行政院國家科學委員會專題研究計畫 成果報告

自由基在糖尿病誘發膀胱病變所扮演角色的探討(2/2)
研究成果報告(完整版)

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Abstract

Patients with diabetes may develop bladder dysfunction (diabetic cystopathy), which can present in a number of ways—from impaired detrusor contractility to detrusor overactivity. The prevalence of diabetic cystopathy was believed to be common, with a reported prevalence of 25% to 87%.Vesical dysfunction develops insidiously so that at first the patient may be completely asymptomatic until progressive decompensation develops, of the asymptomatic diabetic bladder is characterized by marked residual urine, secondary infection, pyelonephritis, sepsis, and azotemia. The pathophysiological mechanism underlying the diabetes induced bladder dysfunction remains unclear. It has been suggested that diabetic urinary bladder dysfunction is initiated by a visceral sensory neuropathy involving the afferent limb of the micturition reflex arc. However, several reports also suggest that alterations may also exist at the level of the bladder smooth muscle itself. These include changes in postsynaptic muscarinic receptor function and possible changes in intracellular second messenger systems.

Over recent years there has been considerable interest in the possibility that altered ROS may be an underlying abnormality in many complications of diabetes mellitus. Accumulating evidences have indicated that the generation of reactive oxygen species (oxidative stress) may play an important role in the etiology of diabetic complications. Several pathways are leading to oxidative stress associated with acute or chronic hyperglycemia, such as the polyol pathway, prostanoid synthesis, glucose autoxidation, and protein glycation by increasing the production of free radicals and each of the three major pathways might be responsible for diabetic damage.

Advanced glycation end products (AGEs) are a heterogeneous group of irreversible adducts resulting from nonenzymatic glycation and oxidation of proteins, lipids, and nucleic acids. AGEs formation has been shown to induce cellular signaling, activation of transcription factors, and gene expression in vitro and in vivo, subsequently alters the structural and functional properties of proteins, lipid components, and nucleic acids and induce pathogenic changes in endothelial cells. Many recent studies have suggested that increased formation of tissue AGEs correlate with early manifestations of renal and retinal complications in patients with diabetes. In addition, administration of AGEs led to the activation of NF-κB, and subsequent VCAM-1 gene expression, resulting in subsequent late diabetic complication.

Although the role of oxidative stress and AGEs formation in late diabetic complications such as diabetic retinopathy, neuropathy, nephropathy and vasculopathy have been demonstrated in several human and animal researches, the

possible involvement of them in diabetic vesicopathy has rarely been studied. In the present study, therefore, we are interested to investigate the possible involvement of ROS in the development of diabetic vesicopathy. Specifically, we will look into the AGEs formation in streptozotocin-induced diabetic rats. First, we will use ELISA method to measure the AGEs formation in diabetic bladder. Secondary, we will use immunohistochemistry method to localize the areas in which AGEs are generated. Through these experimental approaches, we hope to get better insight into the pathopysiology of diabetic vesicopathy.

摘要

慢性糖尿病誘發膀胱病變而造成排尿功能障礙，是臨床上相當常見的問題. 據統計大約25%-85%的糖尿病患者會有不等程度的膀胱功能障礙，輕微者可能只有頻尿及逼尿肌功能亢進等症狀，而較嚴重者造成感覺及收縮功能都發生異常，最後可能惡化成無感覺及無張力的神經性膀胱. 動物研究顯示，自主神經病變可能是造成膀胱功能異常的原因，另一方面，膀胱肌肉細胞內的信息傳遞路徑發生異常，也可能扮演相當重要的角色. 究竟是哪些機轉誘發自主神經病變或是肌肉細胞功能的異常，則有待進一步研究. 本研究計劃將針對自由基與AGEs(advanced glycosylation end products)可能扮演的角色來探討.

自由基對於細胞的傷害，在許多疾病如老化、器官缺血再灌流甚至癌症生成等都扮演很重要的角色. 在動物及人體的研究也顯示，糖尿病患者血液中高濃度血糖過氧化所後產生的自由基對於末稍血管及神經的傷害，可能是造成一些糖尿病患者併發症的主因之一. 近年來許多較深入的研究顯示，在糖尿病的患者或動物血中的高濃度糖分可對某些蛋白質，脂肪，或核酸作用，產生一些AGEs化合物，進而活化血管內皮神經，血球等細胞內的NF-κB. 被活化的NF-κB會催化一些物質如TGF-α, interleukines, adhesion molecules, cyclooxygenase..等的分泌，進而造成一些器官如血管，腎臟，神經及視網膜的病變.

關於自由基與AGEs在糖尿病誘發的一些器官病變如血管，腎臟，神經及視網膜中所扮演的角色的相關研究相當多，但針對於糖尿病造成膀胱功能障礙的深入性研究則相當缺乏. 因此本計畫希望以streptozotocin 注射誘發糖尿病鼠的模式，針對AGEs在糖尿病誘發膀胱功能障礙可能扮演的角色來探討. 主要研究方式是以ELISA方式來分析糖尿病鼠其膀胱組織中AGE濃度的變化. 另外，再以免疫染色方式，來找出自由基與AGE在膀胱組織中分佈的情形，希望藉由此研究，能對糖尿病誘發的膀胱神經與肌肉病變的病理機轉有更深入的了解.

Introduction

Patients with diabetes may develop diabetic cystopathy, characterized mainly by impaired detrusor sensation and contractility [1-2]. Impaired

detrusor contractility may lead to incomplete bladder emptying and subsequently resulting in voiding difficulty, urinary retention, chronic urinary tract infection (UTI), and upper urinary tract damage [1, 3-4]. Although diabetic cystopathy is common, with reported prevalence ranging from 25% to 87% [1], it is frequently unrecognized by patients and physicians due to its insidious development and inconspicuous symptoms. Usually, genitourinary dysfunction in diabetic patients has reached an advanced stage by the time urologists are consulted. Recently, our studies on a cohort of females with diabetes have shown that diabetic patients treated in the diabetic clinic have significantly higher degrees of nocturia, weak urinary stream and decreased maximal urinary flow rate as well as decreased emptying efficiency and consequently, these patients are 4.8 folds as likely to have unrecognized voiding difficulty when compared to aged matched controls. [5-6]. Pressure flow studies indicated that residual urine in diabetics was related to a decrease in the maximum detrusor contraction strength, the fading of detrusor contractility during voiding, and an increase in the size of the bladder [7].

The most common characteristics of bladder dysfunction in animal models of diabetes are increases in bladder mass, urinary frequency, bladder capacity and residual urine [8-10], which mimic the clinical manifestations seen in diabetic patients. The pathophysiological mechanism underlying the diabetes induced bladder dysfunction remains unclear. It has been suggested that diabetic urinary bladder dysfunction is initiated by a visceral sensory neuropathy involving the afferent limb of the micturition reflex arc. The axonal transport in visceral afferent pathways may be disrupted and resulting in a significant reduction of afferent conduction velocities [11-13]. Whilst it is generally accepted that neuropathy plays a role in this organ's impairment [12], there are reports to suggest that alterations may also exist at the level of the bladder smooth muscle itself [14]. These include changes in postsynaptic muscarinic receptor function [15-16] and possible changes in intracellular second messenger systems [17]. Kamata et al [18] suggested that the increased contractile responses of detrusor strips of the bladder to substance P in the diabetic state are due to increased synthesis of prostaglandins and/or thromboxane A2 via the increased activity of phospholipase A2 on the smooth muscle of the diabetic bladder. Other studies also implied that alterations in calcium channel activity, enhanced sensitivity to $[Ca^{2+}]_i$, diminished Na+ pump activity, increased activity of NO in diabetic bladder smooth muscle may have a pathophysiological role in the urinary bladder dysfunction associated with diabetes.[19-21]

Reactive oxygen species (ROS) are involved in a diversity of biological phenomena including radiation damage, carcinogenesis, ischemia-reperfusion injury and diabetes [22]. Accumulating evidences have demonstrated that in patients with diabetes, a single hyperglycemia-induced process of overproduction of ROS, especially superoxide, by the mitochondrial electron-transport chain seems to be the first and key event in the activation of all other pathways involved in the pathogenesis of diabetic complications [23-25]. Several pathways seem to be involved in the development of oxidative stress in the presence of hyperglycemia, namely, glucose autoxidation, protein kinase C (PKC)-dependent activation of arachidonic acids, advanced glycation end product (AGE) formation and the polyol pathway [26]. Superoxide radicals generated by glucose autoxidation may lead to vascular dysfunction by the induction of local inflammation and inactivation of nitric oxide [27].

Advanced glycation end products (AGEs) are a heterogeneous group of irreversible adducts resulting from nonenzymatic glycation and oxidation of proteins, lipids, and nucleic acids. Glucose and other reducing sugars react in a nonenzymatic reaction (Maillard reaction) with the N-terminal residues and/ or e-amino groups of proteins initially forming a Schiff base [28]. The overproduction of AGEs appears to play a key role in the pathogenesis of diabetic complications. AGEs are able to produce ROS via complex biochemical mechanisms. For example, interaction of AGEs with specific receptors (RAGE) leads to the enhanced formation of ROS and the subsequent activation of nuclear factor kappa B (NFκB), which activates the transcription of genes whose proteins are involved in the inflammation process (TGF-α) or in cell proliferation and adhesion (VCAM-1, intercellular cell adhesion molecule type 1 and vascular endothelial growth factor, VEGF), subsequently leading to vascular dysfunction [27, 29]. Several studies have shown that activation of NFκB is also associated with resistance to apoptosis. One mechanism by which NFκB inhibits cell death is to induce the expression of genes that promote resistance to apoptosis. For example, NFκB activation can suppress caspase, mitochondrial cytochrome C release and induce Bcl-2 family members [30].

The overproduction of ROS (e.g., superoxide) may also alter the structural and functional properties of proteins, lipid components, and nucleic acids, which also contribute to the pathogenic changes in endothelial cells [26-29]. Several studies have shown that the deleterious effects of AGEs accumulation in vascular tissue are more likely related to alterations in the connective tissue composition of the microvascular wall resulting in increased tissue rigidity, rather than to functional interference with vascular smooth muscle reactivity [31].

Progressive vascular dysfunction caused by oxidative stress in diabetes may result

in capillary occlusion, ischemia, and organ failure. In diabetic kidneys, AGEs were preferentially localized in vascular lesions, renal cortex, expanded mesangial areas, and the glomerular basement membrane [32]. For example, N-epsilon-(carboxymethyl) lysine (CML), one of the various AGE structures postulated to date, has been shown to accumulate in diabetic kidneys in co-localization with a marker of lipid peroxidation (MDA), which suggests an association of local oxidative stress with the etiology of diabetic glomerular lesions [29]. An increased CML content in serum proteins of diabetic patients and a correlation of serum AGE levels with the progressive loss of kidney function was found [32-33]. Moreover, the increased formation of tissue AGEs has been described to precede and to correlate with early manifestations of renal and retinal complications in patients with diabetes [34].

Material and methods:

Experimental animals.

Group 1: Rats were fed food which was supported by Experimental Animal Center, National Taiwan University.

Group 2: Diabetic rats. They will be induced in female wistar rats weighing 250-300 gm. by feeding high fructose food. Rats were fed fructose food from 1 to 6 months separately.

The animals are housed at the Experimental Animal Center, National Taiwan University at a constant temperature and with a consistent light cycle (light from 07:00 to 18:00). The animal care and experimental protocol are in accordance with the guidelines of the National Science Council of Republic of China (NSC 1997). Blood glucose concentrations are measured in control and diabetic animals prior to STZ administration and again at the time of the experiments.

Whole bladder cystometric studies

We will insert a PE-50 tube into the bladder through the urethra and tie it in place by a ligature around the urethral meatus [Chien et al., 2000a]. The catheter is connected to a pressure transducer and an infusion pump (Infors AG, CH-4103, and Bottmingen, Switzerland) via a T-tube connector. The bladder volume is increased by steady infusion of 0.9% saline (0.10 ml/min) via the infusion pump. The bladders will be filled several times by continuous infusion of 0.9% saline (0.15 ml/min) at room temperature and will be allowed to drain/micturate repeatedly via the urethra. The change in intravesical pressure, bladder capacity, and hemodynamics and oxygen

tension during cystometric studies will be compared between the control and diabetic rats.

In vivo chemiluminescence recording for ROS activity [35]

The ROS generation in response to bladder filling and subsequent emptying is measured from bladder surface by a modified chemiluminescence detection method as described previously [6, 27]. The rat is maintained on a respirator (tidal volume: 1.0-1.5 ml; rate, 80-90 cycles/min; inspiratory pressure, 20-30 cm H_2O) and a circulating water pad at 37°C in a dark box with a shielded plate. Only the bladder window is left unshielded and is positioned under a reflector, which reflected the photons from the exposed bladder surface onto the detector area. The measurement of ROS from the bladder is started by intravenous infusion of a superoxide anion probe, MCLA (0.2 mg/ml/h, TCI-Ace, Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) throughout the experiment by use of a Chemiluminescence Analyzing System (CLD-110, Tohoku Electronic In. Co., Sendai, Japan). The MCLA-enhanced chemiluminescence counts are continuously recorded every 10 sec during bladder pre-filling, AUR, and post-emptied periods. The real-time displayed chemiluminescence signal is registered as the ROS level generated from the bladder surface. To verify the specificity of ROS-enhanced chemiluminescence activity, we will add superoxide dismutase (500 U/ml) in the intravenous solution. Superoxide dismutase is a known scavenger for superoxide anion

Immunoblot analysis for MnSOD, Bax, Bcl-2, CPP32, and Calponin [35, 41]

The method for DNA extraction and electrophoresis was described previously [27]. Total protein is extracted from bladder tissues. We will measure the expression of MnSOD, Bax, Bcl-2, caspase 3, and Calponin in the total protein from bladder subjected to bladder distention.

Antibodies raised against, MnSOD (MnSOD, R&D systems, Inc., Minneapolis, MN,USA), Bax (Chemicon, Temecula, CA, USA), Bcl-2 (Transduction, Bluegrass-Lexington, KY, USA), the activation fragments (32 kD of proenzyme and 17 kD of cleaved product) of caspase 3 (CPP32/Yama/Apopain) (Upstate Biotechnology, Lake Placid, NY, USA), Calponin(Promega, Madison, WI, USA), and β–actin (Sigma, Saint Louis, MI, USA) are used. All these antibodies cross-react with respective rat antigens.

SDS-PAGE is performed on 12.5% separation gels in the absence of urea, and is stained with Coomassie brilliant blue. Proteins on the SDS-PAGE gels, each lane containing 30 μg of total protein, are transferred to nitrocellulose filters. The

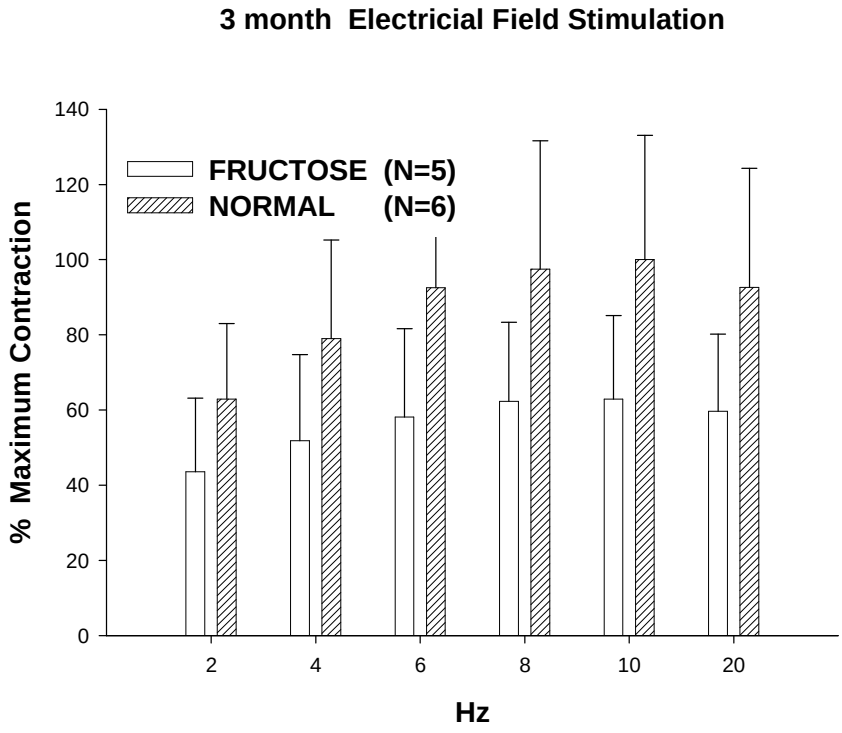
immunoreactive bands are detected by incubation with the antibody described above, followed by secondary antibody-alkaline phosphatase, and finally with NBT and 5-bromo-4chloro-3-indolyl phosphate, toluidine salt (Roche Diagnostic GmbH, Mannheim, Germany) stock solution for 30 min at room temperature.

HE and Masson trichrome stain [40]

The distribution of AGEs in the urinary bladder is studied with *Masson trichrome* techniques. Bladder tissue is immediately snap-frozen, and sections 5μm thick are prepared. Endogenous peroxidase activity is inhibited by a 20-minute incubation with methanol containing 0.6% H_2O_2. Nonspecific staining is blocked by a 10-minute incubation with striptavidin. Nonspecific protein binding sites are blocked by 10 minutes of incubation with 10% normal rabbit serum. Slides are stained with biotin-labeled rabbit anti-AGE polycolnal antibody overnight at 4° C. The slides are washed with PBS and incubated with peroxidase-conjugated streptavidin for 5 minutes. They are then washed in PBS and the color reaction was performed by incubation with aminoethylcalbasole reagent until staining is complete. The reaction is stopped by placing each slide in water, and then Mayer's hematoxylin is added as a counterstain. Negative control staining is performed using the same anti-AGE antibody preincubated with an excess of AGE-BSA.

實驗結果

Fig 1. 果糖餵食3個月及6個月的老鼠膀胱肌肉, 以電刺激進行實驗



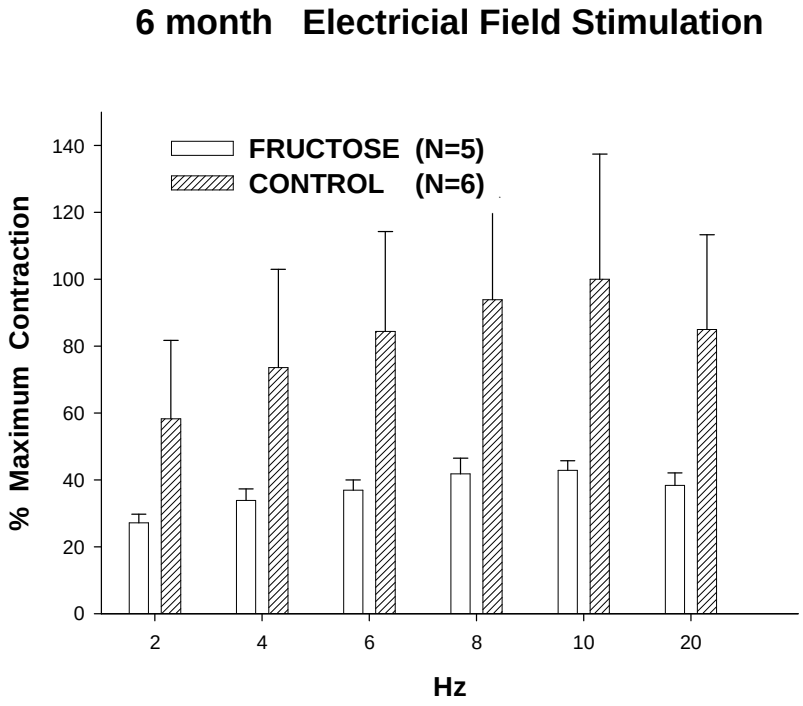
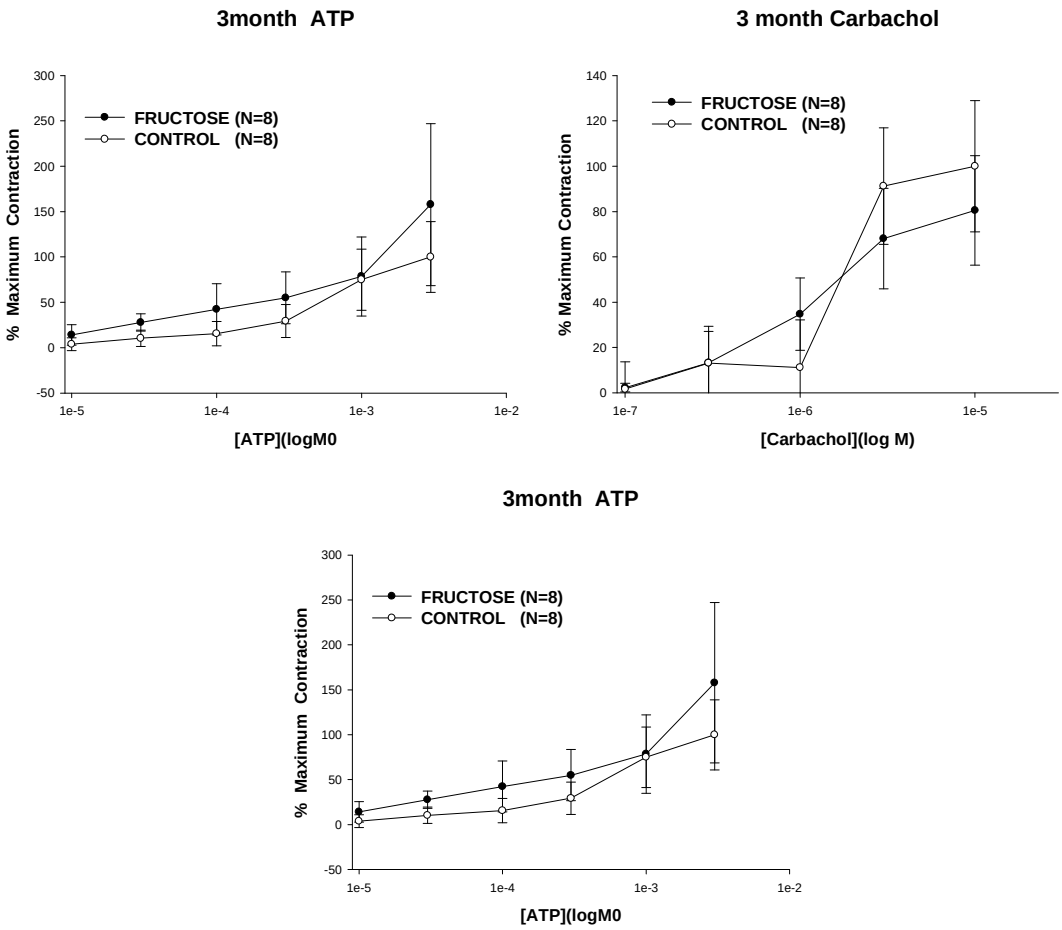


Fig 2 以電刺激模式分別用ATP、KCl和Cabarchol刺激3及6個月老鼠膀胱肌肉



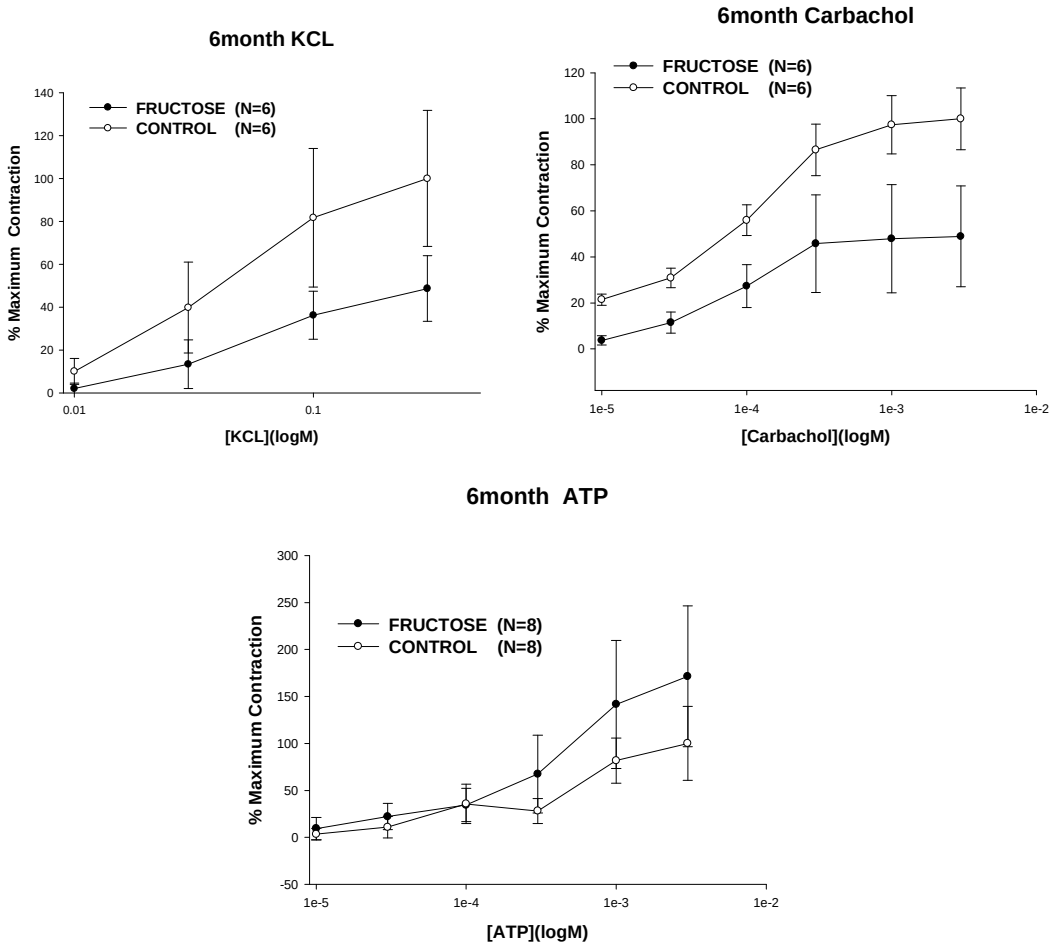
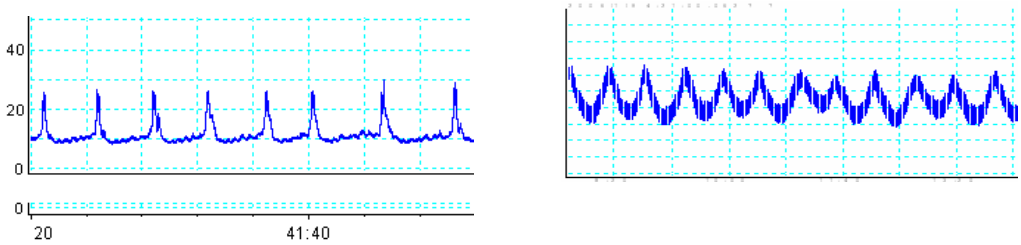
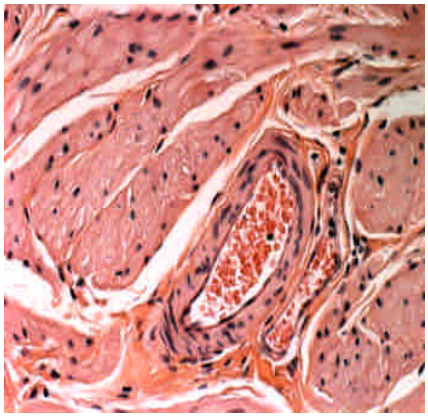


Fig 3活體膀胱收縮波

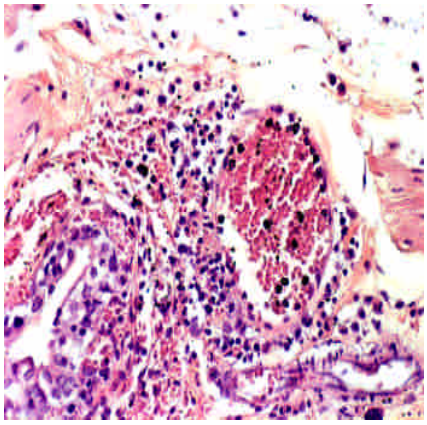


（a）正常對照組出現正常之膀胱收縮波（b）果糖餵養之代謝糖尿病鼠出現過動型收縮

Fig 4 代謝糖尿病鼠合併膀胱收縮不良之膀胱病理切片(HE stain)

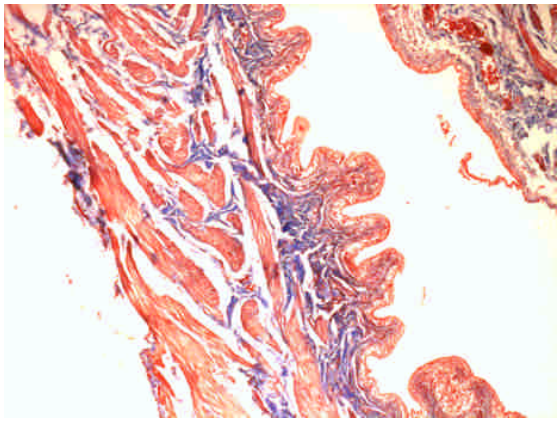


(a)正常對照組之膀胱逼尿肌。

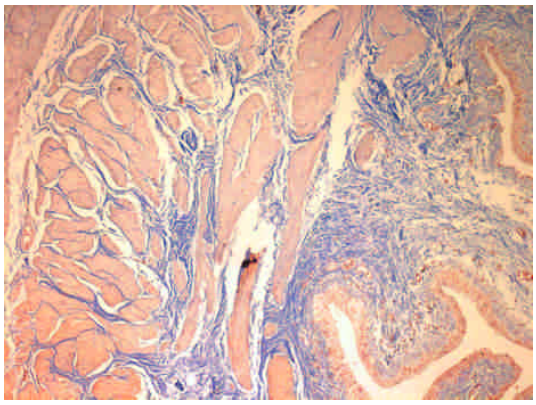


(b)代謝鼠膀胱產生白血球浸潤。

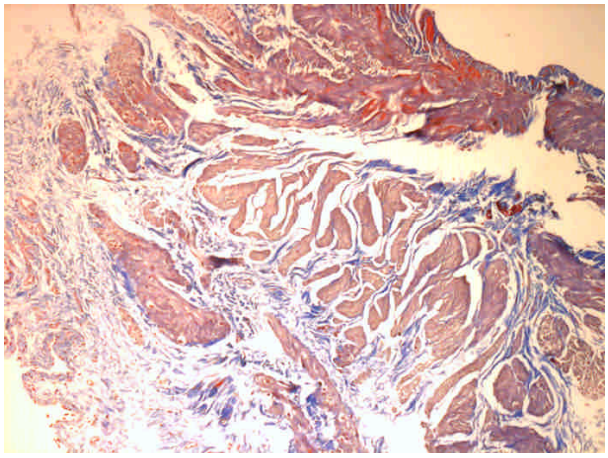
Fig 5 Masson Trichrome



(a) 對照組膀胱染色



(b)餵食果糖3個月糖尿病鼠



(c) 餵食果糖6個月糖尿病鼠

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