Cholinergic regulation of the lower urinary tract

Göteborg University, Sweden

The Sahlgrenska Academy

Institute for Neuroscience and Physiology

Department of Pharmacology

Charles University in Prague

Faculty of Pharmacy in Hradec Králové

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Pavel Jeřábek

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1. Introduction

1.1. Autonomic nervous system

The autonomic nervous system (ANS) regulates the functions of different organs. ANS regulates diameter of blood vessels, heart rate, force of contraction of the heart, diameter of the pupils, salivation, perspiration, bronchiole diameter, peristaltic movements in the intestine, sphincter diameter, erection, ejaculation, and parturition.

The functions that the ANS regulates are typically portrayed as being involuntary, however, they are not completely outside our awareness. It remains open to debate whether the term 'involuntary nervous system' is a precise description of the ANS. While many autonomic functions are beyond conscious control, other functions can result from direct somatic stimulation, for example micturition.

The autonomic nervous system is divided into two subsystems: the sympathetic nervous system (SNS), also known as the adrenergic system due to the use of adrenaline and noradrenaline as neurotransmitters. The parasympathetic nervous system (PS), is also referred as the cholinergic system due to the predominant release of acetylcholine as a neurotransmitter. The SNS and PS may often have opposing effects in the same organs or physiological systems and therefore it is believed that appropriate ANS activity leads to homeostasis.

The SNS is frequently referred to as the "fight or flight" system, as it has a stimulatory effect on organs and physiological systems, responsible for rapid sensory activity (for example pupils in the eye) and movement (skeletal muscle).

The PS instead has a rest and digest activity. Stimulation of the PS results in bradycardia and relaxes many functions of organs and body systems. For example, the PS dilates blood vessels leading to the gastrointestinal tract and these effects may be important immediately following the consumption of food, due to the greater metabolic demands placed on the body by the gut. The PS diverts blood back to the gastrointestinal tract thereby aiding in digestion. The PS constricts the bronchiolar diameter when

the need for oxygen has diminished. During accommodation, the PS causes constriction of the pupil and lens, furthermore the PS stimulates salivary gland secretion and accelerates peristalsis, and thereby, in keeping with the rest and digest functions, appropriate PS activity mediates digestion of food and indirectly, the absorption of nutrients.

The cell bodies of preganglionic autonomic nerve cells are situated in the central nervous system. Those of the SNS arise in the thoracic and lumbar segments of the spinal cord. Preganglionic parasympathetic cell bodies are situated in the brain stem (cranial parasympathetic) and in the sacral spinal cord (sacral parasympathetic).

In order to reach the target organs and glands, the axons of neurons in the SNS and PS often must travel long distances in the body. In both, neurons from the CNS synapse at ganglions; a site where a group of neurons of similar function (presynaptic neurons) connect to another group of neurons (postsynaptic neurons), by means of a synapse. Ganglions allow for the modulation of the presynaptic input before it is sent along the postsynaptic neurons to their effector sites.

The main neurotransmitter that is located at the ganglion is acetylcholine. Acetylcholine is released from the presynaptic neuron and acts on ionotropic postsynaptic nicotinic receptors in both the SNS and PS. Postsynaptic cells pass signals to the effector organs. At the effector organs, SNS postsynaptic neurons release noradrenaline to act on adrenoceptors, with the exception of the sweat glands and the adrenal medulla. At sweat glands, the neurotransmitter is acetylcholine, which acts on muscarinic receptors. At the adrenal cortex, there is no postsynapic neuron. Instead the presynaptic neuron releases acetylcholine to act on nicotinic receptors. These receptors are divided into two subtypes: N_N, which is a neuronal subtype of nicotinic receptor and N_M, which is a muscular subtype of nicotinic receptor. Stimulation of the adrenal medulla releases adrenaline into the bloodstream which will acts adrenoceptors, producing a widespread increase in sympathetic activity. In the PS, postsynaptic cells use acetylcholine as a neurotransmitter, to stimulate muscarinic receptors (M_1 – M_5 subtypes).

The sympathetic axons build a chain of 22 ganglia, paravertebral ganglia, on each side of the spinal column. From these the splanchnic nerves

run to the prevertebral ganglia, which lie in front of the aorta, at the level where its unpaired visceral arteries branch off. The left and right trunks of the sympathetic nerve fuse to form an unpaired ganglion in the pelvic area. Organs innervated by sympathetic fibres include the heart, lungs, esophagus, stomach, small and large intestine, liver, gallbladder and genital organs.

These organs are also innervated by the part of the parasympathetic nervous system. The digestive system distal to the lower part of the colon is regulated by the sacral parasympathetic fibres via the pelvic ganglia. The proximal digestive tract is controlled by the vagus nerve, the largest element of the cranial parasympathetic system. Like those of the vagus, other cranial parasympathetic fibers arise in the brain stem before exiting the skull with various cranial nerves, en route to the cranial parasympathetic ganglia and the innervation of the eye muscles and salivary glands .

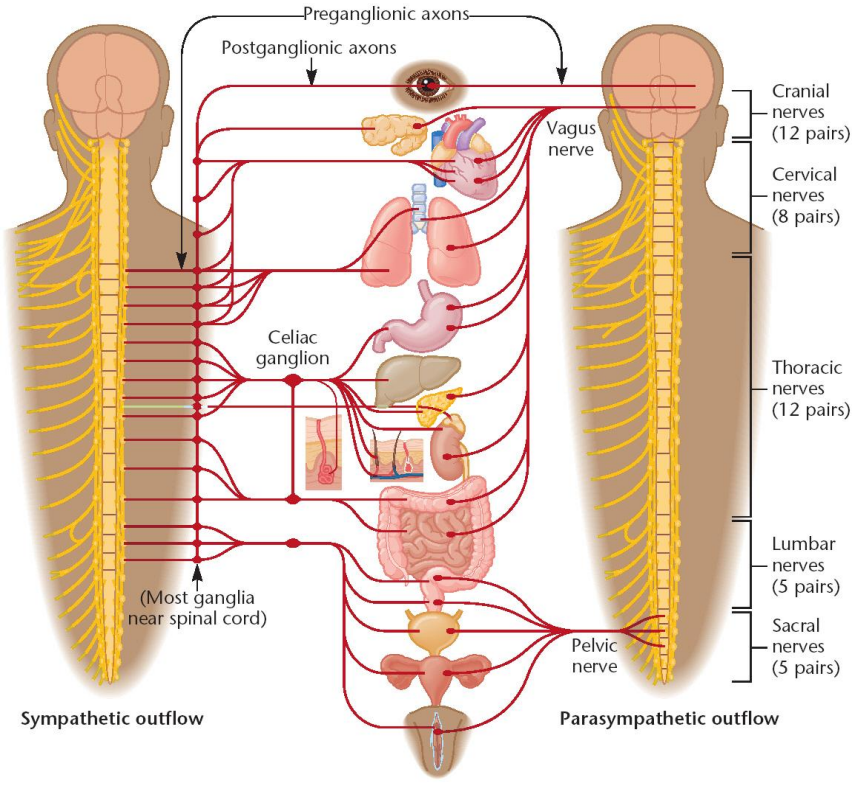

Figure 1:

Overview of autonomic nervous system (Adapted from: http://cti.itc.virginia.edu/~psyc220, 06-10-04)

1.2. Muscarinic receptors and their subtypes

Muscarinic receptors are class I heptahelical G-protein-coupled receptors which, in the human body, mediate the actions of acetylcholine. They are divided into 5 subtypes: M_1-M_5 *(Caulfield et al., 1998).* Further observation techniques also clarified, that these receptors are necessary for mediation of autocrine functions of the acetylcholine. Whilst muscarinic M_2 and M_4 receptors inhibit elevated adenylate cyclase activity, as well as prolonging potassium channel, non-selective cation channel and transient receptor potential (TRP) channels opening *(Zholos et al., 2004),* muscarinic M_1, M_3 and M_5 receptors mobilize phosphoinositides, to generate inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol, via activation of phosphoinositide-specific phospholipase Cβ, thereby increasing intracellular calcium.

Muscarinic M_1 receptors are mostly expressed in brain, especially in the cerebral cortex, hippocampus and striatum. The predominant existence of M_1 subtype in the cerebral cortex and hippocampus may provide further convincing evidence that M_1 subtype is significantly involved in the higher cognitive function *(Oki et al., 2005)*. Muscarinic M_2 receptors are widely expressed in both central and peripheral nervous systems *(Oki et al., 2005)*. Peripheral postjunctional muscarinic M_2 receptors are expressed in the myocardium, and mediate negative chronotropic and inotropic effects of acetylcholine *(Harvey et al., 2003)*. This receptor subtype is also postjunctionally expressed in smooth muscle from several tissues *(Eglen et al., 1996)* and it regulates contractility *(Ehlert, 2003)*. The role of the subtype on prejunctional terminals seems to be the same as an autoreceptor role, regulating the release of acetylcholine and noradrenaline, which is present in several peripheral tissues such as smooth muscle *(Slutsky et al., 2003)*. The muscarinic M_3 receptor subtype is also widely distributed in the central nervous system (CNS), but much less, than other muscarinic receptors *(Felder et al., 2000; Wess, 2004; Wess et al., 2003)*. Some studies on mice have discovered a potential role of this receptor subtype in regulating food intake, but more studies are needed *(Yamada et al., 2001)*. The classical function of M_3 receptors is mediating contraction of smooth muscle, such as in

respiratory, gastrointestinal and genitourinary tracts *(Eglen et al., 1996)*. Muscarinic M_4 receptors are in the CNS distributed in the corpus striatum, being co-localized with dopamine receptors on striatal projecting neurons *(Felder et al., 2000; Oki et al., 2005).* Activation of spinal muscarinic M_2 and M_4 receptors leads to potent anti-nociception, but the precise nature of the receptor subtype mediating the response remains unclear *(Duttaroy et al., 2002; Lazareno et al., 2004; Zhang et al., 2005).* The only muscarinic subtype expressed by the dopamine containing neurones of the substantia nigra pars compacta is the M_5 subtype, but during the process of striatal dopamine release, other subtypes, such as M_4 are involved *(Eglen et al., 2000)*.

1.3. Muscarinic receptors in the urinary bladder

The two most important muscarinic receptors in the urinary bladder smooth muscle, the detrusor, are M_2 and M_3, where M_2 (75%) quantitatively dominates over M_3 (25%), so that the ratio is 3:1. Muscarinic M_3 receptors in the bladder cause direct smooth muscle contraction through mobilizing phosphoinositides and generating inositol 1,4,5-trisphosphate, as mentioned in the paragraph before. It is suggested that muscarinic M_2 receptors also cause smooth muscle contraction, but indirectly, by inhibiting sympathetically mediated relaxation *(Hegde et al., 1999)*. The presynaptic cholinergic receptor, responsible for facilitating the release of acetylcholine is the M_1 subtype. The inhibiting subtypes are M_2 and M_4. The adrenergic inhibiting receptor is probably α_2 (*see Figure 2)*.

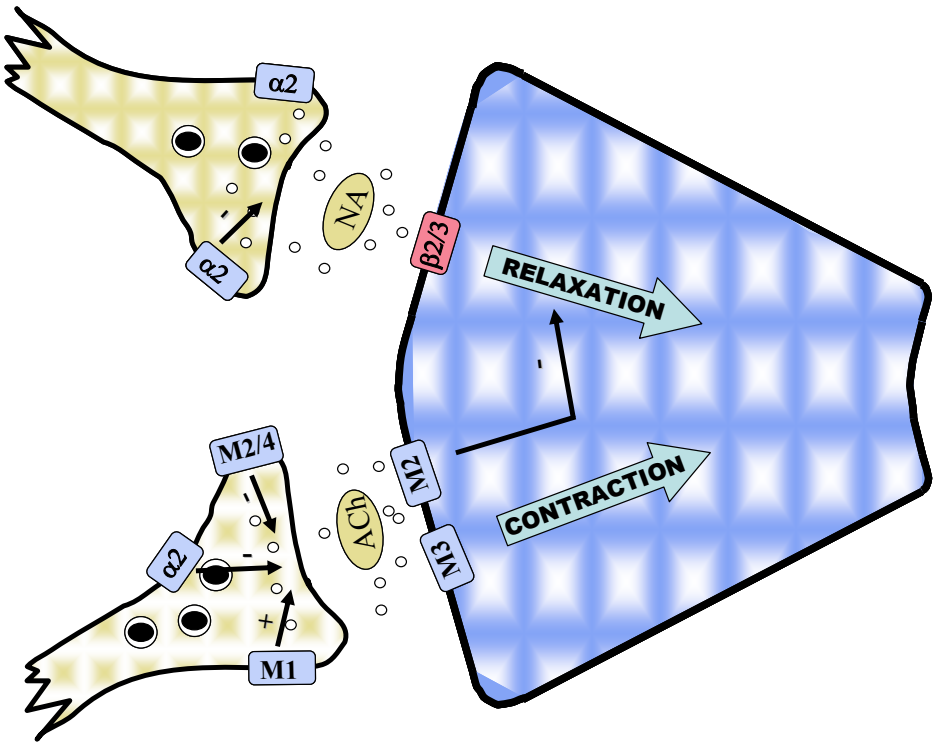

Figure 2:

Synaptic transmission in detrusor smooth muscle

1.4. The anatomy and physiology of the bladder and urethra

The urinary bladder is situated in the lower abdomen and it consists of several muscular layers. In its empty state, it forms a tetrahedral shape and in its filled state, it forms an ovoid shape. In the empty bladder, there is a superior surface, two inferolateral surfaces and a posteroinferior surface or base, where the bladder neck forms the lowest point. The urachus, which is connected at the apex of the bladder, anchors the bladder to the anterior abdominal wall. There are several anatomical differences between male and female bladder setout, but the most important thing is, that the body and fundus of the bladder is highly mobile and distensible and has a large capability of expanding into the abdomen during the filling phase *(Walsh et al., 2002)*. Histological, histochemical and pharmacological differences were found between the bladder neck and the bladder body. The bladder neck is a separated functional unit apart from the bladder body and it is also different in males and females. The bladder outlet is formed by the bladder base, urethra

and external urethral sphincter. Whilst the male urethra is composed of the preprostatic portion, the prostatic urethra, the membranous portion and the penile urethra *(see Figure 3 for outline)*, the female urethra is only 4 cm long, protruding from the anterior wall to the external meatus *(Walsh et al., 2002).*

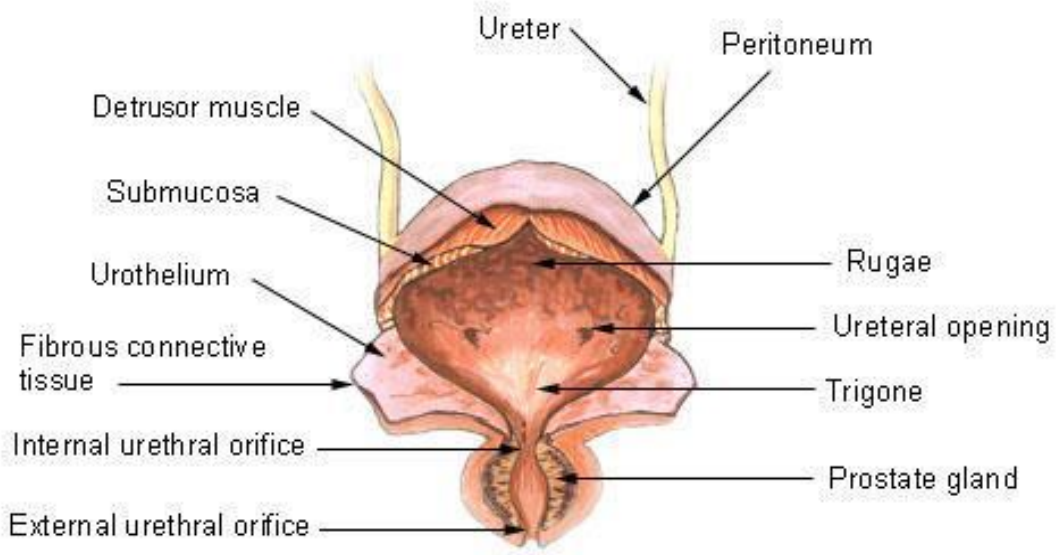

Figure 3:
Outline of the male urinary bladder. Adapted from Wikipedia.
(Wikipedia; 06-10-03, http://en.wikipedia.org/wiki/Image:Illu_bladder.jpg)

The main functions of the bladder are to store urine and also, when the bladder is full, to perform controlled voiding in controlled situations and time. The correct function is a result of cooperation of the somatic, parasympathetic and sympathetic nervous system. The main function of the bladder is divided into two phases, the storage phase and the micturition phase. During the storage phase the bladder distends, while the rise in intravesical pressure is notably small when the bladder volume is under the threshold volume for

inducing voiding *(Torrens et al., 1987; Walsh et al., 2002)*. It has been discovered, that the hypogastric nerve inhibits bladder contractions thereby optimizing bladder filling *(de Groat, 1976)*. Furthermore, when the bladder is distended in response to bladder filling, the vessels need to stretch, whilst still maintaining sufficient blood supply to the smooth muscle *(Brading et al., 1999)*. Unfortunately, there is only partial knowledge about the relaxatory mechanism during the storage phase *(Andersson, 1999)*. After the storage phase comes the voiding (micturition) phase. The physiological capacity of the human bladder is approximately 500 ml but the urge to urinate is already generated through the stretch receptors in the bladder wall at the level of about 200 ml of urine. Impulses from the bladder wall are transduced to the spinal cord via the pelvic nerves, initiating the micturition reflex *(Andersson, 2002; Morrison, 1999).* Furthermore, the hypogastric and pudendal nerve are also involved in the storage and micturition process initiating the micturition reflex *(Andersson, 2002).* The awareness of the bladder fullness is further generated through transmission via projection fibres to the cerebral cortex *(Walsh et al., 2002)*. During the bladder filling and of course increase of urine, the sensation of the bladder fullness becomes more and more acute. The center that controls the micturition is known as the pontine micturition centre (PMC), or “Barrington’s nucleus” *(Barrington, 1921)*. So, the impulse to expel urine from the bladder seems to be a result of pelvic nerve and muscarinic receptor activation together with an increase of the intraabdominal pressure *(Hemat et al., 2003).* During this process a domination of parasympathetic input could be seen while the sympathetic stimulation leads to relaxation of the internal urethral sphincter. The external sphincter, which is innervated by the pudendal nerve, relaxes voluntarily during micturition and after that the bladder is voided.

1.5. The urothelium

The urothelium in vivo is a cell multiayer, that has an endodermal origin. It is composed of 3-6 cell layers and each of them has a different cell type. The basal cell layer is germinal in nature, having cells with diameter of 5-10 μm, the intermediate layer cells have a diameter of 20 μm and the superficial cells, also known as umbrella cells, have a changing diameter, depending on the degree of bladder stretch (50-120 μm across). The cell replacement happens by fusion of the basal cell layer to form intermediate cells and fusion of the intermediate cells to form umbrella cells *(Martin, 1972)*.

There are no special morphological aspects of basal and intermediate cells, whilst the superficial umbrella cells have two unique morphological features. First the apical membrane is covered with scalloped-shaped plaques *(see Figure 4)* that are separated by plasma membrane domains called the "hinge". Because of these plaques, the outer leaflet of the apical membrane appears thicker than the inner leaflet *(Porter et al., 1967)*. The second feature is the high density of cytoplasmatic vesicles of the umbrella cells, which are composed of two apposing plaques joined together by hinge membrane, with an associated cytoskeletal network of fine fibrils *(Hicks, 1975)*. The polygonal-shaped plaques occupy 70 to 90% of the apical surface area, while the hinge membrane, surrounding the individual plaques, occupy the remaining 10-30%. The plaques are composed of subunits, and each plaque contains approximately 1,000 subunits, which have a sixfold symmetry and are composed of an inner and outer ring, each consisting of six particles. There are also two 4 transmembrane domain proteins, UPIa and UPIb and two type 1 proteins, UPII and UPIII. These proteins form an inner and outer ring. The superficial umbrella cells are connected by interconnecting strands, which form tight junctions *(Peter, 1978)*. Resulting in that the combination of umbrella cells and tight junctions offer a physical barrier to the movement of substances between the urine and the blood.

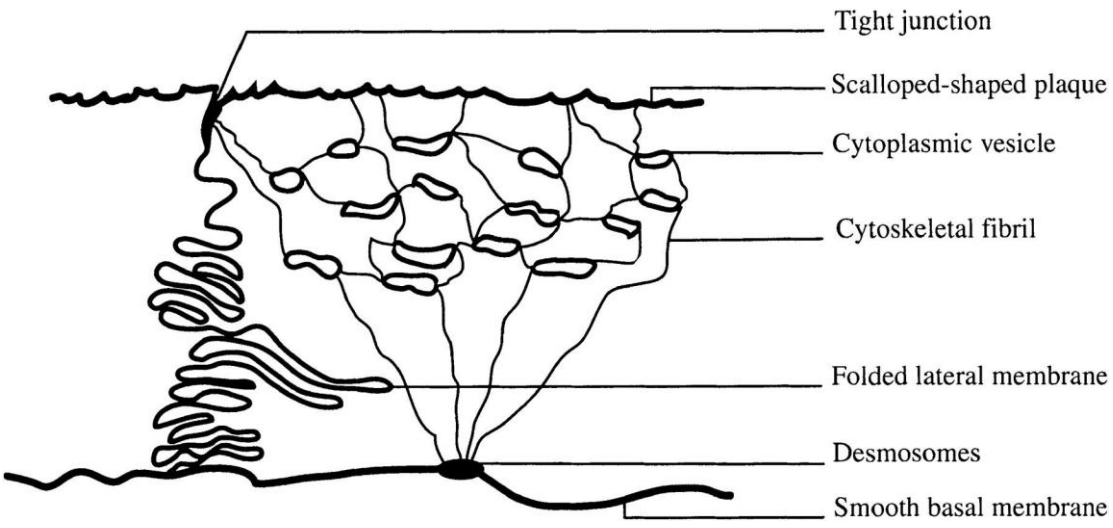

Figure 4:

Schematic of stretched umbrella cell. The basal membrane is smooth, and the lateral membrane is folded. Umbrella cells are joined together by tight junctions. The apical membrane has a scalloped appearance, and the cytoplasm is full of cytoplasmic vesicles joined together by cytoskeletal fibrils. These fibrils coalesce at the tight junctions and desmosomes in the basal membrane.

Adapted from Lewis, Everything you wanted to know about the bladder epithelium but were afraid to ask *(Lewis, 2000)*

1.6. Lower urinary tract symptoms (LUTS)

Problems of the lower urinary tract are common in the population and increase with age. Nowadays, LUTS are divided into two symptom dependent subgroups. The voiding (obstructive) symptoms and storage (irritative) symptoms, relating to the phases of the bladder functions *(Nordling, 2001)*. In the healthy bladder, the walls distend in response to increase in urine volume and urgencies to void occur when the bladder is full. In healthy individuals, signals from the bladder are conveyed to the brain and ensure the awareness of the filled bladder and consequently the urgency to void appears. After that, in the suitable situation, the bladder is voided with a constant urinary flow, until it is completely empty. Furthermore direct urgency to void is reduced during the night time.

Urinary incontinence could be divided into two subtypes, acute and chronic incontinence. Whilst acute incontinence lasts only for a short period of time, chronic incontinence may last for a much longer time period such as weeks, months or years. Chronic incontinence includes overflow incontinence, functional incontinence and the overactive bladder syndrome.

The overactive bladder syndrome (OAB, unstable bladder) and with that connected term detrusor overactivity, occur during the bladder filling. It can occur spontaneously or on provocation, when the patient tries to inhibit micturition *(Underwood, 2003).* The unstable bladder may also arise due to some neurological conditions such as dementia or stroke, which can affect the cerebral cortex, that normally suppress the micturition reflex. Spinal cord injuries, leading to dyssynergia between the bladder and the sphincter, can also cause this dysfunction.

Long-term studies looking for medical treatments revealed that anticholinergics may have effect at the condition of overactive bladder. However, the therapy is associated with only poor improvement of the conditions and mostly bad compliance connected with various side effects *(Lai et al., 2002)*, such as “dry mouth symptom” *(Scully, 2003)*. Resulting in that only few drugs are nowadays used in practice *(see Figure 5)*.

Agent	Mechanisms of action	Evidence	Reference
Oxybutinin	Muscarinic M1/M3 receptor antagonist, calcium antagonist and local anesthetic actions	In vitro smooth muscle relaxant effect (500 times weaker than antimuscarinic activity) Efficacy in OAB shown in clinical studies. Effective on intravesical administration	Reviewed by Andersson & Chapple (2001)
Dicyclomine	Nonselective muscarinic receptor antagonist, calcium antagonist action	Efficacy in OAB shown in clinical studies	Reviewed by Andersson et al. (1999)
Propiverine	Nonselective muscarinic receptor antagonist, calcium antagonist action	Efficacy in OAB shown in clinical studies	Reviewed by Andersson et al. (1999)
Temiverine	Selective muscarinic M3 receptor antagonist, calcium antagonist action	In-vitro inhibition of carbachol- and Ca-induced contractions in human detrusor muscle No published clinical data	Yono et al. (2000)
Terodiline	Nonselective muscarinic receptor antagonist, calcium antagonistic action	Efficacy in OAB shown in clinical studies Induced ventricular arrhythmias (Torsades de Pointes)	Reviewed by Andersson (1984; 1988)

Figure 5:

Outline of muscarinic receptor antagonists with secondary mechanism of action.

Adapted from Abrams, Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder *(Abrams et al., 2006)*

1.7. Cyclophosphamide-induced cystitis and changes in the bladder

Cyclophosphamide *(see Figure 6)* is a cytostatic used for inducing cystitis in experimental animals. Normally it is used in treatment of neoplastic diseases *(Stillwell et al., 1988)*. It is metabolised to acrolein irritating the bladder wall and thereby causing a haemorrhagic cystitis *(Cox, 1979)*. Cyclophosphamide-induced cystitis is a chronic, non-infectious, inflammatory condition of the bladder.

Previous reports of the acutely inflamed bladder induced by cyclophosphamide also have shown changes in postsynaptic down-regulation of muscarinic receptors and P2X purinoceptors. However, a pre-junctional mechanism, increased transmitter release, which compensates for the down regulation of these receptors was discovered in the Suncus murinus (house musk shrew) urinary bladder. This mechanism seems to be nerve-mediated because of it's possible inhibition by tetrodotoxin, but further investigations are

needed to clarify the exact origin of this mechanism *(Mok et al., 2000)*. There is also an evidence that muscarinic receptors located in the urothelium/suburothelium and on afferent nerves may contribute to the pathophysiology of OAB. Blockade of these receptors may also contribute to the clinical efficacy of antimuscarinic agents *(Abrams et al., 2006)*.

The further evidence revealed, that another neurotransmitter or neuromodulator is involved in the function of the bladder during the inflammation *(Chung et al., 1996)*. It is nitric oxide (NO), a toxic gas with free-radical properties. Recent studies in rats indicated the up regulation of NO synthase (NOS) in bladder afferent neurons and so NO, which is produced in the bladder afferent pathways, could influence the regulation of micturition reflex following cystitis. No reports from the bladder function have been made on the topical effect of intravesically administrated NO donors *(Ozawa et al., 1999)*.

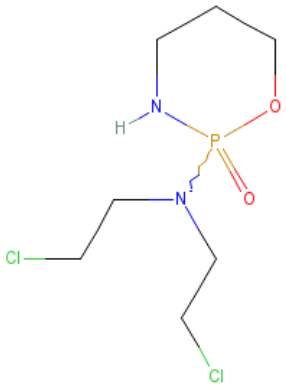

Figure 6: Molecule of cyclophosphamide

2. Materials and methods

All experiments were performed in the metabolic cage (*Harvard Apparatus)* at Göteborg University (Sweden) and were approved by the regional ethical committee at Sahlgrenska hospital. The cage was constructed for a single rat and it supported water supply for the animal during the whole testing period. It consisted of easily dismountable parts (*see Figure 8*) and it prevented urine from washing over and entering the feces tube. All the parts were autoclavable and the urine and feces tubes could be changed without disturbing the animal. Whole cage was placed on a stainless steel stand, which provided an easy access to all parts of the cage. To register drops of urine at each micturition, a sensor was placed below the cage and was pointed to the urine collection tube. The urine was collected and its volume was measured after the 24-hour period. The intake of water by the rat during this period was also measured using the scaled water bottle and a spillage collector. The sensor was connected to the portable PC via USB signal amplifier. To record and storage data, AcqKnowledge software (*Biopac Systems Inc., Goleta, CA 93117*) was used.

In the experiment, 20 male rats (Sprague Dawley, 200-500g) were used. Each rat was used twice. First the rat was placed in the metabolic cage without cystitis and the second time with cystitis, induced by intraperitoneal injection of 100 mg/kg cyclophosphamide (CYP, *Sigma-Aldrich, St. Louis, MO*) with 0,03 ml of Temgesic to relieve local pain after the injection. A 48-hour period for cystitis development was considered the best, because the required effect occurred after 60 hours from injection, which means in the time of the experiment *(see Figure 7)*. This was concluded after the study performed by the research group at the department of pharmacology at The Sahlgrenska Academy in 2005 *(Giglio et al., 2005)*. All experiments took place over the period of 24 hours, starting between 4 and 4,30 pm and lasting until the same time next day. A simulation of diurnal rhythm was induced by keeping the rats in daylight for approximately 1 hour, then in darkness for 16 hours and then in the daylight again for 7 hours. No food was given to the rat during the experiment. The rat was placed in the cage and was given 20

minutes to acclimatize to the surroundings. After that period, outside the cage, an injection of either saline (9% NaCl solution, 0.5 ml) for controls or a dose of 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), which is considered to be an M_1, M_3 and M_5 “selective” antagonist, was administered *(Eglen et al., 2000)*. Three different doses of 4-DAMP were used during the experiment, a low dose (10 µg/kg), a medium dose (100 µg/kg) and a high dose (1 mg/kg). All injections were given intraperitoneally. After the injection the rat was placed in the cage again. Each rat was asphyxiated, after the experiments, using carbon dioxide gas chamber and the dissection was made after that, to ensure, that the urinary bladder was inflamed. The macroscopic signs of the bladder inflammation were dilated, good visible, vessels in the bladder wall and a thickening of the bladder wall, especially by the apex.

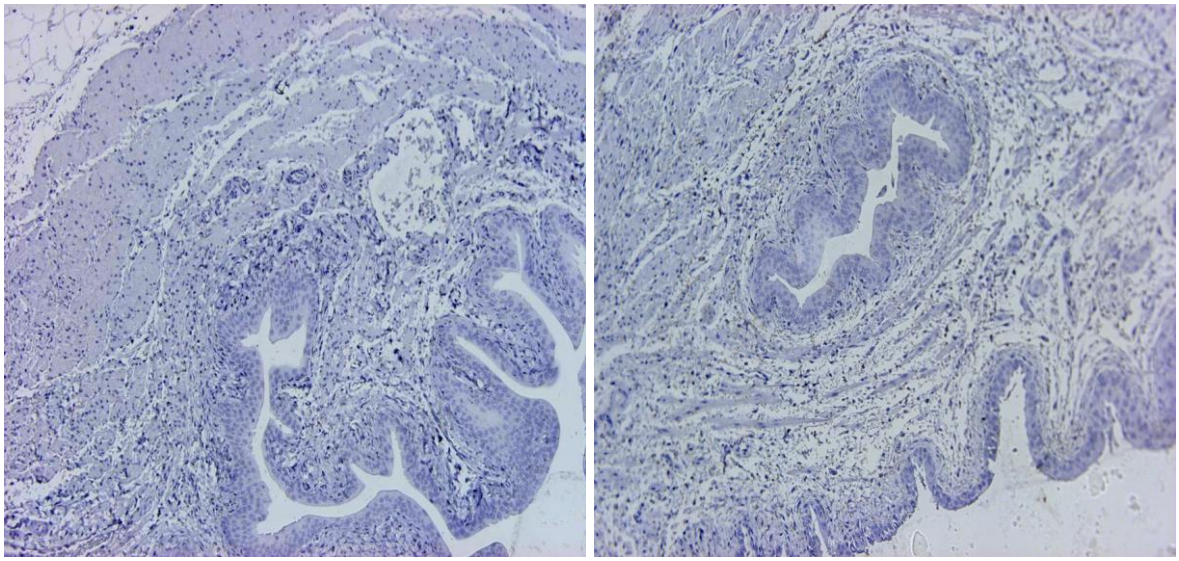

Figure 7:

Microscopic view of normal (left picture) and cyclophosphamide-treated (right picture) urinary bladder after immunohistochemical labeling. Disrupted urothelium, reorganized muscular layers and increased connective tissue could be seen 60h after the injection of cyclophosphamide *(Giglio, 2005).*

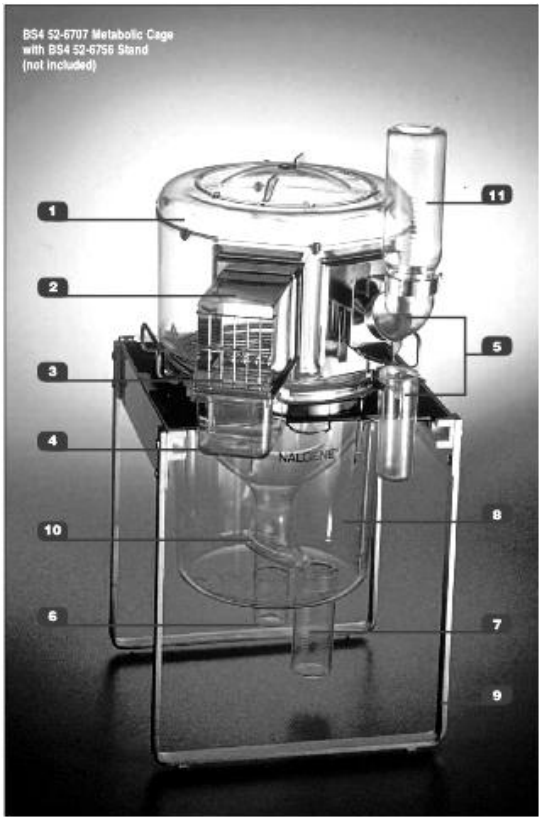

Figure 8:

Metabolic cage. Adapted from Harvard Apparatus web pages (06-09-19, http://www.harvardapparatus.com/webapp/wcs/stores/servlet/product_11051_10001_37596_-1_HAI_ProductDetail_37351____).

Description: 1) Cover; 2) Upper chamber; 3) Feeder chamber; 4) Feeder drawer; 5) Water bottle support & spillage collection 6) Feces collection tube; 7) Urine collection tube; 8) Lower chamber; 9) Stand; 10) Collection funnel and separating cone; 11) Water bottle.

2.1. Results counting and statistical analyse

Results were counted using statistical methods such as one-way ANOVA test, Bonferroni's multiple comparison test and Student's paired t-test. Microsoft Excel *(Microsoft Corp., Redmond, WA)* and Prism *(GraphPad Software Inc., CA)* software was used for creating tables, graphs and necessary calculations. The level of significance was set to $p<0,05$ which represented the probability of non-random results higher than 95%.

3. Results

3.1. Saline solution treatment group

The first group, where differences between normal (without cystitis) and cystitis rats were observed, was the saline (control) group. We compared water intake, urine outflow and number of micturitions between the healthy animals and the animals with inflamed bladders during the whole 24-hour period. In this control group, a significant decrease in the water intake by 7,4 ml (t-test, p=0,0248) as well as in the urine outflow by 8,0 ml (t-test, p=0,0294) was discovered, and also a decrease in number of micturitions by 3,2 (n.s.) in the cystitis rats, compared to the rats without cystitis (*see Figure 9)*.

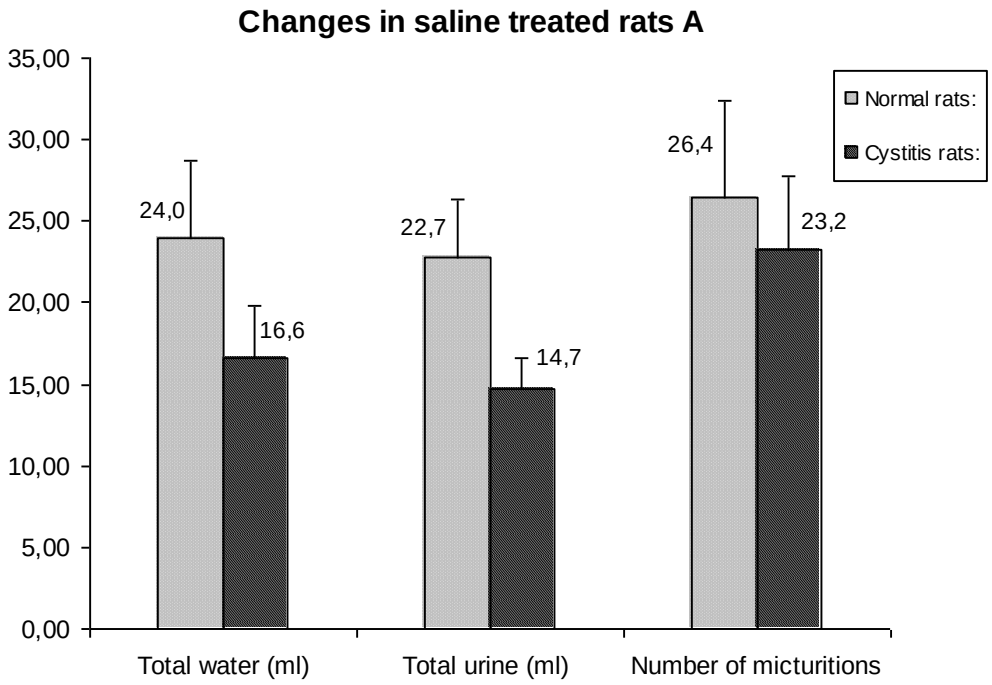

Figure 9:

Changes in saline treated rats, showing significant decrease in water intake, urine outflow and a decrease in number of micturitions in cystitis, compared to healthy animals per 24 hours. The vertical bars represent the S.E.M.

The frequency of micturitions decreased by 0,1 per hour (n.s.), whilst the volume of urine per micturition decreased by 0,13 ml (n.s.) in the cystitis state *(see Figure 10)*.

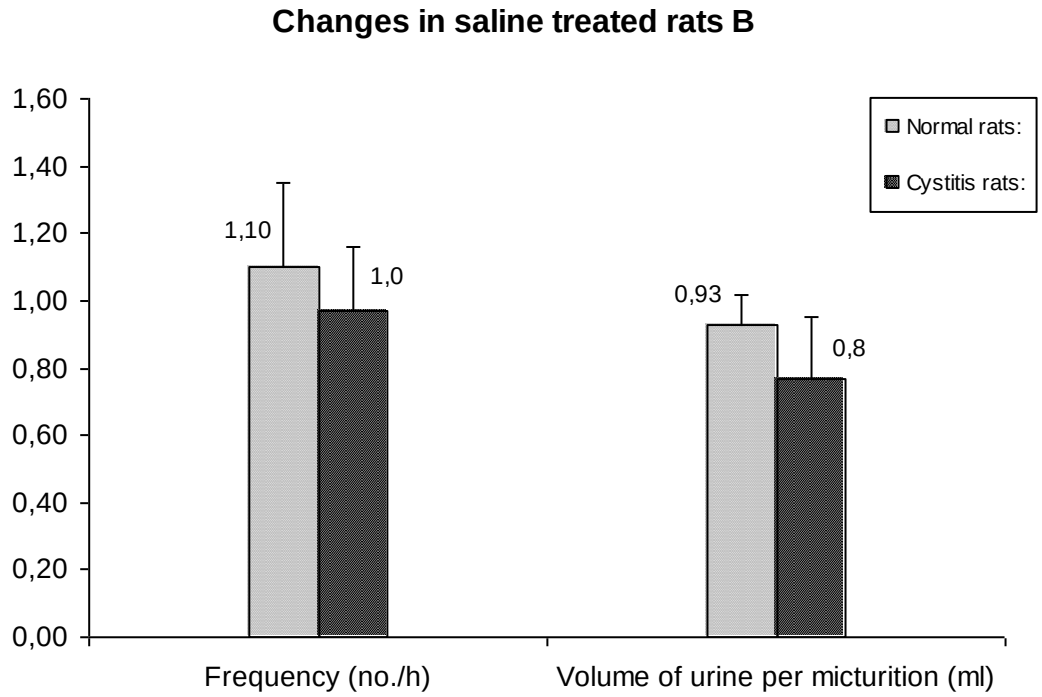

Figure 10:

Changes in saline treated rats, showing the decrease in frequency and volume of urine per micturition in cystitis, compared to healthy animals per 24 hours. The vertical bars represent the S.E.M.

The division of the 24-hour period into four 6-hour periods showed, that in the first two sections the number of micturitions increased in both normal and cystitis cases, then it decreased again in the third and fourth period. Likewise the same shape of the curve could be observed in the urine outflow per period, where the differences between normal and cystitis rats in periods 1 and 3 attained statistical significance (t-test, 1st period: p=0,0279; 3rd period: p=0,0196) and also in the frequency graph.

The volume of urine per micturition graph showed completely different situation. The numbers of both normal and cystitis volumes per micturition remained in all four periods the same *(see Figure 11)*.

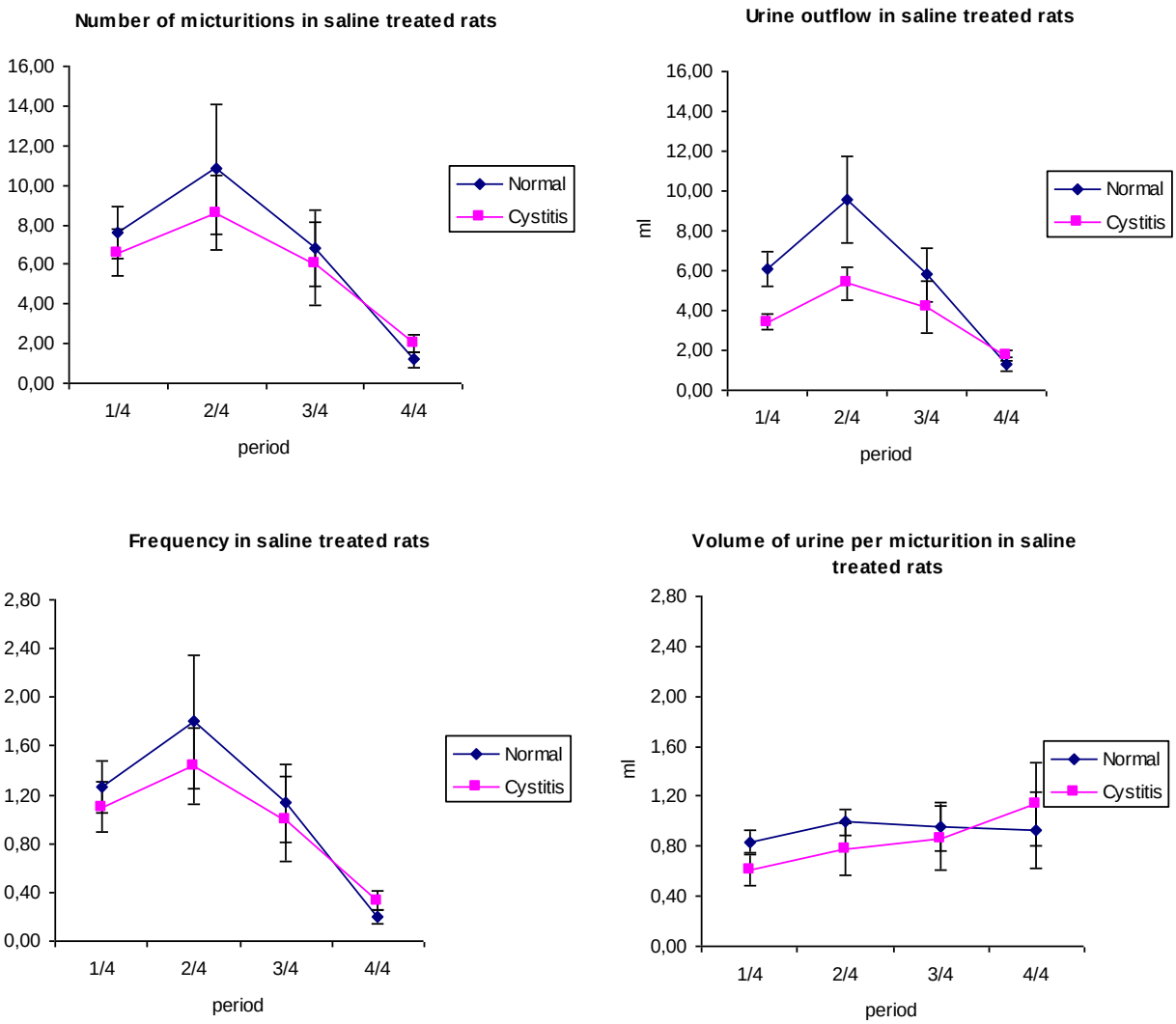

Figure 11:

Changes in cystitis and normal line in saline treated rats, divided into four 6-hour periods. The vertical bars represent the S.E.M.

3.2. 4-DAMP (10µg/kg) treatment group

The next treatment, where we compared the effect of the inflamed bladder was the 10 µg/kg dose of 4-DAMP. We compared the water intake which in cystitis rats decreased by 3,6 ml (n.s.), the urine outflow which decreased by 4,6 ml (n.s.) and number of micturitions decreased by 1,8 (n.s.; *see Figure 12)*.

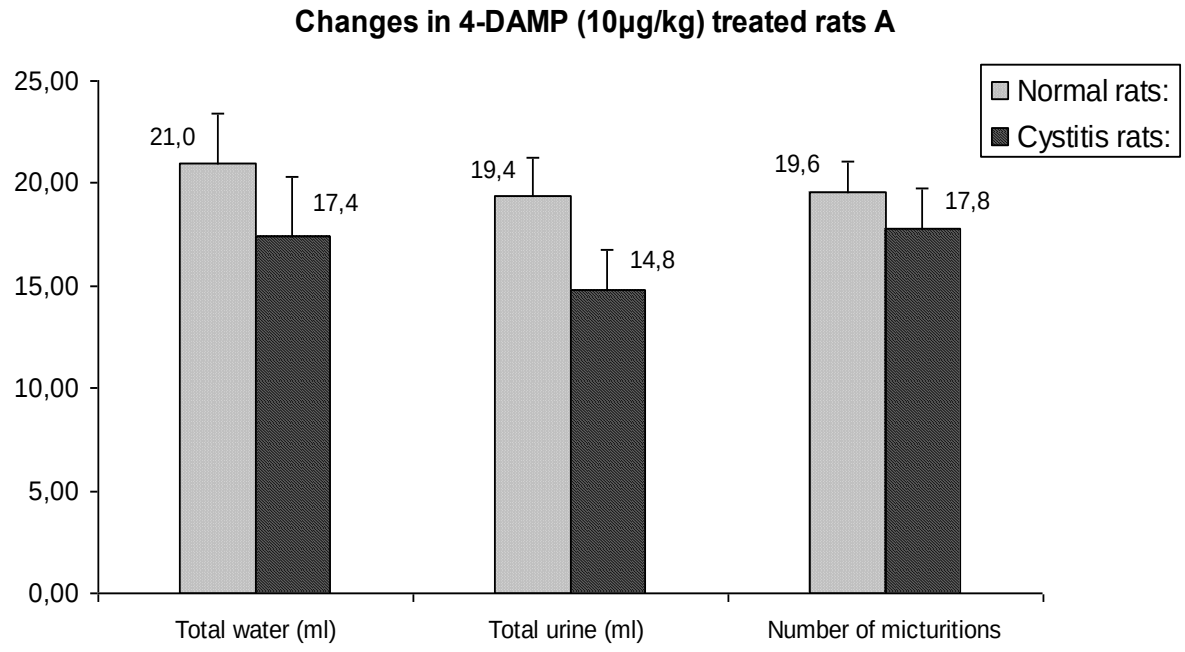

Figure 12:

Changes in 4-DAMP (10µg/kg) treated rats, showing the decrease of the total water intake, urine outflow and number of micturitions per 24 hours in cystitis rats, compared to the normal rats. The vertical bars represent the S.E.M.

The frequency of micturitions decreased by 0,1 micturitions per hour (n.s.), whilst the volume of urine per micturition decreased by 0,2 ml (n.s.) in the cystitis state of the bladder *(see Figure 13)*.

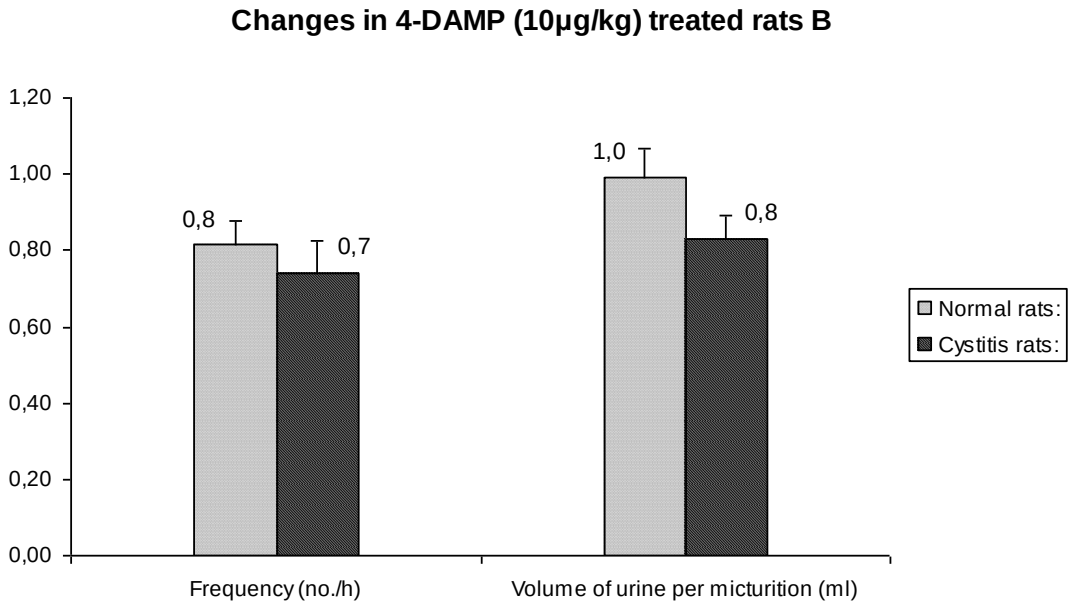

Figure 13:

Changes in 4-DAMP (10µg/kg) treated rats, showing the decrease in frequency and volume of urine per micturition in cystitis, compared to healthy animals per 24 hours. The vertical bars represent the S.E.M.

The comparison of the cystitis rats to the rats without cystitis in number of micturitions discovered that the cystitis rats had lower number of micturitions in the first and second period, but in the third period the numbers aligned and then decreased together in both cystitis and normal states. The same effect could be seen in the urine outflow (t-test, 2nd period: p=0,0498) and in the frequency of micturitions as well.

The volume of urine per micturition graph showed different situation. In the first two periods the cystitis line was lower, in the third period the numbers aligned and then increased together (t-test, 2nd period: p=0,0231; *see Figure 14)*.

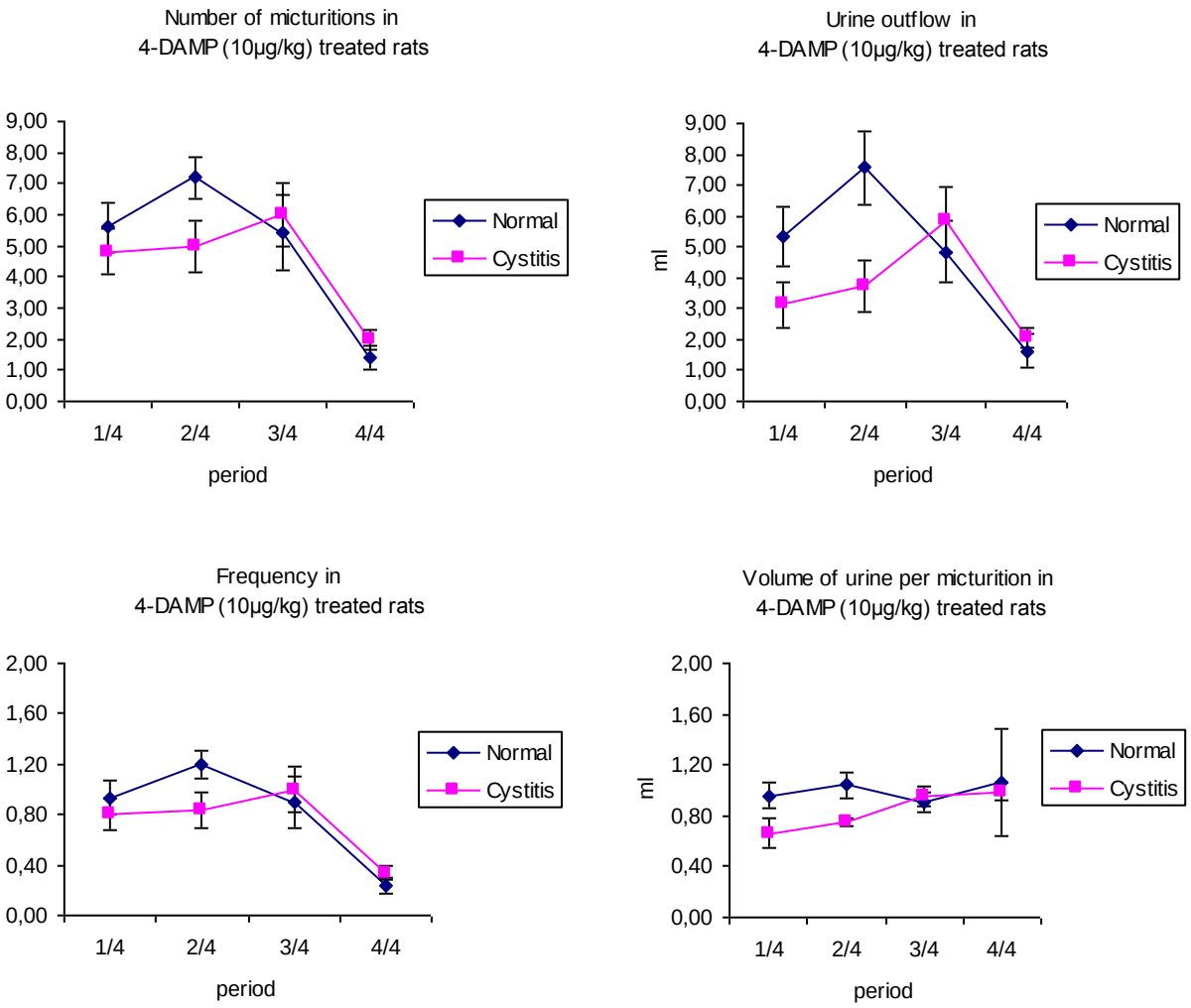

Figure 14:

Changes in cystitis and normal line in 4-DAMP (10µg/kg) treated rats, divided into four 6-hour periods. The vertical bars represent the S.E.M.

3.3. 4-DAMP (100µg/kg) treatment group

In 4-DAMP (100µg/kg) treated rats was discovered an increase in the water intake by 0,4 ml (n.s.), the total urine outflow increased by 0,3 ml (n.s.) and the number of micturitions increased by 2,2 (n.s.) in rats with induced cystitis compared to the normal ones *(see Figure 15)*.

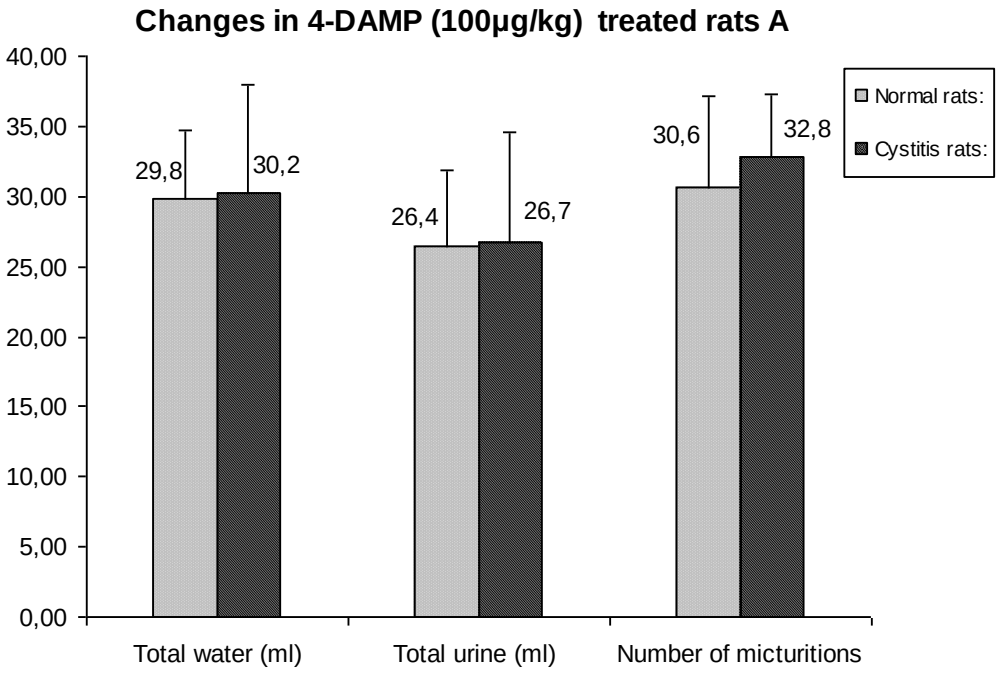

Figure 15:

Changes in 4-DAMP (100µg/kg) treated rats, showing the increase of total water intake, urine outflow and number of micturitions per 24 hours in cystitis rats. The vertical bars represent the S.E.M.

The frequency of micturitions increased by 0,12 micturitions per hour (n.s.), whilst the volume of urine per micturition decreased by 0,11 ml (n.s.) in the rats with inflamed bladders *(see Figure 16)*.

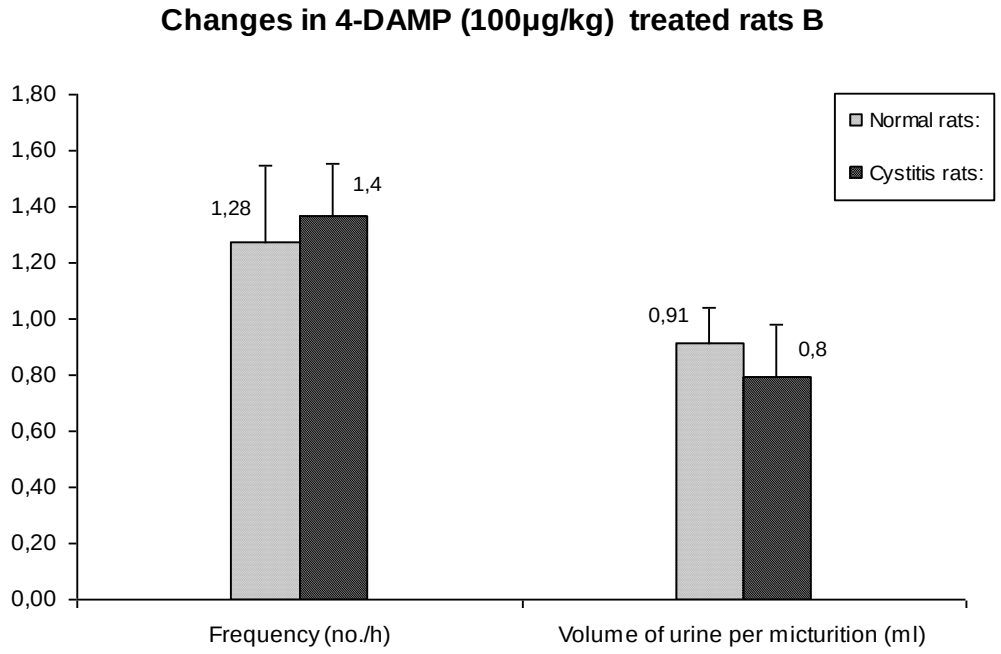

Figure 16:

Changes in 4-DAMP (100µg/kg) treated rats, showing the increase in frequency and decrease in the volume of urine per micturition in cystitis, compared to the normal animals per 24 hours. The vertical bars represent the S.E.M.

The comparison of the cyclophosphamide induced cystitis rats to the rats without cystitis in number of micturitions discovered, that the cystitis rats had slightly lower number of micturitions in the first period, then the number increased in the second period and in the third period it remained higher than the normal number, but started to decrease as well as the normal line. The same shape could be seen in the urine outflow line, but the values were in all four periods about the same. The same effect as by the number of micturitions could be also seen in the frequency graph.

The volume of urine per micturition showed, that the cystitis line remained lower than the normal line in all four periods *(see Figure 17)*.

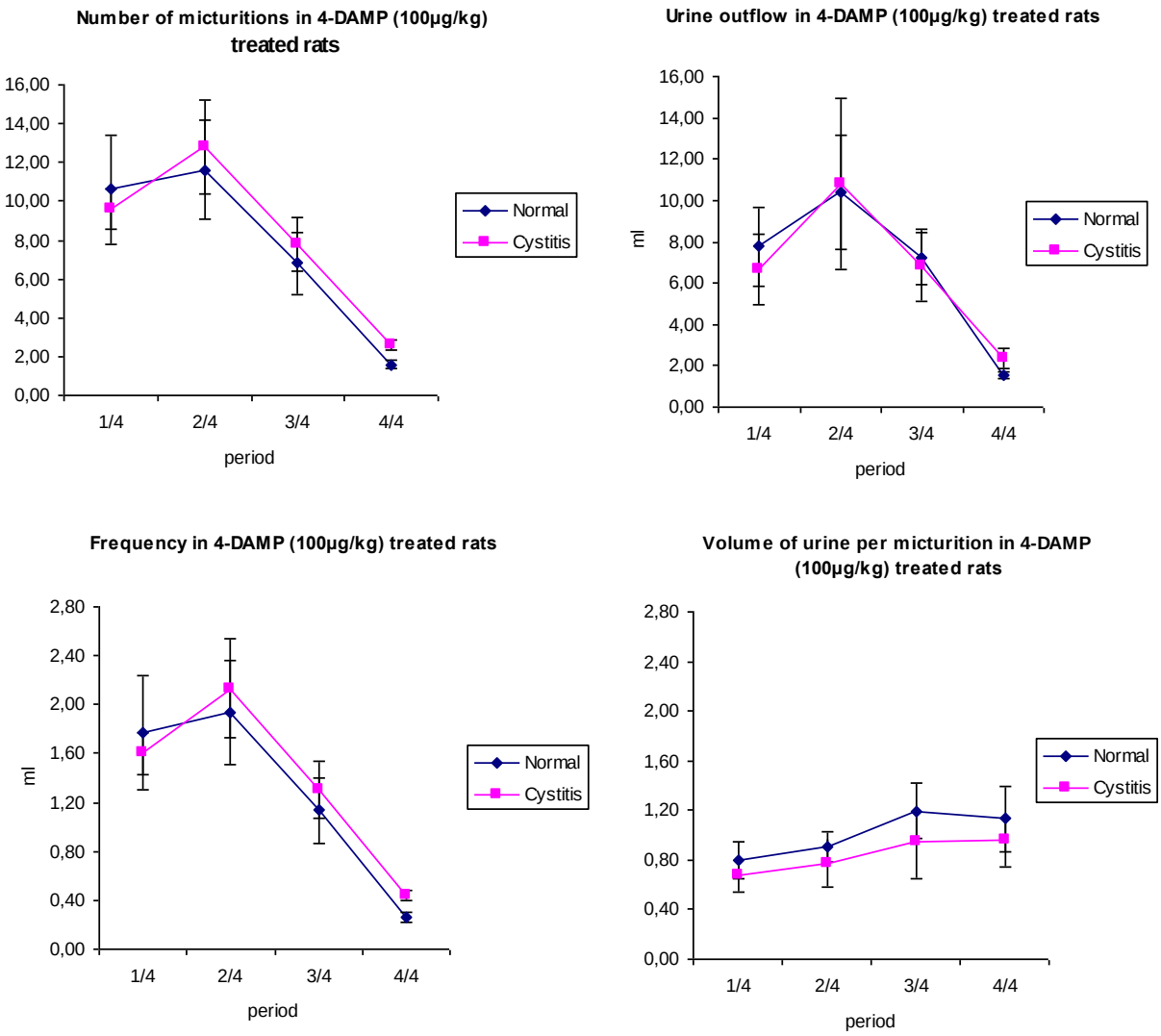

Figure 17:

Changes in cystitis and normal line in 4-DAMP (100µg/kg) treated rats, divided into four 6-hour periods. The vertical bars represent the S.E.M.

3.4. 4-DAMP (1mg/kg) treatment group

In 4-DAMP (1mg/kg) treated rats was observed a decrease in the water intake by 0,2 ml (n.s.), an increase in the total urine outflow by 0,9 ml (n.s.) and also an increase in the number of micturitions by 9,2 (n.s.) in rats with induced cystitis compared to the normal rats *(see Figure 18)*.

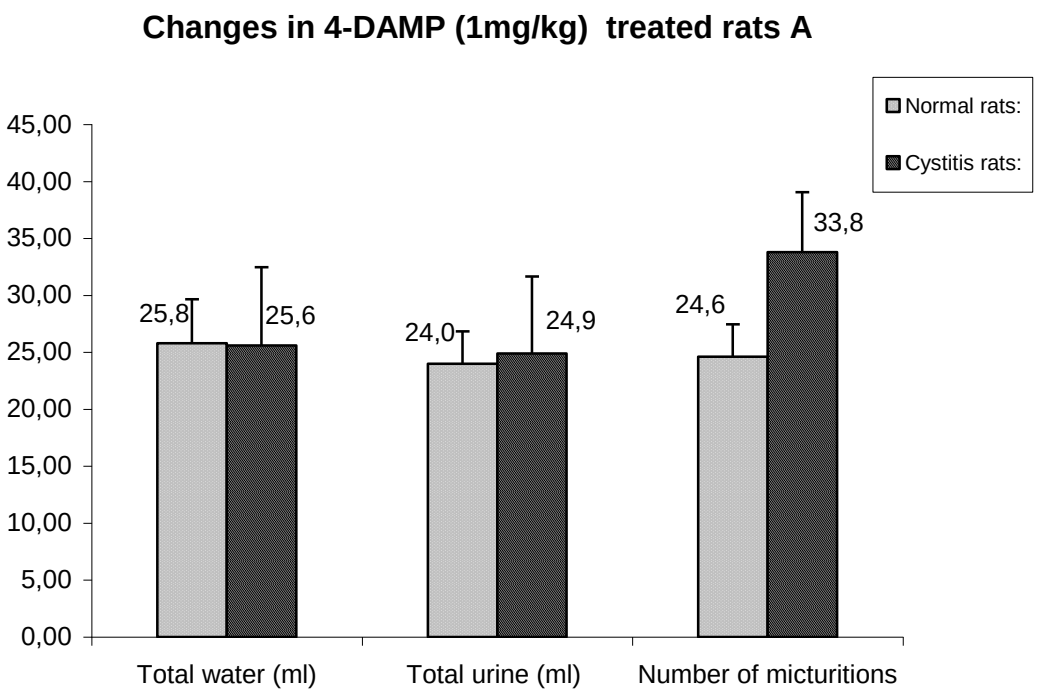

Figure 18:

Changes in 4-DAMP (1mg/kg) treated rats, showing the decrease of total water intake, increase of urine outflow and number of micturitions per 24 hours in cystitis rats. The vertical bars represent the S.E.M.

The frequency of micturitions increased by 0,37 micturitions per hour (n.s.), whilst the volume of urine per micturition decreased by 0,32 ml (n.s.) in the cystitis state *(see Figure 19)*.

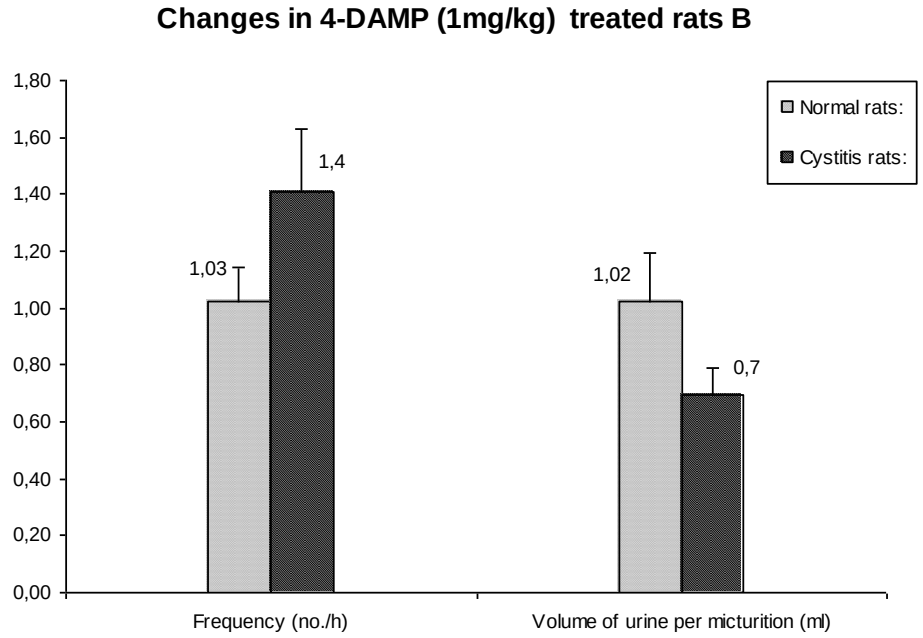

Figure 19:

Changes in 4-DAMP (1mg/kg) treated rats, showing the increase in frequency and decrease in the volume of urine per micturition in cystitis, compared to healthy animals per 24 hours.

The comparison of the cystitis rats to the rats without cystitis within the dose of 4-DAMP (1mg/kg) in number of micturitions discovered, that the cystitis rats had higher number of micturitions in periods 1-3, especially in the second period. In the fourth period the numbers aligned. In the urine outflow graph, we could observe a slight increase in cystitis line in the second period, but the spreading didn't show any major differences between these two lines. The frequency graph showed very similar shape to the graph with the number of micturitions.

The volume of urine per micturition graph showed, that the numbers were about the same in periods 1-2, whilst in periods 3-4 the cystitis line was lower than the normal line (*see Figure 20)*.

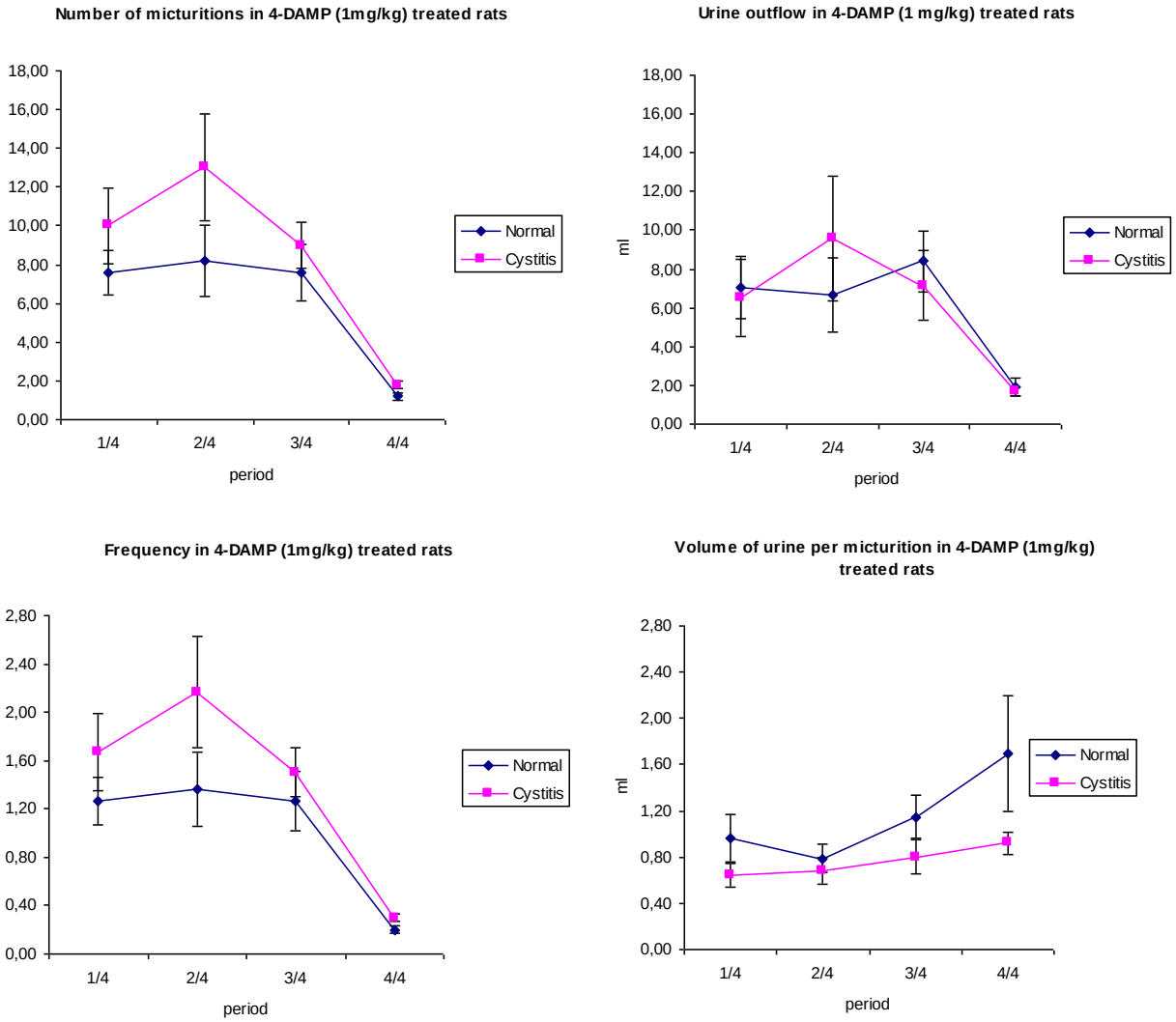

Figure 20:

Changes in cystitis and normal line in 4-DAMP (1mg/kg) treated rats, divided into four 6-hour periods. The vertical bars represent the S.E.M.

3.5. Outline of changes in different treatments and doses

The comparing of graphs with different treatments showed following situation. Differences within drinking habits, total urine outflow and number of micturitions of different treatments in normal rats and also in cystitis rats could be observed *(see Figure 21)*. In number of micturitions in cystitis state was discovered a significance (1-way ANOVA: p=0,0491)

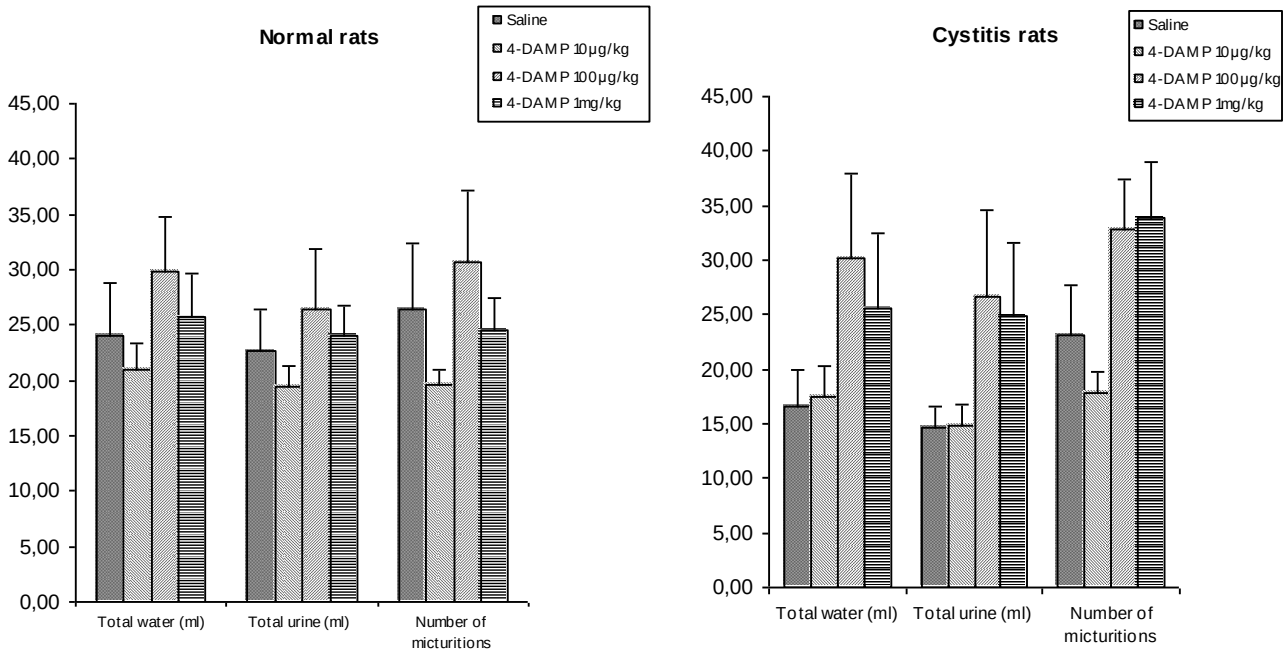

Figure 21:

Comparison of different treatments in normal and cystitis group. The vertical bars represent the S.E.M.

Interesting seems to be the graph with volume of urine per micturition which showed significant difference (t-test, p=0,0096) between all the normal and all the cystitis rats *(see Figure 22)*.

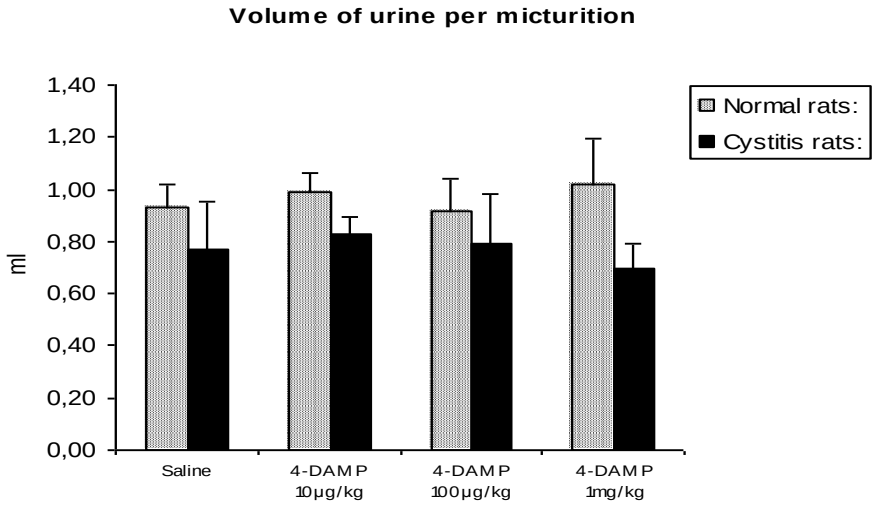

Figure 22:

Graph of volume of urine per micturition, showing differences between normal and cystitis rats as well as between different treatments. The vertical bars represent the S.E.M.

The frequency graphs showed that the saline (control) line was lower during all four periods in the cystitis state, but the profile of the curve was about the same. 4-DAMP (10µg/kg) lowered the curve in periods 1 and 2 in cystitis compared to normal, 4-DAMP (100µg/kg) was about the same in both normal and cystitis cases and in the dose of 4-DAMP (1mg/kg) the frequency increased in periods 1 and 2 in cystitis and then started to decrease again similarly to the normal state.

The volume per micturition in normal rats was approximately equal in all the treatments. Same effect could be observed in the cystitis rats, but the curves have greater tendency to increase in contrast with the normal state *(see Figure 23)*.

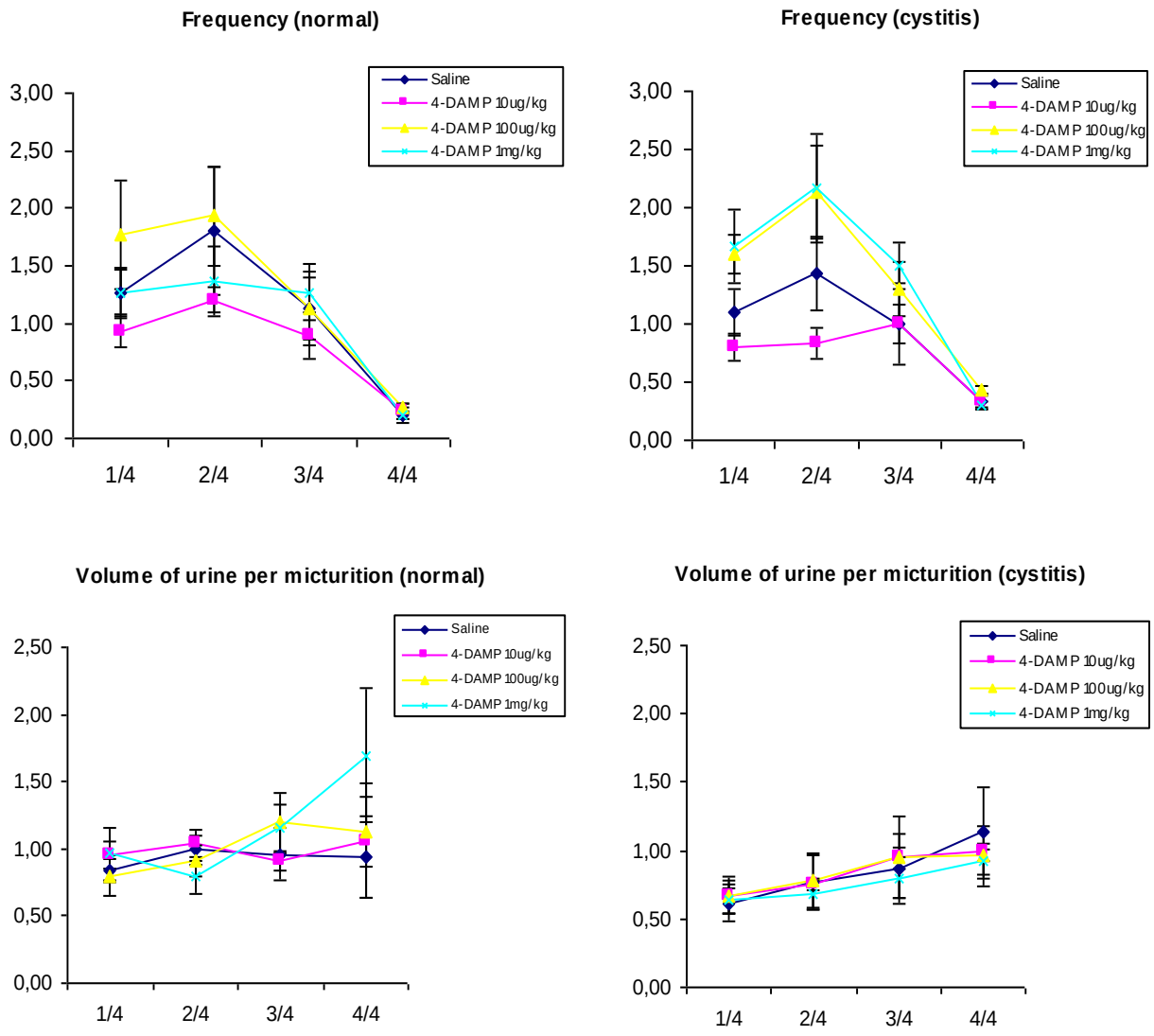

Figure 23:

Overview of time-divided graphs, comparing different treatments in criteria of frequency and volume per micturition and also of normal and cystitis states. The vertical bars represent the S.E.M.

All treatments together in one graph showed no changes in the frequency between normal and cystitis rats in all four periods of the experiment. However the volume of urine per micturition graph, in periods 1 and 2 with all the treatments together, showed significant decrease in cystitis rats (t-test, 1st period: p=0,0062; 2nd period: p=0,0400, *see Figure 24*).

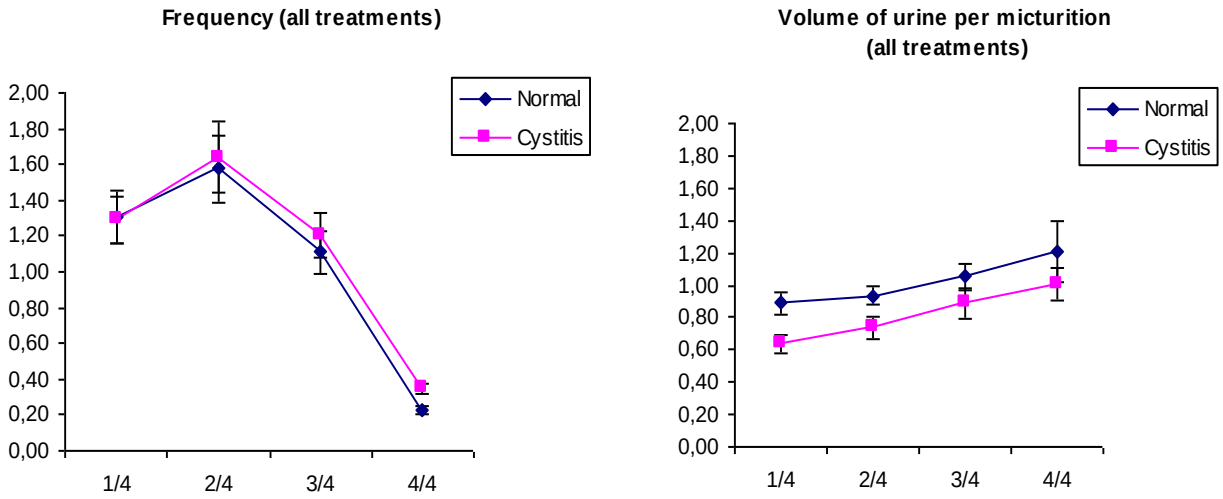

Figure 24:
Graphs showing equal frequency of micturitions in normal and cystitis rats and decreased volume of urine per micturition in cyclophosphamide-induced cystitis rats. The vertical bars represent the S.E.M.

4. Discussion

Our findings from this project could be distorted by inadvertent circumstances which could be caused by different factors. One of these factors could be the technical aspect. The metabolic cage was placed on the stand and the laser beam sensor was pointed in the right-angle to the urine collection tube to register drops. In some cases could happened that the sensor didn't register all the drops because of some reflections from the urine collection tube. Another factor could be the human factor. As was mentioned in the Materials and methods part, data from each experiment were stored in the portable computer and analyzed afterwards. This was conducted by counting peaks of each drop in the PC program visually and dividing the groups of peaks into micturitions. A mistake in grouping peaks into micturitions could occurred due to some uncertain borders between these groups. Another problem could be the injection of the animals. As mentioned before, we injected the rats intraperitoneally and that could resulted in differences in speed of drug absorption according to the position and depth of the injection and so changed the micturition pattern. However, all the rats were injected in the same time, either with saline, 4-DAMP or cyclophosphamide and after each experiment, a dissection, to ensure the inflammation, was made.

Things to be mentioned are that 4-DAMP has effect on the muscarinic receptors in the bladder for approximately 12 hours which means first two periods of the experiment *(Sjogren et al., 1995)*. 4-DAMP, as all antimuscarinic agents in the treatment of OAB, should have effect mostly on number of micturitions, which should decrease. The volume of urine per micturition should thereby increase due to the increment of the threshold for voiding and desensitization of the bladder smooth muscle, the detrusor. We should note that rats have reversed diurnal rhythm compared to humans, so they are more active in the darkness than with the lights on.

In this study, we found a decrease in the water intake in the cystitis rats, compared to normal rats, which could be caused by the bad conditions of the animals after the provoked inflammation of the bladder, which was already

observed in saline-treated rats. The total volume of urine, the number of micturitions and the frequency was thereafter more or less dependent on the water consumption, but the volume of urine per micturition was an independent factor which indicated the increased sensitivity of the urinary bladder by the decrease of the outflow per micturition. The same situation could be seen in the volume of urine per micturition in all 4-DAMP doses, which contributes to the theory of the increased bladder sensitivity during inflammation. However, different effect was observed in the number of micturitions with the increasing dose of 4-DAMP from 10µg/kg to 1mg/kg. The number of micturitions increased with the dose in the cystitis state which denies the theory that the antimuscarinic agents should decrease this parameter by the blockade of the muscarinic receptors *(Abrams et al., 2006)* in the inflamed bladder and evolves another theory of the compensatory mechanisms during the inflammation *(Giglio et al., 2005)*. This was not observed in normal state, where the 4-DAMP treatment caused decrease in the doses of 10µ/kg and 1mg/kg compared to saline and increase in the dose of 100µg/kg which leads to conclusion that a central mechanism was involved after reaching certain concentration of the drug. From which resulted that the dose of 10µg/kg reached the best effect in reducing the number of micturitions as well as therewith connected frequency. Whilst the frequency was lowered by the anticholinergic drug, the volume of urine per micturition increased according to the knowledge about antimuscarinic agents in the treatment of the OAB *(Abrams et al., 2006)*.

The situation within the first two periods of the experiment showed no significant changes between normal and cystitis within the volume of urine per micturition in the saline treated rats, according to our theory of the compensatory mechanism which could be of cholinergic (increased expression of muscarinic M_1 and M_5 receptors) *(Giglio et al., 2005)*, nitric oxide induced *(Korkmaz et al., 2003)* or purinergic origin *(Bolego et al., 1995)*. However this compensation was present in the saline treatment, in the first two periods with 4-DAMP (10µg/kg) treatment this effect diminished and the difference between volume of urine per micturition between normal and cystitis rats increased, which indicates that 4-DAMP affects and reduces the compensatory mechanism of the bladder during the inflammation. Against this

theory stands the fact that in the treatment with increasing doses of 4-DAMP (100µg/kg and 1mg/kg), no similar effects were discovered. However in previous in vitro studies, it has been observed that 4-DAMP may have opposite effect at low and high concentrations *(Giglio et al., 2005) (see Figure 25)*.

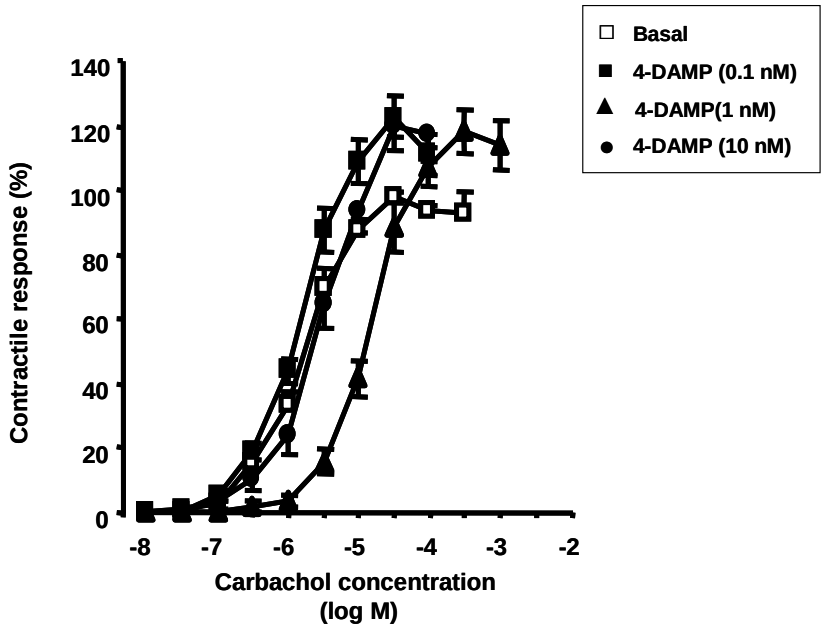

Figure 25:

Graph from in vitro studies, showing left shift of the carbachol curve in the presence of a low dose of 4-DAMP (0,1nM) compared to the basal curve. In the presence of the higher doses of 4-DAMP (1nM resp. 10nM) right shift was observed. The vertical bars represent the S.E.M. Adapted from Giglio, Altered muscarinic receptor subtype expression and functional responses in cyclophosphamide induced cystitis in rats *(Giglio et al., 2005)*

The comparison of all groups together in the normal state showed that the highest water intake as well as the urine outflow and number of micturitions, although not significant, was by the 4-DAMP (100µg/kg). The reason why this effect occurred could possibly be the “Dry mouth syndrome” after the blockade of muscarinic receptors in the salivary gland *(Scully, 2003)*. This effect decreased with the highest dose of 4-DAMP (1mg/kg) possibly due to the predominant influence of the muscarinic blocade in the bladder. Almost similar effect was observed during the inflammation.

As was demonstrated on the outline of the volume of urine per micturition in the Results part, the volume decreased in cystitis state due to the inflamed bladder, but no clue, suggesting any pronounced effect of 4-DAMP was observed, because all the 4-DAMP doses had an equal value in normal as well as in cystitis state.

4.1. Conclusions

The current study shows that there is a contradictory mechanism in the urinary bladder. This mechanism compensates for the inflammation together with the effect of 4-DAMP in the inflamed bladder. This provides further support for previous observations showing a change in the expression of muscarinic receptor subtypes in cystitis. However, further investigations and diagnostic methods such as immunohistochemistry, western blotting as well as more in vivo experiments with directly introduced catheters into the experimental animals are needed to reveal the exact origin and function of this mechanism.

Furthermore only poor effect on the frequency and number of micturitions decrease occured along the treatment with 4-DAMP, which is consistent with the present knowledge about insufficient results from the treatment of OAB incontinence with antimuscarinic agents *(Lai et al., 2002)*.

4.2. Souhrn

Touto studií byla prokázána přítomnost určitého protichůdného mechanismu v močovém měchýři. Tento mechanismus nejspíše kompenzuje probíhající cystitidu společně s účinkem 4-DAMP. Toto podporuje již známý fakt, že během cystitidy dochází ke zvýšené expresi muskarinových receptorů. Přesto je nutno toto zjištění potvrdit dalšími studiemi „in vivo“, případně použitím moderních „in vitro“ diagnostických metod, jako je imunohistochemie a western blotting.

Dále bylo v této studii zjištěno, že antimuskarinikum 4-DAMP vykazuje pouze slabý efekt na snížení frekvence a počtu diurézy, což je v souladu se současnými zkušenostmi s léčbou inkontinence zapříčiněné „přecitlivělým močovým měchýřem“ antimuskarinovými agens.

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