Supplemental Material

A guide to human zinc absorption: general overview and recent advances of *in vitro* intestinal models

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Table 1. Zinc content of the human body.

Tissue	Weight (g) *	Ref.	Zinc Concentration (μg/g tissue wet weight)	Ref.	Total Zinc Content (g)	Proportion of Total Body Zinc * (%)
Eye	7.5	[1]	1.3	[2]	<0.01	<0.01
Liver	1,500	[3]	58	[4]	0.09	3.4
Heart	331	[5]	26.5	[6]	0.01	0.3
Brain	1,407	[3]	11	[6]	0.02	0.6
Lung	840	[3]	16	[6]	0.01	0.5
Kidneys	266	[3]	55	[4]	0.01	0.6
Intestine	2,100	[7]	15.5	[6]	0.03	1.3
Stomach	130	[8]	13.4	[6]	<0.01	0.1
Bone	9,458	[9]	100	[4]	0.95	36.7
Muscle	25,100	[10]	51	[4]	1.28	49.7
Blood plasma	3,437	[11]	1.25 [μg/mL]	[12]	<0.01	0.2
Whole Blood	5,509	[11]	6.81 [μg/mL]	[6]	0.04	1.5
Hair and nails	53.8	[9]	247	[13]	0.01	0.5
Skin	3,405.54	[9]	32	[4]	0.11	4.2
Spleen	139	[3]	14.7	[6]	<0.01	0.1
Pancreas	119	[14]	33.3	[15]	<0.01	0.2
					Σ 2.6	**100**

* calculated for a 60–70 kg adult, based on the respective body weight used as references for tissue weight.

Table 2. Recommended daily allowance for dietary zinc intake for selected life-stages.

WHO [16]				EFSA [17]				DGE [18]						
Age, Sex	**RNI (mg/d)**			**Age**	**PRI (mg/d)**			**Age**	**RDI (mg/d)**					
	High [a]	**Mod** [b]	**Low** [c]											
7–12 mos	0.8 [d]; 2.5 [e]	4.1	8.4	**7–11 mos**	2.9			**<4 mos**	1.5					
1–3 yr	2.4	4.1	8.3	**1–3 yr**	4.3			**4–12 mos**	2.5					
4–6 yr	2.9	4.8	9.6	**4–6**	5.5			**1–4 yr**	3.0					
7–9 yr	3.3	5.6	11.2	**7–10**	7.4			**4–7 yr**	4.0					
10–18 yr, m	5.1	8.6	17.1		**m**	**f**		**7–10 yr**	6.0					
10–18 yr, f	4.3	7.2	14.4	**11–14 yr**	9.4	9.4			**m**			**f**		
19–65 yr, m	4.2	7.0	14.0	**15–17 yr**	12.5	10.4		**Phytate**	Low [f]	Med [g]	High [h]	Low [f]	Med [g]	High [h]
19–65 yr, f	3.0	4.9	9.8	**Age**	**Phytate** (mg/d)			**10–13 yr**		9			8	
> 65 yr, m	4.2	7.0	14.0	**≥ 18 yr**	**300**	9.4	7.5	**13–15 yr**		12			10	
> 65 yr, f	3.0	4.9	9.8	**≥ 18 yr**	**600**	11.7	9.3	**15–19 yr**		14			11	
Pregnancy				**≥ 18 yr**	**900**	14.0	11.0	**≥19 yr**	11	14	16	7	8	10
1st TT	3.4	5.5	11.0	**≥ 18 yr**	**1200**	16.3	12.7	**Pregnancy**						
2nd TT	4.2	7.0	14.0					1st TT				7	9	11
3rd TT	6.0	10.0	20.0	**Pregnancy**			+1.6	2nd–3rd TT				9	11	13
Lactation				**Lactation**			+2.9	**Lactation**				11	13	14
0–3 mo	5.8	9.5	19.0											
3–6 mo	5.3	8.8	17.5											
6–12 mo	4.3	7.2	14.4											

BV, bioavailability; EFSA, European Food Safety Authority; DGE, German Society for Nutrition *(ger.: Deutsche Gesellschaft für Ernährung)*; f, female; m, male; mos, months; PRI, population reference intake; RDI, recommended daily intake; RNI, recommended nutrient intake; TT, trimester; WHO, World Health Organization; yr, years; [a]High bioavailability (50%); [b]Moderate bioavailability (30%); [c]Low bioavailability (15%); [d]exclusively breastfed infants (bioavailability 80%); [e]not exclusively breastfed; [f]300 mg phytate/d; [g]660 mg phytate/d; [h]990 mg phytate/d; Recommendations for adults from EFSA and DGE include different phytate levels using a trivariate model by Miller *et al.* [19] for assessing the relationship between dietary phytate, dietary zinc, and absorbed zinc.

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Table 3. Application of human *in vitro* intestinal models to study zinc-dependent gene expression in enterocytes.

Cell Model	Incubation Parameter	Analysis	Main Outcome	Reference
Caco-2 Differentiation time: 14 d 2D	Recombinant expression of myc-tagged hZnT-5B in Caco-2 cells Addition of $ZnCl_2$ to growth medium: Stepwise increase from 20, 50 and 100 μM each for 7d	Recombinant transfection Gene expression: RT-PCR Immunochemical staining	- highest expression of ZTL1 in mouse kidney, brain, duodenum and jejunum - apical localization of hZTL1 at apical membrane of Caco-2 - hZTL1 (later named ZnT-5B) and MT expression increased in Caco-2-WT cells after prolonged zinc treatment	[20]
Caco-2 Cultivation time: 14 d 2D	Human study: 25 mg $ZnSO_4$/d (placebo Na SO_4); duration: 14 d Caco-2: 100 μM or 200 μM $ZnCl_2$ (in DMEM + 10% FCS) for 3 d	Gene expression: RT-PCR Protein quantification: Immunocytochemistry	- mRNA and protein expression of ZnT-1, ZnT-5, ZnT-5, ZIP4 in enterocytes (biopsies of ileal mucosa) ↓ - *znt-1* ↓ - MT mRNA increased ↑ - mRNA and protein expression in Caco-2 cells was in agreement with human study - localization of ZnT-5 at apical membrane of human enterocytes and Caco-2 cells	[21]
Caco-2 Cultivation time: 24 h 2D	0-100 μM $ZnCl_2$ (in serum-free DMEM)	Transient transfection of Caco-2 cells with pEGFP-ZnT5B	- ZnT-5 variant b is a bidirectional zinc transporter and can operate in an efflux mode, increasing cytoplasmic zinc concentration of Caco-2 cells - upregulation of MT-2 indicates increase of intracellular zinc content in transfected Caco-2 cells	[22]
Caco-2 Cultivation time: 24 h, pre-confluent 2D	0-300 μM $ZnSO_4$ or 0-10 μM TPEN (in n.a.) for 6 or 12 h	Gene expression: *q*PCR	- zinc-dependent mRNA expression of *mt-1*, *dmt-1*, *zip4* and *znt-1* regulates zinc homeostasis in Caco-2 cells - *zip4* ↑ after zinc depletion with TPEN - *mt-1* ↑ *and znt-1* with added zinc concentration	[23]

Caco-2 Differentiation time: 11-13 d 2D	Iron/zinc interaction 0-200 µM $ZnCl_2$ or $FeCl_3$, respectively, (in DMEM) for 2h	Zinc uptake: radioactive zinc (^{65}Zn)	- iron uptake was inhibited dose-dependently by zinc - iron increased cellular zinc uptake - analysis suggests that iron and zinc transport by DMT-1 is not occurring simultaneously	[24]
Caco-2 Cultivation time: 14 d 2D	3-100 µM $ZnCl_2$ (in DMEM+ 10%FCS) for 12 or 24 h	Transcriptomic study: Micro-array Gene expression: *q*PCR	- zinc-regulated genes were analyzed with an micro-array - identification of several genes which are regulated zinc-dependent (such as *mt-1h*, *mt-2a*, *mt-3*, *mtf-1*)	[25]
Caco-2 Cultivation time: 21d 3D Transwell (comparison undifferentiated and differentiated cells)	100-800 µM $ZnCl_2$ (in DMEM + 5% FCS) apical or basolateral incubation) for 24 h	Gene expression: *q*PCR	- influence of polarization and differentiation of Caco-2 cells on zinc tolerance - mRNA expression of *znt-1* ↑, *znt-5*, *zip1*, *zip4*, *mt-1a* ↑, *mt-1x* ↑, *mt-2a* ↑ after exposure with higher zinc concentrations (100-800 µM; apical or basolateral, respectively) - under physiologic zinc concentrations (apical: 100 µM; basolateral: 15 µM zinc) only *mt-1a* ↑	[26]
Caco-2 (1) FHs 74 Int cells (2) Cultivation time (1): Undifferentiated (U) (4 d) Differentiated (D) (12 d) 2D	50 µM $ZnSO_4$ (in serum free medium) for 15 min	Zinc uptake: radioactive zinc (^{65}Zn) Gene expression: *q*PCR Western Blot Biotinylation of surface proteins	- role of zinc exposure on intestinal cells of varying maturity; - zinc uptake in fetal intestinal cells and undifferentiated cells was higher than in differentiated cells - ZnT-1 protein and *znt-1*, *znt-2* as well as *mt-1* ↑, *while zip4* ↑ in U and ↓ in D Caco-2 cells - localization of ZIP4 and ZnT-1 at the plasma membrane of differentiated Caco-2 cells was significantly changed by zinc exposure	[27]
Caco-2 confluent cells 2D; 3D Transwell	0-100 µM $ZnSO_4$ (DMEM +10% FCS) for 7 d	Zinc Uptake: total Zn Western blot	- cellular zinc content increased concentration-dependent (100 µM: 0.4 µg mg^{-1} protein)	[28]

			- expression of TJ protein claudin-2 and tricellulin decreased with added zinc concentration - TEER increased with added zinc concentration	
Caco-2 Cultivation time: Undifferentiated: n.a. Differentiated: 21d 2D; 3D Transwell (EHS-coated matrix membrane)	100 μM $ZnCl_2$ (DMEM +10% FCS) for 48 h	Transient transfection of Caco-2 cells with hZIP1 Gene expression: *q*PCR Zinc uptake: radioactive zinc (^{65}Zn) Immunocytochemistry	- role of hZIP1 in intestinal epithelial cells - hZIP1 tend to localize to the microvilli of Caco-2 cells during differentiation - Caco-2 cells overexpressing hZIP1 accumulated intracellular zinc - hZIP1 might act as a zinc sensing protein	[29]
Caco-2 (1) IPEC-J2 (2) Cultivation time (1): Pre-confluent (2-3 d) Post-confluent (19-21 d) 2D	0-200 μM $ZnSO_4$ (in DMEM +10% FCS) for 6 h and 24 h	Zinc uptake: total Zn Gene expression: *q*PCR	- cellular zinc uptake increases significantly after incubating with 200 μM zinc for 24 h - zinc incubation of post-confluent Caco-2 cells did not change *zip-4* and only showed a trend in *mt1a* and *znt-1* upregulation - enterocyte zinc homeostasis is maintained by expression of these genes	[30]
Caco-2 (1) IPEC-J2 (2) Cultivation time (1): 21 d 3D Transwell	0-200 μM $ZnSO_4$ (apical or basolateral side, in DMEM + 10% FCS) for 24 h	Gene expression: *q*PCR	- *znt-1* and *mt* expression ↑ with higher added zinc concentrations basolaterally - *zip4* expression did not change	[31]
Caco-2 Cultivation time: 24 h	3 or 150 μM zinc (in serum free DMEM) for 24 h	MTF-1 depletion by transient transfection with siRNA MT-2a stable transfection Transiently transfection with ZnT-5 promotor Gene expression: Microarray *q*PCR	- zinc-dependent expression of MTF-1 dependent genes in MTF-1 depleted Caco-2 compared to CTR: *znt-1*) ↓and *mt-1b*) ↓, *mt-1e*) ↓, *mt-1g*) ↓, *mt-1h*) ↓, *mt-1m*) ↓, *mt-2a*) ↓, *mt-1a* , *mt-2a* and *mt-x* did not change - in MTF-1 depleted cells, zinc incubation changed mRNA expression of genes that are normally not affected by increased cellular zinc,	[32]

		indicating that MT and ZnT-1 are buffering their expression - MT-2a overexpressed Caco-2 cells showed higher ZnT-5 promoter activity upon zinc uptake - MTF-1 is controlling intracellular zinc homeostasis by regulating MT and ZnT-1

3D, three-dimensional; DMEM, Dulbecco's Modified Eagles Medium; EHS, Engelbreth-Holm-Swarm cells; (FCS, fetal calf serum; HBSS, Hank's Balanced Salt Solution; n.a., not available; PC, polycarbonate; TEER, transepithelial electrical resistance; TJ, tight junction; Zn, zinc.

Table 4. Application of *in vitro* Caco-2 monocultures to investigate the effect of dietary factors on zinc bioavailability.

Cell Model	Zinc Added	Food Component or Ligand	Quantification	Main Outcome	Reference
Caco-2 Cultivation time: 10-12 d 2D and 3D Transwell	$ZnSO_4$ $FeCl_3$ (apical: HEPES buffer, basolateral: DMEM + 15% FCS) for 1 h (uptake), 1-5 h (transport)	- Inositolphosphates (IP) (phytic acid): IP3, IP4, IP5, IP6	radioactive zinc and iron (^{65}Zn, ^{55}Fe)	- inhibition of iron and zinc transport by phytate in Caco-2 - reduction of zinc uptake and transport rate correlated with level phosphorylation (IP3 to IP6) - cellular uptake was analyzed in 2D, transport with 3D transwell	[33]
Caco-2 Cultivation time: 15-18 d 2D	40.22 µM $ZnCl_2$, 88.24 µM $FeCl_3$ or 823.53 µM $CaCl_2$ respectively (in uptake buffer)	- infant formulas: adapted (milk based) and soy-based - *in vitro* digestion model	total zinc	- lower zinc uptake von soy-based than from milk-based infant formulas - cellular zinc uptake solely observed from digested infant formulas and not from liquid metal solutions	[34]
Caco-2 Cultivation time: 19 – 21 d 3D Transwell (PE membrane)	sample [c] (apical: soluble mineral fraction, basolateral: HBSS buffer) for 2 h	- raw legumes: white beans, chickpeas, lentils - effect on cooking of lentils - *in vitro* digestion model	total zinc	- chickpeas yielded the highest amount of transported zinc - cooking process negatively affected the mineral content of lentils and the soluble zinc fraction decreased	[35]
Caco-2 Cultivation time: 21 d 3D Transwell (PES membrane)	sample [c] (apical: soluble mineral fraction; basolateral: HBSS buffer) for 2 h	- school meals - *in vitro* digestion model	total zinc	- iron, copper, zinc and calcium uptake and transport was analyzed - protein content of meals had no influence on zinc uptake - negative mineral interaction of iron and zinc:	[36]

				soluble iron decreased and transported zinc; soluble zinc and iron retention	
Caco-2 Cultivation time: 14-12 d 2D	25 μM $^{65}ZnCl_2$ (in MEM) for 3 h	- phytic acid, tannic acid, tartaric acid, polyphenols (from tea extract and grape juice), wheat, arginine, methionine, histidine - molar ratio: zinc/dietary ligands (1:1; 1:5; 1:10) - *in vitro* digestion model (use of dialysis membrane for incubation of cells with digested samples)	radioactive zinc (^{65}Zn)	- zinc depletion with TPEN increased zinc uptake, but zinc repletion did not affect uptake - zinc uptake in Caco-2 cells shows a saturable and non-saturable component depending on added zinc concentration - tannic acid (1:50) enhanced zinc uptake from wheat- and rice-food-matrix - histidine, phytate, tartaric acid (1:1) and methionine (1:10) resulted in decreased zinc uptake relative to control cells	[37]
Caco-2 Cultivation time: 14-21 d 3D Transwell (PE membrane)	sample [c] (in salt buffer) for 2 h	- influence of caseinophosphopeptides (CPPs) and milk on zinc uptake from fruit beverages - *in vitro* digestion model	total zinc	- zinc retention, transport and uptake was higher for milk-containing fruit beverages than for CPPs-based fruit beverages	[38]
Caco-2 Cultivation time: 21 d 3D Transwell (PC membrane)	sample [c] (apical: HEPES,MES, glucose, basolateral: HBSS) for 3 h	- cereals and dephytinized cereals (phytase) - *in vitro* digestion model	total zinc	- effect of dephytinization on zinc, iron and calcium bioavailability in Caco-2 cells - zinc and iron solubility and fractional zinc and iron absorption increased after dephytinization of cereals	[39]
Caco-2 Cultivation time: 11-13 d 2D	50 μM $^{65}ZnCl_2$ Iron-zinc interactions: Zn:Fe (1:1)	- ascorbic acid (1 mM) and phytic acid, tannic acid, tartaric acid, cysteine, histidine, methionine (each 500 μM)	radioactive zinc (^{65}Zn)	- ascorbic acid, tartaric acid and tannic acid increased zinc uptake	[40]

	(in DMEM) for 2 h			- phytic acid and histidine decreased cellular zinc uptake - increase of iron uptake in presence of methionine, increased also zinc uptake - without added ligands, zinc inhibited iron uptake into Caco-2 - ligands can modulate iron : zinc-interaction	
Caco-2 Cultivation time: 12-14 d 2D	25 µM $^{65}ZnCl_2$ (in MEM) for 3 h	- polyphenol-rich beverages: red wine, green tea, red grape juice - tannic acid, quercetin, gallic acid, caffeic acid (each 250µM) - *in vitro* digestion model (including a rice matrix)	radioactive zinc (^{65}Zn)	- polyphenol-rich beverages increased cellular zinc uptake from digested rice matrix - tannic acid and quercetin enhanced zinc uptake	[41]
Caco-2 Cultivation time: 21-28 d 3D Transwell (PET-HD membrane)	50 µM zinc (in HEPES buffer) for 1 h	- water soluble vitamins: folic acid, nicotinic acid, ascorbic acid, riboflavin, thiamine, pyridoxine - effect of oxidative species on vitamin-dependent zinc uptake was analyzed - phytic acid and histidine	total zinc	- zinc transport was slightly enhanced by nicotinic acid and slightly decreased by thiamine, riboflavin, and pyridoxine - phytic acid significantly decreased zinc uptake compared to control cells, where histidine resulted in a slight increase of zinc uptake	[42]
Caco-2 Cultivation time: 21 d 3D Transwell (PC membrane)	sample [c] (in apical and basolateral HBSS) for 1 h	- samples from each stage of processing: wheat flour, whole wheat flour; fermented and final product: white bread, whole wheat bread, muffin - *in vitro* digestion model	total zinc	- effect of 'processing' of baking products on bioavailability of calcium, iron and zinc in Caco-2 cells - no differences in zinc uptake from fermented dough and after baking	[43]

Caco-2 Cultivation time: 21 d 2D	0 - 25 µM $ZnCl_2$ (in minimum essential medium) for 24 h	- varying zinc : phytic acid-ratios - *in vitro* digestion model (red beans, fish samples)	stable zinc isotope (n.a.) MT quantification [a]	- MT formation was investigated as a proxy for zinc uptake - phytic acid significantly decreased zinc uptake and MT formation for Zn : phytic acid ratios < 1:5	[44]
Caco-2 Cultivation time: 17 d 3D Transwell (collagen coated)	10 µM $^{65}ZnCl_2$ (apical: HBSS buffer, basolateral: DMEM) for 1-3 h	- bioactive dietary polyphenols: epigallocatechin-3-gallate (EGCG), green tea extract (GT), and grape seed extract (GSE) (each 46 mg/L) - phytate (100 µM)	radioactive zinc (^{65}Zn)	- GSE decreased zinc absorption by inhibiting cellular zinc uptake, similar to phytate - EGCG and GT did not reduce zinc absorption	[45]
Caco-2 Cultivation time: 14 d 2D	sample [c] (in medium) for 6 h	- different rice varieties and zinc biofortified rice (polished or parboiled samples) - *in vitro* digestion model	radioactive zinc (^{65}Zn)	- comparison of zinc uptake from biofortified rice with *in vivo* rat pups - biofortified rice yielded significant higher net absorption *in vitro* and *in vivo* - net absorption was smaller in Caco-2 but showed the same correlation between different rice samples	[46]
Caco-2 Cultivation time: 13 d 2D	sample [c] for 2 h followed by additional incubation for 10 h	- biofortified wheat (low-phytate mutants with varying zinc content) - *in vitro* digestion model	Reporter gene assay [b]	- positive and negative correlations for zinc bioavailability dependent on zinc : phytate-ratios	[47]
Caco-2 Cultivation time: n.a. 2D	sample [c] (in MEM + 3% FCS) for 6 h	- reduction of phytate content in sorghum (genetic modification) - *in vitro* digestion model	radioactive zinc and iron (^{65}Zn, ^{59}Fe)	- comparison of fractional absorption in Caco-2 and *in vivo* suckling rat pup model - phytate reduction significantly increased zinc	[48]

				bioavailability in Caco-2 cells comparable to *in vivo* analysis	
Caco-2 Transwell (PE) Cultivation time: 21 d 3D Transwell (PE membrane)	250 µM $ZnSO_4$ (in DPBS) for 2 h	- GPAGPHGPPG peptide (derived from Alaska pollock)	total Zn	- influence of GPAGPHGPPG peptide on zinc, iron and calcium transport - GPAGPHGPPG peptide significantly increased mineral transport.	[49]
Caco-2 Cultivation time: 10 d 2D	50 µM $ZnCl_2$ (in PBS) for 30 min	- amino acids (AAs): glutamate (Glu), lysine (Lys), methionine (Met) - ZnAAs complexes: ZnGlu, ZnMet, ZnLys	Free zinc (Fluorescent zinc sensor Zinpyr-1)	- ZnAAs are probably absorbed by AAs transporters - zinc uptake into Caco-2 cells is not enhanced by ZnAAS complexes - results suggest that ZnAAs represent a more efficient war for zinc supplementation that zinc salts; especially for AE patients	[50]

3D, three-dimensional; AAs, amino acids BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagles Medium; FCS, fetal calf serum; HBSS, Hank's Balanced Salt Solution; HD, high density; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;; IP, inositolphosphate; MEM, minimum essential medium; n.a., not available; PC, polycarbonate; PE, polyethylene; PES, polyester; Zn, zinc; [a] MT formation was analyzed using a cadmium/hemoglobin assay; [b] reporter gene assay based on the metal response element (MRE)-binding transcription factor-1 (MTF-1) and MRE luciferase, [c] mineral bioavailability from the sample solely was examined; no extra zinc added.

Abbreviations:

2D	two-dimensional
3D	three-dimensional
DGE	German Society for Nutrition; ger. *Deutsche Gesellschaft für Ernährung*
DMEM	Dulbecco's Modified Eagles Medium
DMT-1	divalent metal transporter
EFSA	European Food Safety Authority
EHS	Engelbreth-Holm-Swarm cells
FAAS	flame atomic absorption spectrometry
FCS	fetal calf serum
HD	high density
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP	inositolphosphate
KHB	Krebs-Henseleit buffer
LMW	low molecular weight
mRNA	messenger ribonucleic acid
MEM	minimum essential medium
MT	metallothionein
MTF-1	metal regulatory transcription factor 1
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	polycarbonate
PE	polyethylene
PES	polyester
PET	photo-induced electron transfer
*q*PCR	quantitative real time polymerase chain reaction (PCR)
TEER	transepithelial electrical resistance
TJ	tight junction
TPEN	N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine
WHO	World Health Organization
ZIP	Zrt-, Irt-like protein
Zn	zinc
ZnT	zinc transporter

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