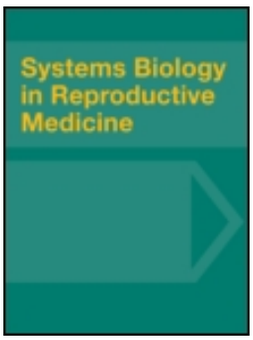

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Research Communication

Expression of Metallothionein-1 (MT-1) mRNA in the Rat Testes and Liver After Cadmium Injection

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Metallothioneins (MTs) belong to the family of stress proteins that are present in the majority of living organisms. The MTs play an important task in detoxifying heavy metals. The mammalian scrotal testis is known to be susceptible to cadmium (Cd) exposure. The present work focuses on the MT-1 isoform and aims to ascertain and confirm previous findings to answer whether rodent testes indeed contain MT-1 mRNA, whether its level is increased with Cd injection in liver and testes, and lastly what is the relative difference in the expression of MT-1 mRNA in liver and testes both with and without Cd injection. Adult male Wistar rats weighing 270–290 g received a subcutaneous injection of 4.0 µmol Cd/kg and were sacrificed by cervical dislocation 6 h later. RNA was isolated from testes as well as the liver. There were 2 replicates per treatment for RNA analyses. MT-1 mRNA levels were determined by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis and then assessed by densitometry scanning. The results of RT-PCR clearly demonstrated that the rodent testes express MT-1 mRNA. The densitometry data shows that the expression of MT-1 mRNA increases with Cd treatment in testes. The relative level of MT1-mRNA is greater in the control-liver than in the control-testes. However, upon Cd injection, the level of testes MT-1 mRNA increases 2.16 fold. These results suggest that the testes respond to Cd for at least 6 h post injection through a transcriptional mechanism.

KEYWORDS cadmium, liver, metallothionein, mRNA, testes

Abbreviations: MT: metallothionein; Cd: cadmium; RT-PCR: reverse transcriptase polymerase chain reaction; DEPC: diethylpyrocarbonate; EDTA: ethylenediaminetetraacetic acid; dNTP: deoxynucleotide triphosphate; oligo dT: oligodeoxythymidylic acid; cDNA: complementary deoxyribonucleic acid; bp: base pair.

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INTRODUCTION

Toxic metals (lead, cadmium, mercury, arsenic, etc.) are widely found in our environment. We are exposed to these metals from numerous contaminated sources. These heavy metals can accumulate in different body parts, including the reproductive organs, to exert their influence on structure and physiology. However, certain defense mechanisms to nullify the

effect of these heavy metals are present. One of the mechanisms by which heavy metals are prevented from exerting their toxic effect is by production of stress proteins.

Metallothioneins (MTs) belong to the family of stress proteins that are present in the majority of living organisms. The MTs play an important role in detoxifying heavy metals by sequestration of the metal as cysteine bound complexes [Suzuki et al. 1998]. However, the mammalian scrotal testis is known to be vulnerable to cadmium (Cd) toxicity. Nominal amounts of this metal can cause histological damage of the testis [Samarawickrama 1979] and Cd can exert a tumorigenic effect [Waalkes and Rehm 1992].

It has been a long-standing controversial issue as to whether or not the male gonads, testis, contain metallothioneins (MTs) [Coogan et al. 1995].

Earlier studies reported that the rodent testis lacks MTs and concluded that this is why the testis is very susceptible to Cd, although other indirect experimental evidence suggests that MTs are present in this organ [McKenna et al. 1996].

There are several functional variants of the MT gene including the primary forms of MT-1, MT-2, and MT-3. The present work was designed to address several issues including whether rodent testes contain MT-1 mRNA, whether its level is increased with Cd injection in liver and testes, and lastly what is the relative difference in the expression of MT-1 mRNA in liver and testes both with and without Cd injection. In part, this work tries to resolve previous studies on metallothionein expression and Cd treatment in rats. Suzuki et al. [1998] isolated metallothionein isoforms MT-1 and MT-2 from rat testes and thus tried to "resolve the long-standing debate" about the presence of MTs in testes. Lee et al. [1999] studied the effects of cadmium on MT-1 and MT-2 mRNA expression in rat ventral, lateral, and dorsal prostate lobes. They showed that Cd treatment induced higher levels of MT-1 and/or MT-2 mRNA in all tissues studied with the exception of lateral lobes of the prostate.

The present study compares two different organs, the liver and the testes, to discern how stress-combating mechanisms respond to heavy-metals. In part this reveals how environmental contaminants interfere with male reproductive function [De Celis et al. 1996] and whether the reproductive system is indeed hyper-susceptible to heavy metal toxicity.

RESULTS AND DISCUSSION

The results of RT-PCR clearly demonstrated that the rodent testes contain a functional MT-1 gene (Fig. 1). The densitometry data show that the expression of MT-1 mRNA increases with Cd treatment in testes (Table 1). Although the relative level of MT-1 mRNA is greater in the control-liver as compared to the control-testes, the testes was responsive to the injection of Cd showing a 2.16 fold increase as compared to liver which essentially remained constant (1.08 times the control value). The *p*-value was significant (0.041) for the differences in the densitometry value in the control and injected testes but not significant (0.325) when compared between the control and injected liver (Table 1) indicative of the overlapping values. This suggests that the testis is responsive to Cd injection at least for 6 h post-injection.

MTs play an essential part in the detoxification of heavy metals typified by Cd. However, there has been a debate whether MT-mRNAs are present in the testes [Tohyama et al. 1993] because of the toxicity which Cd can induce in this organ. Our observation

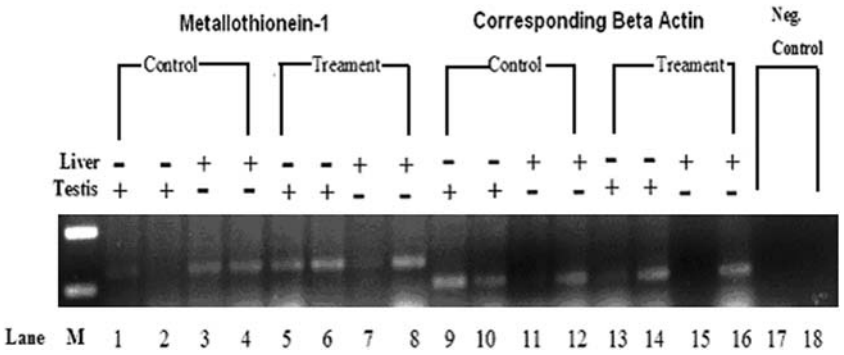

FIGURE 1 **Agarose Gel of PCR products after c-DNA synthesis.**

TABLE 1 **Relative levels of the MT1 and β-ACTIN PCR Products. The density of the electrophorectically resolved RT-PCR products of MT1 and β-ACTIN were determined as the mean. Their relative level was calculated as the ratio of MT1/β-ACTIN. The relative level of MT-1 mRNA before and after cadmium injection in liver and testes including *p*-values are presented.**

P-value (control vs. injected testes)	0.041				
P-value (control vs. injected liver)	0.325	Control (Means ± SD of replicates of actual intermediate-density: MT1/β-ACTIN)	Treated (Means ± SD of replicates of actual intermediate-density: MT1/β-ACTIN)	Increment	Fold increase
Testes		0.655 ± 0.249	1.42 ± 0.214	0.767	2.16
Liver		1.159 ± 0.183	1.26 ± 0.197	0.101	1.08

demonstrates that MT-1 mRNA is expressed in rat testes and liver under normal physiological conditions, which is in accordance with other studies performed in the whole testes of rats and mice [Shiraishi and Waalkes 1994; Shiraishi et al. 1993a,b]. It appears that the relative level of MT-1 mRNA is greater in control-liver than in control-testes. However, after 6 h Cd post-injection the testes continues to show an increased level of MT-1 mRNA compared to that of the liver. The increment of MT-1 mRNA in testes due to Cd is also substantiated in other studies [McKenna et al. 1996; Ren et al. 2003; Abel et al. 1991]. However, there are also reports that indicate a decrease and little or no difference of MT-1 mRNA levels in the whole testes of Cd-injected rats or mice [Shiraishi and Waalkes 1994; Zhou et al. 1999]. This discrepancy may reflect that MT-1 mRNA expression is time and dose dependent. In this regard it should be mentioned that this study does not attempt to establish the comparative susceptibility to Cd. Ren et al. [2003] showed that rat liver MT-1 mRNA peaked in the liver after 3 h followed by a decline whereas in the testicular interstitial MT-1 mRNA peaked at 6 h. This is in accord with the data presented in the above showing a relatively higher level of MT-1 mRNA in testes (2.16 times) after 6 h. The marked increase in the level of MT-1 mRNA in testes after Cd injection suggests that this organ is highly responsive to Cd-toxicity. However, earlier studies indicate that this is not the case since Cd-induced MT mRNA in testes is not followed by an increase in MT synthesis [Ren et al. 2003]. Hypermethylation of the rat testes MT-gene is consistent with this notion [Bhave et al. 1988]. However, regulation at the translational level whether synthesis or degradation of MT should be considered. Vasconcelos et al. [2002], have shown that the levels in liver and testes can also be markedly different through this mechanism [Vasconcelos et al. 1996] perhaps rendering the testes more susceptible to Cd toxicity than liver.

Our observations clearly demonstrate the existence of MT-1 mRNA in rat testes. The testis shows an increased level of MT-1 mRNA as part of a stress response to Cd injection.

This level of production of MT-1 mRNA is greater in testes than in liver as observed after 6 h post Cd injection. A more thorough understanding of the mode of action and expression of stress proteins both at the transcriptional, post-transcriptional, and translational levels is required.

MATERIALS AND METHODS

The study has been approved by University of Calcutta ethical committee.

Animals and Treatment Procedure

Adult male Wistar rats, weighing 270–290 g, raised under similar conditions were obtained from Ghosh Enterprise, Calcutta. Two received a sub-cutaneous injection of 4.0 μmol Cd/kg. Two control animals were treated under similar conditions receiving a single s.c. injection of saline. All of them were sacrificed by cervical dislocation 6 h later.

RNA Isolation

Immediately after sacrificing the rats, the testes and liver were removed, rinsed once with Diethylpyrocarbonate (DEPC) water and a small piece of tissue was kept in 1 ml TRIzol reagent for 15 min. The tissues were minced in TRIzol reagent forming a paste to which 200 μl of chloroform was added and mixed gently. The mixture was centrifuged at

13,000 rpm for 15 min at 4°C. The upper aqueous layer was carefully removed, avoiding the middle layer containing tissue debris, and placed into a 1.5 mL tube. An equal amount of chilled isopropanol was then added and gently mixed and kept in −80°C freezer for 1–2 days. After precipitation the vial was centrifuged at 13,000 rpm for 20 min at 4°C and the supernatant removed. The pellet was washed twice with 500–1000 µl of 70% DEPC alcohol, centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was discarded and dried under vacuum. The pellet was then dissolved in 15–20 uL of DEPC treated water and a quick centrifugation was performed. The pellet was heated to 65°C for 10–15 min until completely dissolved then centrifuged. A total of 8 µl of RNA solution was then placed in a 0.5 mL centrifuge tube and 1 µL (5 units) of DNase (TAKARA BIO INC., Shiga, Japan) and 1 µL of 10X DNase reaction buffer were added to a final concentration of 40 mM of Tris-HCl, pH7.5, 8 mM of $MgCl_2$ and 5 mM of DTT. It was kept at room temperature for 40 min. Subsequently 1 µL of stop solution (EDTA) was added to inactivate DNase activity. The solution was then heated at 65°C for 15 min and quickly transferred to ice.

cDNA Synthesis

A master mix was made by combining 1 µL of dNTP (10 mM conc.), 0.8 µL oligo dT (0.5 µg/µL), and 7.2 µLof DEPC for each tube. To 9 µL of this master mix 4 µL of DNase treated RNA was added. It was heat-chilled by increasing the temperature to 65°C for 5 min then transferred to ice. Following a short centrifugation, 6 µL of 5X Buffer (to a final concentration of 25 mM Tris-HCl (pH 8.3), 37.5 mM KCl, 1.5 mM $MgCl_2$) and a vial of 10 mM DTT in addition to 0.3 µl Superscript II Reverse transcriptase (Invitrogen™, Carlsbad, CA, USA) were added to each tube. Reverse transcription was then carried out with PCR programming: 37°C −2 min, 37°C—50 min and 70°C—10 min and 4°C—forever. The cDNA was kept at −20°C for future use.

PCR of cDNA Products

Rat MT-1 sense primer sequence was 5′ ACTGC-CTTCTTGTCGCTTA-3′ and the antisense primer sequence was 5′-TGGAGGTGTACGGCAAGACT-3′. This targeted a 310 base pair (bp) fragment. Sense and antisense primer sequences for the control β-actin were 5′-CCCATTGAACACGG-CATTG-3′ and 5′-GGTACGACCAGAGGCATACA-3′, respectively, which spanned a 236 bp fragment [Ren et al. 2003]. A 25 µL reaction mixture was prepared containing 0.2 mM of each dNTP, 1 pmol of each sense and antisense primer and 0.625 unit of Taq DNA polymerase (GE Healthcare, Chalfront St. Giles, UK), together with 4 µL of cDNA. The PCR cycle was carried out as follows: initial denaturation at 95°C for 5 min, amplification for 27 cycles which included: denaturation for 1 min at 95°C, annealing for 1 min at 55°C, extension for 1 min at 72°C and a final entension at 72°C for 5 min. Agarose gel electrophoresis was carried out with the PCR products using a 2.0% (w/v) agarose gel, stained with ethidium bromide and visualized under UV light. Parallel β-actin RT-PCR results served as an internal control needed to calculate the actual intermediate density of MT-1 mRNA.

Densitometric Scanning

After RT-PCR was carried out, the levels of MT-1 mRNA in testes and liver tissue with and without Cd injection were determined by scanning densitometry using gel doc Image J (NIH v1.37). The actual intermediate density of mean of replicates of MT-1 mRNA was calculated by obtaining the ratio of MT-1/ β-actin.

Statistical Analysis

For statistical analysis Microsoft Excel (Microsoft office XP) was used. Differences in the levels of MT-1 mRNA between control and injected testes as well as control and injected liver were evaluated by Student's *t*-test (*P*-value <0.05 was considered significant).

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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