Zinc Status Assessment by Analysis of Mononuclear Cell Metallothionein mRNA Using Competitive-Reverse Transcriptase-Polymerase Chain Reaction

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Abstract

Marginal Zn deficiency is prevalent through the world and yet human zinc status has not been properly assessed due to the lack of a reliable diagnostic indicator. One potential possibility for zinc status assessment using Zn-binding protein, metallothionein (MT)-mRNA, has been proposed. The purpose of the present study was aimed to show whether measurement of mononuclear cell (MNC) MT mRNA, using a competitive-reverse transcriptase-polymerase chain reaction (competitive-RT-PCR) assay, could indicate zinc status in human subjects. In this study, MNC MT-mRNA expression was measured using a competitive-RT-PCR to compare before and after 14 days of zinc supplementation (50 mg Zn/d as zinc gluconate). RT-PCR oligonucleotide primers which were designed to amplify both a 278 bp segment of the human MT-2A cDNA and a 198 bp mutant competitor cDNA template from MNCs, were prepared. MT-2A mRNA was normalized by reference to the housekeeping gene, β-actin, mRNA for which was also measured by competitive-RT-PCR. There was considerable inter-individual variation in MT-mRNA concentration and yet, the mean MT-2A mRNA level increased 4.7-fold after Zn supplementation, as compared to before Zn supplementation. This MT-2A mRNA level was shown as the same pattern and, even more sensitive assay, compared to the conventional plasma and red blood cells (RBCs) Zn assessment in which plasma and RBCs zinc levels increased 2.3- and 1.2-fold, respectively (p < 0.05). We suggest that MT competitive-RT-PCR can be a useful assessment tool for evaluating human zinc status.

Key words: Zn assessment, metallothionein, competitive-reverse transcriptase-polymerase chain reaction (competitive-RT-PCR)

INTRODUCTION

Zinc is essential for the structure and function of many enzymes and transcription factors. However, assessment of zinc status is a challenge because there is no reliable diagnostic indicator (1-3). The assessment of nutritional status is usually based on either the level of the nutrient in a blood component or a measurable variable associated with a function that responds to dietary intake or body storage of that nutrient. In the case of zinc nutriture assessment, various biosamples, such as blood, hair, nail and saliva, have been used. However, it has not been proven that any of these biosamples are reliable biomarkers for zinc nutrition status (4).

Measurement of zinc levels in plasma or blood cells, which is the most general tool for zinc nutritional assessment, does not appear to be a reliable and sensitive predictor of zinc status. Plasma zinc concentration is homeostatically regulated at 10~15 µM. When zinc intake is low, body zinc excretion through GI tract decreases in order to conserve zinc and maintain a normal plasma zinc concentration. Unless the body is challenged with severe zinc deficiency, it may not be easy to precisely measure zinc nutritional status. Marginal zinc deficiency, rather than severe zinc deficiency, is thought to be more prevalent in the developed countries, and even in developing countries (3,4).

Metallothionein (MT) has a high zinc-binding capacity. Since dietary zinc intake is directly related to cellular MT mRNA levels, a new approach to assess zinc status using MT mRNA in cells has been proposed (5-9) for a better and more precise assessment of zinc status. The Molecular technique, a double competitive reverse transcriptase-polymerase chain reaction (competitive-RT-PCR), is available that allow detection of small amount of MT mRNAs particularly in mononuclear cells in blood; because

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mature erythrocytes are non-nucleated, MT mRNA levels in erythrocytes cannot be measured. Additionally, mononuclear cells and nucleated blood cells have the highest level of MT protein and MT mRNA of the white blood cells (10). The level of MT mRNA in human mononuclear cells is sufficiently low that RT-PCR is the method of choice for detection of MT mRNA levels in zinc status assessment. Generally, in the procedure of RT-PCR, mRNA is converted to cDNA which is then amplified by PCR and measured following separation of the cDNA products by electrophoresis. The method we describe here for zinc assessment, a double competitive-RT-PCR, was applied to simultaneously amplify both target gene MT cDNA and MT cDNA competitor, whose concentration is already known, and thus determine the MT level quantitatively.

In the present study, we measured zinc status in the selected human subjects using double competitive-RT-PCR for measuring human MT-2A mRNA expression and it was proposed that this method would be a more sensitive tool for zinc assessment. This study is the first trial of zinc status assessment in zinc supplemented Korean subjects using competitive-RT-PCR.

SUBJECTS AND METHODS

Subjects and experimental design

Originally, twelve healthy male and female subjects between the ages of 19~25 y were recruited for this study. The subjects were non-smokers with no history of chronic illness who had normal blood chemistry profiles and were not taking any zinc supplementation during the experimental period. Just before the zinc supplementation started, blood was collected for zinc assessment. The subjects, then, received 50 mg Zn (as zinc gluconate) at the same time each day for 14 days. This intake of zinc is around 4-fold that of the Korean RDA (man 12 mg and woman 10 mg). Out of 12 subjects, only five (2 men, 3 women) finished completely and took the supplements correctly until the end of the 14-day experimental period, and thus only data for five subjects were used in this study. Blood was drawn again at the end of the 14 days of zinc supplementation.

Isolation of mononuclear cells

Venous blood samples (10 mL) were withdrawn into tubes (Becton Dickinson) containing heparin, and mononuclear cells were isolated using Histopaque 1077 (Sigma) according to the manufacturer's directions. The supernatant portion of plasma and pelleted RBCs were removed for zinc analysis. The mononuclear cells were collected at the interface for competitive-RT-PCR analysis.

RNA extraction and reverse transcription of RNA (cDNA synthesis)

RNA extraction: Total RNA was extracted from human mononuclear cells using TRI reagent (MrcGene, USA), which is modified from the acid guanidinium thiocyanin-phenol-chloroform method (11), according to manufacturer's instruction. cDNA was synthesized using a commericial cDNA synthesis kit (Roche, USA) according to manufacturer's instruction. The cDNA reaction solution was diluted to give a known concentration of reverse-transcribed RNA and was either stored at -80°C or used directly for PCR.

Human MT-2A competitive-RT-PCR

The MT-2A competitive-RT-PCR assay used in this study has been reported previously (5,6), with modifications where necessary.

Synthesis of human MT-2A cDNA competitive strand (MT-2A cDNA competitor): MT-2A mutant cDNA template (MT cDNA competitor) was synthesized having the same forward and reverse primer-binding sequences, but containing a deletion so that the PCR product size is different from that of the target MT-2A mRNA. With the primers used in the present study, the target product size for human MT-2A was 278 bp, and a competitive MT-2A cDNA standard containing an 80 bp deletion was made using an MT-2A cDNA template with the following primers (5):

(for MT-2A competitive cDNA template)
Forward primer 5'-GCC CCG CTG GGT CAT GTA AAG AAC-3'
Reverse primer 5'-TGT CCC GTC GTG GAG CAG CAG C-3'

Competitive-RT-PCR for human MT-2A: In this competitive-RT-PCR assay, the original human MT-2A primers were used to simultaneously amplify both the target MT-2A cDNA and the competitor MT-2A cDNA templates. In these experiments, twofold dilutions of the known concentration of the competitor MT-2A cDNA template were added to a constant amount of the mononuclear cell MT-2A cDNA in a PCR reaction volume and co-amplified using the RT-PCR protocol. Target MT-2A cDNA and competitor MT-2A cDNA strands would be competitive for being amplified and the competitor MT-2A cDNA (198 bp) was distinguished from the target MT-2A cDNA (278 bp) by the difference in size of 80 bp deletion, when being separated on an 8% polyacrylamide gel. The primers for human MT-2A cDNA amplification are as below:

(for MT-2A cDNA amplicaton)
Forward primer 5'-GCA ACC TGT CCC GAC TCT AG-3'

Reverse primer 5'-ATC CAG GTT TGT GGA AGT CG-3'

The concentration of MT-2A competitor cDNA that gave a 1:1 signal with the target MT cDNA was used to calculate the concentration of the MT-2A mRNA (5, 12). The amount of amplified PCR products of MT target cDNA was normalized by reference to β-actin which was also assayed by competitive-RT-PCR. The competitive standard for β-actin was synthesized by deletion of 151 bp (392 bp) compared to the normal β-actin PCR product (543 bp). The primers for β-actin cDNA amplification are as shown below:

(for β-actin cDNA amplicaton)
Forward primer 5'-ATC GTG GGG CGC CCC AGG CAC-3'
Reverse primer 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'

The co-amplified competitive-RT-PCR products were separated on an 8% polyacrylamide gel and stained with ethidium bromide, and photographed under UV light. Relative intensities of bands were determined by densitometric scanning. The amount of MT cDNA was determined as the point at which a 1:1 ratio in band density was obtained between the competitive mutant MT-2A cDNA band (198 bp) and the MT-2A cDNA band (278 bp).

Plasma and RBC zinc analysis

Zinc and other divalent cations (Fe, Cu, and Mn) in plasma and red blood cells (RBCs) were measured using inductively coupled plasma (ICP) emission spectroscopy (Boschstrasse 10 Spectro Analytical Instruments, Germany) after concentrated nitric acid wet-digestion and appropriate dilution.

The accuracy and precision of all analytical methods were checked by analyzing a standard reference material (1577b, bovine liver, National Institute of Standards and Technology, Gaithersburg, USA).

Statistics

Statistical analyses were performed with the statistical package SPSS. The mean difference between before and after zinc supplementation was analyzed using a paired Student's t-test.

RESULTS

Plasma and RBC zinc concentration

Plasma and RBC zinc concentrations before and after zinc supplementation are shown in Table 1. Mean plasma zinc concentration increased 2.3-fold after zinc supplementation (258.1 ± 78.7 μg/dL as mean ± SD) than before zinc supplementation (112.2 ± 22.6 μg/dL) (p < 0.05). The increment is within the range of 1.3-through 4.2-fold for each subject. RBC zinc concentration also showed an increase after zinc supplementation compared to before zinc supplementation, but without statistical significance (46.2 ± 19.3 μg/g protein vs 38.0 ± 9.8 μg/g protein), with the range of 1.1-through 1.6-fold for each subject. The increment of zinc concentration after zinc supplementation was higher in the plasma (2.3-fold) than in the RBCs (1.2-fold), which implies that plasma zinc was a more sensitive indicator of zinc status than was RBC zinc.

To determine whether other divalent trace elements are changed by zinc supplementation or not, concentrations of other trace elements (Cu, Mn and Fe) were measured and the results are shown in Table 2. Most of trace element concentrations were not affected by zinc supplementation except plasma Fe level, which increased almost 2.6-fold after zinc supplementation.

MT-2A competitive-RT-PCR

Separation of the target MT-2A cDNA (278 bp) and the competitive MT-2A cDNA (198 bp) products of the PCR was confirmed by polyacrylamide gel electrophoresis. Two clear cDNAs bands were consistent with the sizes expected and they compared with the DNA size markers.

Representative gel separation of MT-2A products following competitive RT-PCR is shown in Fig. 1-1 and 1-2. Fig. 1-1 shows the MT-2A competitive-RT-PCR result of before (a) and after (b) zinc supplementation.

Table 1. Plasma and RBC zinc concentrations of subjects before and after zinc supplementation[1,2]

Subjects	Plasma Zn (μg/dL)			RBC Zn (μg/g protein)		
	Before Zn-supple	After Zn-supple	Increment	Before Zn-supple	After Zn-supple	Increment
1	145.8	190.9	1.3	46.9	73.9	1.6
2	125.1	223.9	1.8	43.8	51.6	1.2
3	98.1	239.0	2.4	44.7	48.8	1.1
4	99.0	242.8	2.5	27.0	24.3	1.1
5	93.0	394.1	4.2	27.8	32.0	1.2
Mean ± SD	112.2 ± 22.6	258.1 ± 78.7*	2.3	38.0 ± 9.8	46.1 ± 19.3	1.2

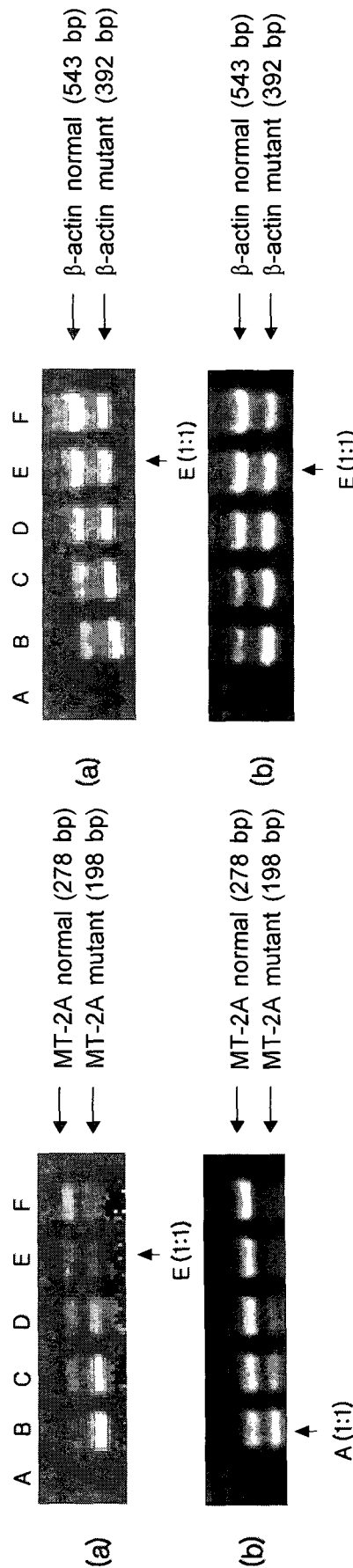[1]Zinc (50 mg Zn as zinc gluconate) was supplemented daily to the subjects for 14 days.
[2]Significantly (*p < 0.05) different compared to 'before zinc supplementation' by Student's paired t-test.

Table 2. Plasma and RBC Cu, Mn and Fe concentrations of subjects before and after zinc supplementation[1,2]

Subjects	Cu				Mn				Fe			
	Plasma (μg/dL)		RBC (μg/g protein)		Plasma (μg/dL)		RBC (μg/g protein)		Plasma (μg/dL)		RBC (μg/g protein)	
	Before Zn-supple	After Zn-supple	Before Zn-supple	After Zn-supple	Before Zn-supple	After Zn-supple	Before Zn-supple	After Zn-supple	Before Zn-supple	After Zn-supple	Before Zn-supple	After Zn-supple
1	31.1	45.8	1.83	2.69	3.6	3.2	0.41	0.46	36.9	49.5	1.20	1.11
2	23.6	34.8	1.75	1.73	3.8	3.4	0.31	0.30	16.4	58.0	1.05	1.34
3	19.0	23.0	1.68	2.01	3.1	3.9	0.28	0.28	14.5	55.0	1.52	1.16
4	25.9	25.5	1.83	1.50	3.6	3.4	0.24	0.20	21.4	66.7	1.14	1.18
5	20.5	25.5	1.64	1.97	3.1	4.2	0.22	0.27	9.8	34.3	1.38	1.07
Mean±SD	24.0±4.7	30.9±8.0	1.75±0.09	1.98±0.45	3.4±0.3	3.6±0.4	0.29±0.08	0.30±0.09	19.8±10.4	52.7±32.5**	1.26±0.19	1.17±0.10

[1] Zinc (50 mg Zn as zinc gluconate) was supplemented daily to the subjects for 14 days.
[2] Significantly (**$p < 0.01$) different compared to 'before zinc supplementation' by Student's paired t-test.

(a)

A B C D E F
E (1:1)
A (1:1)

↓ MT-2A normal (278 bp)
↓ MT-2A mutant (198 bp)

(a)

(b)

A B C D E F
E (1:1)

↓ MT-2A normal (278 bp)
↓ MT-2A mutant (198 bp)

(b)

Fig. 1-1. Metallothionein (MT-2A) competitive-reverse transcriptase-polymerase chain reaction (competitive-RT-PCR). (a) before zinc supplementation and (b) after zinc supplementation. MT-2A competitive mutant cDNA concentrations: lane A, DNA size marker; B, 2400 fg; C, 1200 fg; D, 600 fg; E, 300 fg; F, 150 fg. Monocyte total RNA (200 ng) was reverse transcribed. Two-fold dilutions of the competitive mutant MT-2A cDNA (198 bp) and a fixed quantity of sample mononuclear cell cDNA were amplified simultaneously by PCR. Competitive-RT-PCR product was separated on an 8% polyacrylamide gel and stained with ethidium bromide, and photographed under UV light. Relative intensities of bands were determined by densitometric scanning. The amount of MT-2A cDNA was determined as the point at which a 1:1 relationship occurred between the competitive mutant MT-2A cDNA band (198 bp) and the MT-2A cDNA band (278 bp); at this experiment, E (300 fg) for (a) before zinc supplementation, and A (2400 fg) for (b) after zinc supplementation.

A B C D E F
E (1:1)

↓ β-actin normal (543 bp)
↓ β-actin mutant (392 bp)

A B C D E F
E (1:1)

↓ β-actin normal (543 bp)
↓ β-actin mutant (392 bp)

Fig. 1-2. β-actin competitive-RT-PCR. (a) before zinc supplementation and (b) after zinc supplementation. β-actin competitive mutant cDNA concentrations: lane A, DNA size marker; B, 256 pg; C, 128 pg; D, 64 pg; E, 32 pg; F, 16 pg. Competitive-RT-PCR was analyzed the same as described in Fig. 1-1. The points at which a 1:1 relationship occurred between the competitive mutant β-actin cDNA band (392 bp) and the β-actin cDNA band (543 bp) are E, 32 pg for both (a) before and (b) after zinc supplementation.

Two-fold serial dilutions of the competitive mutant MT-2A cDNA (198 bp) (B; 2400 fg, C; 1200 fg, D; 600 fg, E; 300 fg, F; 150 fg) and a fixed quantity of sample mononuclear cell cDNA were simultaneously amplified by PCR. The amount of MT cDNA was determined as the point at which a 1:1 ratio in band density between the competitive mutant MT-2A cDNA band (198 bp) and the MT-2A cDNA band (278 bp) was obtained. ; at this experiment, E (300 fg MT-2A) in '(a) before zinc supplementation' and A (2400 fg MT-2A) in '(b) after zinc supplementation' (Fig. 1-1).

The β-actin competitive-RT-PCR was also analyzed in order to normalize the MT-2A competitive-RT-PCR cDNA data and a representative gel separation of β-actin competitive-RT-PCR from the same subject in Fig 1-1 is shown in Fig. 1-2. MT-2A competitive-RT-PCR cDNA products were normalized using β-actin competitive-RT-PCR product data (Fig. 1-2). As with the MT-2A competitve-RT-PCR, the β-actin competitive mutant cDNA was serially diluted with equal volumes of water to obtain 5 concentrations (B; 256 pg, 128 pg, D; 64 pg, E; 32 pg, F; 16 pg). The point at which a 1:1 ratio in band density occurred between the competitive mutant β-actin cDNA band (392 bp) and the β-actin cDNA band (543 bp) was E (32 pg β-actin) both '(a) before' and '(b) after zinc supplementation'. Thus, in this subject, MT-2A cDNA increased 2^3-fold after zinc supplementation; E (300 fg MT-2A) in '(a) before zinc supplementation' and A (2400 fg MT-2A) in '(b) after zinc supplementation', which gives 2^3-fold increment (Fig. 1-1). Since the competitive mutant MT-2A was diluted in a two-fold manner, the MT-2A concentration would be changed by an exponential factor of 2.

The mean MT-2A mRNA levels in mononuclear cells in five subjects before and after zinc (50 mg/d) supplementation were calculated and are shown in Table 2. Mononuclear cell MT-2A mRNA intensity increased after zinc supplementation in three out of five subjects; two-fold in subject 1 and four-fold in subject 2 & 3 increased. Subject 4 & 5 showed the same level of MT-2A mRNA intensity before and after zinc supplementation (Table 3). Thus, after normalizing with the calculated β-actin cDNA level, the mean relative MT-2A mRNA intensity increased in response to zinc supplementation (4.45 vs 11.25), even though this result was not significant (Fig. 2). The lack of significance for the comparison of mean values was due to the small sample size, however, the MT-2A competitive-RT-PCR results showed a consistent trend towards increased MT-2A mRNA levels in response to zinc supplementation.

DISCUSSION

There have been considerable efforts to develop meth-

Table 3. Relative MT-2A mRNA intensity in zinc supplemented subjects[1,2]

Subjects	MNC MT-2A mRNA (fg MT-2A mRNA/pg β-actin mRNA)	
	Before Zn-supple	After Zn-supple
1	2.34	4.69
2	1.17	4.69
3	9.38	37.5
4	4.69	4.69
5	4.69	4.69
Mean	4.45	11.25
SEM	1.41	6.56

[1] Values are mean ± SEM (n=5). MT, metallothionein; MNC, mononuclear cells.

[2] Relative MT-2A mRNA intensity in the mononuclear cells of subjects before and after zinc supplementation was calculated. The subjects were zinc supplemented for 14 days with 50 mg zinc as zinc gluconate daily.
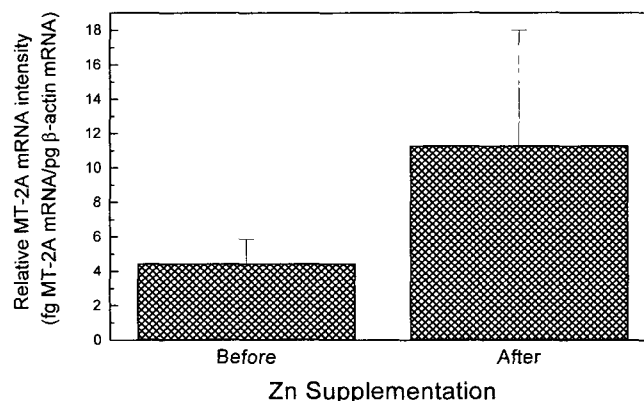
Fig. 2. Relative mononuclear cell MT-2A mRNA intensities in subjects before and after zinc supplementation. Values are mean ± SEM (n=5). The subjects were zinc supplemented with 50 mg Zinc as zinc gluconate for 14 days daily. Mononuclear cell MT-2A mRNA was quantified by competitive-RT-PCR assay. Student's paired t-test didn't show between before and after zinc supplementation.

ods of assessing zinc status more precisely and in a more convenient manner. However, so far, plasma/serum or erythrocytes zinc determination, which may not reflect total body zinc status, are the most commonly used markers for zinc status assessment. Functions of zinc, in general, can be categorized as catalytic (metalloenzymes), structural (such as zinc finger domains of proteins) and regulatory (such as metal response elements of gene promoters) (13,14). Metallothionein (MT) gene expression reflects zinc's regulatory function. Metallothionein (MT) has a high zinc-binding capacity and it is, indeed, one of the strongest biological binding ligands for zinc. MT gene expression is regulated by dietary zinc (15-17), and the close correlation between zinc and MT in zinc-sensitive organs, such as liver or pancreas, is well reported (18,19). MT is also regulated by some other metals or factors (20-22). Dietary zinc intake can be a major factor

determining the level of metallothionein gene expression (18,23,24) and from this knowledge comes the idea of MT would be a reliable marker for zinc assessment.

The MT competitive-RT-PCR assay which is described here has some advantages for overcoming some of the problems of zinc status assessment. Firstly, the MT competitive-RT-PCR can be applied to assess zinc status because very low levels of MT gene product can be measured in mononuclear cells using this technique. The concentration of MT in plasma appears to reflect changes in the metabolic zinc pool, since the plasma MT level is closely related to liver MT level. Thus, plasma MT level has been considered as a diagnostic indicator of zinc status. However, plasma MT levels are low and a diagnostic method based on the use of radioisotopes is not reliable and not convenient for measuring the precise zinc status in the body (5). Competitive-RT-PCR for MT mRNA can potentially be a useful technique to avoid the limitations of conventional MT protein assays. Secondly, the MT-2A competitive-RT-PCR assay is truly quantitative, unlike the semi-quantitative non-competitive PCR assay. Quantification of reverse-transcribed mRNA by non-competitive PCR is unreliable, because measurement of the PCR product may not be made over the linear phase of amplification. This problem can be avoided using competitive-RT-PCR, which involves the addition of a competitive standard cDNA (MT cDNA competitor) that competes with the target gene for PCR amplification. Also under this co-amplication of mutant competitor cDNA and target sample DNA, the competitor cDNA template is amplified within the same tube as the template of interest. Therefore, both two cDNA templates share the same primers and any variable influencing the amplification would affect both the competitor cDNA and the template of interest period. The competitor DNA can serve as an internal standard for PCR. Thirdly, the amount of target cDNA can be quantified from the amount of competitor template that has been added with a known concentration. It is usually possible to determine the target cDNA quantity, when a 1:1 ratio between the competitor cDNA and the target cDNA is achieved.

In the present study, plasma and RBC zinc increased ca 2- and 1.2-fold after zinc supplementation compared to before zinc supplementation (from 112.2 ± 22.6 to 258.1 ± 78.7 µg/mL and from 38.0 ± 9.8 to 46.1 ± 19.3 µg/g protein for plasma and RBCs, respectively), while mean mononuclear cell MT-2A mRNA tended to increase ca 4-fold after zinc supplementation. These results suggest that MT-2A competitive-RT-PCR to measure MT would be more sensitive tool for assessing zinc status compared to conventional zinc assessment tools.

However, apart from the advantages of MT competitive-RT-PCR described above, this assay needs to be developed further for convenience and flexibility.

ACKNOWLEDGMENTS

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