Differential Gene-Expression of Metallothionein 1M and 1G in Response to Zinc in Sertoli TM4 Cells

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ABSTRACT

Background: Zinc (Zn) as an important trace element is essential for testicular development and spermatogenesis. Molecular mechanism of Zn action in the reproductive system may be related to metal binding low-molecular weight proteins, metallothioneins (MT). Our objective was to determine the effect of Zn on two important isoforms of MT, MT1M and MT1G genes expression on testicular sertoli cells. **Methods:** Cultured sertoli TM4 cells were exposed to different concentrations of Zn at different time points. Cellular uptake of Zn was tested using flame atomic absorption spectrometry. The cellular viability and gene expression were assessed by MTT and real-time PCR methods, respectively. **Results:** The treated cells resulted in higher Zn concentration and cellular viability. The expression of MT1M and MT1G genes in the treated cells were greater than those of the untreated cells ($P<0.05$). In the high dosage treated group (100 and 500 μM), Zn concentration and expression of MT1M and MT1G genes increased three h after treatment; MT1G gene expression increased more at sixth h. At 18th h of treatment, the expression of both genes especially MT1G, increased dramatically while Zn concentration decreased. **Conclusion:** Since the increase of MT1G mRNA was coincident with cellular Zn level, it seems that MT1G has a more prominent role than MT1M in the homeostasis of Zn. In addition, Zn at dosage of 50 μM (pharmacologic concentration) may protect cells by increasing the expression of MT genes at longer periods. *Iran. Biomed. J. 14 (1 & 2): 9-15, 2010*

INTRODUCTION

Zinc (Zn) is an important trace element which plays a role in protein and nucleic acid synthesis and participates as a key regulator in all mammalian cells [1-3]. Zn ion has been shown to be essential for testicular development and spermatogenesis [1, 4]. In the male reproductive system, Zn ion facilitates the unique packaging of DNA in spermatocytes and participates in development and function of sperm [3]. Zn deficiency is known to result in oligoazospermia, impotency and hypogonadism in rats and men. Besides, some studies have demonstrated that Zn supplementation reduces astenoazospermia [4, 5].

The underlying molecular mechanism of Zn action in the reproductive system still remains unknown. It has been advocated that Zn and low molecular weight proteins, metallothioneins (MT), might play a role [6, 7]. MT proteins, rich in cysteine residues, are involved in metal homeostasis and scavenging free oxygen radicals [8-10]. By regulating Zn availability to the Zn finger region, this protein can regulate activation of certain transcription factors [11, 12].

On the structural basis, MT have been divided into different classes and their most conspicuous biological feature is their inducibility by a variety of agents and conditions [13, 14]. Between the MT proteins which can act as a reservoir and donor of

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Zn, the actions of two important isoforms, MT1M and MT1G, are very important [9, 15, 16]. In addition, because of the importance of Zn, induction of MT biosynthesis by this ion has been emphasized [17, 18].

Since male genital organs, particularly testis, are extremely susceptible to toxicants like cadmium (Cd), the presence of MT and their induction by Zn may protect spermatogenesis [6, 16, 19, 20]. Sertoli cells as supportive cells in seminiferous epithelium have a critical role in spermatogenesis. These cells can coordinate nutritional and structural support to germ cells [6]. This support could be critical in providing transcription and growth factor compounds which are Zn related [9, 21, 22]. Indeed, sertoli cells form a physiological barrier and limit exposure to potentially toxic substances [23]. In these cells, the induction of these genes by Zn has been debated [15, 24-27]. Thus, in the current study, we have analyzed the effect of different Zn concentrations on the expression of two major isoforms of MT, MT1M and MT1G genes, using different timing in mouse testicular sertoli cells.

MATERIALS AND METHODS

Cell culture and Zn treatment. Sertoli TM4 cells (ATCC number CRL-1715) were cultured as described by the ATCC protocol. Zn dilution was prepared from a 10 mM stock $ZnSO_4$ solution and Zn treatment was performed according to the Chang *et al.* [28] at physiologic (2 μM), pharmacologic (20, 50 and 100 μM) and toxic (500 μM) concentrations. The cultured cells were treated with the designated amount of Zn for 1, 2, 3, and 6 h, except the cells treated with 50 μM (pharmacologic dosage) for 18 h.

Cellular viability assay. Cellular viability in the presence or absence of Zn was determined using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-dimethyl tetrazolium bromide (Sigma, USA)] assay. Briefly, following Zn treatment, 100 μL of culture medium containing MTT solution was added to each well (10×10^5 cells), and the plate was incubated at 37ºC for two h. The medium was then removed and the colored reaction product was solubilized in 100 μL DMSO. Absorbance was measured at 570 nm using an ELISA reader (Anthos 2020, Austria) and the percentage of viability was calculated.

Zn determination. Both treated and untreated cells (10×10^5 cells) were washed twice with ice-cold PBS and then total Zn concentration was determined by flame atomic absorption spectrometry using a Spectra AA-220 (Varian, Australia).

RNA extraction and reverse transcriptase PCR (RT-PCR). The remaining washed cells (10×10^5 cells) were removed and centrifuged twice at 1600 ×g for 5 min. Total RNA was extracted from the cell pellet by the High Pure RNAgents Isolation kit (Promega, UK) as described by the manufacturer. Then, RNA was eluted with 20 μl RNase and DNase free distilled water and stored in aliquots at -80ºC. Purity of RNA was estimated by the absorbance ratio A260/A280 nm (The ratio for RNA was 1.8-2). RNA integrity was confirmed by ethidium bromide staining of ribosomal RNA following gel electrophoresis. Synthesis of cDNA was performed according to the previous report [29]. For primer designing, human sequences for MT1M and MT1G genes and their mouse homologues (Mt1 and Mt2 genes) were aligned. The identical sequences were selected for primer designation (Table 1) and beta actin gene was used as a housekeeping gene. The MT genes were amplified in a multiplex RT-PCR protocol in this way: initial heating at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 62.5°C for 30s, and 72°C for 20s. Amplified products were subjected to electrophoresis in 2% agarose gels and visualized with ethidium bromide (Fig. 1).

Quantitative real-time RT-PCR analysis. The real-time PCR was carried out with QuantiFast SYBR Green Kit (Roche Applied Science, UK) using 1 μl of cDNA (equivalent to 100 ng cDNA) in a 20 μl final volume and 0.4 μM of each primer (final concentration). Quantitative PCR was performed using a Corbett Rotor-Gene 3000 for 35 cycles at 95ºC for 10s and at 60ºC for 20s. Specificity of product for each separate sample was checked with the melting curve analysis and quantification was achieved with the comparative threshold cycle method.

Table 1. Sequences of the primers used in real-time PCR assays.

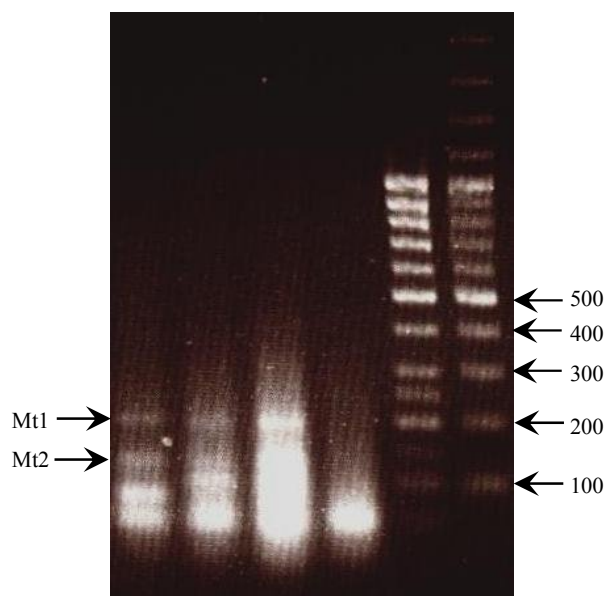| Gene name	Sequence	Product size (bp)
Metallothionein 1	ATGGACCCCAACTGCTCCTG TTCGTCACATCAGGCACAGC	197
Metallothionein 2	ACCCCAACTGCTCCTGTGCC CACTTCGCACAGCCCACGG	127
Beta actin	GCTATGCTCTCCCTCACGCCA CCACTGCCGCATCCTCTTCCT	162

Fig. 1. Gene expression of Mt1 and Mt2 genes in untreated testicular sertoli cells (left side), 100 bp ladder (right side).

Statistical analysis. For statistical analysis, Kruskal-Wallis test, One-Way ANOVA, Mann-Whitney U test and independent t-test were performed. A *P* value of 0.05 was considered for significant difference.

Microscopic observations. Under an inverted microscope (Euromex, Holland), cells treated with physiologic and pharmacologic dosage of Zn did not show any significant morphological changes indicative toxicity. However, cells treated with 500 μM of Zn revealed a transformed round shape cellular morphology (Fig. 2).

RESULTS

Determination of Zn uptake in sertoli cells. The mean of Zn concentration in treated cells was 0.16 ± 0.14 ppm in comparison with 0.08 ± 0.01 ppm in untreated cells ($P<0.05$). For evaluating Zn uptake at different dosages of treatment, we calculated the percentage of concentration. As shown in Figure 3A, Zn concentration inside the treated cells increased by accretion of treatment dosage, although the increase was subtle in cells treated with 2, 20, and 50 μM of Zn. Three and six h after treatment with 100 μM, Zn concentration was about double. At toxic dosage treated cells (500 μM), it increased much more, particularly six h after treatment. According to Figure 3B, in the cells treated with 50 μM

(pharmacologic concentration), Zn level increased by six h of treatment, then at the 18th h, it decreased and reached at its initial value ($P<0.05$).

Effect of Zn treatment on viability of sertoli TM4 cells. The mean value of cellular viability was significantly ($P<0.05$) higher ($158 \pm 52\%$) compared to untreated cells. As seen in Figure 4, at sixth h of treatment, the cellular viability was high for those cells treated with 2, 20 and 50 μM, but it was apparently dropped by increasing dosage (up to 100 μM) and reached near the values of control cells at toxic (500 μM) treatment dosage ($P<0.05$).

Effects of Zn on the MT genes expression in sertoli cells. Relative MT genes expression in the Zn-treated cells (pharmacologic and toxic dosage) was approximately doubled compared to the untreated cells ($P<0.05$). The mean value of relative MT1M gene expression in the treated cells was 1.06 ± 0.75 compared to the untreated cells (0.60 ± 0.33). For MT1G gene expression, this value was 1.79 ± 1.44 compared to the untreated cells (0.77 ± 0.4). As shown in Figure 5A, we considered percentage of relative expression for MT1M and MT1G genes at
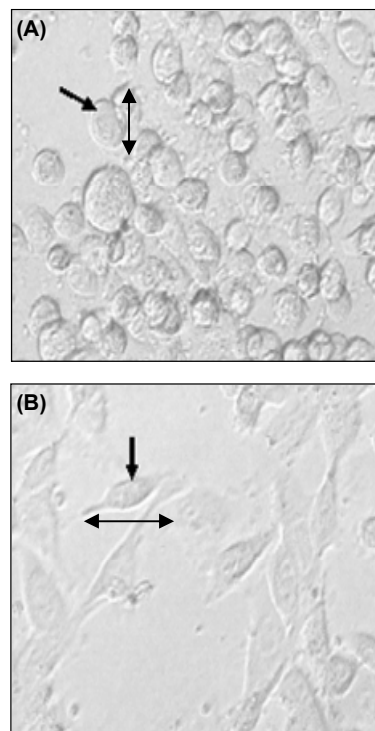

Fig. 2. Toxic dosage of Zn-treated **(A)** and untreated cells **(B)**. The cultured cells six h after treatment with toxic concentration of Zn (500 μM) resulted in a circular morphology (A) compared to the conical shape of untreated cells (B).

different dosages of treatment. There was no obvious difference in the levels of mRNA with low dosage treatment group (2, 20 anf 50 μM) but they increased three h after high dosage (100, 500μM) treatment. After that, at the sixth h of treatment in the cells treated with 100 μM, expression of both genes increased dramatically and in the cells treated with 500 μM, gene expression of MT1M diminished, but in the case of MT1G, it increased. The effect of time on the expression of MT1M and MT1G genes in the cells treated with 50 μM (pharmacologic concentration) revealed that expression of both genes enhanced by increasing duration of treatment (Fig. 5B). The amount of increase in the expression of both genes was significantly high after 18 h post treatment compared to the untreated cells or the first h of treatment (i.e. up to six h) ($P<0.05$).
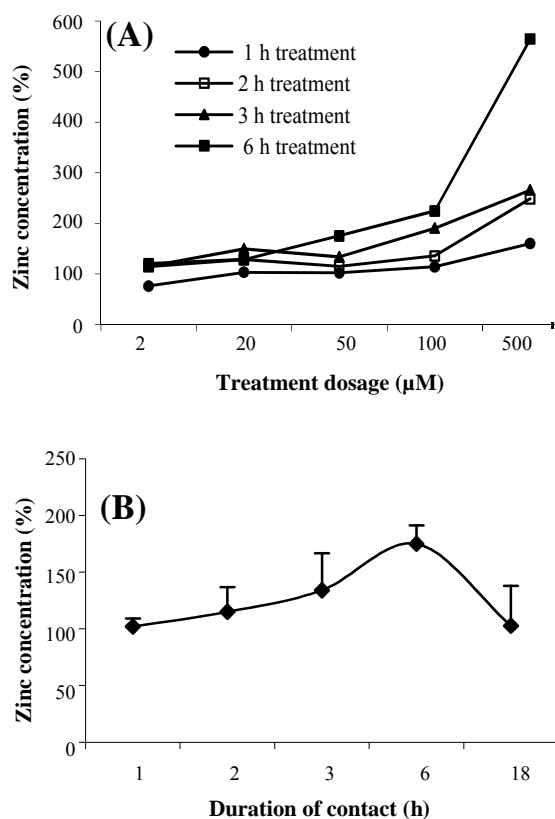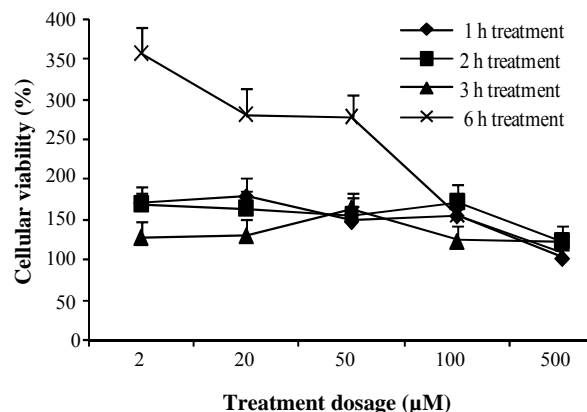

Fig. 4. Zn concentration impacts on viability of sertoli TM4 cells. MTT assay was done for assessing cellular viability when exposing to different concentrations of Zn (2, 20, 50, 100 and 500 μM) for different periods (1, 2, 3 and 6 h). Data are mean ± SE.

DISCUSSION

In this report, we showed that Zn could induce MT genes expression in sertoli cells and also by increasing the Zn dosage, the levels of expression increased. Of course, the induction of MT genes in sertoli cells has been a matter of some controversy, since a number of reports indicated that Zn and Cd caused induction of these genes in sertoli cells, while others showed that these elements could not induce MT genes in sertoli cells whereas they caused their induction in hepatic and ovarian cells [15, 24-27].

The increase in MT genes expression was especially important in the cells exposed to pharmacologic concentration of Zn (50 μM) after long periods of treatment (18 h). Cellular viability and Zn concentration in these cells were about the level of untreated cells. These findings implicate that pharmacologic dosage of Zn can make cells more resistant to subsequent toxicity by an increase in MT genes expression.

Notably, our results showed the degree of accretion was time and dose dependent which was in agreement with the results of Ren *et al*. [6] as they found similar results on the Cd effect on MT genes [6], thus raising speculation on the similarity of effects of Zn and Cd in testis. Cd causes many problems like infertility and tumors in the testis.

On the other hand, Zn is known to provide protective action against the toxic effects of Cd [16, 30]. Although the precise mechanism of its action is unknown, the above explanations bring to mind that the interaction of these two elements on induction of

Fig. 3. Cellular uptake of Zn at different dosage of treatment **(A)** and different time **(B)** in sertoli TM4 cells. Cells exposed to different concentrations of Zn (2, 20, 50, 100 and 500 μM) for different periods (1, 2, 3 and 6 h). Intracellular Zn concentration was determined by atomic absorption spectrometry in different dosage of treatment (A) and it was determined in the cells exposed to pharmacologic concentration of Zn (50 μM) in the longer period (18 h); (B) data are mean ± SE.
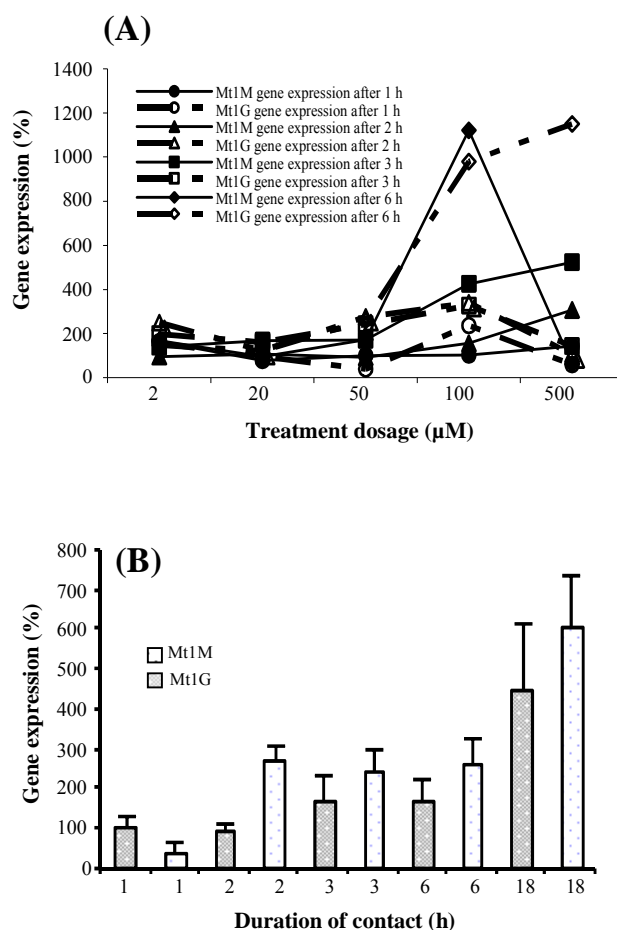
(A)

(B)

Fig. 5. Expression of MT1M and MT1G genes in different dosage of treatments **(A)** and different time **(B)**. Gene expression of cells exposing to different concentrations of Zn (2, 20, 50, 100 and 500 μM) at different periods (1, 2, 3, 6 and 18 h) was determined by real-time PCR method (A). At longer period (18 h), only pharmacologic concentration of Zn (50 μM) was added to the cells and their expression was assessed after 1, 2, 3, 6 and 18 h (B).

MT genes may be the basic mechanism of Zn protection against Cd toxicity [26].

Since in the toxic dosage treated cells, the increase of MT1G mRNA was coincident with cellular Zn level, MT1G might have a more prominent role than MT1M in the homeostasis of Zn. Meanwhile, intensive increasing in MT1G gene expression in the cells treated with 500 μM and simultaneous increase in Zn level could be due to the failure of this mechanism to defeat toxic Zn concentration, which caused a decrease in cellular viability. In this dosage, decrease in the expression of MT1M gene showed that toxic dosage might cease this gene expression.

Data from MTT examination also indicated that cellular viability in the toxic dosage-treated group decreased to basal level after six h of treatment, but other dosages of Zn treatment did not reduce it. Apparently, lack of reduction in cellular viability of non-toxic Zn-treated cells was due to regulation of Zn availability by homeostatic mechanism like MT.

In the presence of toxic levels of Zn in cultured medium, shapes of sertoli cells became round. In fact at this level of Zn, intercellular junctions became loose as indicated by Ren *et al.* [6] with low concentration of Cd. Probably, a toxic dosage of Zn like Cd disrupted the inter sertoli tight junctions. Nevertheless, in humans, because of the existence of a large number of proteins and transporters for Zn homeostasis, toxic doses are very rare and may be achieved only under the most unusual circumstances [31, 32]. These transporters may also affect the rate of Zn transmission in the blood-testis barrier [33].

We also found that MT1M and MT1G mRNA are present in the sertoli cells at the basal level as confirmed by others in relation to Mt1 and Mt2 [6, 27]. Of course, according to the some studies, although testis from sexually mature adults contain high levels of Mt1 and Mt2 mRNA, their localization in different population of cells varies [16, 27, 34]. A number of studies showed that MT mRNA is present in the sertoli cells, while others reported that there were little or no levels of MT RNA in these cells [25, 27, 35, 36].

However, we believe that for an exact examination and for evaluation of the effects of Zn at different dosages especially in pharmacologic dosage, a more comprehensive study over a longer period is needed.

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