



Exacerbation of diabetes-induced testicular apoptosis by zinc deficiency is most likely associated with oxidative stress, p38 MAPK activation, and p53 activation in mice

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ABSTRACT

Since diabetes induces testicular oxidative damage and cell death, and zinc (Zn) plays an important role in the spermatogenesis, the objective of the present study was to define the effects of Zn deficiency on diabetes-induced testicular apoptosis and associated mechanisms. Zn deficiency was induced by chronic treatment of normal and diabetic mice with N,N,N',N'-tetrakis (2-pyridylemethyl) ethylenediamine (TPEN) chelation. After diabetes onset, mice were given intraperitoneally TPEN at 5 mg/kg daily for four months, which, like diabetes, induced a significant decrease in testicular Zn level. TUNEL staining revealed that testicular apoptosis was significantly increased along with an increased Bax/Bcl-2 ratio, in diabetic mice and TPEN-treated normal mice. Zn deficiency significantly exacerbated diabetes-induced testicular apoptosis, along with significantly increased oxidative and nitrosative damage and down-regulation of antioxidant Nrf2 expression. Increased oxidative stress was associated with an increase in activation of p38 MAPK and p53 protein in diabetic testis, which was worsened in the testes of diabetic mice with Zn deficiency. Diabetes also induced a significant increase in endoplasmic reticulum stress and associated cell death, which was not affected by Zn deficiency. These results suggest that like diabetes, chronic depletion of Zn with TPEN induces testicular oxidative stress and damage, along with the activation of p38 MAPK and p53 signaling and mitochondria-related apoptotic cell death. Therefore, prevention of Zn deficiency for diabetic patients is important in order to avoid the exacerbation of diabetic effects on testicular cells death.

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1. Introduction

Infertility is a common complication in diabetic men (Agbaje et al., 2007; Amaral et al., 2008), mainly due to the loss of germ cells by apoptotic cell death (Cai et al., 2000; Koh, 2007; Sainio-Pollanen et al., 1997). However, factors contributing to diabetic induction of testicular apoptosis have not been well described.

Zinc (Zn) is known as an essential trace element required for the maintenance of germ cells, the progression of spermatogenesis, and the regulation of sperm motility (Yamaguchi et al., 2009).

Zn is a cofactor in many enzymes and proteins involved in antioxidant defenses, electron transport, DNA repair and p53 protein expression (Song et al., 2009). Reportedly Zn deficiency induces oxidative stress (Ho et al., 2003; Oteiza et al., 1996; Song et al., 2009). Supplementation with Zn has shown to protect against a range of stress-induced testicular damage (Amara et al., 2008; Ozturk et al., 2003). Oxidative stress occurs when overgeneration of reactive oxygen or nitrogen species (ROS and RNS) overwhelms antioxidant defenses or endogenous antioxidant defense is down-regulated. Oxidative stress induced by Zn deficiency is not only due to the increased generation of ROS and/or RNS, but also due to the impairment of antioxidant capacity (Oteiza et al., 1996; Yousef et al., 2002).

The transcription factor Nuclear Factor-Erythroid 2-Related Factor 2 (Nrf2) regulates basal and inducible transcription of genes encoding protective molecules against various oxidative stresses

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(Ha et al., 2006; Lee and Johnson, 2004; Lee and Surh, 2005). In response to a range of oxidative and electrophilic stimuli including ROS and/or ROS, heavy metals, and certain disease processes, Nrf2 is activated and mediates the induction of a spectrum of cytoprotective proteins including phase II enzymes, such as catalase, NAD(P)H:quinone oxidoreductase, and antioxidant proteins, such as heme oxygenase 1, through the antioxidant response element-dependent pathway. Nrf2 is broadly expressed in tissues and has been recognized to play a critical role in oxidative defense in the testis (Nakamura et al., 2010; Yang et al., 2008). Deletion of the Nrf2 gene was found to cause an age-dependent testicular and epididymal oxidative stress, which disrupts spermatogenesis (Nakamura et al., 2010), suggesting a critical role for the transcription factor Nrf2 in preventing oxidative disruption of spermatogenesis.

p53 plays a critical role in spermatogenesis (Rotter et al., 1993; Yin et al., 1998), and promotes apoptosis, in response to a variety of oxidative stimuli, by transactivation of target genes and by transcription-independent mechanisms (Liu et al., 2007; Rotter et al., 1993; Yin et al., 1998). Wild-type p53 rapidly translocates to mitochondria in response to multiple death stimuli and physically interacts with anti-apoptotic Bcl proteins, induces Bak oligomerization, permeabilizes mitochondrial membranes, and rapidly induces cytochrome *c* release, leading to a mitochondria-dependent cell death cascade (Erster et al., 2004; Erster and Moll, 2004; Li et al., 2009).

P38 MAPK was also found to play a role in maintaining normal spermatogenesis (Ranawat and Bansal, 2009). P38 MAPK acts as one of the p53 up-stream activators in response to oxidative stress to induce p53-dependent and independent cell death pathways (Liu et al., 2010; Papoutsaki et al., 2005). Vera et al. reported that germ cell apoptosis was activated at the 5th day after GnRH antagonist treatment by p38 MAPK activation, followed by increased Bax/Bcl-2 ratio, cytochrome *c* release from mitochondria, and caspase activation (Vera et al., 2006).

In the present study, therefore, we examined the effects of Zn deficiency on diabetes-induced testicular apoptosis and associated mechanistic changes. To this end, we treated mice with streptozotocin (STZ) to induce a type 1 diabetes model. Zn deficiency was induced by chronic treatment with Zn chelator, N,N,N',N'-tetrakis (2-pyridylemethyl) ethylenediamine (TPEN), as used in other studies (Kabu et al., 2006; Yamaguchi et al., 2009). Diabetic mice were treated with and without TPEN for four months, and testicular cell death, oxidative stress and cell death signaling were examined.

2. Materials and methods

2.1. Animals

FVB mice were used for this study. All mice were housed in the University of Louisville Research Resources Center at 22 °C with a 12-h light/dark cycle and provided with free access to standard rodent chow and tap water. All animal procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care.

2.2. STZ-induced diabetes and Zn depletion

Eight-week-old male mice were injected intraperitoneally with multiple low-doses of STZ (Sigma, MO) at 50 mg/kg daily for 5 days to induce type 1 diabetes. Five days after the last injection of STZ, whole blood glucose obtained from mouse tail-vein was measured with a SureStep complete blood glucose monitor (LifeScan, CA). The blood glucose levels higher than 250 mg/dl were considered as diabetic. Once diabetes onset was detected, diabetic mice were injected intraperitoneally with TPEN (Sigma, MO) at 5 mg/kg daily for 4 months to chelate Zn. The selection of TPEN to chronically deplete intracellular Zn is based on several previous studies that have successfully used TPEN to eliminate Zn without significant systemic toxic effects (Kabu et al., 2006; Yamaguchi et al., 2009). At the time of sacrifice, two testes were harvested for the following histopathological and biochemical studies.

2.3. Measurement of testicular Zn levels

Zn levels in the testes were measured by an atomic absorption spectrometer using air-acetylene flame after tissue digestion by nitric acid as described previously (Cai et al., 2002a). By this assay, total Zn in the tissues including free Zn and protein-bound Zn were measured and expressed as µg/g wet tissue.

2.4. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Testis tissue was fixed in 10% formalin, embedded in paraffin and sectioned at 5 µm. The slides were stained for TUNEL with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, CA, USA), as described in previous studies (Cai et al., 2000; Zhao et al., 2010). Briefly, each slide was deparaffinized and rehydrated, and treated with proteinase K (20 mg/l) for 15 min. The endogenous peroxidase was inhibited with 3% hydrogen peroxide for 5 min, and then incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP for 1 h. The TdT reaction was carried out in a humidified chamber at 37 °C. Then 3,3'-diaminobenzidine chromogen was applied. Hematoxylin was used as counterstaining. For negative control, TdT was omitted from the reaction mixture.

Apoptotic cell death was quantitatively analyzed by counting the TUNEL-positive cells selected randomly from ten seminiferous tubule's cross-sections from each of the three slides for each mouse. The apoptotic cells were counted from spermatogonia, primary spermatocytes, secondary spermatocytes, but not spermatid and spermatozoa since total cells of the former can be easily identified for the quantification. Results were presented as TUNEL positive cells per 10³ cells.

2.5. Western blotting

Western blots were performed according to our previous studies (Cai et al., 2002b). Briefly, testicular tissues were homogenized and fractionated on 10% SDS-PAGE gels, and proteins were transferred to a nitrocellulose membrane. The membrane was blocked with a 5% non-fat dried milk for 1 h, and incubated overnight at 4 °C with the following antibodies: anti-Bax, anti-Bcl-2, anti-phospho-p38(Thr180/Tyr182), anti-p38, anti-p53, anti-phospho-p53(Ser15) (1:1000, Cell Signaling, MA), anti-CHOP (1:1000, Santa Cruz, CA), anti-caspase12 (1:1000, Exalpha Biologicals, MA), anti-4-hydroxynonenal-Michael adducts (4-HNE, 1:2000, Calbiochem, CA), anti-3-nitrotyrosine (3-NT, 1:2000, Chemicon, CA), and anti-Nrf2 (1:1000, Abcam, MA). After the unbound antibodies were removed with Tris-buffered saline (pH 7.2) containing 0.05% Tween 20, membranes were incubated with the secondary antibody for 1 h at room temperature. Antigen-antibody complexes were visualized with an enhanced chemiluminescence detection kit (Thermo Scientific, IL). To determine loading, blots were stripped using stripping buffer (Sigma-Gen Lab, MD) and reprobed for β-actin. Quantitative densitometry was performed on the identified bands by using a computer-based measurement system, as employed in previous studies (Cai et al., 2002b).

2.6. Statistical analysis

Data were collected from repeated experiments and were presented as mean ± SD. One-way ANOVA was used to determine if differences exist and if so, a post hoc Tukey's test was used for analysis for the difference between groups, with

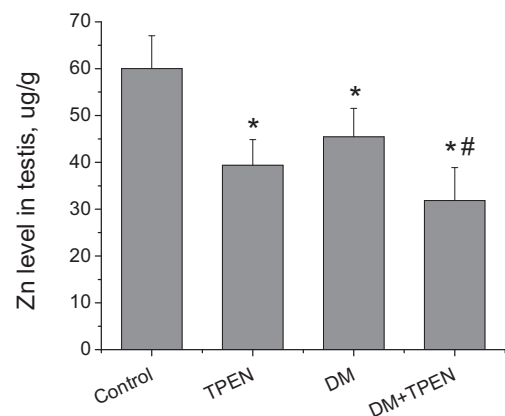


Fig. 1. TPEN- and diabetes-decreased testicular Zn levels. Diabetes was induced with MLD-STZ and treated with and without TPEN at 5 mg/kg daily for 4 months. Testicular Zn levels were measured by atomic absorption spectrometer. Data are presented as mean ± SD ($n=6$ at least in each group). DM: diabetes. * $P<0.05$ vs. control group and # $P<0.05$ vs. DM.

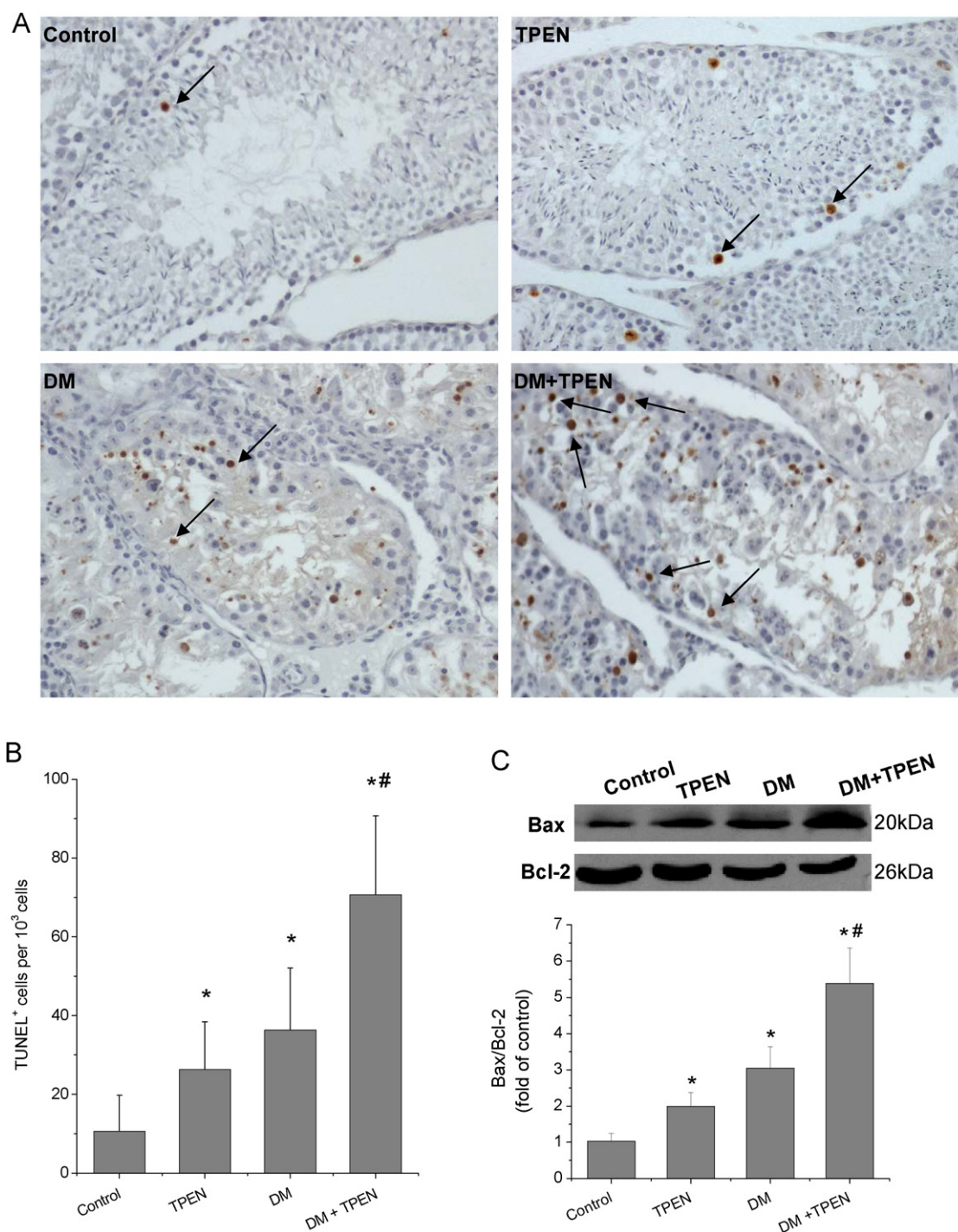


Fig. 2. Diabetes- and TPEN-induced testicular apoptosis. Diabetes was induced with MLD-STZ and treated with and without TPEN at 5 mg/kg daily for four months. Testicular apoptotic cell death was examined by TUNEL staining (A) and TUNEL-positive cells were quantitatively analyzed (B). The expression of Bax and Bcl-2 were detected by Western blotting assay (C), for which the ratio of Bax/Bcl-2 was present. Data are presented as mean \pm SD ($n=6$ at least in each group). DM: diabetes. * $P<0.05$ vs. control group and # $P<0.05$ vs. DM.

Origin 7.5 laboratory data analysis and graphing software. Statistical significance was considered as $p<0.05$.

3. Results

3.1. TPEN-induced testicular Zn deficiency

Normal FVB mice, chronically treated with TPEN at 5 mg/kg daily for 4 months were found to have significantly lower testicular Zn levels than control (Fig. 1). It is noteworthy that diabetes also sig-

nificantly decreased testicular Zn levels, and TPEN-treated diabetic animals showed the lowest testicular Zn levels among the groups (Fig. 1).

3.2. Diabetes- and TPEN-induced testicular apoptosis

A significant induction of testicular apoptosis in diabetic mice at 4 months after diabetes onset, examined by TUNEL staining (Fig. 2A and B), was found along with a significant increase in Bax/Bcl-2 ratio as an index of mitochondrial cell death pathway

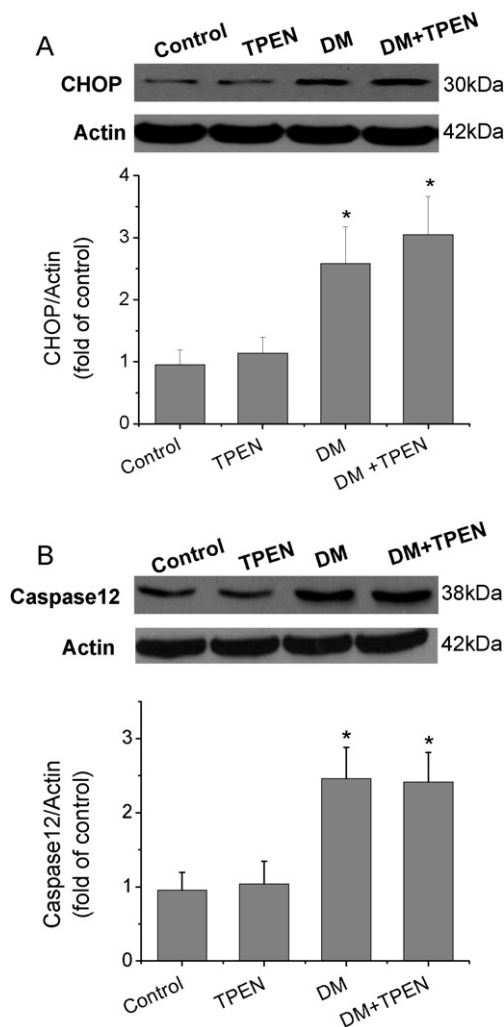


Fig. 3. Diabetes-induced testicular ER stress. Diabetes model and treatment are same as described in Fig. 1. ER stress-associated cell death was examined by Western blotting assay for the expression of CHOP (A) and caspase12 (B). Data are presented as mean \pm SD ($n = 6$ at least in each group). DM: diabetes. * $P < 0.05$ vs. control group and # $P < 0.05$ vs. DM.

(Fig. 2C). Chronic treatment of normal mice with TPEN significantly increased testicular TUNEL-positive cells and Bax/Bcl-2 ratio (Fig. 2C). Chronic treatment with TPEN significantly enhanced diabetes-induced apoptosis and increased the ratio of Bax/Bcl-2 (Fig. 2C). TUNEL-positive cells induced by diabetes and/or TPEN are predominantly spermatogonia and spermatocytes (Fig. 2A).

Next we examined the effect of endoplasmic reticulum (ER) stress in the diabetic testis. Diabetes induced significant increases in CHOP expression (Fig. 3A) and caspase-12 activation (Fig. 3B), indicating diabetic induction of ER stress-related cell death. However, chronic treatment with TPEN did not induce testicular ER stress-related cell death in normal mice and also did not enhance the effect of diabetes on testicular ER stress-related cell death (Fig. 3). These data suggest that diabetes, but not Zn deficiency, induces testicular ER stress-related cell death.

3.3. Diabetes- and TPEN-induced oxidative stress

Oxidative and nitrosative stress in the testis of mice with and without TPEN chronic treatment was examined. Measurement of 4-HNE as an index of lipid peroxide (oxidative damage) by Western blot revealed that there were numerous 4-HNE-modified proteins, at a mass of approximately 30 kDa and 64–145 kDa (Fig. 4A), in the

testis of both diabetic and TPEN-treated normal mice. Similarly, 3-NT assay also revealed a significant increase in several groups of nitrated proteins, at a mass of approximately 22–40 kDa, and 64–145 kDa, in the testis of diabetic mice and TPEN-treated normal mice (Fig. 4B). When diabetic mice were treated chronically with TPEN, there was a significantly synergistic effect on the oxidative and nitrosative damage compared to TPEN-treated controls or diabetic mice alone (Fig. 4A and B).

In the next study, we demonstrated that diabetes significantly decreased testicular Nrf2 expression (Fig. 4C). Although chronic treatment of normal mice with TPEN did not decrease testicular Nrf2 expression, chronic treatment of diabetic mice with TPEN significantly aggravated diabetes-decreased Nrf2 expression (Fig. 4C). This study confirms the diabetic induction of oxidative stress, mirrored by increased oxidative and nitrosative damage and depressed antioxidant Nrf2 expression.

3.4. Diabetes- and TPEN-induced activation of p53 and p38 MAPK

Both p53 and p38 MAPK play important roles in induction of testicular apoptosis by various oxidative stresses (Erster et al., 2004; Erster and Moll, 2004; Li et al., 2009). Therefore, we examined the status of testicular p53 and p38 MAPK activation under diabetic conditions with and without chronic TPEN treatment. A significant increase in p53 activation in the testis of either diabetic mice or TPEN-treated normal mice was found (Fig. 5A). Chronic treatment of diabetic mice with TPEN significantly enhanced diabetes-increased p53 activation. Similarly, phosphorylation of p38 MAPK was increased in the testis of diabetic or TPEN-treated non-diabetic mice (Fig. 5B). Chronic TPEN treatment of diabetic mice also significantly enhanced diabetes-induced p38 phosphorylation (Fig. 5B).

4. Discussion

Diabetes is a global health problem due to its serious complications. The number of young patients with either type 1 or type 2 diabetes is increasing dramatically (Dahlquist and Kallen, 2005; Mayer-Davis et al., 2009). Therefore, infertility of these young diabetic patients has become a concern (Agbaje et al., 2007; Amaral et al., 2008). We have reported the significant induction of apoptotic cell death in the testis of diabetic rats as early as 2000 (Cai et al., 2000), which has been considered as the major cause of the infertility for the men with diabetes. For diabetes-induced testicular apoptotic cell death, a mitochondria-dependent pathway was found to predominate (Amaral et al., 2009; Koh, 2007; Zhao et al., 2010). This notion is further supported by the present study, in which we found a significant increase in Bax/Bcl-2 ratio in the diabetic testis (Fig. 2C).

In a recent study, we demonstrated that oxidative stress is a major cause for diabetic loss of male germ cells since diabetic induction of testicular apoptotic cell death were prevented by treatment with antioxidant N-acetyl-L-cysteine or low-level ionizing radiation that induces up-regulation of testicular antioxidants (Zhao et al., 2010). Consistent with this finding, we observed an induction of oxidative stress and damage in the diabetic testis, reflected by increased 4-HNE contents as lipid peroxidation and 3-NT accumulation as protein nitration (Fig. 4A and B).

One of the novel findings in the present study is the diabetic down-regulation of Nrf2 in the testis (Fig. 4C). Nrf2 has been found to play a critical role in oxidative defense in testis (Nakamura et al., 2010; Yang et al., 2008). The significant down-regulation of Nrf2 may explain why multiple antioxidant expressions in diabetic testis were significantly down-regulated (Zhao et al., 2010). Based on our knowledge the present study is the first to show down-regulation

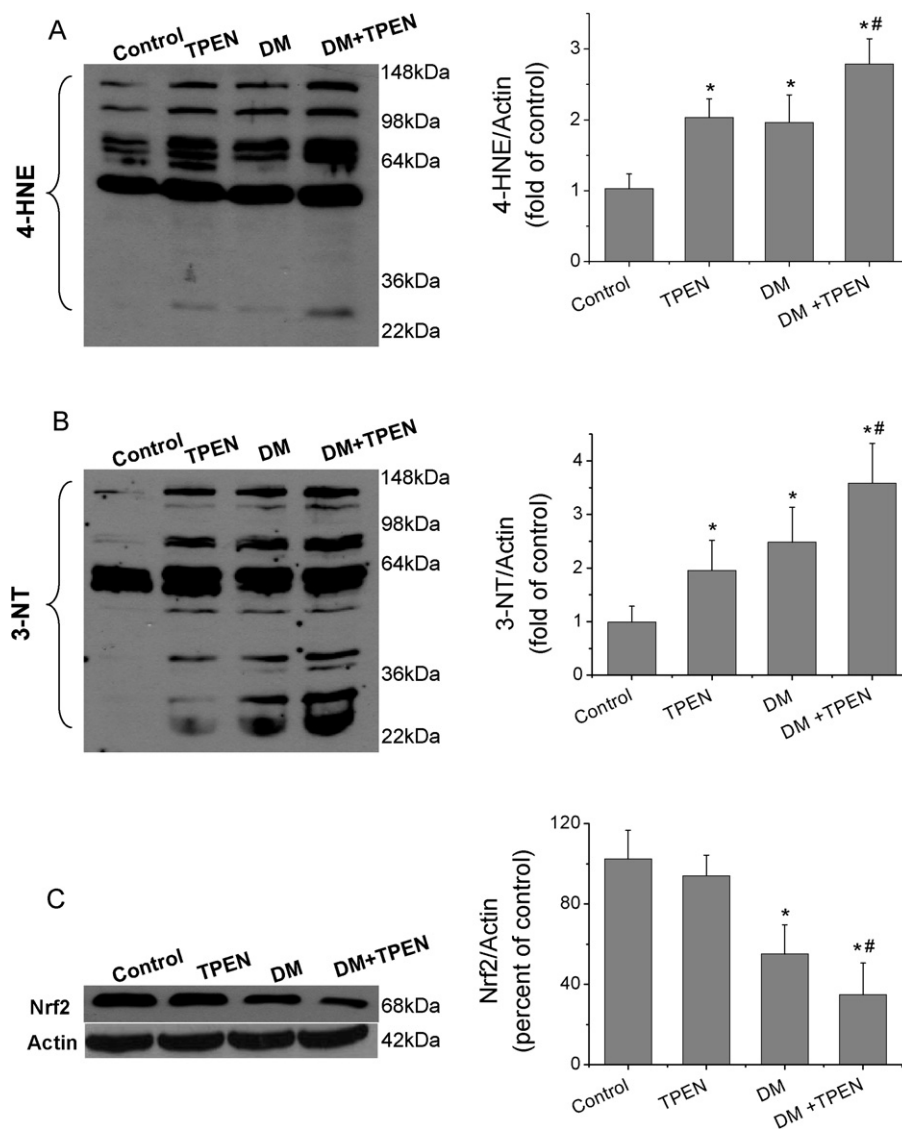


Fig. 4. Diabetes- and TPEN-induced testicular oxidative stress. Diabetes model and treatment are same as described in Fig. 1. Testicular oxidative damage was examined by Western blotting assay for the expression of 4-HNE as an index of lipid peroxidation (A) and 3-NT as an index of protein nitration (B). Since both 4-HNE and 3-NT showed multiple groups of HNE-modified or nitrated proteins within the mass range of an approximate 22–145kDa, when we quantitatively analyze 4-HNE and 3-NT, all bands within the range of mass 22–145kDa were included for the densitometry analysis. Testicular Nrf2 expression was examined by Western blotting assay (C). Since the results of the panels A, B, and C were from same Western membrane, actin expression as the loading control is presented only in panel C. Data are presented as mean \pm SD ($n = 6$ at least in each group). DM: diabetes. * $P < 0.05$ vs. control group and ** $P < 0.05$ vs. DM.

of testicular Nrf2 in diabetic mice. As an adaptive mechanism, Nrf2 is quickly up-regulated in the cells and tissues in response to various stresses, but is down-regulated in cells or tissue exposed to an overwhelming or long-lasting oxidative stress. For instance, low and moderate doses hydrogen peroxide exposure of rat pulmonary microvascular endothelial cells led to nuclear accumulation of Nrf2, increased activity of transcription regulation and up-regulation of antioxidant response element (ARE)-mediated gene expression. In contrast, high doses of hydrogen peroxide exposure led to the nuclear exclusion of Nrf2, decreased activity transcription regulation and down-regulation of ARE-mediated gene expression (Ning et al., 2010). Similarly, rats with chronic renal failure caused by 5/6 nephrectomy exhibited significant increases in oxidative stress and inflammation in the remnant kidney, in which Nrf2 function was mildly reduced at 6 weeks and markedly reduced at 12 weeks after nephrectomy. The products of Nrf2 target genes [catalase, superoxide dismutase, glutathione peroxidase, heme oxygenase-1, NAD(P)H quinone oxidoreductase, and glutamate-cysteine ligase] were all significantly diminished at 12 weeks (Kim and Vaziri,

2010). In addition, acute cigarette smoke exposure led to Nrf2 activation in human macrophages, but Nrf2 expression was significantly decreased in pulmonary macrophages from smokers with chronic exposure to cigarettes (Suzuki et al., 2008). Consistent with these studies, we have demonstrated the increase in Nrf2 in the heart of diabetic mice at 2 weeks after hyperglycemia (He et al., 2009), and decrease in the testis of diabetic mice at 4 months after hyperglycemia (Fig. 4C). As outlined in Fig. 6, therefore, we assume that down-regulation of Nrf2 plays a critical role in the development of diabetic oxidative stress, which induces oxidative damage and activates cell death pathways, in the testis of diabetic mice.

In the present study, we have demonstrated p53 activation in the testes of diabetic mice and non-diabetic mice with Zn deficiency (Fig. 5A), which is consistent with previous observation in other cells and tissues (Corniola et al., 2008; Ho et al., 2003). However, we demonstrate, for the first time, that Zn deficiency significantly enhances diabetic activation of p53 in the testis (Fig. 5A). The synergistic effects of diabetes and Zn deficiency on testicular oxidative stress and p53 activation are predictable since we found

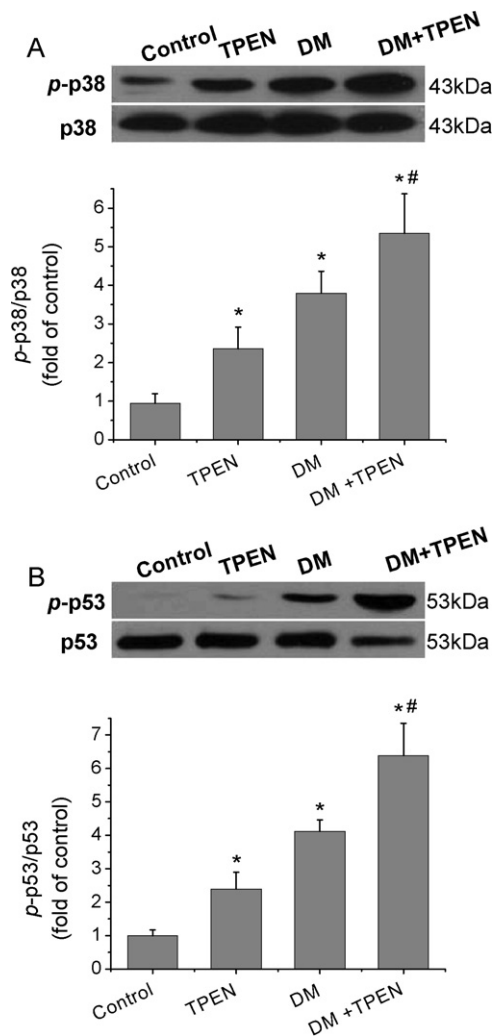


Fig. 5. Diabetes- and TPEN-induced activation of p38 and p53 MAPK. Diabetes model and treatment are same as described in Fig. 1. Testicular expressions of p-p38 and p38 (A), p-p53 and p53 (B) were detected by Western blotting assay, and the ratios of p-p38/p38 and p-p53/p53 were presented, respectively. Data are presented as mean \pm SD ($n=6$ at least in each group). DM: diabetes. * $P<0.05$ vs. control group and # $P<0.05$ vs. DM.

that either of diabetes or Zn deficiency significantly induces these effects. However, it is not always the case that predicted synergistic effects for any two oxidative stressors actually are observed. For instance, either diabetes or ionizing radiation is an inducer for renal inflammation and oxidative stress, but diabetic mice treated with repeated exposure to ionizing radiation showed a significant decrease in renal inflammation and oxidative stress compared to diabetes alone (Zhang et al., 2009). Similar to the changes of p53, p38 MAPK was also activated in the testis of diabetic and TPEN-treated normal mice, and Zn deficiency further enhanced diabetic activation of p38 MAPK (Fig. 5B). Therefore, we assume that diabetic activation of p38 MAPK may be involved in induction of testicular apoptotic cell death that is mediated by either p53-dependent mitochondrial cell death pathway or p53-independent cell death pathway, as outlined in Fig. 6.

Interestingly, chronic depletion of Zn does not affect ER stress-related testicular cell death, examined by CHOP and cleaved caspase-12 (Fig. 3), although diabetes induced both mitochondrial and ER stress-dependent cell death in the testes (Figs. 2 and 3). Therefore, we assumed that diabetic induction of ER stress and associated apoptotic cell death is independent of oxidative stress, as outlined in Fig. 6.

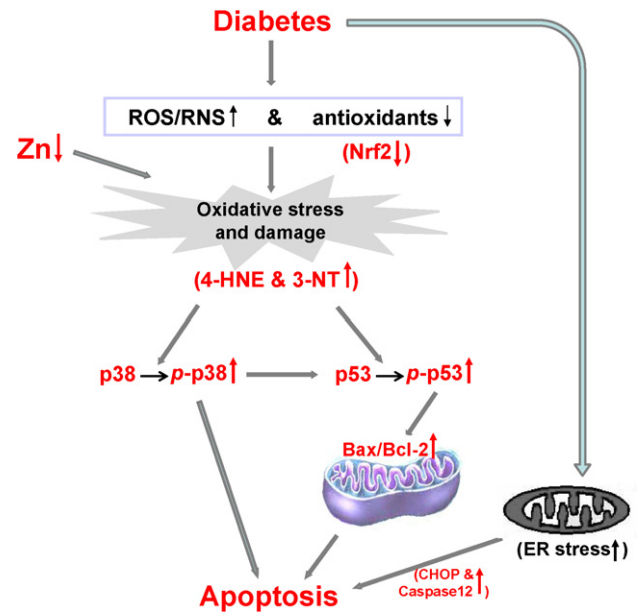


Fig. 6. Illustration of the assumed mechanisms for the exacerbation of diabetes-induced testicular apoptosis by Zn deficiency. Diabetes induces testicular oxidative stress by increasing generation of ROS and/or RNS and also by decreasing antioxidant capacity. The increased oxidative stress may activate p38 MAPK that induces p53-dependent and independent cell death. P53 protein that may be active either due to oxidative stress or via p38 MAPK activation most likely translocates into mitochondria to up-regulate mitochondrial cell death pathway. In addition, diabetes also induces testicular ER stress and associated cell death. Zn deficiency induces oxidative stress, which exacerbates diabetic induction of oxidative stress-activated cell death signaling pathway, but does not affect diabetic induction of ER stress and associated cell death. In this figure, red contents indicate the experimental evidence and gray and black lines represent the well-known pathways based on from literature as discussed in Section 4. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

In summary, since Zn plays important role in maintaining normal spermatogenesis and protecting the testis against oxidative damage, we have investigated for the first time whether Zn deficiency affects diabetic induction of testicular oxidative damage and cell death. We found that diabetes significantly induces testicular oxidative stress and damage, along with the activation of p38 MAPK and p53 signaling and mitochondria-related apoptotic cell death. In addition, diabetes also induces testicular ER stress and associated cell death. Zn deficiency does not affect diabetic induction of ER stress and associated cell death, but Zn deficiency significantly enhances diabetic effects on oxidative stress and damage, mitochondrial cell death and associated p38 MAPK and p53 signaling. Because approximately 12% of Americans do not consume the estimated average requirement for Zn and could be at risk for marginal Zn deficiency (Sandstead et al., 2008; Schneider et al., 2007), this study highlights the importance of proper Zn intake in diabetic patients, which may prevent or delay diabetic complications, including reproductive system damage.

Conflict of interest statement

None declared.

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