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# MECHANISMS OF UROLOGIC COMPLICATIONS IN STREPTOZOTOCIN-INDUCED TYPE 1 DIABETES MELLITUS

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**Key words:**

Diabetes, Streptozotocin, urinary bladder, cation contents.

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**ABSTRACT:** Patients with DM are often afflicted by debilitating urologic complications. The main aim of this study is to investigate the effect of streptozotocin (STZ)-induced type I diabetes on contractile responses to numerous stimuli, intracellular cation concentrations and morphology in the rat. Adult rats (n=12) were humanely killed and detrusor muscles of urinary bladder located and excised rapidly and placed in organ baths. Then urinary bladder detrusor muscles were blotted, weighed and dissolved in concentrated nitric acid for the measurements of cation contents. The diabetic rats had significantly ($P<0.05$) elevated blood glucose compared to control rats. The contractile responses induced by EFS were significantly ($P<0.05$) increased in the diabetic urinary bladder tissue in comparison to the control urinary bladder tissue. The increase in the contractile force was calcium-dependent in both control and diabetic urinary bladder tissues. The results also showed no significant differences in the levels of sodium, potassium, calcium, between the diabetic and control urinary bladder ($p>0.05$). However, there was a significant ($P<0.05$) decrease in magnesium in the diabetic urinary bladder tissue in comparison to the control urinary bladder tissue. The increased responsiveness of the diabetic urinary bladder tissue compared to the control tissue may be due to either alterations in the intracellular cation concentrations, hypertrophy of the diabetic urinary bladder or calcium sensitivity to the myofilaments. The results also raise the possibility that that these factors, in conjunction with other causes may work in tandem to exacerbate diabetic bladder dysfunction.

**INTRODUCTION:** The urologic complications of diabetes mellitus, presenting as bladder dysfunction, sexual and erectile dysfunction, as well as urinary tract infections (UTI) may be held responsible for considerable loss of productivity and burden on healthcare facilities associated with the disease. With DM reaching epidemic proportions and still rising, the impact of these symptoms have been reported to affect the quality of life in equal measures to that of the deadly acquired immunodeficiency syndrome.[1]

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In the literature, there is considerable divergence with regards to electrophysiological and pharmacological studies conducted to identify prospective mechanisms underlying bladder dysfunction in the diabetic state.

There are contradictory results in EFS-induced contractile responses of the diabetic urinary bladder tissue relative to the control urinary bladder tissue; wherein numerous studies have shown the diabetic bladder tissue was more responsive than its control counterpart. [2, 3, 4, 5] On other hand, others report either decreased or little change in EFS-induced contractile response. [6, 7, 8] In the same vein, experiments measuring contractile responses of the bladder to exogenous muscarinic agonists have also produced contradictory results.[9, 10, 11]

With regards to responses to EFS, a number of theories have been postulated and subsequently disproved. Belis *et al* (1997), theorise that the difference in contractile responses in diabetics compared to control is due to increased calcium ($Ca^{2+}$) channel activity but in 2000, Waring and Wendt found no supporting evidence and postulate the difference was due to increased $Ca^{2+}$ sensitivity.[12, 4] Tong *et al* (1999) suggested the increase in EFS-induced contractile responses were due to increased muscarinic receptor density however, contradictory evidence was found by Malmgren *et al* (1989).[13, 9] Moreover, morphological studies are indicative of a progressive hypertrophy of the urothelium of the urinary bladder in diabetes.[14, 15] However, the importance of this on the mechanisms of urologic complications remains ill understood.

Such variability in the observations suggests a complex multi-factorial aetiology of diabetes-induced bladder dysfunction. Therefore, the aim of this study was to investigate the effects of the contractile response to EFS, atropine and verapamil. Furthermore, the same stimulatory parameters were assessed in the presence of varying exogenously administered $Ca^{2+}$ concentrations $[Ca^{2+}]_0$. In addition, morphological studies of the urinary bladder were also conducted.

The main aim of the comparative study is to investigate the role of cations contents compared to the age matched control.

## MATERIALS AND METHODS:

### General Procedures:

All experiments were done using male Wistar rats weighing between 151.1-174.3 ± 1.54 g. One group of animals were rendered diabetic with a single intraperitoneal injection of STZ (60 mg/kg body weight) dissolved in citrate buffer (pH 4.5). Age-matched control rats received 0.3 ml of the citrate buffer alone. The rats were tested for diabetes 4-5 days after injection and on the day when they were used for experiments using a glucose meter.

The rats were humanely killed by a blow to the head followed by cervical dislocation. An incision was made in the lower abdomen and the urinary bladder was located and rapidly excised at the urethra, blotted and weighed. Subsequently the bladder was placed in a Krebs-Henseleit (KH) solution. In experiments involving the perturbation of $[Ca^{2+}]_0$, the same chemical composition of KH was utilised, with the exception of the $Ca^{2+}$ chloride; which was altered to 1.25 mM and 5.00 mM to achieve low and high $[Ca^{2+}]_0$, respectively. The extracellular concentration of 2.56 mM $Ca^{2+}$ was used as the control in these experiments. The study had the relevant ethical clearance from the Faculty of Science and Technology Ethics Committee.

### Bladder Contractility Experiments:

The tissue was gently cut into 10mm long strips, subsequently, the urinary bladder was cleansed thoroughly and one end of the tissue was tied to a hook on the electrode, whilst the other end was tied to an isometric transducer (Model). The tissue was then mounted in the organ bath containing KH solution and kept at a constant temperature of $37^0$C. The pH was maintained at 7.4 and the solution was gassed continuously with 95% oxygen and 5% carbon dioxide. The changes in contractions were measured by an isometric transducer coupled to a preamplifier and subsequently an oscilligraph.

After the tissue stabilized following 1 g calibration, EFS-induced contractions were investigated by using two parallel silver wire electrodes connected to a current amplifier and pulse generator. EFS was induced for a period of 10 seconds at a range of frequencies ranging from 1-100 Hz at 50 volts with 1 msec pulse. The same procedure was repeated in the presence of $10^{-4}$ M atropine and $10^{-4}$ M verapamil. The bladder tissue was incubated for 20 minutes in the required antagonists prior to stimulation. The same series of experiments with the addition of $10^{-4}$ M acetylchline (ACh) were conducted in low (1.25 mM) and high (5.00 mM) extracellular $Ca^{2+}$ concentration. The KH solution was replaced and the urinary bladder tissue was allowed to rest for 30 minutes after each series of stimulation. The contractile responses are expressed as grams/cross sectional area. The cross sectional area was established by mass x (length/density), whereby the density was considered as 1.0 $mm^3$.[4]

**Measurement of Cations:**

The tissue was dissolved in 1 ml of concentrated nitric acid and a volume of 0.2 ml was diluted in 9.8 ml of de-ionized water. The samples were then used to measure the concentrations of sodium ($Na^+$), potassium ($K^+$), calcium ($Ca^{2+}$) and magnesium ($Mg^{2+}$). The corning flame photometer was used to measure $Na^+$ and $K^+$, whilst an atomic absorbance spectrophotometer was used to measure the $Ca^{2+}$and $Mg^{2+}$. The required standard ion solutions ranging from 5-20 mg/ml were used to produce standard curves required to calculate the ion content. All the values are expressed in mM (100 mg tissue) [1].

**Statistical Analysis:**

Results are given as a Mean ±Standard Error of the Mean (SEM). Statistical analysis was performed by using either independent student *t* test and Student's paired *t* tests. The P value less than 0.05 was considered significant (P<0.05), in contrast, a P value greater than 0.05 was considered insignificant (P>0.05).

**RESULTS AND DICUSSION:**

**General Characteristics:**

On arrival in the laboratory, the male Wistar rats weighed 188.13±2.46 g on average but following the introduction of STZ, the diabetic rats gained significantly less (P<0.05) weight than age-matched controls (**Table 1**). The mean plasma glucose concentration was elevated by almost five times in the diabetic group of rats relative to the control rats. The diabetic rats also exhibited the classical symptoms of diabetes, such as polydipsia, polyphagia and polyurea.

**TABLE 1: GENERAL CHARACTERISTICS OF 12-14 WEEK DIABETIC AND AGE-MATCHED CONTROL URINARY BLADDER. NOTE THE SIGNIFICANT INCREASE (*P<0.05) IN BLOOD GLUCOSE AND THE DECREASE (P<0.05) IN WEIGHT GAINED IN THE DIABETIC RATS IN COMPARISON TO THE CONTROL RATS.**

| Parameters | N | Control | Diabetic |
|---|---|---|---|
| Weight of Animals on Arrival (g) | 24 | 188.13 ± 2.46 | - |
| Weight of Animals on Day of Experiment (g) | 12 | 552.20 ± 12.79 | 289.00 ± 8.36* |
| Blood Glucose (mM) | 12 | 5.62 ± 0.21 | 26.84 ± 1.06* |
| Weight of Urinary Bladder at Time of Excision (mg) | 12 | 254.0 ± 0.02 | 468.7 ± 0.02* |

**Response to EFS:**

A frequency-dependent response in the EFS-induced contractions in the control and diabetic urinary bladder tissue is shown in **Fig.1**. The force of contraction induced by EFS was significantly (P<0.05) greater in the diabetic urinary bladder tissue in comparison to the control urinary bladder tissue. The presence of atropine and verapamil caused significant (P<0.05) reductions in both groups of the urinary bladder tissue. Both, atropine and verapamil failed to completely abolish EFS-induced contractile responses in the control and diabetic bladder strips.

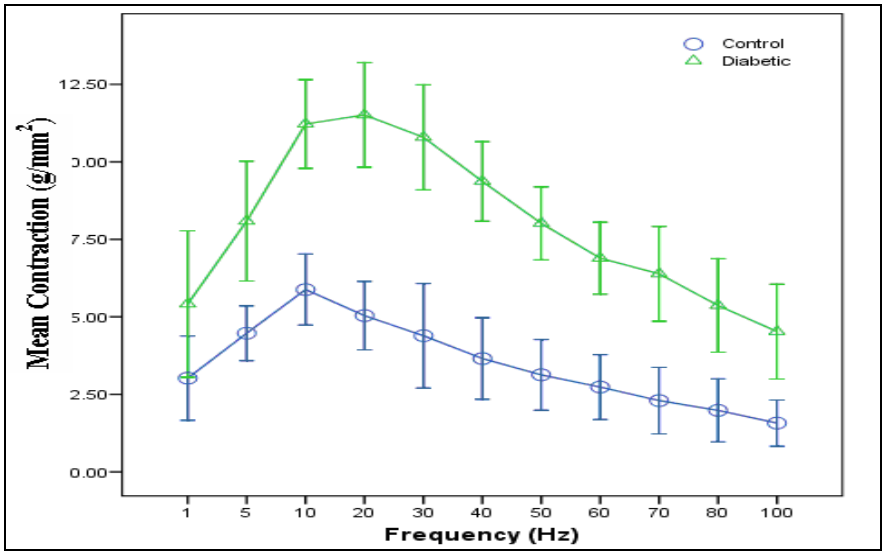


**FIG. 1: EFS-EVOKED CONTRACTILE RESPONSES OF URINARY BLADDER STRIPS IN AGE-MATCHED CONTROL (OPEN CIRCLES) AND STZ-INDUCED (OPEN TRIANGLES) DIABETIC RATS. DATA ARE THE MEAN±SEM, *n*=6, (* P<0.05).**

**Extracellular calcium-dependent responses:**

EFS and ACh-induced contractile force appeared to be a function of extracellular $Ca^{2+}$ concentration in both groups. i.e. the force of contraction induced by EFS was greater in the presence of high extracellular concentration of $Ca^{2+}$ concentration compared to the control extracellular concentration of $Ca^{2+}$. The similar pattern was also seen in contractions induced by exogenous ACh (**Fig.2**).

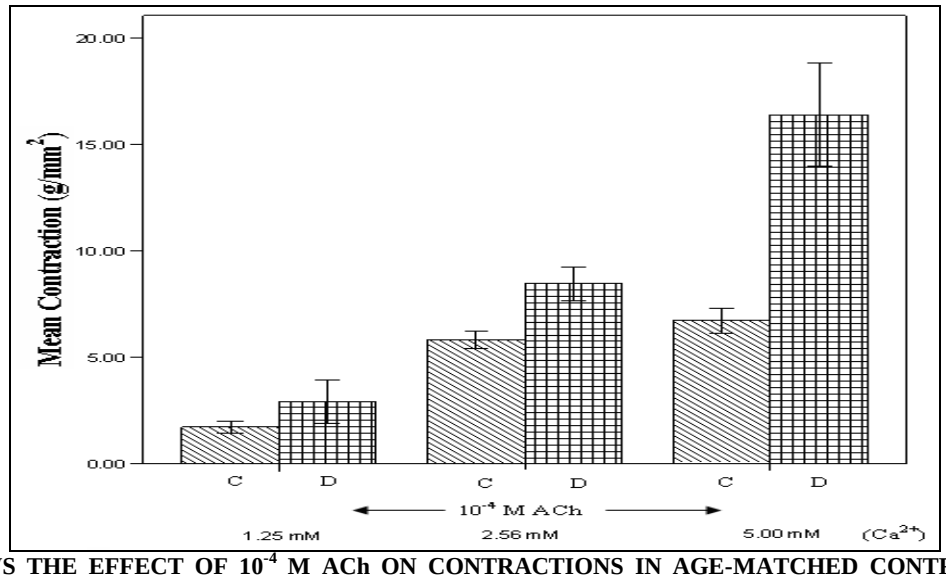


**FIG.2: BAR SHOWS THE EFFECT OF $10^{-4}$ M ACh ON CONTRACTIONS IN AGE-MATCHED CONTROL (C) AND STZ-INDUCED DIABETIC (D) URINARY BLADDER MUSCLE STRIPS IN VARYING $[Ca^{2+}]_o$. DATA ARE THE MEAN±SEM, *n*=6, (* P<0.05).**

**Cation Contents:**

The levels of $Na^+$ and $K^+$ (**Fig.3**) decreased in the un-stimulated diabetic urinary bladder tissues in comparison to the healthy age-match control urinary bladder although the correlation failed to achieve significance (P>0.05. A slight increase was observed in the concentration of $Ca^{2+}$ in the un-stimulated diabetic urinary bladder tissue in comparison to controls (**Fig. 4**). However, the difference was not statistically significant (P>0.05). The results showed the level of $Mg^{2+}$ decreased significantly (P<0.05) in un-stimulated diabetic urinary bladder tissue in comparison to un-stimulated healthy age-match control urinary bladder tissue (**Fig. 5**).

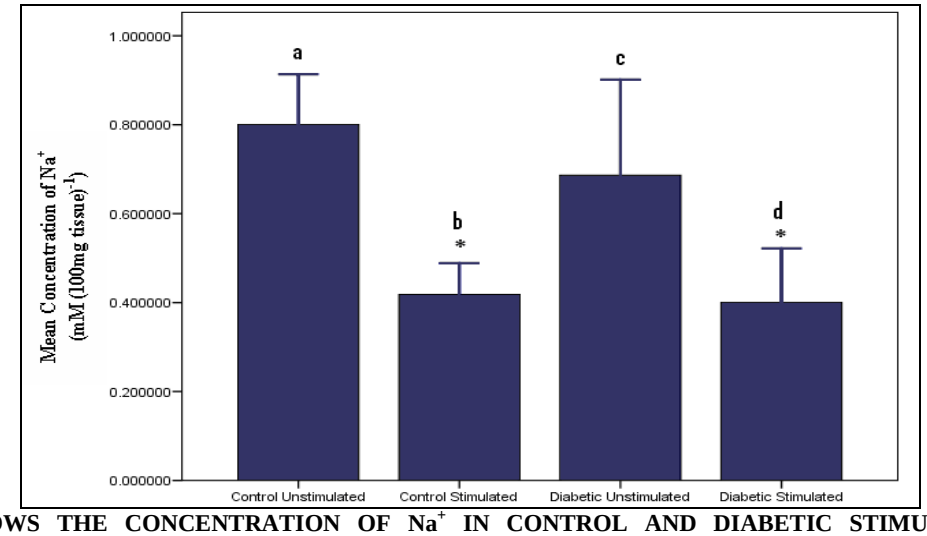


**FIG. 3: BAR SHOWS THE CONCENTRATION OF $Na^+$ IN CONTROL AND DIABETIC STIMULATED AND NON-STIMULATED URINARY BLADDER TISSUE. THE RESULTS SHOW THAT IN BOTH CONTROL AND DIABETIC TISSUES, THE LEVELS OF $NA^+$ DECREASED SIGNIFICANTLY (*P<0.05) AFTER STIMULATION COMPARED TO NON-STIMULATED TISSUES. EACH BAR IS REPRESENTED AS THE MEAN±SEM *n*=5.** Note that A has significantly (*P<0.05) more $Na^+$ than B and D. C has significantly (*P<0.05) more $Na^+$ than D

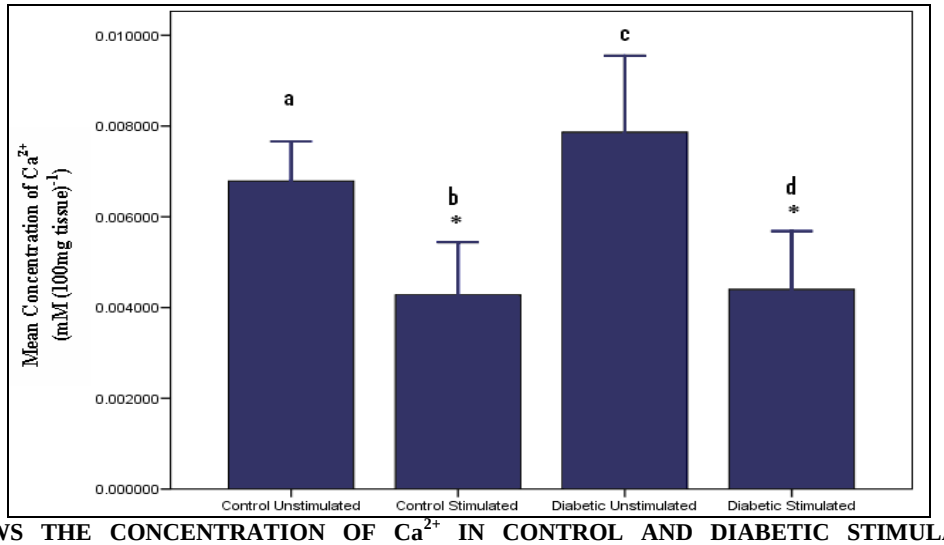


**FIG.4: BAR SHOWS THE CONCENTRATION OF $Ca^{2+}$ IN CONTROL AND DIABETIC STIMULATED AND NON-STIMULATED URINARY BLADDER TISSUE. THE RESULTS SHOW THAT IN BOTH CONTROL AND DIABETIC TISSUES, THE LEVELS OF $Ca^{2+}$ DECREASED SIGNIFICANTLY (*P<0.05) AFTER STIMULATION COMPARED TO NON-STIMULATED TISSUES. EACH BAR IS REPRESENTED AS THE mean±SEM *n*=5.** Note that A has significantly (*P<0.05) more $Ca^{2+}$ than B and D. C has significantly (*P<0.05) more $Ca^{2+}$ than D.

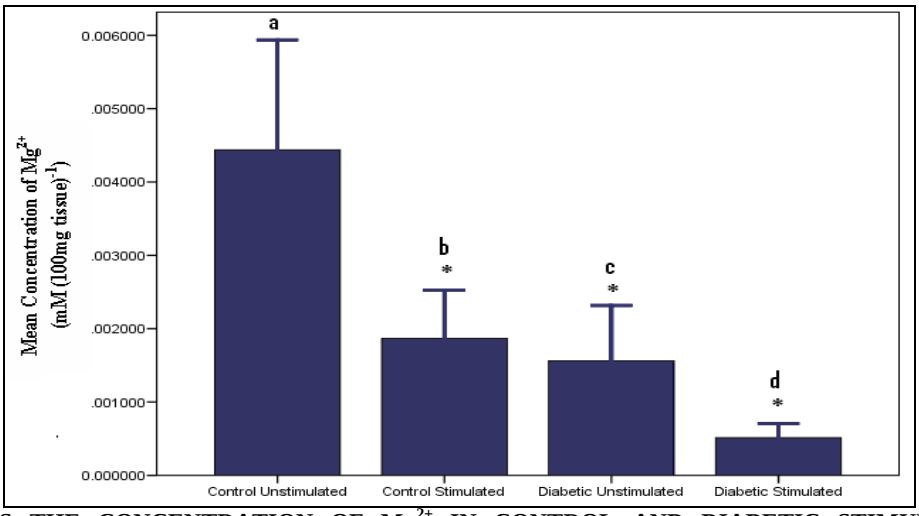


**FIG.5: BAR SHOWS THE CONCENTRATION OF $Mg^{2+}$ IN CONTROL AND DIABETIC STIMULATED AND NON-STIMULATED URINARY BLADDER TISSUE. THE RESULTS SHOW A SIGNIFICANT (*P<0.05) DECREASE IN THE CONCENTRATION OF $Mg^{2+}$ IN THE DIABETIC URINARY BLADDER TISSUE IN COMPARISON TO THE CONTROL TISSUE. MOREOVER, IN BOTH CONTROL AND DIABETIC TISSUES, THE LEVELS OF $Mg^{2+}$ DECREASED SIGNIFICANTLY (*P<0.05) AFTER STIMULATION COMPARED TO NON-STIMULATED TISSUES. EACH BAR IS REPRESENTED AS THE MEAN±SEM** *n*=5. Note that A has significantly (*P<0.05) more $Mg^{2+}$ than B, C and D. C has significantly (*P<0.05) more $Mg^{2+}$ than D.

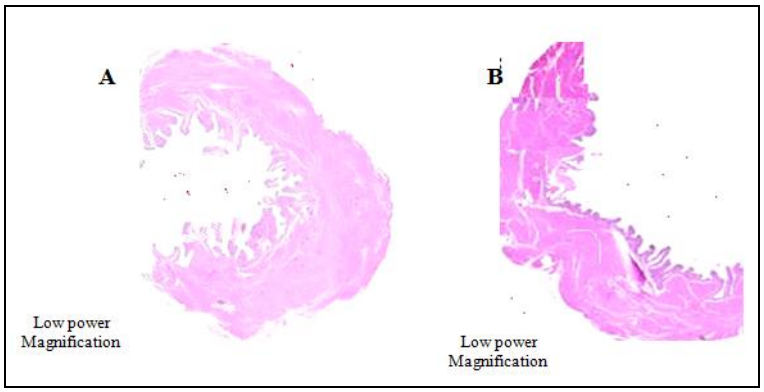


**FIG.6: REPRESENTS IMAGES OF HAEMATOXYLIN AND EOSIN STAINED SECTIONS OF (A) DIABETIC URINARY BLADDER, (B) CONTROL URINARY. THE MICROGRAPHS ARE REPRESENTATIVE OF 3 SUCH EXPERIMENTS.**

The results of this study have demonstrated marked changes in the characteristics, EFS-evoked contraction, cation levels and the morphology of STZ-induced diabetic rats compared to controls.

In this study, numerous observations were made on the general characteristics of both control and STZ-induced diabetic rats were made. On arrival in the laboratory, the male Wistar rats weighed 188.13±2.46 g on average. Following the injection of STZ, the diabetic rats gained significantly less ($P<0.05$) weight than age-matched healthy rats over the experimental periods of 12-14 weeks. In addition, the plasma glucose concentrations were significantly elevated in the diabetic group compared to the control. These observations together with other characteristic changes of the STZ-induced diabetic rat including polyuria, polydipsia and signs of cataract are indicative of the consistency and effectiveness of STZ- in inducing type-1 diabetes in the rat.

Despite the increase in the weight of the diabetic animals, the study also showed that the urinary bladder in the diabetic rats was considerably larger with increased mass. The increased size could be a result of either hypertrophy or hyperplasia, however, both, increased size and mass are thought to compensate for the increase in urine production.[3, 16] Another interesting observation seen in the STZ-induced diabetic rat was the substantial increase in the quantity of urine in the urinary bladder the time of excision in comparison to that of the healthy rat. This could suggest that diabetic rats do not urinate as frequently as healthy rats or they have an increased residual volume of urine. Alternatively, the urinary bladder may fill up with urine a lot faster than their control counterparts, an observation made previously by Eika *et al* (1992).[17]

EFS has proven to be an effective physiological tool to excite intrinsic nerves and to assess contractile and secretary responses to endogenous neurotransmitter release in various tissues.[18] The results of this study have shown that EFS elicits frequency-dependant contractile response in the both control and diabetic tissues and the contractile responses are significantly ($P<0.05$) greater in the diabetic tissue in comparison to control urinary bladder tissue. These results are in agreement with other studies utilizing the same tissue and stimulating technique. [4, 5, 19] EFS-induced contractions are mediated by muscarinic receptors, namely $M_2$ and $M_3$ receptors although $M_1$, $M_4$ and $M_5$ muscarinic receptors are also present in the rat detrusor muscle. [20, 21] The signalling mechanisms for muscarinc receptors vary amongst the different subtypes and species and have yet to be completely understood. Nevertheless, it is thought $M_2$ receptors inhibit adenyl cyclase by reducing cAMP concentrations via the $G_i$ family of proteins, whilst $M_3$ receptors exert the effects through the $G_q$ family of proteins to generate $IP_3$.[22]

The optimum frequency for the production of maximal force of contraction differed for control and diabetic urinary bladder tissue. EFS at 10 HZ evoked maximal contractile force whereas 20 Hz appeared to the optimal frequency for diabetic urinary bladder tissue. Results indicate that EFS-induced contractions were significantly ($P<0.05$) greater in the diabetic urinary bladder tissue in comparison to age-matched control tissue. These results are in agreement with the findings of Tammela et al. (1995) and Waring and Wendt (2000). [3, 4] In contrast, other previous studies have shown little or no change in EFS-induced contractile force of the diabetic urinary bladder relative to controls. In two other studies by Longhurst and Belis (1986) and Ichiyanagi *et al* (2002) a decrease in contractile force in the diabetic urinary bladder tissue was observed in comparison to the controls.[6, 12]

No clear reason for the discrepancies in published results has been ascertained, however, it is thought a poor or a complete lack of standardization in the contractile force generated may be to blame.[5] In addition, discrepancies may arise due to inadequate experimental protocols i.e. usage of different regions of the urinary bladder tissue between both groups, or the length and severity of the disease. This could have implications on the amount of muscarinic receptors present, potentially causing changes in the amount of neurotransmitter release; naturally affecting the contractile force generated. Furthermore, a study conducted by Bezuijen and colleagues showed that the differences in the contractile force could be attributed to an enlarged

bladder size.[23] This may explain the contradictory nature of the results found in the literature. However, the overall consensus remains that diabetes is associated with enhanced contraction of the urinary bladder compared to controls.

The mechanism for the increased contractile force generated by EFS in the diabetic urinary bladder tissue in comparison to the control bladder tissue has yet to be identified, but could be due to the increased density of muscarinic receptors or altered sensitivity to neurotransmitters.[8, 24, 25] Indeed, the former was suggested by Tong *et al* (1999) who reported significant increases in the quantity of $M_3$ mAChR protein in the STZ-induced diabetic rat urinary bladder in comparison to the control urinary bladder. Interestingly, Tong *et al* (1999) also demonstrated an upregulation in $M_2$ receptor mRNA.[13] Nevertheless, its significance in diabetes bladder dysfunction remains ill understood. Another potential mechanism for altered properties of the diabetic urinary bladder tissue could be due to impaired nitric oxide control.[26] These observations reiterate the complex multifactorial aetiology of diabetes-induced bladder dysfunction.

The results of the study have shown that the cholinergic muscarinic antagonist atropine can markedly reduce the EFS-evoked contraction in both control and diabetic bladder tissues indicating that the endogenous neurotransmitter released during EFS is Ach. The finding that atropine failed to completely abolish EFS-evoked contractions in both age-matched control and diabetic urinary bladder tissue is suggestive of the action of atropine-resistant non-cholinerigc, non-adrenergic (NCNA) neurotransmitter.[27]

The NANC-mediated response seen in this study is negligible in comparison to the total EFS-induced contraction, complementing the results found by Sjörgren *et al* (1982).[28] The results so far indicated that EFS can induce contractions via the release of ACh from intrinsic nerves, it was relevant to ascertain whether exogenous application of ACh can elicit urinary bladder contractions. The results indicate that Ach can evoke contractions in both groups in a dose dependent manner. There was no significant ($p > 0.05$) differences in the contractile responses generated between the control and diabetic urinary bladder tissue. However, the Ach-evoked force of contraction was substantially lower than EFS-induced contractions. As expected, Ach-induced contractions diminished but were not completely abolished in the presence of atropine, suggesting a small number of muscarinic receptors are resistant to atropine.

The increase in the force of contraction of diabetic bladder tissue compared to control following EFS may be due to impaired cellular $Ca^{2+}$ homeostasis. Moreover, $Ca^{2+}$ plays a vital role in the ECC process whereby it initiates and promotes muscle contraction.[29] Therefore, it was decided to investigate the effects of varying $[Ca^{2+}]_o$ on both EFS and ACh evoked contraction. The results show that the EFS-evoked force of contraction of both diabetic and control bladder tissue was dependent upon $[Ca^{2+}]_o$. In both control (2.56 mM) and elevated 5.00 mM $[Ca^{2+}]_o$, there were significant increases in force compared to the responses obtained in low 1.4mM $[Ca^{2+}]_o$ The force of contraction in response to applications of exogenous $10^{-4}$ M Ach were not significantly ($p>0.05$) different in the presence of either 1.25 mM or 5mM $[Ca^{2+}]_o$ in the control urinary bladder tissue. However, in the diabetic urinary bladder, there was a significant ($p<0.05$) decrease in contraction with 1.25 mM $[Ca^{2+}]_o$ compared to 2.56 mM and 5.00 mM $[Ca^{2+}]_o$. It is possible that the diabetic urinary bladder tissue is more sensitive to $Ca^{2+}$ compared to control tissue which in turn could be held responsible for the increase in contraction.

In this study, the levels of total tissue $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ were measured in EFS-evoked stimulated and non-stimulated control and diabetic bladder tissues. The rationale was to determine whether non stimulated tissues contain more cations compared to stimulated tissue.

The results show that non-stimulated tissue contained significantly more $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ compared to EFS-evoked stimulated tissues. However, there were no significant changes in the levels of $Na^+$ and $K^+$ in control and diabetic unstimulated or EFS-evoked stimulated muscle tissue. In contrast, the levels of $Mg^{2+}$ decreased significantly in both unstimulated and stimulated diabetic tissues compared to controls. In

comparison, the levels of $Ca^{2+}$ increased in the diabetic non stimulated and EFS-evoked stimulated bladder tissue compared to control. It is well known that both $Ca^{2+}$and $Mg^{2+}$ work reciprocally and $Mg^{2+}$ is a natural antagonist for $Ca^{2+}$. In the presence of low $Mg^{2+}$ tissues take up more $Ca^{2+}$.[30]

The decrease in levels of the four cations following stimulation may be due to damage of the muscles during EFS which can be quantified by measuring levels of lactate dehydrogenase (LDH) in the bathing medium. Alternatively leaching of cations from tissues into the bathing medium may be another possible explanation. In previous studies, we have demonstrated an increase in lactate dehydrogenase in the bathing medium following EFS. Morphological studies have demonstrated that the diabetic urinary bladder tissue exhibits a large increase in the number of smooth muscle and urothelial cells compared to control tissue.

The results of morphological analysis showed that the diabetic urinary bladder tissue had visually, more smooth muscle and urothelium in comparison to age-matched control urinary bladder tissue. However, these experiments fail to use any form of image analysis. Therefore, more accurate morphological studies are required. Nevertheless, the results of histology, in the context of the wider question, suggest differences in the contractile responses may be due to either increased quantity of urothelium or smooth muscle mass. The results of the study conducted by Pinna and colleagues[14] suggest that the diabetic urinary bladder in part, requires prostaglandin release from the urothelium to activate purinergic receptors. Implying the increased urothelium mass may play a role in the abnormalities seen in this, and many other studies employing the diabetic urinary bladder tissue. The importance of the increased smooth muscle layer of the diabetic urinary bladder tissue is not as clear as the urothelial layer.

**CONCLUSION:** The results of this study indicate the diabetic urinary bladder tissue produced significantly increased contractile force in comparison to the control urinary bladder tissue. In addition, the contractile responses of the urinary bladder are mediated by muscarinic receptors and are $Ca^{2+}$dependant. The were no significant differences in the intracellular cation concentrations of sodium, potassium, $Ca^{2+}$ and copper, but the diabetic urinary bladder tissue has significantly less $Mg^{2+}$ as compared to the healthy age-matched control urinary bladder tissue.

The study in its entirety, suggests the marked differences in the contractile force of the diabetic urinary bladder tissue may be due to altered sensitivity to $Ca^{2+}$, decreased intracellular cation concentrations and morphological changes to the diabetic urinary bladder. Further more intricate studies are required to not only to understand the link between these parameters and diabetic bladder dysfunction but also the underlying mechanisms causing changes in these parameters. Ultimately, allowing for new innovative and effective treatment options for diabetes-induced urologic complications.

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**CONFLICT OF INTEREST:** There is no conflict of interest in present work.

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