The influence of dietary components on absorption mechanisms at the intestinal monolayer

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Abstract

The influence of nutrients lactose, magnesium and calcium on the absorption mechanisms of the small intestine was investigated. Two *in-vitro* models of the intestine incorporating Caco-2 cell monolayers were applied to examine the effect of these treatments at concentrations expected *in-vivo* from milk. Effects were examined by measuring changes in intestinal barrier function and nutrient absorption. Fluctuations in paracellular intestinal barrier were detected by epithelial voltohmeter measuring epithelial electrical resistance. The glucose control peaked at 12 ohms at 1 minute then declined. The calcium treated monolayer peaked in resistance at 40 ohms at 10 minutes declining thereafter. The magnesium treatment increased monolayer resistance by 42 ohms at 1 minute, which was sustained for 100 minutes (±10 ohms). The lactose treated cells had sustained increase in resistance reaching 27 ohms at 100 minutes. Transcellular uptake of calcium, magnesium and phosphorus of the intestinal cells at 100 minutes was determined by ICP-AES. Mineral uptake of calcium treated cells was identical to the glucose control. The magnesium treated cells had significantly higher calcium uptake, (+58%) and lactose treatment, a significantly higher phosphorus uptake, (+12%) compared to the glucose control. These results help elucidate the action of dairy components on the functioning intestinal cell and monolayer. Highlighted is the role of lactose whose rate-limited hydrolysis results in gradual release of saccharides to the intestine, causing minimal impact on permeability in contrast to the glucose control. This work further demonstrates Caco-2 cell models are useful tools that may be used for nutritional investigations leading to the formulation of functional foods.

Keywords: intestine, nutrient, absorption, in-vitro, Caco-2, paracellular, transcellular, bioavailability lactose, calcium, magnesium, phosphorus, milk.

Introduction

Milk has been established as key to the development of healthy young mammals (German et al., 2003). The high level of calcium and magnesium combined with lactose in milk are nutritionally unique and this composition is associated with the development and functioning of the healthy intestinal tract.

The biochemical roles of individual nutrients are well understood. Calcium and magnesium play specific roles in the regulation of the internal cellular environment and the functioning of the intestine (Nellans, 1990, Saris *et al.*, 2000). The action of lactose is less well understood however its presence is implicated in stimulating bioavailabilities of a range of nutrients (Shortt and Flynn, 1991, Zitterman *et al.*, 2000, Abrams *et al.*, 2002, Etcheverry *et al.*, 2004). The impact of these components on the health and functioning of the cells of the intestinal tract during digestion events is not fully understood.

The small intestinal wall consists of a monolayer of closely packed intestinal cells which differentiate, proliferate and expire *in-situ* dependant upon diet, health and maturity (Whitfield, 1997). Nutrients pass through the monolayer either transcellularly, through lipid bilayer of the cell or paracellularly, between the spaces between the cells (see Figure 1).

1(a)                                    1(b)
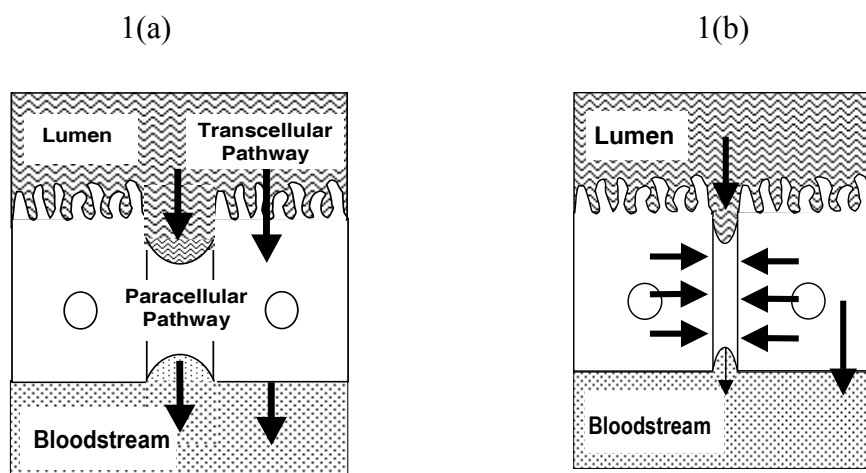


a)  Model of intestinal monolayer showing paracellular and transcellular routes of nutrient transit from intestinal lumen into bloodstream

b)  As nutrients are transported into the cells, the cells swell and constrict the paracellular pathway resulting in increased paracellular resistance.

Figure 1.  Changes in paracellular resistance of the cell monolayer with transport of nutrients. Measured by epithelial voltohmeter (EVOM).

The extent of this transit is affected by the composition of digesta as well as intestinal health (Ballard *et al.*, 1995). Factors impacting intestinal health include paracellular barrier function, transcellular nutrient absorption and performance of brush border enzymes (van der Klis and Jansman, 2002). The operational health of the intestinal tract and these functions can be investigated using *in-vitro* cell culture models that were developed to investigate the cellular functioning of the small intestine (Artursson *et al.*, 1996). The Caco-2 cell line exhibits many of the characteristics of cells of the small intestine, including formation of tight junctions,

glucose transport and enzymic activity when grown to high confluency over periods of 21-30 days (Artursson *et al*., 1996, Bailey *et al*., 1996, Yee, 1997, Seithel *et al*., 2006).

The Caco-2 model has been used in high throughput pharmacokinetic screening of drug candidates to determine intestinal permeability (Artursson and Borchardt, 1997, Seithel *et al*., 2006). Increasingly, Caco-2 model systems have been applied to nutrition studies to investigate the complex transcellular absorption phenomena of minerals (Zodl *et al*., 2005, Etcheverry *et al*., 2005). Caco-2 cells have also been successfully applied to the study of the bioavailability of minerals in sea mustard grass (Kim and Zemel, 1986), iron (Glahn *et al*., 1998) mineral water (Ekmekcioglu *et al*., 1999), and the bioavailability of calcium, iron and zinc in infant formula (Etcheverry *et al*., 2005).

The purpose of this study was to develop a model to identify the effect of lactose and dairy minerals; calcium, magnesium on intestinal barrier function and nutrient uptake. Caco-2 cell monolayers were employed to model paracellular barrier function by measurement of change in transepithelial electrical resistance. Transcellular nutrient transport was investigated by measurement of mineral uptake by the Caco-2 cell monolayer.

Method

The Caco-2 cell line was sourced from The Garvan Institute (Australia). Cells were cultured in an incubator at 37°C in 95% air and 5% $CO_2$ with Gibco BRL Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, California, USA) (low glucose 1.5g/L D-Glucose DMEM containing L-glutamine, 110mg/L sodium pyruvate and pyrdoxine hydrochloride) supplemented with 0.11% $NaHCO_3$, 10% Gibco fetal bovine serum (Invitrogen, USA), 1% non-essential amino acids (Thermotrace, Australia), 4mM Lglutamine, 2% Trace Multicell penicillin–streptomycin (Thermotrace, Australia), buffered at pH 7.2 with 15mM N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic acid (HEPES buffer). Media was changed every other day.

Refined pharmaceutical grade lactose extracted from cheese whey was examined in this study. For test solutions, addition of 3g/L of either D-glucose or lactose from whey was prepared as a stock solution with DMEM powder, 0.11% $NaHCO_3$ and 15mM HEPES buffer.

Concentrations of minerals in the treatment solutions were representative of the level of treatment mineral in one serving of milk (250 mL) diluted in 1500mL, an approximation of the dilution provided by intestinal juices (Ekmekcioglu, 2000). The test solutions prepared on stock media were: 1 = control glucose 4.5g/L (glucose control); 2 = glucose 1.5g/L + lactose 3.0g/L (lactose treatment); 3 = glucose 4.5g/L +calcium (Ca = 400mg/L) (calcium treatment); 4 = glucose 4.5g/L + magnesium (Mg = 32mg/L) (magnesium treatment).

Paracellular model

Viable cells (>85%) at passage 28 were seeded at $5 \times 10^6$/cm$^2$ onto Costar polycarbonate collagen treated cell culture inserts (Corning Incorporated, Acton, Massachusetts USA), surface area 1 cm$^2$, at day 1. One insert was retained as a blank without cells. Transepithelial electrical resistance (TEER) was measured during cell growth phase using an epithelial voltohmeter (EVOM) (World Precision Instruments, US). Once TEER stabilised at day 21, inserts were treated with control stock media containing glucose 4.5 g/L, or one of three test solutions (in triplicate). The blank with glucose based media without cells allowed membrane effects of the insert to be monitored. The first TEER measurement at t = 0 was done immediately before treatment to calibrate the TEER readings, thereafter TEER measurements were recorded at 1, 100 and 1000 minutes.

Transcellular Model

Viable cells (>85%) at passage 28 were seeded onto collagen coated (rat tail collagen, type I Becton Dickinson and Company, US) Costar 6-well plates, surface area 4.71 cm$^2$ (Corning, US) at a density of 6x10$^4$ cells/cm$^2$ at day 1. Media was changed every other day. Cells were treated at day 21 with the test solutions described above (in triplicate). After 100 minutes, the cell monolayer was rinsed three times, twice with a solution of 130 mM NaCl, 5 mM KCl and 5 mM 1,4 Piperazine bis (2-ethanosulfonic acid) (PIPES buffer) at pH 6.7 to remove extracellular minerals followed by a rinse with 2 mM EDTA at pH 8.0 remove surface bound minerals (Etcheverry et al., 2002).

Cell monolayers were prepared for mineral analysis by suspending the treated cells in 2 mL of 5.4mM ethylenediaminetetraacetate (EDTA) at completion of the experiment. The cells were then digested in 5 mL 70% nitric acid in polytetrafluoroethylene (Teflon) digestion containers, sealed and heated at 110°C for 12 hours. After cooling, samples were diluted to 50 mL with Milli-Q Ultra Pure water. The calcium, magnesium and phosphate levels in the cells was determined by inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) (at Australian Government Analytical Laboratories) against a nitric acid blank.

Results and Discussion

Two in-vitro model systems utilising Caco-2 intestinal cell monolayers were applied to measure changes in paracellular resistance and transcellular uptake of minerals. An epithelial voltohmeter (EVOM) was used to detect changes in transepithelial electrical resistance (TEER) of the cell monolayer. Measurement of TEER is an indirect method, measuring changes in paracellular barrier function ( Balda et al., 1996, Morgado-Díaza and de Souzab, 2001, Pappenheimer and Michel, 2003, D'Souza et al., 2003). Increase in TEER is consequent to transcellular transport of ions into the cell and is caused by constriction of tight junctions (see Figure 1) (Pappenheimer and Volpp, 1992). Additionally, transport of nutrients into the cell results in the formation and activation of receptors, transporters, buffers and stores necessary for maintenance of the cellular environment (Thorens, 1993, Saris et al., 2000, Hofer et al., 2004).

Changes in transepithelial electrical resistance (TEER) of the cell monolayer with different treatments over time is shown in Figure 2. Results were analysed using single factor ANOVA (p<0.05).  After one minute of treatment, the glucose control, lactose and calcium had slightly increased monolayer TEER, while the magnesium treatment showed a substantial early increase to 42 ohms. The data for the glucose control, lactose and calcium treatments at one minute indicate that they caused little change in the functioning of the tight junctions. These treatments would be expected to cause a slower uptake of nutrients compared to magnesium. Magnesium is a catalyst in a wide range of enzymic reactions such as the formation of receptors, transporters and buffers (Beyenbach, 1990, Flatman, 1991). To initiate biological effects magnesium needs to be bound deep into protein structure. It appeared the higher level of magnesium present in the treatment the higher the frequency of this occurrence.

Simultaneous to the transcellular movement of the ions, the magnesium treatment caused the monolayer to decrease TEER slightly at 10 minutes. The sudden change in TEER of the calcium treatment is indicative of the influx of calcium ions occurring at that point. Calcium binds to proteins avidly (Kretsinger, 1977). As a consequence, intracellular levels of calcium are strictly controlled to prevent unwanted cell signalling, such as proliferation, differentiation or cell death. Intracellular calcium is therefore buffered, stored as insoluble phosphate or rejected rapidly by the cell (Hofer et al., 2004).

Calcium binds into shallow binding sites in proteins unlike magnesium, which has a large number of coordination spheres (Martell and Hancock, 1996). The decrease in TEER seen in the magnesium treatment may have been due to the effect of calcium occupying more binding sites on the cell surface than at one minute, restricting flow of magnesium into the cell.
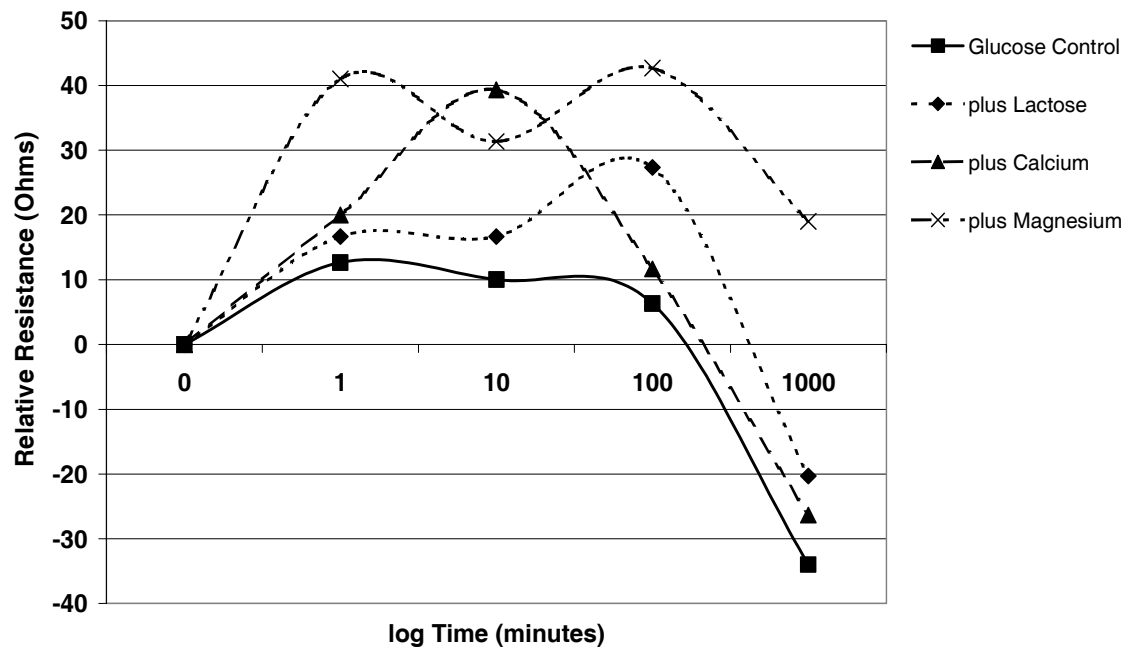


Figure 2. Paracellular results. TEER electrical resistance of Caco-2 cells cultured on transwell inserts exposed to media with added lactose, calcium or magnesium compared to the glucose control.

Lactose-treated cells continued to show moderately increasing TEER at 10 minutes (see Figure 2), indicating continued increases in cellular activity, while the glucose control TEER began to decrease. Lactose is hydrolysed by β−galactosidase formed in the brush border, which is less prolific than the α-galactosidases (Thorens, 1993). Once hydrolysed, the resulting glucose and galactose is metabolised by the cell. The rate-limited hydrolysis of lactose resulted in the gradual increasing TEER observed for the lactose treatment. The glucose control was not subject to such rate limited hydrolysis and TEER decreased after 1 minute, consistent with others observations (Madara and Pappenheimer, 1987).

After 100 minutes the TEER for the calcium treatment had fallen to a level similar to the glucose control (see Figure 2), indicating decreased cellular activity, which is associated with reduced capacity to form products to facilitate receptors and transporters necessary for the uptake of calcium (Hofer *et al.*, 2004).

Meanwhile, the magnesium treated cells maintained a high resistance at 42 ohms and the lactose treated cells continued to rise to reach a maximum of 27 ohms at 100 minutes, consistent with a prolonged maintenance of cellular activity and consequent transcellular uptake of nutrients.

The amount of calcium, magnesium and phosphorus taken up by the cells was determined at 100 minutes to demonstrate the level of transcellular accretion of minerals for the different treatments (see Figure 3). These results show that transcellular calcium uptake was significantly higher with the magnesium treatment (+58%) compared to the glucose control, but there was no difference in transcellular calcium observed for the lactose, calcium and glucose control treatments. The significantly raised calcium levels in the presence of magnesium corresponded with the increased cellular activity identified by the high TEER readings throughout the treatment period.
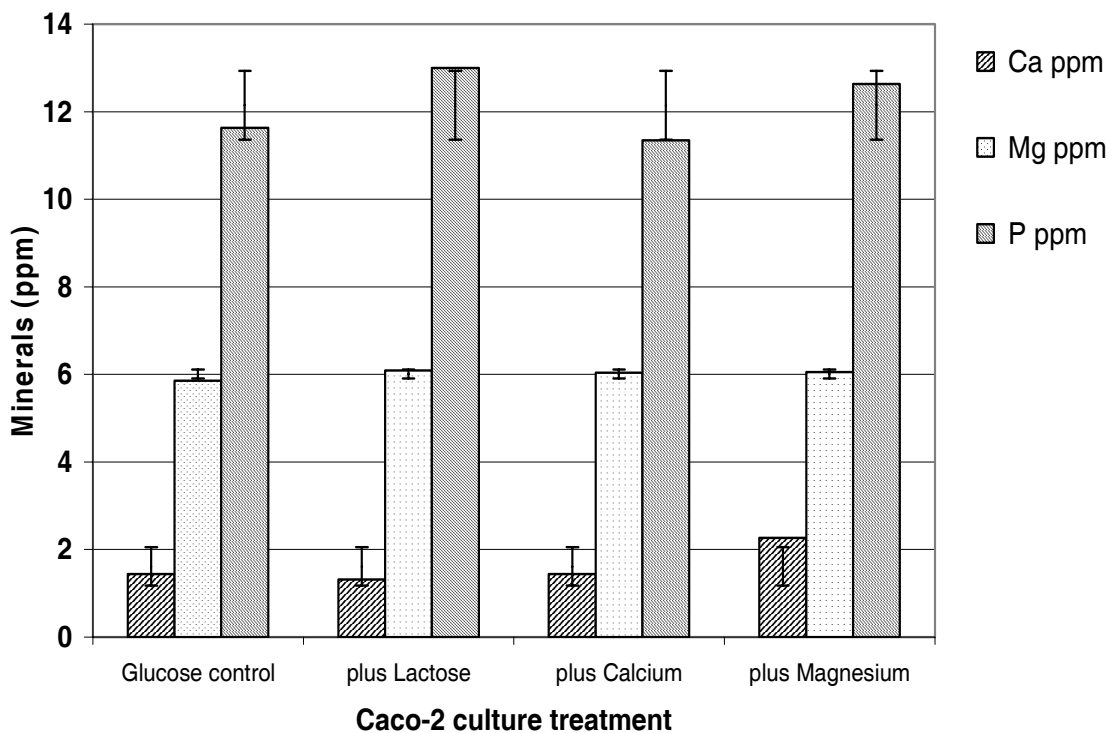


Figure 3: Transcellular Results: Mineral content (ppm +/- SEM) of Caco-cells cultured with added lactose, calcium or magnesium compared to the glucose control, after 100 minutes incubation.

The transcellular phosphorus uptake was significantly higher for the lactose treatment (+12%) compared to the glucose control, but no difference was observed for calcium, magnesium and glucose control treatments (see Figure 3). The steady release of energy to the cell from the hydrolysis of lactose stimulated TEER and cellular activity resulting in the increased uptake of phosphorus, which was not observed in other treatments. Increased phosphorus levels was consistent with protein formation in the cell and the production of β-galactosidase (Thorens, 1993). Production of brush border enzymes is a positive indication of intestinal health (van der Klis and Jansman, 2002).

There was no significant difference in transcellular magnesium uptake with any treatment (see Figure 3). The diffusional transporter maintained intracellular magnesium levels for all treatments regardless of concentration present. Published levels of magnesium recorded in cells (0.5 mM) were close to that of extracellular levels (0.7 mM) (Flatman, 1991, Saris et al., 2000). The small membrane potential that protects the cell from change in cellular concentrations explains this result.

The glucose based control and the calcium treatment (also based on glucose) had similar intracellular mineral profiles and TEER at 100 minutes (see Figure 3). Glucose, in the absence of supplemental magnesium, resulted in a small initial activation of cellular activity, followed by continued decline. Low levels of transcellular absorption indicate less cell activity and combined with low TEER, decreased intestinal barrier function. The reverse is true for the presence of lactose with a steady increase in TEER for 100 minutes.

The final TEER measurements at 1000 minutes had decreased to below zero for all treatments indicating deterioration of tight junctions and structure of the monolayer. Digestion is unlikely to persist for 1000 minutes *in-vivo*, however this outcome may be representative of formulations capable of damaging the intestine integrity over exposures of shorter time periods (Tsukazaki *et al*. 2004).

This work confirms that food compostion can have both positive and negative impacts on the performance of the intestine. This cell culture model system can be applied to perform real-time, rapid assessment of factors affecting intestinal cell functioning. This avenue of food research has much to offer in the development and optimisation of new generation functional foods. Further testing *in-vivo* is desirable to confirm trends.


Conclusions

The functioning and consequent health of the intestine was explored using in-vitro Caco-2 cell models. Intestinal paracellular barrier function was evaluated by changes in transepithelial electrical resistance, while transcellular nutrient transport was evaluated from mineral uptake into the cells.

The results showed that the milk components, calcium, magnesium and lactose had beneficial effects on intestinal paracellular barrier function with sustained increases in resistivity, particularly for treatments including magnesium or lactose. The results also show that transcellular nutrient transport was enhanced in the presence of some dairy components. The magnesium treatment resulted in significant increases in calcium absorption, while the lactose treatment resulted in significant increases in phosphate absorption. Benefits to paracellular resistivity were related to the rate-limited hydrolysis of lactose, in comparison to the glucose control.

Overall, this work contributes to a better understanding of how food formulations impact on intestinal function. The Caco-2 models show potential in application to the formulation of new functional foods.


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