Alteration of Zinc Transporter mRNA Expression in Zinc Depleted Condition by TPEN{N,N,Na2,Na2-Tetrakis(2-Pyridylmethyl)Ethylenediamine}: A Cell-Line Based Study

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Summary Zinc deficiency is rapidly emerging as one of the important concerns in public health nutrition. Early diagnosis of zinc deficiency remains a major challenge. We investigated the expression level of different zinc transporters in zinc-deficient condition induced by TPEN, an intracellular zinc chelator in different cell lines like human monocyte (THP-1), skeletal muscle (RD), bone (Saos-2), liver (HepG2), representing different tissues which play key roles in zinc homeostasis. Cells were exposed to TPEN at various concentrations (2, 5, 10 μM) for 2 to 12 h and mRNA levels of ZnT1 and MT were analyzed using qPCR. Statistical analysis was carried out using one-way ANOVA. ZnT1 expression was significantly different at 4 h with TPEN concentration of 2 μM and 5 μM as compared to untreated controls in THP-1, whereas in HepG2, significant differences were observed at 5 μM and 10 μM TPEN concentration after 6 h. In RD, significant differences were observed at 4 h in presence of 2 μM TPEN and in Saos2 expression was significantly different at 2 h with 2 μM, 5 μM, and 10 μM TPEN as compared to respective controls. Expression of MT in THP1 was significantly different at 2 h and 12 h control in presence of 2 μM, 5 μM and 10 μM TPEN, whereas in HepG2 significant differences were found at 2 μM, 5 μM, and 10 μM TPEN after 6 h of treatment. RD MT expression was significantly different in 10 μM for 12 h. Similarly, Saos2 expression was significantly different in the presence of 5 μM and 10 μM TPEN. Conclusions: This study may help in understanding the molecular cross talks among different zinc tissue storage depots during zinc deficiency and identification of early biomarkers for zinc deficiency.

Key Words zinc, ZnT1, MT, TPEN, nutrigenomics

Dietary zinc deficiency is highly prevalent worldwide (31%) and has been estimated to be responsible for approximately 450,000 global deaths of children less than 5 y old (1). Almost every bodily structure and physiological process is either directly or indirectly require zinc. Zinc is required by most enzymes in the body than all trace minerals combined. It is known to form part of more than 300 metalloenzymes, which cannot function in its absence (2). At cellular level, zinc plays critical catalytic, structural, and regulatory roles. Zinc is required for the proper structure and function of proteins and cell membranes. Zinc helps to regulate gene expression by binding to DNA through zinc finger transcription factors. It plays a role in cell signaling and has been found to influence hormone release and nerve impulse transmission (3). Zinc is also required for proper immune response (due to its role in the activation of T-cells), so zinc deficiency eventually leads to a greater susceptibility to infections (4).

Zinc deficiency is rapidly emerging as one of the important concerns in public health nutrition in the world, as well as in India. Since Indian population mostly consumes cereal-based diet that is poor in bioavailable zinc, mild to moderate zinc deficiency is expected to be highly widespread based on the data available on stunted growth in children (5). Primary risk factors for zinc deficiency include diets low in zinc or high in phytates, malabsorption disorders (including intestinal parasites and diarrheal), impaired utilization of zinc and genetic disorders. Low intake coupled with different life stages such as infants, children, adolescents, and pregnant and lactating women, or conditions such as preterm birth, low birth weight and diarrheal disorders increase the risk of zinc deficiency (6). However, there are no true estimation yet, primarily due to the lack of suitable biomarker (7).

Therefore, early diagnosis of zinc deficiency remains a major challenge for setting up intervention studies. In this study, we investigated the correct dose of TPEN, an intracellular zinc chelator for zinc depletion-repletion studies using THP-1 cell line as an experimental model. This may help us devise better nutrient-specific depletion-repletion study which may lead to indentification of early zinc deficiency-associated molecular biomarkers.
MATERIALS AND METHODS

Chemicals. Roswell Park Memorial Institute (RPMI) 1,640 media was purchased from Thermo Scientific, fetal bovine serum (FBS) was obtained from PAN Biotech, penicillin, streptomycin, glutamine, and trypan blue were purchased from Gibco, Life Technologies. T-25 and T-75 flasks were obtained from Corning. Cell culture plates and centrifuge tubes were purchased from Eppendorf. Reverse transcriptase, oligo-dT Primers, random Hexamer were obtained from Bioline. The membrane-permeable zinc chelator N,N,N,N-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) was purchased from Sigma Chemical. TPEN was stored as a stock solution in dimethyl sulfoxide (DMSO) at −20°C and added to cultures at the appropriate dilution.

For adherent cell lines (HepG2, SaOS2 and RD cell lines), Dulbecco’s Modified Eagle’s Medium (DMEM) modified media was used which contains 4 mM L-glutamine, 4.500 mg/L glucose, 1 mM sodium pyruvate and 1,500 mg/L sodium bicarbonate and was supplemented with 10% FBS.

Cell culture. Human monocyte cells (THP1) were obtained from American Type Culture Collection (ATCC). The cells were maintained in T-25 flasks and shifted to T-75 flasks when they reached sufficient confluency of 1x10^5 cells per mL. During the initial growth, RPMI-1640 medium was supplemented with 20% FBS, 1x antibiotic and glutamine, but subsequently, the acclimatized cells were grown in RPMI medium supplemented with 10% FBS and the culture medium was replaced in alternate days. The cells were grown at 37°C in a humid atmosphere containing 5% CO2 until cell confluency level reached 1x10^6 cells per mL. For all experiments, the cells were seeded in 6 well plates containing RPMI-1640 media free from serum and antibiotic.

HepG2, SaOS2 and RD cells were obtained from American Type Culture Collection (ATCC). The cells were maintained in T-25 flasks and shifted to T-75 flasks when they reached sufficient confluency of about 1x10^5 cells per mL. During initial growth phase, the DMEM medium was supplemented with 20% FBS and 1x antibiotic, sodium pyruvate, and glutamine, and subsequently, the acclimatized cells were grown in DMEM medium supplemented with 10% FBS and the culture medium was replaced in alternate days. The cells were grown at 37°C in a humid atmosphere containing 5% CO2 until cell confluency level reached 1x10^6 cells per mL. For all experiments, the cells were seeded in 6 well plates containing DMEM media free from serum and antibiotic.

Treatment. In this study, we examined the effect of membrane-permeable zinc chelator TPEN has any effect on cell viability. Cell viability was assayed by trypan blue dye exclusion using microscopy at 10X magnification.

For detecting zinc transporter gene expression levels by qPCR, all the cell lines were treated with 2.0, 5.0, 10.0 μM of TPEN for 2, 4, 6 and 12 h and the control consisted of cells treated with DMSO only.

Selection of genes. After an extensive review of literature, evidence strongly suggested that relative expression level of mRNAs in response to zinc depletion by TPEN for Metallothionein (MT) and Zinc transporter1 (ZnT1) were highly prominent. TPEN significantly decreased mRNA expression of ZnT1 and MT.

RNA extraction and qPCR. Total RNA were extracted from cells using the TRI REAGENT LS kit (Sigma Aldrich) according to the manufacturer’s protocol. Total RNA (1 μg) was reverse-transcribed into cDNA using SuperScript Reverse Transcriptase according to the manufacturer’s protocol, using oligo-dT primer and random hexamers provided by Bioline. The final volume of cDNA was 20 μL.

For qPCR, best expressed samples from PCR were pooled for standard preparation and subsequently, 3-fold serial dilutions were carried out to prepare six points of standard of different concentration. The initial sample dilution was 1:20 for all cell lines. Data was normalized using hGAPDH, where the annealing temperature was as follows: ZnT1-56°C, MT-54°C and hGAPDH-54°C.

Protocol used was:
1: 95.0°C for 3:00
2: 95.0°C for 0:10
3: 63.0°C for 0:30 Plate Read
4: GOTO 2, 39 more times
5: Melt Curve 65.0°C to 95.0°C Increment 0.5°C 0:05

Statistical analysis. Statistical analysis was carried out using one-way ANOVA, p value of <0.05 was considered significant.

RESULTS

Expression of ZnT1 in THP-1, RD, Saos-2 and HepG2 cell lines.

ZnT1 expression was significantly different at 4 h with TPEN concentration of 2 μM and 5 μM as compared to untreated controls in THP-1 (Fig. 1). In RD, significant differences were observed at 4 h in the presence of 2 μM TPEN (Fig. 2), while Saos2 expression was significantly different at 2 h with 2 μM, 5 μM, and 10 μM of TPEN as compared to the respective controls (Fig. 3), while in HepG2 significant differences were observed at 5 μM and 10 μM of TPEN concentrations after 6 h (Fig. 4).

Expression of MT in THP-1, RD, SaOS-2 and HepG2 cell lines.

Expression of MT in THP1 was significantly different at 2 h and 12 h control in f 2 μM, 5 μM, and 10 μM TPEN (Fig. 5). In RD cells, MT expression was significantly different at 10 μM at 12 h (Fig. 6). Similarly, Saos2 cells expression was significantly different in the presence of 5 μM and 10 μM TPEN (Fig. 7), whereas in HepG2 cells significant difference was observed at 2 μM, 5 μM, and 10 μM TPEN after 6 h of treatment (Fig. 8).

DISCUSSION

Several studies revealed that total body zinc contents were maintained by tight regulation of intestinal
Fig. 1. ZnT1 expression in THP-1 macrophages.

Fig. 2. ZnT1 expression in RD cell-line.

Fig. 3. ZnT1 expression in Saos-2 cell-line.

Fig. 4. ZnT1 expression in HepG2 cell-line.

Fig. 5. MT expression in THP-1 macrophages.

Fig. 6. MT expression in RD cell-line.

Fig. 7. MT expression in Saos-2 cell-line.

Fig. 8. MT expression in HepG2 cell-line.
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