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# Diabetes Mellitus induces alterations in metallothionein protein expression and metal levels in the testis and liver

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## Abstract

**Objective:** To investigate the effect of diabetes with and without vitamin E treatment on testicular metallothionein (MT) and metal (zinc, copper and iron) changes.

**Methods:** Diabetes was induced with a single intraperitoneal injection (i.p.) of streptozotocin in rats, and diabetic rats were given Vitamin E by i.p. every other day for 4 weeks. MT protein was measured by the cadmium-haeme assay and metal levels were detected by an atomic absorption spectrophotometer.

**Results:** Diabetes did not change testicular MT protein, but significantly increased hepatic MT protein. Diabetes significantly decreased testicular copper, but not hepatic copper. Zinc and iron levels were unchanged in both diabetic testis and liver. Vitamin E significantly enhanced both testicular and hepatic MT, and zinc levels in diabetic rats. Vitamin E slightly decreased the copper levels, but did not change the testicular and hepatic iron in diabetic rats.

**Conclusions:** Testicular MT protein expression was not increased, even though hepatic MT significantly increased independent of metal changes, in diabetic rats. Vitamin E enhanced testicular and hepatic MT, which correlated with increased zinc levels.

## Keywords

Antioxidant, diabetic testis, metallothionein, zinc, copper, iron

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## Introduction

Metallothioneins (MTs) are a group of low molecular weight (6000–7000 Daltons), cysteine-rich (30%) intracellular proteins with high affinity for heavy metals. In mammalian tissues, four major MT isoforms have

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been identified, of which MT-1 and MT-2 are the two major isoforms. MTs are implicated in several important physiological roles including detoxification of potentially toxic metals such as cadmium, and regulation of essential metals, including zinc (Zn) and copper (Cu).[1] MT is constitutively expressed in various species and tissues, including testis, and is considered as one of the stress-response proteins.[2] In the liver, MT protein is induced readily by many stress-associated challenges such as glucocorticoids, bacterial lipopolysaccharide, interferon, alkylating agents and irradiation.[3] These challenges produce reactive oxygen species (ROS) or radicals, which damage tissues and cells, and elicit acute or chronic inflammation. Therefore, many studies have focused on the roles of MTs in metal homeostasis and the molecular mechanisms that control their expression in a variety of diseases such as cancer,[4,5] certain neuronal diseases[6] and diabetes mellitus (diabetes).[7]

Vitamin E is a lipid-soluble antioxidant that includes four tocotrienols and four tocopherols. Among these vitamin E subtypes, α-tocopherol is the most well known for its antioxidant activity.[8] Vitamin E has a particular function of scavenging peroxyl radicals to prevent lipid peroxidation, hence it plays an important role as an antioxidant in several pathological conditions, including cancer, neurodegenerative disorders, cardiovascular diseases and diabetes.[9,10]

Diabetes is a metabolic disorder characterized by hyperglycaemia due to insufficient insulin secretion or insulin resistance. Hyperglycaemia generates ROS, which plays critical roles in the development of diabetic complications.[11] Male sexual and reproductive functions are impaired in diabetic patients and animals.[12] Diabetes has great influence on the fertility of men both directly and indirectly.[13] It has been observed that there are many indirect influences such as low testosterone levels, testicular dysfunction and spermatogenic disruption in the testis of diabetic men and experimental animals induced by diabetes, which lead to erectile dysfunction, and reduced sperm motility and semen volume. Enhanced oxidative stress and changes in antioxidant capacity are considered to play an important role in the pathogenesis of diabetic testis damage.[14]

MT as a potent antioxidant and adaptive protein protects cells and tissues from oxidative stress. The preventive effect of antioxidants including MT and its inducer zinc[15–19] has been extensively reported. Among these studies, zinc deficiency caused by treating diabetic mice with a zinc chelator and zinc supplementation showed exacerbation and protection, respectively, at certain levels from diabetes-induced testicular damage. As zinc is a well-known MT inducer, MT expression has not been examined in most of these studies.[15,17–19] In addition, there is not much information regarding the effect of diabetes or diabetes with antioxidants on the zinc and MT levels. Therefore, in the present study, we investigated the MT protein expression changes and metal levels in the testis, and investigated the effect of vitamin E administration on these alterations in diabetic rats.

## Materials and methods

### *Animals and treatments*

All animal studies were carried out in accordance with the protocols from the Animal Care Committee of the First Hospital of Jilin University, and were reviewed by the Ethics Committee of the First Hospital of Jilin University (No. 2014-066). Male Sprague-Dawley rats (about 200 g) were purchased from Charles River (Beijing, China) and were fed regular rat chow with water *ad libitum*. First, the rats were randomly divided into two groups: non-diabetic control rats (Ctrl; $n = 10$), and rats induced with type 1 diabetes by a single intraperitoneal injection (i.p.) of

streptozotocin (STZ, 65 mg/kg body weight, dissolved in freshly prepared citrate buffer (Sigma, St. Louis, MO)). The control animals received the same amount of citrate buffer intraperitoneally. Whole blood glucose was tested from the tail vein using a SureStep complete blood glucose monitor (LifeScan, Milpitas, CA) on day 5 after STZ injection. Rats with blood glucose level $\geq$250 mg/dL were considered diabetic. Then, the diabetic rats were randomized into two groups: one group (n = 10) was diabetic rats without other treatment (DM); the other group (n = 10) was given Vitamin E (α-tocopherol, 40 mg/kg) by i.p. every other day for 4 weeks (DM/VE). Rats were sacrificed 30 days after diabetes induction with or without Vitamin E.

Rats were sacrificed by cardiac puncture and the blood was collected for the measurement of glycated haemoglobin (HbA1c) (Glycotest$^{TM}$, Pierce, Rockford, IL). Testis and liver were removed for the measurement of MT protein and the levels of metals including the MT-binding metals, Zn and Cu, and the non-MT-binding metal, iron (Fe). Liver as the positive control was selected because it is the commonly chosen tissue for studies on the MT response to various stresses and it has been investigated in diabetic models[20,21].

### *Measurement of MT proteins by the Cd-haeme assay*

MT levels in the testis and liver were determined according to the method described in the Cd-haemoglobin assay.[22] In short, testis or liver tissues were homogenized in 0.25 M sucrose and were centrifuged at 20,000 rpm for 20 min. An aliquot of the supernatant was diluted with 30 mM Tris-HCl buffer (pH 8.0) and was incubated with 10 ppm $^{109}$Cd solution (final concentration) to saturate the MT metal-binding sites. Excessive Cd was removed by heat treatment in a boiling water bath in the presence of rat haemolysate for 10 min. This method leads to the precipitation of Cd-haemoglobin and other Cd-bound proteins, except for MT, which is heat stable. The denatured proteins were removed by centrifugation at 10,000 rpm for 2 min. The above steps were repeated three times. The Cd concentration in the final supernatant was calculated by the radioactivity of $^{109}$Cd measured by a gamma counter (1272 Clinigamma, LKB Wallac, Turku, Finland). The MT concentration was converted on the basis of 7 g-atoms of Cd/MT and expressed as μg/g wet tissue.

### *Measurement of metal levels*

The concentrations of Zn, Cu and Fe in the testis and liver were determined using an atomic absorption spectrophotometry method (Varian Spectra-AA 30, Georgetown, Ontario). Pre-weighed testis samples were digested in nitric acid at room temperature overnight, and then heated to 95°C for 1 h to facilitate complete digestion. The resulting clear liquid was used for analysis. The levels of Zn, Cu and Fe are expressed as μg/g wet tissue.

### *Statistical analysis*

Data are presented as mean ± SE (n = 10). Comparisons between groups were performed by one-way ANOVA. Post hoc pairwise repetitive comparisons were carried out using Tukey's test (Origin 8.0 laboratory data analysis and graphing software, Origin Lab Corp. Northampton, MA). Statistical significance was considered when $P < 0.05$.

## Results

### *Alteration in MT protein in the testis and liver of diabetic rats*

To determine the MT protein expression changes in diabetes, we established rat

diabetes using STZ. Diabetic rats with or without vitamin E treatment showed hyperglycaemia compared with Ctrl rats (Table 1). As shown in Figure 1, the basal level of MT protein was significantly higher (more than 2-fold) in the testis than in the liver. No significant change was detected in the testicular MT level in the DM group compared with that in the Ctrl group. However, diabetes (DM group) significantly increased the hepatic MT protein expression compared with the Ctrl group. These results suggest that the diabetes-induced MT expression is organ-specific in the early stage of diabetes in rats.

**Table 1.** Blood glucose data.

| | Ctrl | DM | DM/VE |
|---|---|---|---|
| Blood glucose (mg/dL) | $103 \pm 2.21$ | $388 \pm 3.17$* | $378 \pm 2.02$*# |

Blood glucose in Ctrl, DM and DM/VE rats on day 5 after STZ injection. Data are presented as mean ± SE.
*, $P < 0.05$ vs. Ctrl; #, $P = 0.233$ vs. DM.
Ctrl: non-diabetic control; DM: diabetic; DM/VE: diabetic rats treated with vitamin E.

### *Alteration in metals in the testis and liver of diabetic rats*

To determine whether the MT protein expression in diabetic rats is associated with metal levels, we measured the concentrations of the MT-binding metals, Zn and Cu, and the non-MT-binding metal, Fe. As shown in Figure 2, no significant change was detected in the testicular or hepatic Zn levels in the DM group compared with the Ctrl group. However, we found a dramatically reduced testicular Cu level, but no change in the hepatic Cu level in the DM group compared with the Ctrl group (Figure 3). In addition, the Fe level in the testis and liver slightly decreased in the DM group compared with the Ctrl group (Figure 4). These results suggest that the induction of hepatic MT in the diabetic rats may not depend on metals.

### *Effect of vitamin E on the alterations in MT and metals in the testis and liver of diabetic rats*

To determine whether vitamin E affects MT protein expression and metal concentration in diabetic rats, we treated diabetic rats with vitamin E. Vitamin E significantly enhanced

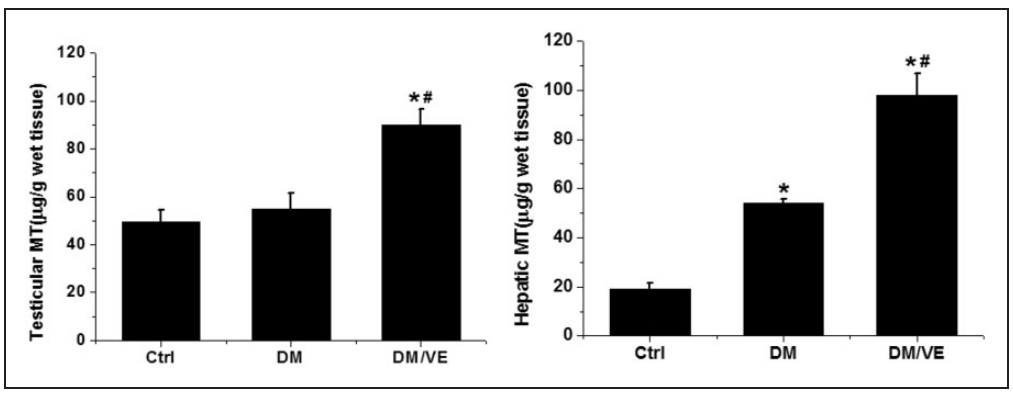


**Figure 1.** MT protein levels in the testis and liver of Ctrl, DM and DM/VE rats at 30 days after diabetes induction. Data are presented as mean ± SE. *, $P < 0.05$ vs. Ctrl; #, $P < 0.05$ vs. DM.
Ctrl: non-diabetic control; DM: diabetic; DM/VE: diabetic rats treated with vitamin E.

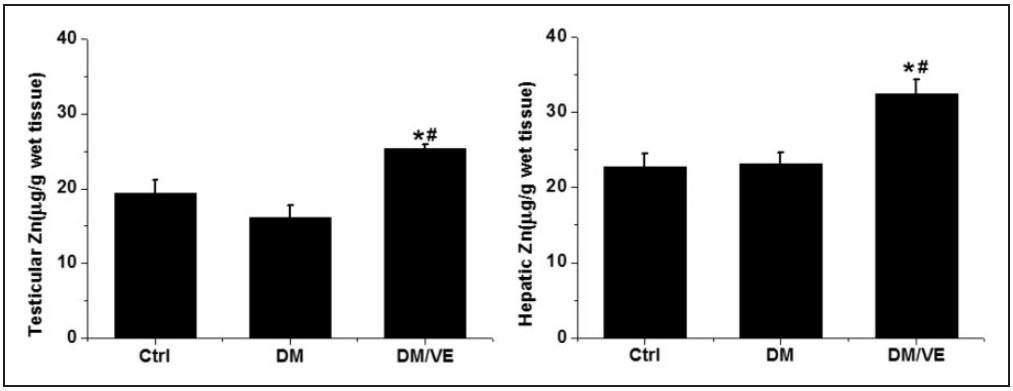


**Figure 2.** Zn levels in the testis and liver of Ctrl, DM and DM/VE rats at 30 days after diabetes induction. Data are presented as mean ± SE. *, $P < 0.05$ vs. Ctrl; #, $P < 0.05$ vs. DM.
Ctrl: non-diabetic control; DM: diabetic; DM/VE: diabetic rats treated with vitamin E.

the testicular and hepatic MT protein (Figure 1), and the Zn levels, while it mildly decreased the testicular and hepatic Cu levels in the DM/VE group compared with the DM group (Figures 2 and 3). However, no significant changes were detected in the testicular and hepatic Fe levels (Figure 4). These results suggest that vitamin E stimulated MT protein synthesis and that this induced testicular and hepatic MT expression was associated with metal level changes, especially increased Zn levels.

## Discussion

Hyperglycaemia has been demonstrated to lead to abnormal metabolism of trace elements and related proteins in various organs.[20,23,24] Recently, it has been shown that MT is one of the top five expressed genes in islets of non-obese diabetic mice compare with the transcriptome profile of islets of age-matched diabetes-resistant NOR mice and C57Bl/6 mice.[25] Increased hepatic MT protein and the MT-related metals, Zn and Cu, have been documented in diabetic animals.[21] However, there is no data on testicular MT protein alteration in diabetic rats. In the present study, although there was an increase in the hepatic MT protein in the diabetic rats, no significant change in the testicular MT protein was detected in diabetic rats after 30 days of diabetes. These data suggest there are organ-specific patterns of diabetes-induced MT expression in the early stage of diabetes in rats. Similar to previous findings,[26,27,28,29] we revealed that the testicular MT protein levels were much higher than the hepatic MT in the Ctrl group, indicating that the antioxidant capacity of the testis may be higher than that of the liver. The basal difference in MT expression between the two organs may explain why we did not observe an obvious change in testicular MT protein in the relatively early stage of diabetes (30 days).

Although we found that hepatic MT protein increased in the DM group, no change was detected in the metal levels. New evidence has revealed that the oxidative stress-induced MT was independent of metal level changes.[30,31] MT protein has also been used as a biomarker of oxidative stress.[32] Therefore, the induction of hepatic MT in the diabetic rats may not depend on metals, but rather on the diabetes-induced oxidative stress and damage in the liver.[33]

Oxidative stress and consequent damage have been considered as the main mechanism

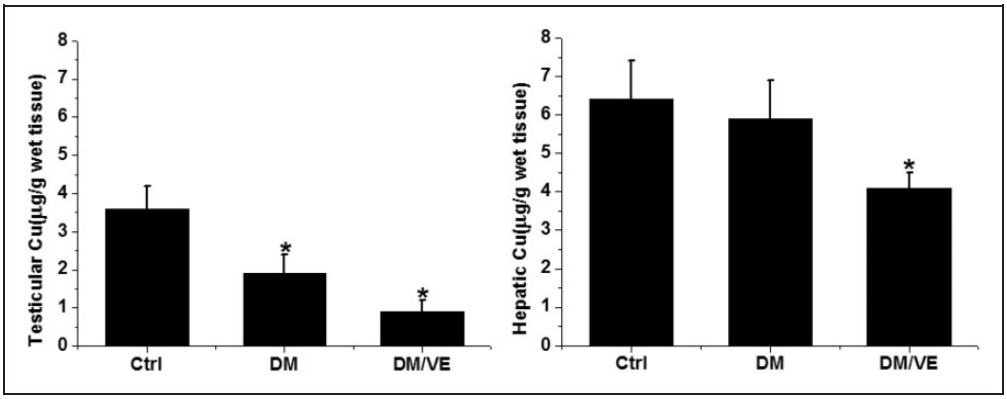


**Figure 3.** Cu levels in the testis and liver of Ctrl, DM and DM/VE rats at 30 days after diabetes induction. Data are presented as mean ± SE. *, $P < 0.05$ vs. Ctrl; #, $P < 0.05$ vs. DM.
Ctrl: non-diabetic control; DM: diabetic; DM/VE: diabetic rats treated with vitamin E.

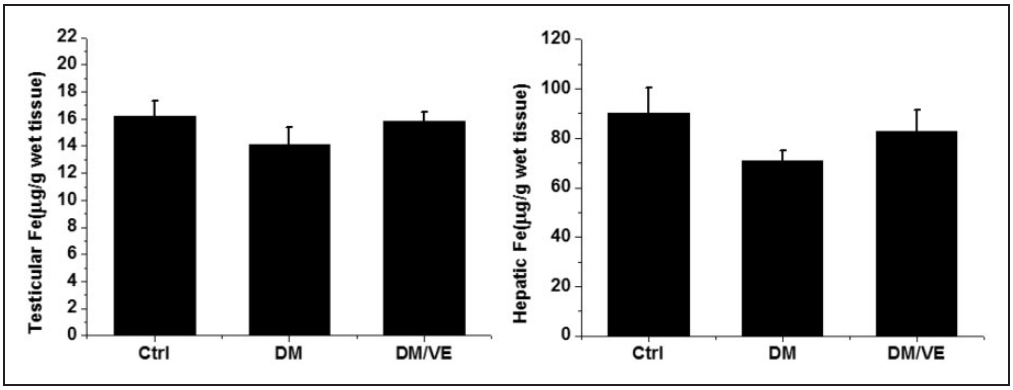


**Figure 4.** Fe levels in the testis and liver of Ctrl, DM and DM/VE rats at 30 days after diabetes induction. Data are presented as mean ± SE. *, $P < 0.05$ vs. Ctrl; #, $P < 0.05$ vs. DM.
Ctrl: non-diabetic control; DM: diabetic; DM/VE: diabetic rats treated with vitamin E.

of diabetic complications in organs including the kidneys and the vascular system.[34,35,36] MT regulates tissue metal homeostasis, and is a potent antioxidant that protects cells and tissues from oxidative damage.[37] Compared with other antioxidants that can specifically protect against certain damages, for example, superoxide dismutase (SOD) against superoxide radicals, catalase against hydrogen peroxide, and glutathione peroxidase against hydrogen peroxide and lipid peroxides, MT is a potent antioxidant against a wide range of free radicals including the most active radicals, hydroxyl radicals and peroxynitrite.[38] Several studies have demonstrated increased oxidative damage in the liver of type 1 diabetic mice.[7,33] Therefore, we concluded that diabetes-induced oxidative stress may play a critical role in hepatic injury, and MT protein as a potent antioxidant may prevent diabetic complications through

increasing its own level to suppress diabetic oxidative damage.

Because oxidative damage results in increased hepatic MT protein, we hypothesized that increased MT expression in the diabetic liver could be prevented by administration of antioxidants. α-Tocopherol, which exhibits the highest vitamin E bioactivity, has been found to be very effective in ameliorating the toxicity produced by xenobiotics, particularly by reducing oxidative damage in terms of decreased lipid peroxidation and improving the antioxidant system.[39] Unexpectedly, we found that vitamin E treatment did not prevent the MT protein induction, and instead, enhanced the MT protein expression in the DM/VE group. Vitamin E also enhanced the testicular MT protein in the DM/VE group. In addition, Vitamin E-enhanced MT protein expression was accompanied by a marked increase in testicular and hepatic Zn, and a mild decrease in the Cu level in the DM/VE group. These results suggest that vitamin E may stimulate MT protein synthesis, and the additional enhancement of the testicular and hepatic MT was associated with metal level changes, especially the increased Zn level. In fact, vitamin E is able to directly induce MT as revealed in cardiomyocytes.[40] It is known that diabetes predominantly increases superoxides because of the impairment of the mitochondrial respiratory function.[41] A study has shown the different responses of MT induction in different tissues, such as liver and kidney. In this study they found that the hepatic level of MT was significantly increased in rats exposed to paraquat (a superoxide-generating compound) and slightly increased in fasted rats, while the renal MT level was significantly increased in fasted rats, but not in paraquat-treated rats.[42] Administration of paraquat increased the hepatic MT protein along with increases in Zn and thiobarbituric acid-reactive substances, indicating the occurrence of lipid peroxidation. Treatment of rats with vitamin E before injection of paraquat only prevented the enhancement of lipid peroxidation without any effect on the increased MT and Zn levels, suggesting the enhanced lipid peroxidation was not required for MT induction.[40] Therefore, this study suggests two mechanisms by which vitamin E increases hepatic and testicular MT: (1) vitamin E can directly stimulate MT expression; (2) vitamin E may reduce STZ-induced toxicity or diabetes-induced toxicity, resulting in more healthy live cells that express more MT in the tissues. These possible mechanisms will be further explored in future studies.

The fact that Zn increases MT transcription via upregulation of metal-responsive elements has previously been extensively investigated. Zn-induced MT expression is most likely the protective mechanism against diabetes-induced liver damage because significantly increased hepatic MT expression was observed in Zn-treated nondiabetic and diabetic mice.[7] Zn deficiency exacerbated diabetes-induced testicular damage,[18,19] and Zn supplementation ameliorated diabetes-associated testicular alterations.[16] Vitamin E significantly increased serum Zn in rats with pulmonary contusion.[43] Ascorbic acid protected antioxidants and increased testis Zn in diabetic rats.[44] We thus considered that vitamin E may enhance MT protein expression by upregulating the Zn level in both the testis and liver.

MT and Zn also play other important roles in many physical and pathological conditions. For example, both MT and Zn may be involved in the alteration in tyrosine phosphatase activity, because under oxidative conditions, MT is oxidized, releasing Zn, which in turn inhibits protein tyrosine phosphatases (PTPs), key regulatory enzymes of cellular phosphorylation signalling.[45,46] Rice et al.[47] have reported that Zn released from MT by ROS activates the p38 mitogen-activated protein kinase (MAPK) signalling pathway in cultured cells. In addition, Zn interacts with Trk

receptor, to activate $STEP_{61}$ at multiple protein kinase A (PKA) sites with a concomitant increase in the phosphorylation of extracellular signal-regulated kinase (ERK) MAPK in brain injury.[48] Consistent with these studies, Zn was also found to inhibit PTP1B, TRB3 and PTEN (Akt negative regulators) function, resulting in Akt activation. Therefore, Zn deficiency exacerbates diabetes-induced downregulation of Akt function because of the lack of the inhibitory effect of Zn on the Akt negative regulators (PTP1B, TRB3 and PTEN), leading to increased diabetes-induced testicular apoptotic cell death.[19] However, whether vitamin E can influence tyrosine phosphatase activity by upregulating Zn and MT levels in diabetic complications needs to be explored in the future.

In summary, in the present study, we demonstrated for the first time that there was no marked increase in the testicular MT protein of diabetic rats compared with the Ctrl. We demonstrated that the increased hepatic MT protein in diabetic rats was not associated with the metal levels, but was associated with oxidative damage. Vitamin E, as an antioxidant, further enhanced the testicular and hepatic MT by increasing the Zn level. Considering that Zn deficiency exacerbates diabetes-induced testicular and hepatic damage, as discussed above, proper administration of Vitamin E may be an ideal approach to indirectly increase testicular and hepatic Zn and MT, to prevent diabetes-induced damage in these organs.

## Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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