mechanism predominates and relies on an active transporter (de Baaij *et al.* 2012, Behar, 1974, Kiela *et al.* 2018). Active magnesium transport is mediated by Transient Receptor Potential Channel Melastatin members (TRPM6 and TRPM7) that possess unusual properties designed to strip away the hydration shell of magnesium. TRPM7 is a high sensitivity sensor that initiates a feedback back loop at high intracellular Mg^{2+} levels that results in saturation and inhibition of transcellular transport, and finally the switch to paracellular transport (Kiela *et al.* 2018, Schlingmann *et al.* 2007, Schmitz *et al.* 2003). This active transport, due to saturability, is only responsible for 10–20 % of total magnesium absorption. The paracellular passive pathway is in mostly mediated by claudin proteins at the tight junctions, that form paracellular channels, in monomeric or heteromeric combinations, which can efficiently transport ions such as calcium and magnesium (Thongon and Chamniansawat 2019, Thongon and Krishnamra 2011, Hou *et al.* 2009). Our study showed that the absorption of Mg^{2+} after 15 minutes and 2 hours of incubation is significantly higher for three salts (magnesium pidolate, magnesium oxide and magnesium carbonate) compared to magnesium citrate, magnesium sulphate and chloride, while magnesium lactate showed no significant difference in magnesium absorption compared to the other treatments. With the aim of comparing our data for bioavailability and bio accessibility of magnesium in different salt compounds to literature data, we conducted a precise analysis of about a hundred published clinical and experimental studies. This analysis shows that the information on the bioavailability of the essential mineral Mg^{2+} is sparse, inconsistent and unsuitable for meta-analysis as well as for direct comparison. Our results seem to be consistent with weak data for pidolate showing good bioavailability properties of this salt in preclinical studies (Coudray *et al.* 2005). Results are however contradictory to the published clinical data for magnesium oxide (Kappeler *et al.* 2017, Lindberg *et al.* 1990, Firoz *et al.* 2001, Walker *et al.* 2003), generally showing a poorer absorption compared to other salts, which might be due to the lower solubility of the compound. Our study model does have its own limitations, however. *In vitro* experimental models in general can approximate the *in vivo* environment to only a limited extend, as it is unfeasible to account for all the variables effecting physiological processes in human body. Since an ideal solubility of all the magnesium salt compounds was ensured in our experimental conditions, this does not necessarily correspond to the *in vivo* situation, where the conditions are not as ideal and magnesium salts are usually dissolved in water. This can in turn significantly obscure the results of the experiment.

In this study we also showed that magnesium oxide has the most pronounced negative effect on cell count and viability in our experimental model. This surprising finding would require further analysis to understand the reason of this difference. Furthermore, we observed a significantly higher Mg^{2+} absorption after 15 minutes and 2 hours' incubation with magnesium pidolate, oxide and carbonate compared to magnesium citrate, compared to magnesium sulphate and chloride, while magnesium lactate showed no significant difference in magnesium absorption compared to the other treatments. Because pidolate has been shown to in preclinical studies

Conclusions

In conclusion, we have demonstrated a good absorption of all magnesium salts tested in the present study, using a new and validated *in vitro* Caco-2 model of intestinal cell barrier. Interestingly, our study showed a significantly higher absorption of magnesium pidolate, carbonate and oxide salts, as compared to the other salts, illustrating the fact that solubilization of the magnesium salts might be a very critical factor in the absorption properties of the salts. However, due to the limitations previously mentioned, further investigation using this promising in vitro model is required, in order to improve the prediction of the *in vivo* bioavailability of Mg^{2+} salts.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Table S1. Comparison of Caco-2 cell counts – after 24 hours of incubation with different magnesium salt treatments. Cell count (cells/ml) after magnesium salt treatment 1/ cell count (cells/ml) after magnesium treatment 2. Analyzed by T-test (paired – unpaired#) p value is listed *p<0.05.

magnesium	citrate	lactate	sulphate	chloride	pidolate#	oxide#	carbonate#
citrate	X	0.0459*	0.2293	0.5720	0.7186	0.0749	0.7416
lactate	0.0459*	X	0.4157	0.1755	0.7257	0.3137	0.7061
sulphate	0.2293	0.4157	X	0.5076	0.9430	0.1477	0.9174
chloride	0.5720	0.1755	0.5076	X	0.7993	0.0704	0.8268
pidolate	0.7186	0.7257	0.9430	0.7993	X	0.1029	0.8127
oxide	0.0749	0.3137	0.1477	0.0704	0.1029	X	0.1014
carbonate	0.7416	0.7061	0.9174	0.8268	0.8127	0.1014	X

Table S2. Pearson's P values for cell counts correlation after different magnesium salt treatments. *p<0.05

magnesium	citrate	lactate	sulphate	chloride	pidolate	oxide	carbonate
citrate	X	0.0004*	0.0024*	0.0002*	0.3768	0.2167	0.2275
lactate	0.0004*	X	0.0046*	0.0044*	0.1973	0.0372*	0.0480*
sulphate	0.0024*	0.0046*	X	0.0183*	0.1927	0.3527	0.3419
chloride	0.0002*	0.0044*	0.0183*	X	0.4359	0.2758	0.2866
pidolate	0.3768	0.1973	0.1927	0.4359	X	0.1600	0.1492
oxide	0.2167	0.0372*	0.3527	0.2758	0.1600	X	0.0107*
carbonate	0.2275	0.0480*	0.3419	0.2866	0.1492	0.0107*	X

Table S3. Comparison of Caco-2 cell viabilities after 24 h of incubation with different magnesium salt treatments. Presented as: cell viability (%) after treatment with magnesium salt 1 / cell viability (%) after treatment with magnesium salt 2, analyzed by T-test (paired – unpaired#), p value is listed *p<0.05.

magnesium	citrate	lactate	sulphate	chloride	pidolate#	oxide#	carbonate#
citrate	X	0.1134	0.6025	0.9100	0.0286*	0.3342	0.4116
lactate	0.1134	X	0.2831	0.0707	0.0327*	0.6805	0.1887
sulphate	0.6025	0.2831	X	0.6107	0.0609	0.4420	0.3844
chloride	0.9100	0.0707	0.6107	X	0.0063*	0.3066	0.3159
pidolate	0.0286*	0.0327*	0.6107	0.0063*	X	0.3025	0.0744
oxide	0.3342	0.6805	0.4420	0.3066	0.3025	X	0.4158
carbonate	0.4116	0.1887	0.3844	0.3159	0.0744	0.4158	X

Table S4. P value for Pearson's cell viabilities correlation after different magnesium salt treatments. *p<0.05.

magnesium	citrate	lactate	sulphate	chloride	pidolate	oxide	carbonate
citrate	X	0.082	0.120	0.167	0.453	0.003*	0.067
lactate	0.082	X	0.088	0.018*	0.355	0.188	0.124
sulphate	0.120	0.088	X	0.484	0.246	0.298	0.234
chloride	0.167	0.018*	0.484	X	0.384	0.160	0.096
pidolate	0.453	0.355	0.246	0.384	X	0.456	0.480
oxide	0.003*	0.188	0.298	0.160	0.456	X	0.064
carbonate	0.067	0.124	0.234	0.096	0.480	0.064	X

Table S5. T-test paired. Correlation of magnesium absorption 0.8, 2.5 and 8 mM magnesium salt treatments after 15-min incubation. *p<0.05.

magnesium	citrate	lactate	sulphate	chloride	pidolate	oxide	carbonate
citrate	X	0.1406	0.5387	0.3508	0.0214*	0.0211*	0.0159*
lactate	0.1406	X	0.2550	0.5361	0.1112	0.1069	0.0952
sulphate	0.5387	0.2550	X	0.1323	0.0035*	0.0035*	0.0030*
chloride	0.3508	0.5361	0.1323	X	0.0056*	0.0058*	0.0024*
pidolate	0.0214*	0.1112	0.0035*	0.0056*	X	0.0153*	0.8995
oxide	0.0211*	0.1069	0.0035*	0.0058*	0.0153*	X	0.4738
carbonate	0.0159	0.0952	0.0030*	0.0024*	0.8995	0.4738	X

Table S6. T-test paired. Correlation of magnesium absorption after 0.8, 2.5 and 8 mM magnesium salt treatments after 2-hour incubation. *p<0.05.

magnesium	citrate	lactate	sulphate	chloride	pidolate	oxide	carbonate
citrate	x	0.1148	0.8158	0.3607	0.0111*	0.0107*	0.0057*
lactate	0.1148	x	0.1957	0.0939	0.0030*	0.0040	0.0009*
sulphate	0.8158	0.1957	x	0.2893	0.0118*	0.0186*	0.0157*
chloride	0.3607	0.0939	0.2893	x	0.0105*	0.0148*	0.0106*
pidolate	0.0111*	0.0030*	0.0118*	0.0105*	x	0.6326	0.3184
oxide	0.0107*	0.0040*	0.0186*	0.0148*	0.6326	x	0.1529
carbonate	0.0057*	0.0009*	0.0157*	0.0106*	0.3184	0.1529	x

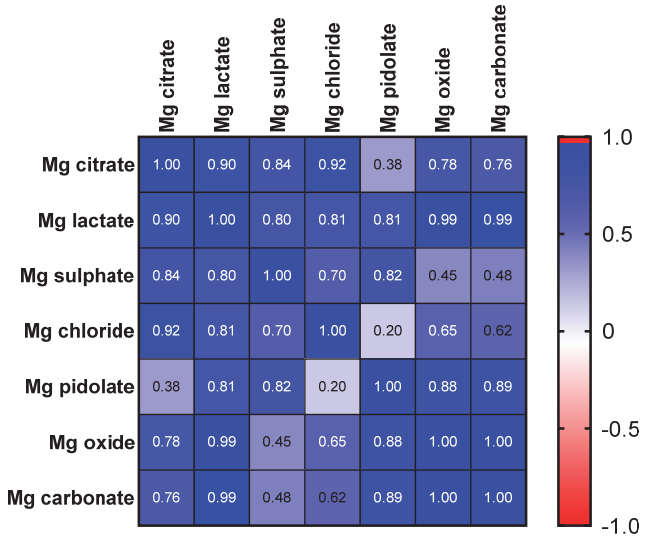

Fig. S1. Pearson's R values for cell counts correlation after magnesium salt treatments.

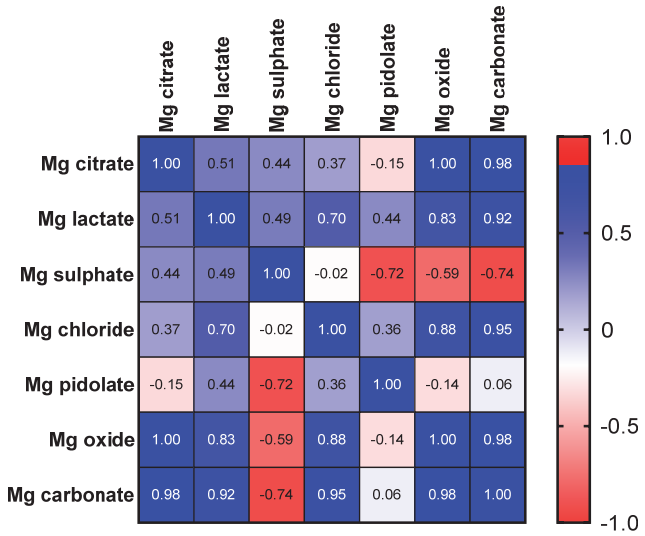

Fig. S2. Pearson's R values for cell viabilities correlation after magnesium salt treatments.