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ORIGINAL CONTRIBUTION

Effect of water soluble vitamins on Zn transport of Caco-2 cells and their implications under oxidative stress conditions

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Abstract
Background The role of different water soluble vitamins in Zn metabolism beyond intestinal Zn absorption is poorly explored.
Aim of the study Using Caco-2 cells, effects of different vitamins on intestinal Zn transport and their implications under oxidative stress (OS) were investigated.
Methods Cells were apically treated with Zn (25 μM) and vitamins (Folic acid (FA), Nicotinic acid (NA), Ascorbic acid (AA), riboflavin, thiamine, pyridoxine) for 60 min. The effect of most promising vitamins on zinc transport, antioxidant enzymes (Catalase, Glutathione peroxidase, and superoxide dismutase), and intracellular OS status (ROS generation and mitochondrial transmembrane potential) were investigated. OS was generated by *tert*-butyl hydro peroxide and results for each vitamin were compared with respective Zn containing controls with and without OS.
Results Without OS, Zn transport was slightly enhanced in presence of NA, while it was significantly reduced by thiamine, riboflavin, and pyridoxine. Under OS, NA significantly ($P < 0.01$) enhanced Zn transport in dose-dependent manner, while, pyridoxine and AA moderately improved it. Under both conditions, Zn transport exhibited decreasing trend with increase of FA. The antioxidant enzyme and OS markers levels varied significantly in Zn + vitamins. With Zn + FA + OS, enzyme activities decreased maximally, with twofold increase in 2′,7′-dichlorofluorescin diacetate (DCF-DA) ($P < 0.01$) and lowering of rhodamine fluorescence ($P < 0.05$). In Zn + AA + OS, DCF-DA fluorescence increased ($P < 0.05$) but with NA, cellular enzymes, and antioxidant profile were improved.
Conclusions Results for the first time demonstrate advantageous effects of NA and deleterious consequences of FA with no effect by AA on Zn transport, especially under OS. These observed changes in the transport of Zn seem to have an impact on OS markers.

Keywords Zn transport · Caco-2 · Water soluble vitamins · Oxidative stress

Abbreviations

DCF-DA	2′,7′-dichlorofluorescin diacetate
NBT	Nitro blue tetrazolium
FA	Folic acid
NA	Nicotinic acid
AA	Ascorbic acid
OS	Oxidative stress
t-BHP	*tert*-Butyl hydroperoxide
EGTA	Ethylene Glycol-bis (beta-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
PMSF	Phenylmethylsulfonyl fluoride
GPx	Glutathione peroxidase
SOD	Superoxide dismutase
ROS	Reactive oxygen species
min	Minutes
Zn − OS	Zn without OS
Zn + OS	Zn with OS
Zn + AA − OS	Zn and AA without OS
Zn + AA + OS	Zn and AA with OS
Zn + FA − OS	Zn and FA without OS
Zn + FA + OS	Zn and FA with OS
Zn + NA − OS	Zn and NA without OS
Zn + NA + OS	Zn and NA with OS

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Introduction

In vertebrates, Zn balance is primarily maintained through a regulated rate of intestinal uptake, fecal elimination of excess Zn, renal reabsorption, and distribution to cells, including intracellular storage [21]. Dietary Zn is presented to the enterocytes, as a constituent of a variety of molecules, with varying binding affinities. About 20–40% of Zn is absorbed from the diet mainly in the form of complex. The absorbed Zn is incorporated into the intestinal epithelium from its apical side [24] and transferred through an intracellular pathway to the basolateral side, from where Zn is released into the portal circulation. There are two mechanisms for the transport of minerals from the lumen of the intestine to portal circulation: transcellular and paracellular transport [12]. Transcellular transport, the movement of zinc across the apical membrane through the cell and exiting at the basolateral membrane, is a carrier-mediated process. Paracellular transport occurs by simple diffusion as the concentration of zinc in the lumen exceeds the ability of transcellular mechanism to transport zinc into intestinal cell at its apical surface. Paracellular transport occurs as zinc diffuses through the tight junctions between the intestinal cells. It is generally assumed that an intraluminal transition occurs that allows Zn to be transported across enterocytes as the free ion. In normal conditions, the amount of absorbable Zn varies inversely with the concentration of dietary Zn for maintaining Zn balance. However, at extremely high intakes, Zn homeostasis operates primarily through excretion rather than absorption.

The gastrointestinal tract (GI) is a major target for oxidative stress damage due to constant exposure of reactive oxygen species (ROS) generated by the luminal contents such as oxidized food debris, transition metals like iron and copper, bacterial metabolites, bile acids, and salivary oxidants [13]. The ROS-mediated injury to the small intestine has been demonstrated in several conditions such as ischemia/reperfusion, inflammatory bowel disease [13, 14], surgical stress [35], radiation enteritis [29], iron supplementation [47], and Zn deficiency [53]. Exposure to free radicals leads to changes in the permeability of Caco-2 cells due to tyrosine phosphorylation of membrane proteins and subsequent disruption of paracellular junctional complexes [36, 37]. There are few reports on protective role of Zn against this damage by stabilizing the membrane structure [30], maintaining cell integrity and barrier functions [17] and improvement in mucosal repair by regulating tight junction permeability [48].

Caco-2 cell line has been widely used as an in vitro model for the small intestine, where cells are grown on a porous membrane to form apical and basolateral chambers. In connection with OS, these cells have been effectively used as an in vitro model for many applications such as screening of drugs for their intestinal absorption potential [33], investigation of toxicants, food mutagens and their mechanisms [31, 54], and studying the absorption of minerals and phenolics [1, 11, 51]. More recently, Caco-2 cell line has been used to demonstrate role of zinc transporters in Zn uptake and homeostasis [43, 52]. Intestinal Zn absorption gets affected by different factors like amino acids, metals, and chelating factors. However, the role of water soluble vitamins in Zn metabolism is least studied and warrants more detailed investigation. Present article investigates the role of six vitamins in intestinal Zn absorption under normal and OS conditions using Caco-2 as cell model.

Methods and materials

Caco-2 cell culture

Caco-2 cells were obtained at passage no. 29, from the National Centre for Cell Sciences (NCCS), Pune, India. The cells (initial cell density 5,600 cells/cm^2) were seeded in 25-cm^2 area cell culture flasks containing 5 ml growth medium. The growth medium used for Caco-2 cell culture and transport study was Dulbecco's modified Eagle's medium with 4.5 g/l glucose (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 20% Fetal Bovine Serum, 1% nonessential amino acids (Sigma Chemical Co. St.Louis, MO) and 1% antimycotic–antibiotic reagent (25 μg/ml of amphotericin B, 100 U/ml penicillin G and 100 μg/ml of streptomycin, Himedia, Mumbai, India). The flasks were incubated at 37 °C, with 5% CO_2 and 95% humidity in a water-jacketed CO_2 incubator (Thermo-Forma, Ohio, USA). The medium was changed twice weekly and growth was monitored using phase contrast inverted microscope (Nikon TS 100, Towa Optics, Tokyo, Japan). At 80–100% confluency, the cells were detached by treatment with 4 ml of Trypsin–Phosphate–Versene–Glucose (TPVG) solution for 10 min and subcultured.

Zn transport in presence of different vitamins

Caco-2 cell monolayer formation on inserts

Caco-2 cells at passages 35–40 were used in all the experiments. Transwell cell culture inserts with PET-HD membranes (6.4-mm diameter, 0.4-μm pore size; Axygen Scientific Pvt. Ltd., India) that divide a well of 24 well plate into apical and basolateral chambers were used for transport study.

Cells were seeded into bicameral chambers at a density of 55,000 cells/cm^2, with 0.7 ml of growth medium in the well (basolateral cell side) and 0.35 ml of medium in the

insert (apical cell side). Before seeding cells on Transwell inserts, the cell viability were examined. The cell viability was judged by trypan blue exclusion assay. More than 95% cells were viable. The cells were incubated to achieve full differentiation and tight junction formation for 21–28 days at 37 °C in an incubator with a 5% CO_2/95% air atmosphere with change of medium every second day. The integrity of the monolayer was assessed by phenol red confluence assay [10] at day 21. For the assay, 100 µl of phenol red (16 µg/ml) was added in the apical medium. After 2 h incubation at 37 °C the optical density (546 nm) of the basal well contents was measured against normal growth medium as a blank to detect any leakage of the phenol red through the intercellular spaces. No increase in the absorbance indicated formation of tight intercellular junctions with uniform monolayer.

Zn transport

A method developed by Bobilya et al. [2] was used to measure zinc transport across the Caco-2 cells. Briefly, cell surfaces of the monolayer were washed four times with HEPES buffer used for uptake experiment (10 mM HEPES, 140 mM NaCl, 7 mM KCl, and 5.6 mM glucose). The vitamins used for assessing the effect on Zn transport were: folic acid (FA), Nicotinic acid (NA), ascorbic acid (AA), riboflavin, thiamine, and pyridoxine. Histidine and phytic acid were used as known promoter and inhibitor of Zn transport. These factors were prepared in HEPES buffer and sterilized by membrane filtration prior to their use. Stocks were diluted 10-fold before transfer of appropriate volume (1%, v/v) to HEPES buffer. Nine experimental sets each for normal and OS conditions along with appropriate control were performed in triplicate. HEPES buffer (0.5 ml) was added to the basolateral chamber, and 0.5 ml solutions in HEPES buffer with factors (Table 1) were added to the apical chamber in different sets. Thus, in apical chamber, the final concentration of vitamins and Zn were at normal physiological plasma levels and 25 µM, respectively. For experimental sets with OS condition, OS was generated by addition of *t*-BHP (*tert*-butyl hydroperoxide) at final concentration of 100 µM. Cell cultures were incubated for 60 min at 37 °C in a humidified air/CO_2 atmosphere. After incubation, cells were washed by HEPES buffer to remove nonspecifically bound zinc and residual buffer. HEPES buffer from both, the apical compartment and basolateral chamber were collected for Zn estimation.

Table 1 The amount of different solutions added to the uptake medium in the apical chamber

Solutions added	Without OS (µl)		With OS (µl)	
	Treatment	Control	Treatment	Control
$ZnSO_4$ (250 µM)	50	50	50	50
t-BHP (1 mM)	–	–	50	50
Factors[a]	50	–	50	–
HEPES buffer	400	450	350	400

[a] Concentrations (10-folds higher, i.e., 10*X*) = NA: 4 µM, FA: 0.25 µM, AA: 800 µM, Riboflavin: 0.85 µM, Thiamine: 1.83 µM, Pyridoxine: 1.27 µM. Histidine and Phytic acid (Zn:Phytic acid/histidine as 1:5 ratio): 1,250 µM

Dose response of various vitamins during Zn transport

From the screening experiment, NA, FA, and AA were identified as factors for next set of experiments which were conducted to evaluate the effect of increasing concentrations of these vitamins on the Zn transport in normal and OS condition. Zn transport assay were performed as described earlier, with uptake media containing 25 µM Zn and NA/FA/AA varying from *X* (physiological levels) to 10*X* levels (NA = 0.4–4 µM, AA = 0.08–0.8 mM and FA = 0.025–0.2 µM).

Antioxidant defense system of Caco-2 cells after Zn − vitamin treatment

Antioxidant profile of Caco-2 cells was monitored through estimation of different enzymes for evaluating the effect of Zn + vitamin (AA, FA, and NA) treatments of cells during OS. For this, cells at a density of 5,600 cells/cm^2 were seeded in 25 cm^2 culture flasks containing 5 ml DMEM + 20% fetal bovine serum and incubated at 37 °C in a CO_2 incubator. After 90% confluency, the cells were exposed to 25 µM Zn and 10 times higher levels of vitamins: NA (4 µM)/AA (0.8 mM)/FA (0.2 µM) in HEPES buffer, with or without *t*-BHP and incubated further for 60 min. After 1 h, the cells were lysed for estimation of different enzymes.

Preparation of cell lysate

After the Zn − vitamin treatment, the medium was removed from the flasks; cells were washed with PBS, trypsinized, and harvested by centrifugation at 900*g* for 4 min. The cells were then suspended in 500 µl of lysis buffer (50 mM Tris–Cl, 5 mM EDTA, 5 mM Ethylene Glycol-bis (beta-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tertaacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100 and 0.1% protease inhibitor cocktail) and centrifuged at 10,000*g* for 15 min at 4 °C [49]. The supernatants were collected and stored at −20 °C for up to 48 h prior to enzyme analysis.

The protein concentration of the cell lysates was determined by the method of Lowry et al. [26]. After incubation, sample absorbance was measured spectrophotometrically

(UV1, Thermo Spectronic Corp., New York, USA) at 660 nm. The amount of protein in the samples was estimated by using bovine serum albumin (BSA) as standard protein.

Catalase assay

Catalase activity was determined by a method of Clairborne and Fridovich [4]. Decrease in absorbance at 240 nm was measured spectrophotometrically and enzyme activity was expressed as nM of H_2O_2 decomposed min^{-1} mg^{-1} protein. The specific activity of catalase was calculated using the molar extinction coefficient for H_2O_2 as 43.6 M^{-1} cm^{-1} at 240 nm in the following equation:

$$\text{Specific activity(units/min/mg protein)} = \Delta A_{240\text{nm}}\,(1\,\text{min}) \times 1,000/43.6 \times \text{mg protein}$$

SOD assay

The activity of SOD in cells was estimated by using the method of Kono [20]. The rate of nitro blue tetrazolium (NBT) reduction was measured at 340 nm for 5 min using a spectrophotometer (UV1, Thermo Electronic Corp., New York, USA). Percentage inhibition in the rate of NBT reduction was calculated and one unit of enzyme was expressed as inverse of the amount of protein required to inhibit the reduction rate of NBT by 50%.

Glutathione peroxidase (GPx) assay

Glutathione peroxidase was assayed by the method of Mohondas et al. [27]. The decrease in the absorbance due to NADPH oxidation was monitored spectrophotometrically at 340 nm. The nonenzymatic reaction rate was correspondingly assayed by replacing the cell lysate with phosphate buffer. The enzyme activity was expressed as nM NADPH oxidized min^{-1} mg^{-1} protein and was calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Intracellular OS marker status

In order to assess the cellular oxidative damages after the treatment with vitamin + Zn under OS condition, cytoplasmic ROS and mitochondrial membrane potential were quantified using DCF-DA and tetra methyl rhodamine fluorescence markers.

Analysis of intracellular ROS generation

Intracellular ROS was detected by DCF-DA (Sigma Chemical Co., St. Louis, MO) [40]. Caco-2 cells were grown and given similar Zn + vitamins treatment as given for antioxidant enzyme measurements. After the treatment, the HEPES buffer was replaced by Hanks' Balanced Salt Solution (HBSS) containing 50 μM DCF-DA (dissolved in DMSO) and cells were further incubated for 1 h at 37 °C in the dark. The cells were then washed with HBSS and solubilized in 1 ml of SDS (0.01% in 0.2 M NaOH). The generation of the fluorescent product 2′,7′-dichlorofluorescin (DCF) was followed in a spectrofluorometer (Hitachi, F-2500, Tokyo, Japan) using an excitation wavelength of 485 nm and emission at 530 nm. The results are expressed in terms of fluorescence arbitrary units/mg protein.

Measurement of mitochondrial membrane potential

Since increase in ROS can lead to damage of mitochondria with reduction in potential, mitochondrial membrane potential during different Zn + vitamin treatments was measured using tetramethyl rhodamine (Sigma Chemical Co., St. Louis, MO) [41], a fluorescent dye that incorporates into mitochondria in a $\Delta\Psi m$-dependent manner. Zn + vitamin-treated Caco-2 cells were further exposed to 3.3 μM tetramethylrhodamine in Hanks' Balanced Salt Solution (HBSS) for 30 min. The cells were washed twice with HBSS and solubilized in 1 ml of SDS (0.01% in 0.2 M NaOH). The fluorescence was determined at 530 nm excitation and 580 nm emission using fluorescence spectrophotometer. The results are expressed in terms of fluorescence arbitrary units/mg protein.

Zn estimation

The apical and basolateral media samples were mineralized in 0.5 ml of 30% HNO_3 at 95 °C for 1 h and after appropriate dilution, zinc concentrations were determined by flame Atomic Absorption Spectrometry (AA 800, Perkin-Elmer, Shelton, USA). The difference between apical and basolateral media Zn before and after incubation was used to estimate Zn transport which was expressed as nM Zn transported/mg of cellular protein. For analysis, Zn specific cathode lamp with 213.9 nm wavelength and 0.7 nm monochromator slit width was used. Commercially available external standards of Zn (Merck, NJ, USA) were analyzed simultaneously with the samples for quantification. Additionally, rice flour samples from National Institute of Environmental Sciences, Japan (NIES) were similarly processed and used as reference biological standards for quality control.

Statistical analysis

All the measurements were made in triplicate. Data were expressed as mean ± SD, with the number of experiments in parentheses. The results were subjected to one way analysis of the variance (ANOVA) followed by Dunnet's

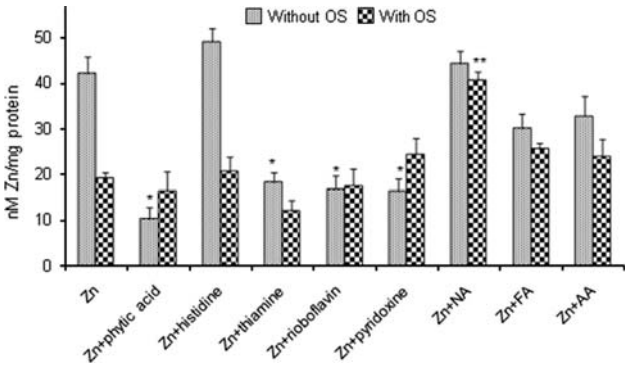

Fig. 1 Differentiated Caco-2 cells were treated with different factors, with and without *t*-BHP for 1 h. Zn transport from apical to basolateral chamber were estimated after 1 h incubation. Results are means ± SD for three experiments. * $P < 0.05$, ** $P < 0.01$ vs. respective controls assessed by one way ANOVA. For the analysis, factors without OS exposure treatment groups were compared to Zn − OS control and factors under OS exposure were examined against respective Zn + OS control

test for multiple comparisons by computing critical difference (C.D.). For the analysis, factors without OS exposure treatment groups were compared to Zn − OS control and factors under OS exposure were examined against respective Zn + OS control. The level of significance were represented as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Results

Effect of different vitamins on Zn transport by Caco-2 cells in the presence and absence of OS

The Zn transport results are expressed in terms of nM Zn/mg cell protein and depicted in Fig. 1. In the control cells with OS, the amount of zinc transported was significantly low as compared to respective control cells without OS ($P < 0.001$). The addition of different vitamins on apical side showed diverse consequences on the Zn transport. Further, for all the factors, the amount of Zn transported was less under OS condition than normal condition except for phytic acid and pyridoxine ($P < 0.001$). In normal condition, Zn transport was slightly enhanced in the presence of histidine and NA as compared to the control ($P > 0.05$). On the other hand, phytic acid, thiamine, riboflavin, and pyridoxine treatment resulted in significant reduction (>50%) in Zn transport. Under OS exposure, there was significant enhancement in Zn transport only in the presence of NA ($P < 0.01$) as compared to respective OS control (Zn + OS). Pyridoxine, AA, and FA treatment caused moderate increase in Zn transport but it was not statistically significant.

Effects of NA, FA, AA on Zn transport by Caco-2 cells under normal and OS conditions

From the screening experiment, NA, FA, and AA were recognized to positively influence Zn transport under OS conditions, which was done at single concentration of vitamins. Therefore, to further understand these interactions, effects of increasing concentrations of these three vitamins on Zn transport by Caco-2 cells were studied under normal and OS conditions in independent sets. The results are shown in Fig. 2. There was a dose-dependent increase in Zn transport with increasing concentration of NA in the apical chamber under OS condition (Fig. 2a), whereas increment in FA resulted in reduced Zn transport under both oxidative and normal conditions (Fig. 2b). There was no significant change in Zn transport due to addition of AA for both, normal conditions and OS (Fig. 2c).

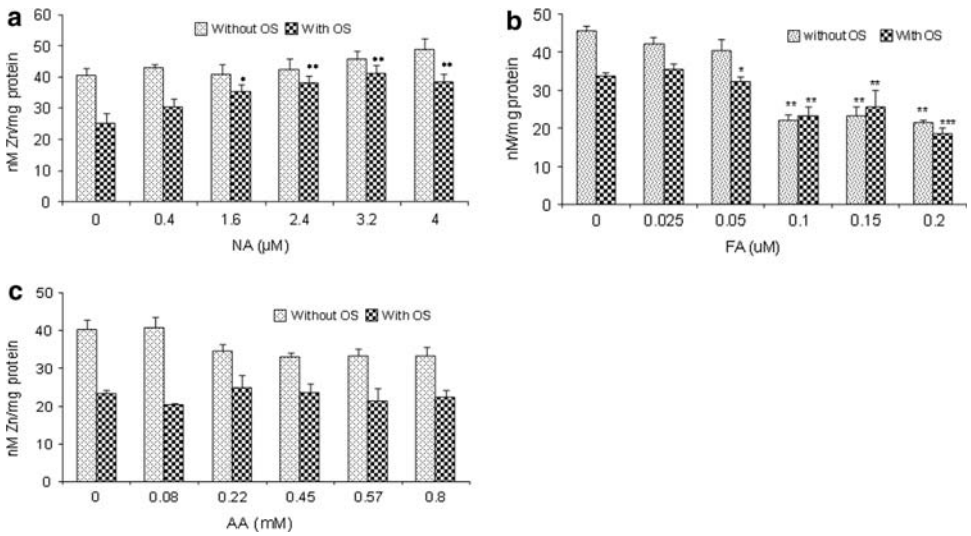

Fig. 2 Differentiated Caco-2 cells were incubated with increasing concentrations of **a** NA, **b** FA, **c** AA with and without *t*-BHP for 1 h. Zn transport from apical to basolateral chamber were estimated after 1 h incubation. Results are means ± SD for three experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. respective controls (i.e., Zn − OS and Zn + OS) assessed by one way ANOVA

Effect of NA, AA, and FA at normal conditions and OS on antioxidant enzymes of Caco-2 cells

The levels of the antioxidant enzymes catalase, SOD and GPx varied significantly depending on the Zn − vitamin treatments. In control cells with OS, all enzymes activities were reduced as compared to control cells with no OS but differences were not statistically significant ($P > 0.1$) (Table 2). In absence of OS, the presence of vitamins in HEPES buffer did not influenced the activities of enzymes ($P > 0.1$). In case of NA exposed cells, the enzyme activities were improved slightly even with externally added *t*-BHP, indicating protection to cellular enzymes by extracellular treatment with NA. However, AA in the presence of OS significantly reduced the activities of catalase ($P < 0.05$), SOD as well as GPx ($P < 0.01$). Further, the presence of FA, under OS, enzyme activities were decreased maximally (SOD—$P < 0.05$, catalase and GPx—$P < 0.01$) as compared to Zn + OS control, suggesting increased oxidative damage to cells. These results about higher levels of FA and AA shows negative consequences under OS, which were different than observed in the initial screening experiment.

Effect of NA, AA, and FA with OS on intracellular OS markers of Caco-2 cells

By exposing cells to 100 μM, *t*-BHP created significant change in cellular oxidative status as the levels of ROS and potential mitochondrial was altered significantly as compared to non-OS-treated control cells. In Zn − OS controls, values for DCF-DA fluorescence were 54.54 ± 10.29 and 96.813 ± 7.57 for rhodamine fluorescence. Figure 3 depicts the effect of various Zn − vitamins treatment on cellular OS indicators. Caco-2 cells incubation with AA + OS, significantly increased DCF fluorescence ($P < 0.05$) compared with Zn + OS-treated cells, however, mitochondrial damage was not observed (no decrease in rhodamine fluorescence).

Upon treatment with Zn + FA + OS, a massive production of ROS (twofold increase as compare to control, $P < 0.01$) as well as lowered rhodamine fluorescence, i.e., decreased $\Delta\Psi m$ ($P < 0.05$) was detected. These results imply higher intracellular OS with depolarized mitochondrial membrane in Caco-2 cells by incubation with FA.

The Zn + NA + OS-treated Caco-2 cells, had not shown altered DCF-DA and rhodamine fluorescence, indicating no change in cellular redox balance.

Table 2 Antioxidant enzyme activities in Caco-2 cells treated with Zn and vitamins (NA, AA, and FA) for 1 h, under normal and OS conditions

Groups	Catalase[a]	GPx[b]	SOD[c]
Zn – OS	46.54 ± 3.97	116.40 ± 4.91	1.02 ± 0.32
Zn + OS	43.14 ± 3.45	108.26 ± 7.81	0.98 ± 0.16
Zn + AA − OS	45.45 ± 5.2	112.47 ± 6.55	1.00 ± 0.27
Zn + AA + OS	34.45 ± 2.2*	88.48 ± 5.37**	0.86 ± 0.34*
Zn + FA − OS	43.14 ± 3.45	109.26 ± 7.81	0.89 ± 0.14
Zn + FA + OS	22.84 ± 1.92**	86.77 ± 7.62**	0.81 ± 0.32*
Zn + NA − OS	49.17 ± 4.25	111.62 ± 6.29	0.99 ± 0.28
Zn + NA + OS	48.82 ± 3.44	99.38 ± 4.48	0.93 ± 0.16

The results are the mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. respective controls (i.e., Zn − OS and Zn + OS) as assessed by one way ANOVA

[a] μmole H_2O_2 decomposed/min/mg protein

[b] nmole NADPH oxidized/min/mg protein

[c] U/mg protein

Discussion

The normal range of plasma zinc concentration is 15–25 μM and that in the gut lumen it could be much higher [39] to reach the optimal plasma zinc concentration. The amount of Zn used in the present transport study (25 μM) therefore can be considered to represent Zn

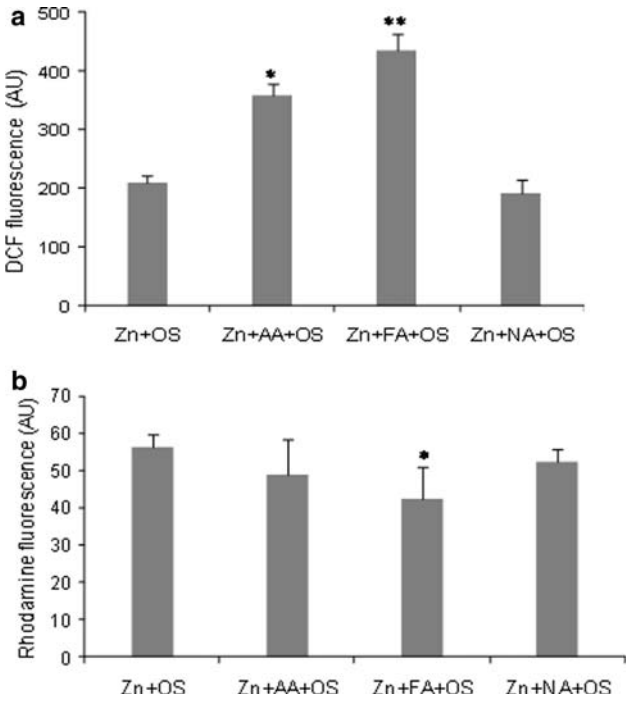

Fig. 3 Effect of NA, AA and FA along with the OS treatment on Caco-2 cells stress markers: **a** DCF-DA, **b** Tetramethyl rhodamine fluorescence. After vitamin − Zn treatments under OS, Caco-2 cells were incubated with 50 μM DCF-DA for 1 h and 3.3 μM tetramethylrhodamine for 30 min. Results are means ± SD for three experiments. * $P < 0.05$, ** $P < 0.01$ vs. respective controls (i.e., Zn − OS and Zn + OS) assessed by one way ANOVA

transport under low Zn condition. Zn Concentrations in the growth medium above 100 μM decrease cell proliferation rate, increase paracellular transport, cause cell damage [38]. Moltedo et al. [28] had reported Zn concentrations up to 200 mM maintain the stability of the membranes and cell integrity. Shen et al. [43] reported that under high zinc levels MT is stimulated, but other zinc cell transporters are susceptible to low extracellular zinc concentration and unregulated to enhance zinc absorption. High concentrations of Zn in growth media also results in MT induction in the cells [15], which consequently causes increased uptake but reduced Zn transport. Therefore, in current experiment the Zn present in the medium and during transport assay may not perhaps induce MT and only result into transcellular transport.

In normal condition, Zn transport was inhibited by 76% with phytic acid and stimulated by 16% with histidine (in 1:5 ratio of Zn: phytic acid/histidine). Han et al. [15] had observed 96% inhibition of Zn transport in Caco-2 cells by inositol hexaphosphate (phytate) in 1:10 molar ratio with Zn as compared to control. Some researchers have reported that higher concentrations of phytic acid and even histidine inhibited Zn uptake by Caco-2 cells [46]. The incubation time, temperature, amount of Zn in culture medium, cellular differentiation period, and Zn composition in uptake medium are the parameters reported which affect the Zn transport of Caco-2. Therefore, it is too difficult to compare the Zn transport rates with earlier reported studies. Nevertheless, within vitamins, higher Zn transport across the Caco-2 cells especially under OS conditions was found only in presence of extracellular NA, while AA and FA treatment caused moderate increase in transport only during screening but showed negative trend during dose response experiments. These responses may be indicative that AA and FA at higher concentrations act synergistically with OS contributing to cellular damage probably due to reduced Zn transport. On the other hand, Zn + NA act as a protective factor under normal condition as well as under OS.

Several studies have reported damage to GI tract due to OS and also involvement of H_2O_2 in the progression of colon cancer. Sreedhar et al. [45] had demonstrated protective role for zinc on intestinal peroxidative damage in rats due to iron-induced OS. Recently, Finamore et al. [8] showed that zinc deprivation induced a decrease of transepithelial electrical resistance and alterations in tight and adherent junctions eventually leading to disruption of membrane barrier integrity. In the current experiment, with the use of polarized Caco-2 cell monolayer grown on filter, beneficial effect of some vitamins on transport of Zn across the enterocytes has been demonstrated. Simultaneously, the findings also imply that OS adversely affects the Zn transport which may be negated by addition of certain vitamins. Metabolic studies have shown that dietary Zn intake influences intestinal absorption, while certain hormones, cytokines, and growth factors influence Zn metabolism in specific organ systems [5, 9, 16, 19]. In present experiment, significant stimulatory effect of NA on transepithelial efflux of apically absorbed Zn was observed from the cell monolayers particularly during OS.

In the current study, in screening experiment at 0.025 μM FA, the Zn transport was found to be enhanced under OS. However, as the levels of FA increased from 0.025 to 0.2 μM (at constant Zn = 25 μM) in dose-response experiment, the molar ratio of Zn:FA decreased (from 1,000 to 125). One possible reason behind this paradoxical observation could be the differences in molar ratios of zinc to FA which decide the equilibrium between loose complex of Zn with FA and zinc transport. Secondly, the differences between 0 FA and 0.025 or 0.05 μM of FA were not statistically significant indicating that action of FA as inhibitor needs certain minimum concentration or molar ratio with zinc. This also could be the reason for various controversial reports about the role of FA on zinc absorption in humans [3, 6, 18, 22]. The inhibitory effects of FA at higher concentrations on Zn transport observed by us are in analogy with previous reports about potential adverse effects of FA supplementation on Zn nutriture [3, 6, 18]. FA at low levels may help in partly alleviating OS through converting homocysteine to glutathione. But at higher level it may show toxicity. If so, FA supplementation during pregnancy which is a common practice may be of special concern as maternal Zn deficiency is common in certain populations consuming vegetarian foods as major source of energy and may lead to intrauterine growth retardation or congenital malformation.

Previously, in our rat liver slice study, we have found that FA showed significant inhibitory effect on zinc cellular uptake under both normal and OS conditions in a dose response manner. Nevertheless, dose response of AA indicated its marked enhancing effect under OS condition [50]. Present results are in agreement with previous results for FA. Also the finding that there was no role for AA in Zn transport which is similar to previous reports [7, 42, 44, 46]. Thus, FA caused inhibitory effect in both intestinal and liver cells, while AA was protective in OS for hepatocyte zinc uptake.

Some of the organic acids present in foods are known to affect the Zn absorption. For example, citrate is present in human milk at 3–5 mM/l and binds a significant part of Zn in breast milk [25]. The addition of organic acids like citrate, to foods can therefore under some conditions enhanced Zn absorption [32]. However, there is paucity of studies on the role of NA in Zn metabolism. The possible mechanism of enhanced Zn transport by NA at low Zn

status may be a weak chelation of Zn by NA, which further keeps the metal ion in solution and facilitate its uptake by the mucosal cell (not specific for NA but appears to be shared by other monocarboxylates).

The susceptibility of tissue to oxidative injuries depends mainly on their antioxidant defense mechanisms. Even with adequate oxidant defense mechanisms, tissues such as gastrointestinal epithelium can be overwhelmed by fluxes of oxidants. Therefore, understanding tissue-mediated defense mechanisms and tolerance levels of enzymes to oxidative damage becomes more important in deciding effective strategies to prevent or reduce cellular oxidative damage. To examine the possibility that higher intracellular Zn along with vitamins will help with better defenses against OS, major antioxidant enzymes activities were estimated after different vitamin + Zn treatments. The antioxidant enzymes SOD, GPx, and catalase limit the effects of oxidant molecules on tissues and are activated in the defense against oxidative cell injury by means of free-radical scavenging [23]. SOD is the first line of defense against oxygen-derived free radicals and functions by dismutating two superoxide ions into H_2O_2. Catalase is a major component in the primary antioxidant enzyme system most of it is located in peroxisomes, which catalyses the decomposition of H_2O_2 to H_2O and shares this function with GPx. GPx on the other hand is located in the cytosol, mitochondrial matrix and catalyses the reduction of H_2O_2, lipidic, and nonlipidic hydroperoxides to oxidized glutathione using two molecules of GSH. Perez and Cederbaum [34] reported that Zn pretreatment prevented the arachidonic acid induced decline in the $\Delta\Psi m$ and ROS production in HepG2 cells. The present results suggest that FA at higher levels inhibited Zn transport across Caco-2 cells, and consequently the Zn deficient cells were more prone to OS-induced damage. With the presence of NA, Zn transport as well as antioxidant profile was found to be better, consequently OS-induced cellular damage was not observed. These results show that with the presence of NA, Zn transport was increased as well cellular antioxidant compositions were slightly improved. This reflects that supplementation of NA with Zn provides compensatory mechanism to prevent OS-induced damage. On the other hand, higher concentration of FA inhibited Zn transport which additionally increased cellular susceptibility toward OS. Caco-2 cells when simultaneously incubated with FA and Zn showed decreased antioxidant enzymes activities with elevated intracellular OS indicators.

In conclusion, the results of the transport studies demonstrated for the first time, the effect of vitamins such as NA for Zn transport at the apical membrane of human intestinal epithelial cells. In addition, the results also suggested possible involvement of ROS-mediated pathway in the regulation of Zn transport by these vitamins. However, it is worth to note that although Caco-2 cells mimic the intestinal environment, they do not represent exact conditions found in the intestinal epithelium because of the absence of nervous and endocrine function. Therefore, further in vivo studies are needed to confirm these findings.

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