Experimental and Molecular Pathology 111 (2019) 104304

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Long-term diabetes causes molecular alterations related to fibrosis and apoptosis in rat urinary bladder

Rania A. Elrashidy[a,b], Guiming Liu[a,*]

[a] Department of Surgery, MetroHealth Medical Center, Case Western Reserve University, Cleveland, OH, USA
[b] Department of Biochemistry, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt

ARTICLE INFO

Keywords:
Diabetes
Bladder
Cystopathy

ABSTRACT

Diabetes induces time-dependent alterations in urinary bladders. Long-term diabetes causes an underactive bladder. However, the fundamental mechanisms are still elusive. This study aimed to examine the histological changes and the potential molecular pathways affected by long-term diabetes in the rat bladder. Diabetes was induced in 8-week-old male Lewis rats by streptozotocin, while age-matched control rats received citrate buffer only. Forty-four weeks after diabetes induction, bladders were harvested for histological and molecular analyses. The expressions of proteins related to fibrosis, apoptosis and oxidative stress as well as the cellular signaling pathway in the bladder were examined by immunoblotting. Histological examinations illustrated diabetes caused detrusor hypertrophy and fibrotic changes in the bladder. Immunoblotting analysis demonstrated higher collagen I but lower elastin expression in the bladder in diabetic rats. These were accompanied by an increase in the expression of transforming growth factor-beta1, along with the downregulation of matrix metalloptoteinase-1, and upregulation of tissue inhibitor of metalloproteinase-1. Diabetic rats showed an increase in nitrotyrosine, but decrease in nuclear factor erythroid-related factor 2 (Nrf2) levels in the bladder. Enhanced apoptotic signaling was observed, characterized by increased expression of Bcl-2-associated X protein (Bax), decreased expression of Bcl-2, in the diabetic bladder. The nerve growth factor level was decreased in the diabetic bladder. A significant suppression in the protein expressions of phosphorylated extracellular signal-regulated kinases 1/2 was found in diabetic bladders. This study demonstrated that long-term diabetes caused molecular changes that could promote fibrosis and apoptosis in the bladder. Oxidative stress may be involved in this context.

1. Introduction

Diabetes mellitus (DM) significantly affects the lower urinary tract leading to bothersome urologic complications such as bladder dysfunction, sexual dysfunction, and urinary tract infections, in up to 80% patients (Arrellano-Valdez et al., 2014). With the high incidence of diabetes, it is anticipated that urological complications will impose an extensive burden on the health and economy over the next few decades (Brown et al., 2005). Diabetic bladder dysfunction (DBD) is the most common urologic complications causing a wide range of storage and voiding problems (Daneshgari et al., 2009; Ellenberg and Weber, 1967; Liu and Daneshgari, 2014).

The previous study showed that DBD in STZ-induced diabetic rodents may include the compensated phase which occurs early after the onset of diabetes, followed by a decompensated phase that develops after a long-term duration of diabetes (Daneshgari et al., 2006; Ellenberg and Weber, 1967; Liu and Daneshgari, 2014). In the early stage, hyperglycemia-induced polyuria leads the hypertrophy and hyperplasia of the detrusor muscle to adapt to the increased volume of urine. Whereas with the continuation of hyperglycemia, polyuria, and their downstream effects, the bladder could decompensate in the late stage, with decreased micturition pressure and voiding efficiency (Ellenberg and Weber, 1967; Liu and Daneshgari, 2014; Liu et al., 2008). Clinical investigations (Kaplan and Blaivas, 1988; Kaplan et al., 1995; Kebapci et al., 2007; Pandey et al., 2018) have shown that patients with long-standing diabetes commonly experienced the symptoms of underactive bladder including poor detrusor contractility, impaired sensation of bladder fullness, incomplete voiding, increased post-void residual urine, and even overflow incontinence, which was first termed as diabetic cystopathy by Frimodt-Moller in 1976 (Moller, 1976a; Moller, 1976b; Moller, 1976c; Moller and Olesen, 1976). The underactive bladder may be related to loss of sensory nerve,

---

[*] Corresponding author at: Department of Surgery, MetroHealth Medical Center, Case Western Reserve University, 2500 Metrohealth Dr, Rammelkamp Research Building, Cleveland, OH 44109, USA.
E-mail address: guiming.liu@case.edu (G. Liu).

https://doi.org/10.1016/j.yexmp.2019.104304
Received 18 January 2019; Received in revised form 23 July 2019; Accepted 30 August 2019

impairment of nerve conduction, decreased detrusor muscle contractility and increased bladder wall fibrosis (Yoshida and Yamaguchi, 2014). Unfortunately, once the advanced stage of DBD is reached, these symptoms can't be reversed and the current therapeutic options are conservative just to relieve symptoms (Gomez et al., 2011; Liu and Daneshgari, 2014).

The change of bladder function is always accompanied by structural and molecular changes, as documented in partial outlet obstruction (Fusco et al., 2018) and spinal cord injury (Foditsch et al., 2017). Regarding the effects of diabetes on the bladder, most of the experimental studies in the literature have focused on short-term diabetes, while little is available on long-term diabetes. Characterization of the histological and molecular changes that occurred in the bladders with long-term diabetes will offer a better understanding of how the disease progressed, and may have a translational value. In this study, we examined the expression of apoptosis and fibrosis-related proteins in bladder with long-term diabetes, since increased apoptosis signaling in bladder indicates the loss of functional cells. Whereas fibrosis can adversely affect the viscoelastic properties of the bladder, resulting in decreased compliance and limited contraction amplitude.

2. Materials and methods

2.1. Animals and experimental design

Eight-week old male Lewis rats (Harlan Laboratories, Indianapolis, IN) were randomly divided into diabetic and age-matched control rats. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ, freshly prepared with citrate buffer [pH 4.5]) at 55 mg/kg of body weight, while age-matched control rats received citrate buffer only. Diabetes was confirmed by measurement of blood glucose (> 300 mg/dl) 72 h after STZ injection. To prevent excessive hyperglycemia, weight loss, and ketonuria, Insulin was given subcutaneously (up to 2 units) as needed to achieve slow weight gain without preventing hyperglycemia. All animals had free access to food and water, and were maintained for 44 weeks.

At the designed time point, non-fasting blood glucose levels were measured using a glucometer (OneTouch SureStep, Johnson & Johnson, Inc., CA). Levels of glycated hemoglobin (HbA1c) were measured using an in2itTM HbA1c analyzer (Bio-Rad, Hercules, CA). The bladders were harvested under 2% isoflurane anesthesia, and then animals were euthanized by intraperitoneal injection of pentobarbital (200 mg/kg). Bladders from some rats were removed at the level of the bladder neck, weighed, and snap-frozen in liquid nitrogen for measuring the protein expressions of oxidative stress markers, apoptotic signaling and fibrosis-related proteins by immunoblotting. While bladders from the remaining rats were sectioned at the equatorial midline. The top half of the bladder was allowed to equilibrate for 15 min at room temperature in the physiological buffer and then fixed in 10% neutral formalin for histological staining. The experimental protocol was approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

2.2. Histological analysis

After fixation, bladder tissues were dehydrated and embedded in paraffin. The cross-sections at the equatorial midline were sectioned and stained with Hematoxylin and Eosin and Masson's trichrome. Urothelium, collagen, and smooth muscle, the main components of the bladder wall, were stained into pink, blue and red respectively in Masson's trichrome stain.

2.3. Immunoblotting analysis

Homogenized bladder tissue was extracted in RIPA extraction buffer (0.5 M Tris-HCl [pH 7.4], 1.5 M sodium chloride, 2.5% deoxycholic acid and 10 mM EDTA) supplemented with protease inhibitor cocktail tablets (Sigma), then centrifuged for 15 min to remove the undissolved material. The protein concentration of the supernatant was measured using the Bradford method.

Aliquots of protein extract containing 30 μg of total protein were electrophoresed on a 4–20% SDS-PAGE gel. After transferring onto PVDF membrane (Immobilon-P, Millipore Co., MA, USA), the membrane was blocked for 1 h with 5% non-fat dried milk in TBS-T and then incubated with primary antibodies against collagen-1 (1:1000 dilution; NB600–408, Novus Biologicals), elastin (1:1000 dilution; SC-17580, Santa Cruz), transforming growth factor-beta1 (TGF-β1, 1:500 dilution; bs-0086R, Bioss), matrix metalloptoteinase-1 (MMP-1, 1:500 dilution; sc-6837, Santa Cruz), tissue inhibitor of metalloproteinase-1 (TIMP-1, 1:1000 dilution; sc-6832, Santa Cruz), nitrotyrosine (1:1000 dilution; sc-65,385, Santa Cruz), Bax (1: 200 dilution; sc-6236, Santa Cruz), Bcl-2 (1:200 dilution; sc-492, Santa Cruz), phosphorylated extracellular signal-regulated kinases 1/2 (ERK1/2, 1:1000 dilution; #9101, Cell Signaling), total ERK1/2 (1:1000 dilution; #9102, Cell Signaling), nerve growth factor (NGF, 1:500 dilution; sc-548, Santa Cruz) overnight at 4 °C, followed by the appropriate secondary antibody at room temperature for 1 h.

After successive washing, the membrane was developed using ECL Plus (Amersham Biosciences, Piscataway, NJ) and exposing the membranes to autoradiographic films. The membrane was then stripped using stripping buffer (Thermo Scientific, Rockford, IL) and reprobed with β-actin antibody (Santa Cruz, sc-47,778, 1:10000). Films were scanned and quantified with Image J Software (National Institutes of Health, Bethesda, MD) and then divided to the corresponding β-actin bands.

2.4. Statistical analysis

All the data were presented as the mean ± standard error of the mean (SEM). Student's *t*-test for unpaired data was used for determining levels of significance between control and diabetic groups. $P < .05$ was considered to indicate statistical significance. Statistical analysis was carried out using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

Table 1

General characteristics of STZ-induced diabetic and age-matched control rats. Data are expressed as mean ± SEM.

Parameters	Control rats (*n* = 4)	STZ-induced diabetic rats (n = 5)	*P* value
Body weight (g)	467.8 ± 26.22	308.8 ± 14.66	0.00082
Bladder weight (mg)	106.7 ± 7.85	164.0 ± 10.33	0.00396
Blood glucose (mg/dl)	97.5 ± 1.66	368.8 ± 36.97	0.00034
HbA1c (%)	3.1 ± 0.06	8.1 ± 0.20	< 0.00001

HbA1c: glycated hemoglobin.

3. Results

3.1. General characteristic of experimental rats

As shown in Table 1, non-fasting blood glucose and HbA1c levels were significantly higher in the diabetic rats than in the age-matched control rats. A significant body weight loss was observed in diabetic rats. Whereas the bladder weights of the diabetic rats were significantly higher than the age-matched controls.

3.2. Fibrotic changes in the bladder in long-term diabetic rats

In age-matched control rats, histological examination revealed that the mucosal surface was lined by urothelium (approximately 1–2 cell layers thick), and thrown into numerous prominent folds. Lumenal diameter and area of lamina propria and connective tissue surrounding muscle fibers in the muscularis were normal. Connective tissue was primarily limited to the core of lamina propria within the folds. In comparison, diabetic bladder showed a dilated luminal diameter, less and broader mucosal folds, comparable urothelium thickness, detrusor smooth muscle hypertrophy, and increased connective tissue in the lamina propria within the folds. Amounts of connective tissue surrounding muscle fibers in the muscularis in diabetic bladders were comparable to those in controls (Fig. 1).

3.3. Effect of long-term diabetes on elasticity- and fibrosis-related proteins in bladder

Since the elastic properties and compliance of urinary bladder are governed by relative amounts of collagen and elastin, it was of interest to evaluate their expressions in bladder tissue in long-term diabetes. A significant increase in the protein expression of collagen I was found in bladder tissue of STZ-induced diabetic rats, versus control rats, after 44 weeks ($P < .05$). Conversely, the protein expression of elastin was significantly lower in bladder tissue of diabetic rats, than their age-matched controls, ($P < .05$), as shown in Fig. 2.

Moreover, STZ-induced diabetic rats showed significant increases in protein expressions of TGF-β1 ($P < .01$) and TIMP-1 ($P < .05$), along with a marked decrease of MMP-1 level ($P < .01$) in bladder tissue of diabetic rats compared with controls (Fig. 3).

3.4. Increased oxidative stress in the bladder in long-term diabetic rats

As shown in Fig. 4, long-term diabetes enhanced the oxidative stress status, evidenced by marked increases in nitrotyrosine-containing proteins levels, as a relevant indicator for oxidative stress, in bladder tissue of diabetic group, compared with age-matched controls, ($P < .05$). The nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is the master regulator of cellular redox homeostasis through inducing the expression of genes coding for antioxidant enzymes. Long-term diabetic rats showed a significant decrease in Nrf2 protein expression in their bladder tissue, ($P < .05$).

3.5. Effect of long-term diabetes on the expressions of proteins regulated apoptosis in bladder

As shown in Fig. 5, STZ-induced diabetic rats showed higher Bax levels ($P < .001$), but lower Bcl-2 levels ($P < .0001$) in their bladder tissue, than in age-matched control rats. Since the protein expressions of Bax and Bcl-2 were measured in the same rat, we were able to calculate the Bcl-2/Bax ratio in the bladder tissue of experimental rats. The Bcl-2/Bax ratio is a good indicator for the susceptibility of cells to apoptotic death (Gillies and Kuwana, 2014). A significant decrease in the mean value of the Bcl-2/Bax ratio was found in the bladder tissue of

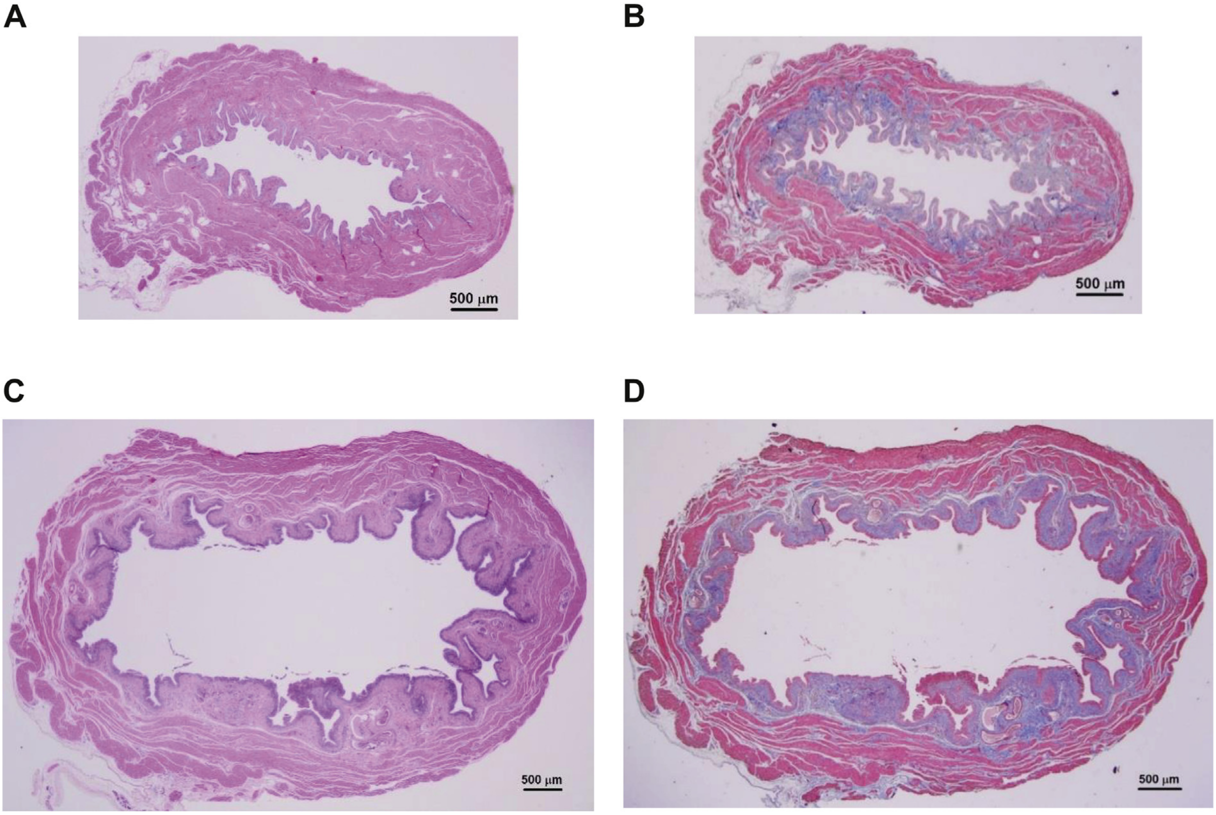

Fig. 1. Images of Hematoxylin and Eosin (A, C) and Masson's Trichrome (B, D) stained sections at equatorial midline of urinary bladders from an age-matched control (A and B) and a STZ-induced diabetic rat (C and D) 44 weeks after induction, revealing increased connective tissue in the lamina propria within the folds in diabetic bladder. Scale bar indicates 500 μm.

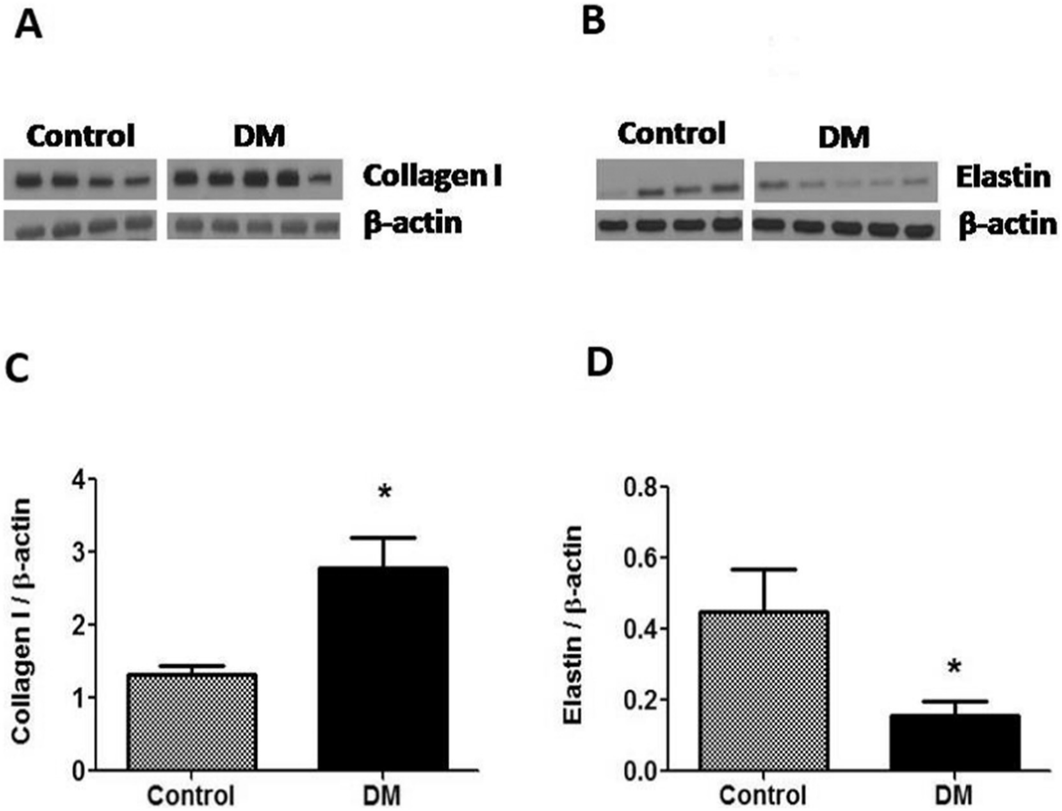

Fig. 2. Immunoblots and quantitative analysis of collagen I (A and C) and elastin (B and D) levels relative to β-actin in bladder tissues from age-matched control ($n = 4$) and STZ-induced diabetic ($n = 5$) rats 44 weeks after induction. Bars with error bars represent means ± SEM. DM, Diabetes mellitus. $^{*}P < .05$ vs age-matched control rats.

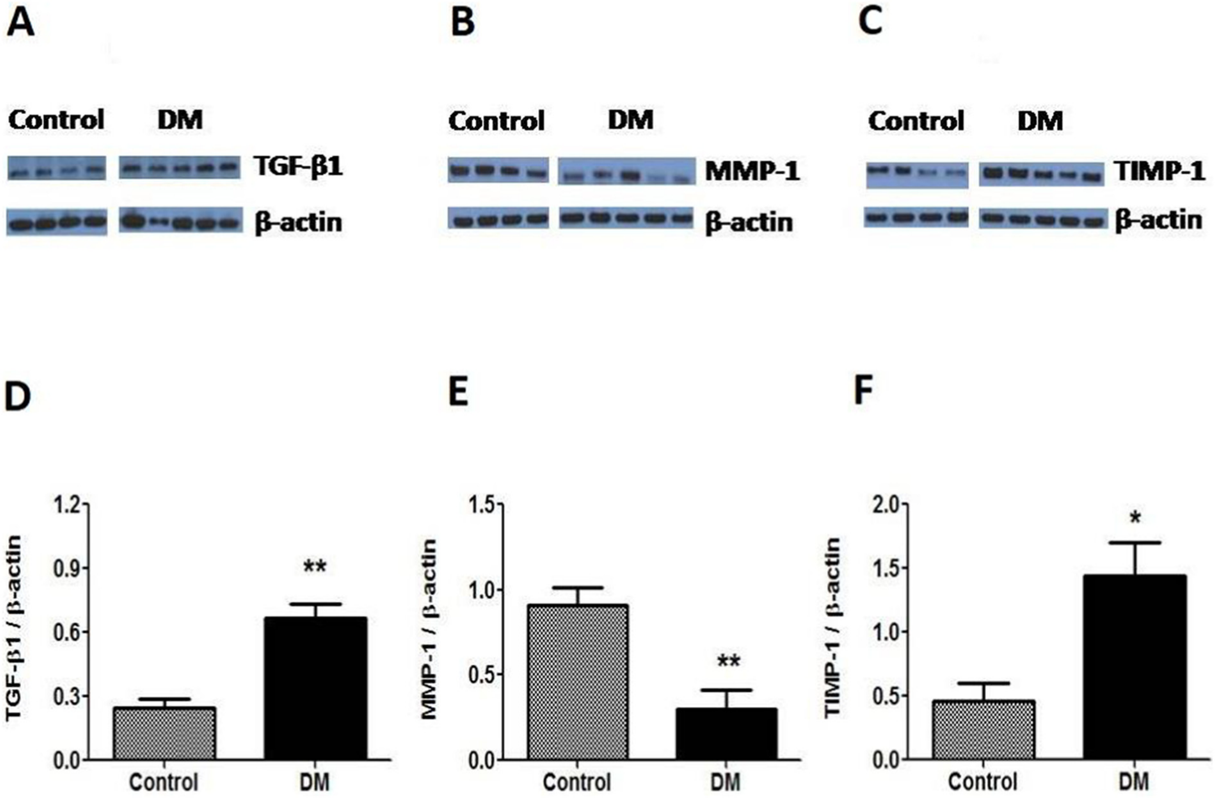

Fig. 3. Immunoblots (A, B, and C) and quantitative analysis (C, D, and E) of TGF-β (A and D), MMP-1 (B and E) and TIMP-1(C and F) relative to β-actin in bladder tissues from age-matched control (n = 4) and STZ-induced diabetic (n = 5) rats 44 weeks after induction. Bars with error bars represent means ± SEM. DM, Diabetes mellitus. $^{*}P < .05$ vs age-matched control rats; $^{**}P < .01$ vs age-matched control rats.

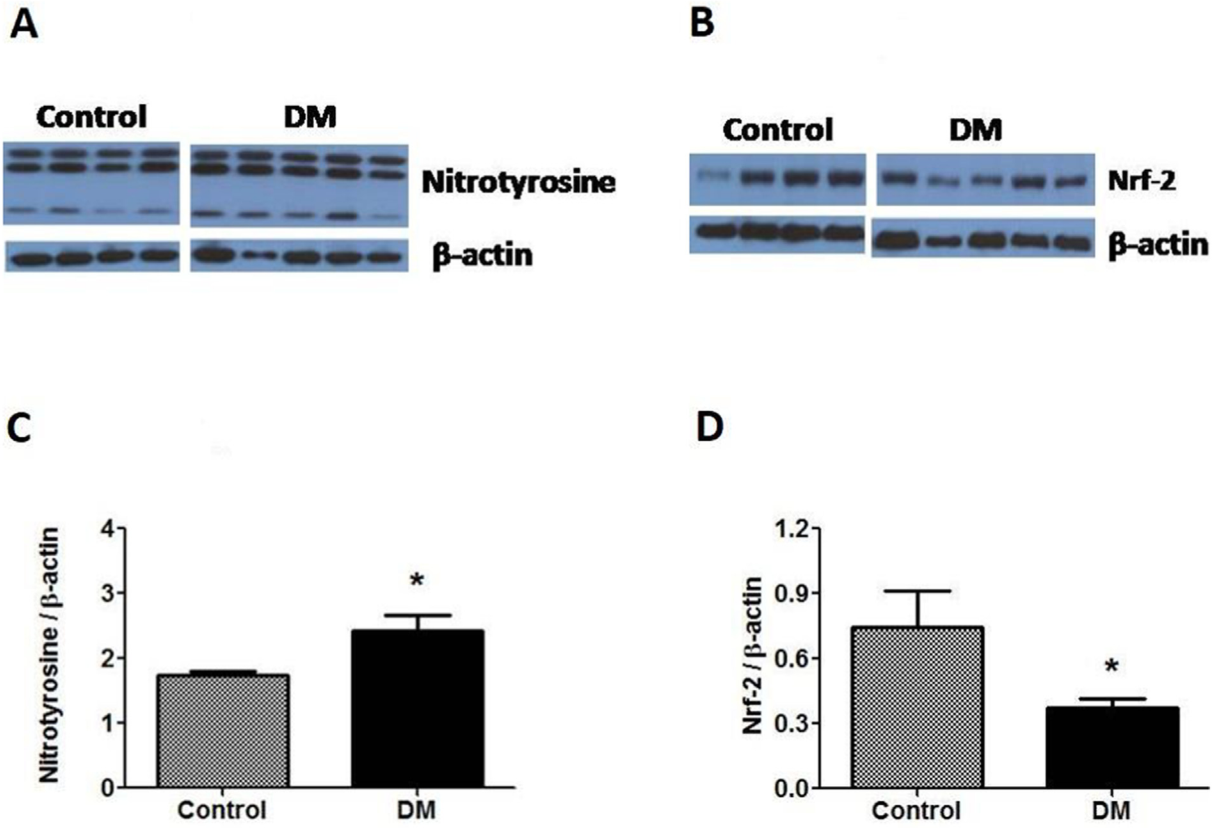

Fig. 4. Immunoblots and quantitative analysis of nitrotyrosine (A and C) and Nrf2 (B and D) relative to β-actin in bladder tissues from age-matched control (n = 4) and STZ-induced diabetic (n = 5) rats 44 weeks after induction. Bars with error bars represent means ± SEM. DM, Diabetes mellitus. *$P < .05$ vs age-matched control rats.

diabetic rats versus age-matched controls.

3.6. Decreased expression of NGF in long-term diabetic rat bladder

NGF is a good marker for neuronal survival and growth (Ceni et al., 2014). As shown in Fig. 6, STZ-induced diabetic rats displayed a marked down-regulation of NGF expression in bladder tissue, compared with age-matched controls, ($P < .0001$).

3.7. Decreased ERK1/2 activation in long-term diabetic rat bladder

As shown in Fig. 7, there was a significant suppression in the protein expression of phosphorylated ERK1/2 in bladder tissue of STZ-induced diabetic rats, compared with age-matched controls ($P < .01$).

4. Discussion

Clinical investigations revealed that long-term diabetes results in diabetic cystopathy including impairment of bladder sensation, decreased detrusor contractility, increased bladder capacity and post-void residual volume (Frimodt-Moller, 1978; Kaplan and Blaivas, 1988; Pandey et al., 2018). The underlying mechanisms involved in these functional changes are not clear. The current study demonstrated that long-term diabetes (44 weeks after diabetes induction), induced alterations of proteins related to fibrosis and apoptosis in the bladder, which may underlie diabetic cystopathy in long-term diabetes, while diabetes-induced oxidative stress might play a fundamental role in those alterations.

One finding of the current study is the fibrotic change in the bladder of long-term diabetic rats. Similar to the early stage of diabetes (Liu and Daneshgari, 2006), bladder hypertrophy was also found in rats with long-term diabetes, represented by a significant increase in bladder weight. Histological examination indicated detrusor smooth muscle hypertrophy along with increased connective tissue in the lamina propria in long-term diabetic rats. We further measured the levels of two important extracellular matrix proteins, collagen I and elastin, and found that collagen I increased, but elastin decreased significantly. These results indicated that long-term diabetes caused fibrotic changes in the bladder. Excessive accumulation of collagen fibers and increased fibrosis adversely affects the elasticity and contractile properties of the bladder, leading to bladder dysfunction. On the contrary, previous studies showed that bladder compliance increased (Wang et al., 2009), along with decreased collagen and increased elastin protein content at the early stage of diabetes: 2 and 8 weeks after diabetes induction (Gray et al., 2008). These observations together suggested that diabetes caused time-dependent alterations in the bladder.

The balance between synthesis and degradation of collagen is mainly regulated by an orchestrated production of growth factors, including TGF-β. TGF-β, a key fibrogenic cytokine, is produced by fibroblast, hematopoietic cells, and many parenchymal cells (Pohlers et al., 2009). TGF-β1 induces fibrosis via promoting the transformation of fibroblasts into myofibroblasts, the proliferation of myofibroblasts, and synthesis of extracellular matrix proteins, but inhibiting extracellular matrix degradation. We found that the level of TGF-β1 in bladder increased significantly in long-term diabetic rats compared to that in control rats. A high level of TGF-β1 could promote the fibrotic changes in the bladder. Howard et al. showed that TGF- β1 treatment of human detrusor smooth muscle cells could induce hypertrophy and collagen I and III syntheses in a dose-dependent manner (Howard et al., 2005). Vice versa, some studies have demonstrated that inhibition of TGF-β1 exerts potent antifibrotic effects in different organs (Gyorfi et al., 2018).

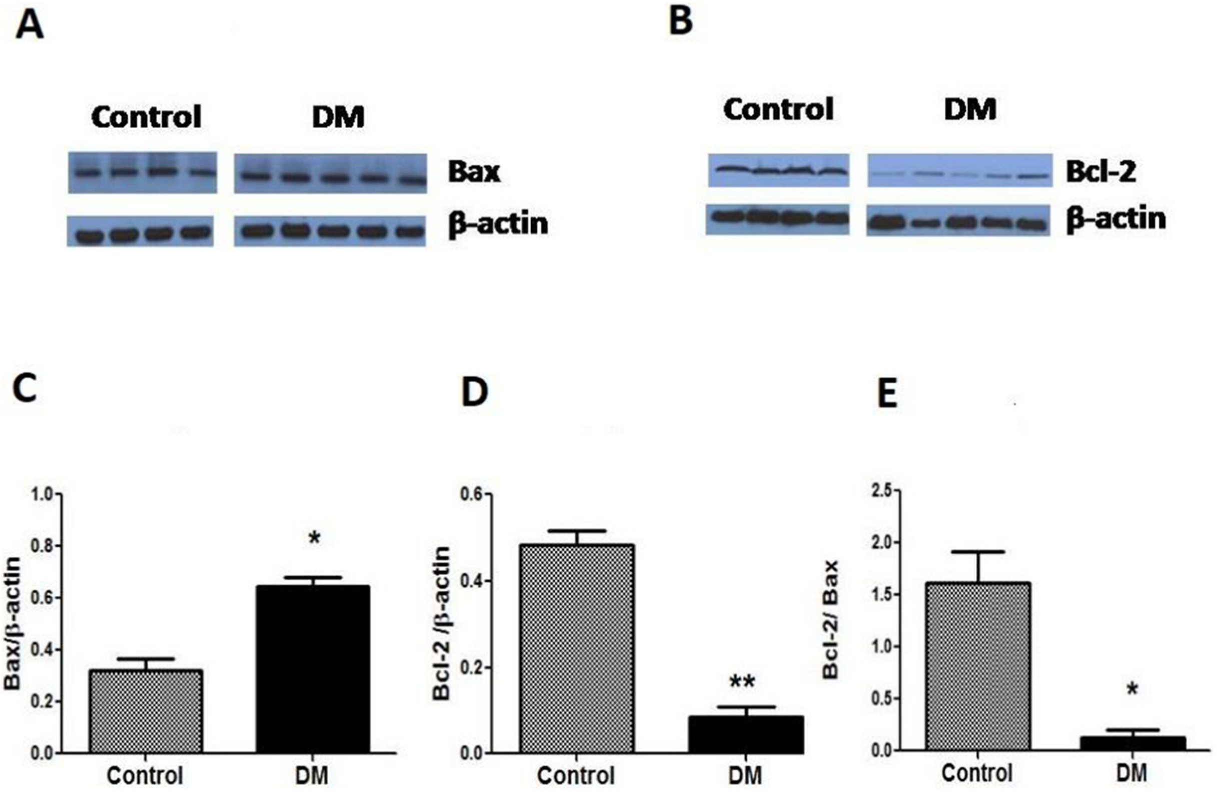

Fig. 5. Immunoblots and quantitative analysis of Bax (A and C), Bcl-2 (B and D) relative to β-actin, as well as Bcl-2/Bax ratio (E) in bladder tissues from age-matched control (n = 4) and STZ-induced diabetic (n = 5) rats 44 weeks after induction. Bars with error bars represent means ± SEM. DM, Diabetes mellitus. $^*P < .001$ vs age-matched control rats; $^{**}P < .0001$ vs age-matched control rats.

The effects of TGF-β1 on the promotion of bladder fibrosis may be partially through modulation of the balance between proteolytic enzymes and their endogenous inhibitors in the extracellular matrix: matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Ma and Chegini, 1999). In our study, MMP-1 was significantly down-regulated in bladder tissue 44 weeks after diabetes induction, while TIMP-1 levels increased. MMPs can degrade the components of the extracellular matrix, including collagens, elastins, gelatin, matrix glycoproteins and proteoglycans (Wang and Khalil, 2018). The MMP-1 can degrade collagen I efficiently (Wang and Khalil, 2018). The activity of MMPs is regulated by their endogenous inhibitors, TIMPs (Wang and Khalil, 2018). Using primary cultures of human myometrial smooth muscle cells, Ma et al. found that TGF-β1 (1 ng/ml) increased the expression of TIMP-1 mRNA, while it reduced the expression of MMP-1 and MMP-3 mRNA (Ma and Chegini, 1999). These regulatory effects were confirmed by other investigators (Hall et al., 2003; Verrecchia and Mauviel, 2002). The imbalance between MMP-1 and TIMP-1 favored the accumulation of extracellular matrix components and hence bladder fibrosis in long-term diabetic rats.

Metabolic abnormalities in diabetes cause excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can impair cellular functions and activate multiple pathological processes (Newsholme et al., 2012). The nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is a member of the cap-n-collar (CNC) subfamily of basic region leucine zipper transcription factors (Ma, 2013). Nrf2 induces multiple genes coding for antioxidant (superoxide dismutases, catalase, glutathione peroxidase, glutathione s-transferase, etc.) and stress enzyme (heme oxygenase-1, ferritins, etc.) and plays an important role in the protection of tissue against oxidative stress (Uruno et al., 2015). The deletion of *Nrf2* in mice substantially increased the susceptibility of livers to various disease conditions related to oxidative stress. Whereas pharmacological inducers of Nrf2 activity protected animals from oxidative damage (Klaassen and Reisman, 2010). In our study, long-term diabetes resulted in a decrease of Nrf2 expression, along with the increase of nitrotyrosine, indicating enhanced oxidative stress. Recent studies have shown that ROS can induce/activate TGF-β1 (Cui et al., 2011; Jaffer et al., 2015). On the other hand, TGF-β1 has been demonstrated to increase ROS production (Abe et al., 2013) and suppress antioxidant enzymes (Michaeloudes et al., 2011). These reciprocal interactions between TGF-β and ROS may play an important role in promoting bladder fibrosis in long-term diabetes (Liu and Desai, 2015). ROS also have been shown to activate MMPs directly and induce their transcription, (Nelson and Melendez, 2004) which may exacerbate the fibrotic process in long-term diabetes.

Another finding of the current study is the enhanced apoptotic signaling seen in the bladder of long-term diabetic rats, represented by a lower Bcl-2/Bax ratio due to significant upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-2**.** Similar alterations were found in skin biopsy in type 2 diabetic patients (Hasnan et al., 2010). These results indicated that long-term diabetes could activate the mitochondrial pathway of apoptosis, which might cause loss of functional cells in the bladder, and ultimately impair the bladder function. An earlier study showed that significantly increased apoptotic cells were detected within the smooth muscle cell layers of the 8-week diabetic bladder but not in 5% sucrose-induced diuretic bladder (Beshay and Carrier, 2004), indicating the key role of hyperglycemia in this process. ROS has been reported to cause the release of cytochrome *c* from mitochondria and the induction of apoptosis through the mitochondrial pathway (Lv et al., 2016). In another study, ROS have been shown to promote apoptosis in T cells by downregulation of Bcl-2 (Hildeman et al., 2003). Nie at al showed that oxidation of conserved cys-62 on the Bax molecule induced Bax activation and mitochondrial permeabilization (Nie et al., 2008).

Accumulating evidence indicates that autonomic neuropathy is implicated in diabetic cystopathy. We have shown that the nerve density decreased in the bladder in rats 20 weeks after diabetes induction

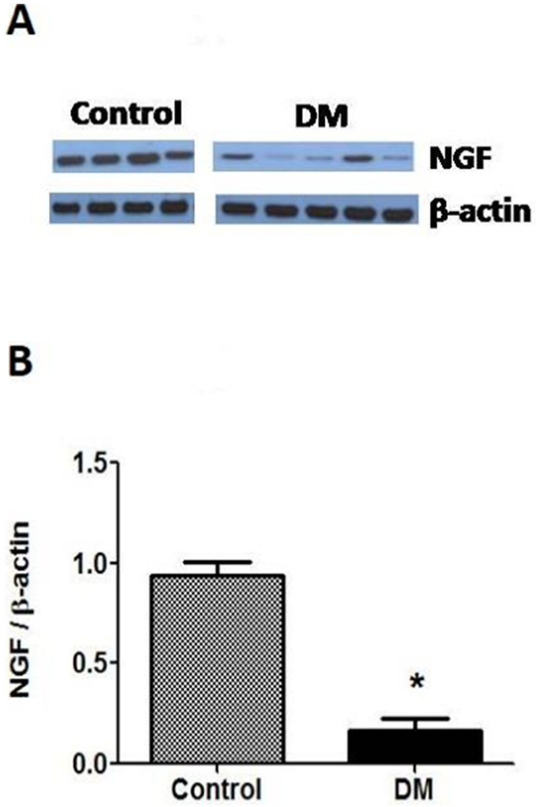

Fig. 6. Immunoblots and quantitative analysis of NGF (A and B) relative to β-actin in bladder tissues from age-matched control (n = 4) and STZ-induced diabetic (n = 5) rats 44 weeks after induction. Bars with error bars represent means ± SEM. DM, Diabetes mellitus. *P < .0001 vs age-matched control rats.

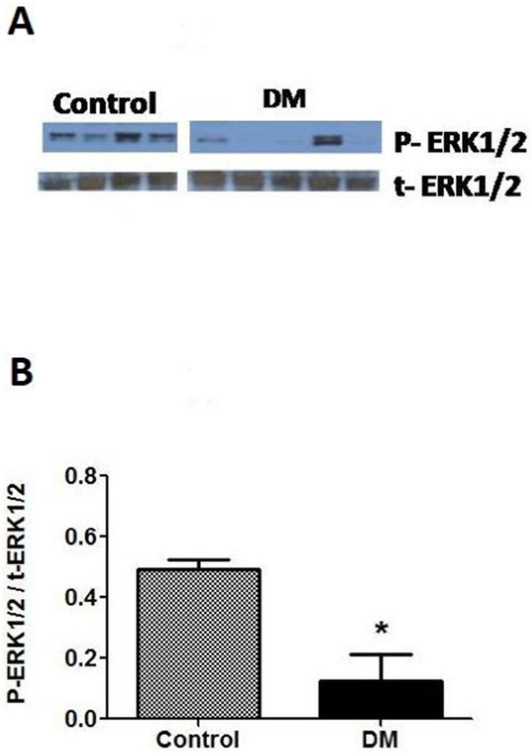

Fig. 7. Immunoblots and quantitative analysis of phosphorylated ERK1/2 relative to total ERK1/2 (A and B) in bladder tissues from age-matched control (n = 4) and STZ-induced diabetic (n = 5) rats 44 weeks after induction. Bars with error bars represent means ± SEM. DM, Diabetes mellitus. *P < .01 vs age-matched control rats.

(Liu et al., 2011). NGF, a member of the neurotrophin family, and a potent pro-survival factor, plays a key role in regulating survival in the developing and injured nervous system (Ceni et al., 2014). Withdrawal of NGF from NGF-dependent neurons results in apoptotic neuronal death (Deshmukh and Johnson Jr., 1997; Kristiansen et al., 2011). Our results showed markedly decreased NGF expression in the bladder of rats with long-term diabetes, which might contribute to neuronal apoptosis, resulting in decreased bladder sensation and incomplete emptying seen in long-term diabetic patients (Kaplan and Blaivas, 1988). Previous studies showed that NGF levels were decreased in the bladder in STZ-induced diabetic rats as early as 12 weeks after diabetes induction, along with increased post-void residual volume and impaired bladder sensation (Sasaki et al., 2002). Neuroprotection by NGF is mainly mediated by the activation of ERK mitogen-activated protein kinases (MAPKs) signaling pathway (Egea et al., 2000; Riccio et al., 1999). NGF activates ERK1/2, phosphorylates the transcription factor cyclic adenosine monophosphate response element binding protein (CREB), thus increases the expression of Bcl-2 and promotes survival of neurons (Egea et al., 2000; Riccio et al., 1999). Increased oxidative stress has been reported to impair neurotrophic support to the peripheral nerves (Hounsom et al., 2001; Obrosova et al., 2001) which may accelerate neurodegeneration in the bladder. On the other hand, NGF is an important modulator of antioxidant mechanisms. NGF can induce expression of antioxidant enzymes, such as superoxide dismutase and catalase (Sampath and Perez-Polo, 1997), thereby neutralizing superoxide anion radicals and hydrogen peroxide. Therefore, NGF deficiency further disrupts the antioxidative defense system.

Hyperglycemia, increased oxidative stress and multiple cytokines released in diabetes can adversely impact cell fate via the modulation of multiple signaling cascades. The MAPKs regulate diverse cellular functions by relaying extracellular signals to intracellular responses (Cargnello and Roux, 2011). ERK signaling, one of the best known classical MAPKs pathways, is triggered by activation of the small G-protein Ras at the cellular membrane. In ype 2 diabetic mouse model, we previously found that ERK1/2 signaling may be critically involved in the pathophysiology of diabetic bladder dysfunction (Tomechko et al., 2015). Activated ERK1/2 signaling regulates cell growth, differentiation, apoptosis and survival through phosphorylation of cytoplasmic targets, and/or transcriptional factors targets in the nucleus (Roskoski Jr., 2012). ERK1/2 has been shown to exert anti-apoptotic effects by downregulating pro-apoptotic molecules (BAD, Bim, etc.) and upregulating anti-apoptotic molecules (Mcl-1, Bcl-XL, etc.) via affecting their activity or transcription (Lu and Xu, 2006). Inhibition of ERK1/2 causes arrest at the G1 phase of the cell cycle and downregulation of the anti-apoptotic molecules of Bcl-2 (Lu and Xu, 2006). However, activated ERK also has been reported to exert a pro-apoptotic role (Dong et al., 2004). The balance among the intensity and duration of pro- versus anti-apoptotic effects induced by ERK1/2 activation determines cell fate (Mebratu and Tesfaigzi, 2009; Pearson et al., 2001). In vitro study showed that both hyperglycemia (Feliers and Kasinath, 2010) and oxidative stress (Kemmerling et al., 2007) can increase the phosphorylation of ERK1/2. Animal studies showed that ERK activity increased in heart at 1 week (Strniskova et al., 2003), liver at 2 and 8 weeks (Martinovic et al., 2012), dorsal root ganglion (DRG) (Purves et al., 2001) and testis (Kong et al., 2016) at 8 weeks, in STZ-induced diabetic rats. However, our study showed that long-term diabetes caused a significant decrease in the activated forms of ERK1/2 in the bladder. This might be due to the sustained effect of hyperglycemia, oxidative stress, and inflammatory cytokines, etc., which could desensitize/exhaust the elements of the ERK activation pathway. Another possible reason might be related to the reduction of extracellular activators such as NGF, which was decreased significantly in long-term diabetes. The pro-survival effect of ERK1/2 may protect the bladder from the injury induced by diabetes. A previous study showed that mice with cardiomyocyte-specific deletion of the *erk2* gene developed deterioration in cardiac function following prolonged pathological hypertrophic stresses, accompanied by increased cardiomyocytes apoptosis (Ulm et al., 2014).

5. Conclusions

We have demonstrated that long-term diabetes causes molecular changes related to fibrosis and apoptosis in the bladder. Increased TGF-β1 and the imbalance between MMP-1 and TIMP-1 contribute to the accumulation of collagen. Diminished Bcl-2/Bax ratio and suppressed ERK1/2 activation promote apoptosis. The decreased production of NGF favors neuronal apoptotic death. Diabetes-induced oxidative stress might be the central mediator of the above pathological changes. The results of this study provide insights into the potential mechanisms of bladder dysfunction in long-term diabetes. Further studies are needed to determine if the prevention or correction of these changes will prevent or delay the occurrence of bladder dysfunction in long-term diabetes.

Declaration of Competing Interest

The authors declare no conflict of interests.

References

Abe, Y., et al., 2013. TGF-beta1 stimulates mitochondrial oxidative phosphorylation and generation of reactive oxygen species in cultured mouse podocytes, mediated in part by the mTOR pathway. Am. J. Physiol. Renal Physiol. 305, F1477–F1490.

Arrellano-Valdez, F., et al., 2014. A comprehensive review of urologic complications in patients with diabetes. Springerplus. 3, 549.

Beshay, E., Carrier, S., 2004. Oxidative stress plays a role in diabetes-induced bladder dysfunction in a rat model. Urology. 64, 1062–1067.

Brown, J.S., et al., 2005. Urologic complications of diabetes. Diabetes Care 28, 177–185.

Cargnello, M., Roux, P.P., 2011. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol. Mol. Biol. Rev. 75, 50–83.

Ceni, C., et al., 2014. Neurotrophins in the regulation of cellular survival and death. Handb. Exp. Pharmacol. 220, 193–221.

Cui, Y., et al., 2011. Oxidative stress contributes to the induction and persistence of TGF-beta1 induced pulmonary fibrosis. Int. J. Biochem. Cell Biol. 43, 1122–1133.

Daneshgari, F., et al., 2006. Time dependent changes in diabetic cystopathy in rats include compensated and decompensated bladder function. J. Urol. 176, 380–386.

Daneshgari, F., et al., 2009. Diabetic bladder dysfunction: current translational knowledge. J. Urol. 182, S18–S26.

Deshmukh, M., Johnson Jr., E.M., 1997. Programmed cell death in neurons: focus on the pathway of nerve growth factor deprivation-induced death of sympathetic neurons. Mol. Pharmacol. 51, 897–906.

Dong, J., et al., 2004. EGFR-independent activation of p38 MAPK and EGFR-dependent activation of ERK1/2 are required for ROS-induced renal cell death. Am. J. Physiol. Renal Physiol. 287, F1049–F1058.

Egea, J., et al., 2000. Nerve growth factor activation of the extracellular signal-regulated kinase pathway is modulated by Ca(2+) and calmodulin. Mol. Cell. Biol. 20, 1931–1946.

Ellenberg, M., Weber, H., 1967. The incipient asymptomatic diabetic bladder. Diabetes. 16, 331–335.

Feliers, D., Kasinath, B.S., 2010. Mechanism of VEGF expression by high glucose in proximal tubule epithelial cells. Mol. Cell. Endocrinol. 314, 136–142.

Foditsch, E.E., et al., 2017. Structural changes of the urinary bladder after chronic complete spinal cord injury in minipigs. Int. Neurourol. J. 21, 12–19.

Frimodt-Moller, C., 1978. Diabetic cystopathy. A review of the urodynamic and clinical features of neurogenic bladder dysfunction in diabetes mellitus. Dan. Med. Bull. 25, 49–60.

Fusco, F., et al., 2018. Progressive bladder remodeling due to bladder outlet obstruction: a systematic review of morphological and molecular evidences in humans. BMC Urol. 18, 15.

Gillies, L.A., Kuwana, T., 2014. Apoptosis regulation at the mitochondrial outer membrane. J. Cell. Biochem. 115, 632–640.

Gomez, C.S., et al., 2011. Bladder dysfunction in patients with diabetes. Curr. Urol Rep. 12, 419–426.

Gray, M.A., et al., 2008. Time-dependent alterations of select genes in streptozotocin-induced diabetic rat bladder. Urology. 71, 1214–1219.

Gyorfi, A.H., et al., 2018. Targeting TGF-beta signaling for the treatment of fibrosis. Matrix Biol. 68-69, 8–27.

Hall, M.C., et al., 2003. The comparative role of activator protein 1 and Smad factors in the regulation of Timp-1 and MMP-1 gene expression by transforming growth factor-beta 1. J. Biol. Chem. 278, 10304–10313.

Hasnan, J., et al., 2010. Relationship between apoptotic markers (Bax and Bcl-2) and biochemical markers in type 2 diabetes mellitus. Singap. Med. J. 51, 50–55.

Hildeman, D.A., et al., 2003. Control of Bcl-2 expression by reactive oxygen species. Proc. Natl. Acad. Sci. U. S. A. 100, 15035–15040.

Hounsom, L., et al., 2001. Oxidative stress participates in the breakdown of neuronal phenotype in experimental diabetic neuropathy. Diabetologia. 44, 424–428.

Howard, P.S., et al., 2005. Transforming growth factor-beta1-induced hypertrophy and matrix expression in human bladder smooth muscle cells. Urology. 66, 1349–1353.

Jaffer, O.A., et al., 2015. Mitochondrial-targeted antioxidant therapy decreases transforming growth factor-beta-mediated collagen production in a murine asthma model. Am. J. Respir. Cell Mol. Biol. 52, 106–115.

Kaplan, S.A., Blaivas, J.G., 1988. Diabetic cystopathy. J. Diabet. Complicat. 2, 133–139.

Kaplan, S.A., et al., 1995. Urodynamic findings in patients with diabetic cystopathy. J. Urol. 153, 342–344.

Kebapci, N., et al., 2007. Bladder dysfunction in type 2 diabetic patients. Neurourol. Urodyn. 26, 814–819.

Kemmerling, U., et al., 2007. Calcium release by ryanodine receptors mediates hydrogen peroxide-induced activation of ERK and CREB phosphorylation in N2a cells and hippocampal neurons. Cell Calcium 41, 491–502.

Klaassen, C.D., Reisman, S.A., 2010. Nrf2 the rescue: effects of the antioxidative/electrophilic response on the liver. Toxicol. Appl. Pharmacol. 244, 57–65.

Kong, W.Y., et al., 2016. The calcium-sensing receptor participates in testicular damage in streptozotocin-induced diabetic rats. Asian J. Androl. 18, 803–808.

Kristiansen, M., et al., 2011. Global analysis of gene expression in NGF-deprived sympathetic neurons identifies molecular pathways associated with cell death. BMC Genomics 12, 551.

Liu, G., Daneshgari, F., 2006. Temporal diabetes- and diuresis-induced remodeling of the urinary bladder in the rat. Am. J. Physiol. Regul. Integr. Comp Physiol. 291, R837–R843.

Liu, G., Daneshgari, F., 2014. Diabetic bladder dysfunction. Chin. Med. J. 127, 1357–1364.

Liu, R.M., Desai, L.P., 2015. Reciprocal regulation of TGF-beta and reactive oxygen species: a perverse cycle for fibrosis. Redox Biol. 6, 565–577.

Liu, G., et al., 2008. External urethral sphincter activity in diabetic rats. Neurourol. Urodyn. 27, 429–434.

Liu, G., et al., 2011. Temporal diabetes and diuresis-induced alteration of nerves and vasculature of the urinary bladder in the rat. BJU Int. 107, 1988–1993.

Lu, Z., Xu, S., 2006. ERK1/2 MAP kinases in cell survival and apoptosis. IUBMB Life 58, 621–631.

Lv, B., et al., 2016. Crocin protects retinal ganglion cells against H2O2-induced damage through the mitochondrial pathway and activation of NF-kappaB. Int. J. Mol. Med. 37, 225–232.

Ma, Q., 2013. Role of nrf2 in oxidative stress and toxicity. Annu. Rev. Pharmacol. Toxicol. 53, 401–426.

Ma, C., Chegini, N., 1999. Regulation of matrix metalloproteinases (MMPs) and their tissue inhibitors in human myometrial smooth muscle cells by TGF-beta1. Mol. Hum. Reprod. 5, 950–954.

Martinovic, V., et al., 2012. Activation level of JNK and Akt/ERK signaling pathways determinates extent of DNA damage in the liver of diabetic rats. Cell. Physiol. Biochem. 30, 723–734.

Mebratu, Y., Tesfaigzi, Y., 2009. How ERK1/2 activation controls cell proliferation and cell death: is subcellular localization the answer? Cell Cycle 8, 1168–1175.

Michaeloudes, C., et al., 2011. TGF-beta regulates Nox4, MnSOD and catalase expression, and IL-6 release in airway smooth muscle cells. Am. J. Physiol. Lung Cell Mol. Physiol. 300, L295–L304.

Moller, C.F., 1976a. Diabetic cystopathy. III:urinary bladder dysfunction in relation to bacteriuria. Dan. Med. Bull. 23, 287–291.

Moller, C.F., 1976b. Diabetic cystopathy.I: a clinical study of the frequency of bladder dysfunction in diabetics. Dan. Med. Bull. 23, 267–278.

Moller, C.F., 1976c. Diabetic cystopathy.II: relationship to some late-diabetic manifestations. Dan. Med. Bull. 23, 279–287.

Moller, C.F., Olesen, K.P., 1976. Diabetic cystopathy. IV: micturition cystourethrography compared with urodynamic investigation. Dan. Med. Bull. 23, 291–294.

Nelson, K.K., Melendez, J.A., 2004. Mitochondrial redox control of matrix metalloproteinases. Free Radic. Biol. Med. 37, 768–784.

Newsholme, P., et al., 2012. Reactive oxygen and nitrogen species generation, antioxidant defenses, and beta-cell function: a critical role for amino acids. J. Endocrinol. 214, 11–20.

Nie, C., et al., 2008. Cysteine 62 of Bax is critical for its conformational activation and its proapoptotic activity in response to H2O2-induced apoptosis. J. Biol. Chem. 283, 15359–15369.

Obrosova, I.G., et al., 2001. Taurine counteracts oxidative stress and nerve growth factor deficit in early experimental diabetic neuropathy. Exp. Neurol. 172, 211–219.

Pandey, S., et al., 2018. Chronic urinary retention due to diabetic cystopathy masquedering as mesenteric cyst. BMJ Case Rep. 2018.

Pearson, G., et al., 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr. Rev. 22, 153–183.

Pohlers, D., et al., 2009. TGF-beta and fibrosis in different organs - molecular pathway imprints. Biochim. Biophys. Acta 1792, 746–756.

Purves, T., et al., 2001. A role for mitogen-activated protein kinases in the etiology of diabetic neuropathy. FASEB J. 15, 2508–2514.

Riccio, A., et al., 1999. Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science. 286, 2358–2361.

Roskoski Jr., R., 2012. ERK1/2 MAP kinases: structure, function, and regulation. Pharmacol. Res. 66, 105–143.

Sampath, D., Perez-Polo, R., 1997. Regulation of antioxidant enzyme expression by NGF.

Neurochem. Res. 22, 351–362.

Sasaki, K., et al., 2002. Diabetic cystopathy correlates with a long-term decrease in nerve growth factor levels in the bladder and lumbosacral dorsal root ganglia. J. Urol. 168, 1259–1264.

Strniskova, M., et al., 2003. Mitogen-activated protein kinases in the acute diabetic myocardium. Mol. Cell. Biochem. 249, 59–65.

Tomechko, S.E., et al., 2015. Tissue specific dysregulated protein subnetworks in type 2 diabetic bladder urothelium and detrusor muscle. Mol. Cell. Proteomics 14, 635–645.

Ulm, S., et al., 2014. Targeted deletion of ERK2 in cardiomyocytes attenuates hypertrophic response but provokes pathological stress induced cardiac dysfunction. J. Mol. Cell. Cardiol. 72, 104–116.

Uruno, A., et al., 2015. The Keap1-Nrf2 system and diabetes mellitus. Arch. Biochem. Biophys. 566, 76–84.

Verrecchia, F., Mauviel, A., 2002. Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. J. Invest. Dermatol. 118, 211–215.

Wang, X., Khalil, R.A., 2018. Matrix metalloproteinases, vascular remodeling, and vascular disease. Adv. Pharmacol. 81, 241–330.

Wang, C.C., et al., 2009. Diabetes-induced alternations in biomechanical properties of urinary bladder wall in rats. Urology. 73, 911–915.

Yoshida, M., Yamaguchi, O., 2014. Detrusor underactivity: the current concept of the pathophysiology. Low Urin. Tract. Symptoms. 6, 131–137.