

UNIVERSITY OF KWAZULU-NATAL

EFFECTS OF ACETYLCHOLINE ON ISOLATED URINARY BLADDERS OF  
NORMAL AND STREPTOZOTOCIN-TREATED DIABETIC RATS

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NORMAL AND STREPTOZOTOCIN-TREATED DIABETIC RATS

BY

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## ABSTRACT

This study was prompted by the inconsistent reports and apparent controversies that exist in the biomedical literature on the responses of diabetic bladder strips to cholinergic nerve stimulation or exogenous administration of muscarinic agonists, especially acetylcholine (ACh), *in vitro*. In the present study, acetylcholine-induced contractions of urinary bladders isolated from normoglycaemic (normal) and streptozotocin-treated, diabetic Wistar rats were examined under physiological conditions. Mechanical contractile changes of the isolated urinary bladders of STZ-treated, diabetic rats in response to bath-applied acetylcholine were compared with those obtained from isolated urinary bladders of normal, age-matched, control rats. Results obtained show that urinary bladders from diabetic rats consistently weighed more, and were always more spontaneously active after mounting, than those of the age-matched normal, control rats.

ft A

Acetylcholine (ACh,  $10^{-10}$  -  $10^{-6}$  M) provoked concentration-related, atropine-sensitive contractions of the isolated urinary bladders of both diabetic and age-matched normal, control rats. However, acetylcholine always induced more powerful and greater contractions of the diabetic bladders compared with bladders from the age-matched normal, control rats. The enhanced contractile responses of the diabetic bladder strips to bath-applied ACh were detected soon after induction of diabetes, and the magnitude and/or intensity of the enhanced contractile responses to ACh continued to increase as the diabetic state of the animals progressed. Although this preliminary study could not establish the mechanism of the increased contractile responsiveness of the diabetic



bladders to the muscarinic agonist (ACh) used, the results tend to suggest that alterations in diabetic urinary bladder synaptosomal, vesicle-bound neurotransmitter (ACh) concentrations and the compensatory increase in the density of muscarinic M3-receptor population in diabetic bladders are two of the most attractive plausible mechanisms of the increased diabetic bladder responsiveness to bath-applied acetylcholine

# CHAPTER ONE

## BACKGROUND INFORMATION AND LITERATURE REVIEW

### Anatomy and Physiology of the Urinary Bladder

Studies on the anatomy and physiology of urinary bladder are important because they help to understand how diseases that involve this organ occur (pathophysiology), how treatments and attempts to discover new drugs directed at these diseases are conducted (physiopharmacology). Urinary incontinence is one of the conditions that disturb diabetic patients, especially when diabetes has not been well controlled for a long period. Investigations on this condition require knowledge of histology and cytology of the urinary bladder in order to detect alterations caused by the disease. By knowing how urinary bladder contraction and relaxation are initiated, and the neurotransmitters involved, one may be able to see the difference that exists between a normal and a diseased urinary bladder.

#### Anatomy of the urinary bladder

The urinary bladder is a hollow organ that serves as a temporary reservoir of urine. It is located in the anterior part of the pelvis. Its position, shape and size depend on the amount of urine that it contains. When it is full of urine, the urinary bladder expands into abdominal cavity, and may reach the umbilicus. When the bladder is empty, it is enclosed within the pelvis, and as it fills with urine, it changes from tetrahedral to ovoid shape, and it can contain up to 0.5 L of urine. The bladder has an apex, a base or fundus, and a neck (Kolta *etal*, 1985).

### **The apex**

The apex is the portion of the bladder directed forwards, and it is connected by ligaments to the umbilical cord.

### **The base**

The structure of the base depends of the sex of the individual. In the female, it is related to the upper wall of vagina, while in the male, the base is separated from the rectum by seminal vesicles and the recto-vesical pouch.

### **The neck**

The neck is the lowest part of the bladder. It is located behind the pubic symphysis, and it is perforated by urethral orifice. In the male, urethral tube passes through the prostate. In the female, it forms pelvic fascia that exists on the urethra. The bladder wall is made of detrusor muscle that is composed of smooth muscle fibers. In the male bladder, there is an involuntary internal urethral sphincter towards the neck, and this sphincter contracts when there is ejaculation in order to prevent reflux of semen into the bladder. The ureteric orifices are encircled by a ring-like detrusor muscle that stops the reflux of urine to the bladder's ureters. The bladder interior is lined in greater part, by the mucous membrane attached to a muscular coat. The same muscle is folded when the bladder is empty. Orifices of the ureters are situated at the postero-lateral angles of the trigonum vesicae, while internal urethral orifice is connected to the apex of trigonum vesicae. The muscle of the bladder's neck is called internal sphincter. By its natural contraction, it prevents the emptying of the bladder before the required pressure inside the bladder is attained. Urethra crosses a urogenital diaphragm made by a layer of muscles, united together to form an external sphincter of the bladder. The external sphincter is made of

skeletal muscle which is under voluntary control, and it is used to prevent uncontrolled urination.

## The structure of urinary bladder

The urinary bladder is composed of four layers:

(a) **Serous coat** (*tunica serosa*); derived from the peritoneum. It covers the superior and the upper parts of lateral surfaces.

(b) **Muscular coat** (*tunica muscularis*) made up of 3 layers: external, middle and internal layers named detrusor urinary muscle. This is a thick smooth muscle, deep layer that forms the internal wall of urinary bladder.

(c) **Submucous coat** (*tela submucosa*); a layer made of areolar tissue, which joins together the muscular and mucous coat.

(d) **Mucus coat** (*tunica mucosa*); thin and smooth. It is also found in ureters, in the lining membrane of the renal tubules and in urethra. This thin layer is also called **urothelium**.

Urothelium being an epithelial lining of the urinary tract, its role was initially seen as a simple barrier between the urinary tract and the urine. Today, it is known that urothelium plays an important role in the regulation of bladder's activity, and it is involved in causing bladder dysfunction. The urothelium of urinary bladder is made of 3 distinct layers: *the basal, intermediate and the superficial layers* directly lining the surface of the bladder. It is also known as the umbrella cells stratum, with tight junctions on its surface. It is covered by a sheet of crystalline proteins called "**uroplakins**". Urothelium has an important role in bladder contraction and relaxation because it acts as sensory neurones. Urothelium contains receptors for different neurotransmitters such as nicotinic, muscarinic and adrenergic. When it is stimulated, it releases neurotransmitters. When the

bladder is filling with urine, it stretches without losing its barrier property. This property is maintained by vesicles' movement from cytoplasm to the plasmic membrane of the umbrella cells, and by increasing the surface area of the cells. The trafficking of vesicles is mediated by cAMP, protein kinase A, and protein secretion (De Groat, 2004). The movement of vesicles is also accompanied by the release of nitric oxide (NO) and adenosine triphosphate (ATP) which bind to the receptors of sub-mucosa of the bladder (Fry *et al.*, 2004). The sensory nerves located at that area of the bladder, generate input to the spinal cord. Other stimuli of the sensory nerves are hydrostatic pressure and pH change (De Groat, 2004).

## Innervations of the urinary bladder

The urinary bladder is innervated by parasympathetic, sympathetic and somatic nerves.

The central pathway is located in the brain, spinal cord and pons.

### Efferent nerves

**a. Parasympathetic fibres** play a major role in the bladder function. It is formed by preganglionic fibres from central nervous system (CNS) and postganglionic fibres. The preganglionic fibres originate from S2-S4 (sacral outflow), and are responsible for the motor stimuli to the bladder. Activation of parasympathetic nerves induces contraction of the urinary bladder detrusor muscle. All preganglionic and postganglionic fibres release acetylcholine (ACh) as neurotransmitter, and are also called cholinergic neurones. The preganglionic neurones synapse with postganglionic fibres in ganglia that are situated within the bladder.

**b. Sympathetic fibres** that innervate urinary bladder are derived from T10 to L2, and pass through superior and inferior hypogastric plexi in order to reach urethral sphincter. It

releases ACh by pre-synaptic fibres and noradrenaline by postganglionic fibers. Noradrenaline induces contraction of the bladder base and urethral smooth muscle via its action on vesicle and urethral  $\alpha_1$ -receptors. The action of noradrenaline on  $\beta_2$ -receptors causes relaxation of the bladder's body (Moffett, 1993).

**c. Somatic nerves** (skeletal motor fibres)

These nerves originate from S2-S4, and pass through pudendal nerves to innervate skeletal muscles of external urethral sphincter. During voiding, there is inhibition of these nerves, which leads to relaxation of external sphincter. Somatic nerves release ACh, its action on nicotinic receptors induces contraction of external sphincter. This skeletal muscle also receives noradrenaline from sympathetic nerves. The combination of sympathetic and somatic actions increases resistance of urinary bladder outlet, which strengthens urinary continence (Souter, 2005).

### Afferent pathways

When the bladder is full of urine, the fullness is conveyed to the spinal cord by afferent nerves. Afferent nerve fibres are sensitive to the pressure exerted by urine on the bladder walls, owing to tension receptors situated in the urothelium layer. The micturition reflex in humans is mediated by myelinated axons because unmyelinated fibers are unresponsive to chemicals or cold stimuli. When the bladder is filling, there is stretching of urothelium; this situation causes cellular changes in order to accommodate the increasing quantity of urine. Urothelium being a tissue with neurone-like properties, releases neurotransmitters such as ATP and NO that stimulate sub-mucosal afferent nerves, and leads to transmission of sensations from bladder to spinal cord (De Groat, 2004).

## Blood vessels of urinary bladder

The urinary bladder receives blood from the superior, middle and the inferior vesical arteries originating from the anterior trunk of hypogastric, the obturator and inferior gluteal arteries which branch from the vagina in the female. Vessels that drain blood from the bladder are those from plexi of the inferior surface and hypogastric area (Marieb, 2004).

## Cytology of urinary bladder smooth muscle

Smooth muscles are divided into two types, namely single unit- and the multi-unit smooth muscles. This study focuses on a single unit smooth muscle (SUSM), since it is the one found in the urinary bladder. SUSM contains fibres that have synchronous electrical and mechanical activities, and when it is stimulated, it contracts as a single unit. These fibres are attached to other adjacent cells by gap junctions: channels that facilitate the flow of ions from one cell to another, leading to the propagation of action potentials (AP). These gaps are formed by trans-membrane proteins known as connexins. The contractile response of the smooth muscles may be due to the stimuli brought by nerves, hormones and other factors such as heat and change in pH. When the smooth muscle stretches, it increases the luminary volume which may also induce contraction caused by reflex system stimulation. The smooth muscle is made of proteins that play a role in the urinary bladder contraction and relaxation, made of actin latices (formed by globular actin molecules); myosin filaments (contractile units); dense bodies and intermediate filaments that interconnect the dense bodies (Sugi, 1992).

**Thin filament**

Thin filaments are mainly constituted by actin and tropomyosin, actin filaments itself being made by two stranded helix of actin monomers. Tropomyosin is found in the groove on either side of actin filaments (Berne and Levy, 2004).

**Myosin (thick filament)**

Myosin is made up of two pairs of heavy chains formed by a head and a tail structure that assumes a filamentous form during the contraction-relaxation process (Fozzard and Jennings, 1991). The cross bridge between actin and myosin occurs at the region of the head of heavy chain.

**Calmodulin**

This is a cytosolic protein that contains four Ca<sup>2+</sup> binding areas. The binding induces molecular conformational changes and becomes activated, ready to bind to the target cell (Sugi, 1992).

**Myosin light chain kinase**

This is an enzyme that depends on calcium-calmodulin complex to get activated. It regulates the phosphorylation of the light chain present on the head of the myosin (Berne and Levy, 2004).

**Dense bodies**

Dense bodies play a role of actin anchorage site; the same role is played by Z-line in skeletal muscles. In smooth muscles, contractile apparatus is not organized into myofibrils; instead, they are simply replaced by dense bodies in myoplasm. In sarcolemma, dense bodies are attached to one another as patches on the inner side of plasma membrane (Berne and Levy, 2004).



### **Sarcoplasmic reticulum (SR)**

SRs are small diameter sarcotubules that surround the myofibrils'. They have no basement membrane, and are often close to the surface of sarcolemma where they form dreads. SRs extend throughout the cell, and at some points, abut regions of sarcolemma (Sugi, 1992).

### **Intermediate filaments**

These filaments are made up of insoluble proteins that form the skeletal frame-work of cytoplasm. They are divided into two different forms: (a) filaments that contain actin, desmin and filamin; and (b) filaments that contain actin, myosin, caldesmon and tropomyosin (Berne and Levy, 2004).

### **Gap junction**

They are responsible for electrical and chemical communications by diffusion of low molecular weight compounds, and facilitate the propagation of action potentials (AP) from one cell to another (Bullock *et al*, 1995).

### **Caveoli**

Caveoli are invaginations of smooth muscle membranes (Berne and Levy, 2004; Lai *et al*, 2004).

## **CHAPTER TWO**

### **PHYSIOLOGY OF URINARY BLADDER**

#### **Peripheral nervous system**

In this section, the nervous system will be described because it is one of the components that play a crucial role in urinary system. The description made focus on the autonomic nervous system in order to understand the functions of the urinary bladder. I will also examine three components of the nervous system: parasympathetic, sympathetic and somatic nervous systems. Parasympathetic and sympathetic nervous systems are known as autonomic nervous or involuntary nervous system because they are not controlled by will.

The contraction of urinary bladder and relaxation of internal sphincter are involuntary actions. On the contrary, the skeletal muscle that plays the role of external sphincter is controlled by the brain, thus voluntarily controlled (somatic innervations).

#### **Parasympathetic nervous system**

Parasympathetic nervous system is made up of pre- and post-ganglionic fibres. The pre-ganglionic fibres originate from CNS (midbrain, medulla oblongata and spinal cord). The parasympathetic fibres that innervate the urinary bladder are those from sacral area of the spinal cord. They pass through pelvic nerves before synapsing with post-ganglionic nerve, and abut to the urinary bladder. The pre-ganglionic fibers are longer than the post-ganglionic ones which cause the innervated organs to lie near the ganglion.

The parasympathetic nervous system plays an important role in energy conservation and maintenance of organ function when less energy is required. The pre- and post-ganglionic

nerves release acetylcholine, respectively, in ganglion and in the neuro-effector junction of innervated organs (Rhoades and Pflanzner, 1989).

### **Synthesis of acetylcholine (ACh)**

Acetylcholine is synthesized from acetyl co-enzyme A and choline by an enzyme called choline acetyltransferase. Acetyl co-enzyme A is a compound formed during glycolysis, catalysed by pyruvate dehydrogenase. This synthesis takes place in the mitochondria, and then transported by citrate into cytoplasm where the complex is hydrolyzed by citrate lyase. After its synthesis, ACh is stored in synaptic vesicles. This process requires energy that is provided by ATPase, which pumps proton inside the vesicle. The release of  $H^+$  from the vesicles provides the required energy for the uptake of ACh. ACh base being positively charged, ATP and heparin sulphate proteoglycan help in the molecular neutralization, and thus, facilitate the packaging (Rang, 2003).

### **The release of ACh**

When an action potential reaches the end of efferent nerves, there is a release of ACh as a result of fusion between synaptic vesicles with plasma membrane following depolarization that opens  $Ca^{+}$ -dependant channels. This synaptic transmission is also known as electrochemical transmission that allows ACh to be released into synaptic cleft, and to bind to nicotinic receptors that are present in ganglions. The binding of ACh to postsynaptic receptors causes stimulation of postsynaptic neurones, called "direct ligand-gated conductance". When two ligands bind to a nicotinic receptor, they cause a change in conformation, followed by the opening of ionic channel that is selective to  $K^{+}$  and  $Na^{+}$ , and depolarization of the postsynaptic neurons. The duration of the binding is limited to

less than 10 ms, and ACh is degraded by acetylcholinesterase (AChE) (Goodman and Gilman, 2001).

### **Acetylcholinesterase (AChE)**

Acetylcholinesterase is an enzyme involved in the degradation of ACh. There are two types of AChE, namely: (i) acetylcholinesterase or true cholinesterase, and (ii) butylcholinesterase (BuChE) that is also known as 'pseudo-cholinesterase' (Goodman and Gilman, 2001). The first one is formed by simple homeric oligomers in catalytic subunits, while the other one is formed by heteromeric catalytic subunits. The mode of action of AChE resides in its ability to hydrolyze ACh, thus reducing the action of Ach (Golan *et al.*, 2005).

### **Activation of nicotinic receptors at neuro-muscular junction**

The binding of ACh to nicotinic receptors of a cell membrane causes depolarization of the motor-end-plate, and triggers muscle contraction. ACh also binds to different types of muscarinic receptors, and causes different outcomes according to the organs concerned (Goodman and Gilman, 2001).

### **Physiology of cholinergic transmission**

The parasympathetic system is mainly formed by neurones that discharge discrete and localized neurotransmitters. Its role is to maintain the organ's function and energy conservation during the period of low activity. Parasympathetic nervous system stimulation excites the GIT, reduces the heart rate, decreases the blood pressure and contracts the urinary bladder smooth muscle. In order to assume that task, the post-synaptic neurons release ACh to innervated organs (Golan *et al.*, 2005).

## **Steps in ACh transmission**

**It** is important to understand the steps involved in ACh transmission because they help in understanding ACh regulation. When the axon is at rest, its interior is about -70mv compared to its exterior. This difference in potential is maintained by the high concentration of  $K^+$  in the axoplasm. The extracellular area is highly concentrated in  $Na^+$  and  $Cl^-$  ions. The gradient of these ions is maintained by the active transport that requires energy from ATP by  $Na^+/K^+$ ATPase. During depolarization, there is initiation of action potential (AP) by increased permeability of axonal membrane to  $Na^+$  through voltage-sensitive  $Na^+$  channels. Inside the axon, there is an increase in positive charges. The second phase involves the closure of  $Na^+$  channels, and the delayed opening of  $K^+$  channels that allows  $K^+$  to leave the axon. Depolarization is maintained by constant inward movement of  $Ca^{2+}$  ions; all three events initiate electrical circuit in the axon, which, in turn, propagates to the area of axon that was not previously affected. When the action potential reaches the axonal terminal, it causes excitation or inhibition on synapse or effectors organs (Goodman and Gilman, 2001).

## **Nicotinic ACh receptors**

Nicotinic ACh receptors are known to be ligand-gated ionic channels. When they are activated, they cause an increase in permeability of host cells to  $Na^+$  and  $Ca^{2+}$ . The sequence of their amino acids have been found by the process of molecular cloning which has revealed the existence of two species of nicotinic receptors that belong to different families of proteins. They exist in a pentameric form, comprising one to four different subunits built around an ionic channel. Nicotinic acetylcholine receptors are molecules that weigh about 280 KDa, and are made of five subunits that form a central channel.

Because of the similarity with GABA, serotonin and glycine receptors, they are in one group called "nicotinoid receptor family". Nicotinic receptors have twelve subunits made up of  $\alpha$  and  $\beta$  units that combine together to make a pentamer. For example, nicotinic receptors on skeletal muscles comprise of four different subunits ( $\alpha 1, \alpha 2, \beta 1, \beta 2$ ). When ACh binds to these receptors, there is initiation of end plate potential (**EPP**) and excitation of postsynaptic potential in the neurone (EPSP) (Goodman and Gilman, 2001).

### **Muscarinic receptors**

Five subtypes of muscarinic receptors have been identified, using DNA cloning method (Eglen and Nahorski, 2000).

These five muscarinic subtypes are differently localized in the body.

The pharmacological classification of muscarinic receptors is based on selectivity of agonists and antagonists. McNA343 and oxotremorine display selectivity to  $M_1$ -receptors, and among antagonist, pirenzepine is selective on  $M_1$ -receptors.  $M_1$ -receptors are found mainly in CNS and in peripheral neurons of gastric parietal cells. They are also found in ganglia and in secretory glands.  $M_1, M_3$  and  $M_5$  subtypes exert their activity by stimulating G-protein called  $G_q$  coupled to phosphatidylinositol polyphosphate of cell membranes, resulting in formation of inositol polyphosphates. This process leads to the release of  $Ca^{2+}$  from endoplasmic reticulum, which initiates the contraction of smooth muscles. Another product of hydrolysis is diacyl glycerol (DAG), which activates protein kinase C.

-  $M_2$ - and  $M_4$ -muscarinic receptor activations lead to interactions of the receptors with G-proteins ( $G_i$  and  $G_o$ ), resulting in inhibition of adenylate cyclase (AC), activation of receptor-operated K channels, and suppression of the activity of voltage-gated Ca

channels (Uustare *et al.*,2003). The  $\alpha$ -subunits of G-protein ( $G_i$ ) possess the following subtypes  $\alpha_i$  ( $\alpha_{i1}$ ,  $\alpha_{i2}$ ,  $\alpha_{i3}$ ). When M2 mACh R is stimulated, adenylate cyclase previously stimulated becomes inhibited through activation of  $G_i$  proteins (Uustare *et al.*, 2004). The contraction of urinary bladder is mediated by parasympathetic nerves through the binding of ACh to M2 and M3 receptors (Tong and Cheng, 1990). Studies on bladder dome have revealed that M2 receptors are more in number compared with M3, but the contractile response of detrusor muscle is mediated mainly by M3-receptor subtype  $G_o$  (Uustare *et al.*, 2001).

### Interaction between M<sub>2</sub> and M<sub>3</sub> receptors

It has been shown that M3-receptor stimulation has a direct contractile effect on bladder smooth muscles through mobilization of  $Ca^{2+}$  inside the smooth muscle cells, while M2-receptor action is mediated through M3-receptor potentiation (Ehler, 2003). M2-receptor stimulation may also potentiate other  $Ca^{2+}$  mobilizing receptors, which suggests that M2-receptors alone cannot mediate a direct contraction of smooth muscles because those contractions require  $Ca^{2+}$  mobilization. The inhibition of cAMP production through M2 receptor stimulation leads neither to calcium mobilization nor to direct contraction of smooth muscles. Because M2-receptor stimulation can inhibit p-adrenoreceptor-mediated effect that leads to cAMP production, thus, inhibition of smooth muscle contraction, it is clear that M2-receptors can stop inhibition of contraction resulting from the relaxant effects generated by P-adrenoceptor activation. This is why contractions generated by stimulation of M2- and M3-receptors in the presence of P-agonist are far stronger compared to the contractions generated by activation of M3-receptors alone. When a smooth muscle is at rest, the  $Ca^{2+}$  concentration inside its cells is low; therefore, little

effect will be attained by stimulation of M2-receptors because this effect is supposed to result from  $\text{Ca}^{2+}$  mobilization. But if M<sub>3</sub>-receptors are stimulated first, there will be  $\text{Ca}^{2+}$  mobilization, and then stimulation of M2-receptors will generate contraction through stimulation of ionic conductance  $I_{\text{cat}}$  and  $\text{BKCa}$  (channels that depend on  $\text{K}^+$  and  $\text{Ca}^{2+}$  respectively, in order to open and close). Calcium mobilization will stop the efflux of  $\text{K}^+$ , and thus inhibit relaxation. This observation may be the explanation for the fact that contraction of urinary bladder is due to the stimulation of M3-receptors, even though studies on urinary bladder dome revealed that the density of M2-receptors is higher than that of M3-receptors, and the contractile action of the bladder is being triggered through M3-receptor stimulation. When competitive, selective antagonists on muscarinic receptors are used, the blockade of contractions is consistent with M3-receptor blockade instead of M2-receptors, but when non-selective antagonists are used, contractions that result from stimulation of M2- and M<sub>3</sub>-receptors together are far greater than the response obtained from stimulation of M3-receptors alone (Ehlert, 2003). The studies of Giglio *et al.* (2001) and Ehlert (2003), suggest that other neurotransmitters, apart from acetylcholine, are involved in detrusor muscle excitation because, atropine, which is a non-selective muscarinic receptor antagonist, failed to inhibit totally, contractions of urinary bladder. It has been found that purine nucleotide ATP, with its action on P<sub>2</sub>-purinoceptors, may be responsible for contractions that are resistant to atropine's inhibitory effect. Adenosine, a purine nucleoside from ATP, causes relaxation of detrusor muscle via subtype A<sub>i</sub> or P<sub>r</sub> purinoceptors. It is now clear that contraction and relaxation of urinary bladder smooth muscles are caused by interaction of numerous substances that act on different receptors. Because ATP is co-released with acetylcholine in the



parasympathetic nervous system innervating urinary bladder, and its metabolite, adenosine, has a relaxant effect on urinary bladder detrusor muscle, it can be assumed that ATP has an indirectly relaxant effect on urinary bladder. This is probably why when ATP is administered to an isolated urinary bladder; it causes an initial contraction followed by a long duration relaxation. These relaxations can be terminated by adenosine receptor antagonists. In this scenario, M<sub>3</sub>-receptor stimulation may interfere with the relaxant effect of cAMP produced during stimulation of purinergic receptors by adenosine. The effect of M<sub>3</sub>-receptors is largely due to activation of adenylate cyclase.

### The role of G-proteins

G-proteins are molecules that interact with guanine nucleotides GTP and GDP. They consist of 3 subunits  $\alpha$ ,  $\beta$  and  $\gamma$ . When guanine nucleotide (GTP) binds to an  $\alpha$ -subunit, there is conversion of GTP to GDP, while  $\beta$   $\gamma$ - complex remains unchanged. The three subunits are attached to phospholipids of the cell membrane, and are coupled to G-protein by a reaction called 'phosphorylation'. When no ligand is attached to the receptor, the G-protein consists of unseparated  $\alpha\beta\gamma$ -complex with GDP attached to  $\alpha$ -subunit. When an agonist settles on these receptors, there is a conformational change in cytoplasmic domain that becomes coupled to  $\alpha\beta\gamma$ -complex (Goodman and Gilman, 2001). This event allows GDP that was previously bound to the  $\alpha$ -subunit to exchange with GTP, followed by dissociation of the  $\alpha\beta\gamma$ -complex into  $\alpha$ -GTP and  $\beta\gamma$ -complex (Golan *et al.*, 2005). The newly formed fragments are active forms that are ready to diffuse and activate ionic channels and different proteins inside the cell. This action will be terminated by the hydrolysis of GTP to GDP, catalyzed by GTP-ase followed by reunification of  $\alpha$ -GDP and  $\beta\gamma$ -complex. The selectivity of G-protein is due to the difference in the affinity that

the various classes of G-proteins (Gi, Gs and Gq) have with respect to the receptors and the effectors involved. Activation of Gs and Gi leads, respectively, to the stimulation and inhibition of adenylate cyclase (Golan *et ai*, 2005; Rang *et ah*, 2003; Goodman and Gilman, 2001).

## Second messengers

Second messengers are cytoplasmic compounds, whose synthesis, release and degradation are responsible for changes seen when ligands bind to G-protein receptors on cell membranes. In this section, attention will only be focused on cAMP, Ca<sup>2+</sup>, inositol phosphate, and diacylglycerol. All second messengers influence one another, which make their study more complex.

### 1. cAMP and adenylate cyclase

Cyclic adenosine monophosphate (cAMP) is a nucleotide that is synthesized in the cell by action of adenylate cyclase on adenosine triphosphate (ATP). Its existence is terminated by a group of enzymes called 'phosphodiesterases', which transform cAMP into 5'-AMP. cAMP is involved in regulating cellular functions such as ion transport, contraction of smooth muscle, and modulation of ionic channel. All these functions are due to its ability to activate protein kinases. Protein kinases use ATP as source of phosphate group in order to phosphorylate proteins' serine and threonine residues. In urinary bladder smooth muscle, protein kinase phosphorylates an enzyme involved in muscle contraction known as myosin light chain kinase. When the receptor linked to Gi is activated, they inhibit adenylate cyclase which leads to the reduction in cAMP synthesis. For example, stimulation of M2-receptors of urinary bladder detrusor muscle by acetylcholine leads to inhibition of adenylate cyclase (Rang, 2003).

## 2. Inositol phosphate and phospholipase C

Inositol phosphate is a second messenger generated by the breakdown of membrane phospholipids. This messenger increases the concentration of intracellular  $\text{Ca}^{2+}$  in smooth muscles. Phosphatidyl inositol 4, 5-bisphosphate (PIP<sub>2</sub>) is a substrate of PLC $\beta$ , which dislocates into diacyl glycerol (DAG) and inositol 1, 4, 5- triphosphate (IP<sub>3</sub>) that functions as second messengers. IP<sub>3</sub> triggers the release of  $\text{Ca}^{2+}$  from intracellular stores. IP<sub>3</sub> being water soluble, acts on IP<sub>3</sub> -receptors in cytosol, which is a ligand-gated calcium channel that exists on the endoplasmic reticulum membrane. Inside the cell, IP<sub>3</sub> is converted into 1, 3, 4, 5-tetraphosphate (IP<sub>4</sub>) by a kinase. IP<sub>4</sub> is involved in facilitating  $\text{Ca}^{2+}$  entry into cytoplasm. DAG, formed from PLC $\beta$ , diffuses inside the cell membrane, and activates protein kinase C (PKC), which phosphorylates protein inside the cell. DAG is generated during hydrolysis of inositol phosphate, and is involved in the activation of membrane bound protein kinase, followed by phosphorylation of different intercellular proteins. Being a lipophilic compound, it is able to diffuse through the cell membrane. When GAD activates specific protein, there is an increase in intracellular  $\text{Ca}^{2+}$  concentration (Rang, 2003).

### Sympathetic nervous system

In urinary bladder function, sympathetic neurones are derived from T<sub>11</sub>, L<sub>1</sub> and 2, and they innervate internal sphincter. The action of noradrenaline on  $\alpha$ -receptors causes contraction of urinary bladder sphincter. When noradrenaline is released by sympathetic neurones, its binding on  $\beta_2$ -receptors of the bladder base causes relaxation (Vignes, 2005).

### **Synthesis, action and degradation of noradrenaline**

Noradrenaline is a neurotransmitter released by sympathetic postganglionic neurons. It is synthesized from tyrosine by a series of reactions. Tyrosine reacts with tyrosine hydroxylase to give dopa. By the action of dopa decarboxylase, dopa is transformed to dopamine; the action of dopamine  $\beta$ -hydroxylase on dopamine generates noradrenaline. Noradrenaline is changed into adrenaline by phenylethanolamine N-methyltransferase. The release of noradrenaline from vesicles is triggered by the arrival of action potential (AP) impulses which stimulate the discharge of  $\text{Ca}^{2+}$  into synaptic vesicles, followed by the release of noradrenaline (Golan *et al.*, 2005)

### **Beta-adrenergic receptors**

There are three types of  $\beta$ -adrenoceptors with similarities in 60% of their amino acid sequence. They are membrane spanning receptors, and noradrenaline binds to their external domain. The three  $\beta$ -adrenergic receptors stimulate adenylate cyclase by their interaction with Gs proteins. When these receptors are stimulated, there is accumulation of cAMP, followed by activation of protein kinase. Gs may also modulate the voltage-sensitive  $\text{Ca}^{2+}$  channels. Stimulation of urinary bladder P2-receptors leads to relaxation caused by the increase in cAMP synthesis and release. The relaxation of this smooth muscle is also due to the extrusion of  $\text{Ca}^{2+}$  from muscle cells, and the binding of the remaining  $\text{Ca}^{2+}$  inside sarcoplasmic reticulum store (Culter *et al.*, 1994).

### **Alpha-adrenergic receptors**

Stimulation of  $\alpha$ -adrenoceptors by agonists triggers mobilization of  $\text{Ca}^{2+}$  in the cytoplasm from endoplasmic reticulum stores by phospholipase C. Phospholipase C hydrolyses phosphoinositides to produce diacyl glycerol (DAG) and inositol-1, 4, 5-triphosphate

(IP<sub>3</sub>), which activate protein kinase C, followed by the release Ca<sup>2+</sup> from endoplasmic reticulum stores (Culter *et al*, 1994).

## Other neurotransmitters involved in urinary bladder function

### **Adenosine Triphosphate (ATP)**

ATP is a co-transmitter in terminal nerves. In the urinary bladder, ATP is responsible for the bladder contractions initiated by parasympathetic nerves. Its action is blocked by suramin, which antagonizes ATP at its purinergic receptors (Giglio, 2001). When urothelium of the urinary bladder is stimulated by stretch, hydrostatic pressure or pH change, it releases ATP, which binds to purinergic receptors P<sub>2</sub>X, and mediates mechanosensory transduction in the bladder afferent nerves (Rang *et al*, 2003; Benko *et al*, 2003).

### **Vanilloid receptors**

Vanilloid receptors are present in afferent neurones. They inherited this name on the basis of the fact that they are stimulated by capsaicin, an alkaloid found in chilli peppers, with vanillic acid structure (De Groat, 2004). Vanilloid receptors are sensitive to heat, pH variation (such as the transient receptor potential channel vanilloid receptor subunit 1 TRPV1). When TRPV1 is activated by heat above 50°C, it stimulates sensory neurones. Vanilloid receptors are also sensitive to mechanical stimuli, and their excitation is mediated by different cation channels (Golan *et al*, 2005). When an agonist such as capsaicin binds to these receptors, there is opening of Ca<sup>2+</sup> and Na<sup>+</sup> channels, followed by depolarization, resulting in the propagation of action potential (AP). Other compounds that act on vanilloid receptors are: anandamide and resiniferatoxin. When capsaicin is applied to urinary bladder, it causes degeneration of afferent nerve terminals (Fry *et al*,

2004). This is why it is used to treat incontinence (inhibition of C-fibers of afferent nerves). Stimulation of urinary bladder by capsaicin results in the release of nitric oxide (NO) mediated by calcium-dependant nitric oxide synthetase (Fry *et al.*, 2004; Culter *et al.*, 1994).

### **Prostaglandins**

The epithelium of urinary bladder is not a passive barrier between the urine component and the blood. Contrary, it releases a second messenger such as PG (F2a; F2) and other non-prostanoic compounds that participate in the bladder function. Prostaglandins are second messengers that regulate the bladder's physiological and pathological functions. Increases in synthesis of prostanoid compounds, for example, in the case of epithelium irritation, causes hyperactivity of detrusor muscle. In diabetic rats, the amount of PGF<sub>2α</sub> and PGF<sub>2</sub> are greater compared to the urinary bladder of normal rats. Hyperactivity induced by the increase of prostanoic compounds can be treated successfully with indomethacin. This situation seems to be due to the direct action of prostanoic compounds in urinary bladder smooth muscle, rather than on sensory neurone endings. The PGF<sub>2α</sub> and PGF<sub>2</sub> production is due to the cyclo-oxygenase 2-induced response to pro-inflammatory cytokinines and lipopolysaccharides (Yamaguchi, 2004). Prostaglandin in this case, is increased as compensation against diabetic neuropathy of motor and sensory nerves (Yamaguchi, 2004)

## The micturition reflex

Even if micturition reflex is an automatic action, in an adult person, it can be inhibited by the appropriate centre in the brain (the centre that is located in pons and cerebral cortex). The higher centre will inhibit micturition until the individual has a desire to urinate, and it may control and inhibit micturition even if there is a desire to urinate. In due time, the cortical centre will facilitate micturition reflex and inhibit contraction of external sphincter. Urine is brought in to the bladder by small smooth muscle tubes called ureters. They are innervated by both sympathetic and parasympathetic nervous systems. The flow of urine in the ureters is facilitated by peristaltic waves traveling along the ureters. When the bladder is empty, intravesical pressure is zero, and the pressure rises as the bladder progressively receives urine. As the bladder fills up with urine, micturition contraction starts to appear as a result of stretch of urinary bladder that stimulates the sensory receptor of the bladder wall. These sensory signals are brought to the spinal cord by pelvic nerves, and return to the bladder through parasympathetic fibres. The first contraction, self generated, induces further activation of receptors which cause increase in reflex contraction of the bladder; the scenario is repeated until the bladder has reached a high degree of contraction. The micturition reflex is a cycle made up of 3 phases:

- (1) progressive and rapid rise in internal pressure
- (2) constantly raised pressure, and
- (3) drop in basal tonic pressure of the bladder.

When micturition pressure has not succeeded in emptying the bladder, the second reflex may come after a period of about 30 minutes to 1 hour. As the urine continues to flow inside the bladder, the contraction becomes more and more stronger, and often painful.

As micturition becomes stronger, another reflex is generated, and passes along pudendal nerves to the external sphincter in order to inhibit it. If the voluntary contractor signals of external sphincter from the brain cannot overcome pudendal reflex, involuntary urination may occur (Souter, 2005; Viges *et al.*, 2005).

### Contraction of the smooth muscle of urinary bladder

The urinary bladder smooth muscle functions as other smooth muscles found elsewhere in the body, with a slight difference in how it is stimulated. Smooth muscles in general, differ from skeletal muscles because the former is not under voluntary control. The structures of these two types of muscles differ also. Smooth muscles have no striation found in skeletal muscles. In urinary bladder, cells are irregularly shaped and bind together by intermediate junctions. At these junctions, cells are thick and separated by gaps that contain dense granular materials. Through these gaps, proteinous tubes connect adjacent cells, and give a passage to ions and small molecules. When urinary bladder smooth muscles are at rest, their cells have a membrane potential that is between -20 and 65 mv. This resting membrane potential is maintained by the relative permeability of the cell membrane to ions through ionic pumps like  $\text{Na}^+\text{-K}^+$  ATPase. The most important and useful channel in cell excitation is the voltage-gated calcium channel. This channel can be blocked by nifedipine or verapamil. Another channel that plays a role in the contraction of urinary bladder is the  $\text{K}^+$ -channel. The membrane of the bladder cells also has  $\text{Na}^+\text{-Ca}^{2+}$  exchange mechanism that allows calcium to enter into the cell, and to expel  $\text{Na}^+$  ions to outside of the cell.

As indicated previously, smooth muscle cells contain thin, thick and intermediate filaments. The thin filaments contain actin, while the thick filaments contain myosin.



Intermediate filaments are made of proteins called 'desmin' and 'vimentin' in smooth muscle cells. Thin filaments are organized into bundles inserted through dense bodies, which play a role comparable to the one played by Z lines in skeletal muscles. Thin filaments that pass through dense bodies have opposite polarities at their two extremities, which allow them to react with myosin. As skeletal muscles have T-tubules that are useful in providing electrical links to the sarcoplasmic reticulum (SR), smooth muscles use alveoli that assume the same role. They increase the surface volume ratio of the cell where  $\text{Ca}^{2+}$  enters the cell through sarcolemma that contains voltage-gated calcium channels. SR is also a reservoir of free  $\text{Ca}^{2+}$  and plays a role in excitation-contraction (E-C) coupling by regulating free  $\text{Ca}^{2+}$  concentration in the myoplasm. Ryanodine receptors (RyR) are important in assuming the role of ligand-gated, Ca-channels that are situated at the junctional area of SR, where  $\text{Ca}^{2+}$  is expelled after action potential (Frank and Bianchi, 1992). The release of  $\text{Ca}^{2+}$  from SR into myoplasm can be induced by neurotransmitters, hormones or drugs that bind to receptors on the sarcolemma. Ryanodine receptors are activated by an increase in intracellular  $\text{Ca}^{2+}$ . Its release from SR is important in balancing the concentration of free  $\text{Ca}^{2+}$ . It is a physiological regulation in which free cytoplasmic  $\text{Ca}^{2+}$  is kept at a controlled level in the centre of SR, and at the junction of SR with plasma membrane. Contrary to skeletal muscles where  $\text{Ca}^{2+}$ -dependant channels can be opened potentially to release  $\text{Ca}^{2+}$  from T- cells, in smooth muscles, the release of  $\text{Ca}^{2+}$  from SR must be initiated by chemicals (Sugi, 1992). Contractions are produced by  $\text{Ca}^{2+}$  entry through voltage- or ligand-gated  $\text{Ca}^{2+}$  channels, or by  $\text{Ca}^{2+}$  released from SR initiated by IP3 (Frank and Bianchi, 1992; Rang *et al.*, 2003). Contraction of smooth muscles is generated when the myosin light chain is

phosphorylated by myosin light chain kinase, leading to its separation from actin filaments. This process of phosphorylation is catalyzed by myosin light chain kinase, and activated by  $\text{Ca}^{2+}$ -calmodulin complex. Myosin light chain kinase catalyses the phosphorylation of myosin P light chain, leading to the activation of myosin by actin. The end result of this series of reactions is the cross bridge and sliding of filaments, resulting in muscle contraction. During the interaction between phosphorylated light chain myosin and actin, ADP and inorganic phosphate (Pi) are liberated from myosin, allowing ATP formation. ATP reduces the affinity of myosin to actin, followed by the release of myosin from actin. Energy released in this process is used to change the conformation of myosin head, and another cycle of contraction is ready to start (Berne and Levy, 2004). Relaxation of smooth muscles requires a second enzyme called 'myosin phosphatase', which reverses previous phosphorylation.

## CHAPTER THREE

### Diseases of the urinary bladder

#### 1. Cystitis

Cystitis is an inflammation of the urinary bladder that may be the result of bacterial infection, or interstitial cystitis which is not caused by infection. When it is caused by infection (normally a coliform bacterium transmitted from the bowel to urinary bladder passing through urethra), it may be treated by antibiotics and the patient is advised to drink a lot of fluid which helps to flush out the remaining of unkilld bacteria from the bladder, then the bladder may recover after a certain period of time. Drugs that increase urinary pH, such as potassium citrate, are also given to the patient in order to relieve symptoms.

**Hemorrhagic cystitis** is an inflammation of the bladder that may result into hemorrhage. It may be caused by chemotherapy, viral infection, or radiations.

**Interstitial cystitis** is a disease of urinary bladder which causes intense pain of the bladder and the pelvis in general. It also induces frequent urination and painful sexual intercourse. It is caused by autoimmune factors, neurologic, genetic and allergic conditions. It damages the protective layer of the bladder (Rosamilia and Franzog, 2005). Treatment of interstitial cystitis involves modification of diet (avoid irritants), bladder coating (astringent, e.g. silver nitrate that kills infecting agents), antihistamines that control the released histamine from mast cells, and the use of antidepressants such as amitriptyline, that stop neuro-inflammation. When interstitial cystitis is caused by pelvic floor dysfunction, the treatment will be to relax pelvic floor muscles.

## **2. Ureterocele**

Ureterocele is a congenital abnormality of the urinary bladder that involves the distal ureter in the opening to the bladder. The patient may present with the following symptoms: urinary retention, haematuria, abdominal pain and urethral calculus. The cause of ureterocele is not yet well identified. If it is not treated, however, it may lead to kidney stones formation, increased uric acid stones, and poor kidney function (Gotta *et al*, 1998).

## **3. Urinary bladder incontinence**

Incontinence is an involuntary excretion of urine. There are many types of urinary incontinence (Michel *et al*, 2005):

### **a. Urge incontinence**

It is an involuntary urination which occurs without any reason. It results from contractions of urinary bladder detrusor muscles. It includes idiopathic detrusor over-activity and neurogenic detrusor over-activity. This incontinence normally occurs when the patient has taken fluid before going to sleep. Contractions of the bladder may occur because of the damage to the neurones that innervate urinary bladder, or to the spinal cord. This damage may be caused by surgical operation, Alzheimer's disease, and so on.

### **b. Stress incontinence**

This is an incontinence that results from an increased internal pressure from coughing, laughing, exercising, or even pregnancy. In females, it may be caused by a decrease in oestrogen that occurs before menstrual period. This decrease lowers the force of urethral sphincter, resulting in leakage. This kind of incontinence may be aggravated by menopause.

### **c. Overflow incontinence**

When the bladder is full of urine, it may leak as a result of the weakness of urinary bladder detrusor muscle that is not able to empty the bladder completely. Blockade of urethra may be responsible for this type of incontinence. Diabetic autonomic neuropathy may also cause overflow incontinence by the mechanism already explained earlier.

### **Treatment of urinary bladder incontinence**

Several options are available to treat urinary bladder incontinence. They include exercise, electrical stimulation that strengthens the muscles of lower pelvis, bladder training, and medications such as oestrogen, oxybutynin, darifenacin, propantheline, tolteroline, solifenacin tropsium, imipramine, and so on (Michel *et al*, 2005).

## CHAPTER FOUR

### Diabetes mellitus

**Definition** — Diabetes mellitus is a group of hormonal and metabolic syndromes characterized by hyperglycaemia and altered metabolism of lipid, carbohydrates and proteins (Goodman and Gilman, 2001). "Diabetes" means in Greek "pass through"; and "mellitus" "honeyed or sweet". It is named diabetes mellitus because patients with diabetes mellitus produce urine that contains sugar. This syndrome results from relative or absolute lack of insulin. It may also result from relative insufficiency of insulin production, or insulin resistance.

Another type of diabetes is called 'gestational diabetes': It occurs during pregnancy, and it is caused by resistance of the body cells to insulin. The increase in glucose is sometimes proportional to the increase in foetal weight (polyhydramnios). There are two main types of diabetes mellitus, known as 'type 1' (or juvenile onset, insulin-dependent diabetes mellitus, i.e., IDDM) and 'type 2' (or adult/maturity onset, non-insulin-dependent diabetes mellitus, i.e., NIDDM), respectively.

### Diagnosis

The diagnosis, in the first instance, is clarified by some symptoms such as polyuria, polydipsia and polyphagia that become increasingly worse as the disease progresses. Screening of the blood will reveal high glucose concentrations than normal ( $>5$  mmol/L). Confirmation of diagnosis requires the fasting blood glucose testing, and insulin and glucose tolerance testing. Some risk factors are associated with diabetes, and may guide physicians in its diagnosis. These factors are: high blood pressure, coronary artery

diseases, hepatic stenosis, and so on. Patients who are on chronic medication with glucocorticoids and/or thiazide diuretics may also develop diabetes mellitus (Shargel *et al*, 1997).

## Aetiology

Type 1 diabetes mellitus is usually caused by immunological and environmental factors, while type 2 diabetes mellitus is caused by endocrine disorder, pancreatic disease, drugs, genetic and environmental factors.

## Laboratory results

A patient is classified as diabetic, when his/her fasting blood glucose measurement gives a concentration greater than 7.0 mmol/L or when his/her random blood glucose measurement gives results that are greater than 11.0 mmol/L. The higher concentration of the patient's blood glucose must be obtained more than once in order to qualify him/her as diabetic. In order to measure the patient's compliance to the treatment, it is necessary to check the level of glucose bound to haemoglobin (glycosylated hemoglobin, HbA<sub>1c</sub>). When the result (HbA<sub>1c</sub>) is >7%, it means that the patient has not been compliant for the last three months (Shargel *et al*, 1997).

## Diabetic ketoacidosis (DKA)

Diabetes ketoacidosis (DKA) is one of the complications caused by uncontrolled diabetes mellitus, and is a medical emergency. DKA is accompanied by dehydration, abdominal pain, lethargy and coma. This complication is experienced by type 1 diabetic patients who totally lack insulin necessary to protect the degradation of fatty acid. Some type 2 diabetic patients may also experience DKA as their diabetic state becomes worse, because the pancreas may stop to produce insulin. In type 2 diabetic patients, a similar

complication called "hyperosmolar diabetic coma" occurs. Elevated blood glucose drives out water from cells into blood stream and finally reaches the kidney where it is eliminated with urine. High blood glucose concentration will exacerbate dehydration, increase in blood osmolarity, electrolyte imbalance, and coma (Shargel *et al.*, 1997).

### Complications of diabetes

Chronic complications of diabetes affect different organs of diabetic patients when glycaemia has not been well controlled. These complications are classified into two groups according to their locations: macro- and micro-angiopathy. When complications result from small vessels, they lead to the following complications:

- retinopathy (damage to retina) that may lead to blindness
- peripheral neuropathy that causes foot ulcers, and
- diabetic nephropathy that causes kidney failure.

When complications result from large vessels, the patient may experience ischaemic heart diseases (condition that is caused by imbalance between oxygen demand and supply to the myocardium). Diabetes mellitus can also be caused by genetic factors, P-cells defect, and peripheral insulin receptor defect that lead to insulin resistance. While type 1 DM is caused by genetic, environment and autoimmune diseases; type 2 diabetes is caused by other conditions such as endocrine disorders, pancreatic disease, pregnancy or drugs such as thiazide diuretics and corticosteroids. Insulin reduces the amount of blood glucose by inhibiting its production by the liver, stimulating its uptake and metabolism in skeletal and adipose tissues (Vinik *et al.*, 2003).



## **Anatomy of the Pancreas**

The pancreas is an organ that has exocrine and endocrine glands. The endocrine gland is also called 'Islets of Langerhans' which has three types of secretory cells, namely:  $\alpha$ -cells that secrete glucagon,  $\beta$ -cells that release insulin, and  $\delta$ -cells that secrete somatostatin and gastrin. Glucagon promotes glycogenolysis, gluconeogenesis and lipolysis of adipose tissues. Insulin promotes uptake of glucose, amino acids and fatty acids. It increases glycogen synthesis and storage in the liver from glucose. Somatostatin regulates the release of insulin and glucagons. It decreases the motility of gastrointestinal tract and growth hormones secretion. In the fed state, carbohydrates are broken down into glucose, fructose and galactose by gastrointestinal tract enzymes. These simple sugars are transported actively and passively by basal membrane transporters from epithelial cells into capillaries. When glucose reaches the blood stream, it is taken up into pancreatic  $\beta$ -cells. The increased concentration of glucose inside  $\beta$ -cells stimulates the release insulin that diffuses into portal vein, where it is transported by blood into the liver at the same time with digested food. Insulin thus secreted acts on tissues that store energy, such as liver, skeletal and adipose tissues. In the fasting state, glucose concentration decreases, pancreatic  $\alpha$ -cells release glucagon, and pancreatic  $\beta$ -cells reduce the amount of insulin secreted. Glucagon mobilizes glucose from the liver by increasing glycogenolysis and gluconeogenesis. When the individual continues with fasting, catecholamine and glucocorticoid concentrations increase. This situation leads to the release of fatty acid; break down of protein and adipose tissue (Golan *et al.*, 2005).

## **Insulin synthesis and secretion**

Insulin is synthesized in endoplasmic reticulum of pancreatic P-cells as a preproinsulin. It is then transported to Golgi apparatus where it is cleaved by proteolytic enzymes into insulin and c-peptide. The quantity of insulin to be released is proportional to the concentration of glucose in the blood. Two phases of insulin release are rapid secretion of stored insulin, and delayed secretion of synthesized insulin. This response is impaired in a diabetic patient (Goodman and Gilman, 2001).

### **Secretion**

When pancreatic  $\beta$ -cells are in contact with glucose, there is an increase in the ratio of ATP/ADP that stimulates insulin release. Glucose enters  $\beta$ -cells down concentration gradient, and gets phosphorylated by hexokinase into glucose-6-phosphate, then enters into glycolytic cycle that ends up by citric acid and ATP increases. This ratio modulates the activity of ATP-sensitive  $K^+$  channel. The opening of this channel hyperpolarizes the cell by increased outward of  $K^+$ . This channel is inhibited by ATP, and activated by ADP. The increased ATP/ADP ratio inside the cells shuts  $K^+$ -channel, and induces depolarization followed by activation of voltage-gated  $Ca^{2+}$ -channel. The increase in intracellular  $Ca^{2+}$  stimulates insulin release. In the case of low glucose,  $K^+$ -channel remains open because of insufficient ATP/ADP ratio, and  $\beta$ -cells will be in hyperpolarized state, which inhibits  $Ca^{2+}$  entry into the cells. Without influx of  $Ca^{2+}$  inside the cells, vesicles that contain insulin will not be able to release insulin. When insulin is released from vesicles of  $\beta$ -cells, it binds to receptors of target cells that exist on the cell membrane. A high density of insulin receptors is located on the surface of energy-storing tissues such as liver, adipose tissue, and striated muscles. These receptors are made up of

$\alpha$ - and  $\beta$ - subunits, located at the cell surface. Two  $\alpha$ -subunits are exclusively located at the surface of all membranes, while two  $\beta$ -subunits are transmembranous; one portion being outside, and the other one inside the cell membrane. When insulin binds to these receptors, it activates the tyrosine kinase domains of  $\beta$ -subunits, leading to receptor's autophosphorylation that initiates a cascade of reactions resulting in glucose transport, protein and glycogen synthesis. Insulin is also involved in visceral growth by stimulating cell proliferation (Golan *et al.*, 2005).

### **Glucagon synthesis and secretion**

#### **Synthesis of glucagon**

Glucagon synthesis occurs in  $\alpha$ -cells of the pancreas, and a small quantity is also synthesized in the upper part of GIT. Glucagon synthesis is stimulated by increased concentration of amino acids, especially arginine. Its secretion is induced by a protein meal, and a low concentration of blood glucose. When it is released from  $\alpha$ -cells, it increases blood glucose concentration. It has the same action as  $\beta$ -adrenergic compounds; and acts on specific receptors to stimulate adenylate cyclase (Goodman and Gilman, 2001).

## Treatment of Diabetes mellitus

Patients with type 1 diabetes mellitus must be treated with insulin. The insulin dose should be calculated on the basis of the patient's weight, and his/her plasma glucose concentration.

### Management, control and/or treatment of type 2 diabetes mellitus

#### 1. Sulphonylureas (e. g., glibenclamide)

Sulphonylureas are also called "insulin secretagogues". Sulphonylureas such as glibenclamide and gliclazide, stimulate insulin release from pancreatic  $\beta$ -cells. They inhibit  $K^+$ /ATP-channel, and they also increase insulin release by displacing  $Mg^{2+}$ -ADP, which activates  $K^+$ /ATP-channel. Because of their insulin release stimulation, sulphonylureas cause hypoglycaemia as their major side-effect. By their action on adipose tissues, sulphonylureas may cause patient's weight gain (Shalgel *et al.*, 1997).

#### 2. Biguanides (e. g., metformin).

The major action of biguanides is to increase insulin sensitivity by their action on adenosine monophosphate-dependant protein kinase (AMPPK). Biguanides activate AMPPK to block fatty acid degradation and gluconeogenesis. They also increase the sensitivity of insulin at the receptors. By decreasing the formation of glucose from fatty acid metabolites, they cause lactic acidosis as side-effect (Das *et al.*, 1996).

### 3. **Thiazolidinediones** (e. g., rosiglitazone)

These compounds decrease blood glucose by increasing insulin action at its receptors. In diabetic patients, the level of glucagons may be elevated, a situation that opposes the effect of insulin because glucagons stimulate glycogenolysis and gluconeogenesis (Das *et al*, 1996).

## Complications of diabetes mellitus

When diabetes mellitus is not well controlled, it causes complications that involve several organs and systems. Some complications are acute, while others are chronic.

### **Acute complications**

- diabetic ketoacidosis (DKA)
- hyperglycaemic hyperosmolar non-ketotic coma (HHNC)

### **Chronic complications**

- cardiovascular diseases
- ocular complications
- diabetic nephropathy
- skin and mucous membrane complications, and
- diabetic neuropathies

The focus here will be on diabetic neuropathy, because it is linked to the objective of the present my study.

## Diabetic autonomic neuropathy (DAN)

Diabetic autonomic neuropathy is a complication that results from uncontrolled diabetes mellitus. This complication is not well understood, even though it has a negative impact on the quality of life of diabetic patients. It is also one of the conditions that increase medical expenses, and long duration of hospitalization. DAN can involve all ANS, motor or sensory nerves. This condition may involve one or more organs at a time, and may be clinical or sub-clinical. Here are some of the organs involved:

- cardiovascular system
- sudomotor
- ocular, and
- genito-urinary system

When the vagus nerve is affected, because of its widespread nature in the body, DAN may cause a great damage to many organs at a time (Niakan *et al.*, 1986; Vinik *et al.*, 2003).

### **Epidemiology**

Diabetes neuropathy is a common complication of uncontrolled diabetes mellitus. It is a big cause of morbidity and mortality, with its prevalence of 20% in diabetic patients. It is also a cause of 75% amputation that results from non-traumatic causes. Hyperglycaemia is the main risk factor, and diabetes neuropathy is proportional to the duration of diabetes, age, hypertension and hyperlipidemia (Goodman and Gilman, 2001).

## **Pathology and Pathogenesis**

Factors that are involved in the development of diabetic neuropathy are: microvascular diseases, protein kinase C, polyol pathway and advanced glycation end product (Vinik *et al.*, 2003).

### **Aetiology of diabetic autonomic neuropathy**

#### **1. Microvascular diseases**

There is interdependence between blood vessels and nerve functions because the functions of blood vessels are controlled by nerves, and nerves are fed with oxygen and other nutrients by the blood carried in the vessels. Microvascular vasoconstriction, when it is not treated, causes nerve damage that results into neuronal dysfunction because of reduced blood supply around neurones and inevitable hypoxia. Neuronal ischaemia is one of the characteristics of diabetes neuropathy, and one of the clinical changes seen in diabetic patients with uncontrolled diabetes (Niakan *et al.*, 1986).

#### **2. Protein kinase C (PKC)**

Protein kinase C is an enzyme that adds phosphate groups to other proteins. This addition causes a functional change of the substrate. PKC is one of the enzymes that regulate cellular functions such as signal transduction. It removes inorganic phosphate from ATP and attaches it to hydroxyl group of amino acids (serine, tyrosine, and threonine). Its activity is turned off or on respectively, when it binds to protein inhibitor or protein activator. PKC is one of the proteins implicated in diabetic neuropathy because, the increase in blood glucose level leads to activation of diacyl glycerol, followed by activation of PKC that reduces nervous conductivity and blood flow around neurones (Vinik *et al.*, 2003).

### **3. Advanced glycated end-products**

When there is a high level of glucose inside the cell, this condition may lead to the establishment of covalent bond between glucose and cell proteins, resulting in alteration and inactivation of targeted proteins. Because of the change in structure, this also exacerbates diabetes neuropathy (Beshay, 2004).

### **4. Polyol pathway**

This pathway is also known as *sorbitol-aldose-reductase* pathway. It is a pathway that generates products involved in damaging micro-vessels that supply blood to nervous tissues. When blood glucose is high, there is activation of other biochemical pathways that have, as a consequence, reduction in glutathione and formation of oxygen radicals, by the action of enzyme called *aldose reductase*. Normally, body cells need insulin in order to utilize glucose, except retinal neuronal tissue and kidney cells. This means that without insulin, these glucose-dependent cells will continue to use glucose to produce the required energy. Excess glucose will be pushed into polyol pathways to generate sorbitol. As the level of blood glucose increases, the affinity of aldose reductase for glucose will also increase, stimulating the production of more sorbitol, and reduction in the level of NADPH. Sorbitol, being hydrophilic, cannot enter the cells easily, and its accumulation causes water retention. NADPH is involved in nitric oxide (NO), glutathione and fermentation of oxygen molecules. NO is a vasodilator of blood vessels, while glutathione protects cells against haemolysis resulting from oxidative stress. Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) which is also produced in the pathway, plays a role in preventing the formation of reactive oxygen species (ROS) that damage the cells. Consequently, when there is activation of polyol pathway, there is damage to the



vessels that supply blood to the kidney, retina and the neurones. Diabetic autonomic neuropathy (DAN) has not only one cause; it has a complex aetiology, among which are:

- autoimmune damage of neurones;
- deficiency in neuro-hormonal growth factors; and
- metabolic insult to nerve fibres.

Diabetes mellitus being a condition manifested by increased blood glucose, activates polyol pathway that causes an increase in the amount of sorbitol, and an imbalance in NAD: NADH ratio. This leads to the damage of neurones, and to a reduced blood flow. This situation is complicated by activation of protein kinase C that further causes vasoconstriction and reduction of blood flow around neurones. There is also an increased amount of free radical production, which reduces NO production and endothelium damage. When there is a reduction in neurotrophic growth factors, reduction of fatty acid and advanced glycosylation end-products, all these situations participate in the reduction of blood flow, leading to hypoxia that further damages neurones (Beshay and Carrier, 2004).

### **Clinical manifestations of DAN**

All diabetic patients must be screened in order to assess the progression of DAN because sometimes the patient may be in a sub-clinical state of the complication. Here are some complications classified according to the organs or systems involved.

### **1. Gastro-intestinal complications**

- gastroparesis diabeticorum
- constipation
- oesophageal dysmotility
- diarrhoea, and
- faecal incontinence

### **2. Metabolic disorders**

- hypoglycaemia unawareness
- hypoglycaemia-associated autonomic failure

### **3. Cardiovascular disorders**

- tachycardia
- exercise intolerance
- orthostatic hypotension

### **4 .Genito-urinary disorders**

- neurogenic bladder
- erectile dysfunction
- female sexual dysfunction
- retrograde ejaculation (Kolta *et al.*, 1985).

## **Cardiovascular autonomic neuropathy (CAN)**

Cardiovascular autonomic neuropathy is a complication of untreated diabetes mellitus that results from damage of neurones that innervate the blood vessels and the heart. This damage causes change in heart rate and blood flow. In diabetic patients with CAN, most

of the time, they manifest with orthostatic hypotension and exercise intolerance (Goodman and Gilman, 2001).

### Orthostatic hypotension

This is a fall in blood pressure when the patient changes position from supine to standing. Orthostatic hypotension is a result of damage caused to an efferent nerve of the sympathetic nervous system. Normally, when an individual changes his/her position from supine to standing, there is an increase in noradrenaline release in order to maintain the blood pressure. In a patient with CAN, this response is not as efficient as it is seen in a normal individual. This failure causes a sudden drop in blood pressure, and a reduction in heart rate. An individual with orthostatic hypotension usually presents with lightheadedness, dizziness, fatigue and neck pain (Niakan *et al.*, 1986)

### Exercise intolerance

A patient with CAN may experience reduction in heart rate and fall in blood pressure during exercise. This condition may lead to reduced cardiac output and impaired oxygen perfusion as well as metabolic requirement of various organs in the body (Vinik *et al.*, 2003).

### Gastro-intestinal autonomic neuropathy

Gastro-intestinal autonomic neuropathy complications are commonly present in patients with diabetes mellitus, and the severity of this condition is aggravated by other underlining conditions. Oesophageal dysfunction is associated with heart burn, delayed gastric emptying, anorexia, and nausea and vomiting. This disorder is caused by vagal neuropathy that leads to uncontrolled oesophageal peristalsis and impaired gastric

emptying. Because of the delay in bowel movements, there is overgrowth of bacteria that may contribute or initiate diarrhoea (Vinik *et al.*, 2003).

### Genito-urinary autonomic neuropathy

Diabetic autonomic neuropathy causes neurogenic bladder as a result of the damage caused on sensory and motor nerves. Deterioration of parasympathetic neurones may increase the supersensitivity of muscarinic receptors and increase bladder contraction. The damage of efferent nerve fibres to urinary bladder causes hesitancy, dribbling and weak/poor urine stream. The reduction in detrusor muscle activity causes increased post voiding residual urine, and subsequent urinary retention. The involvement of internal and external sphincter, owing to denervation of these muscles, results in overflow incontinence. Urinary bladder dysfunction predisposes the patient to urinary tract infection and pyelonephritis, and may even lead to renal failure (Spigt *et al.*, 2003; Spigt *et al.*, 2004).

### Erectile dysfunction

Erectile dysfunction (ED) is a consistent inability to have penile erection sufficient to sustain a satisfactory sexual intercourse. This condition must, at least, be present for several months to qualify as an erectile dysfunction. The cause of ED is multifactorial; it ranges from vascular disease, neuropathy, to metabolic disorder. Hyperglycaemia is responsible for glycation of fibres which impair the relaxation of *corpora cavernosa*,

causing failure of erectile mechanism. Many drugs have been used to reverse this situation, and these include sildenafil citrate (Viagra®). Erectile dysfunction may also be caused by dyslipidemia and endothelial dysfunction of sinusoidal cells, which decrease nitric oxide production, and thus reducing relaxation of penile muscles. In females, sexual dysfunction is characterized by a decreased sexual desire and dyspareunia during intercourse, due to inadequate vaginal lubrication (Vinik *et al.*, 2003).

### Pathophysiology of urinary bladder in diabetic patient

Diabetes mellitus is a syndrome that causes metabolic changes in the body and these changes are responsible for several complications that diabetic patients often experience. Usually, there is an increase in the amount of muscarinic receptors present in diabetic bladder. This condition leads to an increased contractility of detrusor muscles (receptor supersensitivity) (Kolta *et al.*, 1985; Nakamura *et al.*, 1992). More than 80% of diabetic patients suffer from urinary bladder dysfunction resulting from:

- (1) axonopathy of autonomic neurone of the urinary bladder, a myopathy caused by diuresis; and
- (2) alterations in adrenergic and muscarinic receptors caused by metabolic changes.

Studies by Tong *et al.* (1999) on STZ-treated diabetic rats demonstrated the change in response of bladder detrusor muscle to different muscarinic agonists. This change was attributed to an increase in muscarinic receptors in the dome of urinary bladder of diabetic rats when compared with age-matched controls. The supersensitivity of M-receptors is caused by deterioration of peripheral autonomic nerves, resulting from segmental demyelination and axonal degeneration.

## Modulation of detrusor muscle hyperactivity

Many factors are involved in the aetiology of urinary bladder hyperactivity. The central nervous system, neuronal extremity defect, and the pathology of urinary bladder muscle may be the major causes of urinary bladder hyperactivity. When sensory neurones are damaged, there is an increased frequency in micturition reflex, causing stimulation to occur at low volume of urine in the bladder. Fry *et al.* (2004) showed that stretch of the urothelium leads to release of adenosine triphosphate (ATP), which induces sensation of bladder fullness. For the control and stabilization of urothelium tissue sensitivity in the urinary bladder, detrusor muscle hyperactivity treatment must be of priority. Neurotransmitters such as acetylcholine, ATP, noradrenaline and their respective receptors are involved in the contraction of detrusor muscles. Normally, sensations of bladder fullness are propagated through pelvic and hypogastric nerve fibres; some of which are myelinated fiber-A8, while others are unmyelinated fibres-C. Investigations on these nerves, in normal individuals and in comparison with those of diabetic subjects, may reveal differences in their conductivity. Vanilloid receptors are involved in the sensory activity of urinary bladder to pH and temperature changes. Abnormal sensations may lead to hyperactivity or to relaxation of urinary bladder smooth muscle. Another tissue that has attracted attention of researchers is myofibroblast, because it plays a role in bladder fullness sensations. Myofibroblast is a layer interposed between nerve endings and urothelium, and it acts as an intermediary layer during the transmission of sensations. In diabetic patients, this layer may be impaired. This impairment leads to hypersensitivity of bladder fullness sensations, even with less urine in the bladder. In this tissue, there is a high density of non-neuronal P2X3 and VR1 receptors on interstitial cells. These two

structures play a role in the sensation of bladder fullness. Microscopic observation of myofibroblasts revealed the following characteristics: fine cytoplasmic filaments linked to dense bodies, rough endoplasmic reticulum, vacuoles and Golgi apparatus. These structures are also innervated by neurones that release nitric oxide (NO) and GMP, mediators that are absent in detrusor muscle. This observation shows that there is another network of nerves that connect myofibroblasts that are not present in detrusor muscle. ATP and change in pH may stimulate the myofibroblast, and initiate micturition reflex (Liu and Daneshgari, 2005; Matsutomo *et al.*, 2004).

## Drugs that affect urinary bladder

Most of the drugs used in the treatment of urinary bladder dysfunction are chosen because of their mode of action, and are not specifically conceived for urinary bladder dysfunction.

### **Anticholinergic agents**

Anticholinergic drugs are widely used in a variety of conditions that require treatment by inhibition of parasympathetic nervous system action. Some anticholinergic drugs are not selective for different sub-types of muscarinic M-receptors, and when used, they may cause more side-effects. Some anticholinergic compounds are natural, such as atropine and hyoscine found respectively in *Atropa belladonna* and *Datura stramonium*. Some of the anticholinergic drugs used in the treatment of urinary incontinence are tolterodine and oxybutynin, and they act by binding to muscarinic receptors. When ACh concentration increases at the receptor site, it displaces atropine which is a reversible antagonist. Because most anticholinergic agents are not specific on muscarinic receptors, their administration causes diverse side-effects (Kim *et al.*, 2005).

### **Central Nerves System (CNS)**

Muscarinic M-receptor antagonists cause CNS depression that is characterized by amnesia, reduction of rapid eye movement sleep (REMS), drowsiness and euphoria. When atropine is used in a high dose, it causes hallucinations and delirium. Anticholinergic drugs are also used to treat side-effects resulting from the use of antipsychotic drugs such as haloperidol and chlorpromazine.

### **Cardiovascular system**

Anticholinergic drugs increase heart rate (i.e., they cause tachycardia), and this is due to the inhibitory action of the drugs on vagal nerve through blockade of M2-receptors on the SA nodal pacemaker.

### **Gastro-intestinal tract**

Anticholinergic drugs are used as antispasmodic agents, and in the treatment of gastric ulcers, owing to their inhibitory properties on vagal nerve. Salivary gland secretions are mediated through M3-receptors, and when selective M3-receptor blockers or non-selective anticholinergics are used, the patient may experience dryness of mouth and reduction in gastric acid release. Anticholinergic drugs also reduce GIT motility and cause sphincter constriction, leading to constipation.

### **Urinary bladder muscle**

Anticholinergic drugs reduce normal contraction (both tone and amplitude) of ureters and urinary bladder smooth muscles. To achieve these effects, the drugs must be able to inhibit muscarinic M2- and M3-receptors, and cause unwanted effects in other organs.



## **Respiratory tract**

Parasympathetic stimulation causes bronchoconstriction by the action of ACh on nicotinic and muscarinic receptors in post ganglionic fibers. This mode of action makes the drug to be useful in the treatment of asthma and chronic obstructive pulmonary disease. By inhibiting the secretions, anticholinergic agents help during procedures that involve the use of anaesthetic agents in order to prevent secretion and accumulation of fluid in the upper respiratory tract.

## **Classification of muscarinic receptor antagonists**

### **a. Quaternary ammonium compounds**

ipratropium

oxitropium bromide

triotropium bromide

methylscopolamine

homatropine methyl bromide

propantheline

glycopyrolate

### **b. Tertiary amines**

homatropine hydrobromide

cyclopentolate hydrochloride

benzotropine mesylate

trihexyphenioly hydrochloride

dicyclomine hydrochloride

flavoxate hydrochloride

oxybutynin chloride

tolterodine

### **Selective muscarinic antagonists**

Triptamine and darifenacin are selective for muscarinic M<sub>2</sub>- and M<sub>3</sub>-receptors, and are used for the treatment of over-active bladder.

#### **Darifenacin**

Darifenacin has a higher affinity for muscarinic receptors. Its affinity for muscarinic M<sub>s</sub>-receptors is about 32-fold higher than for muscarinic M<sub>2</sub>-receptors. It is a muscarinic receptor antagonist with pseudo-irreversible activity. This drug is used for the treatment of bladder hyperactivity because it has an inhibitory effect on the contraction induced by non-muscarinic receptor agonists such as ATP. Darifenacin is metabolized by CYP2D6 enzymes.

#### **Solifenacin**

Solifenacin has a 13-fold affinity for muscarinic M<sub>3</sub>-receptors than for muscarinic M<sub>a</sub>-receptors. It also binds to muscarinic M<sub>i</sub>-receptors. This drug is used to treat incontinence, and it is able to reduce the number of urgency episodes and nocturia. The side-effects that accompany the use of solifenacin are: constipation and blurred vision.

#### **Trospium**

Trospium is an anticholinergic drug that has affinity for muscarinic M<sub>i</sub>-, M<sub>2</sub>- and M<sub>s</sub>-receptors. It is a quaternary amine, and may also be used for the treatment of bladder incontinence. It has lower side-effects compared to solifenacin and darifenacin, but its

efficacy is quite the same as for those two drugs. It has a higher potency than oxybutynin in the treatment of bladder incontinence (Michel *et al*, 2005).

### **Tolterodine**

Tolterodine is an antimuscarinic drug that is commonly used in the treatment of incontinence. It acts on muscarinic M<sub>2</sub>- and M<sub>3</sub>-receptors. Even if it is not selective, it has less side-effect than oxybutynin, which acts only on M<sub>3</sub>, because it has a great affinity for urinary bladder smooth muscles (Michel *et al*, 2005).

### **Duloxetine**

Duloxetine is used in the treatment of diabetic peripheral neuropathy and stress urinary incontinence. It exerts its action by inhibiting re-uptake of serotonin and noradrenaline (Raskin *et al*, 2006).

## **Drugs affecting sympathetic pathways**

The urinary bladder is innervated by sympathetic nervous system from the rostral lumbar of spinal cord that release noradrenaline to the bladder dome and urethra. Noradrenaline has both excitatory and inhibitory input on the bladder and urethra. Before reaching the urinary bladder, sympathetic nerves from spinal cord release acetylcholine at the ganglionic level (pre-synaptic neurones), ACh thus released binds to nicotinic receptors and stimulate post-synaptic neurones that now noradrenaline to the urinary bladder and urethra. Noradrenaline contracts the bladder base and the urethral smooth muscle by its action on p- and ai-adrenergic receptors, respectively. Based on the above phenomenon, one may predict the effect of sympathomimetic and sympatholytic agents on urinary bladder and urethral internal sphincter (Goodman and Gilman, 2001).

### **Ganglionic blocking agents**

ACh is the neurotransmitter involved in stimulating the post-synaptic neurones owing to its action on nicotinic receptors. Agents like hexamethonium and trimethaphan block this pathway, thus preventing excitation of post-synaptic neurones which normally increase excitatory post-synaptic potential (EPSP). Inter-neurones store and release noradrenaline, some peptides and amino acids. EPSP is generated by the influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  ions inside neurones through nicotinic receptor channels. The effects of ganglionic blocking agents depend on the division of autonomic nervous system that exerts a dominant control on a given organ. In the urinary bladder, this blockade causes atony of detrusor muscles. The non-selectivity of these agents often limits their clinical use and restricts their experimental usage. Some of the side-effects accompanying the use of ganglion blocking agents is cycloplegia, xerostomia, diminished perspiration, postural hypotension, and so on. Nevertheless, these agents are used in the management of autonomic hyper-reflexia, especially in patients with injuries to the upper spinal cord that causes excessive sympathetic discharge (Golan *et al.*, 2005).

### **Sympathomimetic agents**

The response of an organ to sympathomimetic agents depends on the presence of  $\alpha$ - and  $\beta$ -adrenergic receptors on that particular organ. The extent of the effect is proportional to the density of these receptors and the affinity of these agents on different receptors. For example, agents that have a high affinity for  $\beta_2$ -adrenergic receptors, have a greater effect on urinary bladder than those that have low affinity. The action of sympathomimetics on urinary bladder  $\alpha$ -adrenergic receptors leads to the contraction of the trigone and internal sphincter of urethra. The use of these agents results in hesitancy in urination and in

urinary retention. Sympathomimetic agents have a limited use in the treatment of incontinence because of their severe side-effects on the heart and blood vessels. Agents such as minodrine, ephedrine and norephedrine act by direct stimulation of  $\alpha$ - and  $\beta$ -adrenoceptors, and can also induce the release of noradrenaline from sympathetic nerve terminals. The use of  $\beta$ -adrenoceptor antagonists is based on the fact that these agents reduce the effect of noradrenaline on  $\alpha$ -adrenoceptors of urethral muscle. Imipramine, which inhibits reuptake of noradrenaline in nerve terminals, enhances the contractile effects of noradrenaline on urethra through  $\alpha$ -adrenoceptors that exist on urethral smooth muscle (Golan *et al.*, 2005).

## CHAPTER FIVE

### BASIS OF THE PROJECT

The mammalian urinary bladder is innervated and functionally regulated by the autonomic nervous system (ANS). Activation of the parasympathetic motor fibres to the bladder causes an intense stimulation of the muscarinic M3-receptors in the bladder body, resulting in a strong and efficient bladder contraction, which in turn causes emptying of the bladder. Urinary bladder dysfunction is a common complication of chronic diabetes mellitus. Vesical dysfunction is a well-known manifestation of the peripheral autonomic neuropathy that accompanies diabetes mellitus (Kamata *et al.*, 1992). It has been suggested that the initial bladder distension seen in diabetic patients is a consequence of impaired sensory transmission, and sensory neuropathy has been shown to correlate with bladder neuropathy (Kamata *et al.*, 1992; Hukovic *et al.*, 1965). Contractile responsiveness of isolated bladder muscles from streptozotocin (STZ)-treated, diabetic rats to cholinergic nerve stimulation or administration of muscarinic agonist drugs has been studied by various investigators (Kamata *et al.*, 1992; Carrier and Aronstam, 1990; Luheshi and Zar, 1991). Many studies have attempted to clarify the pathogenesis of the urinary bladder dysfunction seen in diabetes mellitus (Nakamura *et al.*, 1992). It has been suggested that urinary bladder dysfunction in chronic diabetes might be due to an altered response of the organ to autonomic stimuli (Kamata *et al.*, 1992). Segmental demyelination and axonal degeneration of the peripheral autonomic nerves have also been reported to cause dysfunction of lower urinary bladder tract (Van Poppel *et al.*, 1988). Several studies have been undertaken to investigate contractile responses of the bladder to muscarinic drugs in diabetic animals. It has been shown that in diabetic

animals, contractile responses of the urinary bladder to muscarinic agonists are inconsistent. Some investigators have reported increased responses (Kamata *et al.*, 1992; Kolta *et al.*, 1985; Latifpour *et al.*, 1988 ; Tong and Cheng, 2002), while others have reported decreased responses (Longhurst and Belis, 1986 ; Lunghurst *et al.*, 1990) or no change in contractile responses (Luheshi and Zar, 1991 ; Lincoln *et al.*, 1984) of diabetic urinary bladder smooth muscle strips to muscaric agonists. The present study was, therefore, prompted by the existing controversies and inconsistencies in the biomedical literature on the responses of diabetic urinary bladders to muscarinic agonists.

## STUDY OBJECTIVE

The core aim of this study was, therefore, to investigate the responses of urinary bladders isolated from normal (normoglycaemic) and STZ-treated, diabetic Wistar rats to acetylcholine (ACh), a prototype muscarinic agonist.

## EXPERIMENTAL PROTOCOL AND PROCEDURES

### MATERIALS AND METHODS

The experimental protocol and procedures used in this study were approved by the Ethics Committee of the University of KwaZulu-Natal, Durban 4000, South Africa; and conform with the "*Guide to the Care and Use of Animals in Research and Teaching*" [published by the Ethics Committee of the University of Durban-Westville, Durban 4000, South Africa].

## Animals and induction of diabetes

Thirty (30) healthy, young adult, male Wistar rats (*Rattus norvegicus*) weighing 250-300 g, were used. The animals were kept and maintained under laboratory conditions of temperature, humidity, and light; and were allowed free access to food and water *ad libitum* one week before the commencement of my experiments. The rats were divided into two groups of fifteen age-matched, normal (non-diabetic) and fifteen STZ-treated, diabetic animals; and were kept in separate cages. Diabetes was induced in the diabetic group of rats by intraperitoneal injections of STZ (75 mg/kg). The STZ-treated rats were kept in their cages for 7-10 days under laboratory conditions, to allow diabetes to develop in the animals. Two-to-ten-week diabetic rats were used in this study.

## Preparation of Bladder Tissues

STZ-treated rats with blood glucose concentrations  $>18$  mmol/L were considered to be diabetic and used in this study. The normal (non-diabetic) and STZ-treated diabetic rats were sacrificed by decapitation, and the whole (entire) Urinary bladder of each rat was removed, opened up longitudinally to form a semi-rectangular piece of tissue, tied with cotton thread at the upper and lower ends, and suspended in 30-ml 'Ugo Basile Two-Chambered Organ Baths' (model 4050) containing Krebs-Henseleit physiological solution (of composition, in g/litre: NaCl, 6.29; KCl, 0.34;  $\text{NaH}_2\text{PO}_4$ , 0.15;  $\text{NaHCO}_3$ , 2.1;  $\text{MgCl}_2$ , 0.11;  $\text{CaCl}_2$ , 0.26; and glucose, 1.00) maintained at  $32 \pm 1^\circ\text{C}$  and continuously aerated with carbogen (i.e., 5% carbon-dioxide + 95% oxygen gas mixture) under an applied resting tension of 1 g. Each bladder muscle strip was allowed to equilibrate for 45-60 minutes, during which time the bathing physiological solution was changed every



15 minutes, before it was challenged with graded concentrations of ACh (and other drugs used).

### Acetylcholine (ACh) Treatment

Each isolated urinary bladder muscle strip was challenged with graded concentrations of acetylcholine (ACh,  $10^{-8}$ - $10^{-4}$  M) in the absence, and in the presence, of atropine (ATR,  $10^{-7}$ - $10^{-5}$  M). The tissues were washed out 3-5 times after the maximal contractile response to each ACh concentration was obtained, and thereafter allowed to equilibrate for 5-10 minutes before sequential addition of the next higher concentration of acetylcholine. Isolated urinary bladder strips from diabetic and normal, age-matched control animals were always set-up in parallel under the same experimental conditions, in order to make allowance for adequate comparison of the tissues' contractile responses to acetylcholine. The ACh-induced contractile responses of the tissues were recorded isometrically by means of 'Ugo Basile' force displacement transducers and pen-writing 'Gemini' recorders (model 7070).

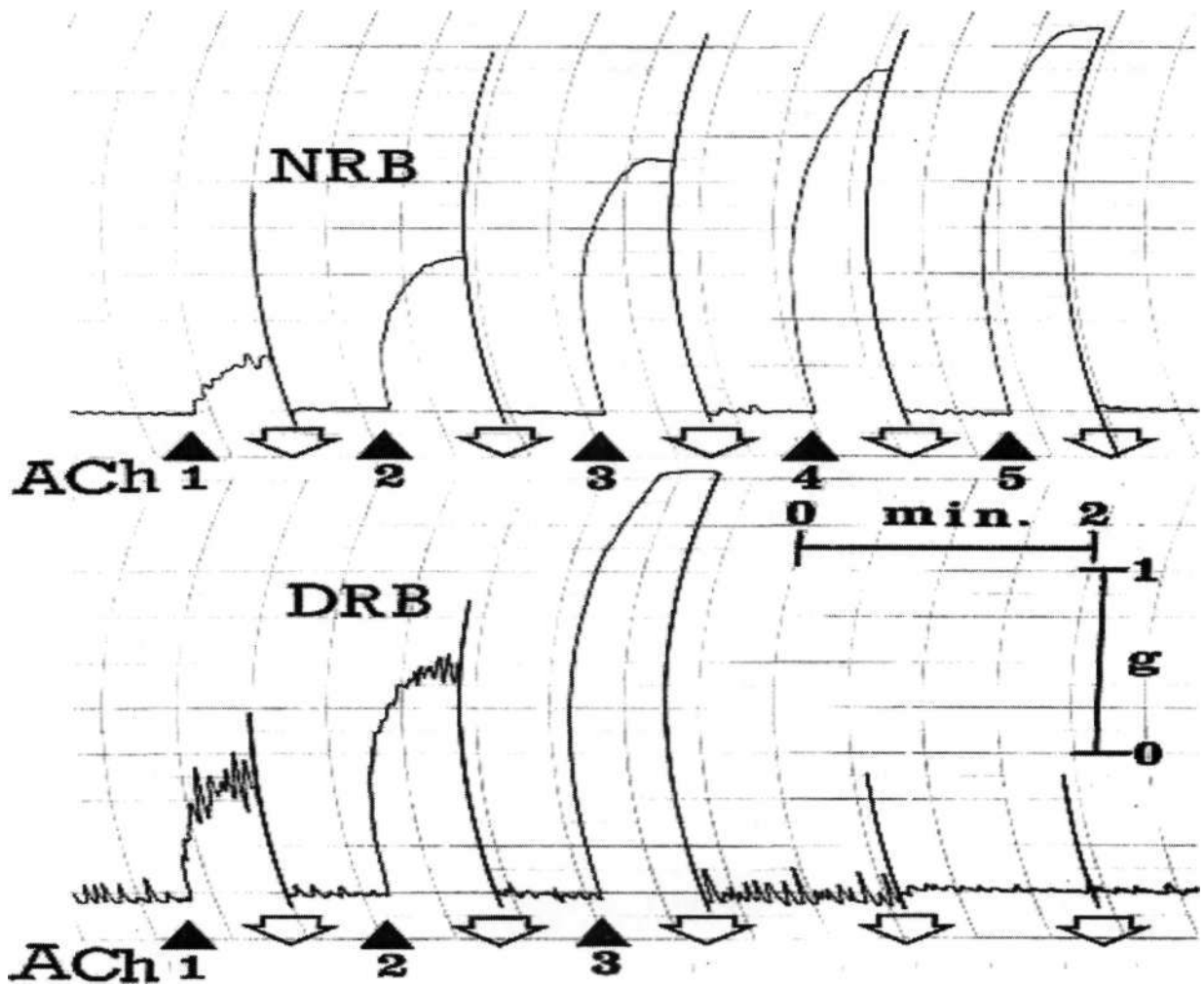
### Data Analysis

Data obtained are presented as means ( $\pm$ SEM) of the contractile responses of the bladder tissues to the various ACh concentrations. Statistical evaluation of the data was done by means of 'Student t-test' for unpaired data. Values of  $P < 0.05$  were taken to be statistically significant.

## RESULTS

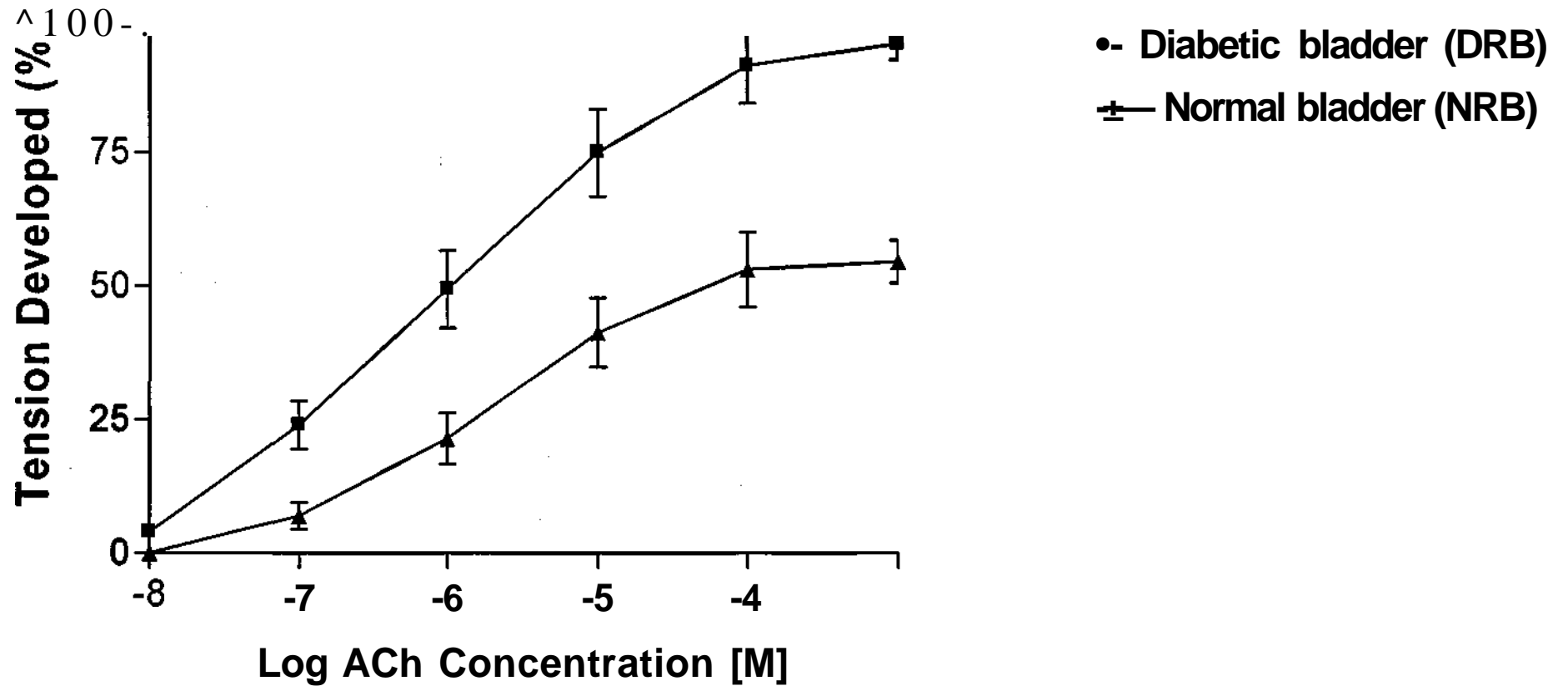
### Changes in blood glucose concentrations, body and bladder weights

One to ten weeks after treatment with STZ, the fasting blood glucose concentrations of the STZ-treated, diabetic rats were significantly elevated ( $P < 0.05-0.001$ ), compared with those of the normal (non-diabetic), age-matched, control rats (Table 1). Furthermore, the body weights of the STZ-treated, diabetic rats decreased significantly ( $P < 0.05-0.01$ ) after six weeks of STZ treatment, compared with the normal (non-diabetic), age-matched, control rats. The bladders of the STZ-treated, diabetic rats were visibly more distended and larger than those of the normal, age-matched control rats at the time of dissection. Moreover, the wet weights of the bladders from diabetic rats were significantly greater ( $P < 0.05-0.01$ ) than those from normal (non-diabetic), age-matched control rats (Table 1).



**Figure 1.**

Effects of graded concentrations of acetylcholine (ACh) on isolated urinary bladder muscle strips from a normal rat bladder (NRB, upper trace) and a diabetic rat bladder (DRB, lower trace) respectively. ACh A 1, 2, 3, 4 and 5 denote acetylcholine,  $5.0 \times 10^{-8}$ ,  $5.0 \times 10^{-7}$ ,  $5.0 \times 10^{-6}$ ,  $5.0 \times 10^{-5}$  and  $5.0 \times 10^{-4}$  M respectively, sequentially added to the bath-fluid at the solid dots (A), and washed out 3-5 times at the adjacent, open right-hand-side, downward-pointing arrows.



**Figure 2.**

Comparative effects of graded concentrations of acetylcholine (ACh) on tension developed by isolated urinary bladder tissues from normal (A, NRB) and diabetic (•, DRB) rats. Each point represents the mean of 15 preparations, while the vertical bars denote standard errors of the means.

Table 1.

Fasting blood glucose (FBG) levels, body weights and wet weights of isolated bladders of normal (non-diabetic) and STZ-treated, diabetic rats at week 10 of the study period.

Parameter	Control, normal rats (n=15)	Diabetic rats (n =15)
Fasting blood glucose (FBG) levels (in mmol/L)	4.10 (3.8-4.6 mmol/L)	15.06 (8.2-21.1mmol/L)
Body weights (g)	310±15	203±12
Bladder wet weights (g)	0.63±0.11	0.98±0.14

### Acetylcholine-induced contractile responses of the isolated bladders

Acetylcholine (ACh,  $10^{-8}$ - $10^{-4}$  M) caused concentration-dependent contractions of bladders isolated from both non-diabetic and STZ-treated, diabetic rats. However, acetylcholine always induced stronger, more powerful and greater contractions of the diabetic bladders compared with bladders from the non-diabetic, age-matched control rats. Figure 1 illustrates typical traces obtained, while Figure 2 summarises the results obtained. The enhanced contractile responses of the diabetic bladder strips to bath-applied ACh were detected soon after induction of diabetes, and the magnitude or

intensity of the enhanced contractile responses to ACh continued to increase steadily and linearly as the diabetic state of the animals progressed from one week to ten weeks. Atropine (ATR,  $10^{-7}$ - $10^{-5}$  M), a muscarinic antagonist, inhibited the contractile responses of the isolated bladder preparations to bath-applied acetylcholine in a concentration-dependent manner. The mean EC<sub>50</sub> values for ACh on the isolated whole bladders of diabetic and age-matched, non-diabetic control rats were found to be  $0.01 \pm 0.08 \times 10^{-6}$  M (n=15) and  $7.21 \pm 0.10 \times 10^{-5}$  M (n=15), respectively.

## DISCUSSION

The results of the present study show that acetylcholine (ACh) induced stronger, more powerful and much greater contractions of isolated bladders from STZ-treated, diabetic rats compared with bladders isolated from normal (non-diabetic), age-matched, control rats. It is now firmly established that the sympathetic and parasympathetic nervous systems of the autonomic nervous system (ANS) regulate the functions of the lower urinary bladder in different ways. During the storage phase, the roles of the sympathetic nervous system are relaxation of the bladder body, and contraction of the bladder base through  $\alpha$ - and P-adrenoceptors, respectively; whereas, the role of the parasympathetic nervous system is a contraction of the detrusor muscle through stimulation of the muscarinic M<sub>3</sub>-receptors in the bladder body to expel urine (Nakamura *et al.*, 1992; Frimondt, 1976). Lincoln *et al.* (1984) showed significant increases in the activities of choline acetyltransferase and choline esterase in the rat bladder after two to eight weeks of diabetes, suggesting that cholinergic nerve activity is increased in the urinary bladder

during diabetes. The investigators (Lincoln *et al.*, 1984) further observed that norepinephrine content in the bladder showed a tendency to decrease in diabetes.

Previous investigators have shown that in diabetic animals, the contractile response of the urinary bladder to muscarinic agonists is inconsistent. Some investigators have reported increased contractile responses (Kamata *et al.*, 1992; Kolta *et al.*, 1985; Latifpour *et al.*, 1988), while others have reported either decreased responses (Longhurst and Belis, 1986) or no change in contractile responses (Luheshi and Zar, 1991; Nakamura *et al.*, 1992; Lincoln *et al.*, 1984) of the urinary bladder smooth muscle to muscarinic agonists. Latifpour *et al.* (1988) and Tong and Cheng (2002) have also reported an increased number of muscarinic M3-receptors associated with increased contractile responses to muscarinic agonists in the bladder dome of STZ-treated diabetic rats. On the contrary, Carrier and Aronstam (1990) have reported an increased muscarinic responsiveness and a decreased density of muscarinic receptors in ileal smooth muscles from STZ-treated, diabetic rats. Kamata *et al.* (1992) have also shown that detrusor strips of urinary bladders from STZ-treated diabetic rats exhibit an enhanced contractile response to acetylcholine, and associated the increased responses to ACh with an increased population of muscarinic receptors in the tissues. However, findings of this study are in agreement with the observations of Kolta *et al.* (1985); Latifpour *et al.* (1988); and Kamata *et al.* (1992) who have reported increased contractile responses of diabetic bladders to muscarinic agonists. The discrepancies in the findings of the various earlier investigators who have examined contractile responses of isolated urinary bladders from diabetic mammals to acetylcholine (or parasympathetic nerve stimulation) are unlikely to be due to differences in species, strains and ages of the experimental animals used

because my preliminary studies also showed enhanced, increased contractile responses of isolated bladders from all available diabetic species and strains of mice, rats, guinea-pigs and rabbits of different ages to bath-applied acetylcholine. However, the duration of hyperglycaemia, the segment of the bladder used the breeders of the animals and the experimental conditions employed, might contribute significantly to the differences in the findings of the earlier investigators. Nilvebrant *et al.* (1986) and Tong and Cheng (2002) noted "supersensitivity" to muscarinic agonists, and an increased density of muscarinic M3-receptors. Nilvebrant *et al.* (1986) also noted no change in affinity for  $^3\text{H}$ -QNB with hypertrophied bladders following peripheral parasympathetic denervation of the urinary bladders. Latifpour *et al.* (1988) have also shown that STZ-induced diabetic state causes an increased maximum contractile response to muscarinic agonist, an increased density of muscarinic receptors, and no change in the affinity of the bladder dome smooth muscle for  $^3\text{H}$ -QNB. However, in their study, Kamata *et al.* (1992) found that muscarinic receptors in the detrusor strips from STZ-treated diabetic rats had a lower affinity for  $^3\text{H}$ -QNB than the detrusor strips from normal, control rats. The latter investigators (Kamata *et al.*, 1992) concluded that although it is unclear why the affinity for  $^3\text{H}$ -QNB was lower in diabetic state, it is unlikely that the decreased affinity of muscarinic receptors to acetylcholine is related to the increased contractile response to acetylcholine (ACh).

The findings of the present study are in agreement with the works of Kolta *et al.* (1985) and Tammela *et al.* (1995) who observed enhanced and significantly greater responses of bladder strips from diabetic rats to acetylcholine and carbachol respectively, soon after induction of diabetes with STZ. The investigators (Kolta *et al.*, 1985; Tammela *et al.*, 1995) further observed that the magnitude and/or intensity of the acetylcholine- and



carbachol-induced contractile responses of the diabetic bladder strips continued to increase as the diabetic state of the animals progressed. The increased diabetic bladder mass observed in the present study is also in consonance with the findings of Tammela *et al.* (1995) who found a significantly increased diabetic bladder mass in their study, and concluded that effects of diabetes (and sucrose consumption) on contractile bladder function are related to diuresis-induced increases in bladder mass.

In conclusion, the findings of the present study have demonstrated that urinary bladders from STZ-treated diabetic rats exhibit an increased contractile responsiveness to bath-applied acetylcholine. Although the present study could not establish the mechanism of the increased contractile responsiveness of the diabetic bladders to the muscarinic agonist (ACh) used, my findings tend to suggest that the alterations in diabetic urinary bladder synaptosomal, vesicle-bound neurotransmitter (ACh) concentrations reported by Tong *et al.* (1996), and the compensatory increase in the density of muscarinic M3-receptor population reported by Latifpour *et al.* (1988) would appear to be two of the most plausible mechanisms of the increased diabetic bladder responsiveness to acetylcholine.

## CHAPTER SIX

### Study Constraints and Recommendations for Future Studies

This study, like many other experimental studies, encountered a number of obstacles and constraints, without which its outcome would probably have been much better. The restriction on the number of experimental animals allowed in this study meant that only a limited number of rats (30) could be used for the study. This restriction may pose a threat to statistical analysis of results. In this study, only one animal species (rat) was used. It would have been better to use other animal species (e. g., guinea-pigs, rabbits, etc) as well for meaningful comparison. Because of time and financial constraints, I was unable to carry out receptor studies and cloning experiments, which would have shed more light on the mechanism of action of ACh in diabetic rat bladders, and improve the quality of the present experimental findings generally - probably by identifying the muscarinic receptor subtypes and population/s in diabetic rat bladders. The sensitivity and accuracy of the recording equipment used can only be assumed for the measurement of the small differences that existed between the two experimental animal groups.

### Recommendations for future studies

1. It would be interesting to extend this study to other animal species (especially, mice, guinea-pigs and rabbits) to see if 'species variation' affects the responsiveness of diabetic bladders to ACh.
2. Receptor studies should be undertaken to throw more light on the mechanism of the enhanced responsiveness of diabetic bladders to ACh. The mechanistic studies should include determination of muscarinic receptor subtype population and/or density in the bladders of diabetic and non-diabetic animals.
3. The effects of other neurotransmitters, especially noradrenaline and adenosine triphosphate (ATP), on diabetic and non-diabetic bladders should also be investigated in great detail.

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