PLOS ONE

RESEARCH ARTICLE

Fe^{3+} opposes the $1,25(OH)_2D_3$-induced calcium transport across intestinal epithelium-like Caco-2 monolayer in the presence or absence of ascorbic acid

Sukpapohn Phummisutthigoon[1,2], Kornkamon Lertsuwan[2,3], Nattapon Panupinthu[1,2], Ratchaneevan Aeimlapa[1,2], Jarinthorn Teerapornpuntakit[2,4], Wasutorn Chankamngoen[2,5], Jirawan Thongbunchoo[1,2], Narattaphol Charoenphandhu[1,2,6,7]*, Kannikar Wongdee[2,8]

1 Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand, **2** Center of Calcium and Bone Research (COCAB), Faculty of Science, Mahidol University, Bangkok, Thailand, **3** Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand, **4** Department of Physiology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand, **5** Graduate Program in Molecular Medicine, Faculty of Science, Mahidol University, Bangkok, Thailand, **6** Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand, **7** The Academy of Science, The Royal Society of Thailand, Dusit, Bangkok, Thailand, **8** Faculty of Allied Health Sciences, Burapha University, Chonburi, Thailand

* naratt@narattsys.com

Citation: Phummisutthigoon S, Lertsuwan K, Panupinthu N, Aeimlapa R, Teerapornpuntakit J, Chankamngoen W, et al. (2022) Fe^{3+} opposes the $1,25(OH)_2D_3$-induced calcium transport across intestinal epithelium-like Caco-2 monolayer in the presence or absence of ascorbic acid. PLoS ONE 17(8): e0273267. https://doi.org/10.1371/journal.pone.0273267

Editor: Rajeev Kapila, ICAR - National Dairy Research Institute, INDIA

Received: April 20, 2022

Accepted: August 5, 2022

Published: August 30, 2022

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: Our study was supported by grants from Thailand Science Research and Innovation (TSRI)–Mahidol University (Fundamental Fund/Basic Research Fund: fiscal year 2022; to NC), the National Research Council of Thailand (NRCT)–Mahidol University (Distinguished Research

Abstract

Although iron is an essential element for hemoglobin and cytochrome synthesis, excessive intestinal iron absorption—as seen in dietary iron supplementation and hereditary disease called thalassemia—could interfere with transepithelial transport of calcium across the intestinal mucosa. The underlying cellular mechanism of iron-induced decrease in intestinal calcium absorption remains elusive, but it has been hypothesized that excess iron probably negates the actions of 1,25-dihydroxyvitamin D [$1,25(OH)_2D_3$]. Herein, we exposed the $1,25(OH)_2D_3$-treated epithelium-like Caco-2 monolayer to $FeCl_3$ to demonstrate the inhibitory effect of ferric ion on $1,25(OH)_2D_3$-induced transepithelial calcium transport. We found that a 24-h exposure to $FeCl_3$ on the apical side significantly decreased calcium transport, while increasing the transepithelial resistance (TER) in $1,25(OH)_2D_3$-treated monolayer. The inhibitory action of $FeCl_3$ was considered rapid since 60-min exposure was sufficient to block the $1,25(OH)_2D_3$-induced decrease in TER and increase in calcium flux. Interestingly, $FeCl_3$ did not affect the baseline calcium transport in the absence of $1,25(OH)_2D_3$ treatment. Furthermore, although ascorbic acid is often administered to maximize calcium solubility and to enhance intestinal calcium absorption, it apparently had no effect on calcium transport across the $FeCl_3$- and $1,25(OH)_2D_3$-treated Caco-2 monolayer. In conclusion, apical exposure to ferric ion appeared to negate the $1,25(OH)_2D_3$-stimulated calcium transport across the intestinal epithelium. The present finding has, therefore, provided important information for development of calcium and iron supplement products and treatment protocol for specific groups of individuals, such as thalassemia patients and pregnant women.

Professor Grant; to NC), National Science and Technology Development Agency (NSTDA; to NC), TSRI/Thailand Research Fund (TRF) through the International Research Network Program (IRN60W0001; to NC, KW, and WC), TRF–Office of the Higher Education Commission Research Grant for New Scholar (MRG6280198; to JTe), TSRI–Burapha University (Fundamental Fund; to KW), and Faculty of Science, Mahidol University (CIF/CNI Grant; to NC, NP and KL). SP was supported by a scholarship from Science Achievement Scholarship of Thailand (SAST). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Daily iron and calcium requirement normally increases during pregnancy and lactation [1]. Since iron is believed to inhibit the intestinal calcium absorption, a combined calcium and iron supplementation is presently considered ineffective and not recommended [2–4]. In addition, there are certain conditions in which the intestinal iron absorption is markedly enhanced, for example, in a disease called thalassemia—a hereditary anemic disorder caused by globin gene mutation [5], in which calcium absorption may be compromised and bone disorder has been reported [6, 7]. Up until now, the underlying cellular mechanism of iron-induced inhibition of calcium absorption has been elusive. Since cellular uptake of iron and calcium occurs through completely different sets of transporting proteins (please see below), it is unlikely that iron interfere directly with transepithelial transport of calcium. Therefore, we hypothesized that iron probably hinders the stimulatory effect of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] on calcium absorption.

Under normal conditions, $1,25(OH)_2D_3$ enhances cellular calcium uptake across the apical membrane of enterocytes by upregulating the expression and activity of divalent ion channel transient receptor potential vanilloid subfamily member 6 (TRPV6) [8]. Meanwhile, it also accelerates the cytoplasmic calcium translocation and plasma membrane Ca^{2+}-ATPase-1b ($PMCA_{1b}$)-mediated calcium extrusion across the basolateral membrane [8]. In other words, $1,25(OH)_2D_3$ exerts its positive effects on all steps of the transcellular calcium absorption, particularly in the proximal small intestine (duodenum and proximal jejunum) and proximal large intestine (cecum) [8–10]. Although there are several factors that potentially reduce intestinal calcium absorption, such as calcitonin and stanniocalcin [11–13], only a few have been reported to diminish the $1,25(OH)_2D_3$-induced calcium absorption. For example, fibroblast growth factor (FGF)-23—either from the systemic circulation or local cellular production—is a known inhibitory factor for $1,25(OH)_2D_3$ signaling as well as the $1,25(OH)_2D_3$-stimulated calcium absorption [14, 15]. Besides stimulating the transcellular calcium transport, $1,25(OH)_2D_3$ also enhances calcium movement across the paracellular pathway by reducing the intercellular resistance and increasing tight junction permselectivity, which represents an ability of the intestinal epithelium to discriminate ions with different size and charge, including calcium [8]. Thus, in the presence of high-calcium concentration in the intestinal lumen, $1,25(OH)_2D_3$ is able to upregulate both transcellular and paracellular calcium transports and becomes an important regulator of calcium absorption.

Dietary compositions, such as oxalate, phytate, quercetin, and iron can modulate intestinal absorption of minerals [16, 17]. It is well established that iron is normally transported across the apical and basolateral membrane of enterocyte by divalent metal transporter (DMT)-1 and ferroportin-1, respectively [18], and iron transport mechanism is probably not directly related to that of calcium uptake. Hence, the explanations of iron-induced inhibition of calcium transport are often based on iron/calcium physicochemical interaction in aqueous environment, change in calcium solubility, or an increase in cellular free radical production, the last of which was reported to reduce intestinal calcium transport [19–21]. On the other hand, other molecules, e.g., ascorbic acid, has long been used to increase calcium solubility and reduce cellular oxidative stress, but whether it can promote calcium absorption in the presence of iron remains unclear. Nevertheless, the fact that $1,25(OH)_2D_3$ is the salient stimulator of calcium absorption ushers us to postulate that iron, by compromising $1,25(OH)_2D_3$ action, is probably a potent inhibitor of the $1,25(OH)_2D_3$-induced calcium transport.

Therefore, the objectives of the present study were (*i*) to investigate the effects of ferric ion (Fe^{3+}) from iron(III) chloride ($FeCl_3$) on the transepithelial calcium transport across the intestinal epithelium-like Caco-2 monolayer with or without $1,25(OH)_2D_3$ pre-treatment, (*ii*) to

determine the acute response of Caco-2 cells to ferric ion exposure, and (*iii*) to demonstrate whether ascorbic acid was able to revert the action of ferric ion. Under normal conditions, DMT1 transports only Fe^{2+}; therefore, Fe^{3+} used in the present experiment (i.e., $FeCl_3$) must be reduced to Fe^{2+} by Dcytb (ferric reductase) prior to apical uptake by DMT1. In addition, $FeCl_3$ has previously been used to study iron uptake in Caco-2 cells [22]. Furthermore, we avoided using iron salts consisting of anions with $\geq$2 negative charges (e.g., sulfate, citrate or ethylenediaminetetraacetate) since the anions may bind to or form insoluble complexes with Ca^{2+}. Caco-2 monolayer was used in the present study because it has been shown to have functional characteristic of small intestine, including expression of transcellular calcium transporters (e.g., TRPV6 and calbindin-D_{9k}), presence of the brush border, expression of sucrase-isomaltase enzyme, and responses to vitamin D [23–25].

Materials and methods

Cell culture

Intestinal epithelium-like Caco-2 cells obtained from American Type Culture Collection (ATCC no. HTB-37; RRID CVCL_0025) were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 15% fetal bovine serum (FBS) GIBCO, Grand Island, NY), 1% L-glutamine (GIBCO), 1% non-essential amino acid (Sigma), 100 U/mL penicillin-streptomycin (Sigma), and 0.25 μL/mL amphotericin B (Sigma). Cells were propagated in a 75-cm^2 T flask (Corning, NY, USA) under humidified atmosphere containing 5% CO_2 at 37˚C and subcultured as described in the ATCC's protocol. Thereafter, Caco-2 cells (420,000 cells/well) were grown on a porous polyester membrane, i.e., Snapwell with a diameter of 12 mm and pore size of 0.4 μm (catalog no. 3801; Corning), as reported previously [26]. Culture media was changed daily, and monolayers were incubated at 37˚C for 3 days in a humidified atmosphere containing 5% CO_2. Under normal conditions, Caco-2 cells that form a confluent monolayer will develop microvilli and tight junction with abundant expression of calcium-transporting proteins, e.g., TRPV6, calbindin-D_{9k} and $PMCA_{1b}$, similar to the small intestinal epithelial cells [24, 27].

Experimental design

Unless otherwise specified, Caco-2 monolayers were incubated with culture media containing 0, 1, 10 or 100 nM $1,25(OH)_2D_3$ (catalog no. 71820; Cayman Chemical, MI, USA) on both apical and basolateral compartments for 72 h. Thereafter, each Snapwell was transferred into Ussing chamber for determination of transepithelial calcium flux and epithelial electrical parameters. To demonstrate the negative effect of ferric ion on $1,25(OH)_2D_3$-induced transepithelial calcium transport, the $1,25(OH)_2D_3$-treated monolayers were exposed for 24 h to 100 μM $FeCl_3$ in the basolateral compartment (catalog no. 157740; Sigma-Aldrich, Saint Louis, MO, USA).

In some experiments, Caco-2 monolayers were pre-incubated for 24 h with 0.5 mM ascorbic acid (catalog no. A8960; Sigma-Aldrich, Saint Louis, MO, USA) to demonstrate whether ascorbic acid was able to counterbalance the action of ferric ion on calcium transport. The concentration ranges of $FeCl_3$ and ascorbic acid were the optimal concentration without causing toxicity to the cells and consistent with previous reports [22, 28]. $FeCl_3$ treatment protocol was sub-divided into (*i*) an acute exposure protocol, in which $FeCl_3$ was directly added into the apical hemichamber during Ussing chamber study, and (*ii*) a prolonged exposure protocol, in which Caco-2 monolayers were incubated in culture media containing $FeCl_3$ in both apical and basolateral compartments. We added $FeCl_3$ in the basolateral compartment to ensure that even though a prolonged exposure to Fe^{3+} might increase extracellular Fe^{3+} concentration in

the close vicinity to the basolateral membrane, it could not decrease the baseline calcium flux. In other words, in a condition with high serum free iron, it was likely affected the 1,25 $(OH)_2D_3$-induced calcium flux rather than the baseline calcium flux.

Measurement of transepithelial calcium transport using radioactive tracer

Ussing chamber technique was used to determine the transepithelial calcium flux, as previously described [7]. In brief, Caco-2 monolayer was first mounted and equilibrated between apical and basolateral hemichambers for 10 min in isotonic bathing solution, which was comprised of (in mM) 118 NaCl, 4.7 KCl, 1.1 $MgSO_4$, 1.25 $CaCl_2$, 23 $NaHCO_3$, 12 D-glucose and 2 mannitol (all purchased from Sigma). The solution was continuously gassed all the time with humidified 5% CO_2 in 95% O_2, and maintained at 37˚C and pH 7.4. The osmolality was 290–293 mmol/kg water as measured by a freezing point-based osmometer (model 3320; Advanced Instruments, Norwood, MA, USA). Thereafter, the bathing solution in the apical hemichamber was replaced with fresh bathing solution containing ^{45}Ca at the initial amount of 0.451 Ci/mL and final specific activity of 90 mCi/mol (catalog no. NEZ013; PerkinElmer, Boston, MA, USA), while the basolateral side was replaced with fresh normal bathing solution. The ^{45}Ca radioactivity in counts per minute was analyzed by a liquid scintillation spectrophotometer (model Tri-Carb 3100; Packard, Meriden, CT, USA). Radiotracer samples were collected from Ussing chamber, and the unidirectional calcium flux in the apical-to-basolateral direction was calculated as previously described [26].

Measurement of epithelial electrical parameters

The epithelial electrical parameters, i.e., transepithelial potential difference (PD or voltage), short-circuit current (I_{sc}) and transepithelial resistance (TER), were determined as described previously [6]. In brief, PD and I_{sc} were recorded by two pairs of electrodes made of Ag/AgCl half cells connecting with Ussing chamber through salt bridges (2 M KCl in 3 g% agar). The PD-sensing electrodes were placed near the Caco-2 monolayer, connected to a preamplifier (model EVC-4000; World Precision Instruments, Sarasota, FL, USA) and PowerLab digital recording system (model 4/30; ADInstruments, Colorado Springs, CO, USA). An I_{sc}-passing electrode was located at the rear end of each hemichamber, connected in series to the EVC-4000 current-generating unit and PowerLab 4/30 operated with Chart version 5.2.2. Fluid resistance was subtracted by the EVC-4000 system. TER was calculated from Ohm's equation.

Quantitative real-time PCR

The mRNA expression levels of ascorbic acid transporters (SVCT1 and SVCT2), TRPV6, calbindin-D_{9k}, $PMCA_{1b}$ and DMT1 in Caco-2 monolayers were measured by real-time PCR. Total RNA was prepared by using TRIzol extract reagent (Invitrogen, Carlsbad, USA), as previously described [29]. Total RNA concentration was determined by NanoDrop-2000c spectrophotometer (Themo Specific, Waltham, MA, USA) and the 260/280-nm ratio ranged 1.8–2.0. One microgram of total RNA was then reverse-transcribed into cDNA by iScript cDNA synthesis kit (Bio-rad, Hercules, CA, USA). PCR and melting curve analyses were operated by QuantStudio 3 real-time PCR system (Applied Biosystems, MA, USA) with glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene) or other primers (Table 1). The mRNA expression levels were calculated based on the method of Livak and Schmittgen [30].

Cell viability assay

Viability of Caco-2 cells treated with various concentrations of $FeCl_3$ was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. In

Table 1. ***Homo sapiens*** **primers used in real-time PCR.**

Gene	Accession no.	Primer (Forward/Reverse)	Product size (bp)	Annealing temperature (˚C)
Vitamin C transporters				
SVCT1	NM_005847	5′-TCATCCTCTTCTCCCAGTACCT-3′	141	57
		5′-AGAGCAGCCACACGGTCAT-3′		
SVCT2	NM_005116	5′-GCACCCAGCTTTCTTCACTC-3′	163	61
		5′-CAGACTGCCAGTGCTATCCA-3′		
Calcium transport related genes				
TRPV6	AF365928	5′-TCTGACTGCGTGTTCTCAC-3′	144	56
		5′-ACATTCCTTGGCGTTCAT-3′		
Calbindin-D_{9k}	NM_004057	5′-TAGCTGTTTCACTATTGGGCA-3′	127	56
		5′-TTCATCCTTTGACAACTGGTCT-3′		
$PMCA_{1b}$	NM_001001323	5′-AGAAGGTGGAGATGGTGATGA-3′	179	56
		5′-CCCAGAAGGTGTCAATGACA-3′		
Iron transporter				
DMT1	NM_001174125	5′-CTTTGCCCGAGTGGTTCTGA-3′	185	56
		5′-AGTCACTCATTACTGGCCGC-3′		
Housekeeping gene				
GAPDH	NM_001289746	5′-TTGTTGCCATCAATGACCC-3′	166	53
		5′-ATTTTGGAGGGATCTCGCT-3′		

SVCT1, solute carrier family 23 member 1 (SLC23A1); SVCT2, solute carrier family 23 member 2 (SLC23A2); TRPV6, transient receptor potential cation channel subfamily V member 6; $PMCA_{1b}$, plasma membrane Ca^{2+}-ATPase-1b; DMT1, divalent metal transporter-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

https://doi.org/10.1371/journal.pone.0273267.t001

brief, Caco-2 cells were plated in 96-well plate at 25,000 cells/well for 24 h, and were then treated with $FeCl_3$ at concentrations ranging from 0 to 200 μM for 24, 48 and 72 h. To assess cell viability, the MTT solution (catalog no. M5655; Sigma) was added to obtain a final concentration of 0.5 mg/mL for 4 h to generate formazan crystals, which were dissolved with dimethyl sulfoxide. The color was measured at the absorbance of 540 nm with a microplate spectrophotometer.

Statistical analysis

The results are expressed as means ± standard errors. Two-group data were compared by unpaired Student's *t*-test. One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used for multiple sets of data. All analyses were performed by using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). The level of significance for all statistical tests was $P < 0.05$.

Results

Prior to investigating the effects of $FeCl_3$ on the $1,25(OH)_2D_3$-induced calcium transport, the Caco-2 cells were verified for the normal response to $1,25(OH)_2D_3$ and the expression of sodium-vitamin C co-transporters (i.e., SVCT1 and SVCT2), which are essential for cellular ascorbic acid uptake [31, 32]. Quantitative real-time PCR analysis showed that Caco-2 cells were able to express both SVCT1 and SVCT2 transcripts with the mRNA level of SVCT1 being greater than that of SVCT2 (Fig 1A). Moreover, after exposure to 1, 10 or 100 nM $1,25(OH)_2D_3$, the transepithelial calcium fluxes were significantly enhanced across the Caco-2 monolayers in a dose-dependent manner (Fig 1B). Since we performed the Ussing chamber experiment in an absence of transepithelial calcium gradient—i.e., both apical and basolateral

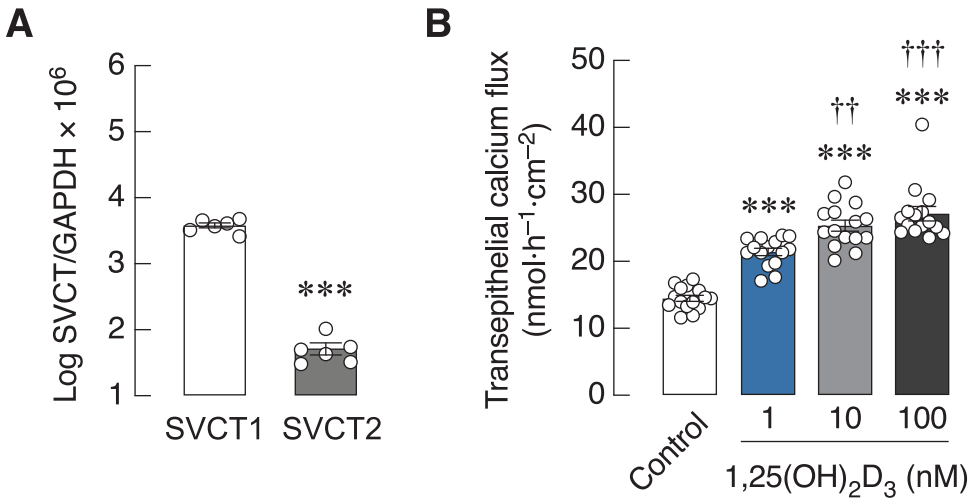

Fig 1. mRNA expression of vitamin C transporters and $1,25(OH)_2D_3$-induced calcium transport in Caco-2 monolayer. Expression of sodium-vitamin C co-transporters (SVCT and SVCT2) in Caco-2 cells (A). GAPDH is a housekeeping gene for normalization. (n = 6; ***, $P < 0.001$ compared with the SVCT1 group. (B) Transepithelial calcium flux across the $1,25(OH)_2D_3$-treated Caco-2 monolayers in Ussing chamber in the absence of transepithelial calcium gradient. ***$P < 0.001$ compared with the control group (white bar); ††$P < 0.01$; †††$P < 0.001$ compared with the 1 nM $1,25(OH)_2D_3$-treated group (blue bar).

https://doi.org/10.1371/journal.pone.0273267.g001

hemichambers contained equal free-ionized calcium concentration of 1.25 mM, the observed calcium flux represented the transcellular calcium transport in an apical-to-basolateral direction. We also verified that Caco-2 cells responded to $1,25(OH)_2D_3$ by increasing the transcellular calcium transport, similar to that observed in the proximal small intestine [9].

Thereafter, a series of experiments was performed to demonstrate that 24-h exposure to ferric ion had an inhibitory effect on calcium transport across Caco-2 monolayer pre-treated with 10 nM $1,25(OH)_2D_3$ for 72 h (Fig 2A). The results revealed that, despite the absence of both $1,25(OH)_2D_3$ and $FeCl_3$ in Ussing chamber, the transepithelial calcium flux of $FeCl_3$ and $1,25(OH)_2D_3$-treated monolayer was less than that of the monolayer treated with $1,25(OH)_2D_3$ alone (Fig 2B). Meanwhile, $FeCl_3$ significantly decreased I_{sc} and increased TER with no effect on PD (Fig 2C–2E). Thus, the actions of $1,25(OH)_2D_3$ and $FeCl_3$ during the pre-treatment of the Caco-2 cells persisted although Caco-2 cells in Ussing chamber no longer exposed to both agents.

In Fig 3A, we further explored whether acute exposure to ferric ion in Ussing chamber was capable of diminishing calcium transport in Caco-2 monolayer pre-treated for 72 h with $1,25(OH)_2D_3$, and whether ascorbic acid pre-treatment could revert the diminished calcium flux. As depicted in Fig 3B, $FeCl_3$ significantly decreased calcium transport in 10 nM $1,25(OH)_2D_3$-treated Caco-2 monolayer, but not in monolayer without $1,25(OH)_2D_3$ treatment. $FeCl_3$ also reverted the $1,25(OH)_2D_3$-induced changes in I_{sc} and TER to control levels [i.e., cells without $1,25(OH)_2D_3$ and $FeCl_3$], with no PD changes (Fig 3C–3E). However, ferric ion did not affect the epithelial electrical parameters of cells without $1,25(OH)_2D_3$ treatment (Fig 3C–3E). In addition, although ascorbic acid has been known to increase the solubility of calcium compounds, as shown in the present results (S1 Fig), 24 h ascorbic acid pre-treatment did not alter the electrical parameters or transepithelial calcium transport across Caco-2 monolayer with or without exposure $1,25(OH)_2D_3$ (Fig 3).

We further investigated whether the inhibitory action of $FeCl_3$ would still be observed after a prolonged 72 h exposure to ferric ion, or whether cells could eventually adapt to prolonged

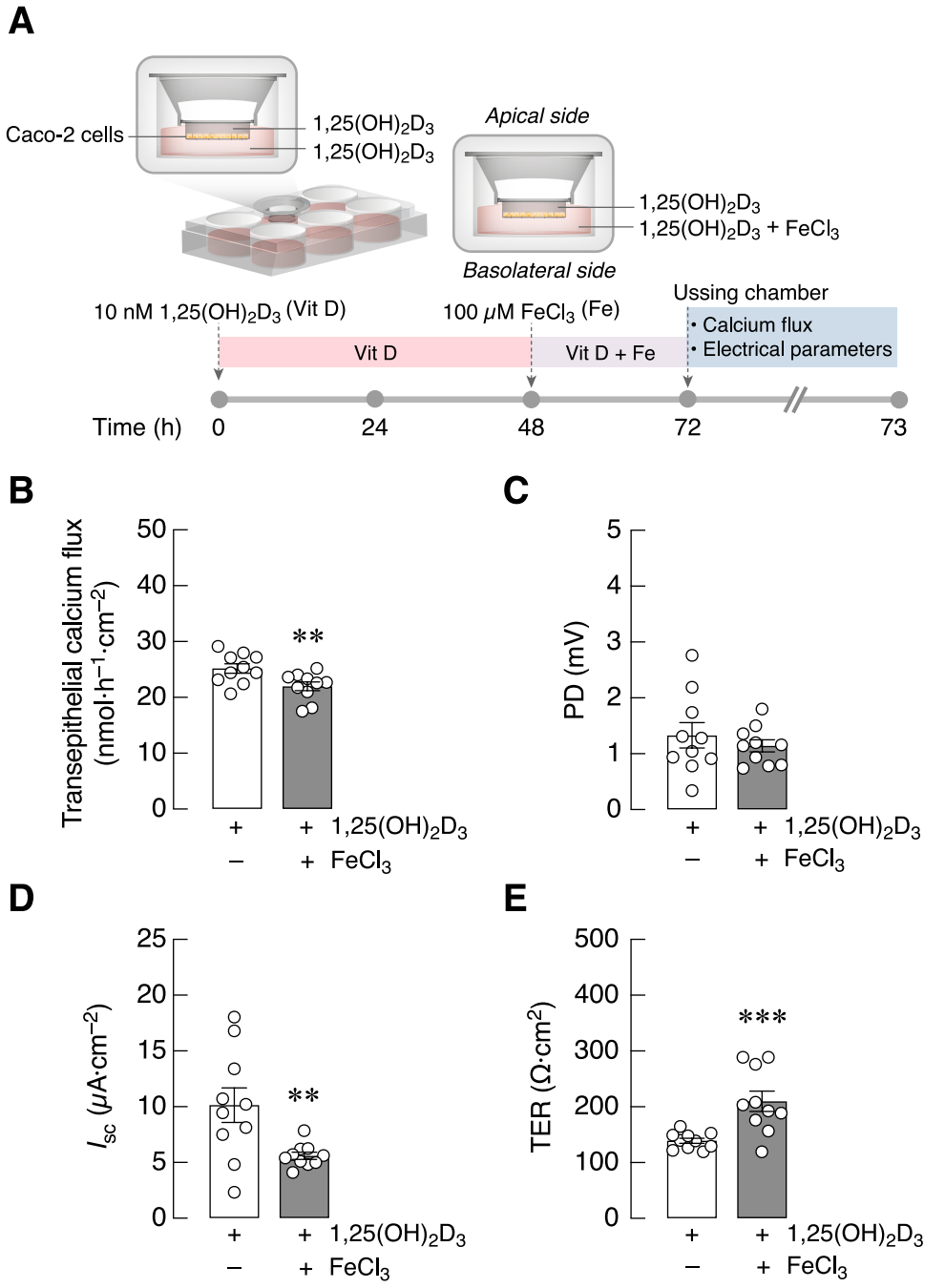

Fig 2. Transepithelial calcium flux of $FeCl_3$ and $1,25(OH)_2D_3$-treated Caco-2 monolayer. (A) Experimental timeline of $1,25(OH)_2D_3$ and $FeCl_3$ treatment (please see text for detail). (B–E) Transepithelial calcium transport and epithelial electrical parameters (PD, I_{sc}, and TER) in $1,25(OH)_2D_3$-treated Caco-2 monolayers with or without 100 μM $FeCl_3$. PD values were the magnitudes of potential difference (the apical side being negative with respect to the basolateral side), and glucose made the apical side more negative. (n = 10; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the control group (white bar).

https://doi.org/10.1371/journal.pone.0273267.g002

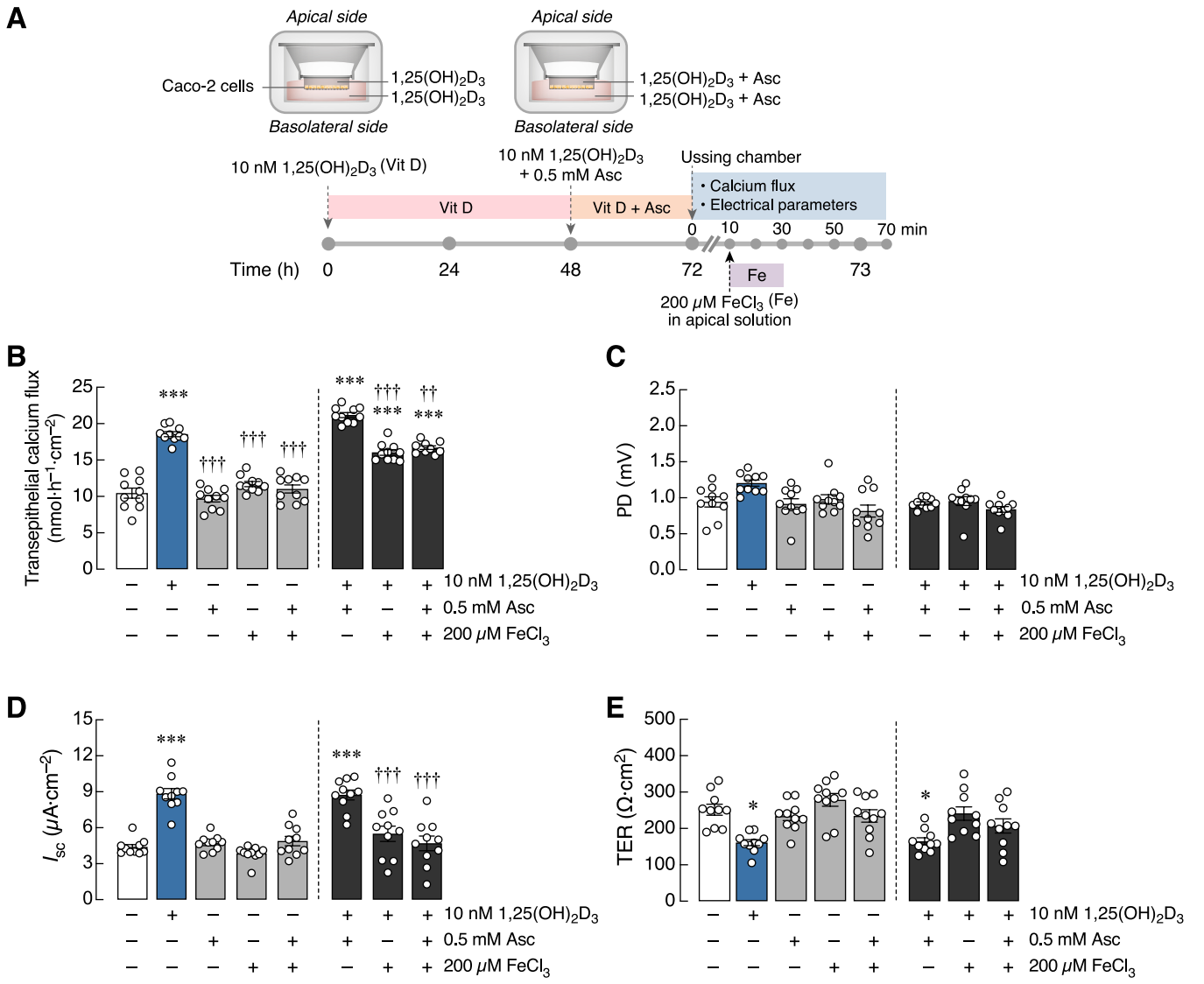

Fig 3. The role of $FeCl_3$ on calcium transport across Caco-2 monolayer pre-treated with $1,25(OH)_2D_3$ and ascorbic acid (Asc). (A) Experimental timeline (please see text for detail). (B–E) Transepithelial calcium transport and epithelial electrical parameters (PD, I_{sc}, and TER) in Caco-2 monolayers with or without 10 nM $1,25(OH)_2D_3$, 200 μM $FeCl_3$, and 0.5 mM Asc. PD values were the magnitudes of potential difference (the apical side being negative with respect to the basolateral side), and glucose made the apical side more negative. (n = 10; $^{*}P < 0.05$; $^{***}P < 0.001$ compared with the control group (white bar); $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ compared with the 10 nM $1,25(OH)_2D_3$-treated group (blue bar).

https://doi.org/10.1371/journal.pone.0273267.g003

high-iron milieu by decreasing the inhibitory action of $FeCl_3$ and maintaining the $1,25(OH)_2D_3$-induced calcium transport (Fig 4A). As shown in Fig 4B, 72-h $FeCl_3$ exposure was able to diminish the $1,25(OH)_2D_3$-induced calcium transport. Nevertheless, the $FeCl_3$ action on I_{sc} and TER was trivial compared to that observed in the acute $FeCl_3$ exposure experiment (Fig 4C–4E). Similar to the earliest experiment, ascorbic acid showed no effect on either epithelial electrical parameters or calcium transport (Fig 4B–4E).

The last series of experiments aimed to demonstrate whether $FeCl_3$ affect baseline calcium flux in the presence or absence of ascorbic acid, Caco-2 monolayers were pre-incubated for 24 h with 0.5 mM ascorbic acid in both apical and basolateral compartments. The results confirmed that

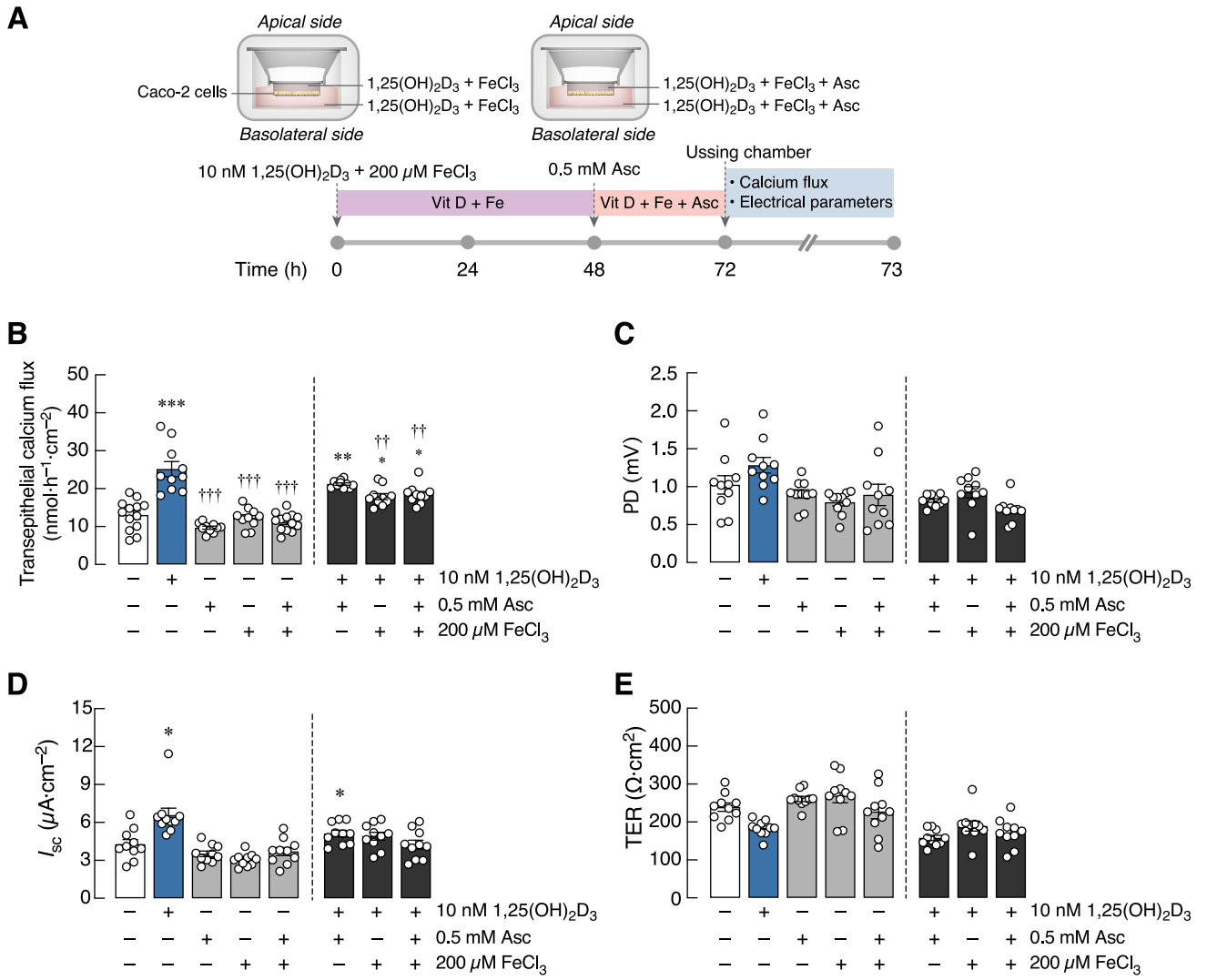

Fig 4. Calcium transport across Caco-2 monolayer combine treated with $1,25(OH)_2D_3$, ascorbic acid (Asc), and $FeCl_3$. (A) Experimental timeline (please see text for detail). (B–E) Transepithelial calcium transport and epithelial electrical parameters (PD, I_{sc}, and TER) in Caco-2 monolayers with or without 10 nM $1,25(OH)_2D_3$, 200 μM $FeCl_3$, and 0.5 mM Asc. PD values were the magnitudes of potential difference (the apical side being negative with respect to the basolateral side), and glucose made the apical side more negative. (n = 10; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the control group (white bar); $^{\dagger\dagger}P < 0.01$; $^{\dagger\dagger\dagger}P < 0.001$ compared with the 10 nM $1,25(OH)_2D_3$-treated group (blue bar).

https://doi.org/10.1371/journal.pone.0273267.g004

acute exposure to 20, 100 or 200 μM $FeCl_3$ in Ussing chamber—either on the apical or basolateral side—did not affect the baseline calcium transport (Fig 5A and 5B). In addition, ascorbic acid pre-treatment did not alter the transepithelial calcium transport across the Caco-2 monolayer. As shown in Fig 6, exposure to 20, 100 or 200 μM $FeCl_3$ for 24–72 h did not affect Caco-2 cells viability or the mRNA levels of TRPV6 and $PMCA_{1b}$. Nevertheless, $FeCl_3$-exposed Caco-2 cells exhibited downregulation of calbindin-D_{9k} and DMT1 mRNA expression.

Discussion

Under normal conditions, calcium and bone metabolism is tightly regulated by several hormones, e.g., $1,25(OH)_2D_3$, estrogen and prolactin [29, 33–35]. Regarding intestinal calcium

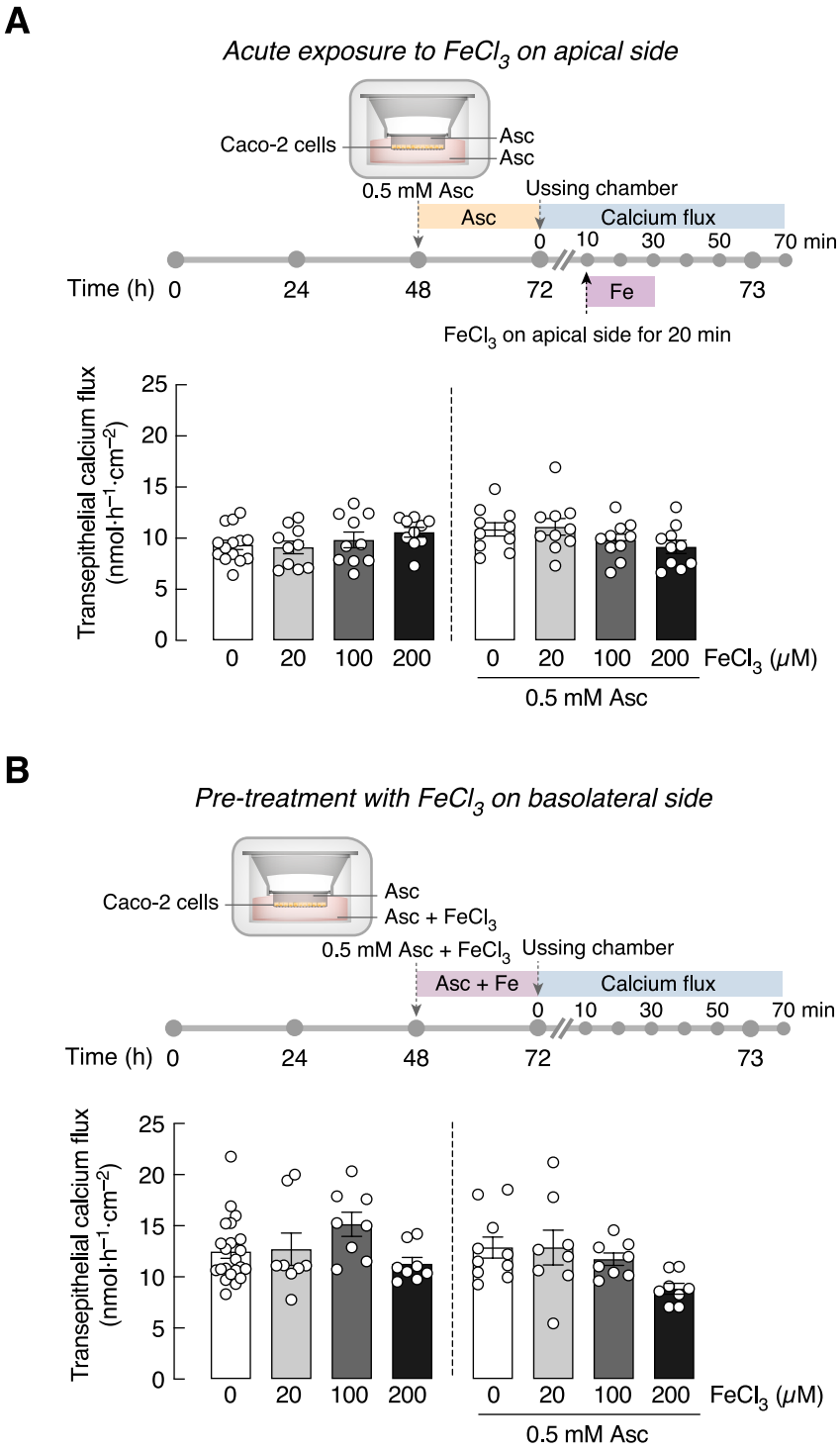

Fig 5. Acute and long-term effects of $FeCl_3$ exposure on calcium transport in Caco-2 monolayer pre-treated with ascorbic acid (Asc). (A) Transepithelial calcium transport across Caco-2 monolayers with or without 0.5 mM Asc pre-treatment and acute exposure with different doses of $FeCl_3$ (i.e., 0, 20, 100, 200 μM) on apical side. (B) Transepithelial calcium transport across Caco-2 monolayers with or without 0.5 mM Asc with different doses of $FeCl_3$ (i.e., 0, 20, 100, 200 μM) pre-treatment on basolateral sides (n = 10).

https://doi.org/10.1371/journal.pone.0273267.g005

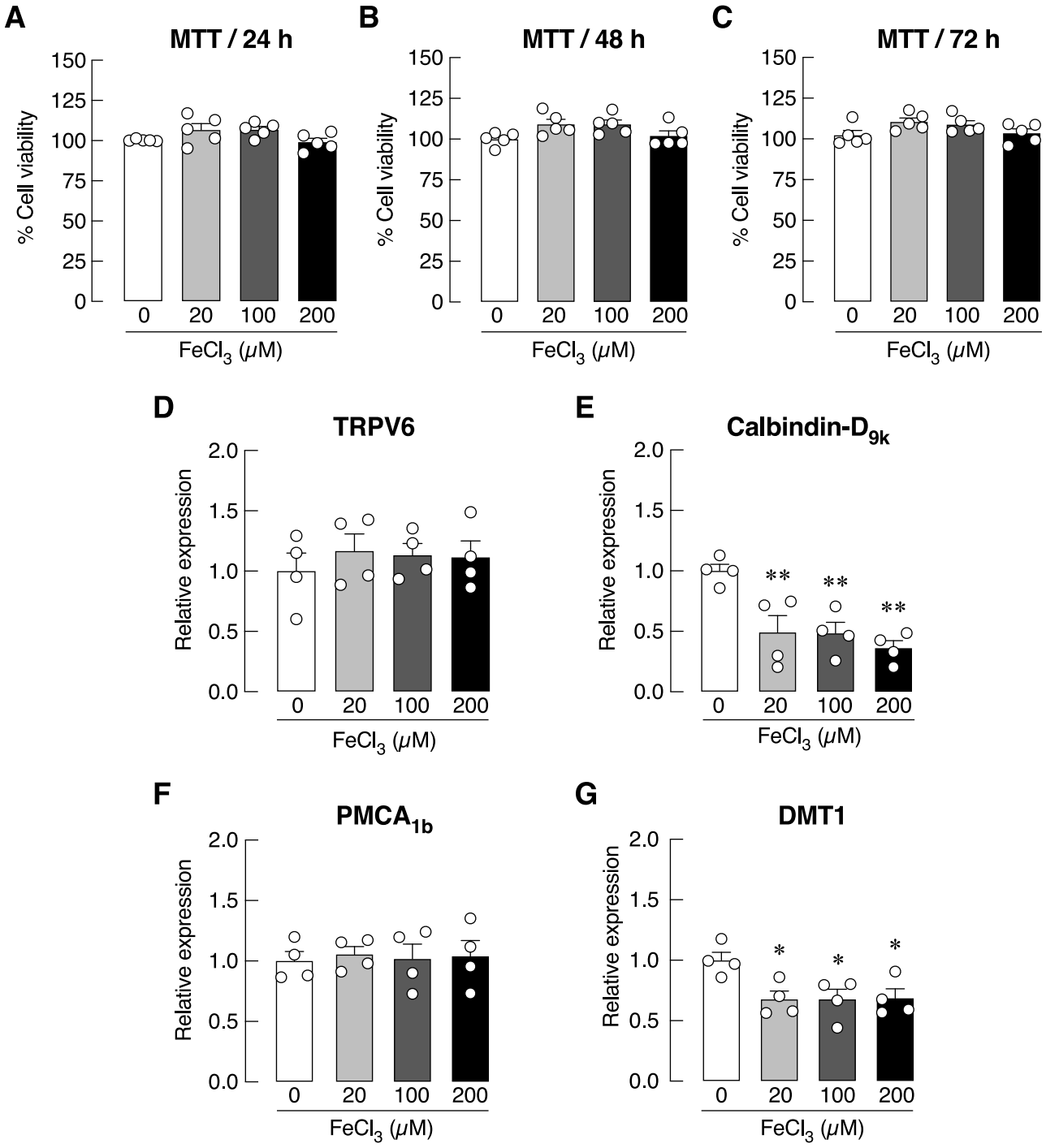

Fig 6. Viability and mRNA expression of calcium transport-related genes and DMT1 gene in Caco-2 cells treated with $FeCl_3$. (A–C) Cell viability of Caco-2 cells treated with various concentrations of $FeCl_3$ (i.e., 0, 20, 100, 200 μM) for 24, 24 and 72 h. (D–G) mRNA expression of calcium transport-related genes (i.e., TRPV6, calbindin-D_{9k}, $PMCA_{1b}$), and iron transporter DMT1 gene in Caco-2 cells treated with various concentrations of $FeCl_3$ (i.e., 0, 20, 100, 200 μM) for 24 h.

https://doi.org/10.1371/journal.pone.0273267.g006

uptake, ~15–30% of dietary calcium is absorbed into the circulation [34, 36]. In other words, after ingesting 1,000 mg/day elemental calcium, the net intestinal calcium uptake into the body is ~150–300 mg/day. This relatively low fractional calcium absorption is often thought to be due to low transporting capacities of the apical calcium channels and/or basolateral calcium transporters rather than the presence of calcium transport inhibitors—such as iron or FGF-23 [2, 3, 14, 15]. Herein, we elaborated the inhibitory effect of ferric ion on the $1,25(OH)_2D_3$-induced transcellular calcium transport across the intestinal epithelium-like Caco-2 monolayer. Since the present data showed that ferric ion reverted both transcellular calcium flux and electrical parameters (i.e., I_{sc} and TER), which indicated paracellular permeability, it was unlikely that ferric ion directly inhibited the transcellular calcium transporters, but it probably compromised overall $1,25(OH)_2D_3$ actions in a similar manner to those of other inhibitory factors, such as FGF-23 (for review, please see [37]).

As mentioned earlier, $1,25(OH)_2D_3$ has been known to stimulate every step of the intestinal transcellular calcium transport—i.e., TRPV6-mediated apical calcium influx, calbindin-D_{9k}-assisted cytoplasmic calcium translocation and $PMCA_{1b}$-mediated basolateral calcium efflux [8–10]. Therefore, blockade of $1,25(OH)_2D_3$ action almost abolishes the transcellular calcium transport [38, 39]. We have previously demonstrated that FGF-23 was capable of downregulating the $1,25(OH)_2D_3$-induced transcellular calcium transport across mouse intestinal epithelium and Caco-2 monolayer as well as the expression of calbindin-D_{9k}, which was considered a cellular biomarker of $1,25(OH)_2D_3$ repletion [14, 15, 26]. Because FGF-23 activates the intracellular catabolism of $1,25(OH)_2D_3$ by upregulating the 24-hydroxylase expression [37, 40], the presence of FGF-23 would reduce the cytoplasmic level of $1,25(OH)_2D_3$, thereby reducing its binding to vitamin D receptor (VDR). It is noteworthy that enterocytes, including Caco-2 cells, do express FGF-23, which probably helps prevent excessive calcium absorption during $1,25(OH)_2D_3$ stimulation [26, 41].

As depicted in Fig 6, ferric ion did not directly affect the mRNA levels of TRPV6 and $PMCA_{1b}$. However, downregulation of calbindin-D_{9k} mRNA expression might somewhat deteriorate capability of Caco-2 cells to translocate intracellular calcium ions, but this genomic or transcriptional change was not large enough to alter calcium flux (Fig 5), consistent with the existence of transcellular calcium transport in calbindin-D_{9k} knockout mice [42]. Although the exact cellular and molecular mechanism(s) of ferric ion-induced inhibition of the vitamin D-stimulated calcium transport remains elusive, cellular oxidative stress induced by cellular iron uptake and the resultant reactive oxygen species (ROS) production could be at least partially responsible for the inhibitory effect of the ferric ion on $1,25(OH)_2D_3$-induced calcium transport. More evidence supporting the impact of oxidant-antioxidant balance on cellular function was provided by experiment in rat renal proximal tubular cells. Hydrogen peroxide, which is ROS, was found to upregulate 24-hydroxylase expression [43], leading to an increase in intracellular $1,25(OH)_2D_3$ degradation. ROS not only impaired VDR, but also suppressed transcriptional activation of retinoic acid receptor/retinoid X receptor (RXR) [44], which forms a heterodimer and translocates to interact with specific vitamin D response elements (VDREs) in vitamin D-responsive genes [45]. Acute and prolonged exposure to pro-oxidants, such as menadione, is also known to directly inhibit mitochondrial function and cellular energy-dependent calcium transporters [20]. Furthermore, we previously provided evidence that, in thalassemic mice with intestinal iron hyperabsorption, iron could interfere with the cytoplasmic vesicular calcium uptake, thereby slowing down the transcellular calcium transport across the small intestinal epithelium [7].

Interestingly, the epithelial electrical parameters, I_{sc} and TER, were also altered by $1,25(OH)_2D_3$. The absence of PD changes suggested that an increase in I_{sc} might have resulted from an increased paracellular permeability rather than the increased electrogenic ion

transport. TER apparently decreased under $1,25(OH)_2D_3$-exposed conditions, consistent with an increase in I_{sc}. A decrease in TER indeed favors paracellular calcium movement. Specifically, in the presence of transepithelial calcium gradient (e.g., high luminal calcium concentration during calcium supplementation), $1,25(OH)_2D_3$ is able to enhance the paracellular calcium absorption by increasing expression of claudin-2 and -12. Both claudins normally form cation-selective tight junction pores, thereby enhancing paracellular cation movement as represented by a reduction in TER, and increasing tight junction permeability to calcium as well [46]. It was herein apparent that ferric ion negated $1,25(OH)_2D_3$ action, thus reverting I_{sc} and TER to the control levels. In other words, it was likely that ferric ion exposure was able to reduce the paracellular transport of calcium and some other cations (e.g., sodium), as indicated by greater TER and lower I_{sc} in 200 μM $FeCl_3$+10 nm $1,25(OH)_2D_3$ group vs. 10 nm $1,25(OH)_2D_3$ alone (Fig 3D and 3E). Although cellular oxidative stress could exert a negative effect on tight junction and paracellular calcium transport [20], cellular oxidative stress due to iron exposure in this study did not alter the epithelial electrical parameters in the absence of $1,25(OH)_2D_3$. Therefore, ferric ion and/or ROS predominantly interfered with $1,25(OH)_2D_3$ action rather than producing a direct effect on the tight junction function.

Besides having many health benefits such as being an anti-oxidant, ascorbic acid is able to increase the solubility of certain calcium compounds, such as calcium carbonate (S1 Fig); therefore, it was often added in calcium supplement formulations to help accrue free-ionized calcium in the intestinal lumen. In human and rodent intestine, luminal calcium must be solubilized into free-ionized form before being absorbed into the body via transcellular and paracellular pathways [11]. In the present study, we aimed to determine whether ascorbic acid did have other actions in the SVCT1/2-expressing Caco-2 cells by exposing cells to ascorbic acid well before the calcium absorption experiment in Ussing chamber. After being transported into the cells, ascorbic acid was able to exert pro- and/or anti-oxidant actions depending on the intracellular iron level and pH [47]. In the presence of both ascorbic acid and ferric ion, intracellular production of oxygen radicals probably increased through Fenton reaction [48]. However, we found that ascorbic acid did not affect transepithelial calcium transport in $FeCl_3$-exposed Caco-2 monolayer. Therefore, the negative effect of ferric ion on $1,25(OH)_2D_3$ action was rather specific and robust, and was not simply alleviated by generic anti-oxidant like ascorbic acid.

Regarding the limitations, the present study focused on ferric ion rather than ferrous ion (Fe^{2+}); therefore, future experiments are required to confirm that both ferrous and ferric ions are able to inhibit the $1,25(OH)_2D_3$-induced calcium absorption in vivo. Since the iron transporter DMT1 only uptakes ferrous ions, but not ferric ions, a ferric reductase namely Dcytb serves to reduce ferric ions into ferrous ions prior to absorption. In other words, ferrous ions were the majority of ionic iron moving across the apical membrane, and thus ferrous treatment might similarly induce an inhibitory effect on $1,25(OH)_2D_3$-induced calcium transport. It was noteworthy that exposure to $FeCl_3$ for 24–72 h significantly downregulated DMT1 expression in Caco-2 cells, suggesting a compensatory or negative feedback response during excessive iron uptake. Indeed, DMT1 was reportedly modulated by extracellular calcium. Shawki and Mackenzie demonstrated that extracellular calcium was a noncompetitive DMT1 inhibitor, which could reduce cellular iron uptake; however, DMT1 itself did not uptake calcium into the cytoplasm [3].

In conclusion, ferric ion was found to completely diminish the $1,25(OH)_2D_3$-enhanced calcium transport but not the baseline calcium transport, and could retain its inhibitory action even though cells were no longer in the presence of $FeCl_3$ (Fig 5B). The inhibitory action of ferric ion was rapid as demonstrated by its effects on calcium flux and electrical parameters being observed after 20-min exposure (Fig 5A). The finding that ascorbic acid did not increase

calcium transport in $FeCl_3$-exposed Caco-2 monolayer suggested that it did not have a significant role as a pro- or anti-oxidant under these conditions. Although more investigation is required to reveal the molecular mechanism of ferric ion-induced inhibition of $1,25(OH)_2D_3$ actions, the present study has provided evidence to help explain how iron diminishes intestinal calcium transport and to support a notion that oral iron and calcium supplement should be given separately to avoid calcium absorption being compromised by iron.

Supporting information

S1 Fig. Effects of ascorbic acid on solubility of CaCO3. Results are expressed as mean ± SE. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with the control group (open circle). $^{\dagger}P < 0.05$ compared with ascorbic acid group (black circle). $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ compared with HCl (pH 4) group (black square with dash line).
(EPS)

Acknowledgments

We thank Prof. Nateetip Krishnamra for critical comments and Thitapha Kiattisirichai for the artworks.

Author Contributions

Conceptualization: Kornkamon Lertsuwan, Nattapon Panupinthu, Ratchaneevan Aeimlapa, Jarinthorn Teerapornpuntakit, Narattaphol Charoenphandhu, Kannikar Wongdee.

Data curation: Sukpapohn Phummisutthigoon, Narattaphol Charoenphandhu.

Formal analysis: Sukpapohn Phummisutthigoon, Kornkamon Lertsuwan, Nattapon Panupinthu, Ratchaneevan Aeimlapa, Jarinthorn Teerapornpuntakit, Wasutorn Chankamngoen, Narattaphol Charoenphandhu, Kannikar Wongdee.

Funding acquisition: Sukpapohn Phummisutthigoon, Kornkamon Lertsuwan, Nattapon Panupinthu, Narattaphol Charoenphandhu, Kannikar Wongdee.

Investigation: Sukpapohn Phummisutthigoon, Ratchaneevan Aeimlapa, Wasutorn Chankamngoen, Jirawan Thongbunchoo.

Methodology: Narattaphol Charoenphandhu.

Project administration: Narattaphol Charoenphandhu.

Resources: Narattaphol Charoenphandhu.

Supervision: Kornkamon Lertsuwan, Narattaphol Charoenphandhu, Kannikar Wongdee.

Validation: Sukpapohn Phummisutthigoon, Nattapon Panupinthu, Jarinthorn Teerapornpuntakit, Narattaphol Charoenphandhu, Kannikar Wongdee.

Visualization: Narattaphol Charoenphandhu.

Writing – original draft: Sukpapohn Phummisutthigoon, Jarinthorn Teerapornpuntakit, Narattaphol Charoenphandhu, Kannikar Wongdee.

Writing – review & editing: Sukpapohn Phummisutthigoon, Kornkamon Lertsuwan, Nattapon Panupinthu, Ratchaneevan Aeimlapa, Jarinthorn Teerapornpuntakit, Narattaphol Charoenphandhu, Kannikar Wongdee.

References

1. National Institutes of Health Office of Dietary Supplements. Iron 2021 [updated 30 March 2021; cited 1 March 2022]. Available from: https://ods.od.nih.gov/factsheets/Iron-HealthProfessional/.
2. Beck KL, Conlon CA, Kruger R, Coad J. Dietary determinants of and possible solutions to iron deficiency for young women living in industrialized countries: a review. Nutrients. 2014; 6(9):3747–76. https://doi.org/10.3390/nu6093747 PMID: 25244367.
3. Shawki A, Mackenzie B. Interaction of calcium with the human divalent metal-ion transporter-1. Biochem Biophys Res Commun. 2010; 393(3):471–5. https://doi.org/10.1016/j.bbrc.2010.02.025 PMID: 20152801.
4. Wawer AA, Harvey LJ, Dainty JR, Perez-Moral N, Sharp P, Fairweather-Tait SJ. Alginate inhibits iron absorption from ferrous gluconate in a randomized controlled trial and reduces iron uptake into Caco-2 cells. PLoS One. 2014; 9(11):e112144. https://doi.org/10.1371/journal.pone.0112144 PMID: 25391138.
5. Muncie HL Jr., Campbell J. Alpha and beta thalassemia. Am Fam Physician. 2009; 80(4):339–44. PMID: 19678601.
6. Charoenphandhu N, Kraidith K, Teerapornpuntakit J, Thongchote K, Khuituan P, Svasti S, et al. 1,25-Dihydroxyvitamin D_3 -induced intestinal calcium transport is impaired in β-globin knockout thalassemic mice. Cell Biochem Funct. 2013; 31(8):685–91. https://doi.org/10.1002/cbf.2956 PMID: 23371483.
7. Kraidith K, Svasti S, Teerapornpuntakit J, Vadolas J, Chaimana R, Lapmanee S, et al. Hepcidin and $1,25(OH)_2D_3$ effectively restore Ca^{2+} transport in β-thalassemic mice: reciprocal phenomenon of Fe^{2+} and Ca^{2+} absorption. Am J Physiol Endocrinol Metab. 2016; 311(1):E214–23. https://doi.org/10.1152/ajpendo.00067.2016 PMID: 27245334.
8. Christakos S, Dhawan P, Porta A, Mady LJ, Seth T. Vitamin D and intestinal calcium absorption. Mol Cell Endocrinol. 2011; 347(1–2):25–9. https://doi.org/10.1016/j.mce.2011.05.038 PMID: 21664413.
9. Christakos S, Dhawan P, Ajibade D, Benn BS, Feng J, Joshi SS. Mechanisms involved in vitamin D mediated intestinal calcium absorption and in non-classical actions of vitamin D. J Steroid Biochem Mol Biol. 2010; 121(1–2):183–7. https://doi.org/10.1016/j.jsbmb.2010.03.005 PMID: 20214989.
10. Favus MJ, Angeid-Backman E. Effects of $1,25(OH)_2D_3$ and calcium channel blockers on cecal calcium transport in the rat. Am J Physiol. 1985; 248(6 Pt 1):G676–81. https://doi.org/10.1152/ajpgi.1985.248.6.G676 PMID: 2408484.
11. Kopic S, Geibel JP. Gastric acid, calcium absorption, and their impact on bone health. Physiol Rev. 2013; 93(1):189–268. https://doi.org/10.1152/physrev.00015.2012 PMID: 23303909.
12. Madsen KL, Tavernini MM, Yachimec C, Mendrick DL, Alfonso PJ, Buergin M, et al. Stanniocalcin: a novel protein regulating calcium and phosphate transport across mammalian intestine. Am J Physiol. 1998; 274(1):G96–102. https://doi.org/10.1152/ajpgi.1998.274.1.G96 PMID: 9458778.
13. Xiang J, Guo R, Wan C, Wu L, Yang S, Guo D. Regulation of intestinal epithelial calcium transport proteins by stanniocalcin-1 in Caco2 cells. Int J Mol Sci. 2016; 17(7). https://doi.org/10.3390/ijms17071095 PMID: 27409607.
14. Khuituan P, Teerapornpuntakit J, Wongdee K, Suntornsaratoon P, Konthapakdee N, Sangsaksri J, et al. Fibroblast growth factor-23 abolishes 1,25-dihydroxyvitamin D_3-enhanced duodenal calcium transport in male mice. Am J Physiol Endocrinol Metab. 2012; 302(8):E903–13. https://doi.org/10.1152/ajpendo.00620.2011 PMID: 22275752.
15. Khuituan P, Wongdee K, Jantarajit W, Suntornsaratoon P, Krishnamra N, Charoenphandhu N. Fibroblast growth factor-23 negates $1,25(OH)_2D_3$-induced intestinal calcium transport by reducing the transcellular and paracellular calcium fluxes. Arch Biochem Biophys. 2013; 536(1):46–52. https://doi.org/10.1016/j.abb.2013.05.009 PMID: 23747333.
16. Amalraj A, Pius A. Bioavailability of calcium and its absorption inhibitors in raw and cooked green leafy vegetables commonly consumed in India–an *in vitro* study. Food Chem. 2015; 170:430–6. https://doi.org/10.1016/j.foodchem.2014.08.031 PMID: 25306367.
17. Lesjak M, Hoque R, Balesaria S, Skinner V, Debnam ES, Srai SK, et al. Quercetin inhibits intestinal iron absorption and ferroportin transporter expression *in vivo* and *in vitro*. PLoS One. 2014; 9(7):e102900. https://doi.org/10.1371/journal.pone.0102900 PMID: 25058155.
18. Gulec S, Anderson GJ, Collins JF. Mechanistic and regulatory aspects of intestinal iron absorption. Am J Physiol Gastrointest Liver Physiol. 2014; 307(4):G397–409. https://doi.org/10.1152/ajpgi.00348.2013 PMID: 24994858.
19. Areco V, Rodriguez V, Marchionatti A, Carpentieri A, Tolosa de Talamoni N. Melatonin not only restores but also prevents the inhibition of the intestinal Ca^{2+} absorption caused by glutathione depleting drugs. Comp Biochem Physiol A Mol Integr Physiol. 2016; 197:16–22. https://doi.org/10.1016/j.cbpa.2016.03.005 PMID: 26970583.

20. Diaz de Barboza G, Guizzardi S, Moine L, Tolosa de Talamoni N. Oxidative stress, antioxidants and intestinal calcium absorption. World J Gastroenterol. 2017; 23(16):2841–53. https://doi.org/10.3748/wjg.v23.i16.2841 PMID: 28522903.

21. Marchionatti AM, Diaz de Barboza GE, Centeno VA, Alisio AE, Tolosa de Talamoni NG. Effects of a single dose of menadione on the intestinal calcium absorption and associated variables. J Nutr Biochem. 2003; 14(8):466–72. https://doi.org/10.1016/s0955-2863(03)00078-0 PMID: 12948877.

22. Yeung CK, Glahn RP, Miller DD. Inhibition of iron uptake from iron salts and chelates by divalent metal cations in intestinal epithelial cells. J Agric Food Chem. 2005; 53(1):132–6. https://doi.org/10.1021/jf049255c PMID: 15631519.

23. Fleet JC, Eksir F, Hance KW, Wood RJ. Vitamin D-inducible calcium transport and gene expression in three Caco-2 cell lines. Am J Physiol Gastrointest Liver Physiol. 2002; 283(3):G618–25. https://doi.org/10.1152/ajpgi.00269.2001 PMID: 12181175.

24. Yee S. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man–fact or myth. Pharm Res. 1997; 14(6):763–6. https://doi.org/10.1023/a:1012102522787 PMID: 9210194.

25. Zweibaum A, Triadou N, Kedinger M, Augeron C, Robine-Leon S, Pinto M, et al. Sucrase-isomaltase: a marker of foetal and malignant epithelial cells of the human colon. Int J Cancer. 1983; 32(4):407–12. https://doi.org/10.1002/ijc.2910320403 PMID: 6352518.

26. Rodrat M, Wongdee K, Panupinthu N, Thongbunchoo J, Teerapornpuntakit J, Krishnamra N, et al. Prolonged exposure to $1,25(OH)_2D_3$ and high ionized calcium induces FGF-23 production in intestinal epithelium-like Caco-2 monolayer: A local negative feedback for preventing excessive calcium transport. Arch Biochem Biophys. 2018; 640:10–6. https://doi.org/10.1016/j.abb.2017.12.022 PMID: 29317227.

27. Murphy EF, Hooiveld GJ, Muller M, Calogero RA, Cashman KD. Conjugated linoleic acid alters global gene expression in human intestinal-like Caco-2 cells in an isomer-specific manner. J Nutr. 2007; 137(11):2359–65. https://doi.org/10.1093/jn/137.11.2359 PMID: 17951470.

28. Wawer AA, Sharp PA, Perez-Moral N, Fairweather-Tait SJ. Evidence for an enhancing effect of alginate on iron availability in Caco-2 cells. J Agric Food Chem. 2012; 60(45):11318–22. https://doi.org/10.1021/jf3031309 PMID: 23101614.

29. Wongdee K, Tulalamba W, Thongbunchoo J, Krishnamra N, Charoenphandhu N. Prolactin alters the mRNA expression of osteoblast-derived osteoclastogenic factors in osteoblast-like UMR106 cells. Mol Cell Biochem. 2011; 349(1–2):195–204. https://doi.org/10.1007/s11010-010-0674-4 PMID: 21116687.

30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001; 25(4):402–8. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609.

31. Boyer JC, Campbell CE, Sigurdson WJ, Kuo SM. Polarized localization of vitamin C transporters, SVCT1 and SVCT2, in epithelial cells. Biochem Biophys Res Commun. 2005; 334(1):150–6. https://doi.org/10.1016/j.bbrc.2005.06.069 PMID: 15993839.

32. Maulen NP, Henriquez EA, Kempe S, Carcamo JG, Schmid-Kotsas A, Bachem M, et al. Up-regulation and polarized expression of the sodium-ascorbic acid transporter SVCT1 in post-confluent differentiated CaCo-2 cells. J Biol Chem. 2003; 278(11):9035–41. https://doi.org/10.1074/jbc.M205119200 PMID: 12381735.

33. Charoenphandhu N, Teerapornpuntakit J, Methawasin M, Wongdee K, Thongchote K, Krishnamra N. Prolactin decreases expression of Runx2, osteoprotegerin, and RANKL in primary osteoblasts derived from tibiae of adult female rats. Can J Physiol Pharmacol. 2008; 86(5):240–8. https://doi.org/10.1139/y08-037 PMID: 18432284.

34. Charoenphandhu N, Tudpor K, Thongchote K, Saengamnart W, Puntheeranurak S, Krishnamra N. High-calcium diet modulates effects of long-term prolactin exposure on the cortical bone calcium content in ovariectomized rats. Am J Physiol Endocrinol Metab. 2007; 292(2):E443–52. https://doi.org/10.1152/ajpendo.00333.2006 PMID: 17003239.

35. Nakkrasae LI, Thongon N, Thongbunchoo J, Krishnamra N, Charoenphandhu N. Transepithelial calcium transport in prolactin-exposed intestine-like Caco-2 monolayer after combinatorial knockdown of TRPV5, TRPV6 and $Ca_v1.3$. J Physiol Sci. 2010; 60(1):9–17. https://doi.org/10.1007/s12576-009-0068-0 PMID: 19885716.

36. Hall JE, Hall ME. Guyton and Hall Textbook of medical physiology 2021. p. 991–1010.

37. Martin A, David V, Quarles LD. Regulation and function of the FGF23/klotho endocrine pathways. Physiol Rev. 2012; 92(1):131–55. https://doi.org/10.1152/physrev.00002.2011 PMID: 22298654.

38. Song Y, Kato S, Fleet JC. Vitamin D receptor (VDR) knockout mice reveal VDR-independent regulation of intestinal calcium absorption and ECaC2 and calbindin D_{9k} mRNA. J Nutr. 2003; 133(2):374–80. https://doi.org/10.1093/jn/133.2.374 PMID: 12566470.

39. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, Stockmans I, Van Herck E, Kato S, et al. Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. Proc Natl Acad Sci USA. 2001; 98(23):13324–9. https://doi.org/10.1073/pnas.231474698 PMID: 11687634.

40. Kagi L, Bettoni C, Pastor-Arroyo EM, Schnitzbauer U, Hernando N, Wagner CA. Regulation of vitamin D metabolizing enzymes in murine renal and extrarenal tissues by dietary phosphate, FGF23, and 1,25 $(OH)_2D_3$. PLoS One. 2018; 13(5):e0195427. https://doi.org/10.1371/journal.pone.0195427 PMID: 29771914.

41. Wongdee K, Rodrat M, Keadsai C, Jantarajit W, Teerapornpuntakit J, Thongbunchoo J, et al. Activation of calcium-sensing receptor by allosteric agonists cinacalcet and AC-265347 abolishes the 1,25 $(OH)_2D_3$-induced Ca^{2+} transport: Evidence that explains how the intestine prevents excessive Ca^{2+} absorption. Arch Biochem Biophys. 2018; 657:15–22. https://doi.org/10.1016/j.abb.2018.09.004 PMID: 30217510.

42. Lee GS, Lee KY, Choi KC, Ryu YH, Paik SG, Oh GT, et al. Phenotype of a calbindin-D_{9k} gene knockout is compensated for by the induction of other calcium transporter genes in a mouse model. J Bone Miner Res. 2007; 22(12):1968–78. https://doi.org/10.1359/jbmr.070801 PMID: 17696760.

43. Shankar K, Liu X, Singhal R, Chen JR, Nagarajan S, Badger TM, et al. Chronic ethanol consumption leads to disruption of vitamin D_3 homeostasis associated with induction of renal 1,25 dihydroxyvitamin D_3-24-hydroxylase (CYP24A1). Endocrinology. 2008; 149(4):1748–56. https://doi.org/10.1210/en.2007-0903 PMID: 18162528.

44. Guleria RS, Choudhary R, Tanaka T, Baker KM, Pan J. Retinoic acid receptor-mediated signaling protects cardiomyocytes from hyperglycemia induced apoptosis: role of the renin-angiotensin system. J Cell Physiol. 2011; 226(5):1292–307. https://doi.org/10.1002/jcp.22457 PMID: 20945395.

45. Qin X, Wang X. Role of vitamin D receptor in the regulation of *CYP3A* gene expression. Acta Pharm Sin B. 2019; 9(6):1087–98. https://doi.org/10.1016/j.apsb.2019.03.005 PMID: 31867158.

46. Fujita H, Sugimoto K, Inatomi S, Maeda T, Osanai M, Uchiyama Y, et al. Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca^{2+} absorption between enterocytes. Mol Biol Cell. 2008; 19(5):1912–21. https://doi.org/10.1091/mbc.E07-09-0973 PMID: 18287530.

47. Shen J, Griffiths PT, Campbell SJ, Utinger B, Kalberer M, Paulson SE. Ascorbate oxidation by iron, copper and reactive oxygen species: review, model development, and derivation of key rate constants. Sci Rep. 2021; 11(1):7417. https://doi.org/10.1038/s41598-021-86477-8 PMID: 33795736.

48. Kaźmierczak-Barańska J, Boguszewska K, Adamus-Grabicka A, Karwowski BT. Two faces of vitamin C-antioxidative and pro-oxidative agent. Nutrients. 2020; 12(5). https://doi.org/10.3390/nu12051501 PMID: 32455696.