INTRAVESICAL THERAPY OF INTERSTITIAL CYSTITIS

by

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ABSTRACT

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Interstitial cystitis (IC) is an inflammatory disorder of bladder which affects middle-aged Caucasian women. Intravesical administration of drugs is a mainstay in its treatment either as adjunct to oral therapy or as second-line therapy. The vehicles currently used for this route of administration are ideally suited for hydrophobic drugs and typically maintain drug exposure in the bladder for very short duration of time. The present dissertation project was aimed at investigating the use of alternative vehicles for improving intravesical drug delivery of hydrophobic small molecular weight drugs such as capsaicin misoprostol in addition to the delivery of large molecular weight peptide nucleic acid (PNA) for antisense based therapy. The hydrophobic drugs selected for this study were delivered by intravesical route using liposomes, thermosensitive hydrogel and TAT peptide. The efficiency of drug delivery was assessed by measuring the physiological response of normal and diseased rat bladder by metabolic cages and the method of cystometrogram (CMG). Histology and immunohistochemistry of bladder and spinal cord sections was done to corroborate the response measured in the physiological measurement. Liposomes were demonstrated to be a superior vehicle for capsaicin and thermosensitive hydrogel was able to sustain the exposure of a hydrophobic drug in the bladder for prolonged time and increase the efficacy of misoprostol in rat model of cystitis induced by cyclophosphamide. An interesting observation made during the study was that liposomes in

absence of drug were able to modulate physiological response of bladder and this observation was further investigated to define the charge on the lipid headgroup and the structural requirements of hydrophobic backbone in the lipids for reducing bladder hyperactivity induced by sequential infusion of protamine sulfate and high concentration of KCl. Overexpression of βNGF in cyclophosphamide induced cystitis was downregulated in the urothelium of rat bladder using antisense based therapy with PNA, which was delivered with the aid of TAT peptide. Overall, the study concluded that liposomes cannot only be a treatment option but can also be used for delivery of hydrophobic drugs. The potential of hydrogels and cell penetrating peptides for intravesical drug delivery needs further investigation.

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PREFACE

Interstitial cystitis (IC) is an inflammatory bladder syndrome of obscure etiology and pathogenesis.A patient's response to therapy is fraught with variation owing to the multifactorial nature of IC, and current treatments are largely empirical achieved through a variety of oral and intravesical therapies. The strategies used in therapeutic management of IC patients in the clinic are reviewed. Intravesical agents have been used for many years as adjuncts to oral treatment regimens or as second-line therapies for IC. The efficiency of intravesical drug delivery is a critical factor in the often incomplete and variable response from conventional formulations used in intravesical therapy in IC. Recent developments in the field of improved intravesical delivery are reviewed and the results of experiments designed to study application of liposomes and hydrogel as novel vehicles for drugs used in IC are reported.

It has been suggested that neuroinflammation plays a role in painful bladder disorders of uncertain etiology, such as IC. The role of inflammation in the pathogenesis of IC is reviewed. Inflammation in the bladder can results from disruption in the permeability barrier of urinary bladder from agents in the urine. Acrolein, a metabolic product of cyclophosphamide (CYP) is known to cause cystitis by making pores in the apical membrane of umbrella cells present in bladder luminal lining. The two animal models for IC used in these studies for evaluating novel intravesical therapies are based on disruption of permeability barrier either through agents in urine or by agents instilled into bladder. The ultimate goal of this work is the development of new and improved intravesical therapeutic strategies for cystitis. New therapeutic strategies for cystitis that were evaluated are liposomes and intravesical antisense delivery, the studies described in chapters 2 and 5. Improvements through the use of liposomes and hydrogel in

existing intravesical therapy of vanilloids and prostaglandins are described in chapters 3 and 4. CYP-induced cystitis results in a dramatic reorganization of micturition reflex circuitry characterized by changes in neurochemical, electrophysiological, and organizational properties. These changes suggest considerable reorganization of reflex connections of bladder afferents after bladder inflammation. Previous studies have demonstrated alterations in mRNA and/or protein following CYP injection. CYP-induced cystitis was used as the animal model in the studies described in chapters 4 and 5.

Rat model of bladder injury was the other animal model used in the study described in chapter 2. Selective damage to the upper layers of the urothelium or umbrella cells is induced in this model by infusion of protamine sulfate and later high concentration of potassium chloride is infused to demonstrate bladder hyperactivity in a cystometrogram (CMG). A prior study from our lab using the same model of hyperactive bladder noted the novel treatment option of instilling liposomes as a therapeutic option. This novel treatment option was further explored in the present dissertation project by experiments designed to define the charge and structural elements of lipids necessary for efficacy in bladder injury. The study is described in chapter 2. Capsaicin-induced nerve desensitization of bladder afferents is a viable intravesical treatment option for IC patients, which is currently achieved by instilling the hydrophobic drug in saline using ethanol as a cosolvent. The study described in chapter 3 employed liposomes and hydrogel as vehicles for intravesical delivery of capsaicin for reducing the toxicity incurred from vehicle in normal rats without compromising efficacy of capsaicin.

Other labs have achieved slow and sustained release of drugs in the urine by using prolonged infusion of drugs into the bladder. The same objective was attained in this work by instilling thermosensitive hydrogel as a matrix filled with drug for sustained intravesical delivery

of fluorescent probe in urine. The efficacy of this delivery system was studied by assessing the effect of misoprostol in the study described in chapter 4. The luminal lining of urinary bladder facing the urine, is called the urothelium, it is covered by uroplakin and together with tight junctions represent the toughest water tight barrier to drug delivery known so far. The composition of this permeability barrier in context of drug delivery and the use of liposomes as a treatment option is reviewed. Inflammation of the bladder seen in IC is characterized by heightened pain sensitivity and this hyperalgesia is the consequence of the release of inflammatory mediators, cytokines and growth factors from the bladder. A key participant in this process is the induction of the neurotrophin nerve growth factor (NGF). Prior studies suggest that therapies aimed at abating NGF may help treat IC. Study described in chapter 5 is about the improvement in the intravesical delivery of agents for blocking NGF expression by helping them cross the permeability barrier of urothelium. Experiments were designed to explore antisense based therapy with peptide nucleic acid (PNA) for downregulation of NGF in acute cystitis following CYP injection. The permeability of PNA across urothelium was enhanced by using the cell-penetrating peptide TAT.

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Pradeep Tyagi

1. INTRODUCTION

Interstitial cystitis (IC) is a debilitating chronic inflammatory disorder of the bladder, which predominantly affects middle-aged Caucasian women. It is a multifactorial syndrome of pelvic/perineal pain, urinary frequency and urgency (1). However, these clinical hallmarks are also shared by other pelvic disorders and complicate the diagnosis of IC, a term first used in 1878 by Skene to describe unexplained pelvic pain. It is ten times more common in women than men. Its diagnosis is often made from the combination of symptoms, cystoscopic findings, bladder biopsies and by exclusion of a vast array of other possibilities, including carcinoma and detrusor hyperreflexia.

There are two subtypes of IC, classic and nonulcer IC, with similar symptoms but different outcomes with respect to clinical course and response to treatment (2). Histologically, there are fundamental differences between the two subtypes, classic IC presenting a severe abnormality of the urothelium and characteristic inflammatory cell infiltrates while inflammation is scant in nonulcer IC (3). Recent study showed that patients with classic IC had higher nitric oxide (NO) content in their bladder compared to patients with non ulcer IC and steroid treatment reduced NO content in bladder of classic IC (4). This attribute of IC is akin to asthma, where inflammation in patients is measured by concentration of NO in the airways to modulate the dose of inhaled steroids (5). Moreover, localization of afferent nerves adjacent to the urothelium argue for a important role in the development of afferent excitability leading upto IC symptoms (6).

1.1. Pathophysiology

The pathophysiology is IC incompletely understood, and numerous theories have been proposed to explain the pathogenesis and progression of IC symptoms including mast cell

activation, altered bladder epithelial permeability and afferent nerve up-regulation. Leading theories for the pathogenesis of IC include (1) changes in urothelial permeability; (2) mast cells infiltration; (3) neural-immune mechanisms; (4) plasticity in the nervous system; and (5) infection., toxic urinary agents, deficiency in bladder lining and neurogenic causes.

1.1.1.1. Changes in Urothelial Permeability

Most cellular membranes possess a very high permeability for a wide range of small molecules like water and urea, but the cells in luminal lining of urinary bladder have excessively low permeability to these molecules (7). In spite of being constantly exposed to urine, that is so different from blood in its ionic composition and osmolality, bladder lining maintains a water tight barrier between the blood and the contents of urine. This tight permeability barrier of urinary bladder is essential in storing urine for prolonged periods and to help kidneys in maintaining optimal plasma osmolality and ionic strength (8). The permeability barrier of bladder for enduring such steep gradients is believed to be erected by the uppermost layer of bladder epithelium also called urothelium (9).

The uppermost layer of urothelium is made up by umbrella cells, whose apical plasma membrane facing the urine has a unique lipid composition and transmembrane uroplakin proteins, both of which seem to play important role in reducing the permeability of the apical membrane to water, ammonia, protons and urea (10-12). In its healthy state, the bladder epithelium is almost impermeable to irritants present in urine by virtue of tight junctions joining the uroplakin covered umbrella cells (13, 14). The rigid plaques covering 90% of the surface area of these cells are composed of four major uroplakins, i.e., UPIa (27 kDa), UPIb (28 kDa), UPII (15 kDa) and UPIII (47 kDa) (12). It is now well accepted that the permeability of the urothelium

is further augmented by the mucin layer composed of proteoglycans present on the surface of umbrella cells (15, 16).

Glycosaminoglycans (GAG) include chondroitin 4 and 6 sulfate, dermatan sulfate, heparan sulfate, and hyaluronic acid, which are present in the GAG layer on the bladder surface (17, 18). The theory of changes in urothelial permeability arising from defects in GAG layer as the cause of IC was popularized by Parsons and coworkers (19, 20). Despite its popularity, it has few shortcomings that have been recently reported in the concept of defective bladder epithelium. Electron microscopic studies on bladder biopsies failed to show any differences in the GAG layer of normal and IC patients diagnosed according to National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases criteria (21). Bladder permeability of IC patients measured by serum uptake of radionuclide was comparable in 70% of patients with their normal counterparts and it was increased in only 3 out of 10 patients (22). Serum radioactivity was taken as the index of bladder permeability, determined after infusion of 10 ml saline containing 5 mCi of radionuclide (^{99}technetium-diethylenetriaminepentaacetic acid, ^{99}Tc-DTPA) followed by bladder distension to 80% of its functional capacity with saline. Functional capacity is the volume of saline at which patients ask the fluid inflow into the bladder to be stopped. Potassium sensitivity test used in IC for a diagnosing mucosal leakage showed that instillation of potassium chloride into bladder provoked symptoms in only 70% of IC patients and 4.5% of normal volunteers (23). It seems that a defective GAG layer is the cause of IC in only a subpopulation of IC patients.

1.1.1.2. Inflammation & Mast cells

In recent reports however, evidence is mounting in support of a central role for inflammation in the propagation of IC symptoms. Inflammation has the potential to induce and

perpetuate neurotrophic changes in the bladder (24). Peptidergic and sympathetic innervation of the submucosa and muscular layers of the bladder are increased in patients with interstitial cystitis (2, 25). The observation of inflammation in the bladder of some IC patients parallels the biochemical interactions between inflammatory cells and the nervous system in murine models of cystitis (26, 27). In multiple animal models the stimulation of sensory nerves releases inflammatory neuropeptides, that induce inflammation through mast cell activation (26, 27).

Mast cells derived from bone marrow are ubiquitous and are responsible for generating allergic reactions in various tissue by releasing numerous vasodilatory, nociceptive and pro-inflammatory molecules in response to stimulation by immunoglobulin E (IgE) and specific antigen (28). Mast cells in the bladder have been often found in juxtaposition to neurons, where they can be activated by neuropeptides such as bradykinin and substance P and also by acute psychological stress (29). Activation of mast cells without typical exocytosis has been observed in the urothelium and suburothelium tissue of IC bladder (2, 30). Vasoactive and proinflammatory molecules released from mast cells may cause normally unexcitable C-fiber afferents to become hyperexcitable and spawn the symptoms of IC (31, 32). Inflammation can be intiated by mast cell induced expression of cell adhesion molecules such as interacellular adhesion molecule ICAM-1 and P-selectin (33, 34). These effects of mast cells can explain the absence of cystitis in mice genetically manipulated to be deficient in mast cell, following instillation of lipopolysaccharide LPS, whereas frank inflammation was observed in wild-type mice having functional mast cells (35).

1.1.1.3. Neurogenic Causes

It has been suggested that neuroinflammation plays a important role in painful bladder disorders of uncertain etiology, such as IC (36, 37). Inflammatory cells can act as an extension of

the peripheral nervous system in the bladder and mediate IC symptoms (38). Levels of nerve growth factor (NGF) were found to be increased in the bladder of IC patients (39). Increased (NGF) production and morphological changes in afferent neurons innervating the bladder during bladder inflammation argue for a complex association between inflammatory cells and the nervous system (24). Therapies aimed at abating NGF have shown promise in the clinic.

Continence or control over micturition requires regulation of lumbosacral afferent fibers (pelvic afferents) and afferents in the hypogastric and pudendal nerves (40). The afferent pathway in the continent state is mediated largely by Aδ-fibers, which ultimately send information about the state of bladder fullness or wall tension detected by mechanoreceptors to the pontine micturition center via the periaqueductal gray matter in spinal cord, while the C fibers mainly detect noxious signals and initiate painful sensations (32). These afferent nerves have been identified in the urothelim, suburothelially as well as in the detrusor muscle (6). Afferent nerves can form a plexus in suburothelium, which is particularly dense in the bladder neck and the trigone region (41). Increased density of afferent fibers in bladder of IC patients can be the result of neuroplastic changes induced by inflammation (42, 43). The neuroplasticity may explain the mechanisms by which chronic irritative symptoms and pain may persist even after the removal of an inflammatory stimulus. Clinically, this corresponds to a subset of patients with IC who continue to have pain without inflammation or patients feeling IC associated pain even after removal of bladder (44).

Bladder inflammation can bring about functional alterations in bladder afferent pathways comprising of small myelinated Aδ fibers and unmyelinated C fibers (45). Following bladder inflammation, capsaicin-sensitive C-fiber afferents can take over the role of Aδ-fibers and they convey the signal of bladder fullness at reduced bladder capacity (45). This shift in afferent arm

of micturition reflex has been modulated by instillation of botulinum toxin in a inflamed bladder, which only attenuated the afferent response from bladder without impairing the efferent bladder function (46). Alteration of afferent pathways can lead to a reduction in pain threshold (allodynia) and an amplification of painful sensations (hyperalgesia). Increased pain sensitivity can result from changes in peripheral nociceptor afferents or changes in the central nervous system mechanisms that process nociceptive inputs. The link between symptoms of IC and C fibers has not been clearly established and changes in afferent signaling bought about by inflammation may help in establishing the connection. Therefore modulation of the inflammatory cascade presents a logical therapeutic target for treating cystitis.

1.2. Treatment of IC

The postulated etiologies mentioned above have led to a variety of treatment regimens, none of which uniformly eradicates the symptoms of urinary frequency, urgency, nocturia, and/or pain (47). The response to therapy is fraught with variation owing to the multifactorial nature of IC, and clinical approaches to treatment have been largely empirical and can involve a variety of oral and intravesical therapies (48). Oral medication is usually the first line of treatment for IC, but a multipronged treatment strategy is often selected as an appropriate therapy and drugs treating GAG layer dysfunction, mast cell abnormalities, and neurogenic inflammation are frequently combined with each other (Fig.1.1) (49). Overdistension of bladder has nevertheless remained one of the widely used empirical method of treatment, despite its cytodestructive effects (50, 51). The diversity of IC therapies underscores the lack of

understanding about the treatment of this syndrome (52). The schematic chart shown in Fig.1.1 shows the current approved treatment of IC and new treatment options evaluated in this project.

1.2.1. Oral Therapy of IC

Oral treatments of IC include pentosan polysulfate, tricyclic antidepressants and antihistamines (47). In the past, patients with long-standing disease have been given agents such as tricyclic antidepressants, and narcotics to suppress symptoms of hyperactivity (53, 54). Amitriptyline is thought to act via various mechanisms such as blockade of acetylcholine receptors and inhibition of reuptake of released serotonine and norepinephrine. As amitriptyline is known to block the histamine H_1 receptor, it is also believed to act via sedation, possibly via its H_1–antagonism. The clinical efficacy of hydroxyzine in reducing IC symptoms may be explained by its ability to inhibit bladder mast cell activation by neurogenic stimuli along with its anticholinergic, anxiolytic and analgesic properties (31).

Defective GAG layer is believed to be present in a subset of IC patients, which prompted intravesical administration of heparin, hyaluronic acid, or pentosan polysulfate for restoring the GAG layer on the surface of transitional cells of bladder (55, 56). Moreover, sodium pentosanpolysulfate was also able to manipulate the microenvironment between umbrella cells and urine after prolonged oral administration and alleviate the symptoms of IC in patients (19, 57). The beneficial response in these patients was maintained for extended over time (58).An open multicenter study done on 87 patients found a differnece in the therapeutic benefit obtained from pentosanpolysulfate in two types of IC. Patients without bladder ulceration had much favorable response than the patients with bladder ulcer with significantly reduced frequency of micturition and increased mean daily urine volume per void in the patients with non-classical IC

(59). In many other studies patients taking the drug reported subjective improvement in pain, urgency, frequency and nocturia compared to placebo (56, 60, 61). However, a recent study done on 121 patients randomized over 18 months a failed to show any distinct gain in terms of therapeutic benefits in patients treated with sodium pentosanpolysulfate over patients taking hydroxyzine (62).

Oral administration of pentosan polysulfate sodium is effective in one third of IC patients, and continued administration for several months or more is required for reduction in the symptoms of pain and urgency. Apart from restoring GAG layer, it also appears to be a potent inhibitor of allergic and nonimmune mast cell stimulation (63). Adverse effects from pentosanpolysulfate are few and transient, but rare cases of serious bleeding complications have been reported following its use (64). Orphan drug status of sodium pentosanpolysulfate allowed it to be tested in the National Toxicology Program (NTP) for its chronic toxicity and carcinogenicity in mice and rats. Carcinogenic potential of sodium pentosanpity was not detected in rats, but its carcinogenic activity in mice was revealed by increased incidences of liver hemangiosarcoma, hepatocellular neoplasms (predominantly adenomas), and malignant lymphomas. Escalating doses of sodium pentosanpolysulfate also produced elevated occurrences of nonneoplastic lesions in multiple organs of mice and rats (65). Increased proliferation in human breast cancer cell lines has also been reported at several concentrations of pentosanpolysulfate (66).

Recently, a dietary supplement formulated with the natural GAG components, chondroitin sulfate and sodium hyaluronate was evaluated in an open label study done on 37 female patients refractory to all forms of therapy. The dietary formulation also contained the flavonoid quercetin which has anti-inflammatory properties and shown to inhibit activation of

mast cells. This formulation sold as CystoProtek was taken for 6 months by IC patients at the dose of six capsules per day. Patient assessment following reatment revealed a decrease in Global assessment scale and OLeary/Sant Symptom index (67).

The oral administration of anticholinergic agents for treating the symptom of urinary urgency in IC is justified by the physiology of voluntary or involuntary contraction of bladder, which involves stimulation of the muscarinic receptors in the detrusor by acetylcholine released from activated cholinergic nerves. However, approximately 20% to 40% of patients cannot tolerate anticholinergics due to the troublesome side effects such as excessive dry mouth, constipation or blurred vision (68). The systemic side effects of oxybutynin, a important drug in this class are suggested to be caused by the high serum level of its active metabolite, N-desethyl-oxybutynin (DEOB) (69). Moreover, not all patients respond clinically to oral anticholinergic therapy despite maximal dosage.

1.2.2. Intravesical Treatments for IC

For patients who do not respond to oral therapy or for those patients who suffer from a flare and require additional treatment, several intravesical agents are available. Intravesical agents have been used for many years as adjuncts to oral treatment regimens or as second-line therapies for IC (70). On the contrary, intravesical therapy of urinary bladder cancer has been more widely used to accomplish multiple goals such as eradication of existing disease, prevention of recurrences and tumor progression (71). The incidence of local recurrence of superficial transitional cell carcinoma of the bladder is reduced by intravesical adjuvant therapy after transurethral resection (TUR) (72). Intravesical therapy consists of drugs being placed directly into the bladder through a urethral catheter.

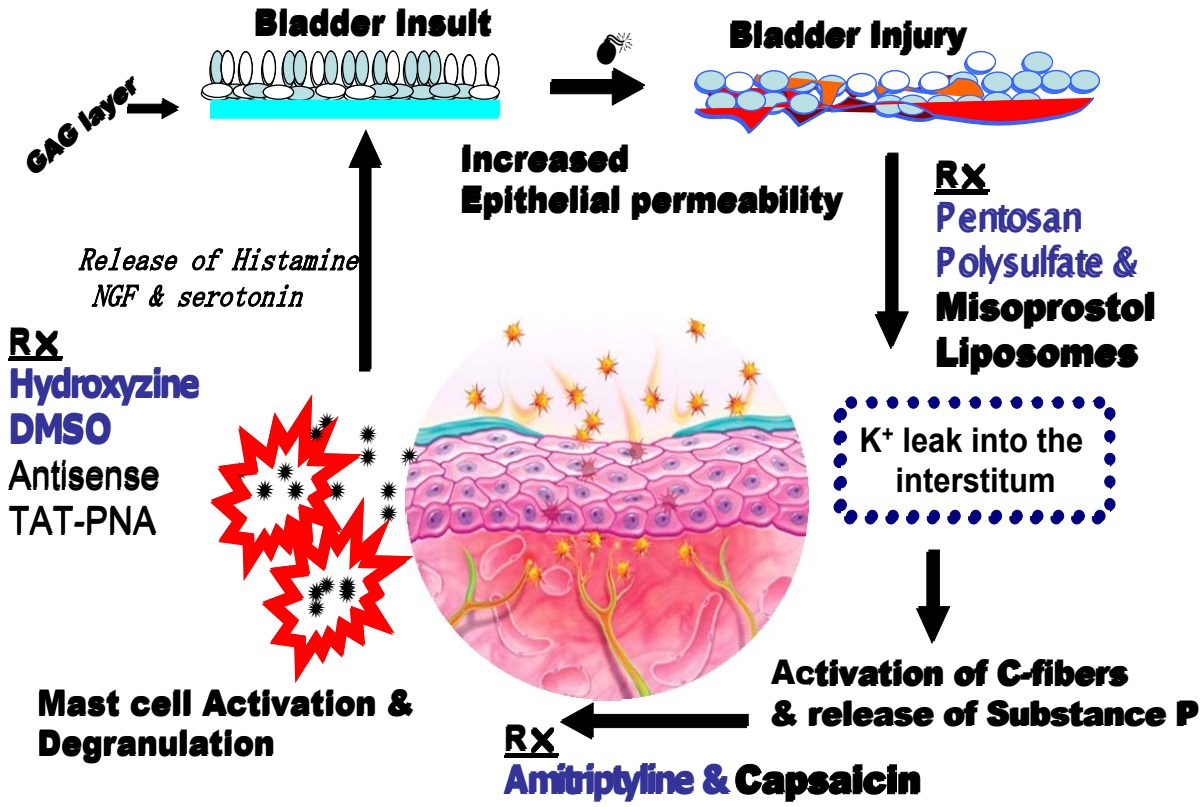


Figure 1.1: The role of multipronged therapy of IC in relation to the multifactorial etiology of IC.

The current approved therapies and the therapies studied in this dissertation are shown with respect to their mechanism of action.

High local drug concentrations in the bladder can be achieved with low risk of systemic side effects. The problem of low levels of drugs being excreted into the urine in its active form can also be eliminated with intravesical administration as illustrated by misoprostol. The first report on intravesicular pharmacotherapy of IC appeared in 1967 with the instillation of DMSO, dimethyl sulphoxide into the bladder (73). DMSO was approved by FDA, as 50% solution (Rimso-50) for intravesical treatment of IC in 1978 (74). DMSO is an amphipathic molecule with a highly polar domain and two apolar methyl groups. Its mechanism of action in IC has not been fully elucidated, and symptomatic relief in about two-thirds of patients probably occurs

through its anti-inflammatory and mast cell stabilizing property (75). DMSO can disrupt hydrogen bonds, affect intercellular electrical uncoupler and scavenge hydroxyl radicals which is believed to be an important trigger of inflammatory process (76, 77). In controlled crossover trial done on 33 patients with biopsies suggestive of interstitial cystitis, DMSO proved superior to placebo in the objective and subjective improvement (78).

DMSO has now become a standard treatment for intravesical therapy for IC (70). Administration can be done either in the clinic or at home by patients capable of self-catheterization and it is generally administered weekly for at least 6 weeks (79). DMSO can stimulate bladder afferent pathways and induce NO release from afferent neurons which may be involved in desensitization of nociceptive pathways (80). A recent study done on isolated rat bladder strips revealed that a 50% aqueous solution of DMSO can adversely affect muscle contractility and decrease its compliance (81). Notwithstading the multiple effects of DMSO, its treatment is associated with a low frequency of serious adverse effects with only rare cases of systemic contact dermatitis and eosinophillic cystitis being reported (82, 83).

Intravesical instillation of Bacillus Calmette-Guerin (BCG) is an established therapy for recurrent superficial (papillary) bladder carcinoma and carcinoma *in situ* (84). BCG instillation can delay tumor progression, decrease the need for subsequent cystectomy and overall survival rate in cancer patients is improved (85). The mechanism of action is still unclear, although a greater rate of cell turnover has been postulated. BCG triggers a variety of local immune responses that appear to correlate with antitumor activity (86, 87). The immunmodulatory activity of BCG prompted its evaluation for immunotherapy of IC and it showed a favorable outcome in refractory IC patients (88). Later, a randomized, double-blind, placebo controlled study done on 30 patients showed a 60% response rate in patients receiving 6 weekly instillations

of Tice strain BCG against a 27% in placebo and adverse events in both groups were mostly similar (89, 90).

BCG instillation is now considered an alternative option for symptomatic treatment of IC (91). The BCG therapy is thought to modulate urothelial immune responses and downregulation of an interleukin-6 driven type 2 T helper cell response could be the mechanism of BCG action in IC (92). Intravesical BCG immunotherapy can lead to a host of adverse effects such as malaise, low-grade fever, cystitis, and hematuria in generally up to 5% of patients (93, 94). Recently, BCG instillation was reported to have caused a rare severe complication of arthralgia and arthritis (95). A prospective double-blind study was conducted to compare the benefits from weekly instillation of either DMSO or BCG in IC patients (96). Maximal functional capacity of IC patients was not changed by either treatment, but DMSO was better than BCG in reducing pain and urinary frequency of patients with classic IC and similar reduction was absent in patients instilled with BCG (96).

Although DMSO is the principle intravesical agent approved by FDA, numerous other agents have been used. Intravesical sodium hyaluronate has been used to treat interstitial cystitis due to its possible replenishment of bladder glycosaminoglycans (97, 98). Hyaluronic acid (HA) is an important component in the urothelium. It inhibits adherence of immune complexes to polymorphonuclear cells, leukocyte migration and aggregation. Hyaluronic acid binds to lymphocytes and endothelial cells expressing ICAM-1 and it presumably alleviates the inflammatory processes by blocking the ICAM-1 receptors, but repeated instillations are needed to maintain the response (34). In a recent open label study, 18 patients with classic features of IC were instilled with 0.2% chondroitin sulfate (40 ml), once a week for four weeks and then once a month for 12 months (99). Patients who were positive for potassium sensitivity test reported

benefit from the treatment. Similar results were obtained following HA instillations in a recent study done on 48 patients having positive 0.4 M potassium sensitivity test (100).

Heparin is another GAG analogue effective in approximately 50% of patients following its instillation (101). As with the oral heparin analogues, intravesical heparin may also take several months to produce symptomatic relief. In a recent study done on mice, the expression of inflammatory cytokines expressed by inflamed bladder following LPS instillation was blocked by a novel synthetic peptide RDP58 (NH2-arg-norleucine (nle)-nle-arg-nle-nle-nle-gly-tyr-CONH2) (102). The peptide blocked the early signal transduction pathways involved in expression of inflammatory cytokines.

Intravesical treatment of particularly severe or long-standing cases of IC requires addressing the significant upregulation of afferents in the bladder of patients (103). C-fiber afferents involved in aberrant micturition reflex of IC are believed to be silent under normal conditions, but are activated after bladder irritation and spinal cord injury (104, 105). Downregulation of sensory nerves by using neurotoxin such as capsaicin or resiniferatoxin RTX has proven itself a viable approach in urology (48., 106). Successful treatment of neurogenic incontinence with intravesical capsaicin or its ultrapotent non pungent analog, RTX (40, 107) has revealed the role of capsaicin-sensitive C-fibers in the triggering of bladder hyperactivity and bladder pain (108). Increased suburothelial nerve density seen in the bladder of such patients can be reversed by successful treatment with intravesical vanilloids (42, 43). However, aqueous insolubility of these neurotoxins necessitates the use of ethanol as a cosolvent with saline for the instillation of these drugs in the treatment of IC. A recent study demonstrated the superiority of nonalcoholic solvents for capsaicin when compared to RTX delivered in alcohol (109).

1.2.3. Improvements in Intravesical Therapy

The response of intravesical therapy in bladder cancer and cystitis is often incomplete and variable among patients from conventional formulations typically maintained in the bladder for only a short duration (*i.e.*, 2 hours). Variable and incomplete response may be partly explained by resistant drug target and partly by unsuccessful drug delivery to diseased tissue. Besides poor penetration of drug through urothelium, inadequate intravesical drug delivery may also arise from the immediate dilution of drug concentration by residual urine in the bladder, in addition to subsequent sustained dilution by constant urine production during the 2-hour treatment. Effect of physiologic variable of urine on intravesical therapy can be reduced by complete bladder emptying just before dose administration and rate of urine production can be reduced by restricting fluid intake (110).

Attempts to overcome inherent drawbacks of intravesical instillation has been reported from various labs by using the approach of a slow and sustained release of drugs using various methods has been reported from various labs. Prolonged intravesical instillation of RTX was recently demonstrated to be a feasible procedure for treating IC patients (111). RTX was infused through sovrapubic 5Fr mono Pigtail catheter for 10 days at the flow rate 25µl/h with the help of infusion pump. Patients were evaluated after 30 days from the end of infusion (primary end point, PEP) and after three months (secondary end point, SEP). A 30% decrease in frequency and a 3 fold reduction of nocturia with significant reduction of symptoms of pelvic pain for at least six months after the end of infusion were observed. Similar approach has been previously applied for local therapy with prostaglandins in the treatment of cyclophosphamide-induced cystitis in patients (112-114). A 100 ml irrigation of 5 µg/ml PGE2 into the bladder for 3h completely freed the 4 year old patient of all the symptoms within 24 hours (115).

Intravesical therapy can also be improved by helping drugs cross the permeability barrier of urothelium. However, access to urothelium is limited by a layer of glycosaminoglycans covalently attached to cell membrane proteins (116). In a recent phase III trial enhanced penetration of mitomycin C across the bladder urothelium nearly doubled the recurrence-free rate in superficial bladder cancer patients (117).Weekly instillations of drugs were given for 6 weeks and drug concentration in urine demonstrated a linear relationship with its penetration into bladder tissue (118). Increased concentration in urine can improve the efficacy of drug by acting in the bladder without significant enhancement in toxicity (110). A novel delivery system for administration of mitomycin for immediate and safe delivery after tumor resection has been recently reported (119). Intravesical electromotive administration of mitomycin increased its bladder uptake, and improved response rate in high risk superficial bladder cancer (120). DMSO has been previously used to enhance the penetration of paclitaxel from cremophor micelles across the swine urothelium (121). Iontophoresis has also been recently used for intravesical delivery of drugs for the treatment of IC (122, 123).

Recently, certain peptides called "cell penetrating peptides" (CPP) or "protein transduction domains" (PTD) have been shown to possess the ability to translocate across the plasma membrane (124, 125). However these peptides lack the ability to be cell selective in their ability and are therefore a poor choice for drug targeting (126). We explored the potential of using shortlength TAT peptide derived from human immunodeficiency virus for regional therapy of large molecular weight drugs known as peptide nucleic acid (PNA) following intravesical administration. Peptide nucleic acids (PNAs) have been used for their antisense effect in various studies, because they form stable duplexes with the target mRNA and arrest translation (127, 128). PNA was chosen in this study owing to its superior binding properties, and higher stability

in biological media over a wide pH range, compared to traditional oligos and ribozymes (129, 130). Antisense effect of PNA against βNGF delivered by coupling with TAT peptide using solid phase synthesis was investigated as a treatment option for cystitis.

Various labs have reported application of bioadhesion for improving intravesical drug delivery. Controlled release of the paclitaxel at the urothelium/urine interface of mouse bladder was achieved following intravesical administration of poly(methylidene malonate-2.1.2) bioadhesive microspheres (131). Spherical 5 μm thick microspheres adhered to the mouse urothelium for upto 2 days after instillation and mice having bladder cancer survived for significantly longer time following instillation of bioadhesive microspheres loaded with 5% w/w paclitaxel compared to similar doses of the conventional paclitaxel formulation. The microspheres survived in the mouse bladder through 12 to 15 cycles of bladder filling/emptying (132).

In another study employing similar approach, a fibrinogen-based bioadhesive loaded with 5-fluoruracil was used for preventing tumor recurrences in resected tumor beds of mouse bladder (133). Storage-phosphor autoradiography was used to quantify drug retention in the bladder after administration. In a recent study, magnetic resonance imaging MRI was used for temporal and spatial monitoring of bioadhesive polymeric microparticles following intravesical delivery into mouse (134). The polymeric microparticles encapsulated with MRI contrast agent gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) for measuring T1 relaxation rate of particles untill 5 days after instillation. Mitomycin-C loaded alginate and chitosan bioadhsive carriers were recently evaluated in mice for the post-operative chemotherapy in bladder cancer (135). A gelatin based sustained drug delivery system was able to release adriamycin for 12 days in rabbit bladder (136). Retention of doxorubin in dog bladder following instillation was

increased by instilling microparticles called as magnetic targeted carriers (MTC) composed of metallic iron and doxorubicin adsorbed onto activated carbon. Externally applied magnetic field was used to achieve extended retention of magnetic targeted carriers following instillation (137). Isolated porcine urinary bladder was used to evaluate mucoadhesive intravesical drug delivery using polymers such as chitosan and polycarbophil for a hydrophilic drug. Drug distribution in to the bladder wall was determined by sectioning the frozen bladder and extracting the drug from tissue slices for analysis (138).

A mucosal adhesion substance, hydroxypropylcellulose (HPC), was added to the oxybutynin chloride solution (5%w/w) to improve intravesical delivery of oxybutinin in patients of overactive bladder (139). The mucoadhesive solution was instilled twice daily via the catheter used for bladder emptying at a dosage of 0.5 mg/ml. CMG was performed on patients before starting treatment and at 1 week and 3 years after the first instillation of oxybutynin. A significant increase in bladder capacity was observed in 4 of 6 patients. Intravesical delivery of oxybutinin is suitable for patients who suffer from side effects of its metabolite N-desethyloxybutynin (DEOB) following oral administration. This intravesical oxybutynin therapy is thought to depend on three mechanisms that prevent or improve urge incontinence: a direct effect on the bladder muscle, a topical anesthetic effect, and the indirect effect of absorbed oxybutynin and its metabolites (83).

Aqueous solutions of poly(ethylene glycol-b-[DL-lactic acid-co-glycolic acid]-b-ethylene glycol) (PEG-PLGA-PEG) triblock copolymers form a free-flowing sol at room temperature and become a gel at body temperature of 37°C (140). Thermosensitive hydrogel formed by these polymers have been used for *in situ* gel formation of drug depot of hydrophobic and hydrophilic drugs following subcutaneous administration in rats (141). The triblock copolymer was used for

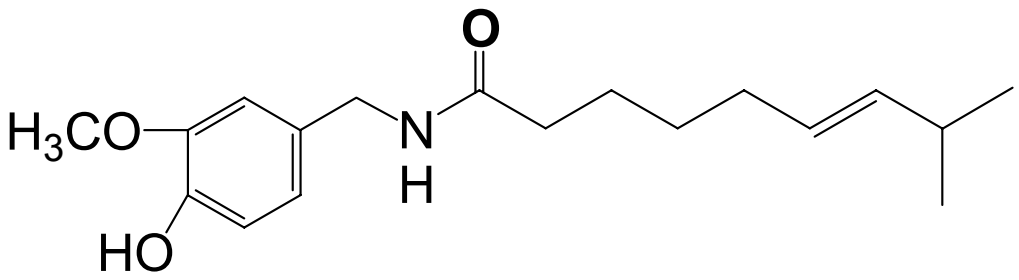
sustaining the residence time of hydrophobic drugs in the urine by instilling aqueous solution of polymer at room temperature.

Bladder cancer cell lines were used initially to study the intracellular delivery of anticancer drugs and biologics using liposomes (142, 143). Use of multilamellar liposomes proved favorable in cell culture studies and the antiproliferative capacity of IFN-α in resistant bladder cancer cell line was improved with liposomes (144). Instillation of liposome encapsulated radiolabeled IFN-α or radiolabeled liposomes into mouse bladder was able to achieve localized therapy with negligible penetration to other organs (145). Recently, liposomes have been used for intravesical gene therapy; intravesical instillation of murine interleukin-2 (IL-2) gene plasmid with cytofectins, dimyristoyl rosenthal inhibitor ether DMRIE and dioleoylphosphatidylethanolamine (DOPE) was used to treat 3 day-old pre-established orthotopic bladder tumors (146). In a recent study, plasmid DNA for IFN-α and GM-CSF was transfected into implanted tumors in mouse bladder with cationic liposome, N-[1-(2,3-dioleoyloxyl)propyl]-N,N,N-trimethylammoniummethyl sulfate and methyl-beta-cyclodextrin-solubilized cholesterol (147). Liposomes have been used to deliver hydrophobic drugs for cancer treatmemt. Capsaicin is also a hydrophobic drug and lipsomes were used in this dissertation as vehicles for its delivery into the bladder. A prior study from our lab has demonstrated therapeutic beneficial of instilling liposomes in the absence of any drug in a bladder injury model. In the current dissertation project the requirements of charge and structural elements required in the lipids for making biologically active liposomes in a bladder injury model were studied.

2. URODYNAMIC AND IMMUNOHISTOCHEMICAL EVALUATION OF INTRAVESICAL CAPSAICIN DELIVERY USING THERMOSENSITIVE HYDROGEL & LIPOSOMES

Capsaicin is the pungent chemical obtained from red pepper has been used in urology to treat voiding dysfunction and bladder pain (48, 106, 148). A derivative of homovanillic acid, capsaicin is 8-methyl-N-vanilyl-6-nonenamide, possessing an aromatic ring, amide bond and a hydrophobic side chain as seen in the chemical structure of capsaicin making it a highly hydrophobic molecule, insoluble in water (Fig.3.1). Therefore, for intravesical application, capsaicin is usually formulated in normal saline solution (NSS) using 30% ethanol (149). This vehicle has been reported to aggravate the adverse histological changes such as, epithelium thining and submucosal edema produced by capsaicin (150).

Thus, there is a need for better and safer vehicles for the water insoluble vanilloids. The undesirable use of ethanol to dissolve vanilloids, prompted the present investigation into the potential of hydrogel and liposomes as alternative vehicles. The hydrophobic nature of capsaicin makes it amenable for entrapment in the lipid bilayer of liposomes. The success of creams and gels as vehicles of capsaicin in topical treatment of peripheral neuropathy and herpes zoster neuralgia, guided us in considering hydrogel as an alternative vehicle for intravesical capsaicin (151). A polymer suitable for intravesical administration of capsaicin should be in the fluid state at the time of instillation and switch to the gel state after instillation. The triblock thermosensitive polymer PEG-PLGA-PEG selected for our experiments converts to gel owing to sol-gel transformation at body temperature (152). The polymer accomplishes the twin goals of semisolid consistency once inside the bladder and ability to be injected through a PE-50 catheter.



Capsaicin

Figure 2.1: Chemical structure of capsaicin having an aromatic ring, amide bond and a hydrophobic side chain

Pharmacological effect of capsaicin results from its action on vanilloid receptors VR-1, expressed on afferent nerves and urothelial cells (153). Activation of VR-1 produces a biphasic response, with initial stimulation driven by influx of Na^{+} and Ca^{2+} ion, and a delayed desensitization underlying the paradoxical analgesia produced by capsaicin (103, 154). C-fiber afferents involved in the micturition reflex are believed to be silent under normal conditions, but are activated after bladder irritation and spinal cord injury, to cause neurogenic detrusor overactivity (104, 105). Successful treatment of neurogenic incontinence with intravesical capsaicin or its ultrapotent non pungent analog, resinferatoxin (40, 107, 155) has revealed the role of capsaicin-sensitive C-fibers in the triggering of bladder hyperactivity and bladder pain (107, 108).

2.1. Methods

2.1.1. Preparation of formulations for instillations

2.1.1.1. *Preparation of liposome*:

Liposomes were prepared as previously reported from our lab (156). Phosphatidylcholine, cholesterol and capsaicin in 2:1:1 mole ratio were coated inside the glass tube and dried in vacuum overnight to form a thin film inside the tube. The lipids were hydrated with normal saline the next day to form multilamellar vesicles.

2.1.1.2. *Preparation of Hydrogel*:

Required amount of PEG-PLGA-PEG polymer was dispersed in 0.1 M phosphate buffer pH 7.4 to form a 30% w/v aqueous dispersion at room temperature. Aqueous dispersion of the polymer was prepared by constant vortex at room temperature. The prepared polymer dispersion was then added to the glass tube containing 1 mM capsaicin, with stock solution solvent ethanol previously removed by air drying.

2.1.1.3. *Preparation of Ethanolic Solution*:

Capsaicin was added from its stock solution to normal saline containing 30% ethanol to produce a 1mM of capsaicin solution.

2.1.2. Instillation of Formulations of Capsaicin:

1mM capsaicin entrapped in lipid bilayer of liposome, dispersed in the polymer or dissolved in ethanolic saline was instilled intravesically into the female Sprague-Dawley rats (200-300 g) (n= 8 for each group) under halothane anaesthesia (157). The volume for intravesical instillation was 0.5 ml for each formulation of capsaicin. Subsequent to instillation, the urethra was ligated to prevent evacuation and to allow enough time for gel formation in the bladder, the dwell time for all instillation was 30 min. Subsequently, bladders were emptied by pressing the lower abdomen and washed with 0.5 ml of saline. All the animals were also treated with antibiotic (Pen-strep, 30mg/kg, s.c.) to prevent infection.

2.1.3. Cystometry

Animals were anesthetized with urethane (1.2 g/kg s.c.) before transurethral cystometry, 48 h after intravesical instillation. PE50 tubing (Clay-Adams, Parsippany, NJ) was inserted into the bladder through the urethra. By means of a three way stopcock the catheter was connected to a pressure transducer for recording intravesical pressure and to a syringe pump for infusing

saline into the bladder. The catheter system was filled with 0.9% w/v saline. After the bladder was emptied, a cystometrogram (CMG) was performed by filling with a constant infusion (0.04 ml/min) of saline. The amplitude, pressure threshold and frequency of reflex bladder contractions per minute were recorded. Measurements in each animal represented the average of 3 to 5 bladder contractions.

2.1.4. Histopathological analysis

After cystometry, whole bladders were harvested, fixed in 10% buffered formalin, and cryopreserved. Tissue blocks were blind coded and sectioned (20 μm thickness) for haematoxylin and eosin (H& E) staining and immunohistochemistry of CGRP staining.

*Statistical analysis***:** Quantitative data are expressed throughout this paper as means plus or minus standard error. Multiple comparisons among the different groups were analyzed by a single-factor ANOVA, followed by post hoc comparisons with Newman Keuls test, according to the Graph Pad prism v. 3.0 (GraphPad Software, San Diego, CA). Differences among groups were considered significant at $p < 0.05$.

2.2. Results

2.2.1. Effects of intravesical capsaicin on cystometrogram

In the cystometric studies in rats under urethane anaesthesia, with 30% ethanol and liposome as vehicles, capsaicin was able to produce blockade of micturition reflex shown in tracings F and G of Fig. 3.2, respectively. Absence of periodic bladder contractions in the CMG represents blockade of micturition reflex following capsaicin treatment, and raised plateau in intravesical pressure reflect urinary retention. The mean bladder contraction frequency was

considered zero for such animals, which was observed in 6 and 4 rats of the capsaicin treated groups using 30% ethanol (NSS) and liposomes, respectively (n=8).

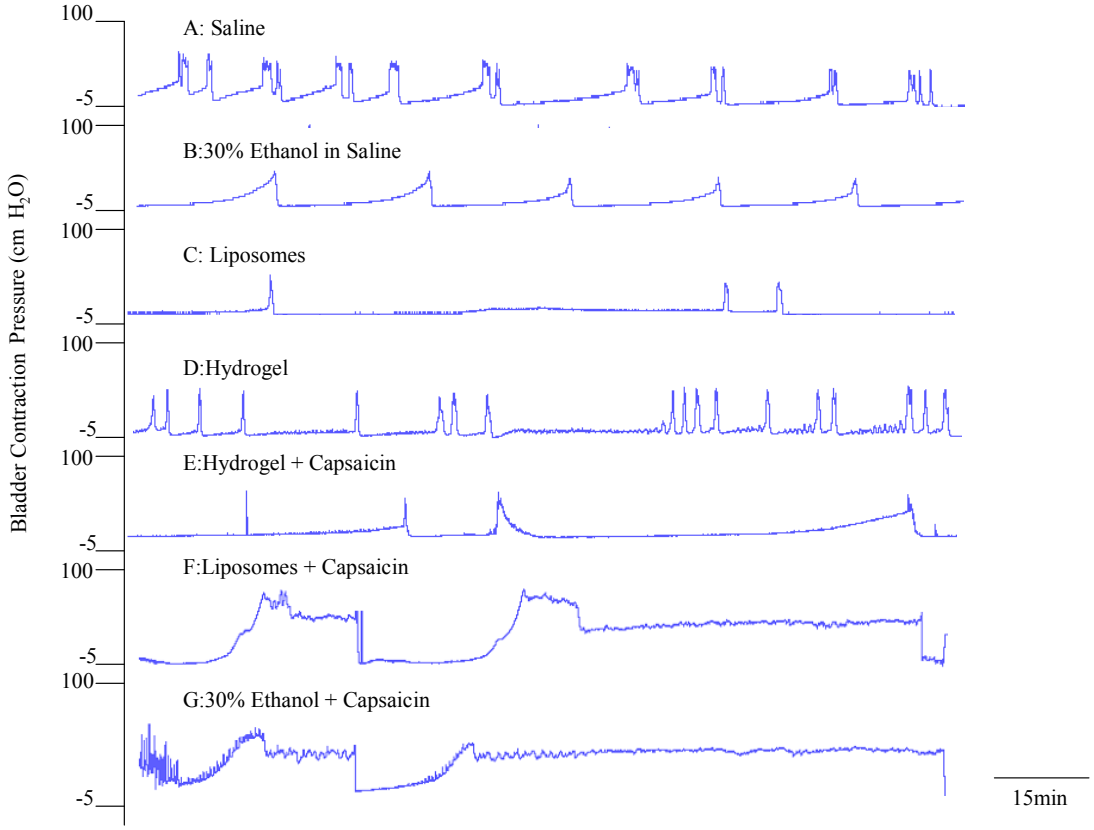


Figure 2.2: Representative tracings of CMG from various groups.

Starting from top tracing A is of normal saline treated rats, showing periodic micturition events under urethane anesthesia. Tracing B and C represents 30% ethanol and liposomes treated rats, respectively, in absence of capsaicin revealing the dissimilar effects of ethanol and liposomes on bladder afferents by decrease in bladder contraction frequency. Tracings D and E are hydrogel treated rats in absence and presence of capsaicin, respectively, revealing a decrease in bladder contraction frequency in presence of capsaicin. Tracings F and G are from rats treated with liposomes and 30% ethanol, respectively, in presence of capsaicin showing complete blockade of micturition reflex in both cases.

The remaining rats in capsaicin treated groups using ethanol (NSS) and liposomes showed decreased mean bladder contraction frequency with no significant difference between the two groups (0.01 ± 0.006 vs 0.01 ± 0.007, $p>0.05$). Representative tracing of CMG from various groups are shown in (fig. 2). Hydrogel in presence of capsaicin significantly reduced

mean bladder contraction frequency compared to hydrogel alone (0.10±0.021 vs 0.25±0.033), a similar significant difference of mean bladder contraction frequency was observed in ethanol (NSS) treated groups in presence and absence of capsaicin. Raised plateau of bladder contraction pressure reflects urinary retention. (0.01±0.00654 vs 0.12±0.021) (Fig.3.2). However, liposome treated groups failed to show significant difference in the mean bladder contraction frequency in presence and absence of capsaicin (0.01±0.007 vs 0.08±0.025).

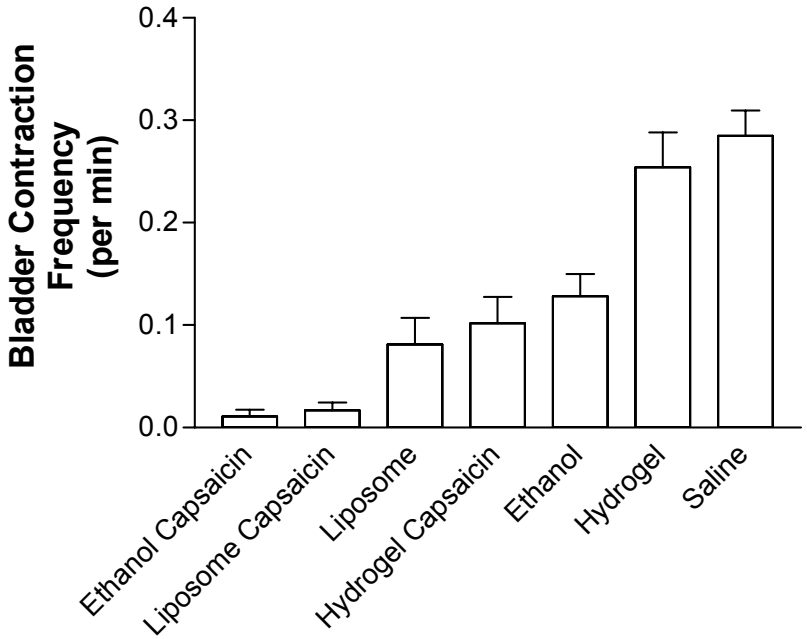


Figure 2.3: Rat bladder contraction frequency 48 h after administration of capsaicin in various vehicles.

All capsaicin treated groups showed significant difference from saline treated group ($p < 0.05$). Comparison of hydrogel treated group in absence of capsaicin with saline treatment; ethanol and liposome in presence of capsaicin; and liposome in presence and absence of capsaicin, respectively, was not significant ($p > 0.05$).

Mean bladder contraction frequency (0.25±0.033) of hydrogel treated rats in absence of capsaicin was lower but not significantly different from saline treated rats (0.28±0.02491) ($p>0.05$). Other CMG parameters such as pressure threshold and amplitude of bladder contractions were not affected by capsaicin treatment in various vehicles (data not shown). All capsaicin treated groups showed significant difference from saline treated group ($p < 0.05$).

Comparison of hydrogel treated group in absence of capsaicin with saline treatment; ethanol and liposome in presence of capsaicin; and liposome in presence and absence of capsaicin, respectively, was not significant ($p > 0.05$).

2.2.2. Gross bladder morphology:

None of the animals in our study, showed any signs of urinary tract infection after instillation of the intravesical solutions. However, as shown in (fig. 3.4), bladders treated with 30% ethanol showed sign of severe redness throughout the bladder tissue, which turned to bleeding within the walls and ulceration at the bladder lumen, in presence of capsaicin (Panel C &D), in marked contrast to untreated bladders (panel A). Only dilatation of blood vessels at the bladder dome was visible in other vehicle treated groups (Panel B, E and G) in absence of capsaicin, which become more prominent in presence of capsaicin (Panel F and H).

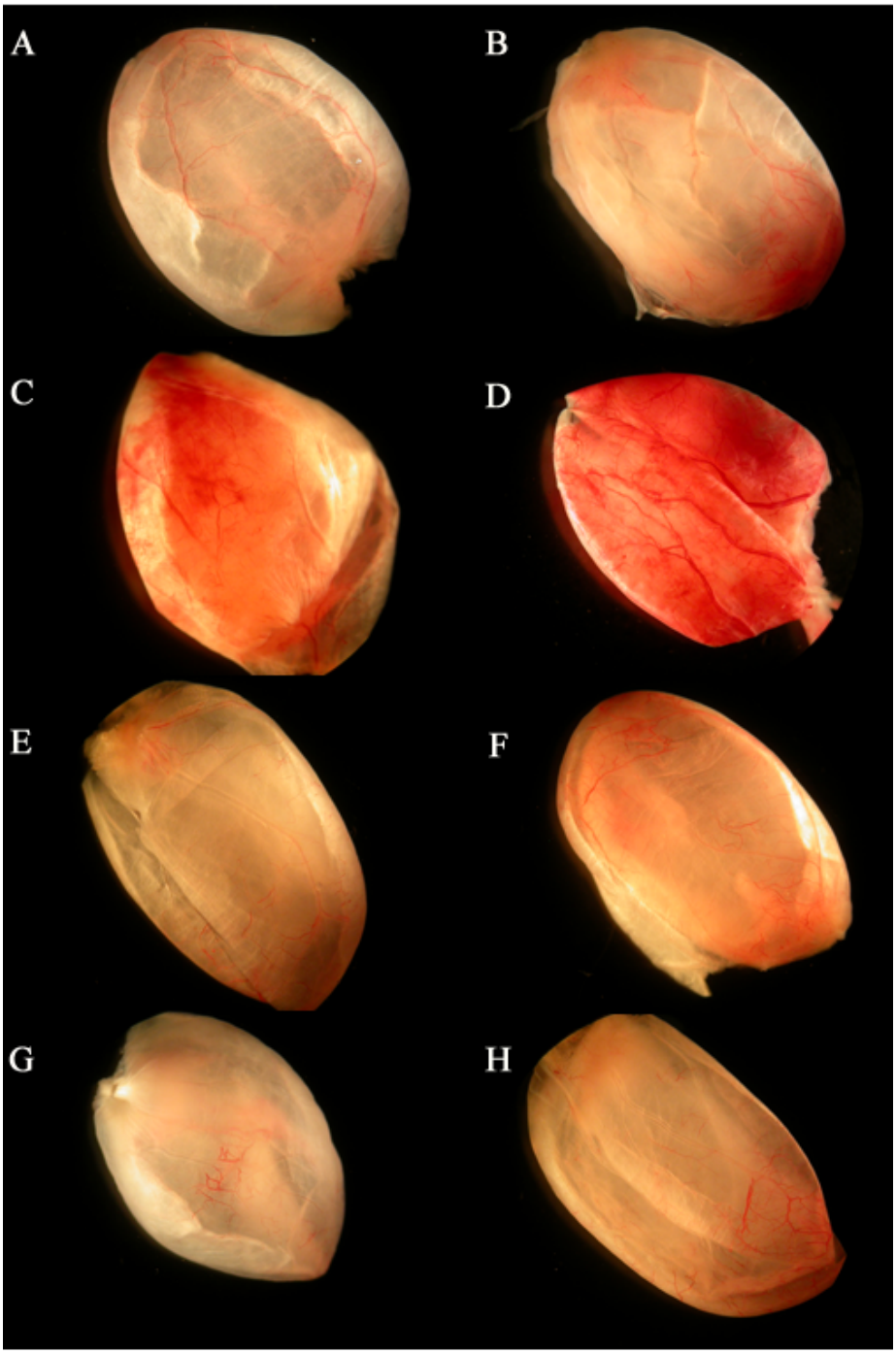


Figure 2.4: Gross bladder morphology of rat bladder 48 h after administration of capsaicin in various vehicles.

Panels a) and b) are of untreated and saline treated bladder, respectively. Panels c) and d) are of 30% ethanol in normal saline (NSS) treatment group in the absence and presence of 1mM capsaicin, respectively. Panels e) and f) are from liposome treated group in the absence and presence of 1mM capsaicin, respectively. Panels g) and h) are of hydrogel treated group in the absence and presence of 1mM capsaicin, respectively. Photographs of previously fixed bladders were taken through dissecting microscope at 1X magnification.

2.2.3. Immunohistochemistry

CGRP staining was performed to assess for depletion of CGRP by capsaicin. We found that capsaicin caused significant depletion of CGRP with both liposomes and ethanol as vehicles (Panel B and D of Fig.3.5). However, capsaicin in hydrogel failed to produce the depletion observed in liposome and ethanol treatment groups (Panel F). Primary rabbit polyclonal antibody bound to the CGRP containing nerve fibers is localized by Cyanine-3 fluorescently labeled secondary goat antibody.

2.2.4. Effect on bladder histology

Hydrogel and lipsosome without capsaicin demonstrated similar bladder mucosal histology (Panel C and E, respectively, of Fig.3. 6). Intravesical instillation of ethanol alone revealed distinct histological changes including, thinning and denuding of epithelium, submucosal edema and vascular congestion (Panel A). These changes were further aggravated by capsaicin and acute mucosal injury was visible in capsaicin with 30% ethanolic in normal saline treatment group (Panel B). On comparison, histological changes produced by capsaicin delivered using liposome and hydrogel appeared mild (Panel C to F, respectively), with urothelium remaining intact in presence of capsaicin.

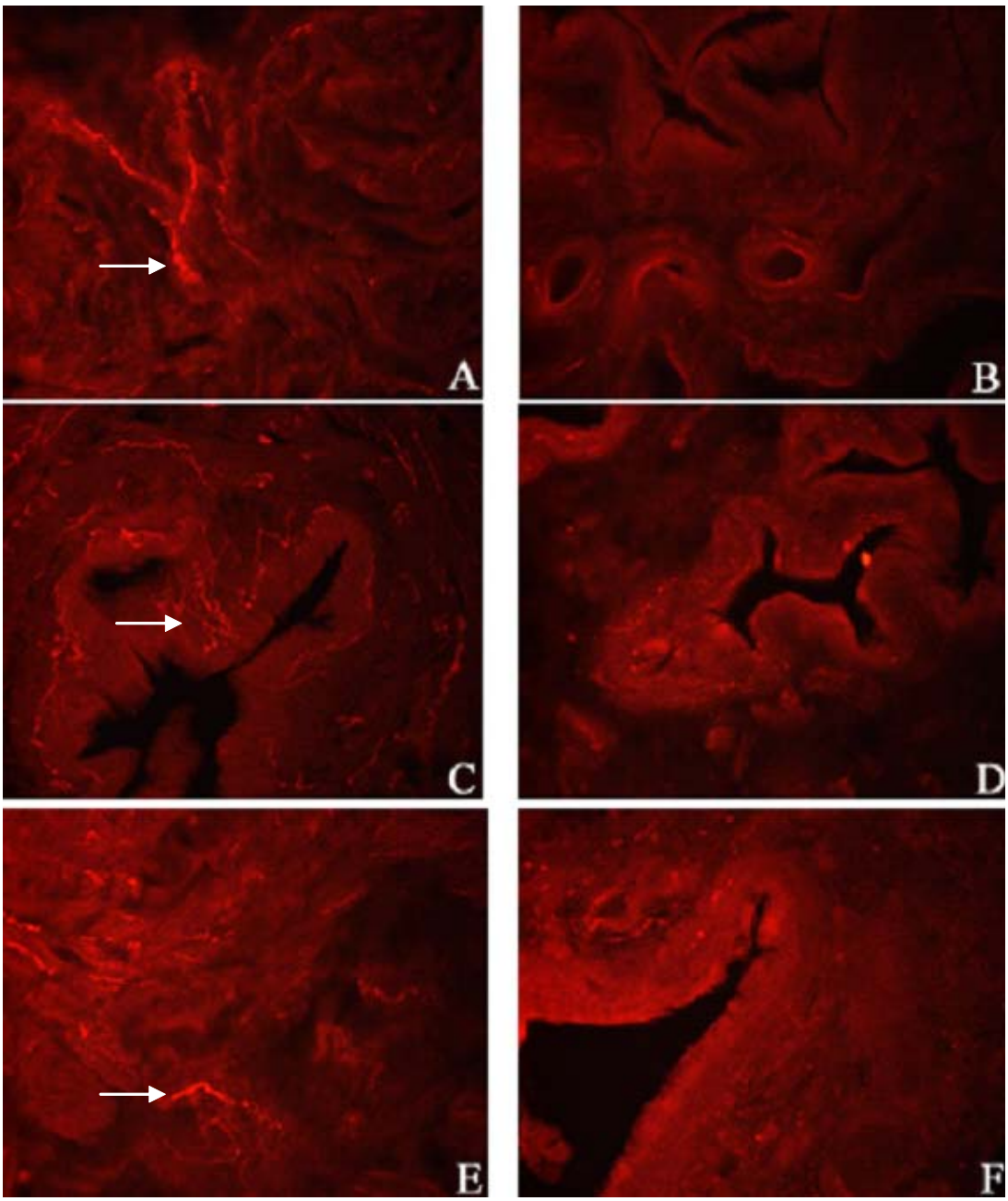


Figure 2.5: Photographs of CGRP staining in rat bladder 48 h after administration of capsaicin in various vehicles.

CGRP fibers are visible as bright red lines in the bladder photographs against a dull red tissue auto-fluorescence (marked by horizontal white arrows in the sections visible). Panel a) is control untreated rat bladder and panel b) is of 30% ethanol in normal saline (NSS) treatment group in presence of 1mM capsaicin. Panels c) and d) are from liposome treated groups in the absence and presence of 1 mM capsaicin, respectively. Panels e) and f) are of hydrogel treated group in the absence and presence of 1mM capsaicin, respectively. Magnification was 20X in all panels.

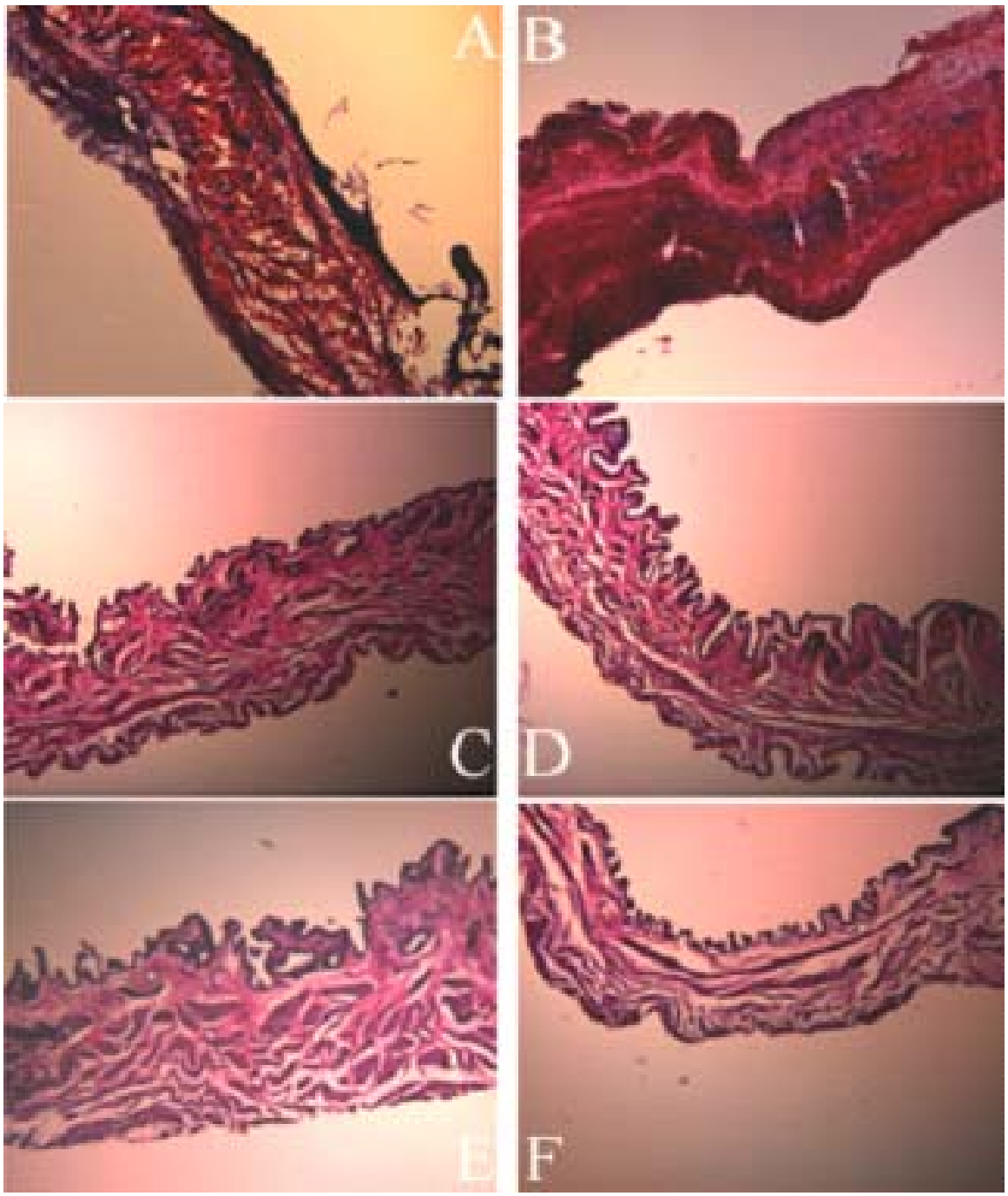


Figure 2.6: Photographs of H& E staining of rat bladder 48h after administration of capsaicin in various vehicles.

Lumen side of bladder is facing upwards in all photographs (marked by horizontal white arrow in all panels). Panels a) and b) are of 30% ethanol in normal saline (NSS) treatment group in the absence and presence of 1 mM capsaicin, respectively, showing partial to complete urothelial denudation and vascular congestion. Panels c) and d) are from liposome treated group in the absence and presence of 1 mM capsaicin, respectively, showing normal appearance of urothelium. Panels e) and f) are of hydrogel in the absence and presence of 1 mM capsaicin, respectively, revealing intact urothelium. Magnification was 10X in all panels.

2.3. Discussion

Traditional anticholinergic therapies of hyperactive bladder target the efferent branch of micturition reflex. Treatment on the afferent branch of micturition reflex by using C-fiber neurotoxin capsaicin is an attractive alternative that would avoid systemic anticholinergic side effects. The role of capsaicin-sensitive bladder afferents in micturition control and bladder irritation is collectively supported by both close apposition of VR-1-expressing fibers with bladder smooth muscle cells as well as urothelial expression of VR-1 (153, 158). Intravesical instillation of capsaicin and RTX for treatment of detrusor overactivity not only reduces the problem of systemic neurological toxicity, intrinsic property of neurotoxins (159), but also eliminates their significant first pass effect (160). The presently accepted vehicle for intravesical capsaicin is 30% ethanol in saline. Unfortunately, the ethanol vehicle alone was observed to be just as irritating to the bladder mucosa as capsaicin itself in a study done on spinal cord injured patients (161-163). The pain and autonomic dysreflexia reported by some patients during and sometimes after the instillations has hindered wider application of intravesical vanilloid's clinical use.

In the present study, normal adult female rats were used to investigate the efficacy of capsaicin entrapped in the lipid bilayer of liposomes or multilamellar vesicles (MLV) and in the hydrophobic matrix of thermosensitive hydrogel. Micturition in such rats with an intact neuraxis is dependent upon a spinobulbospinal reflex activated by Aδ bladder afferents and their employment as animal model for comparison of efficacy for capsaicin formulations under urethane anaesthesia is based on extensive information published by various laboratories. Maggi et al (1990) reported that the facilitatory action of capsaicin-sensitive nerves on micturition threshold is more evident in anesthetized than awake rats and that capsaicin-resistant bladder

afferents are more sensitive to the depressant action of urethane than the capsaicin-sensitive afferents (164). Electrophysiological experiments showed that micturition reflex triggered by bladder distension during CMG conducted under urethane-anesthesia requires activation of a supraspinal reflex pathway (165). A recent study by Chuang et al showed that afferent limb of the micturition reflex under urethane anaesthesia can be blocked by intravesical administration of RTX, another vanilloid drug (166).

Given this knowledge of micturition control of normal rats under urethane anaesthesia, we expected to see blockade of the micturition reflex, as a successful endpoint of capsaicin delivery from various vehicles. Moreover, by using normal rats, we can avoid the variable influence of disease on the uptake of capsaicin from the different delivery systems. Damaged urothelium is more permeable than normal urothelium and it is possible that pathological conditions will induce a variation in study designed to examine capsaicin uptake from various vehicles (167).

Aqueous solutions of poly(ethylene glycol)-b-poly (DL-lactic acid-co-glycolic acid)-b-poly(ethylene glycol) (PEG-PLGA-PEG) triblock copolymers form a free-flowing sol at room temperature which becomes a gel at body temperature (168). The amphiphilic nature of the polymer used in our study might be helpful in the permeation of drug substances through biological membranes. The slow erosion of this material in aqueous environments gives it a biodegradable and ultimately more biocompatible nature. We have chemically modified this polymer, to improve the robustness of the hydrogel to withstand urine constituents such as urea and electrolytes. We compared the effect of hydrogel and liposomes containing capsaicin with the usual vehicle (30% alcohol in saline) as a positive control in our study. The three vehicles, liposomes, hydrogel and 30% ethanolic saline were also tested in our study in absence of any

capsaicin. CMGs done after 48 h following intravesical instillation, demonstrated, that liposomal capsaicin was as successful as ethanolic capsaicin in blocking the micturition reflex in urethane anaesthesized normal rats.

In a previous study, a large dose of capsaicin (50 mg/kg s.c.) elicited an acute block of bladder activity that persisted for 8-15 h (169). The longer duration of capsaicin (48 h) effect reported in the present study might be explained by high local concentration of capsaicin following intravesical administration, which would prevent first pass effect. The prolonged desensitizing effect of capsaicin in our present work corroborates the earlier report documenting the initial excitation produced by capsaicin following its intravesical delivery using liposomes of similar composition. As previously reported from our laboratory (156), liposomes alone in absence of capsaicin were able to reduce the bladder contraction frequency in normal rats and in bladder injury model. Due to lack of any previous studies in the literature on the interaction of liposomes with urothelium surface, at this time we can only speculate on this observation in normal rats. Perhaps liposome treatment produces some alteration in the biophysical properties of urothelium and thereby affecting the afferent branch of micturition reflex. Compared to hydrogel and saline treated animals, 30 % ethanol (NSS) in absence of capsaicin reduced bladder contraction frequency as well as produce adverse histological changes in bladder. Possible damage to the afferents located near the urothelium by ethanol might explain both CMG and histological observations.

Hydrogel with capsaicin was effective in reducing the bladder contraction frequency compared to vehicle controls of hydrogel and liposomes alone, but failed to produce overflow continence, a feature observed, when capsaicin was delivered using 30% ethanol and liposomes (170). The difference seen with of capsaicin delivery in hydrogel treated animals may be related

to tight binding of capsaicin with hydrophobic chains of the polymer, rendering it unavailable for immediate effect. However, this study was done in normal bladders, and it would be interesting to see, how the biocompatible and emollient nature of liposomes and hydrogel affect capsaicin activity in a rat model of cystitis compared to 30% ethanol.

At the cellular level, capsaicin acts by releasing CGRP stored in afferent fibers (171) and it was reported to produce reduction in suburothelial nerve densities in the bladder of patients with detrusor hyper-reflexia. This may explain its prolonged beneficial effect in these patients (103). Capsaicin delivered in liposome and ethanol vehicles was able to reduce the CGRP staining in bladders removed following the completion of CMG studies. H&E staining on formalin fixed bladder sections revealed disruptive effect of existing ethanolic vehicle on urothelium even in absence of capsaicin. Our results agree with those reported previously (150) as well as explain the discomfort felt by the patients following intravesical administration in an ethanol vehicle.

Conclusions:

The findings of our study support that liposomes are a superior vehicle for intravesical administration of capsaicin than 30% ethanol in terms of safety and comparable in efficacy. The evidence gathered in favour liposomal formulation of capsaicin in this pre-clinical study argues for clinical translation of this work, which is yet to be determined. Hydrogel can be a safe alternative option for capsaicin delivery. The study failed to show any advantage of using hydrogel as a vehicle for capsaicin delivery. Thermosensitive hydrogel was further studied to demonstrate its utility as a vehicle for sustained intravesical drug delivery in chapter 4 of this dissertation. Next chapter 3 describes the study done to define the charge and structural elements required in liposomes for efficacy in hyperactive bladder.

3. CHARGE AND STRUCTURAL ELEMENTS REQUIRED IN LIPOSOMES FOR REDUCING BLADDER HYPERACTIVITY

Liposomes are vesicles composed of concentric lipid bilayers separated by aqueous compartments. They can be adsorbed on to cell surfaces and create a molecular film on cell surfaces, which prompted their use in promotion of wound healing (172, 173). Studies done on canine kidney cells showed that low permeability of epithelial cell membranes results from the low fluidity of the apical membrane of superficial cells (174). It can be argued that the special composition and structural packing of lipids present in apical membrane of umbrella cells is a key factor in urothelial barrier function (175, 176). Moreover, umbrella cells can further strengthen their permeability barrier against urine by establishing asymmetry in the lipid composition of its apical membrane also called asymmetrical unit membrane (AUM) with different lipids in its outer exoplasmic and inner cytoplasmic leaflets (177). Uroplakins may organize and rigidify the lipids in the outer leaflet and maintain the different composition of lipids in the two leaflets (178, 179). Phospholipid flippases can restrict intermingling of lipids assigned to the two leaflets by trans-orienting phospholipids in an energy-dependent process from one leaflet to the other (180). Tight junctions present in urothelium can also assist in establishing this bilayer asymmetry by helping the segregation of exofacial and cytoplasmic leaflet of the apical membrane in addition to maintaining epithelial polarity by segregating basolateral membrane from apical membranes (181, 182).

The surface area of the apical plasma membrane is much smaller than that of the basolateral membrane and apical membrane of umbrella cells is proposed to have different lipid composition in its two leaflets the based on studies done on apical membrane of canine kidney cells (183). The outer leaflet seems to have glycosphingolipid GSL (such as cerebrosides),

sphingomyelin (SPM), phosphatidylcholine (PC), and cholesterol whereas the inner cytoplasmic leaflet has phosphatidylserine and phosphatidylethanolamine (11, 184-186). The proposed asymmetric lipid composition agrees with earlier reports showing preponderance of choline-containing lipids in the exofacial leaflet and localization of amino-containing phospholipids at the cytoplasmic side (187). The importance of bilayer asymmetry in apical membrane of epithelial cells such as gastric apical vesicles has been demonstrated and changes in the lipid composition of artificial apical membrane markedly increased the water, proton, and nonelectrolyte permeabilities (188). A similar study done on liposomes having lipid composition derived from the exofacial leaflet of apical membrane in dog kidney cell also showed an increased permeability towards solutes following removal of SPM and GSLs from the lipid composition of liposomes (184).

Disruption in the permeability barrier of urinary bladder has been one of the accepted theories to explain the pathogenesis of IC (23). Previously, our lab has reported the development of an effective rat model of bladder injury which can simulate the condition of breached bladder permeability barrier observed in IC patients (189, 190). This reproducible method involves inducing selective damage to the upper layers of the urothelium or umbrella cells by infusion of protamine sulfate PS followed by potassium chloride to exhibit bladder hyperactivity in cystometrogram (CMG) of bladder, a prominent symptom of IC. It has been shown that PS can induce nonselective pores in the apical membrane, leading to irreparable cell damage in rabbit bladder (191, 192). PS is a cationic peptide found in the sperms of animals, which can interact with anionic charged layer of surface glycosaminoglycans found on urothelial cells or specific lipids present in the apical, but not in its basolateral, membrane (10, 193, 194). The induced damage following intravesical instillation can be restricted to only the umbrella cell layer by

optimizing the concentration of protamine sulfate and its duration of infusion into the bladder (195).

A dysfunctional urothelium can allow the transepithelial migration of solutes such as potassium, which can depolarize subepithelial afferent nerves and can initiate and provoke inflammation (11). The resultant increased afferent excitability and tissue irritation can lead to urinary frequency and urgency so as to reduce the effect of urine on the bladder wall (11, 196). The permeability barrier of bladder has been demonstrated to be disturbed in a feline model of IC and a majority of IC patients (22, 194). In a previous report from our lab, liposomes formulated with egg PC and cholesterol in 2:1 molar ratio were effective in reducing bladder hyperactivity in protamine sulfate bladder injury model (156). Liposomes of similar composition were used as vehicles for delivery of capsaicin in uninjured bladders, in the study described in chapter 2 of this dissertation project. As discussed earlier liposomes in absence of capsaicin were able to decrease the bladder contraction frequency of rats under urethane anesthesia (197).

The present study was prompted to identify the bioactive component in egg PC that is responsible for reducing bladder hyper-activity, as the egg PC used in our earlier study was only 60% pure. Experiments were designed to elucidate the charge and structural elements necessary in the lipids for affecting the physiological response of bladder. We evaluated the effect of liposomes prepared from various pure natural and synthetic lipids in the bladder injury model. The effect of removing cholesterol in the liposomal formulation was also studied by assessing the effect on CMG. Synthetic lipids having acyl chain lengths similar to natural lipids but varying in degree of saturation and charge on the headgroup were also tested for efficacy in the bladder injury model (Fig.3.1 & 3.2).

3.1. Methods

3.1.1. Preparation of Liposomes

L-α-phosphatidylcholine (egg), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (OSPC), sphingomyelin, sphingosine and ceramide were obtained from Avanti Polar Lipids and dihydrosphingomyelin was obtained from bio-labs, Israel.

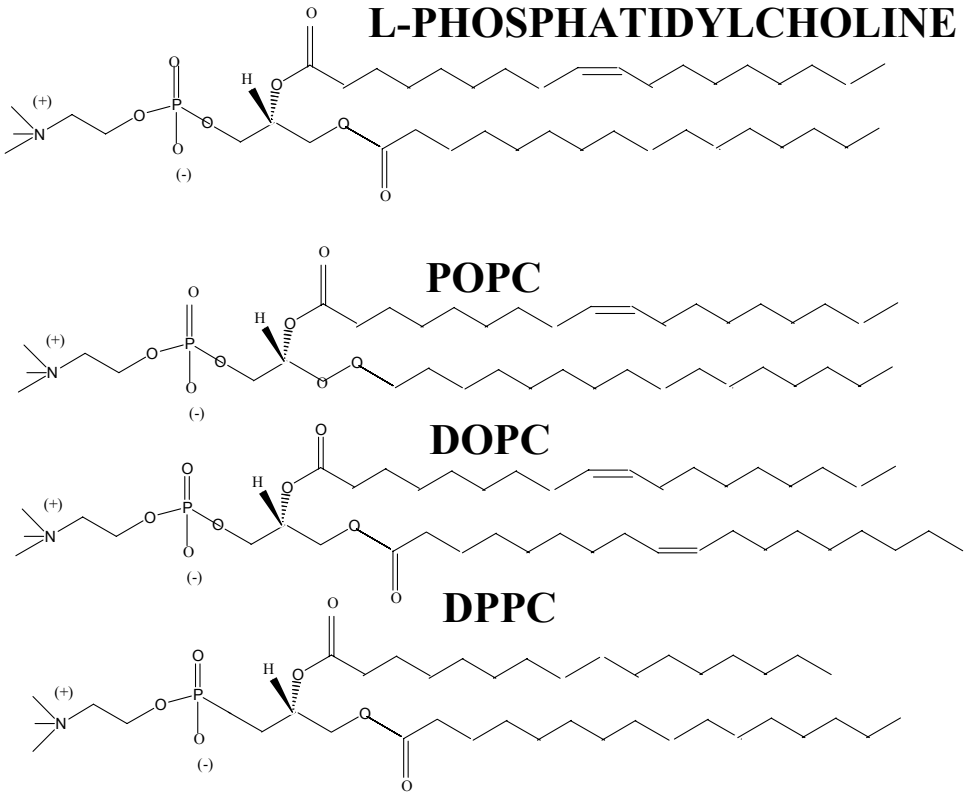


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Figure 3.1:Pure natural lipid egg PC and its synthetic analogues varying in acyl chain length and saturation.

L-α-Phosphatidylcholine(egg), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)

Liposomes were prepared by adding the lipids in chloroform around the round bottom flask, solvent was removed under a stream of nitrogen and then lipids were dessicated under vacuum for 4h. Dried lipids were hydrated with saline containing 500 mM KCl at the temperature above their respective transition temperatures by sonication for ~5 min. Liposomes were prepared at a final lipid concentration of 2.6mM (2 mg/ml).

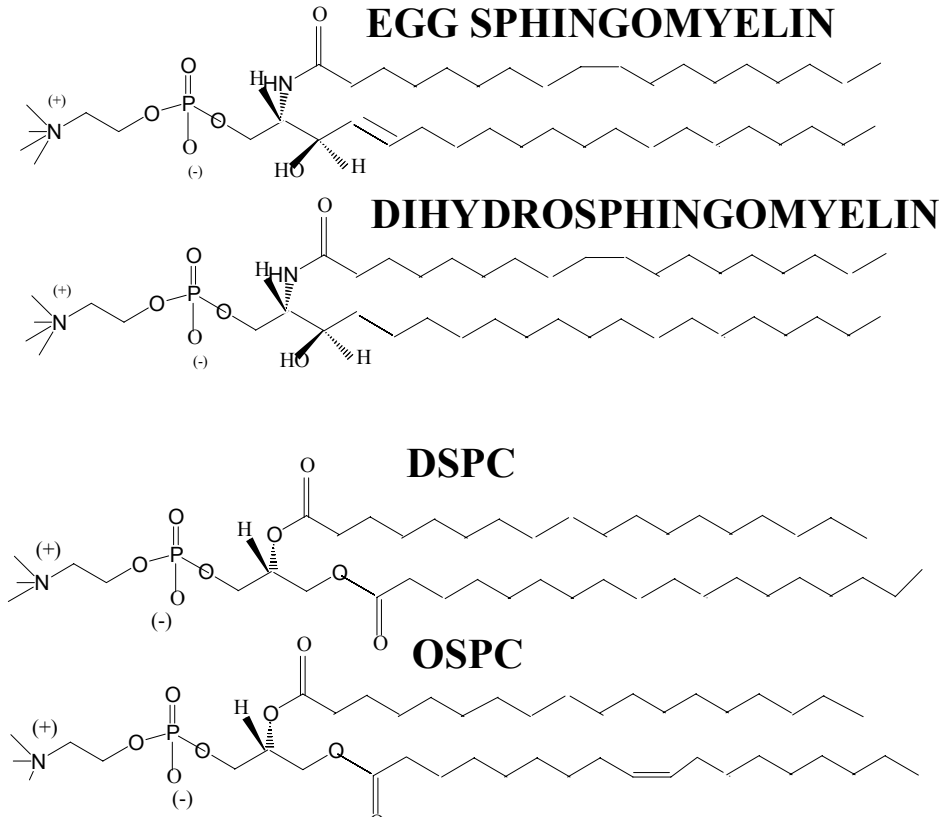

Figure 3.2: Natural lipid egg sphingomyelin, dihydrosphingomyelin and pure synthetic lipids having one acyl chain derived from stearic acid.

1,2-distearoyl-sn-glycero-3-phosphocholine(DSPC),1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (OSPC).

3.1.2. Cystometry and drug application.

Bladder reflex activity of Sprague-Dawley rats (200-250 g) was studied by cystometry performed under urethane anesthesia (1.2 g/kg, s.c.). Their body temperature was maintained in the physiologic range using a heating lamp. A transurethral bladder catheter (PE-50) connected

by a three-way stopcock to a pressure transducer and to a syringe pump was used to record intravesical pressure and to infuse solutions into the bladder. A control cystometrogram (CMG) was performed by slowly filling the bladder with saline (0.04 ml/min) to elicit repetitive voiding. The bladder contraction frequency of the reflex bladder contractions was recorded.

3.1.3. Induction of a hyperactive bladder

After performing control CMGs with saline infusion for 3h, PS (Sigma Chemical; 10 mg/ml) was infused at rate of (0.04 ml/min) for 1h to increase epithelial permeability followed by infusion of KCl (500 mM) for 1 h. Subsequently liposomes prepared in KCl were infused for 2 hours. The KCl concentration was within the range of concentrations present in normal rat urine (198).

3.1.4. C-*fos* Immunohistochemistry

Activation of nociceptive primary sensory neurons resulting in pain-related behavior in animals evokes enhanced c-*fos* expression in the spinal cord (199). Rats were perfused through the ascending aorta with 400 ml of 0.9% w/v saline followed by 400 ml of 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. The L6 spinal segments were removed and post-fixed for 12h into the same fixative at 4°C followed by immersion overnight in 30% sucrose in phosphate buffer. Spinal segments were cut by a freezing microtome into 40 µm transverse sections. After sectioning of spinal cords from all the animals, sections were washed with PBS and incubated in the same bath with a polyclonal antiserum against the c-fos protein raised in rabbit (diluted at 1:15000; Santa Cruz Biotechnology) for 72h. The immunoreaction was visualized by the ABC-HRP method (Vector Elite kit; Vector Laboratories, Burlingame, CA). Spinal c-*fos* expression

was analyzed by calculating the mean mean number of fos-immunoreactive cells found in the appropriate four animals.

3.1.5. Statistical analysis

Quantitative data are expressed as means plus or minus standard error. Multiple comparisons among the different groups were analyzed by a single-factor ANOVA, followed by post hoc comparisons with Newman Keuls test, according to the Graph Pad prism v. 3.0 (GraphPad Software, San Diego, CA). Differences among groups were considered significant at $p < 0.05$.

3.2. Results

3.2.1. Effect of Charge on the Headgroup

We first determined the effect of charge carried by lipids in reducing bladder activity.

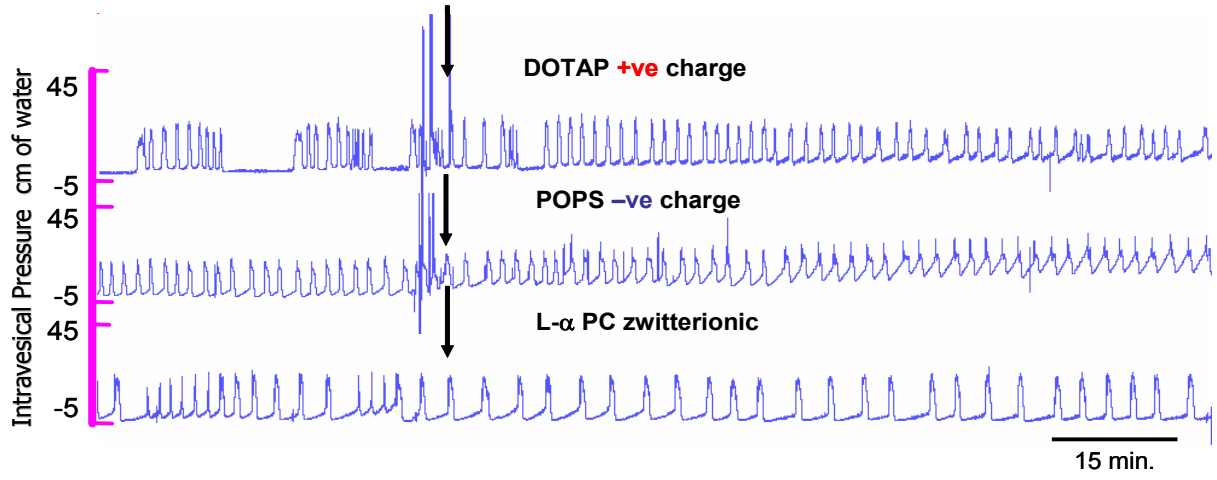

Figure 3.3: Effect of Charge carried on lipid headgroup in reducing bladder hyperactivity.

The black arrow marks the start of infusion of liposomes in presence of the irritant 500 mM KCl. Zwitterionic lipid egg PC were significantly better than lipids having negative or positive charge in their headgroup, L-α-phosphatidylserine (POPS) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP).

Liposomes prepared with zwitterionic lipid egg PC were compared against lipids having positive or negative charged polar headgroups. 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) and L-α-phosphatidylserine (POPS) represented the positively and negatively charged lipid, respectively. Liposomes made from lipids with phosphaditylcholine (PC) head group were able to suppress chemically induced bladder hyperactivity to a significantly greater extent than lipids having cationic or anionic charge such as DOTAP and phosphatidylserine, i.e. POPS (Fig.3.3). L-□- PC was able to produce a three fold higher (33 + 5.3%) reduction in the bladder contraction frequency over its pretreatment control compared to DOTAP (10 $\pm$ 0.09%) and POPS (5 $\pm$ 0.02%), number of rat in each group was 5 ($p<0.01$)

3.2.2. Effect of Cholesterol on Bladder activity:

Lipsomes prepared from pure natural lipids by exclusion of cholesterol were able to reduce bladder hyper activity.

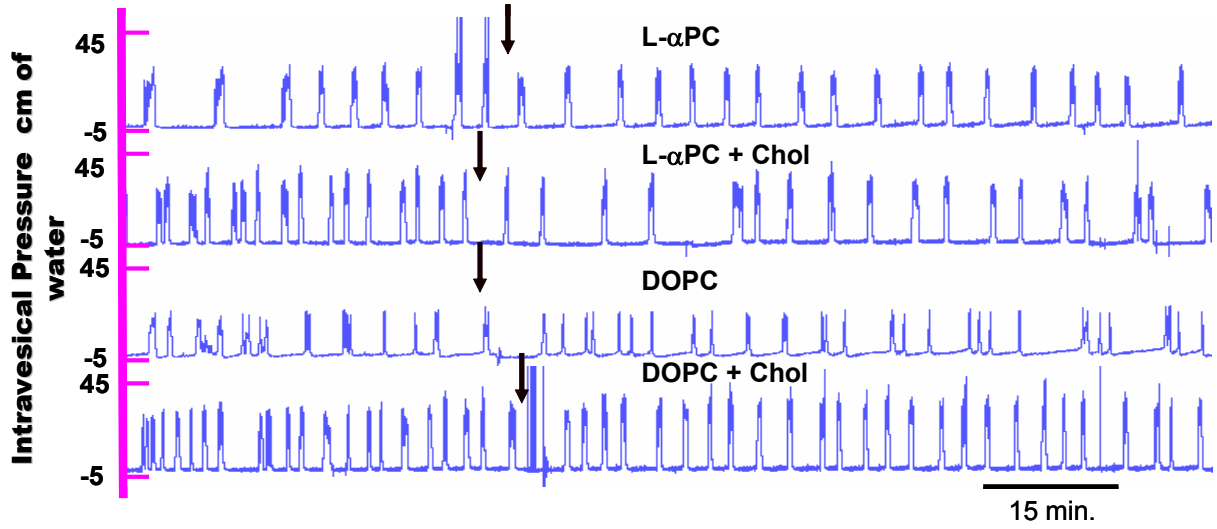

Figure 3.4: Effect of cholesterol inclusion in the liposomes prepared from synthetic and natural lipid

The black arrow marks the start of infusion of liposomes in presence of the irritant 500mM KCl and liposomes in absence of cholesterol were able to reduce bladder hyperactivity as evident from decreased bladder contraction frequency following the start of liposome infusion compared to the bladder contraction frequency prior to the black arrow. L-α phosphatidylcholine L-α-PC and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). $p>0.05$

Inclusion of cholesterol in the liposomal formulation of pure synthetic lipids at 2:1 molar ratio did not significantly alter the protection afforded by liposomes prepared without including cholesterol in their formulation (Fig. 3.4).

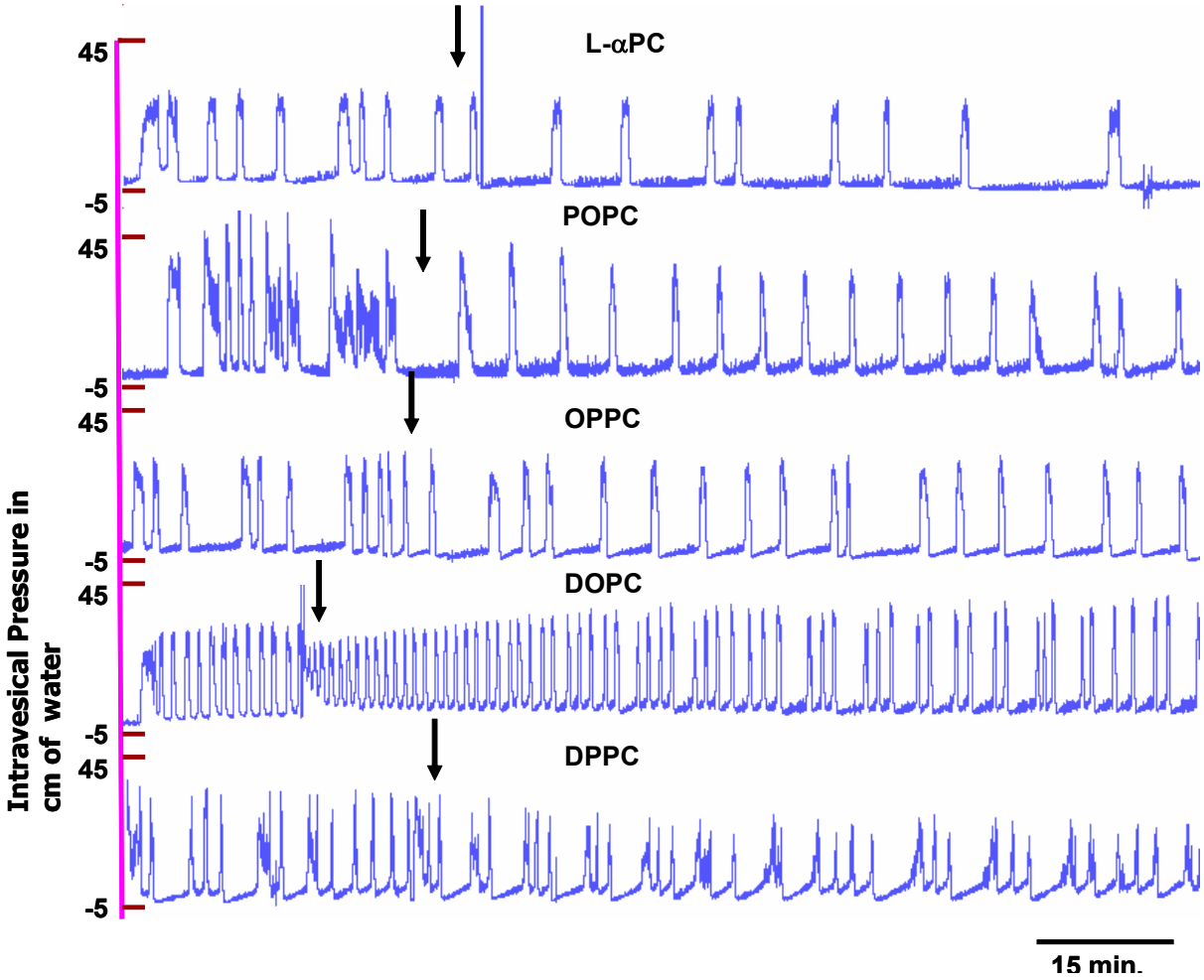

Figure 3.5: Natural egg PC and analogue synthetic lipids infused into hyperactive bladder in absence of cholesterol.

Synthetic lipids having saturation pattern similar to natural egg PC such as POPC and OPPC were effective in reducing bladder hyperactivity as evident from decreased bladder contraction frequency following the start of liposome infusion compared to the bladder contraction frequency prior to the black arrow. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine (OPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)

Infusion of PS (10 mg/ml) did not significantly change the CMGs. However, subsequent infusion of KCl resulted in an irritative effect after a delay of 30 to 40 minutes. Liposomes were

infused in presence of KCl to demonstrate immediate benefit of liposomes on injured urothelium in the presence of an insult (KCl).

3.2.3. Pure Natural & Synthetic Lipids

Pure natural and synthetic lipids were used to make liposomes in 500mM KCl with concentration of total lipids being 2mg/ml. Synthetic lipids possessing average saturation state in acyl chains similar to natural lipids were preferred in reduction of bladder hyperactivity.

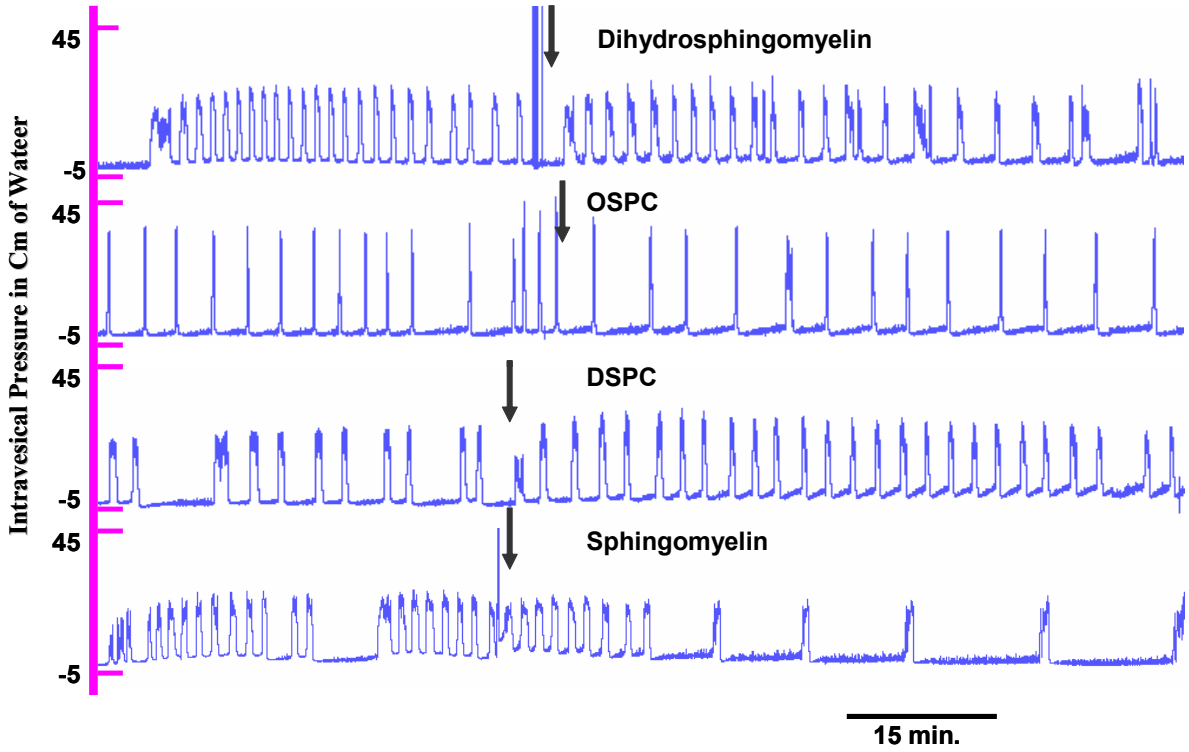

Figure 3.6: Liposomes prepared from SPM, DHSPM and pure synethetic lipid having one of acyl chain derived from stearic acid

Sphingomyelin was able to reduce the bladder hyperactivity in presence of KCl significantly more than dihydrosphingomyelin and pure synthetic lipids as evident from decreased bladder contraction frequency following the start of liposome infusion compared to the bladder contraction frequency prior to the black arrow. 1,2-distearoyl-sn-glycero-3-phosphocholine(DSPC), 1-Oleoyl-2-Stearoyl-*sn*-Glycero-3-Phosphocholine (OSPC).

Lipids having either both chains of unsaturated acyl chains or saturated acyl chains showed significantly reduced activity (Fig.3.5). Egg PC does not contain fatty acyl chains shorter

than 16 carbons, but it does contain minor amounts of lipids having one of the acyl chains derived from stearic acid. Therefore, liposomes prepared from lipids having steraoyl acyl chains were also tested to study their effect on bladder hyperactivity. We observed a similar pattern of difference in biological activity with respect to the degree of saturation in the chains (Fig.3.6).

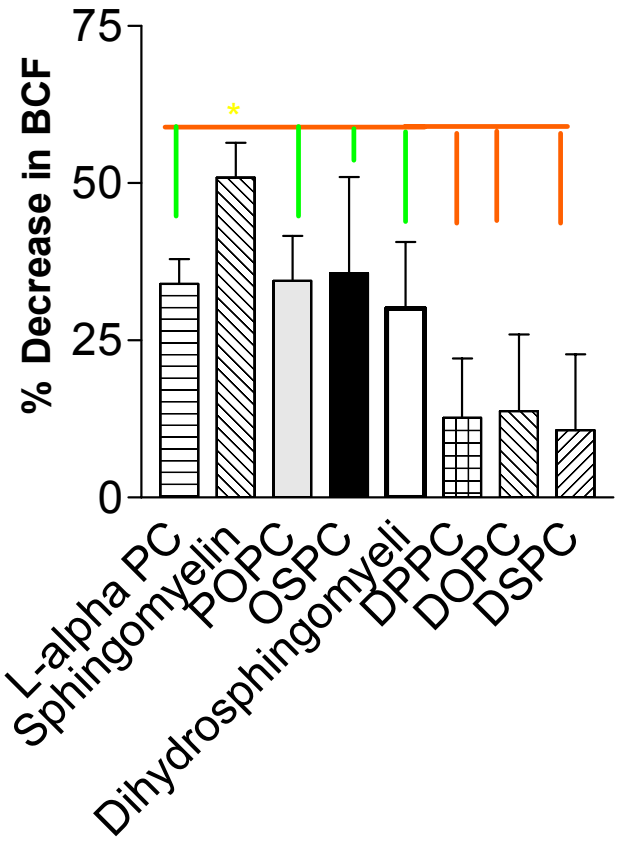

Figure 3.7: Percent decrease in bladder contraction frequency of rat bladder with liposomes.

Sphingomyelin was able to significantly increase the percent decrease in bladder contraction frequency (BCF) compared to dihydrosphingomyelin and other lipids tested in the study. *$p<0.05$; n=7

Optimal activity was found when only one of the linked acyl chains was unsaturated. Switching the position of unsaturation from one chain to the other *sn*-2 to *sn*-1 did not produced a significant difference in the efficacy of 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (OPPC) compared to POPC ($p>0.05$, n=5) Another lipid present as one of the impurities in the

egg PC is sphingomyelin SPM, which contains the same polar headgroup phosphocholine, but differs in hydrophobic backbone. Sphingomyelin was found to be the best among the lipids with PC head group in reducing the bladder contraction frequency (Fig. 3.7).Unlike other natural lipids, sphingomyelin possess a *trans* double bond and we investigated the efficacy of SPM in absence of that double bond i.e. dihydrosphingomyelin(DHSPM) demonstrated efficacy similar to natural lipids and synthetic lipids having saturation in only one of the acyl chains (Fig. 3.6, Fig.3.2).

3.2.4. Fluorescencent Lipids

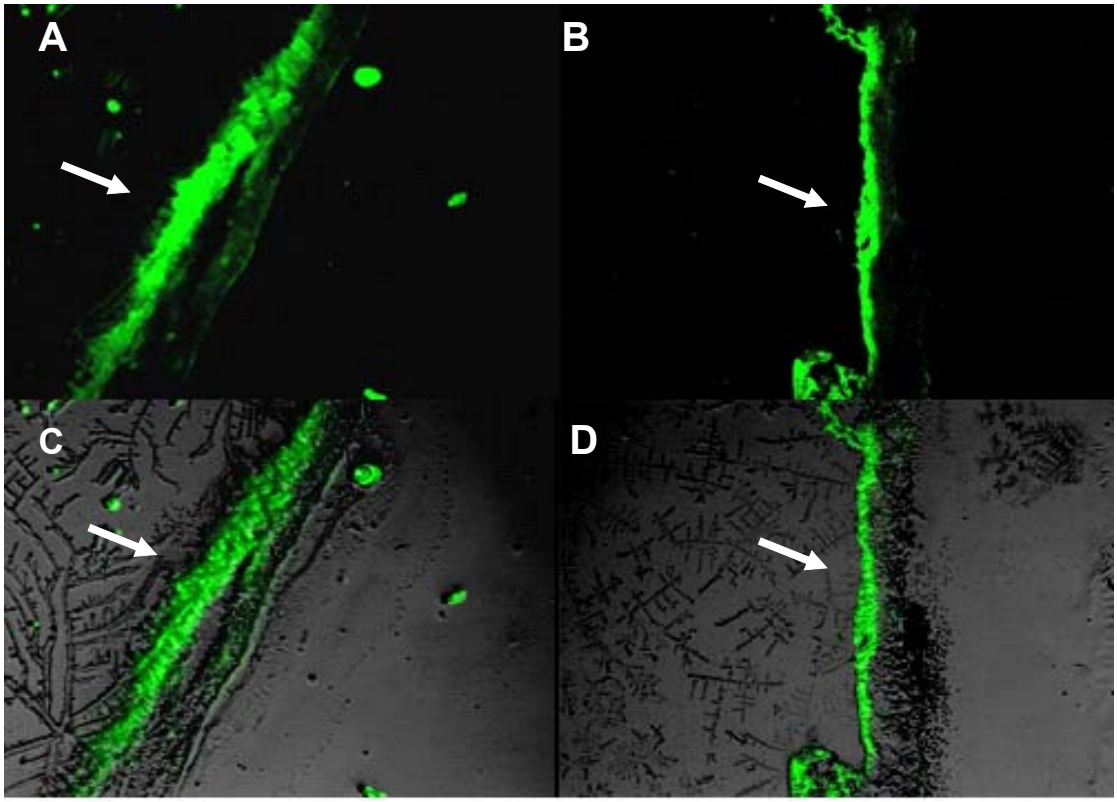

Figure 3.8: Photographs of cryosections of injured rat bladder following administration of liposomes

Panel A is liposome prepared with DOPC +1 mol%1-oleoyl-2-[6-{(7-nitro-2-1,3-benzoxadiazol-4-yl)amino}hexanoyl]-sn-glycero-3-phosphocholine, C-6 NBD PC and panel B is SPM liposomes with 1mol% N-[6-{(7-nitro-2-1,3-benzoxadiazol-4-yl)amino}dodecanoyl]-sphingosine-1-phosphocholine (C12-NBD Sphingomyelin). Bright green fluorescence of NBD was visible in both the treatment groups represented in. panel A & B. Panel C & D are merged images of A&B on their respective bright field images, respectively. Lumen side of urothelium having fluorescence is marked by white arrows and magnification is 10X.

We initially suspected that activity or inactivity of liposomes is related to their ability to adhere or coat the bladder surface. To evaluate the ability to adhere to the bladder surface, the liposomes were prepared having 1mol% of fluorescent lipid having the fluorescent label in the hydrophobic backbone. Liposomes of the most active lipid SPM were compared against the inactive lipid DOPC, following the addition of 1mol% lipid N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sphingosine-1-phosphocholine (C12-NBD Sphingomyelin) and 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine, C-6 NBD PC respectively. The cryosections of rat bladder taken after completion of CMG showed a layer of bright green fluorescence from NBD adhering to the bladder surface (Fig.3.8).

3.2.5. Inclusion of SPM Metabolites

Data shown in Fig, 3.8 ruled out our initial assumption that active lipids have the better ability to coat the bladder surface than inactive lipid.

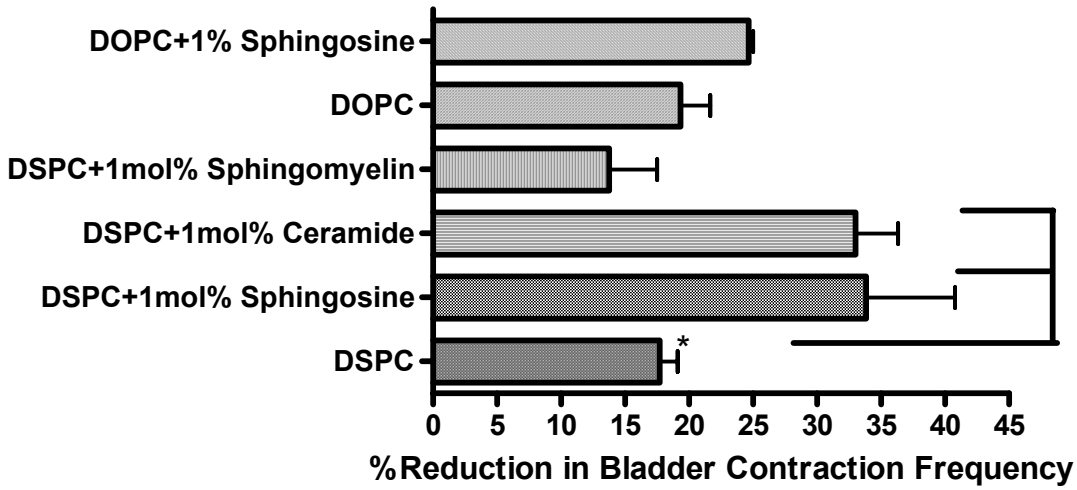

Figure 3.9: Effect of adding SPM and its metabolites to DSPC and DOPC liposomes.

Addition of sphingosine and ceramdie at 1mol% to DSPC liposomes significantly increase the percentage reduction of bladder contraction frequency but there was only slight increase in DOPC liposomes. Adddition of SPM to DSPC metabolites did not alter the efficacy of DSPC liposomes.

The active lipid SPM devoid of its phosphorylcholine head group is ceramide which is a well-known lipid second messenger that mediates a wide range of cellular responses to external stimuli and also used for the biosynthesis of SPM and glycosphingolipids. Since ceramide cannot form stable liposomes, we have to test its effect on hyperactive bladder by including it at 1 mol% in an inert lipid such as DSPC or DOPC.We studied the changes in the efficacy of DSPC following inclusion of C18 ceramide.As shown in Fig.3.9, ceramide was able to significantly increase the efficacy of DSPC liposomes.

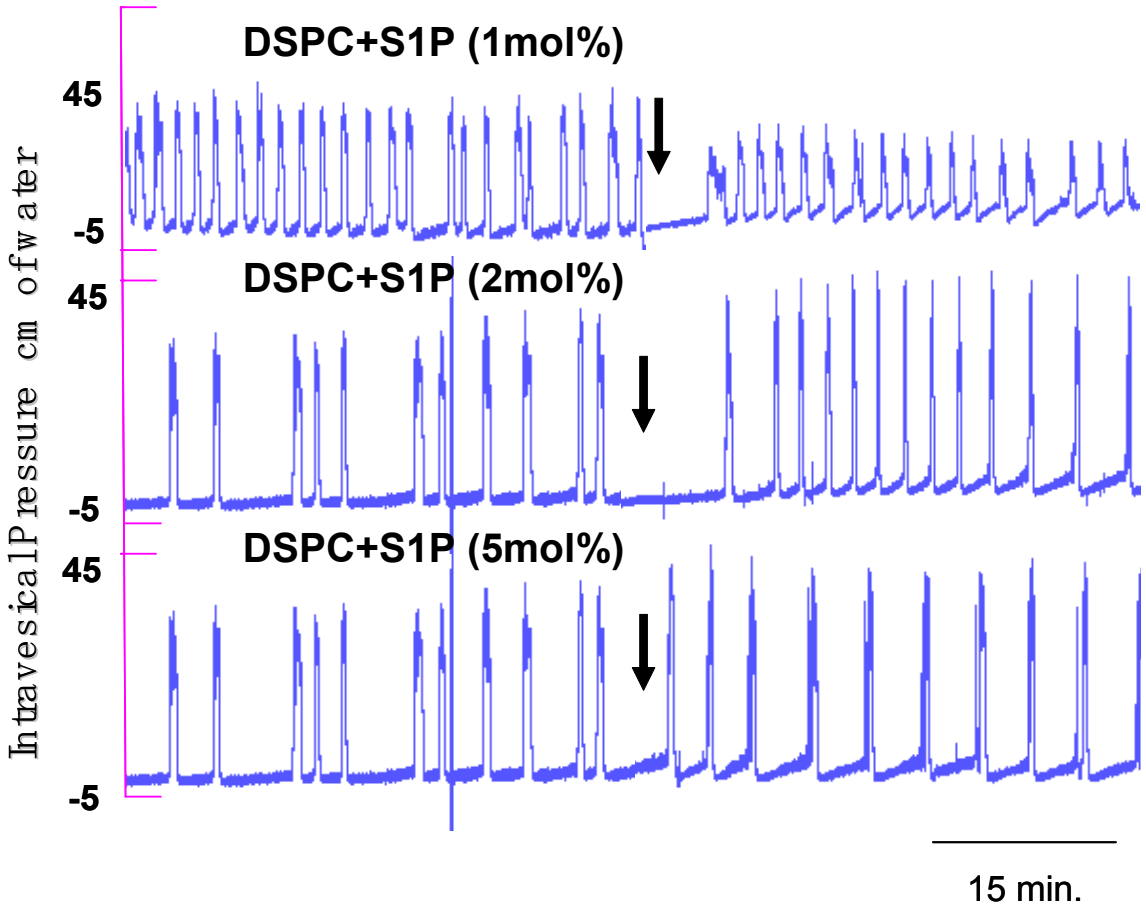

Figure 3.10: Effect of including Sphingosine-1-phosphate on efficacy of DSPC liposomes.

Higher concentration of S1P was required for improving efficacy of DSPC liposomes and S1P at lower mole percentages did not have positive effect on the efficacy of DSPC liposomes.

Inclusion of 1mol% of sphingosine to DSPC liposomes also had similar effect, but addition of sphingosine to DOPC caused only a modest increase in their efficacy. The difference in the DOPC and DSPC liposomes to the addition of sphingsine indicates a hydrophobic mismatch in DOPC liposomes with sphingosine. In our experiments, addition of sphingomyelin at 1mol% to the DSPC liposomes did not increase the efficacy of DSPC liposomes. These observations seem to indicate that metabolites of SPM at the low concentration of 1mol% are far more active than the parent compound itself. Interestingly, inclusion of sphingosine-1-phosphate S1P, another SPM metabolite at 1 and 2 mol% as used for ceramide and sphingosine did not produce similar changes in the efficacy of DSPC liposomes. S1P is a bioactive lysophospholipid, whose diverse can involve both intracellular and extracellular actions (Fig. 3.10). S1P has to be included at 5 mol% to demonstrate any increase in the efficacy of DSPC liposomes. Perhaps at higher concentration, the lysophospholipid can cross the cell membrane and act intracellulary to produce the beneficial effect.

3.2.6. Spinal C-fos expression

Immunohistochemical staining of the L6 spinal cord revealed that KCl infusion following the protamine sulfate infusion in rat significantly increased the total number of Fos-positive cells in the L6 spinal cord. Fos-positive cells were identified as dark spots within nuclei and positive cells were distributed in three main areas, lamina I, intermediolateral gray matter and dorsal commissure. Infusion of sphingomyelin liposomes in presence of KCl was able to significantly reduce the number of Fos-positive neurons (Fig. 3.11 & 3.12). DOTAP liposomes failed to reduce the number of Fos-positive cells compared to KCl infusion. Liposomes prepared with DHSPM also produced reduction in Fos-positive cells, but the difference was not significant with respect to SPM.

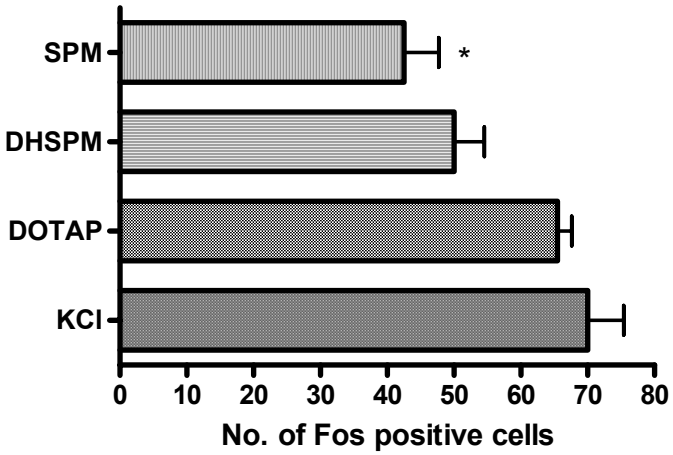

Figure 3.11: Number of Fos-positive cells in spinal cord of rat with hyperactive bladder

Intravesical infusion of KCl following protamine sulfate infusion was able to significantly increase the spinal *c-fos* expression in the L6 segment. Sphingomyelin was able to prevent the KCl evoked increase in the number of Fos-immunopositive cells. *p<0.05.Error bars indicate SEM, n= 4.

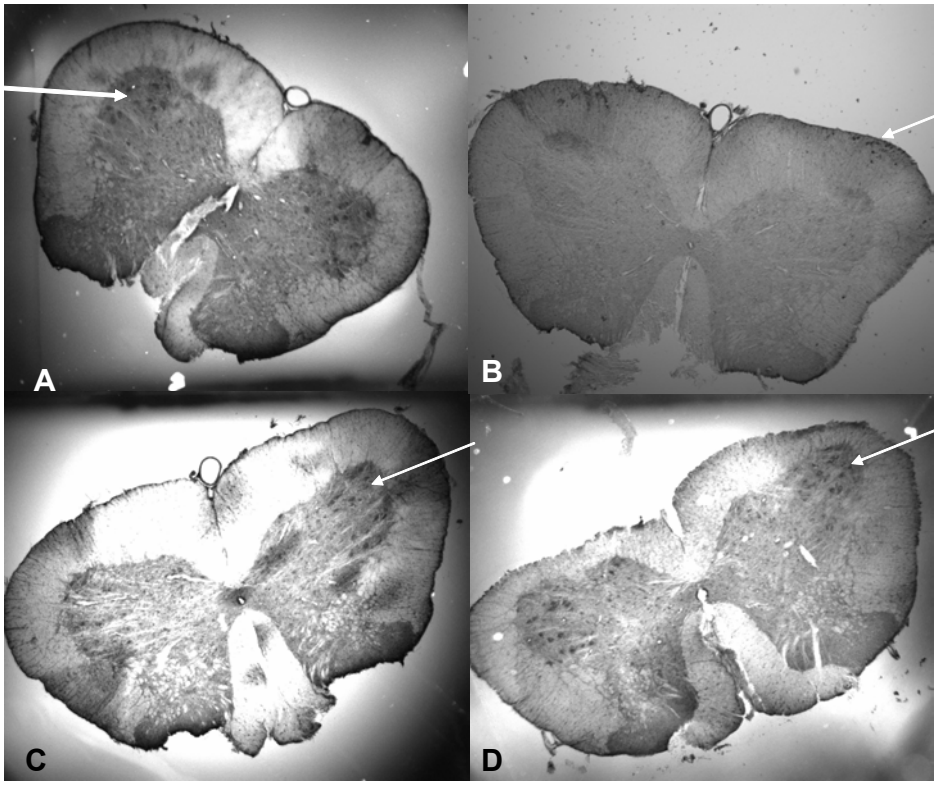

Figure 3.12: Representative transverse sections of spinal cord of rats following liposome instillation.

Sections were obtained immediately after infusion of either potassium chloride alone or in presence of liposomes. The number of dark sports (identified by white arrows) are significantly higher in Panel A of rat infused with KCl. Panel B is SPM and panel C is DOTAP and panel D is DHSPM.

3.3. Discussion

One of the most impermeable and tightest membrane barrier in the human body is erected by tight junctions and uroplakins of umbrella (transitional) cells in concert with exceptionally low permeability of their apical membrane (186, 200-203). Investigations into the primary antibacterial defense mechanisms of the bladder revealed that luminal mucopolysaccharide, the glycosaminoglycans (GAG) layer on the surface of transitional cells is the principal barrier to permeability (204-206). Exposure to protamine sulfate, can compromise the GAG layer as revealed by a precipitous decrease in transepithelial resistance and marked increase in permeability of rat urothelium (195). Scanning and transmission electron microscopic examination of bladder treated with PS showed necrosis and sloughing of sheets of umbrella cells (207).

It is believed that a subset of patients with IC seem to have defective GAG layer, and goal of restoring it back and palliation of IC symptoms in the process prompted intravesical administration of heparin, hyaluronic acid, or pentosan polysulfate (55, 56). Although effective in one third of IC patients, oral administration of heparinoid-based therapy (pentosan polysulfate sodium) may require several months or more to provide any relief of pain and urgency/frequency. Intravesical administration of GAG analogues such as pentosan polysulfate sodium also fails to produce immediate and sustained relief of IC symptoms (208).

The present study was undertaken for studying the key structural elements necessary for efficacy of liposomes in hyperactive bladder. Previous report published from our lab, showed that infusion of liposomes produces an immediate decrease in bladder contraction frequency even in the presence of provoking agent, KCl (156). In the current study, liposomes were

prepared from lipids with variation in the charge carried by the polar headgroup and variation in hydrocarbon domain of lipid with respect to saturation and length. As reported earlier also, we found that neutral zwitterionic lipids are significantly effective in reducing bladder activity compared to anionic charged and cationic charged liposomes. Moreover, infusion of liposomes prepared with only pure natural lipid egg PC also produced reduction in bladder contraction frequency comparable to liposomes composed of pure egg PC and cholesterol. We observed similar results with infusion of liposomes from pure synthetic lipids in absence or presence of cholesterol. This led us to conclude that cholesterol is not necessary for the action of liposomes in hyperactive bladder.

The experimental design of this study also sought to identify the active component in the liposomes used in earlier study, which were formulated with impure egg PC and cholesterol in 2:1 molar ratio. The fatty acid distribution of egg PC is heterogeneous with a degree of unsaturation varying on average up to 0.35 *cis*-double bonds per molecule. A ratio of approx. 0.82 exists between saturated and unsaturated fatty acids with 34% of palmitic, 32% of oleic acid and 11 % of stearic acid and 16% of linoleic acid. The bladder injury model used in our earlier study was also used in the current study to evaluate the liposomes prepared from lipids possessing homogenous distribution of fatty acids. Liposomes prepared with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were able to reduce bladder hyperactivity with efficacy similar to pure egg PC.

POPC has the chemical structure identical with the representative structure of the major lipid present in pure egg PC, indicating that pure synthetic lipids possessing similar acyl chain length and saturation can demonstrate the activity of natural egg PC. Absence or presence of saturation in the acyl chain linked at *sn*-2 position of POPC led to parallel changes in the activity

of liposomes. Presence of saturation in the acyl chain linked to the *sn*-1 and *sn*-2 position was detrimental to the efficacy of liposomes as is evident from liposomes prepared with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) having both chains saturated. Same was true for lipids having both chains unsaturated, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) as DOPC liposomes were significantly less effective in reducing bladder hyperactivity than POPC or pure egg PC. Switching the position of unsaturation from one chain to the other did not drastically reduce the efficacy of POPC as was evident from the activity of 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (OPPC). Similarly, when both chains of DOPC were saturated as in 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC) , the efficacy was minimal. However, efficacy of 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine OSPC was comparable to POPC, as saturation was absent in one of the chains.

We observed that infusion of micelles prepared with platelet activating factor PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine at same lipid concentration as used for liposomes in hyperactive bladder further increased the bladder frequency (data not shown). Moreover, liposomes prepared from DMPC also failed to reduce the bladder hyperactivity in our studies (data not shown). DMPC has saturation in both acyl chains and they are also shorter than those found in egg PC. It was determined that there is a requirement for optimal acyl chain length in the efficacy of liposomes in hyperactive bladders. It appears that liposomes prepared from lipids having acyl chain lengths shorter than those found in natural lipids in cell membranes do not have efficacy in hyperactive bladder.

Phospholipids with saturated fatty acid chains are known to form more rigid and stable membranes than natural phospholipids. Presence of double bonds imposes different packing constraints, creates disorder in hydrophobic region of the lipid bilayer and influence fluidity,

surface curvature, and nonlamellar phase forming of liposomes such as reverse hexagonal H_{II} phase (209). In a previous study, liposomes prepared from phospholipids of natural origin (egg PC) could inhibit generation of superoxide anion O-2 in neutrophils to a far greater extent than liposomes prepared with saturated lipids such as DPPC (210). The activation of neutrophils is in part dependent on protein kinase C (PKC) and reduction in free radical seems to be a result from modification in the activity of NADPH oxidase and PKC. It is known that interaction of phospholipids with various forms of membrane associated protein kinase C (PKC) is drastically influenced by the saturation in the acyl chains of phospholipids. Symmetrically saturated phospholipids such as DPPC cannot inhibit PKC in nuetrophils, whereas natural lipids such as egg PC having asymmetric saturation in its acyl chains can inhibit PKC (211). It is a accepted fact that activation of PKC is greatly enhanced by unsaturated phospholipids such as DOPC than by saturated phospholipids and micelles of PC can activate PKC to greater extent than PC liposomes (212, 213). A specific lipid-protein interaction was ruled out by the unchanged activity of PKC following alteration in the degree of acyl chain unsaturation and positioning of the double bond (213). Addition of cholesterol to both saturated and unsaturated systems did not affect the PKC activation indicating fluidity is not a factor (213).

Our observations on reduction of bladder hyperactivity with regard to liposomes prepared with lipids having asymmetric saturation in acyl chains and lack of benefit from inclusion of cholesterol seems to agree with previously reported work done on interaction of phospholipids with PKC. Exogenous administration of liposomes having saturation in only one of the acyl chains seems to reduce bladder hyperactivity by modulating the activity of PKC in addition to suppressing the activity of free radical generating enzymes in the inflamed condition of rat bladder (214). Symmetrically saturated DPPC and DSPC liposomes failed to show activity in our

study, perhaps due to decreased affinity for binding with PKC (215). DOPC liposome having unsaturation in both acyl chains is most suitable to bind and activate the PKC activity, although PKC activation may already be maximal in the injured bladder following potassium chloride infusion and PKC activity cannot be increased any further (216).

PKC is a key element of signal transduction and it is representative of a class of extrinsic membrane proteins that show lipid dependent interactions with cell membranes. Translocation from the cytosolic to membrane fraction of the tissue lysate following tissue injury indicates activation PKC (217). PKC appears to be a central player in orchestrating the inflammation and the role of PKC isoforms, specifically PKCδ and PKCε in mediation of intestinal inflammation has been previously reported (218). The phorbol ester induced nuclear translocation of annexin A1 , a calcium-dependent, phospholipid binding protein, can be inhibited by the PKCδ- specific inhibitor rottlerin, indicating that inhibition of PKCδ reduces inflammation by inhibiting nuclear translocation of the cleaved annexin A1 (219).

SPM is one of the several lipids constituting the 13% of other neutral lipids in impure egg PC. SPM belongs to the class of sphingolipids, a member of a diverse family of phospholipids and glycolipids mediating cell-cell interactions through different signal transduction pathways (220). Interestingly, more than half of the total phospholipid content in eukaryotic membrane lipids is constituted by SPM and PC, residing mostly in the outer leaflet of plasma membranes (221). Egg PC and SPM shares the same polar headgroup, but they differ in the interfacial and hydrophobic parts of the molecules such as the higher average saturation state of SPM's acyl chains and the greater capacity of SPM to form inter- and intramolecular hydrogen bonds, which can lead to significant deviations in the macroscopic properties of respective bilayers (liposomes) (Fig.1 & 2) (222).

Of all the pure lipids evaluated in our study, SPM was able to significantly reduce the bladder activity compared to other treatment groups. SPM contains both hydrogen bond donating and accepting groups, while PC only has hydrogen bond accepting groups (222). SPM molecules present in mammalian systems usually constitute a mixed population of different amide linked acyl chains, their length ranging from 16 to 24 carbons. The most common base in mammalian SPM is sphingosine (1,3-dihydroxy-2-amino-4-octadecene), with a chain length of 18 carbon atoms and a *trans* double bond between C4 and C5. Loss of C4-C5 double bond significantly reduced the efficacy, as evident from the CMG of rat treated with liposomes prepared with dihydrosphingomyelin DHSPM (Fig.2.7). Differences in the efficacy of liposomes prepared from SPM and DHSPM was also reflected in the number of cells stained positive for c-fos in the L6 spinal cord of rat. SPM liposomes were able to reduce the number of Fos-positive cells. C-fos is a generic marker for trans-synaptic activity in the spinal cord and the increased number in the rats instilled with DOTAP and KCl alone signify bladder irritation (223). The decreased fos-positive cells in the spinal cord of rats treated with SPM indicate suppression of irritation in prescence of KCl.

It has been shown that lack of the trans-double bond C4-C5 in DHSPM allows formation of more ordered membranes with a higher melting temperature (T_m) compared to acyl-chain-matched SPM (224). Lipid bilayers of DHSPM pack more tightly, and NMR spectral trends suggest DHSPM have stronger intermolecular interactions in the lipid bilayer and SPM form more stable intramolecular hydrogen bonds between OH group in sphingosine moiety and the phosphate ester oxygen (225, 226). DHSPM and SPM interact differently with water molecules and the intramolecular hydrogen bond can be stabilized by two tightly bound water molecules participating in flip-flop interactions with the hydroxyl group (227).

Further, NMR study revealed that absence of double bond in biologically inactive dihydroceramide produced from DHSPM has reduced stabilization of intramolecular hydrogen bond than bioactive ceramide (228). Intramolecular hydrogen bond in ceramide is formed by hydrogen donating NH group on C1 of the sphingosine backbone and hydrogen accepting OH groups on carbon C3. The differences in the hydrogen bonding behavior of SPM and DHSPM is known to influence the fusion of Semliki forest virus with liposomes as only SPM can fuse with the virus (229, 230). Among other notable difference in the biophysical properties of DHSPM in lipid bilayers is the dramatic alteration in the transbilayer distribution of galactosylceramide in SPM/POPC vesicles following inclusion of 2 and 10 mol% DHSPM (231, 232).

Moreover, it has been well established that metabolites of sphingomyelin, such as ceramide, sphingosine and sphingosine-1-phosphate (S1P) are bioactive lipids mediating essential biological functions such as chemotactic motility, calcium homeostasis, cell growth, cell death, and differentiation. Ceramide is formed by the hydrolysis of sphingomyelin by sphingomyelinase, which is activated probably as a result from agonist-induced activation of several receptors for tumor necrosis factor-α, γ-interferon, and interleukin-1 (233). Enzymatic formation of ceramide from sphingomyelin can result in aggregation and partial fusion of liposomes to cell membranes (234). Generation of ceramide from sphingomyelin can be catalyzed by at least five different sphingomyelinases; neutral sphingomyelinase in plasma membrane and cytosol, acid sphingomyelinases in endosome and lysosomes (235).

Sphingosine can be generated from ceramide by action of ceramidase is also a negative effector of PKC action like ceramide (Fig. 2.13), which has been recently shown to reduce interleukin-2 production in Jurkat cells by inhibiting PKC-mediated activation of NF-κB (236). Emerging evidence also suggests that sphingomyelin-enriched lipid domains or "rafts" may serve

as substrate pools for sphingomyelinase-induced formation of ceramide microdomains that act as platforms from which these signal transduction cascades originate (237). Sphingosine can prevent activation of PKC by platelet-derived growth factor, bradykinin, and phorbol esters (238) Higher efficacy of SPM in reducing bladder hyperactivity observed in our study seems to emerge from its effect via dual pathways, SPM can act through the suppression of free radicacl generation and PKC inhibition similar to egg PC in addition to the metabolites of SPM further augmenting the effect produced by PKC inhibition. The DHSPM do not have the added effect of its metabolite in reduction of bladder hyperactivity and therapeutic benefit of DHSPM in hyperactive bladder is perhaps the results of its action via the pathway of egg PC. DHSPM is probably suppressing the generation of free radicals in the injured urothelium or it is modulating the activity of PKC. Translocation of PKC βII to the nucleus in phorbol ester stimulated HEK 293 cells was inhibited by the addition of exogenous ceramides or formation of endogenous ceramide by the action of bacterial sphingomyelinase (239).

We hypothesized that SPM higher activity of SPM over PC and other synthetically active lipids can be explained by the generation of metabolites. To test the hypothesis, we studied the effects on CMG following the inclusion of ceramide and sphingosine at low mole ratios in inactive lipids determined earlier such as DOPC and DSPC. Sphingosine has a long hydrophobic tail and a polar head containing a proton trapping amino group which allow it to act as a detergent on protonation (Fig.3.13). The charged form of sphingosine will predominate at the acidic pH of the lysosome and subsequent lysosome rupture can release proteases to induce apoptosis (240). Apoptosis of neutrophils and other inflammatory cells can contribute to the beneficial effect seen in the CMG of animals infused with ceramide and sphingosine. Inclusion of sphingosine was able to significantly increase the protection afforded by DSPC but not in

DOPC. It appears that hydrophobic mismatch of the acyl chains may be responsible for these observations. Similar changes in efficacy of DSPC were obtained with ceramide at 1 mol%. Therapeutic benefit observed with inclusion of ceramide and sphingosine is probably in part mediated by inhibition of PKC as previously discussed for egg PC and synthetic lipids having asymmetric saturation. Interestingly, inclusion of sphingosine-1-phosphate at 1 and 2 mol% as used for ceramide and sphingosine did not produce similar changes in the efficacy of DSPC liposomes (Fig. 2.9). S1P is a bioactive lysophospholipid, whose diverse extracellular effects are clearly mediated through the activation of five specific G protein coupled receptors, called S1P receptor 1-5 (previously known as endothelial differentiation gene receptor Edg), which are expressed on cell surface. S1P is known to act on S1P2 receptor to produce contraction of coronary artery smooth muscles through S1P(2)/Rho signaling and play a role in pathogenesis of ischemic heart disease (241).

It is possible that increased bladder contraction frequency produced by S1P at lower mole percentages is acting through similar mechanism or through degranulation of mast cells as shown for airway smooth muscle cells (242). It has been widely reported that signaling induced by S1P is complex and can involve both intracellular and extracellular actions (243). Sphingosine-1-phosphate has to be included at 5 mol% to demonstrate any increase in the efficacy of DSPC liposomes. Perhaps at higher concentration, the lysophospholipid can cross the cell membrane and act intracellulary to produce the beneficial effect. Besides being inducers of apoptosis, ceramide and sphingosine are also known to dampen mast cell responsiveness, whereas S1P can act as a chemoattractant and up-regulate some effector responses of mast cells (244). Mast cells, are well known to play a role in bladder inflammation and they represent a prototype for the "sphingolipid rheostat" hypothesis because of the multiple functions of S1P both as an

intracellular messenger and autocrine/paracrine mediator (245). Failure of SPM to increase the efficacy of DSPC liposomes, when added at 1mol% indicates that metabolite is more potent than the parent drug in altering the efficacy of DSPC liposomes. It is also possible that SPM in absence of its metabolic products can also influence the mast cell function which may be responsible for reduction in bladder hyperactivity as it has been reported that exogenously administered SPM analogs such as gangliosides (GSL) can inhibit the release of preformed secretory mediators from mast cells activated via Fc epsilon receptor (246). Bladder injury by protamine sulfate is known to increase levels of malondialdehyde in rat bladder, which is an end product of lipid peroxidation by free radicals (207).

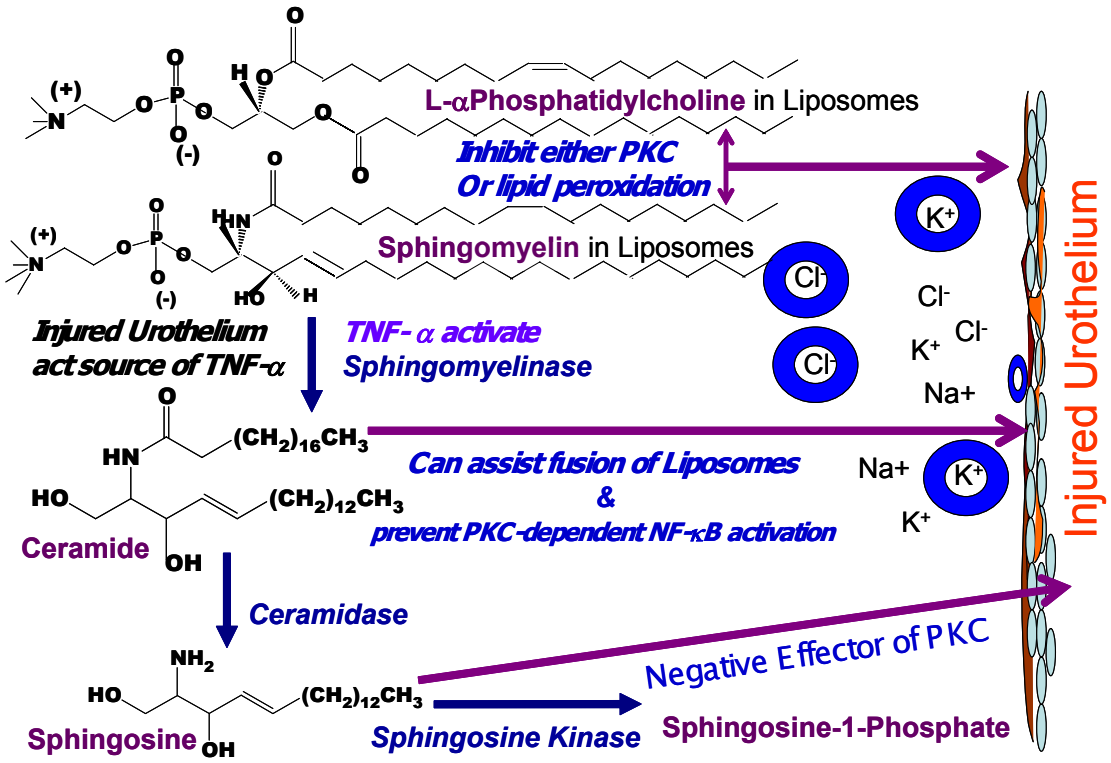

Figure 3.13: Proposed mechanism for activity of egg PC and SPM liposomes.

Egg PC and synthetic lipids having asymmetric saturation in acyl chains can inhibit free radical mediated lipid peroxidation or inhibit PKC to reduce inflammation and decrease bladder hyperactivity in bladder injury induced by protamine sulfate. DHSPM probably act through the pathway of egg PC and SPM can either act through the pathway of egg PC at the injured urothelium or its efficacy can be further augmented by the effect of one of its metabolites.

Thiobarbituric acid reactive materials following exposure to hydroxyl free radicals are not formed by SPM, although they are formed by phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE) liposomes in the order PS > PC > PE (247). It is postulated that mostly saturated long chain hydrocarbon groups of SPM can protect against oxidative damage and SPM can also impede the lateral propagation of the lipid peroxides by decreasing membrane fluidity (Fig. 3.13) (220). SPM can also reduce phospholipase degradation of phospholipids by inhibiting the rate limiting step in generation of arachidonic acid from membrane lipids by inhibiting phospholipase A_2 (248). Therefore, either in isolation or collectively, these molecular interplays of SPM or its metabolites may be contributing in reducing the bladder hyperactivity in SPM liposomes.

Conclusion

Sphingomyelin is able to reverse the bladder hyperactivity in bladder injury model significantly better than egg PC and synthetic lipids. Bioactive metabolite of SPM seems to be responsible for this effect. No currently available intravesical IC therapy achieves immediate symptom relief without destroying the nerve endings. Unlike neurotoxins discussed in the previous chapter, sphingomyelin does not destroy the nerves. Neurotoxins such as vanilloids potentially may afford short term relief, but the nerves may then regenerate, with a "sprouting" effect that could possibly results in an increased density of nerve endings and augmented neural activity when the nerves eventually recover. Since SPM and other active PC are not toxic on intravesical application, their potential use in treating hyperactive bladder in clinic should be seriously considered.

4. SUSTAINED INTRAVESICAL DRUG DELIVERY USING THERMOSENSITIVE HYDROGEL

A polymer chosen to serve as a vehicle for sustained intravesical drug delivery should be in the fluid state at the time of instillation and switch to the gel state after instillation. It is expected that hydrogel once formed inside the bladder can act as a matrix filled with drug for maintaining a prolonged exposure of drugs at the urothelium. The dispersion of a thermosensitive triblock co-polymer (PEG-PLGA-PEG), in 0.1M phosphate buffer exists in a sol state at room temperature or below but converts to a gel state at elevated temperature such as 37°C (249). We propose to employ this thermosensitive polymer as a vehicle for extending drug exposure in the bladder beyond the voiding of urine post-instillation. Temperature sensitive gelation of the instilled polymeric dispersion is expected to happen inside the bladder, as rat body temperature is 37°C. Successful sustained intravesical drug delivery can eliminate multiple catheterizations leading to improved patient compliance, so important for the success of any form of therapy.

Unlike systemic chemotherapy, responses to intravesical therapy are directly proportional to drug concentration rather than drug dose as shown for topical chemotherapy (250). However, duration of drug exposure so critical for therapeutic response is usually limited by bladder capacity and it is short with the use of conventional vehicles in intravesical therapy (251). Ueda et al showed that doxorubicin remained longer within the urinary bladder tissue of patients, when administered together with a mucoadhesive agent hydroxypropylcellulose and higher concentrations of drugs were achieved in tumorous tissue than in normal tissue (252).

Inadequate drug delivery by conventional vehicles justifies the search for new agents to overcome the limitations inherent in intravesical route of drug administration. The thermo-

sensitive and biodegradable characteristics of the triblock co-polymer, selected for our study have been widely reported (141, 168, 253, 254). As previously reported from our lab, a polymer of similar chemical composition was used as a matrix for a controlled gene delivery system (152). For the purpose of intravesical application, we modified the above polymer, by increasing its hydrophobic content to improve the robustness of the formed hydrogel in the bladder to endure the urine constituents such as urea and electrolytes.

We failed to show any therapeutic benefit of using hydrogel as a vehicle in the study done on capsaicin probably because the drug itself has prolonged therapeutic effect. In the, present study, we first used Fluorescein isothiocyanate (FITC) as a model drug to demonstrate sustained intravesical drug delivery. Further validation of the concept of sustained intravesical delivery was done with the efficacy studies of misoprostol delivered using the hydrogel. Intravesical administration of prostaglandins is a promising treatment of hemorrhagic cystitis (114). Misoprostol is a stable analogue of PGE1 a cytoprotective eicosanoid (cyclooxygenase mediated arachidonic acid metabolite) having several anti-inflammatory effects, including down-regulation of the cytokine response of both macrophages and lymphocytes (255). Intravesical instillation of prostaglandin E2 was effective in the treatment of cyclophosphamide induced hemorrhagic cystitis in bone marrow transplant patients (256). In a similar study done on patients having grade 3 or 4 refractory hemorrhagic cystitis, intravesical infusion of a prostaglandin analogue, carboprost at the rate 0.8 mg/dL for 60 minutes every 6 hours, was effective in 15 out of 24 patients (257-259). Misoprostol is being widely used in the clinic for the treatment of gastric and duodenal ulcer induced by chronic consumption of NSAIDs (nonsteroidal anti-infllammatory drugs) such as aspirin and ibuoprofen. The native drug is a racemate of four stereoisomers, available as a viscous liquid form, which is difficult to formulate due to its

chemical instability. Misoprostol rapidly de-esterifies to its active form, misoprostolic acid after oral administration, which is excreted in urine as its inactive metabolites with an elimination half-life of approximately 30 minutes.

4.1. Materials & Methods

4.1.1. Synthesis of Polymer:

Modified thermosensitive triblock co-polymer, (polyethylene glycol- poly[lactic acid-co-glycolic acid]-polyethylene glycol) PEG-PLGA-PEG were synthesized by ring-opening polymerization according to the modified procedure previously described (168),(260). Briefly, ring-opening polymerization of D,L-lactide (DLLA) and glycolide (GA) onto monomethoxy poly(ethylene oxide) mPEG 750 was done using 1% stannous octoate as a catalyst in toluene under reflux conditions at 150~170 ^{0}C, followed by coupling of the resulting diblock copolymer (mPEG 750-PLGA) using HMDI as a coupling agent) in toluene at 60 °C for 12 h, followed by reflux at 140 °C for 6 h. The resulting PEG-PLGA-PEG triblock copolymers were dried under reduced pressure. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 30°C with a Bruker DPX-300 NMR spectrometer operating at 300 MHz by using $CDCl_3$ as solvent. The molecular weight and molecular weight distribution of the triblock copolymers were monitored by GPC system with Waters 1515 isocratic HPLC pump and Waters 2410 refractive index detector. The GPC data were calibrated with polystyrene standards with molecular weights in the range of 640~1,010,000. Styragel® HR 4 and HR 2 columns (Waters) were used in series. The molecular weights were calculated using monodispersed polystyrene standard (Waters). All of the determinations were carried out in THF at a flow rate of 1.0 ml/min at 35 °C. The molecular weight of diblock copolymers was controlled at around 3,000~4,500. PEG-PLGA-PEG triblock

copolymers were synthesized and its structure was confirmed by ^{1}H NMR. The weight average molecular weight (M_w) of triblock copolymer was 13,981 with a polydispersity index of 1.52 as determined by GPC. The aqueous solutions (30%, w/v) of this polymer flow freely at room temperature but form a gel at 37°C (5).

4.1.2. Preparation of solutions for Instillations:

The control animals were instilled with a 0.02%w/v solution of free FITC in 0.1 M phosphate buffer in absence of any polymer. Equivalent amount of FITC in 0.1M phosphate buffer pH 7.2 was added to a 30% w/v solution of PEG-PLGA-PEG polymer at room temperature by constant shaking in dark for 3h. Female Sprague-Dawley rats in the weight range of 200- 250g were used in the present study. 6 animals were included in each group.

Before intravesical instillation, animals were lightly anaesthetized with halothane. Volume of instillation into rat bladder was kept at 0.5ml in both groups of animals instilled with either free FITC solution or FITC entrapped in hydrogel. Intravesical administration was done using a PE-50 tubing (Clay-Adams, Parsipanny, NJ), inserted into the bladder through the urethra and tied in place by a ligature around the urethral orifice. Under continuing halothane anesthesia, the urethra was left tied for 30min after instillation to prevent evacuation by the animal, as well as to allow enough time for sol-gel transformation to occur inside the bladder. All animals were given subcutaneous injection of antibiotic (Pen-strep, 30mg/kg) to prevent any infection as a result of the procedure. All protocols involving the use of animals in this study adhered to “Principles of Laboratory Animal Care” (NIH publication #85-23) and were approved by the IACUC of the University of Pittsburgh.

After recovery from halothane anesthesia, rats were kept in metabolic cages with food and water *ad libitum* for next 24 h to study the kinetics of FITC excretion in the urine. Urine was

collected at time points chosen arbitrarily. Cumulative urine output was measured from the volume of urine collected at these time points. Animals were sacrificed by carbon dioxide euthanasia and bladders were removed and immediately frozen for cryosections. Collected urine was diluted several fold for fluorescence measurement in order to reduce the background noise from extraneous urine constituents. Fluorescence emitted by FITC was measured using Perkin Elmer spectrofluorimeter at λ_{Ex}= 496nm and λ_{Em} = 512nm.

4.1.3. Efficacy studies:

Adult female Sprague Dawley rats were injected intraperitoneally with a high dose of cyclophosphamide (150mg/kg) under halothane anaesthesia to induce cystitis (261). Chemical cystitis induced by cystitis is characterized by marked increase in erosions, ulcers in bladder including inflammatory cell infiltration, haemorrhages and increased micturition frequency (262). Rats injected with cyclophosphamide were divided into 4 groups and 3 of which served as controls were either instilled with saline, hydrogel or misoprostol independently (n=3). The main treatment group was instilled with hydrogel containing misoprostol. Due to the unstable nature of misoprostol in its native form, we used the drug available in commercially available Cytotec tablets from Searle. Tablets were homogenized to form a powder in mortar pestle and powdered tablet containing equivalent of 50µg misoprostol was administered with or without hydrogel in 0.5ml volume of instillation. Rats were kept in the metabolic cage after instillation for 24h followed by cystometric measurement (CMG) under urethane anaesthesia (1.2g/kg, subcutaneous s/c) to measure frequency of micturition.

CMG was done as previously reported from our lab (156) briefly, PE50 tubing (Clay-Adams, Parsippany, NJ) was inserted into the bladder through the urethra and using a three way stopcock the catheter was connected to a pressure transducer for recording intravesical pressure

and to a syringe pump for infusing saline into the bladder. The catheter system was filled with 0.9% w/v saline. After the bladder was emptied, a cystometrogram (CMG) was performed to measure micturition frequency, which was the number of voiding contractions occurring during a 60-min time period of transurethral saline infusion at the rate of 0.04ml/min. No. of contractions (peaks) divided by time period (60min) gave the number used to compare efficacy of treatment. After completion of CMG, bladders were isolated, fixed in Zamboni fixative and then cryosectioned for staining with haemtoxylin and eosin (H&E).

Statistical analysis: All values in the text and figures are mean values $\pm$ SEM. Student t test was used to test for significance between unpaired groups.

4.2. Results

4.2.1. Kinetics of FITC excretion in urine

Urine collected at various time points from rats in metabolic cages was used to calculate cumulative urine output over a 24h time period (Fig.4.1). Mean cumulative urine output of rats instilled with free FITC was 21.35+2.73ml and that of rats instilled with FITC entrapped in hydrogel was 19.55+2.72ml (number of animal in each group was 6). Difference in the cumulative amount of urine excreted for 24h after instillation was not significant (*p> 0.05). (n=6).

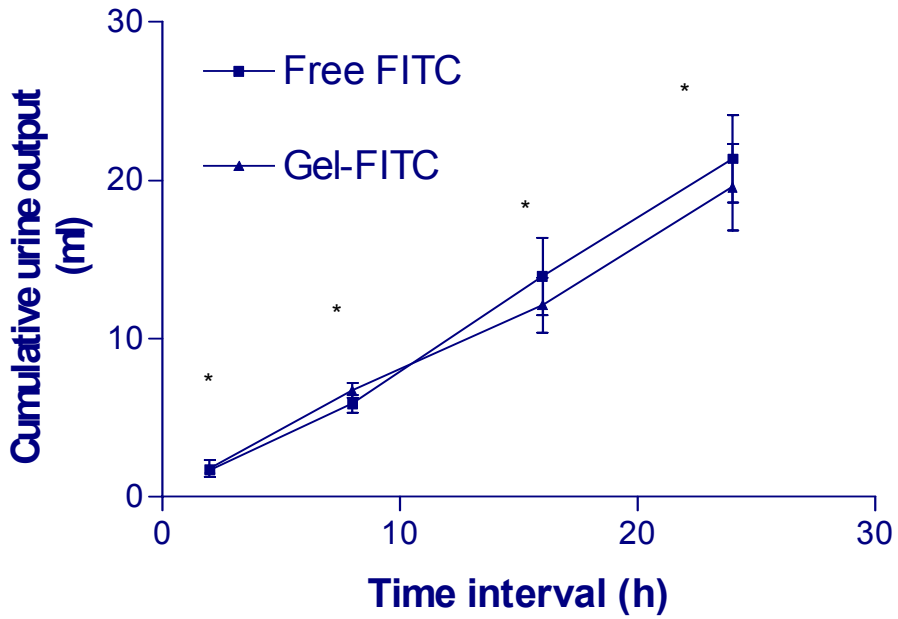

Figure 4.1: Cumulative urine output of the rats instilled with either free FITC solution or FITC entrapped in a hydrogel formed by a thermosensitive polymer.

Difference in the cumulative amount of urine excreted for 24h after instillation was not significant (*p> 0.05). (n=6).

The fluorescence intensity of urine measured at different time points from the collected urine is plotted (Fig. 4.2). Fluorescence intensity was normalized for the volume of urine collected at each time point. A significantly higher fluorescence signal was measured in the urine collected at the first time point of 2h from the rats instilled with free FITC solution (*p<0.01). Urine was diluted several folds before measuring the fluorescence of FITC at 512nm in the Perkin Elmer spectrofluorimeter with excitation at 498nm (mean+SEM; n=6) *p<0.01. However, at the later time point of 8, 16 and 24h, a higher signal was observed in the urine of rats instilled with FITC entrapped in hydrogel with statistical significance for later two time points (p<0.01).

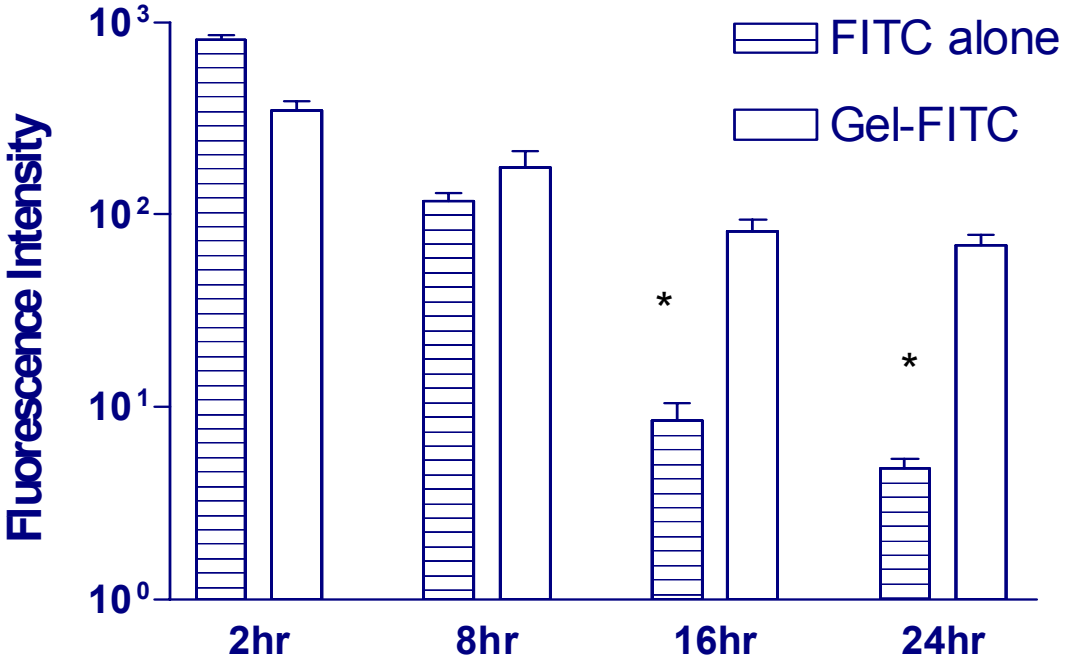

Figure 4.2: Semi-logarithmic plot of fluorescence intensity of the urine measured at various time points after instillation of free FITC and FITC entrapped in hydrogel.

A higher fluorescence signal was observed in the urine of rats instilled with FITC entrapped in hydrogel with statistical significance for later two time points (*$p<0.01$) (n=6).

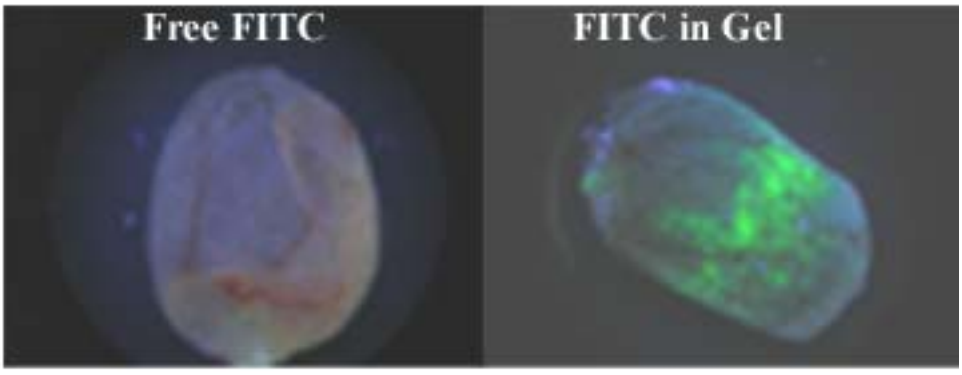

Figure 4.3: Photographs of the representative rat bladders instilled with either free FITC or FITC entrapped in hydrogel.

Bladders were cut opened longitudinally before viewing under ultraviolet light with the aid of a dissecting microscope at 24h after instillation of free FITC solution (left) and FITC entrapped in hydrogel (right). In contrast to the bright green fluorescence of FITC seen in bladder with hydrogel, only a background signal was visible with free FITC (left). (Magnification at 1X).

Bladders from both groups were isolated 24h after instillation for examination under ultra violet light with the help of a dissecting microscope to detect the presence of any adhering FITC with the bladder. Bladders instilled with FITC entrapped in hydrogel exhibited bright green fluorescence of FITC whereas only background fluorescence was visible in the rats instilled with free FITC (Fig.3). The figure also serves as visual evidence for the formation of hydrogel inside the bladder after instillation, because the hydrogel was stained with the dye.

4.2.2. Therapeutic effect following Intravesical delivery of misoprostol:

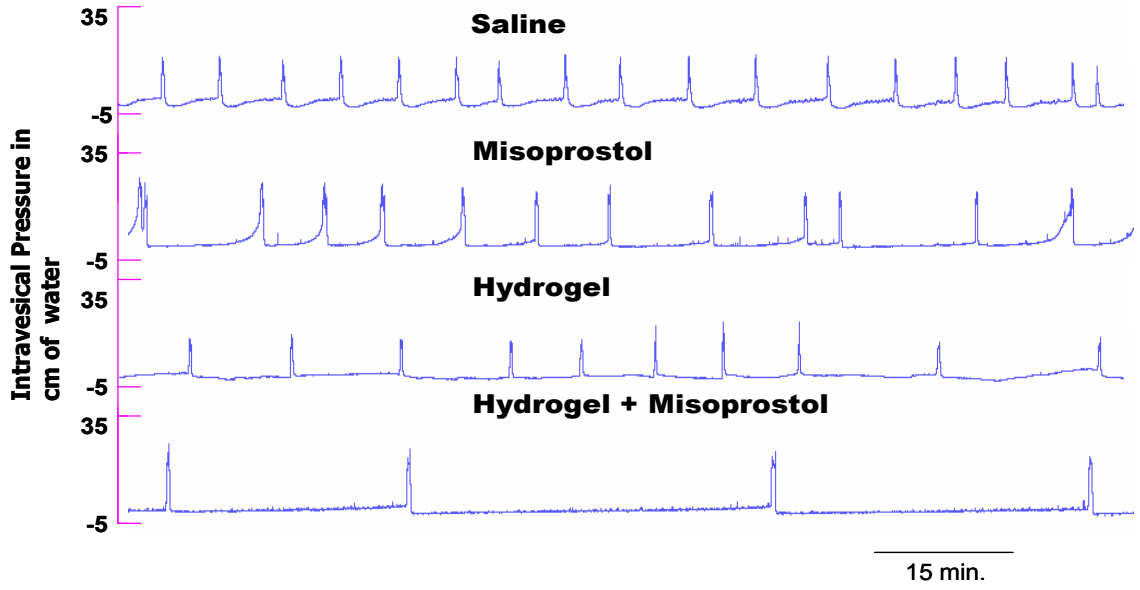

Figure 4.4: Representative cystometry recordings illustrating the effect of intravesically delivered misoprostol in rats treated with cyclophosphamide.

Cyclophoshamide administration markedly increased micturition frequency as seen in the topmost panel representing saline treated rats. Cyclophosphamide induced increased micturition frequency was dramatically reduced in the rats instilled with misoprostol entrapped in hydrogel (lowermost panel). Second and third panel from the top are from rats treated with misoprostol in saline and hydrogel alone, respectively both of which offered only marginal improvement over saline instillation. Scale on vertical axis is intravesical pressure in cm of water (cm/w) and time is on the horizontal axis.

Cystitis induced by injection of cyclophosphamide leads to urinary incontinence in rats marked by wet and dirty perineal region around urethra. Rats instilled with saline had similar

appearance during their stay in the metabolic cage (data not shown). Rats kept in metabolic cages after being instilled with misoprostol entrapped in hydrogel appeared to be continent after cyclophosphamide injection. Cystometric measurement performed under urethane anaesthesia evaluated the micturition reflexes induced by saline infusion with a transurethral catheter at a rate of 0.04ml/min, which approximates the rate of physiological bladder filling with urine. When the bladder was continuously filled, multiple contractions were elicited and the number of micturitions (indexed by peaks in the cystograph) evoked over a 60 min infusion period was used to describe micturition frequency.

The micturition frequency, which was used to compare treatment groups with control groups. CMGs performed on rats 24h after cyclophosphamide injection showed a dramatic decrease in the frequency of micturition in the rats instilled with misprostol in hydrogel (0.04167 ± 0.01014) compared to rats instilled with saline alone (0.1583 ± 0.04640 n=3 $p<0.05$)(Fig.4.4). Histological assessment done by H&E staining revealed severe ulcerative cystitis in the saline instilled rats after cyclophosphamide injection, Panel A of (Fig.4.5).

Epithelium was denuded leaving an ulcerated area with submucosal edema, inflammation and vascular ectasia and congestion. Top left Panel A showed severe lesions in the epithelium, extensive submucosal edema, and multiple bleeding vessels in subepithelium in saline instilled rats. In tissue sections obtained from rats treated with misoprostol, shown in Panel B and hydrogel separately Panel C, hisotological changes were less severe which included infiltration of inflammatory cells and edema of subepithelial tissue layer responsible for its slightly enlarged appearance. Lower right panel D reveals the resistance afforded by misoprostol entrapped in hydrogel from the chemical cystitis induced by cyclophosphamide with only slightly enlarged subepithelial layer due to edema (Fig.4.5).

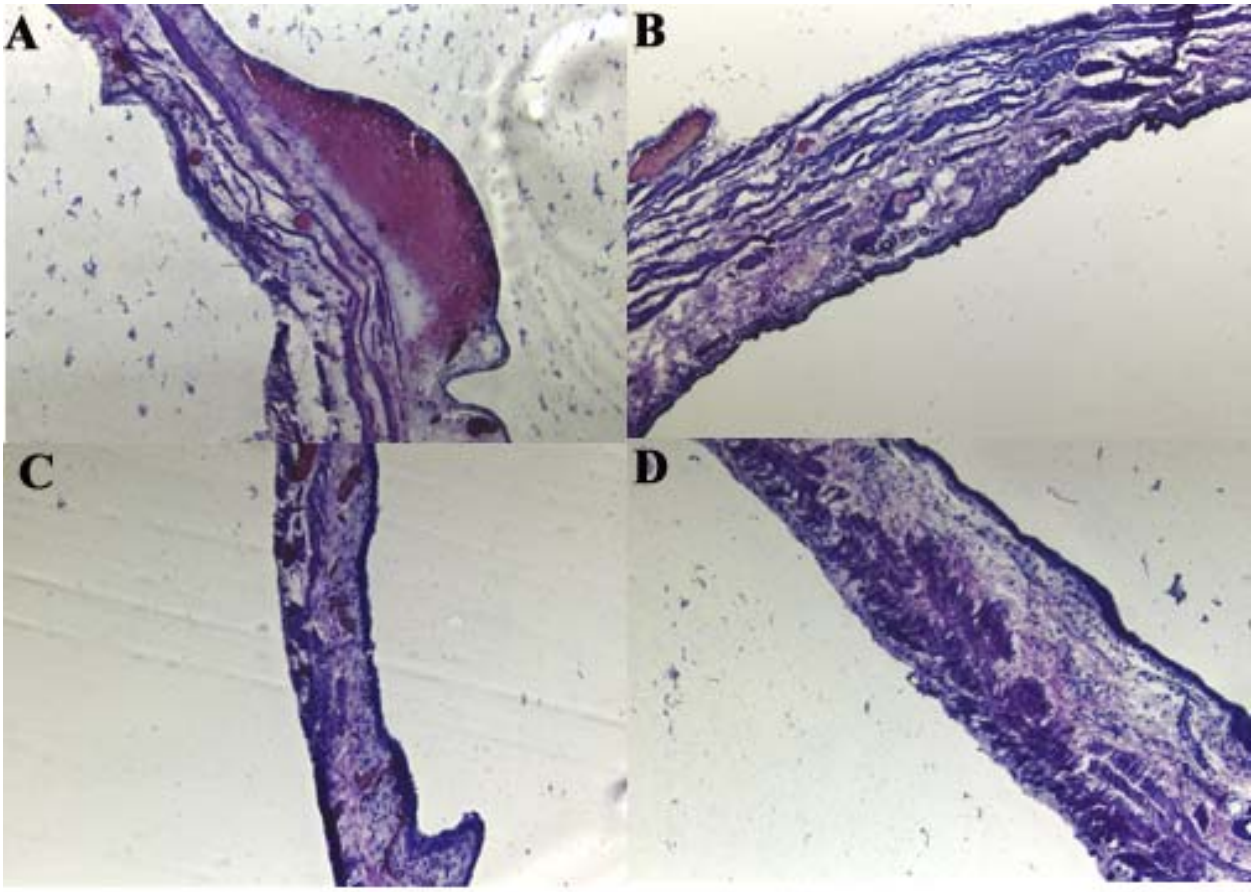

Figure 4.5: Haemtoxylin and Eosin staining of cyclophosphamide treated rat bladders in representative cross sections.

Sections in top right panel B and lower left panel C were from rats instilled with misoprostol and hydrogel *alone*, respectively, showing changes such as edema, moderate vascular ectasia and vascular congestion compared to saline treated rats. Treatment with misoprostol in hydrogel showed drastic improvement, which was evident by lack of any ulcerations in the normal appearing epithelium with slightly enlarged appearance in panel D (lumen side and bleeding spots are marked by white arrow in all sections and magnification is 10X.

4.3. Discussion

Intravesical drug delivery continues to remain an ideal treatment option for therapy of superficial bladder cancer; since drug exposure is restricted only to the disease site and systemic exposure to toxic anticancer drugs is avoided (250). In recent years, intravesical route has been exploited for effective therapy of other bladder diseases such as neurogenic bladder (263). Instillation of C-fiber neurotoxin into the neurogenic bladder of spinal cord injury patients, limits

the potent action of capsaicin only to the afferent fibers in the bladder wall and circumvents its possible systemic neurotoxicity (159, 264).

However, conventional vehicles used for the intravesical route of administration fail to provide a sustained exposure of drug to the urothelium. Duration of exposure for instilled drugs does not last beyond the first voiding of urine after instillation. Increase in efficacy of drugs used for intravesical therapy would be expected, if the duration for direct contact between the drug and the abnormal urothelium were increased (145). Polymers that display a physicochemical response to stimuli have been widely explored as potential drug delivery systems. Therefore, in our present work, we explored the effectiveness of a modified PEG-PLGA-PEG polymer as a feasible hydrogel matrix for sustained delivery by the intravesical route. The thermosensitive polymer used in our study offers several advantageous over common drug delivery systems. First, the formulation is simple and requires no organic solvent. Second, the products from bioerosion of this nontoxic biocompatible polymer are polyethylene glycol (PEG), glycolic acid and lactic acid, all of which are nontoxic, making the polymer biocompatible (265). It was found that water penetration within the hydrogel changes with the change in hydrophilic PEG content of the polymer (266).

We observed no significant difference in urine output with the treatment of hydrogel during the 24h period (Fig.4.1) indicating that the soft gel did not obstruct the elimination of urine from the bladder. However, the difference observed in the cumulative urine output between two groups did not proved to be statistically significant. The picture shown in Fig. 4.3 provides the most plausible explanation for the data shown in Fig.4.1. The photograph of the bladder taken 24h after instillation indicates that hydrogel did not completely fill the bladder, rather it attach itself as a smooth layer on the inner surface of the bladder. The presence of the gel inside

the bladder after instillation of a free flowing liquid indicates that this vehicle could produce a prolonged drug exposure to the urothelium, even after multiple voidings post-instillation of the hydrogel.

It follows naturally from the retention inside the bladder of hydrogel delivery system, that it can withstand the hostile environment of rat urinary bladder. The hydrogel could also resist being washed away by the flush of urine during the multiple voidings that occurred during 24h. The higher fluorescence signal in the control group at the initial time point of 2h demonstrates the drawback of conventional vehicles used for intravesical drug delivery (Fig.4.2). Animals instilled with FITC entrapped inside a hydrogel showed a lower fluorescence at the early time point presumably because FITC did not exist in the bladder as free FITC, but rather it was sequestered in the hydrogel and was released over a period of time.

According to earlier reports on release profile of drugs contained in the sol-gel transition system, it is probable that FITC is released at first by diffusion, and later by the combination of both diffusion and degradation mechanisms (140). The reversible nature of gelation process of a thermosensitive hydrogel may also facilitate easy removal of the gel from the bladder if desired at any time after administration by simply rinsing the bladder with sterile water at room temperature. A lower temperature of instilled water should convert from the gel state of the polymer back to its sol state allowing it to be voided in the urine.

To answer the question, whether a drug entrapped in the hydrogel is available for its therapeutic action, we tested intravesical misoprostol in a rat model of cyclophosphamide cystitis. A previous study done on the same model reported reduction in cyclophosphamide induced ulceration, inflammation and edema in bladder walls of male rat following long term oral administration of misoprostol (267). A daily oral dose of 600µg administered chronically for

3 months was also effective in patients with refractory interstitial cystitis (268). Local therapy with prostaglandins has been shown useful for cyclophosphamide-induced cystitis in patients (112-114). A 100ml irrigation of 5µg/ml PGE2 into the bladder for 3h completely freed the 4 year old patient of all the symptoms within 24 hours (115). In our study, we chose to use a fraction of the oral dose of misoprostol for a single intravesical administration in the same rat model of cystitis. Urinary incontinence induced by cyclophosphamide was observed during the 24h metabolic cage stay of the rats. Simultaneous instillation of misoprostol in hydrogel enabled rats to maintain continence following intraperitonal injection of cyclophosphamide. Cystometric measurements showed that misoprostol entrapped in hydrogel is available for its biological action as is evident from the decreased bladder contraction frequency in misoprostol treated rats (Fig.4.5). Rats instilled with hydrogel without misoprostol offered improvement in micturition frequency over saline instillation and benefit was comparable to rats instilled with suspension of misprostol (tablet powder). The beneficial effect observed in rats instilled with hydrogel itself without any drug might be a consequent of its emollient properties as hydrogel with similar composition have shown excellent wound dressing activity (269). It is possible that intravesically administered hydrogel might help in healing of ulcers induced by cyclophosphamide and wound healing effect of hydrogel might underlie the benefit seen in Fig.4.4.

As previously reported from our lab that in absence of cyclophosphamide injection, instillation of hydrogel in normal rats did not significantly change micturition frequency compared to rats instilled with saline (197). Effect of misoprostol alone in improving micturition frequency is expected from the local action of PGE1 analogue on the urothelium mucosa. Histological examination (Fig.4.5) seem to support our explanation for Fig.3 as well as affirms that the uroprotective action of misoprostol is retained when delivered intravesically using a

hydrogel in rat model of cystitis. Results of our efficacy study agree with observation reported from another lab following oral administration of misoprostol in cyclophosphamide cystitis (267).

Conclusions

The first successful use of a modified thermosensitive PEG-PLGA-PEG polymer as a suitable matrix for sustained drug delivery by intravesical route is described in this present chapter. Modifications introduced into the polymer in our lab, enable the sol-gel transformation of this thermosensitive polymer to occur even in the presence of typical urine constituents, urea and excess electrolytes to extend its application for intravesical therapy of bladder diseases. Safe and long-acting intravesical delivery of misoprostol may be a desirable new treatment for interstitial cystitis. However, further safety studies of the polymer following intravesical instillation are needed before it becomes a clinically feasible drug delivery system. Successful delivery of small molecular weight drugs such as capsaicin and misoprostol prompted us to investigate the delivery of peptide nucleic acid a large molecular weight drug.in the next chapter of this dissertation. TAT peptide has been used to deliver large mol.wt drugs such proteins and oligos in studies done earlier. However its non-selective penetration following systemic delivery is a concern that can be obviated by regional administration. TAT peptide was used in the following chapter to study intravesical antisense therapeutics.

5. INTRAVESICAL ANTISENSE THERAPY OF CYSTITIS USING TAT-PNA CONJUGATES

Overexpression of nerve growth factor (NGF), a neurotrophic factor, has been implicated in mediation of persistent pain states such as interstitial cystitis (IC) (104). The present study was aimed at delivering antisense molecule to this key putative molecular target identified in IC. A member of a growth factor family known as neurotrophins, NGF was originally identified by its ability to promote the survival of sensory and sympathetic neurons during development (270). It is a 13.6 kD natural protein, composed of a high-molecular-weight 7S NGF complex a dimmer of two α and a low-molecular-weight protein called βNGF, or 2.5S NGF, dimer of two β subunits (271). The biologically active β subunit is proteolytically cleaved from a precurosor called prepro NGF, which is translated from the 3′ exon of NGF mRNA (104).

Adenoviral mediated transfection of NGF gene in rat bladder was able to transiently increase NGF expression in rat bladder, which was responsible for sensitizing afferent pathways and bladder overactivity in the absence of any inflammation (272). Microarray analysis done on rodent models of IC also implicated overexpression of βNGF gene as a primary cause of the disorder (273). Moreover, increased NGF followed the increase in the level of NGF mRNA in response to chemically induced bladder inflammation in rats (274). NGF can either activate mast cells via its trkA receptor or it can cause their degranulation to produce hyperalgesia through sensitization of nociceptive sensory neurons as well as increase their proliferation (274, 275). Elevated levels of NGF protein were also found in the bladder of IC patients (39).

Most groups have demonstrated bladder urothelium to be the primary source of NGF in neurogenic inflammation of murine bladder and in patients with idiopathic sensory urgency (36, 39). In a study done on patients with idiopathic detrusor instability, NGF was determined to be

produced by bladder smooth muscle (276). Neutralization of NGF by antibody has been demonstrated to be effective in blocking bladder afferent sensitization in spinal cord injured rats (277). Recombinant antibody against NGF is effective in reducing the severity of IC in patients (Abstract # 363; AUA abstract book 2004). Although short term therapeutic use of monoclonal antibody in humans appears to be relatively safe, there are anecdotal accounts of antibodies used in disorders unrelated to IC causing severe side effects in a few patients (278). Moreover, development of anti- antibody responses cannot be ruled out, which can diminish efficacy on chronic use for even "humanized" antibodies (279).

In the present study, we explore an alternative approach for downregulation of NGF expression by using antisense approach. Antisense based therapy has begun to gain acceptance from the clinicians and this technology has emerged as a major tool in deciphering function of new genes in the era of post- human genome project. Peptide nucleic acids (PNAs) have been used for their antisense effect in various studies, because they form stable duplexes with the target mRNA and arrest translation (127, 128). PNA was chosen in this study owing to its superior binding properties, and higher stability in biological media over a wide pH range, compared to traditional oligos and ribozymes (129, 130). PNAs offer an exciting option for silencing gene expression in mammalian cells, if strategies can be developed to improve their poor intracellular delivery into mammalian cells (280). Non-selective cellular uptake of macromolecules including oligonucleotides has been reported to be facilitated by a protein transduction domain (PTD) of the TAT protein of human immunodeficiency virus (280). Moreover, a 11-mer **(RRRQRRKKRGY)** cell penetrating peptide derived from its PTD can even penetrate blood brain barrier and most other tissues (124). TAT peptide has been previously used to enhance the penetration of antisense PNA molecule in cell cultures (281). In the present study

we use the same peptide to deliver PNA across the rat urothelium, after intravesical instillation, for blocking NGF overexpression.

5.1. Materials and Methods

5.1.1. Design of Antisense Sequence.

We utilized minimum free energy algorithm available on the web to predict RNA secondary structure (using *m*fold server with *m*fold version 3.1 (282) of the 3′exon of rat βNGF mRNA (283). The predicted secondary structure estimation of local folding and possible unpaired regions such as loops and bulges is shown in Fig.1a and b). The following sequences were selected for *in vitro* evaluation. Sequence K – TAACGATAGACACATGCC was complementary to the region close to the adjoining 5′ intron from nt 347-364. Similar was the case for sequence Q- GCCCGAGACGCCTCCCGA from 427-444, sequence B-TACACCTTCTGACCCAC from 661-678, sequence T- TGGAGGTCCGTCGTCGGA from 939-956 and sequence A-AGTACGTCAGGAATATTA from 1097 to 1114 in the 3′ UTR . A randomly scrambled control of identical length was also used in all the experiments.

5.1.2. Identification of Active Antisense Oligonucleotide.

The activity in the panel of candidate oligos having theoretical binding affinity to discrete sites in 3′ exon of NGF mRNA was evaluated in Griptite HEK293 cells (Invitrogen) transfected with cDNA coding for rat βNGF. The rat βNGF cDNA was a gift from Dr. William Goins, University of Pittsburgh. Griptite HEK293 cells were grown in DMEM medium supplemented with 10% fetal bovine serum and 5% non-essential amino acids. Cells were plated overnight in 24 well plates. Phosphorothioate (PS) oligonucleotides with different sequences were procured from Integrated DNA Technology Inc. Cationic liposomes composed of DOTAP (1,2-dioleoyl-3-

trimethyl-ammonium-propane) were used as transfection reagent for co-transfecting antisense oligos and cDNA coding for rat βNGF for a transient production of NGF. Forty eight hrs after transfection, NGF secreted into the cell medium was measured by ELISA. The isolated culture media samples were stored at -20°C until assay. The samples were assayed in triplicate in an antigen capture ELISA Emax ImmunoAssay System (Promega, Madison, Wisconsin) according to manufacturer instructions. ELISA plates were read at 450 nm on an Elx800 microplate reader (Bio-Tek Instruments, Winooski, Vermont).

5.1.3. Synthesis of TAT-PNA

The protein transduction domain (PTD) from the HIV-1 TAT protein, residue 47 -57, GGGGYGRKKRRQRRR-COOH known as TAT peptide was synthesized at the University of Pittsburgh Peptide Synthesis Facility. The peptide had its 9-fluorenylmethyloxycarbonyl (Fmoc) protection intact on its N-terminal and was not cleaved from the synthetic resin. The TAT peptide bound to the resin was extended by Fmoc solid-phase synthesis of PNA by Expedite 8909 instrument (Applied Biosystems, Farmington MA). The first round of coupling linked the C-terminal residue of the PNA to the N-terminal of TAT peptide. The synthesized TAT-PNA conjugates were cleaved from the resin after synthesis, purified by reverse phase HPLC. Synthesis of full length TAT-PNA conjugate was confirmed by analyzing the HPLC purified compound by MALDI-TOF. The obtained mass numbers were nearly equal to the theoretical mass numbers.

5.1.4. Rat Bladder Uptake of TAT-PNA Post-Instillation.

To label the PNA, TAT-PNA was chemically coupled to fluorescent probe rhodamine isothiocyanate (TRITC, Molecular Probes) before cleaving the TAT-PNA conjugate from the

resin. To determine the bladder uptake of TAT-PNA conjugates, Sprague-Dawley rats (150-200g) were anaesthetized by halothane inhalation and instilled with 30μM of either labeled PNA or TAT-PNA -Rhodamine conjugate in a volume of 0.2 ml through a PE-50 plastic tubing (Clay-Adams, Parsippany, NJ) inserted into rat urethra. Bladder outlet was tied with a suture thread for 1h and 3h after instillation, the bladders were drained with 0.9% saline and animals were allowed to recover from the anaesthesia. Twenty four hours later, animals were sacrificed and bladders were isolated and cryopreserved. Six micron thick bladder sections were prepared by cryostat for confocal microscopy (Olympus, Fluoview). Sections were counterstained with sytox green dye.

5.1.5. Efficacy Evaluation of TAT-PNA conjugates.

Rats were injected with cyclophosphamide (CYP) (100mg/kg i.p.) to induce acute cystits. Under halothane anaesthesia, either saline, TAT-PNA conjugates with selected antisense sequence including its scrambled control were instilled into the bladder at the concentration of 100μM, 30 min prior to injection of cyclophosphamide. The volume for intravesical instillation was 0.5 ml in each animal. Two hrs after cyclophosphamide injection, rats were injected with urethane (1.2g/kg s.c.) to perform open transurethral cystometry. PE50 tubing (Clay-Adams, Parsippany, NJ) was inserted into the bladder through the urethra. The catheter system was filled with 0.9% w/v saline and cystometrogram (CMG) was performed by filling the bladder with a constant infusion (0.04 ml/min) of saline by a syringe pump. The gradual rise in the internal pressure of bladder (intravesical pressure) caused by this slow filling was monitored by a pressure transducer connected to a side arm of the filling catheter. The frequency of reflex bladder contractions per minute was recorded. Measurements in each animal represented the

average of 3 to 5 bladder contractions. All animal experimental protocol was approved by the institutional animal care and use committee (IACUC) of University of Pittsburgh.

5.1.6. Histopathological analysis

After cystometry, whole bladders were harvested from the animals, fixed in 10% buffered formalin, and cryopreserved. Tissue blocks were sectioned (20 μm thickness) for haematoxylin and eosin (H& E) staining and immunohistochemistry for NGF. The polyclonal antibody against NGF was raised in goat and bound antibody in the tissue sections was detected with anti-rabbit antibody labeled with Cy3.

5.1.7. Statistical analysis

Quantitative data are expressed throughout this paper as means $\pm$ standard error. Multiple comparisons among the different groups were analyzed by a single-factor ANOVA, followed by post hoc comparisons with Newman Keuls test, according to the Graph Pad prism v. 3.0 (GraphPad Software, San Diego, CA). Differences among groups were considered significant if $p< 0.05$.

5.2. Results

5.2.1. Selecting the Antisense Sequence

The minimum free energy algorithm helped to predict the secondary structure of rat βNGF mRNA (using *m*fold server with *m*fold version 3.1) (Fig. 5.1 & 5.2) (284). The sequence elements were then chosen and evaluated by *in vitro* screening using a transient transfection assay.

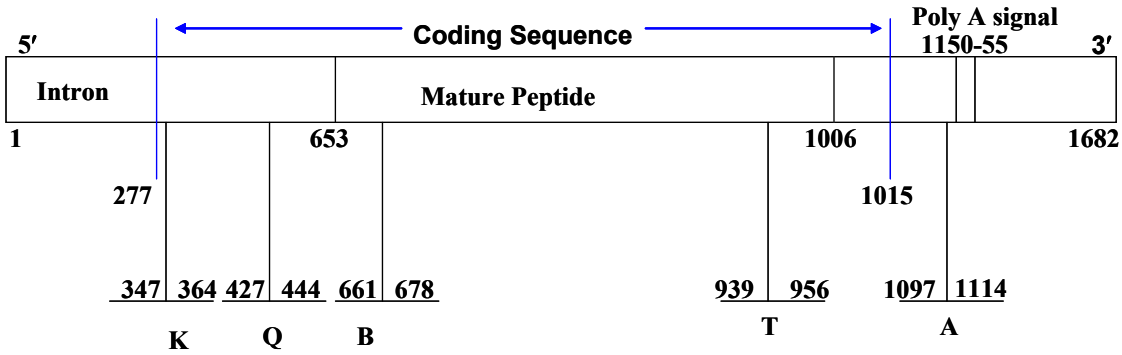

Figure 5.1: Schematic diagram of 3′ exon of rat NGF gene and the regions selected for designing antisense oligos.

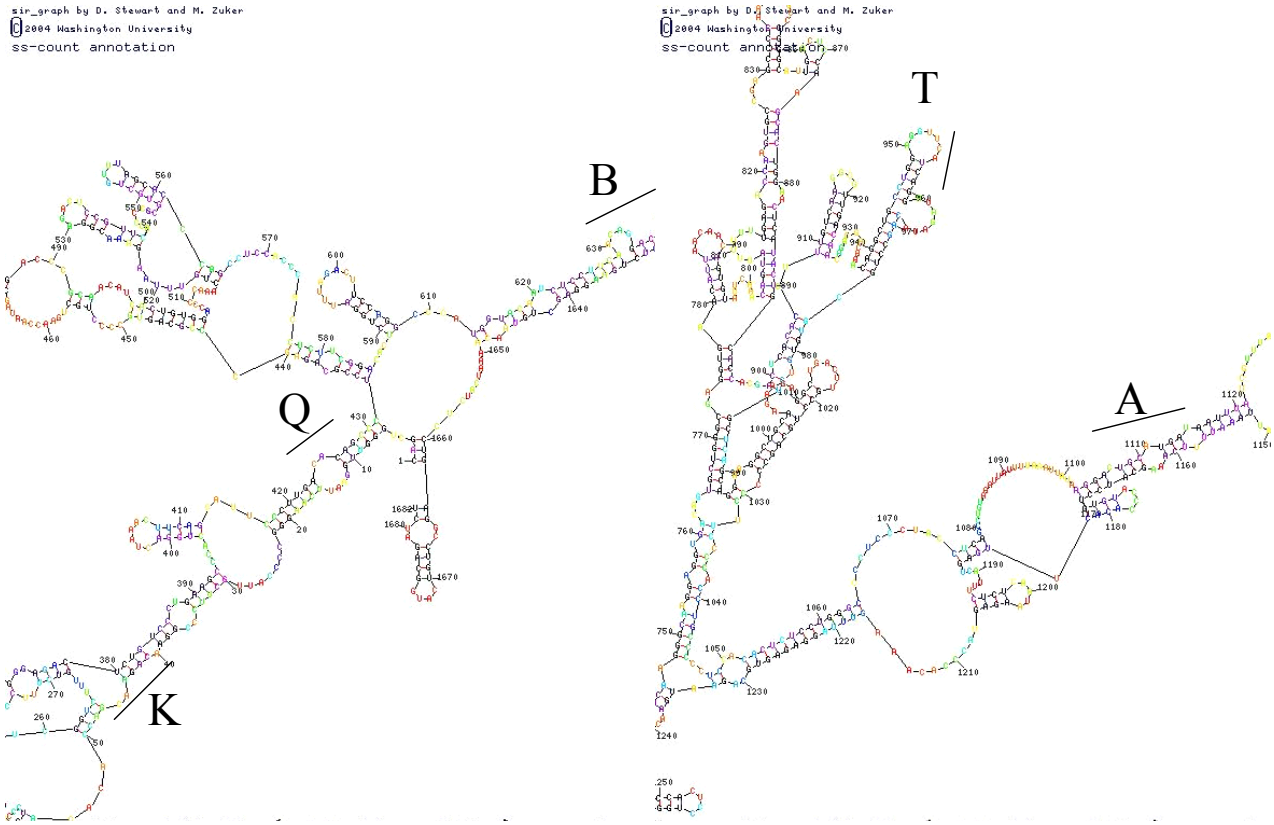

Figure 5.2: Folded structure of NGF mRNA as predicted by minimum free energy algorithm and local folding of selected regions.

Regions selected for binding are identified in the diagram are underlined by letter A,B,Q,K and T.

The oligos with sequence T, Q and A were able to decrease the NGF expression in transfected cells (Fig. 5.3) and sequence T was identified to be the most effective in blocking the NGF expression with minimal effect on cell viability measured by MTT assay. The decrease in NGF protein was sequence specific as scrambled control did not show activity.

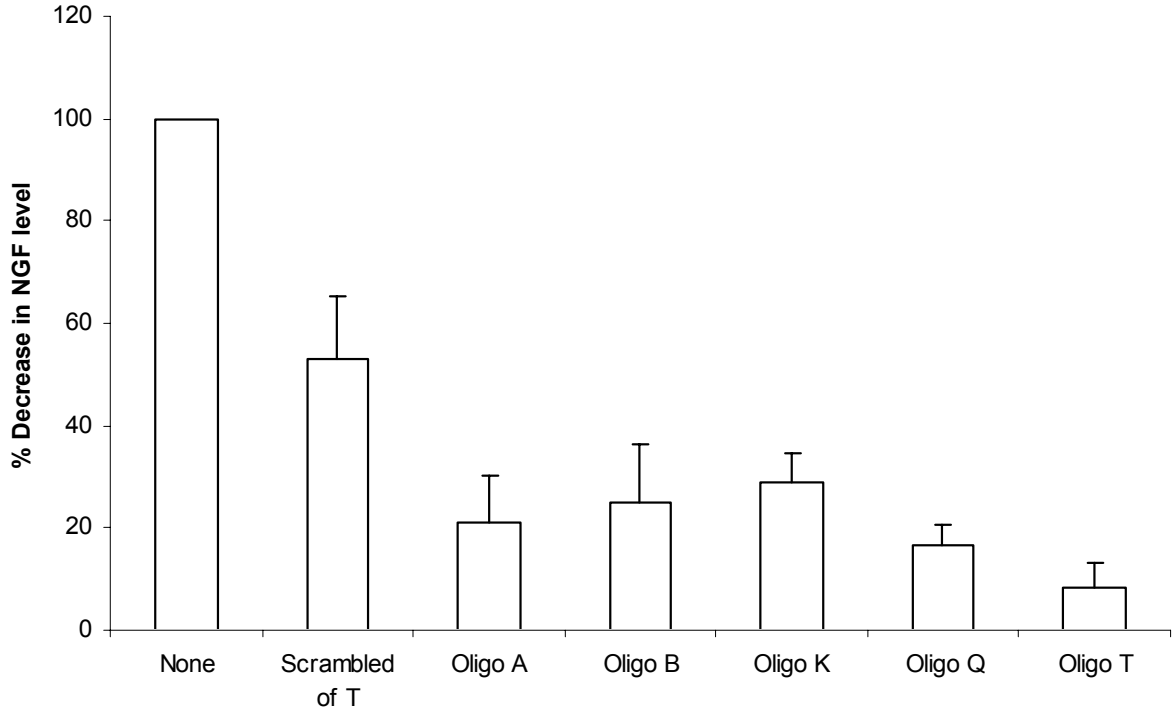

Figure 5.3: The decrease in NGF expression measured by ELISA in the cell media of HEK293 co-transfected with NGF cDNA and antisense phosphorothioate oligos.

5.2.2. Bladder Uptake

The confocal images of 6 μm bladder sections taken from the bladder sections were able to localize the rhodamine fluorescence alongside the fluorescence from nuclear counterstain, 24 h after instillation (Fig. 5.4). Co-localization of green fluorescence from nuclear counterstain with the red fluorescence from the rhodamine probe used in our experiment, indicate that PNA covalently tethered to the 11-mer cell penetrating peptide could successfully penetrate into the

cells of the urothelium. The penetration of TAT-PNA into top cellular layers of urothelum is evident from the overlap yellow color seen in panel C and F of Fig.2 due to the merge of the red fluorescence of rhodamine with green fluorescence of sytox counterstatin

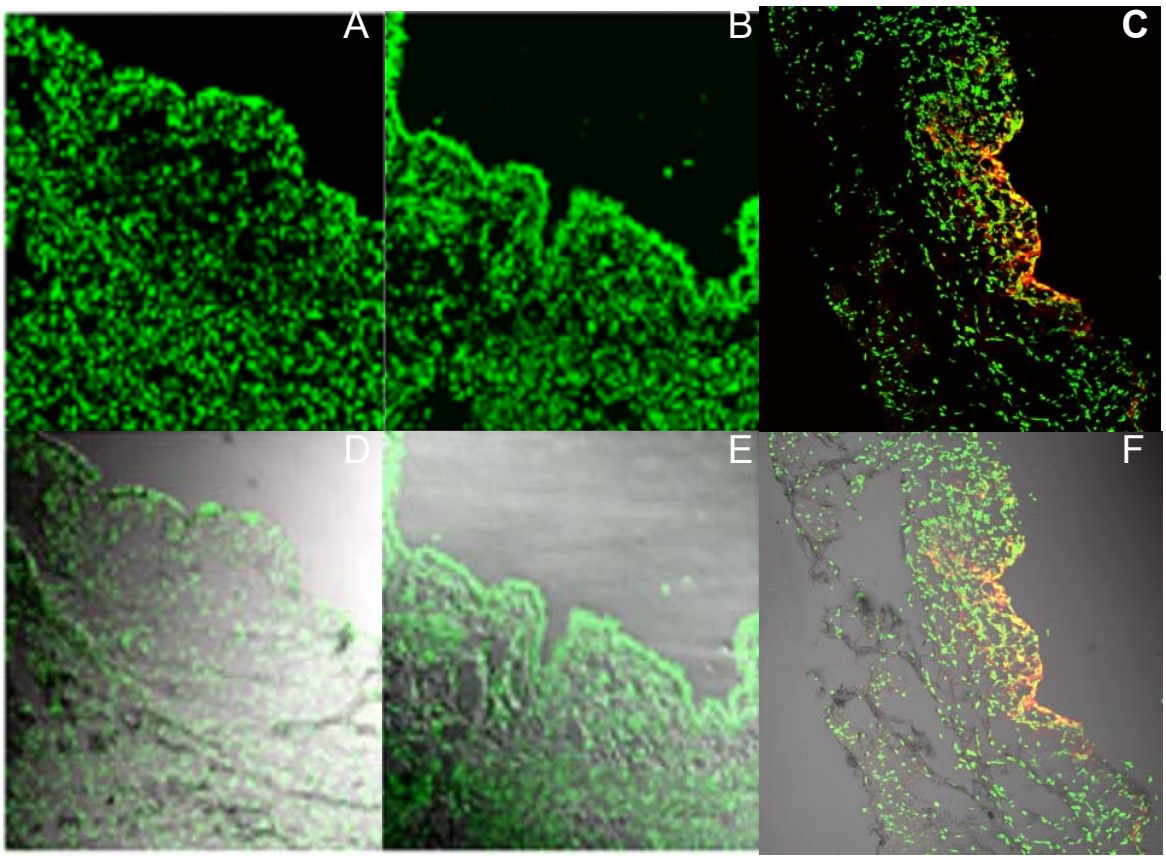

Figure 5.4: Confocal microscopic images depicting bladder uptake of TAT-PNA.

Photomicrogaphs of rat bladder taken with confocal microscopy after instillation of rhodamine labeled PNA alone or covalently tethered with TAT peptide. Cryopreserved rat bladder sectioned into 6 micron thick sections were counterstained with sytox green dye to stain nuclei. Top panel A and B show absence of any red fluorescence, which represents untreated rat bladder and bladder instilled with free PNA conjugated with rhodamine, respectively. Red fluorescence of rhodamine is seen overlapping with green fluorescence in panel C, which represent bladder instilled with rhodamine labeled PNA conjugated with the TAT peptide. Panel D, E and F are overlay images of panel A, B & C with their respective bright field images. Magnification is 20X in all sections.

Bladders treated with PNA alone without the tethered TAT did not show any rhodamine fluorescence (Panels B and E, Fig. 5.4) indicating TAT was required for successful delivery of PNA to the cells in the urothelium.

5.2.3. *In vivo* Efficacy

The activity of sequence T was further confirmed *in vivo* by evaluating the efficacy of antisense TAT-PNA having exactly the same sequence as oligo T in cyclophosphamide induced cystitis in rat.

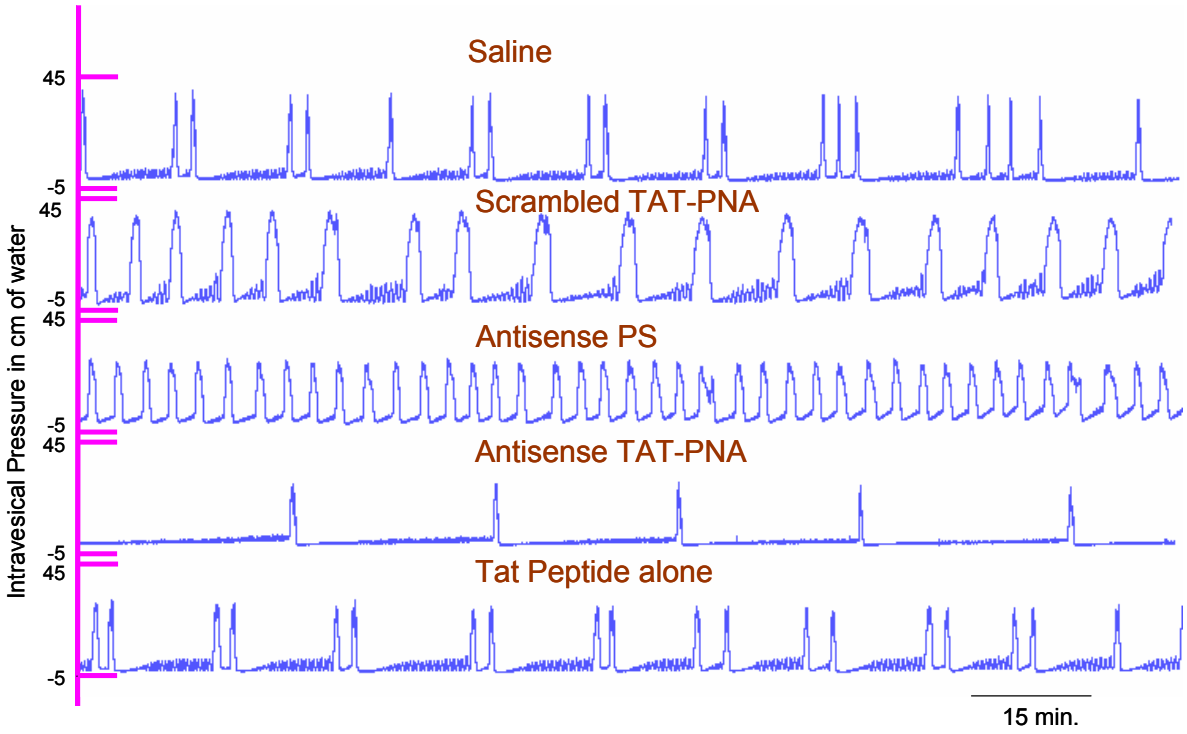

Figure 5.5: Effects of intraperitoneal injection of cyclophosphamide (100mg/kg) on cystometrograms in rats instilled with antisense sequence against rat βNGF mRNA.

Note that in rats instilled with antisense TAT-PNA, the cyclophosphamide induced bladder hyperactivity was suppressed.

Cystometrograms (CMG) performed on rats was one of the outcome measures for the efficacy. The slow filling of bladder during CMG gradually raises the internal pressure of bladder, known as intravesical pressure and micturition contractions are elicited. These reflex contractions are triggered by afferent activity of the bladder wall. It is suggested that, hyperexcitablity of afferent bladder reflexes is partly mediated by NGF during inflammation

induced by cyclophosphamide (285). Pre-treatment of antisense PNA conjugated with TAT was effective in attenuating the bladder hyperreflexia induced by cyclophosphamide (Fig.5.5).

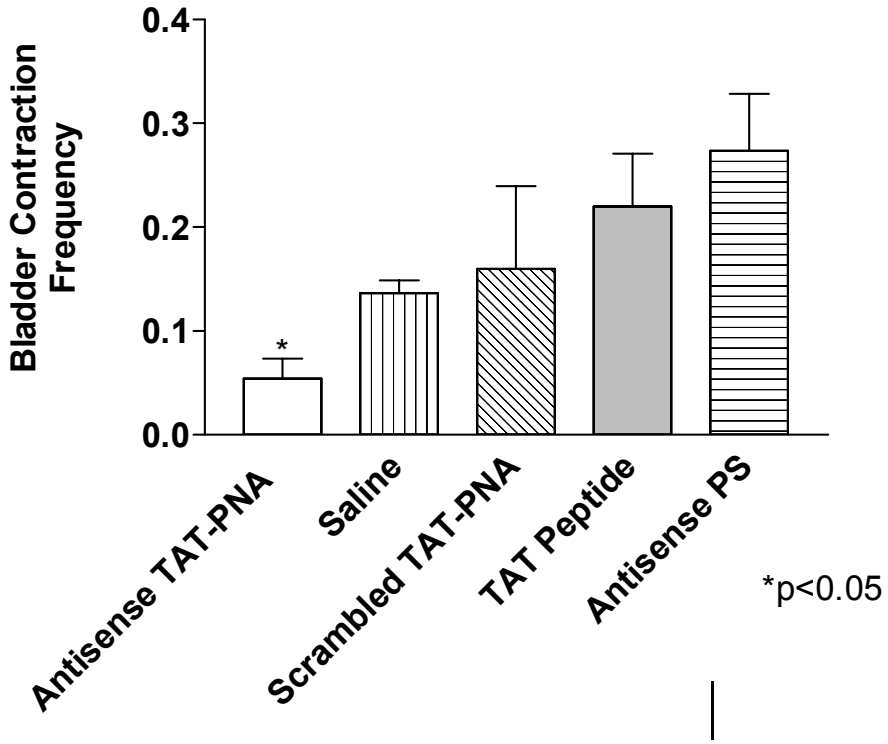

Figure 5.6: Bladder contraction frequency of rats with induced cystitis instilled with antisense sequence against rat βNGF mRNA using either phosphothioate (PS) oligonucleotide or TAT-PNA.

The bladder contraction frequency (BCF) was significantly decreased in rats instilled with antisense TAT-PNA compared to saline and other controls (*$p<0.05$, $n=5$). The rats instilled with TAT peptide alone and scrambled control showed BCF slightly higher than rats instilled with saline but the difference was not statistically significant.

The bladder contraction frequency in the antisense treatment group was significantly reduced compared to rats instilled with other treatments such as scrambled sequence or saline or TAT peptide alone (Fig. 5.6). Rats instilled with TAT peptide alone and scrambled TAT-PNA sequence showed a slightly higher bladder contraction frequency than the rat group instilled with saline, but the difference was not statistically significant. Phosphorothioate oligonucleotide of the antisense sequence and TAT-PNA with a scrambled sequence served as control as a control. The higher bladder contraction frequency observed in rats instilled with phosphothioate oligos is

probably due to non-specific interaction of polyanionic oligos leading to aggravation of bladder irritation caused by CYP injection.

5.2.4. Effect on Bladder NGF Levels

The presence of NGF in urothelium was detected by immunofluorescence. Twenty micron thick rat bladder sections were incubated overnight at 4^{o}C with anti-NGF antibody and then the bound primary antibody was localized with secondary antibody labeled with Cy3. The bright red fluorescence of Cy3 was visible in deeper layers of rat bladder in all treatment groups, indicating lack of change in expression of NGF in deeper layers (Fig. 5.7).

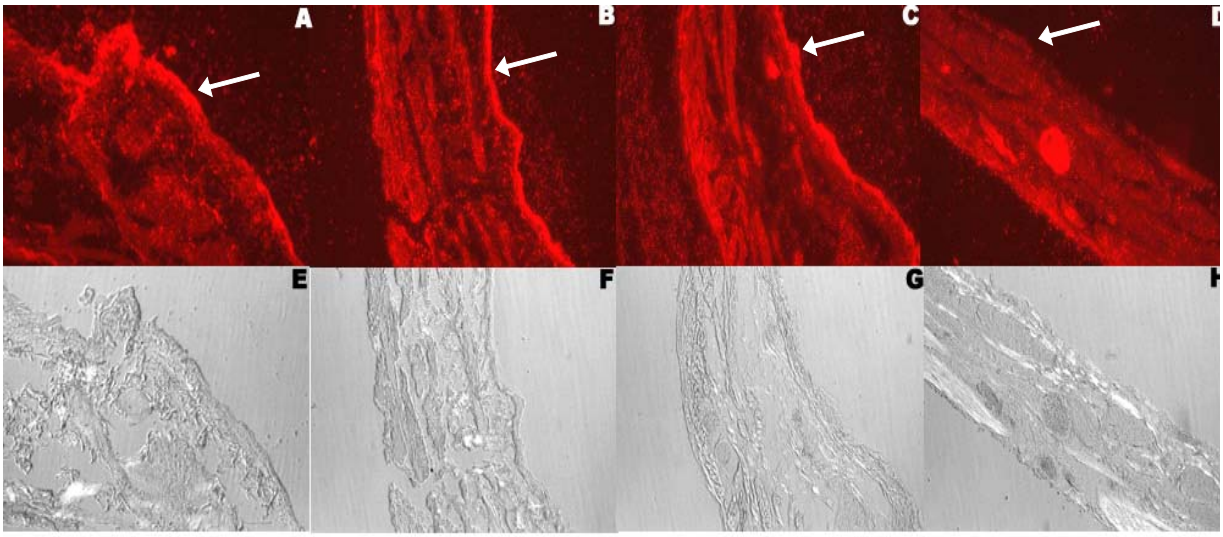

Figure 5.7: Effects of TAT-PNA antisense against βNGF mRNA on immunoreactivity of βNGF in rat urothelium.

Panel A is rat instilled with saline, panel B is rat instilled with Phosphothioate oligo, panel C is rat instilled with TAT-PNA of scrambled sequence. Bladder instilled with TAT-PNA of the antisense sequence is shown in panel D. The decrease in immunoreactivity of βNGF is apparent from decrease in the intensity of Cy3 stain in the urothelium (shown by white arrows) of rat instilled with TAT-PNA of the antisense sequence. Magnification in all of the sections was 20X.

The red fluorescence was seen in the urothelium (indicated by arrow) of all sections except in the urothelium (panel D) of bladder sections isolated from rat bladder instilled with TAT-PNA of the antisense sequence, indicating effective downregulation of NGF by TAT-PNA antisense. The activity was specific, because the bladder treated with TAT-PNA of a scrambled sequence showed high level of NGF in the urothelium (Panel C).

5.2.5. Effect on Bladder Histology

H&E staining of bladder sections revealed submucosal bleeding in most groups following cyclophosphamide injection (Fig. 5.8). The number of bleeding spots appearing red in the submucosa of bladder sections (marked by arrows) were similar in rats instilled with either saline, TAT peptide alone or TAT-PNA of a scrambled sequence (Panel B, D & E respectively).

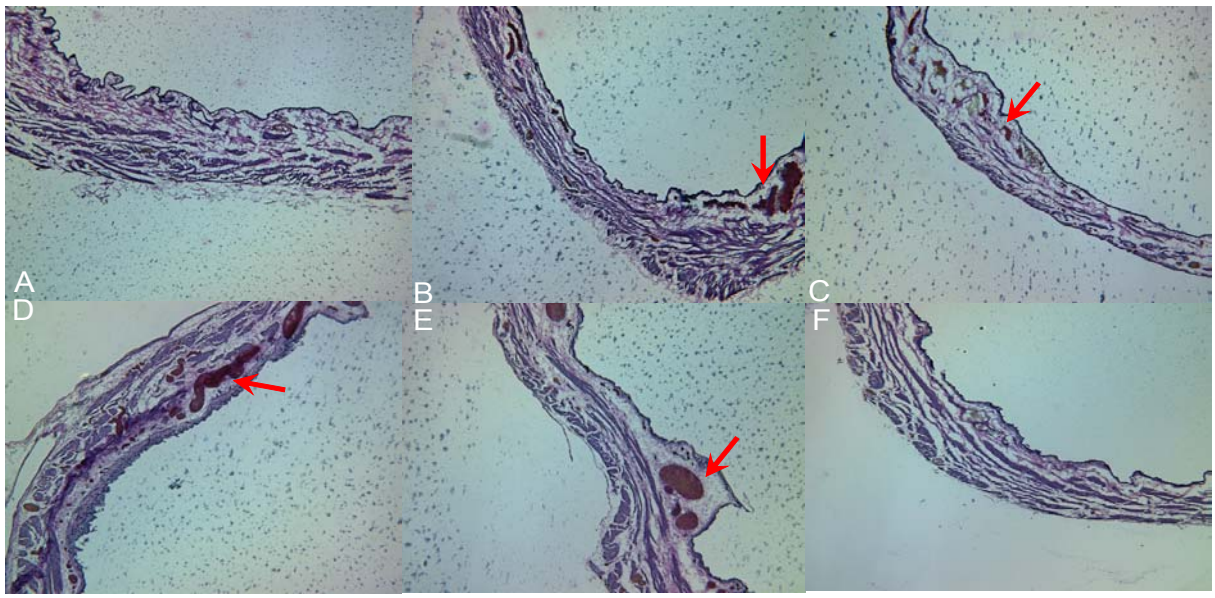

Figure 5.8: Bladder histology of untreated and cyclophosphamide treated rat bladders instilled with antisense against rat βNGF mRNA

Histology of bladder instilled with antisense TAT-PNA from a cyclophosphamide injected rat (Panel F) appeared similar to the bladder untreated with cyclophosphamide (Panel A). Bladder in rest of the panels were from rat injected with cyclophosphamide, Panel B saline, Panel C is phosphothioate oligo , Panel D and E are rat instilled with TAT peptide alone, and a scrambled TAT-PNA, respectively all showing evidence of vascular ectasia and vascular congestion. Magnification is 10X in all sections.

Bladders instilled with phosphothioate oligo with the antisense sequence were slightly better with respect to reduced areas of bleeding spots. The submucosal bleeding spots seen in rats treated with cyclophosphamide is indicated by red arrows and antisense treatment could afford near normal histology of the rat bladder.These results indicate that bladders instilled with antisense TAT-PNA were able to resist the inflammatory changes induced by cyclophosphamide. Such resistance activity appeared to be specific for the antisense effect, as it was absent in bladders instilled with the scrambled TAT-PNA.

5.3. Discussion

PNA is a second generation of antisense agents first reported by Nielsen et al (286). In a recent study, expression of common neurotrophin receptor p75NTR was reduced following intraperitoneal administration of an 11-mer antisense PNA and the age associated neurodegeneration was delayed in transgenic mice with amyotrophic lateral sclerosis (287). The present study is the first to report the delivery of TAT-PNA conjugates by intravesical route for blocking βNGF overexpression in the bladder. Systemic administration of any drug or an agent blocking NGF expression is bound to have adverse effects on long term use as NGF signaling subserves multiple neuroprotective and repair functions both in the central and peripheral nervous systems (288). Local delivery at the disease site could be a viable approach for blocking NGF expression with reduced toxicity. Indeed, therapeutic advantage of drug administration by intravesical route is underlined by high local drug concentrations with minimal systemic exposure.

In our study, the process of selecting a biologically active antisense sequence against βNGF was done in two steps: first, most favorable target site was selected with the aid of an

algorithm followed by a second and more focused, experimental procedure to identify the effective antisense sequence positioned within the predicted accessible target sites in mRNA (289, 290). Traditional PS oligonucletides were used for determining antisense sequence against βNGF in an *in vitro* screening assay using transiently transfected HEK 293 cells. NGF secreted into the cell media by transfected cells was measured by ELISA. Surprisingly, the selected T sequence was located near the 3'-UTR of the βNGF mRNA and it has been previously reported that AU nucleotide-rich sequence present around that region affects βNGF mRNA stability (291). The sequence of T oligo, which was effective in reducing NGF expression with minimal effect on cell viablity, was selected for *in vivo* experiments. PNA was selected to substitute for PS oligos in evaluating the therapeutic efficacy in cyclophosphamide cystitis.

The charged phosphothioester backbone in PS oligo is replaced by the uncharged N-(2-aminoethyl-glycine) units in PNA (127). The absence of a repetitive charged backbone avoids the intrastrand repulsion during hybridization to their target mRNA. The affinity of association obeys Watson-Crick hydrogen bonding rules and is independent of salt concentration (292). The achiral polyamide backbone not only provides resistance against nucleases and proteases (292), but nonspecific interactions arising from PNA binding to proteins that normally recognize polyanions are also prevented by the neutral backbone (293). However, antisense PNA uses a steric blocking of RNA translation or processing to decrease protein expression (294) Unlike the first generation antisense agents, PNA-RNA hybrids are not substrates for RNase H (129, 295).

The problem of poor cellular uptake of PNA has been resolved by conjugating cell penetrating peptides to inhibit gene expression in primary and transformed human cells (129, 294, 296). This approach is uniquely suitable for PNA, as most of the penetrating peptides are

highly positively charged which can form an intramolecular complex with the highly negatively charged oligos. In our study we examined the *in vivo* potential of tethering TAT peptide to aid translocation of PNA across the urothelium. Red fluorescence of rhodamine tagged to PNA was only visible in the rat bladder section administered the TAT peptide-PNA conjugates. Although, it has been previously reported that conjugation with cell-penetrating peptides such as TAT enhances delivery of PS oligonucleotides without interfering with their base-pairing function (126), but this observation was obtained from the study of physical interaction and effect on physiological activity was not determined. The exact mechanism of TAT transduction is unknown, but recently, convincing biochemical and genetic findings has established that the full-length TAT protein was internalized in cells via binding with the ubiquitous heparan sulfate proteoglycans (125, 297). The presence of the glycoaminoglycan layer at the apical surface of urothelium in rats and humans has been extensively demonstrated (18, 298, 299). Whether the action of TAT-PNA in the bladder is facilitated by the glycosaminoglycans is presently unknown.

A well established model of cystitis was used to evaluate the *in vivo* efficacy of TAT-PNA conjugates and bladder reflex activity was recorded by CMG, which is a suitable index for integrated neuronal response to inflammation induced by CYP. The physiological response of animals treated with antisense was evaluated by CMG, 4h after injection of CYP. Concentration of acrolein, an irritant CYP metabolite is at it peak around that time and gene expression of other mediators has been shown to reach its peak around 4h after CYP injection in rats (300-302). Rats instilled with antisense PNA showed a significantly reduced bladder contraction frequency compared to rats instilled with saline alone or other controls. Rats instilled with phosphothioate oligo exhibited significantly increased bladder contraction frequency compared to saline. It is

possible that nonspecific interaction of polyanionic oligos could aggravate the irritation of urothelium caused by acrolein produced from CYP. It was recently reported that full length TAT protein can upregulate Id1 expression and inhibit the nerve growth factor (NGF)-induced neuronal differentiation of PC12 cells (303). It is highly unlikely that a peptide having only the PTD of the TAT protein will have such deleterious effects. Moreover, such potential concerns for systemic toxicity from TAT peptide argue for local delivery of TAT-PNA conjugates. However, instillation of TAT peptide alone in CYP treated rats produced a physiological response similar to rat instilled with saline, indicating that the observed pharmacological effect of TAT-PNA antisense was not due to TAT, but resulted from the antisense effect of PNA.

Immunohistochemistry of the bladder sections cryopreserved after CMG showed a decrease in immunoreactive NGF only in the urothelium of rat bladder following instillation with TAT-PNA conjugates. This technique has been used in many studies to demonstrate depletion of NGF in bladder of rodents after instillation of LPS or IC urine (304, 305). However, in our study, when whole bladders were used for measuring total NGF by ELISA, we failed to show any difference between the treatment groups (data not shown). It is possible that pre-formed NGF residing in tissue layers lying below urothelium contributes to the bulk of the total NGF content of the bladder. Nevertheless, our study was able to demonstrate that modulating the NGF content in the urothelium can bring about changes in the physiological response of the bladder. Besides, histological studies performed on bladders also corroborated with the therapeutic benefit accrued from treating cyclophosphamide injected bladders with antisense PNA.

Our studies show that intravesical instillation of TAT-PNA conjugates are a feasible approach for determining and modulating molecular determinants of interstitial cystitis.

6. CONCLUSIONS AND FUTURE DIRECTIONS

The clinical approach to treatment of interstitial cystitis is currently largely empirical and can involve a variety of oral and intravesical therapies (48). Intravesical agents have been used for many years as adjuncts to oral treatment regimens or as second-line therapies for IC. Intravesical drugs delivery has potential and real benefits for patients having morbid adverse effects from oral administration. It is also advantageous for patients who have developed tolerance to drug effects and require higher levels of drug in bladder for therapeutic benefit. Compartively high drug concentration can be achieved in the urinary bladder with intravesical drug delivery and the incidence of systemic side effects can be lowered due to the relatively poor absorption of the drug from the bladder. These characteristics should ensure maximal therapeutic benefit to occur at the site most desired. However, the only FDA approved intravesical treatment option for IC available in USA is DMSO (70). DMSO has multiple effects, whose instillation leaves discomforting bad breath in patients and it has also been associated with rare cases of systemic contact dermatitis and eosinophillic cystitis (82, 83). Other intravesical treatment options such as BCG and heparin analogues failed to show effectiveness in phase III clinical trials.

Treatment of IC by intravesical route can be expensive if the treatment costs also include the costs of physician visits required in the treatment. Cost of treatment will also be higher if the man hours wasted in getting the treatment are counted and cost of catheters and lubricant is included. Insertion of catheter in the urethra for intravesical drug administration can be a cause of discomfort in some patients. There can be a risk of urinary tract infection and potential risk of a transient chemical cystitis from some drugs.

Intravesical Administration of Liposomes

A prior study from our lab reported a promising intravesical treatment option by administration of liposomes prepared with egg phosphatidylcholine and cholesterol in 2:1 molar ratio. Liposomes were able to provide immediate symptomatic relief by reducing bladder contraction frequency of hyperactive bladder induced by protamine sulfate and potassium chloride. Here we report the identification of chief therapeutically active lipid in the liposomal formulation used in the earlier study. It was determined that use of cholesterol is not necessary in reducing bladder hyperactivity and zwitterionic lipids were significantly better in reducing bladder hyperactivity than lipids having anionic or cationic charged headgroup. Liposomes prepared with natural lipids were found to have higher efficacy than synthetic lipids and synthetic lipids having unsaturation ratio in acyl chains similar to efficacy similar to natural lipids.

We found that a minor component of egg PC known as sphingomyelin was able to reduce bladder hyperactivity in bladder injury model. Moreover, metabolites of SPM added at low molar ratios were also able to increase the efficacy of inactive synthetic lipids. It seems efficacy of SPM in hyperactive bladder is augmented by the effects of its metabolites. Further studies are needed to determine the exact mechanism of action of SPM using sphingomyelinase knockout mouse or in different models of cystis. We measured physiological response of the bladder following liposome administration in the prescence of anaesthesai and future studies using animals in the awake condition are required to be done to rule out the variation from anaesthesia. The existing intravesical therapy for IC are capable of achieving immediate symptom relief only through destruction of nerve endings by administering neurotoxins such as vanilloids or

botulinum toxin in the bladder or using psychotropic drugs. The regeneration of nerves with a "sprouting" effect can not only restrict the relief from neurotoxins as short term, and subsequent recovery could possibly led to genesis of a higher density of nerve endings and cause hyperalgesia from enhanced neural activity. Liposomes prepared from egg PC and SPM are not neurotoxic unlike vanilloids, and therefore they need to be evaluated as a treatment option in the clinical treatment of IC.

Intravesical Administration of Capsaicin

The approach toward intravesical drug therapy that has probably received the most attention has been the administration of capsaicin into the bladder. Capsaicin obtained from chilli peppers acts on the afferent C-fiber vanilloid receptors TRPV1. Bladder inflammation can bring about functional alterations in bladder afferent pathways, which in the continent state is constituted by small myelinated Aδ fibers (45). Aδ-fibers ultimately send information about the state of bladder fullness or wall tension detected by mechanoreceptors to the pontine micturition center via the periaqueductal gray matter in spinal cord. In the healthy state unmyelinated C fibers mainly detect noxious signals and initiate painful sensations (32). Neuroinflammation seen in IC causes considerable reorganization of reflex connections of bladder afferents and capsaicin sensitive C-fiber afferents take over the role from Aδ-fibers and convey the signal of bladder fullness at reduced bladder capacity (45). Therefore, capsaicin induced nerve desensitization of bladder afferents is a logical and viable intravesical treatment option for IC patients. However, instillation of capsaicin in bladder is currently achieved by using ethanol as a cosolvent for saline owing to the hydrophobic nature of capsaicin.

We demonstrated that liposomes could be a suitable vehicle for capsaicin as they had comparable efficacy to the standard vehicle for capsaicin. Moreover, the morphology and histology studies on instilled bladder revealed liposomes were far superior vehicle for capsaicin than 30% ethanol in saline. We suggest clinical evaluation of liposoems as a vehicle for capsaicin based on the work done here. We failed to show any benefit from using hydrogel as a vehicle for capsaicin, although instillation of thermosensitive hydrogel in rat bladder proved to be safe. In future studies, we suggest evaluation of hydrogel as sustained delivery of capsaicin by loading higher amounts of drugs in gel. Higher amounts of drug will saturate the binding sites for capsaicin in hydrogel and sustained release of capsaicin would be observed during the slow degradation of polymer.

Sustained Intravesical Administration of Misoprostol

Intravesical administration may solve some of the intrinsic shortcomings in oral treatment of IC by overcoming the drug or formulation specific vagaries in absorption, metabolism and renal excretion (ADME). In a previous study the cytoprotective drug misoprostol was used in the clinic for treatment of IC. However it was to be administered for 6 months with thrice daily administration for achieving therapeutic benefit. Perhaps the low amounts of drug excreted in the urine following oral administration might explain the need for prolonged regimen for demonstrating efficacy.

Sustained intravesical delivery of drugs can ensure continuous presence of drug in the bladder and avoid intermittent catheterization. The concentration of drug in the bladder would be

constant without the peaks and valleys. Moreover, it should provide predictable, reliable therapy over an extended period of time.

In this work sustained intravescial drug delivery in rat bladder was achieved using thermosensitive hydrogel formed by the polymer PEG-PLGA-PEG. Fluorescien Isothiocyanate (FITC) was loaded in the hydrogel as a model hydrophobic drug and kinetics of its excretion in the rat urine was studied to demonstrate sustained intravesical drug delivery. FITC was replaced with the misoprostol to demonstrate therapeutic benefit from sustained drug delivery afforded by thermosensitive hydrogel as a vehicle. Misoprostol delivered by hydrogel was able to to protect the rat bladder in cystitis induced by cyclophosphamide injection. Effect of misoprostol delivered for the first time in the rat bladder via intravesical route was assessed by CMG and bladder histology. Misoprostol was able to decrease the bladder contraction frequency and bladder histology revealed protection against tissue damage caused by acrolein produced from cyclophosphamide.

Future studies in different model of cystitis are needed to support our observations and higher animals such as cats, dogs or pigs need to be used to confirm the efficacy of hydrogel administration. Work done here supports the evaluation of hydrogel in other bladder diseases such as overactive bladder and bladder cancer. Intravesical administration of oxybutinin for the treatment of refractory overactive bladder has been desperately seeking a vehicle for its intravesical administration and thermosensitive hydrogel would be option that certainly merit evaluation based on our work.

Intravesical Administration of Antisense TAT-PNA.

Intrvesical delivery allows site-specific drug delivery with a reduced side effect profile as compared to oral delivery systems either by avoiding first pass metabolism or by obtaining a local effect. NGF is shown to have beneficial and pathological effects. Peripheral administration of NGF is neuroprotective in multiple sclerosis and many labs have reported that intravesical administration of NGF can cause bladder hyperactivity.

Therefore any agent that aim to inhibit NGF action or downregulate NGF overexpression in the bladder of IC patients should ideally be administered locally to avoid deleterious effects arising from blocking the effects of NGF on neurons innervationg healthy tissues. NGF is a target tissue dervid neurotrophic factor and is produced in the bladder during inflammation and then migrates to the spinal cord to reorganize the micturition reflex. In the work presented here, we identified an antisense sequence against rat βNGF mRNA by using its thermodynamically stable secondary structure and evaluating the five selected regions in a co-transfection assay done on Griptite HEK 293 cells using NGF cDNA. Short length TAT peptide was conjugated with PNA to produce the antisense construct TAT-PNA with the ability to cross urothelium. TAT-PNA with effective anstisense sequence indentical to the sequence of active PS oligo from contransfection assay was synthesized using Fmoc solid phase synthesis.

The utility of coupling TAT peptide to the PNA sequence was demonstrated in bladder uptake studies done using TAT-PNA conjugated with rhodamine isothiocyanate. Bright red fluorescence visible in the cryosections of rat urothelium instilled with TAT-PNA and not with rhodamine labeled PNA.The in vivo efficacy of antisense TAT-PNA was shown in acute rat cystitis model induced by cyclophosphamide. NGF expression is maximal around 2 to 4 h following cyclophosphamide injection and mRNA for rat βNGF has short half life of 2h. Peptide

nucleic acid (PNA) produces its antisense effect by translation arrest and short life of βNGF mRNA justified half an hour prior administration of antisense TAT-PNA for demonstrating its efficacy in this animal model. Antisense TAT-PNA was able to suppress the bladder hyperactivity induced by cyclophosphamide and NGF immunoreactivity was reduced in the urothelium. Bladder histology studies showed reduced inflammation in rats treated with antisense TAT-PNA. The experiments failed to show a decrease in the NGF content of bladder by antisense treatments. This is most likely due to the preformed NGF existing in the deeper layers of urinary bladder.

Owing to the compromised antisense effect of PNA, we suggest use of small interfering RNA (siRNA) in future experiments. The bladder uptake experiments showed that uptake of TAT-PNA is only limited to urothelium and other cell penetrating peptides should be evaluated for deeper penetration. Chronic animal models of cystitis need to be used to demonstrate clearly the therapeutic benefit of intravesical antisense therapy.

BIBLIOGRAPHY

1. D. Porru, R. Politano, M. Gerardini, G. L. Giliberto, S. Stancati, L. Pasini, C. Tinelli, and B. Rovereto. Different clinical presentation of interstitial cystitis syndrome. *Int Urogynecol J Pelvic Floor Dysfunct* **15**: 198-202 (2004).
2. R. Peeker and M. Fall. Treatment guidelines for classic and non-ulcer interstitial cystitis. *Int Urogynecol J Pelvic Floor Dysfunct* **11**: 23-32 (2000).
3. Y. R. Logadottir, I. Ehren, M. Fall, N. P. Wiklund, R. Peeker, and P. M. Hanno. Intravesical nitric oxide production discriminates between classic and nonulcer interstitial cystitis. *J Urol* **171**: 1148-50; discussion 50-1 (2004).
4. A. Hosseini, I. Ehren, and N. P. Wiklund. Nitric oxide as an objective marker for evaluation of treatment response in patients with classic interstitial cystitis. *J Urol* **172**: 2261-5 (2004).
5. P. E. Silkoff, M. Carlson, T. Bourke, R. Katial, E. Ogren, and S. J. Szefler. The Aerocrine exhaled nitric oxide monitoring system NIOX is cleared by the US Food and Drug Administration for monitoring therapy in asthma. *J Allergy Clin Immunol* **114**: 1241-56 (2004).
6. L. Birder. Role of the urothelium in bladder function. *Scand J Urol Nephrol Suppl* 48-53 (2004).
7. R. Hicks, Ketterer B, and W. RC. The ultrastructure and chemistry of the luminal plasma membrane of the mammalian urinary bladder: a structure with low permeability to water and ions. *Philos Trans R Soc Lond B Biol Sci* **268**: 23-38 (1974).
8. M. L. Zeidel. Low permeabilities of apical membranes of barrier epithelia: what makes watertight membranes watertight? *Am J Physiol Renal Physiol* **271**: F243-245 (1996).
9. S. T. Truschel, W. G. Ruiz, T. Shulman, J. Pilewski, T.-T. Sun, M. L. Zeidel, and G. Apodaca. Primary Uroepithelial Cultures. A model system to analyze umbrella cell barrier function. *J Biol Chem* **274**: 15020-15029 (1999).
10. C. Stubbs, Ketterer B, and H. RM. The isolation and analysis of the luminal plasma membrane of calf urinary bladder epithelium. *Biochem Biophys Acta* **558**: 58-72 (1979).
11. H. O. Negrete, J. P. Lavelle, J. Berg, S. A. Lewis, and M. L. Zeidel. Permeability properties of the intact mammalian bladder epithelium. *Am J Physiol Renal Physiol* **271**: F886-894 (1996).
12. M. Born, I. Pahner, G. Ahnert-Hilger, and T. Jons. The maintenance of the permeability barrier of bladder facet cells requires a continuous fusion of discoid vesicles with the apical plasma membrane. *Eur J Cell Biol* **82**: 343-50 (2003).
13. G. Hohlbrugger. The vesical blood-urine barrier: a relevant and dynamic interface between renal function and nervous bladder control. *J Urol* **154**: 6-15 (1995).
14. C. L. Parsons, C. Stauffer, and J. D. Schmidt. Bladder-surface glycosaminoglycans: an efficient mechanism of environmental adaptation. *Science* **208**: 605-7 (1980).
15. C. L. Parsons, D. Boychuk, S. Jones, R. Hurst, and H. Callahan. Bladder surface glycosaminoglycans: an epithelial permeability barrier. *J Urol* **143**: 139-42 (1990).

16. C. L. Parsons. The therapeutic role of sulfated polysaccharides in the urinary bladder. *Urol Clin North Am* **21**: 93-100 (1994).
17. H. Tay, C. L. Parsons, and P. C. Stein. Electrophysiologic monitoring of the effects of soluble virulence factors produced by Escherichia coli infection in urine. *Urology* **48**: 389-92 (1996).
18. J. M. de Deus, M. J. Girao, M. G. Sartori, E. C. Baracat, G. Rodrigues de Lima, H. B. Nader, and C. P. Dietrich. Glycosaminoglycan profile in bladder and urethra of castrated rats treated with estrogen, progestogen, and raloxifene. *Am J Obstet Gynecol* **189**: 1654-9 (2003).
19. C. L. Parsons, G. Benson, S. J. Childs, P. Hanno, G. R. Sant, and G. Webster. A quantitatively controlled method to study prospectively interstitial cystitis and demonstrate the efficacy of pentosanpolysulfate. *J Urol* **150**: 845-8 (1993).
20. C. L. Parsons. A model for the function of glycosaminoglycans in the urinary tract. *World J Urol* **12**: 38-42 (1994).
21. J. S. Dixon, M. Holm-Bentzen, C. J. Gilpin, J. A. Gosling, E. Bostofte, T. Hald, and S. Larsen. Electron microscopic investigation of the bladder urothelium and glycocalyx in patients with interstitial cystitis. *J Urol* **135**: 621-5 (1986).
22. M. J. Chelsky, S. I. Rosen, L. C. Knight, A. H. Maurer, P. M. Hanno, and M. R. Ruggieri. Bladder permeability in interstitial cystitis is similar to that of normal volunteers: direct measurement by transvesical absorption of 99mtechnetium-diethylenetriaminepentaacetic acid. *J Urol* **151**: 346-9 (1994).
23. C. L. Parsons, M. Greenberger, L. Gabal, M. Bidair, and G. Barme. The role of urinary potassium in the pathogenesis and diagnosis of interstitial cystitis. *J Urol* **159**: 1862-6; discussion 1866-7 (1998).
24. M. C. Dupont, J. M. Spitsbergen, K. B. Kim, J. B. Tuttle, and W. D. Steers. Histological and neurotrophic changes triggered by varying models of bladder inflammation. *J Urol* **166**: 1111-8 (2001).
25. T. J. Christmas, J. Rode, C. R. Chapple, E. J. Milroy, and R. T. Turner-Warwick. Nerve fibre proliferation in interstitial cystitis. *Virchows Arch A Pathol Anat Histopathol* **416**: 447-51 (1990).
26. M. R. Saban, R. Saban, T. G. Hammond, M. Haak-Frendscho, H. Steinberg, M. W. Tengowski, and D. E. Bjorling. LPS-sensory peptide communication in experimental cystitis. *Am J Physiol Renal Physiol* **282**: F202-10 (2002).
27. R. Saban, N. P. Gerard, M. R. Saban, N. B. Nguyen, D. J. DeBoer, and B. K. Wershil. Mast cells mediate substance P-induced bladder inflammation through an NK(1) receptor-independent mechanism. *Am J Physiol Renal Physiol* **283**: F616-29 (2002).
28. J. Kitaura, T. Kinoshita, M. Matsumoto, S. Chung, Y. Kawakami, M. Leitges, D. Wu, C. A. Lowell, and T. Kawakami. IgE- and IgE/antigen-mediated mast cell migration in an autocrine/paracrine fashion. *Blood* **105**:3222-9 (2005).
29. T. C. Theoharides, D. Kempuraj, and G. R. Sant. Mast cell involvement in interstitial cystitis: a review of human and experimental evidence. *Urology* **57**: 47-55 (2001).
30. T. J. Christmas and J. Rode. Characteristics of mast cells in normal bladder, bacterial cystitis and interstitial cystitis. *Br J Urol* **68**: 473-8 (1991).
31. T. C. Theoharides and G. R. Sant. Hydroxyzine therapy for interstitial cystitis. *Urology* **49**: 108-10 (1997).

32. N. Yoshimura, S. Seki, M. B. Chancellor, W. C. de Groat, and T. Ueda. Targeting afferent hyperexcitability for therapy of the painful bladder syndrome. *Urology* **59**: 61-7 (2002).
33. M. Green, A. Filippou, G. Sant, and T. C. Theoharides. Expression of intercellular adhesion molecules in the bladder of patients with interstitial cystitis. *Urology* **63**: 688-93 (2004).
34. M. Leppilahti, P. Hellstrom, and T. L. Tammela. Effect of diagnostic hydrodistension and four intravesical hyaluronic acid instillations on bladder ICAM-1 intensity and association of ICAM-1 intensity with clinical response in patients with interstitial cystitis. *Urology* **60**: 46-51 (2002).
35. D. E. Bjorling, T. J. Jerde, M. J. Zine, B. W. Busser, M. R. Saban, and R. Saban. Mast cells mediate the severity of experimental cystitis in mice. *J Urol* **162**: 231-6 (1999).
36. D. E. Bjorling and Z. Y. Wang. Estrogen and neuroinflammation. *Urology* **57**: 40-6 (2001).
37. N. Yoshimura and W. C. de Groat. Increased excitability of afferent neurons innervating rat urinary bladder after chronic bladder inflammation. *J Neurosci* **19**: 4644-53 (1999).
38. A. Elbadawi. Interstitial cystitis: a critique of current concepts with a new proposal for pathologic diagnosis and pathogenesis. *Urology* **49**: 14-40 (1997).
39. E. M. Lowe, P. Anand, G. Terenghi, R. E. Williams-Chestnut, D. V. Sinicropi, and J. L. Osborne. Increased nerve growth factor levels in the urinary bladder of women with idiopathic sensory urgency and interstitial cystitis. *Br J Urol* **79**: 572-7 (1997).
40. C. J. Fowler. Bladder afferents and their role in the overactive bladder. *Urology* **59**: 37-42 (2002).
41. P. Y. Jen, J. S. Dixon, and J. A. Gosling. Immunohistochemical localization of neuromarkers and neuropeptides in human fetal and neonatal urinary bladder. *Br J Urol* **75**: 230-5 (1995).
42. X. Pang, J. Marchand, G. R. Sant, R. M. Kream, and T. C. Theoharides. Increased number of substance P positive nerve fibres in interstitial cystitis. *Br J Urol* **75**: 744-50 (1995).
43. A. Apostolidis, C. M. Brady, Y. Yiangou, J. Davis, C. J. Fowler, and P. Anand. Capsaicin receptor TRPV1 in urothelium of neurogenic human bladders and effect of intravesical resiniferatoxin. *Urology* **65**: 400-5 (2005).
44. L. S. Baskin and E. A. Tanagho. Pelvic pain without pelvic organs. *J Urol* **147**: 683-6 (1992).
45. C. J. Fowler. The perspective of a neurologist on treatment-related research in fecal and urinary incontinence. *Gastroenterology* **126**: S172-4 (2004).
46. V. M. Vemulakonda, G. T. Somogyi, S. Kiss, N. A. Salas, T. B. Boone, and C. P. Smith. Inhibitory effect of intravesically applied botulinum toxin A in chronic bladder inflammation. *J Urol* **173**: 621-4 (2005).
47. J. Nordling. Interstitial cystitis: how should we diagnose it and treat it in 2004? *Curr Opin Urol* **14**: 323-7 (2004).
48. M. B. Chancellor and N. Yoshimura. Treatment of interstitial cystitis. *Urology* **63**: 85-92 (2004).
49. J. R. Dell and C. L. Parsons. Multimodal therapy for interstitial cystitis. *J Reprod Med* **49**: 243-52 (2004).

50. K. J. Turner and L. H. Stewart. How do you stretch a bladder? A survey of UK practice, a literature review, and a recommendation of a standard approach. *Neurourol Urodyn* **24**: 74-6 (2005).
51. E. Rovner, K. J. Propert, C. Brensinger, A. J. Wein, M. Foy, A. Kirkemo, J. R. Landis, J. W. Kusek, and L. M. Nyberg. Treatments used in women with interstitial cystitis: the interstitial cystitis data base (ICDB) study experience. The Interstitial Cystitis Data Base Study Group. *Urology* **56**: 940-5 (2000).
52. V. Ratner and L. Perilli. Interstitial cystitis: an updated overview. *Urol Nurs* **23**: 107-10; quiz 111 (2003).
53. A. van Ophoven, S. Pokupic, A. Heinecke, and L. Hertle. A prospective, randomized, placebo controlled, double-blind study of amitriptyline for the treatment of interstitial cystitis. *J Urol* **172**: 533-6 (2004).
54. P. M. Hanno. Amitriptyline in the treatment of interstitial cystitis. *Urol Clin North Am* **21**: 89-91 (1994).
55. C. L. Parsons, T. Housley, J. D. Schmidt, and D. Lebow. Treatment of interstitial cystitis with intravesical heparin. *Br J Urol* **73**: 504-7 (1994).
56. J. C. Nickel, J. Forrest, J. Barkin, C. Payne, and P. Mosbaugh. Safety and efficacy of up to 900 mg/day polysulfate sodium (elmiron) in patients with interstitial cystitis. *Urology* **57**: 122-3 (2001).
57. D. P. Lubeck, K. Whitmore, G. R. Sant, S. Alvarez-Horine, and C. Lai. Psychometric validation of the O'leary-Sant interstitial cystitis symptom index in a clinical trial of pentosan polysulfate sodium. *Urology* **57**: 62-6 (2001).
58. P. M. Hanno. Analysis of long-term Elmiron therapy for interstitial cystitis. *Urology* **49**: 93-9 (1997).
59. A. Fritjofsson, M. Fall, R. Juhlin, B. E. Persson, and M. Ruutu. Treatment of ulcer and nonulcer interstitial cystitis with sodium pentosanpolysulfate: a multicenter trial. *J Urol* **138**: 508-12 (1987).
60. J. J. Bade, M. Laseur, A. Nieuwenburg, L. T. van der Weele, and H. J. Mensink. A placebo-controlled study of intravesical pentosanpolysulphate for the treatment of interstitial cystitis. *Br J Urol* **79**: 168-71 (1997).
61. C. L. Parsons, J. Forrest, J. C. Nickel, R. Evans, L. K. Lloyd, J. Barkin, P. G. Mosbaugh, D. M. Kaufman, J. M. Hernandez-Graulau, L. Atkinson, and D. Albrecht. Effect of pentosan polysulfate therapy on intravesical potassium sensitivity. *Urology* **59**: 329-33 (2002).
62. G. R. Sant, K. J. Propert, P. M. Hanno, D. Burks, D. Culkin, A. C. Diokno, C. Hardy, J. R. Landis, R. Mayer, R. Madigan, E. M. Messing, K. Peters, T. C. Theoharides, J. Warren, A. J. Wein, W. Steers, J. W. Kusek, and L. M. Nyberg. A pilot clinical trial of oral pentosan polysulfate and oral hydroxyzine in patients with interstitial cystitis. *J Urol* **170**: 810-5 (2003).
63. G. Chiang, P. Patra, R. Letourneau, S. Jeudy, W. Boucher, M. Green, G. R. Sant, and T. C. Theoharides. Pentosanpolysulfate inhibits mast cell histamine secretion and intracellular calcium ion levels: an alternative explanation of its beneficial effect in interstitial cystitis. *J Urol* **164**: 2119-25 (2000).
64. S. Gill, S. C. Naiman, A. Jamal, and L. M. Vickars. Massive bleeding on a bladder protectant: a case report of pentosan polysulfate sodium-induced coagulopathy. *Arch Intern Med* **162**: 1644-5 (2002).

65. K. M. Abdo, J. D. Johnson, and A. Nyska. Toxicity and carcinogenicity of Elmiron in F344/N rats and B6C3F1 mice following 2 years of gavage administration. *Arch Toxicol* **77**: 702-11 (2003).
66. S. Zaslau, D. R. Riggs, B. J. Jackson, F. C. Adkins, C. C. John, S. J. Kandzari, and D. W. McFadden. *In vitro* effects of pentosan polysulfate against malignant breast cells. *Am J Surg* **188**: 589-92 (2004).
67. T. C. Theoharides and G. R. Sant. A pilot open label study of Cystoprotek(R) in interstitial cystitis. *Int J Immunopathol Pharmacol* **18**: 183-188 (2005).
68. A. J. Wein. Practical uropharmacology. *Urol Clin North Am* **18**: 269-81 (1991).
69. Y. Abramov and P. K. Sand. Oxybutynin for treatment of urge urinary incontinence and overactive bladder: an updated review. *Expert Opin Pharmacother* **5**: 2351-9 (2004).
70. J. Parkin, C. Shea, and G. R. Sant. Intravesical dimethyl sulfoxide (DMSO) for interstitial cystitis--a practical approach. *Urology* **49**: 105-7 (1997).
71. P. U. Malmstrom. Intravesical therapy of superficial bladder cancer. *Crit Rev Oncol Hematol* **47**: 109-26 (2003).
72. D. L. Lamm. Long-term results of intravesical therapy for superficial bladder cancer. *Urol Clin North Am* **19**: 573-80 (1992).
73. B. H. Stewart, L. Persky, and W. S. Kiser. The use of dimethyl sulfoxide (DMSO) in the treatment of interstitial cystitis. *J Urol* **98**: 671-2 (1967).
74. S. W. Shirley, B. H. Stewart, and S. Mirelman. Dimethyl sulfoxide in treatment of inflammatory genitourinary disorders. *Urology* **11**: 215-20 (1978).
75. G. M. Ghoniem, D. McBride, O. P. Sood, and V. Lewis. Clinical experience with multiagent intravesical therapy in interstitial cystitis patients unresponsive to single-agent therapy. *World J Urol* **11**: 178-82 (1993).
76. S. J. Childs. Dimethyl sulfone (DMSO2) in the treatment of interstitial cystitis. *Urol Clin North Am* **21**: 85-8 (1994).
77. N. C. Santos, J. Figueira-Coelho, J. Martins-Silva, and C. Saldanha. Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects. *Biochem Pharmacol* **65**: 1035-41 (2003).
78. R. Perez-Marrero, L. E. Emerson, and J. T. Feltis. A controlled study of dimethyl sulfoxide in interstitial cystitis. *J Urol* **140**: 36-9 (1988).
79. R. D. Biggers. Self-administration of dimethyl sulfoxide (DMSO) for interstitial cystitis. *Urology* **28**: 10-1 (1986).
80. L. A. Birder, A. J. Kanai, and W. C. de Groat. DMSO: effect on bladder afferent neurons and nitric oxide release. *J Urol* **158**: 1989-95 (1997).
81. D. Melchior, C. S. Packer, T. C. Johnson, and M. Kaefer. Dimethyl sulfoxide: does it change the functional properties of the bladder wall? *J Urol* **170**: 253-8 (2003).
82. M. Nishimura, Y. Takano, and S. Toshitani. Systemic contact dermatitis medicamentosa occurring after intravesical dimethyl sulfoxide treatment for interstitial cystitis. *Arch Dermatol* **124**: 182-3 (1988).
83. Y. Abramov, R. P. Goldberg, M. McGuire, B. Golden, S. Gandhi, and P. K. Sand. Eosinophilic cystitis after bladder instillation with dimethyl sulfoxide. *Urology* **63**: 1182-3 (2004).
84. T. M. de Reijke, K. H. Kurth, R. J. Sylvester, R. R. Hall, M. Brausi, K. van de Beek, K. E. Landsoght, and P. Carpentier. Bacillus Calmette-Guerin versus epirubicin for primary, secondary or concurrent carcinoma in situ of the bladder: results of a European

Organization for the Research and Treatment of Cancer--Genito-Urinary Group Phase III Trial (30906). *J Urol* **173**: 405-9 (2005).
85. W. Kassouf and A. M. Kamat. Current state of immunotherapy for bladder cancer. *Expert Rev Anticancer Ther* **4**: 1037-46 (2004).
86. D. P. Poppas, C. P. Pavlovich, J. Folkman, E. E. Voest, X. Chen, A. D. Luster, and M. A. O'Donnell. Intravesical bacille Calmette-Guerin induces the antiangiogenic chemokine interferon-inducible protein 10. *Urology* **52**: 268-75; discussion 275-6 (1998).
87. J. J. Patard, F. Saint, F. Velotti, C. C. Abbou, and D. K. Chopin. Immune response following intravesical bacillus Calmette-Guerin instillations in superficial bladder cancer: a review. *Urol Res* **26**: 155-9 (1998).
88. E. J. Zeidman, B. Helfrick, C. Pollard, and I. M. Thompson. Bacillus Calmette-Guerin immunotherapy for refractory interstitial cystitis. *Urology* **43**: 121-4 (1994).
89. K. Peters, A. Diokno, B. Steinert, M. Yuhico, B. Mitchell, S. Krohta, B. Gillette, and J. Gonzalez. The efficacy of intravesical Tice strain bacillus Calmette-Guerin in the treatment of interstitial cystitis: a double-blind, prospective, placebo controlled trial. *J Urol* **157**: 2090-4 (1997).
90. K. M. Peters, A. C. Diokno, B. W. Steinert, and J. A. Gonzalez. The efficacy of intravesical bacillus Calmette-Guerin in the treatment of interstitial cystitis: long-term followup. *J Urol* **159**: 1483-6; discussion 1486-7 (1998).
91. J. C. Lukban, K. E. Whitmore, and G. R. Sant. Current management of interstitial cystitis. *Urol Clin North Am* **29**: 649-60 (2002).
92. K. M. Peters, A. C. Diokno, and B. W. Steinert. Preliminary study on urinary cytokine levels in interstitial cystitis: does intravesical bacille Calmette-Guerin treat interstitial cystitis by altering the immune profile in the bladder? *Urology* **54**: 450-3 (1999).
93. L. E. Resel Folkersma, J. A. Belon Lopez Tomasetti, S. Isorna Martinez de la Riva, and J. A. Caminero Luna. [Complications of endovesical treatment with BCG in a series of 200 patients]. *Arch Esp Urol* **52**: 957-65 (1999).
94. C. Parker, S. Steele, R. Raghavan, G. Evans, A. Cranney, S. Robb, and J. C. Nickel. Hypersensitivity reaction associated with intravesical bacillus Calmette-Guerin for interstitial cystitis. *J Urol* **172**: 537 (2004).
95. E. Tinazzi, V. Ficarra, S. Simeoni, D. Peterlana, and C. Lunardi. Reactive arthritis following BCG immunotherapy for bladder carcinoma. *Clin Rheumatol* (2005).
96. R. Peeker, M. A. Haghsheno, S. Holmang, and M. Fall. Intravesical bacillus Calmette-Guerin and dimethyl sulfoxide for treatment of classic and nonulcer interstitial cystitis: a prospective, randomized double-blind study. *J Urol* **164**: 1912-5; discussion 1915-6 (2000).
97. W. S. Boucher, R. Letourneau, M. Huang, D. Kempuraj, M. Green, G. R. Sant, and T. C. Theoharides. Intravesical sodium hyaluronate inhibits the rat urinary mast cell mediator increase triggered by acute immobilization stress. *J Urol* **167**: 380-4 (2002).
98. J. Nordling, S. Jorgensen, and E. Kallestrup. Cystistat for the treatment of interstitial cystitis: a 3-year follow-up study. *Urology* **57**: 123 (2001).
99. G. Steinhoff, B. Ittah, and S. Rowan. The efficacy of chondroitin sulfate 0.2% in treating interstitial cystitis. *Can J Urol* **9**: 1454-8 (2002).
100. L. K. Daha, C. R. Riedl, D. Lazar, G. Hohlbrugger, and H. Pfluger. Do cystometric findings predict the results of intravesical hyaluronic Acid in women with interstitial cystitis? *Eur Urol* **47**: 393-7 (2005).

101. C. L. Parsons. Current strategies for managing interstitial cystitis. *Expert Opin Pharmacother* **5**: 287-93 (2004).
102. R. R. Gonzalez, T. Fong, N. Belmar, M. Saban, D. Felsen, and A. Te. Modulating bladder neuro-inflammation: RDP58, a novel anti-inflammatory peptide, decreases inflammation and nerve growth factor production in experimental cystitis. *J Urol* **173**: 630-4 (2005).
103. P. Dasgupta, V. A. Chandiramani, A. Beckett, F. Scaravilli, and C. J. Fowler. The effect of intravesical capsaicin on the suburothelial innervation in patients with detrusor hyper-reflexia. *BJU Int* **85**: 238-45 (2000).
104. F. Cruz. Mechanisms involved in new therapies for overactive bladder. *Urology* **63**: 65-73 (2004).
105. F. Cruz. Vanilloid receptor and detrusor instability. *Urology* **59**: 51-60 (2002).
106. T. W. Cannon and M. B. Chancellor. Pharmacotherapy of the overactive bladder and advances in drug delivery. *Clin Obstet Gynecol* **45**: 205-17 (2002).
107. C. J. Fowler. Intravesical treatment of overactive bladder. *Urology* **55**: 60-4; discussion 66 (2000).
108. M. de Seze, L. Wiart, M. P. de Seze, P. A. Joseph, B. Brochet, J. M. Ferriere, J. M. Mazaux, and M. Barat. [Reiterated intravesical instillation of capsaicin in neurogenic detrusor hyperreflexia: a 5-years experience of 100 instillations]. *Ann Readapt Med Phys* **44**: 514-24 (2001).
109. M. de Seze, L. Wiart, M. P. de Seze, L. Soyeur, J. P. Dosque, S. Blajezewski, N. Moore, B. Brochet, J. M. Mazaux, M. Barat, and P. A. Joseph. Intravesical capsaicin versus resiniferatoxin for the treatment of detrusor hyperreflexia in spinal cord injured patients: a double-blind, randomized, controlled study. *J Urol* **171**: 251-5 (2004).
110. J. L. Au, R. A. Badalament, M. G. Wientjes, D. C. Young, J. A. Warner, P. L. Venema, D. L. Pollifrone, J. D. Harbrecht, J. L. Chin, S. P. Lerner, and B. J. Miles. Methods to improve efficacy of intravesical mitomycin C: results of a randomized phase III trial. *J Natl Cancer Inst* **93**: 597-604 (2001).
111. M. Lazzeri, M. Spinelli, P. Beneforti, S. Malaguti, G. Giardiello, and D. Turini. Intravesical infusion of resiniferatoxin by a temporary in situ drug delivery system to treat interstitial cystitis: a pilot study. *Eur Urol* **45**: 98-102 (2004).
112. M. Shurafa, E. Shumaker, and S. Cronin. Prostaglandin F2-alpha bladder irrigation for control of intractable cyclophosphamide-induced hemorrhagic cystitis. *J Urol* **137**: 1230-1 (1987).
113. M. E. Trigg, J. O'Reilly, S. Rumelhart, D. Morgan, M. Holida, and P. de Alarcon. Prostaglandin E1 bladder instillations to control severe hemorrhagic cystitis. *J Urol* **143**: 92-4 (1990).
114. L. J. Miller, S. W. Chandler, and C. M. Ippoliti. Treatment of cyclophosphamide-induced hemorrhagic cystitis with prostaglandins. *Ann Pharmacother* **28**: 590-4 (1994).
115. T. Saito, Y. Ikeda, E. Ito, K. Miyano, K. Kawauchi, M. Yokoyama, and Y. Sato. [Bladder irrigation with prostaglandin E2 in cyclophosphamide-induced hemorrhagic cystitis]. *Gan To Kagaku Ryoho* **15**: 155-7 (1988).
116. R. E. Hurst and R. Zebrowski. Identification of proteoglycans present at high density on bovine and human bladder luminal surface. *J Urol* **152**: 1641-5 (1994).
117. J. L. Au, S. H. Jang, and M. G. Wientjes. Clinical aspects of drug delivery to tumors. *J Control Release* **78**: 81-95 (2002).

118. X. Gao, J. L. Au, R. A. Badalament, and M. G. Wientjes. Bladder tissue uptake of mitomycin C during intravesical therapy is linear with drug concentration in urine. *Clin Cancer Res* **4**: 139-43 (1998).
119. N. Thiruchelvam and H. Mostafid. A novel device for reconstituting and delivering intravesical chemotherapy. *BJU Int* **92**: 492 (2003).
120. S. M. Di Stasi, A. Giannantoni, R. L. Stephen, G. Capelli, P. Navarra, R. Massoud, and G. Vespasiani. Intravesical electromotive mitomycin C versus passive transport mitomycin C for high risk superficial bladder cancer: a prospective randomized study. *J Urol* **170**: 777-82 (2003).
121. D. Chen, D. Song, M. G. Wientjes, and J. L. Au. Effect of dimethyl sulfoxide on bladder tissue penetration of intravesical paclitaxel. *Clin Cancer Res* **9**: 363-9 (2003).
122. C. R. Riedl, M. Knoll, E. Plas, and H. Pfluger. Electromotive drug administration and hydrodistention for the treatment of interstitial cystitis. *J Endourol* **12**: 269-72 (1998).
123. C. R. Riedl, R. L. Stephen, L. K. Daha, M. Knoll, E. Plas, and H. Pfluger. Electromotive administration of intravesical bethanechol and the clinical impact on acontractile detrusor management: introduction of a new test. *J Urol* **164**: 2108-11 (2000).
124. S. R. Schwarze, A. Ho, A. Vocero-Akbani, and S. F. Dowdy. *In vivo* protein transduction: delivery of a biologically active protein into the mouse. *Science* **285**: 1569-72 (1999).
125. E. Vives, J. P. Richard, C. Rispal, and B. Lebleu. TAT peptide internalization: seeking the mechanism of entry. *Curr Protein Pept Sci* **4**: 125-32 (2003).
126. A. Astriab-Fisher, D. Sergueev, M. Fisher, B. R. Shaw, and R. L. Juliano. Conjugates of antisense oligonucleotides with the Tat and antennapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences, and biologic actions. *Pharm Res* **19**: 744-54 (2002).
127. V. L. Marin, S. Roy, and B. A. Armitage. Recent advances in the development of peptide nucleic acid as a gene-targeted drug. *Expert Opin Biol Ther* **4**: 337-48 (2004).
128. P. E. Nielsen. Gene targeting using peptide nucleic acid. *Methods Mol Biol* **288**: 343-58 (2005).
129. M. Pooga, T. Land, T. Bartfai, and U. Langel. PNA oligomers as tools for specific modulation of gene expression. *Biomol Eng* **17**: 183-92 (2001).
130. A. Ray and B. Norden. Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. *FASEB J* **14**: 1041-60 (2000).
131. C. Le Visage, N. Rioux-Leclercq, M. Haller, P. Breton, B. Malavaud, and K. Leong. Efficacy of paclitaxel released from bio-adhesive polymer microspheres on model superficial bladder cancer. *J Urol* **171**: 1324-9 (2004).
132. A. L. Burnett, D. C. Calvin, S. L. Chamness, J. X. Liu, R. J. Nelson, S. L. Klein, V. L. Dawson, T. M. Dawson, and S. H. Snyder. Urinary bladder-urethral sphincter dysfunction in mice with targeted disruption of neuronal nitric oxide synthase models idiopathic voiding disorders in humans. *Nat Med* **3**: 571-4 (1997).
133. S. S. Singh, K. M. Smith, and D. M. Brown. Drug retention following intravesical delivery of fluorouracil therapeutic adhesive in C3H mouse bladder. *Anticancer Drugs* **7**: 507-13 (1996).
134. H. H. Chen, C. Le Visage, B. Qiu, X. Du, R. Ouwerkerk, K. W. Leong, and X. Yang. MR imaging of biodegradable polymeric microparticles: A potential method of monitoring local drug delivery. *Magn Reson Med* **53**: 614-20 (2005).

135. E. Ozturk, M. Eroglu, N. Ozdemir, and E. B. Denkbas. Bioadhesive drug carriers for postoperative chemotherapy in bladder cancer. *Adv Exp Med Biol* **553**: 231-42 (2004).
136. Z. Ye, J. Chen, X. Zhang, J. Li, S. Zhou, W. Yang, and Y. Zhang. Novel gelatin-adriamycin sustained drug release system for intravesical therapy of bladder cancer. *J Tongji Med Univ* **21**: 145-8 (2001).
137. T. Leakakos, C. Ji, G. Lawson, C. Peterson, and S. Goodwin. Intravesical administration of doxorubicin to swine bladder using magnetically targeted carriers. *Cancer Chemother Pharmacol* **51**: 445-50 (2003).
138. I. Grabnar, M. Bogataj, and A. Mrhar. Influence of chitosan and polycarbophil on permeation of a model hydrophilic drug into the urinary bladder wall. *Int J Pharm* **256**: 167-73 (2003).
139. M. Saito, T. Watanabe, F. Tabuchi, K. Otsubo, K. Satoh, and I. Miyagawa. Urodynamic effects and safety of modified intravesical oxybutynin chloride in patients with neurogenic detrusor overactivity: 3 years experience. *Int J Urol* **11**: 592-6 (2004).
140. B. Jeong, Y. H. Bae, and S. W. Kim. Drug release from biodegradable injectable thermosensitive hydrogel of PEG-PLGA-PEG triblock copolymers. *J Control Release* **63**: 155-63 (2000).
141. B. Jeong, Y. H. Bae, and S. W. Kim. In situ gelation of PEG-PLGA-PEG triblock copolymer aqueous solutions and degradation thereof. *J Biomed Mater Res* **50**: 171-7 (2000).
142. J. W. Johnson, R. Nayar, J. J. Killion, A. C. von Eschenbach, and I. J. Fidler. Binding of liposomes to human bladder tumor epithelial cell lines: implications for an intravesical drug delivery system for the treatment of bladder cancer. *Sel Cancer Ther* **5**: 147-55 (1989).
143. R. B. Egerdie, G. Reid, and J. Trachtenberg. The effect of liposome encapsulated antineoplastic agents on transitional cell carcinoma in tissue culture. *The Journal Of Urology* **142**: 390-398 (1989).
144. J. J. Killion, D. Fan, C. D. Bucana, D. N. Frangos, J. E. Price, and I. J. Fidler. Augmentation of antiproliferative activity of interferon alfa against human bladder tumor cell lines by encapsulation of interferon alfa within liposomes. *J Natl Cancer Inst* **81**: 1387-92 (1989).
145. D. N. Frangos, J. J. Killion, D. Fan, R. Fishbeck, A. C. von Eschenbach, and I. J. Fidler. The development of liposomes containing interferon alpha for the intravesical therapy of human superficial bladder cancer. *J Urol* **143**: 1252-6 (1990).
146. Y. Horiguchi, W. A. Larchian, R. Kaplinsky, W. R. Fair, and W. D. Heston. Intravesical liposome-mediated interleukin-2 gene therapy in orthotopic murine bladder cancer model. *Gene Ther* **7**: 844-51 (2000).
147. Q. Wu, R. Mahendran, and K. Esuvaranathan. Nonviral cytokine gene therapy on an orthotopic bladder cancer model. *Clin Cancer Res* **9**: 4522-8 (2003).
148. M. B. Chancellor and W. C. de Groat. Intravesical capsaicin and resiniferatoxin therapy: spicing up the ways to treat the overactive bladder. *J Urol* **162**: 3-11 (1999).
149. A. Giannantoni, S. M. Di Stasi, R. L. Stephen, P. Navarra, G. Scivoletto, E. Mearini, and M. Porena. Intravesical capsaicin versus resiniferatoxin in patients with detrusor hyperreflexia: a prospective randomized study. *J Urol* **167**: 1710-4 (2002).

150. D. S. Byrne, A. Das, J. Sedor, B. Huang, D. A. Rivas, H. J. Flood, W. DeGroat, M. L. Jordan, M. B. Chancellor, and P. McCue. Effect of intravesical capsaicin and vehicle on bladder integrity control and spinal cord injured rats. *J Urol* **159**: 1074-8 (1998).
151. P. G. Jensen and J. R. Larson. Management of painful diabetic neuropathy. *Drugs Aging* **18**: 737-49 (2001).
152. Z. Li, W. Ning, J. Wang, A. Choi, P. Y. Lee, P. Tyagi, and L. Huang. Controlled gene delivery system based on thermosensitive biodegradable hydrogel. *Pharm Res* **20**: 884-8 (2003).
153. L. A. Birder, A. J. Kanai, W. C. de Groat, S. Kiss, M. L. Nealen, N. E. Burke, K. E. Dineley, S. Watkins, I. J. Reynolds, and M. J. Caterina. Vanilloid receptor expression suggests a sensory role for urinary bladder epithelial cells. *Proc Natl Acad Sci U S A* **98**: 13396-401 (2001).
154. R. M. Craft, S. M. Cohen, and F. Porreca. Long-lasting desensitization of bladder afferents following intravesical resiniferatoxin and capsaicin in the rat. *Pain* **61**: 317-23 (1995).
155. C. J. Fowler, R. O. Beck, S. Gerrard, C. D. Betts, and C. G. Fowler. Intravesical capsaicin for treatment of detrusor hyperreflexia. *J Neurol Neurosurg Psychiatry* **57**: 169-73 (1994).
156. M. O. Fraser, Y. C. Chuang, P. Tyagi, T. Yokoyama, N. Yoshimura, L. Huang, W. C. De Groat, and M. B. Chancellor. Intravesical liposome administration--a novel treatment for hyperactive bladder in the rat. *Urology* **61**: 656-63 (2003).
157. A. E. Cohen and H. M. Oliver. Urethral Catheterization of the Rat. *Lab Anim Care* **14**: 471-3 (1964).
158. A. Avelino, C. Cruz, I. Nagy, and F. Cruz. Vanilloid receptor 1 expression in the rat urinary tract. *Neuroscience* **109**: 787-98 (2002).
159. S. Ritter and T. T. Dinh. Age-related changes in capsaicin-induced degeneration in rat brain. *J Comp Neurol* **318**: 103-16 (1992).
160. J. Donnerer, R. Amann, R. Schuligoi, and F. Lembeck. Absorption and metabolism of capsaicinoids following intragastric administration in rats. *Naunyn Schmiedebergs Arch Pharmacol* **342**: 357-61 (1990).
161. M. de Seze, L. Wiart, P. A. Joseph, J. P. Dosque, J. M. Mazaux, and M. Barat. Capsaicin and neurogenic detrusor hyperreflexia: a double-blind placebo-controlled study in 20 patients with spinal cord lesions. *Neurourol Urodyn* **17**: 513-23 (1998).
162. M. Trevisani, D. Gazzieri, F. Benvenuti, B. Campi, Q. T. Dinh, D. A. Groneberg, M. Rigoni, X. Emonds-Alt, C. Creminon, A. Fischer, P. Geppetti, and S. Harrison. Ethanol causes inflammation in the airways by a neurogenic and TRPV1-dependent mechanism. *J Pharmacol Exp Ther* **309**: 1167-73 (2004).
163. M. Trevisani, D. Smart, M. J. Gunthorpe, M. Tognetto, M. Barbieri, B. Campi, S. Amadesi, J. Gray, J. C. Jerman, S. J. Brough, D. Owen, G. D. Smith, A. D. Randall, S. Harrison, A. Bianchi, J. B. Davis, and P. Geppetti. Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. *Nat Neurosci* **5**: 546-51 (2002).
164. C. A. Maggi and B. Conte. Effect of urethane anesthesia on the micturition reflex in capsaicin-treated rats. *J Auton Nerv Syst* **30**: 247-51 (1990).
165. B. Mallory, W. D. Steers, and W. C. de Groat. Electrophysiological study of micturition reflexes in rats. *Am J Physiol* **257**: R410-21 (1989).

166. Y. C. Chuang, M. O. Fraser, Y. Yu, J. M. Beckel, S. Seki, Y. Nakanishi, H. Yokoyama, M. B. Chancellor, N. Yoshimura, and W. C. de Groat. Analysis of the afferent limb of the vesicovascular reflex using neurotoxins, resiniferatoxin and capsaicin. *Am J Physiol Regul Integr Comp Physiol* **281**: R1302-10 (2001).
167. X. Gao, C. A. Buffington, and J. L. Au. Effect of interstitial cystitis on drug absorption from urinary bladder. *J Pharmacol Exp Ther* **271**: 818-23 (1994).
168. B. Jeong, Y. H. Bae, D. S. Lee, and S. W. Kim. Biodegradable block copolymers as injectable drug-delivery systems. *Nature* **388**: 860-2 (1997).
169. C. L. Cheng, C. P. Ma, and W. C. de Groat. Effects of capsaicin on micturition and associated reflexes in rats. *Am J Physiol* **265**: R132-8 (1993).
170. P. Santicioli, C. A. Maggi, and A. Meli. The effect of capsaicin pretreatment on the cystometrograms of urethane anesthetized rats. *J Urol* **133**: 700-3 (1985).
171. P. Tucci, M. G. Evandri, and P. Bolle. Tachykinin-independent activity of capsaicin on in-vitro lamb detrusor. *J Pharm Pharmacol* **54**: 1111-5 (2002).
172. S. Girod de Bentzmann, O. Bajolet-Laudinat, F. Dupuit, D. Pierrot, C. Fuchey, M. C. Plotkowski, and E. Puchelle. Protection of human respiratory epithelium from Pseudomonas aeruginosa adherence by phosphatidylglycerol liposomes. *Infect Immun* **62**: 704-8 (1994).
173. P. Wutzler, A. Sauerbrei, A. Hartl, and K. Reimer. Comparative testing of liposomal and aqueous formulations of povidone-iodine for their angioirritative potential at the chorioallantoic membrane of ex ovo cultivated chick embryos. *Dermatology* **207**: 43-7 (2003).
174. M. C. Giocondi and C. Le Grimellec. Water permeation in Madin-Darby canine kidney cells is modulated by membrane fluidity. *Biochim Biophys Acta* **1064**: 315-20 (1991).
175. C. Venien and C. Le Grimellec. The involvement of cytoskeletal proteins in the maintenance of phospholipid topology in renal brush-border membranes. *Biochim Biophys Acta* **946**: 307-14 (1988).
176. C. Venien and C. Le Grimellec. Phospholipid asymmetry in renal brush-border membranes. *Biochim Biophys Acta* **942**: 159-68 (1988).
177. J. M. Caruthers and M. A. Bonneville. The asymmetric unit membrane (AUM) structure in the lumenal plasma membrane of urothelium: the effect of trypsin on the integrity of the plaques. *J Ultrastruct Res* **71**: 288-302 (1980).
178. G. van Meer and K. Simons. Lipid polarity and sorting in epithelial cells. *J Cell Biochem* **36**: 51-8 (1988).
179. T. T. Sun, H. Zhao, J. Provet, U. Aebi, and X. R. Wu. Formation of asymmetric unit membrane during urothelial differentiation. *Mol Biol Rep* **23**: 3-11 (1996).
180. A. Zachowski, J. P. Henry, and P. F. Devaux. Control of transmembrane lipid asymmetry in chromaffin granules by an ATP-dependent protein. *Nature* **340**: 75-6 (1989).
181. P. R. Dragsten, R. Blumenthal, and J. S. Handler. Membrane asymmetry in epithelia: is the tight junction a barrier to diffusion in the plasma membrane? *Nature* **294**: 718-22 (1981).
182. B. L. Blazer-Yost, J. C. Vahle, J. M. Byars, and R. L. Bacallao. Real-time three-dimensional imaging of lipid signal transduction: apical membrane insertion of epithelial Na(+) channels. *Am J Physiol Cell Physiol* **287**: C1569-76 (2004).

183. G. Hansson, K. Simons, and G. van Meer. Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. *EMBO J.* **5**: 483-489 (1986).
184. W. G. Hill and M. L. Zeidel. Reconstituting the Barrier Properties of a Water-tight Epithelial Membrane by Design of Leaflet-specific Liposomes. *J Biol Chem* **275**: 30176-30185 (2000).
185. W. G. Hill, R. L. Rivers, and M. L. Zeidel. Role of leaflet asymmetry in the permeability of model biological membranes to protons, solutes, and gases. *J Gen Physiol* **114**: 405-14 (1999).
186. M. Lande, J. Donovan, and M. Zeidel. The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. *J Gen Physiol* **106**: 67-84 (1995).
187. J. A. F. Op den Kamp. Lipid Asymmetry in Membranes. *Annual Review of Biochemistry* **48**: 47-71 (1979).
188. M. B. Lande, N. A. Priver, and M. L. Zeidel. Determinants of apical membrane permeabilities of barrier epithelia. *Am J Physiol Cell Physiol* **267**: C367-374 (1994).
189. Y. C. Chuang, M. B. Chancellor, S. Seki, N. Yoshimura, P. Tyagi, L. Huang, J. P. Lavelle, W. C. De Groat, and M. O. Fraser. Intravesical protamine sulfate and potassium chloride as a model for bladder hyperactivity. *Urology* **61**: 664-70 (2003).
190. M. O. Fraser, Y. C. Chuang, J. P. Lavelle, N. Yoshimura, W. C. de Groat, and M. B. Chancellor. A reliable, nondestructive animal model for interstitial cystitis: intravesical low-dose protamine sulfate combined with physiological concentrations of potassium chloride. *Urology* **57**: 112 (2001).
191. S. A. Lewis, J. R. Berg, and T. J. Kleine. Modulation of epithelial permeability by extracellular macromolecules. *Physiol Rev* **75**: 561-589 (1995).
192. C. J. Tzan, J. R. Berg, and S. A. Lewis. Mammalian urinary bladder permeability is altered by cationic proteins: modulation by divalent cations. *Am J Physiol* **267**: C1013-26 (1994).
193. C. J. Tzan, J. Berg, and S. A. Lewis. Effect of protamine sulfate on the permeability properties of the mammalian urinary bladder. *J Membr Biol* **133**: 227-42 (1993).
194. J. P. Lavelle, S. A. Meyers, W. G. Ruiz, C. A. Buffington, M. L. Zeidel, and G. Apodaca. Urothelial pathophysiological changes in feline interstitial cystitis: a human model. *Am J Physiol Renal Physiol* **278**: F540-53 (2000).
195. J.P. Lavelle, S.A. Meyers, R. Ramage, S. Bastacky, D. Doty, G. Apodaca, and M. L. Zeidel. Bladder permeability barrier: recovery from selective injury of surface epithelial cells. *Am J Physiol Renal Physiol* **283**: F242-53 (2002).
196. J. P. Lavelle, G. Apodaca, S. A. Meyers, W. G. Ruiz, and M. L. Zeidel. Disruption of guinea pig urinary bladder permeability barrier in noninfectious cystitis. *Am J Physiol Renal Physiol* **274**: F205-214 (1998).
197. P. Tyagi, M. B. Chancellor, Z. Li, W. C. de Groat, N. Yoshimura, M. O. Fraser, and L. Huang. Urodynamic and immunohistochemical evaluation of intravesical capsaicin delivery using thermosensitive hydrogel and liposomes. *J Urol* **171**: 483-9 (2004).
198. M. Ohnishi, H. Yajima, T. Takeuchi, M. Saito, K. Yamazaki, T. Kasai, K. Nagano, S. Yamamoto, T. Matsushima, and T. Ishii. Mechanism of Urinary Tract Crystal Formation Following Biphenyl Treatment. *Toxicology and Applied Pharmacology* **174**: 122-129 (2001).

199. S. P. Hunt, A. Pini, and G. Evan. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. *Nature* **328**: 632-4 (1987).
200. L. A. Staehelin, F. J. Chlapowski, and M. A. Bonneville. Lumenal plasma membrane of the urinary bladder: I. Three-Dimensional Reconstruction from Freeze-Etch Images. *J Cell Biol* **53**: 73-91 (1972).
201. R. M. Hicks and B. Ketterer. Hexagonal lattice of subunits in the thick luminal membrane of the rat urinary bladder. *Nature* **224**: 1304-5 (1969).
202. R. Romih and K. Jezernik. Reorganisation of the urothelial luminal plasma membrane in the cyclophosphamide treated rats. *Pflugers Arch* **431**: R241-2 (1996).
203. X. R. Wu, J. H. Lin, T. Walz, M. Haner, J. Yu, U. Aebi, and T. T. Sun. Mammalian uroplakins. A group of highly conserved urothelial differentiation-related membrane proteins. *J Biol Chem* **269**: 13716-24 (1994).
204. C. L. Parsons, S. G. Mulholland, and H. Anwar. Antibacterial activity of bladder surface mucin duplicated by exogenous glycosaminoglycan (heparin). *Infect Immun* **24**: 552-7 (1979).
205. C. L. Parsons, C. Stauffer, and J. D. Schmidt. Impairment of antibacterial effect of bladder surface mucin by protamine sulfate. *J Infect Dis* **144**: 180 (1981).
206. J. D. Lilly and C. L. Parsons. Bladder surface glycosaminoglycans is a human epithelial permeability barrier. *Surg Gynecol Obstet* **171**: 493-6 (1990).
207. S. Cetinel, F. Ercan, S. Sirvanci, O. Sehirli, Y. Ersoy, T. San, and G. Sener. The ameliorating effect of melatonin on protamine sulfate induced bladder injury and its relationship to interstitial cystitis. *J Urol* **169**: 1564-8 (2003).
208. J. F. Metts. Interstitial cystitis: urgency and frequency syndrome. *Am Fam Physician* **64**: 1199-206 (2001).
209. J. A. Szule, N. L. Fuller, and R. P. Rand. The effects of acyl chain length and saturation of diacylglycerols and phosphatidylcholines on membrane monolayer curvature. *Biophys J* **83**: 977-84 (2002).
210. F. Bellemare and H. Rocheleau. Modulation of noninduced and phorbol ester-induced generation of superoxide anion by free liposomes and liposomes containing dexamethasone. *Immunopharmacol Immunotoxicol* **19**: 121-34 (1997).
211. K. Yoshida and V. Mohsenin. Inhibition of protein kinase C of human neutrophils by phosphatidylcholines. *Life Sci* **54**: 515-24 (1994).
212. J. Walker and J. Sando. Activation of protein kinase C by short chain phosphatidylcholines. *J Biol Chem* **263**: 4537-4540 (1988).
213. E. J. Bolen and J. J. Sando. Effect of phospholipid unsaturation on protein kinase C activation. *Biochemistry* **31**: 5945-51 (1992).
214. D. H. Gong, B. Turner, K. R. Bhaskar, and J. T. Lamont. Lipid binding to gastric mucin: protective effect against oxygen radicals. *Am J Physiol* **259**: G681-6 (1990).
215. D. J. MacEwan, R. Mitchell, M. S. Johnson, F. J. Thomson, E. M. Lutz, R. A. Clegg, and K. Connor. Evidence that protein kinase C alpha has reduced affinity towards 1,2-dioctanoyl-sn-glycerol: the effects of lipid activators on phorbol ester binding and kinase activity. *Eur J Pharmacol* **246**: 9-18 (1993).
216. S. J. Slater, M. B. Kelly, F. J. Taddeo, C. Ho, E. Rubin, and C. D. Stubbs. The modulation of protein kinase C activity by membrane lipid bilayer structure. *J Biol Chem* **269**: 4866-71 (1994).

217. T. Seki, H. Matsubayashi, T. Amano, Y. Shirai, N. Saito, and N. Sakai. Phosphorylation of PKC activation loop plays an important role in receptor-mediated translocation of PKC. *Genes Cells* **10**: 225-39 (2005).
218. M. Conte, B. Soper, Q. Chang, and B. Tepperman. The role of PKCdelta and PKCepsilon in the neonatal rat colon in response to hypoxia challenge. *Pediatr Res* **55**: 27-33 (2004).
219. Y. S. Kim, J. Ko, I. S. Kim, S. W. Jang, H. J. Sung, H. J. Lee, S. Y. Lee, Y. Kim, and D. S. Na. PKCdelta-dependent cleavage and nuclear translocation of annexin A1 by phorbol 12-myristate 13-acetate. *Eur J Biochem* **270**: 4089-94 (2003).
220. P. V. Subbaiah and R. M. Sargis. Sphingomyelin: a natural modulator of membrane homeostasis and inflammation. *Med Hypotheses* **57**: 135-8 (2001).
221. Y. Barenholz and T. E. Thompson. Sphingomyelin: biophysical aspects. *Chem Phys Lipids* **102**: 29-34 (1999).
222. B. Ramstedt and J. P. Slotte. Membrane properties of sphingomyelins. *FEBS Letters* **531**: 33-37 (2002).
223. L. A. Birder and W. C. de Groat. Increased c-fos expression in spinal neurons after irritation of the lower urinary tract in the rat. *J Neurosci* **12**: 4878-89 (1992).
224. T. K. M. Nyholm, M. Nylund, and J. P. Slotte. A Calorimetric Study of Binary Mixtures of Dihydrosphingomyelin and Sterols, Sphingomyelin, or Phosphatidylcholine. *Biophys J* **84**: 3138-3146 (2003).
225. M. Kuikka, B. Ramstedt, H. Ohvo-Rekila, J. Tuuf, and J. P. Slotte. Membrane properties of D-erythro-N-acyl sphingomyelins and their corresponding dihydro species. *Biophys J* **80**: 2327-37 (2001).
226. C. M. Talbott, I. Vorobyov, D. Borchman, K. G. Taylor, D. B. DuPre, and M. C. Yappert. Conformational studies of sphingolipids by NMR spectroscopy. II. Sphingomyelin. *Biochim Biophys Acta* **1467**: 326-37 (2000).
227. S. R. Ferguson-Yankey, D. Borchman, K. G. Taylor, D. B. DuPre, and M. C. Yappert. Conformational studies of sphingolipids by NMR spectroscopy. I. Dihydrosphingomyelin. *Biochim Biophys Acta* **1467**: 307-25 (2000).
228. L. Li, X. Tang, K. G. Taylor, D. B. DuPre, and M. C. Yappert. Conformational characterization of ceramides by nuclear magnetic resonance spectroscopy. *Biophys J* **82**: 2067-80 (2002).
229. L. Moesby, J. Corver, R. K. Erukulla, R. Bittman, and J. Wilschut. Sphingolipids activate membrane fusion of Semliki Forest virus in a stereospecific manner. *Biochemistry* **34**: 10319-24 (1995).
230. J. Corver, L. Moesby, R. K. Erukulla, K. C. Reddy, R. Bittman, and J. Wilschut. Sphingolipid-dependent fusion of Semliki Forest virus with cholesterol-containing liposomes requires both the 3-hydroxyl group and the double bond of the sphingolipid backbone. *J Virol* **69**: 3220-3 (1995).
231. P. Mattjus, B. Malewicz, J. T. Valiyaveettil, W. J. Baumann, R. Bittman, and R. E. Brown. Sphingomyelin modulates the transbilayer distribution of galactosylceramide in phospholipid membranes. *J Biol Chem* **277**: 19476-81 (2002).
232. B. Malewicz, J. T. Valiyaveettil, K. Jacob, H. S. Byun, P. Mattjus, W. J. Baumann, R. Bittman, and R. E. Brown. The 3-Hydroxy Group and 4,5-trans Double Bond of Sphingomyelin are Essential for Modulation of Galactosylceramide Transmembrane Asymmetry. *Biophys J* **88**:2670-80 (2005).

233. Y. A. Hannun. Functions of ceramide in coordinating cellular responses to stress. *Science* **274**: 1855-9 (1996).
234. M. B. Ruiz-Arguello, G. Basanez, F. Goni, and A. Alonso. Different Effects of Enzyme-generated Ceramides and Diacylglycerols in Phospholipid Membrane Fusion and Leakage. *J Biol Chem* **271**: 26616-26621 (1996).
235. G. Liu, L. Kleine, and R. L. Hebert. Advances in the signal transduction of ceramide and related sphingolipids. *Crit Rev Clin Lab Sci* **36**: 511-73 (1999).
236. N. Abboushi, A. El-Hed, W. El-Assaad, L. Kozhaya, M. E. El-Sabban, A. Bazarbachi, R. Badreddine, A. Bielawska, J. Usta, and G. S. Dbaibo. Ceramide inhibits IL-2 production by preventing protein kinase C-dependent NF-kappaB activation: possible role in protein kinase Ctheta regulation. *J Immunol* **173**: 3193-200 (2004).
237. R. T. Dobrowsky. Sphingolipid signalling domains Floating on rafts or buried in caves? *Cellular Signalling* **12**: 81-90 (2000).
238. E. M. Raeder, P. J. Mansfield, V. Hinkovska-Galcheva, L. Kjeldsen, J. A. Shayman, and L. A. Boxer. Sphingosine blocks human polymorphonuclear leukocyte phagocytosis through inhibition of mitogen-activated protein kinase activation. *Blood* **93**: 686-93 (1999).
239. K. P. Becker, K. Kitatani, J. Idkowiak-Baldys, J. Bielawski, and Y. A. Hannun. Selective inhibition of juxtanuclear translocation of protein kinase C betaII by a negative feedback mechanism involving ceramide formed from the salvage pathway. *J Biol Chem* **280**: 2606-12 (2005).
240. K. Kagedal, M. Zhao, I. Svensson, and U. T. Brunk. Sphingosine-induced apoptosis is dependent on lysosomal proteases. *Biochem J* **359**: 335-43 (2001).
241. T. Ohmori, Y. Yatomi, M. Osada, F. Kazama, T. Takafuta, H. Ikeda, and Y. Ozaki. Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via S1P2. *Cardiovasc Res* **58**: 170-7 (2003).
242. P. S. Jolly, H. M. Rosenfeldt, S. Milstien, and S. Spiegel. The roles of sphingosine-1-phosphate in asthma. *Mol Immunol* **38**: 1239-45 (2002).
243. S. Spiegel and S. Milstien. Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. *Biochem Soc Trans* **31**: 1216-9 (2003).
244. T. Baumruker and E. E. Prieschl. Sphingolipids and the regulation of the immune response. *Semin Immunol* **14**: 57-63 (2002).
245. A. Olivera and J. Rivera. Sphingolipids and the balancing of immune cell function: lessons from the mast cell. *J Immunol* **174**: 1153-8 (2005).
246. L. Draberova, L. Dudkova, M. Boubelik, H. Tolarova, F. Smid, and P. Draber. Exogenous administration of gangliosides inhibits Fc epsilon RI-mediated mast cell degranulation by decreasing the activity of phospholipase C gamma. *J Immunol* **171**: 3585-93 (2003).
247. R. Brett and M. G. Rumsby. Susceptibility of myelin glycerophospholipids and sphingolipids to oxidative attack by hydroxyl free radicals as measured by the thiobarbituric acid test. *Neurochem Int* **24**: 241-51 (1994).
248. E. Klapisz, J. Masliah, G. Bereziat, C. Wolf, and K. S. Koumanov. Sphingolipids and cholesterol modulate membrane susceptibility to cytosolic phospholipase A(2). *J Lipid Res* **41**: 1680-8 (2000).

249. P. Tyagi, Z. Li, M. Chancellor, W. C. De Groat, N. Yoshimura, and L. Huang. Sustained intravesical drug delivery using thermosensitive hydrogel. *Pharm Res* **21**: 832-7 (2004).
250. D. L. Lamm and J. G. Griffith. Intravesical therapy: does it affect the natural history of superficial bladder cancer? *Semin Urol* **10**: 39-44 (1992).
251. M. C. Walker, J. R. Masters, C. N. Parris, P. J. Hepburn, and P. J. English. Intravesical chemotherapy: in vitro studies on the relationship between dose and cytotoxicity. *Urol Res* **14**: 137-40 (1986).
252. K. Ueda, H. Sakagami, Y. Masui, and T. Okamura. Single instillation of hydroxypropylcellulose-doxorubicin as treatment for superficial bladder carcinoma. *Cancer Chemother Pharmacol* **35 Suppl**: S81-3 (1994).
253. B. Jeong, Y. K. Choi, Y. H. Bae, G. Zentner, and S. W. Kim. New biodegradable polymers for injectable drug delivery systems. *J Control Release* **62**: 109-14 (1999).
254. Y. J. Kim, S. Choi, J. J. Koh, M. Lee, K. S. Ko, and S. W. Kim. Controlled release of insulin from injectable biodegradable triblock copolymer. *Pharm Res* **18**: 548-50 (2001).
255. E. M. Bulger and R. V. Maier. Lipid mediators in the pathophysiology of critical illness. *Crit Care Med* **28**: N27-36 (2000).
256. D. Laszlo, A. Bosi, S. Guidi, R. Saccardi, A. M. Vannucchi, L. Lombardini, G. Longo, R. Fanci, A. Azzi, R. De Santis, and et al. Prostaglandin E2 bladder instillation for the treatment of hemorrhagic cystitis after allogeneic bone marrow transplantation. *Haematologica* **80**: 421-5 (1995).
257. C. Ippoliti, D. Przepiorka, R. Mehra, J. Neumann, J. Wood, D. Claxton, J. Gajewski, I. Khouri, K. van Besien, B. Andersson, and et al. Intravesicular carboprost for the treatment of hemorrhagic cystitis after marrow transplantation. *Urology* **46**: 811-5 (1995).
258. L. A. Levine and D. M. Kranc. Evaluation of carboprost tromethamine in the treatment of cyclophosphamide-induced hemorrhagic cystitis. *Cancer* **66**: 242-5 (1990).
259. L. A. Levine and D. F. Jarrard. Treatment of cyclophosphamide-induced hemorrhagic cystitis with intravesical carboprost tromethamine. *J Urol* **149**: 719-23 (1993).
260. B. Jeong, S. W. Kim, and Y. H. Bae. Thermosensitive sol-gel reversible hydrogels. *Adv Drug Deliv Rev* **54**: 37-51 (2002).
261. P. J. Cox. Cyclophosphamide cystitis-identification of acrolein as the causative agent. *Biochem Pharmacol* **28**: 2045-9 (1979).
262. D. J. Grinberg-Funes, C. Sheldon, and M. Weiss. The use of prostaglandin F2 alpha for the prophylaxis of cyclophosphamide induced cystitis in rats. *J Urol* **144**: 1500-4 (1990).
263. Y. Igawa, T. Satoh, H. Mizusawa, S. Seki, H. Kato, O. Ishizuka, and O. Nishizawa. The role of capsaicin-sensitive afferents in autonomic dysreflexia in patients with spinal cord injury. *BJU Int* **91**: 637-41 (2003).
264. S. Ritter and T. T. Dinh. Capsaicin-induced neuronal degeneration: silver impregnation of cell bodies, axons, and terminals in the central nervous system of the adult rat. *J Comp Neurol* **271**: 79-90 (1988).
265. B. Ronneberger, W. J. Kao, J. M. Anderson, and T. Kissel. In vivo biocompatibility study of ABA triblock copolymers consisting of poly(L-lactic-co-glycolic acid) A blocks attached to central poly(oxyethylene) B blocks. *J Biomed Mater Res* **30**: 31-40 (1996).
266. K. J. Zhu, X. Lin, and S. Yang. Preparation, characterization, and properties of polylactide (PLAO-poly(ethylene glycol)(PEG) copolymers: potential drug carriers. *J. Appl. Polym. Sci* **39**: 1-9 (1990).

267. K. J. Gray, U. H. Engelmann, E. H. Johnson, and I. J. Fishman. Evaluation of misoprostol cytoprotection of the bladder with cyclophosphamide (Cytoxan) therapy. *J Urol* **136**: 497-500 (1986).
268. J. D. Kelly, M. R. Young, S. R. Johnston, and P. F. Keane. Clinical response to an oral prostaglandin analogue in patients with interstitial cystitis. *Eur Urol* **34**: 53-6 (1998).
269. P. Y. Lee, Z. Li, and L. Huang. Thermosensitive hydrogel as a TGF-beta1 gene delivery vehicle enhances diabetic wound healing. *Pharm Res* **20**: 1995-2000 (2003).
270. S. R. Whittemore, T. Ebendal, L. Larkfors, L. Olson, A. Seiger, I. Stromberg, and H. Persson. Development and regional expression of beta nerve growth factor messenger RNA and protein in the rat central nervous system. *Proc Natl Acad Sci U S A* **83**: 817-21 (1986).
271. E. M. Shooter. Early days of the nerve growth factor proteins. *Annu Rev Neurosci* **24**: 601-29 (2001).
272. K. Lamb, G. F. Gebhart, and K. Bielefeldt. Increased nerve growth factor expression triggers bladder overactivity. *J Pain* **5**: 150-6 (2004).
273. M. R. Saban, N. B. Nguyen, T. G. Hammond, and R. Saban. Gene expression profiling of mouse bladder inflammatory responses to LPS, substance P, and antigen-stimulation. *Am J Pathol* **160**: 2095-110 (2002).
274. N. Dmitrieva, D. Shelton, A. S. Rice, and S. B. McMahon. The role of nerve growth factor in a model of visceral inflammation. *Neuroscience* **78**: 449-59 (1997).
275. K. C. Dines and H. C. Powell. Mast cell interactions with the nervous system: relationship to mechanisms of disease. *J Neuropathol Exp Neurol* **56**: 627-40 (1997).
276. R. Tanner, P. Chambers, M. H. Khadra, and J. I. Gillespie. The production of nerve growth factor by human bladder smooth muscle cells in vivo and in vitro. *BJU Int* **85**: 1115-9 (2000).
277. S. Seki, K. Sasaki, Y. Igawa, O. Nishizawa, M. B. Chancellor, W. C. De Groat, and N. Yoshimura. Suppression of detrusor-sphincter dyssynergia by immunoneutralization of nerve growth factor in lumbosacral spinal cord in spinal cord injured rats. *J Urol* **171**: 478-82 (2004).
278. J. S. de Bono, S. Y. Rha, J. Stephenson, B. C. Schultes, P. Monroe, G. S. Eckhardt, L. A. Hammond, T. L. Whiteside, C. F. Nicodemus, J. M. Cermak, E. K. Rowinsky, and A. W. Tolcher. Phase I trial of a murine antibody to MUC1 in patients with metastatic cancer: evidence for the activation of humoral and cellular antitumor immunity. *Ann Oncol* **15**: 1825-33 (2004).
279. R. M. Sharkey, A. Brenner, J. Burton, G. Hajjar, S. P. Toder, A. Alavi, A. Matthies, D. E. Tsai, S. J. Schuster, E. A. Stadtmauer, M. S. Czuczman, D. Lamonica, F. Kraeber-Bodere, B. Mahe, J. F. Chatal, A. Rogatko, G. Mardirrosian, and D. M. Goldenberg. Radioimmunotherapy of non-Hodgkin's lymphoma with 90Y-DOTA humanized anti-CD22 IgG (90Y-Epratuzumab): do tumor targeting and dosimetry predict therapeutic response? *J Nucl Med* **44**: 2000-18 (2003).
280. M. C. de Koning, G. A. van der Marel, and M. Overhand. Synthetic developments towards PNA-peptide conjugates. *Curr Opin Chem Biol* **7**: 734-40 (2003).
281. U. Koppelhus, S. K. Awasthi, V. Zachar, H. U. Holst, P. Ebbesen, and P. E. Nielsen. Cell-dependent differential cellular uptake of PNA, peptides, and PNA-peptide conjugates. *Antisense Nucleic Acid Drug Dev* **12**: 51-63 (2002).

282. M. Zuker. M fold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31**: 3406-15 (2003).
283. S. R. Whittemore, P. L. Friedman, D. Larhammar, H. Persson, M. Gonzalez-Carvajal, and V. R. Holets. Rat beta-nerve growth factor sequence and site of synthesis in the adult hippocampus. *J Neurosci Res* **20**: 403-10 (1988).
284. D. H. Mathews, J. Sabina, M. Zuker, and D. H. Turner. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol* **288**: 911-40 (1999).
285. M. A. Vizzard. Changes in urinary bladder neurotrophic factor mRNA and NGF protein following urinary bladder dysfunction. *Exp Neurol* **161**: 273-84 (2000).
286. P. E. Nielsen, M. Egholm, R. H. Berg, and O. Buchardt. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* **254**: 1497-500 (1991).
287. B. J. Turner, I. K. Cheah, K. J. Macfarlane, E. C. Lopes, S. Petratos, S. J. Langford, and S. S. Cheema. Antisense peptide nucleic acid-mediated knockdown of the p75 neurotrophin receptor delays motor neuron disease in mutant SOD1 transgenic mice. *J Neurochem* **87**: 752-63 (2003).
288. M. V. Sofroniew, C. L. Howe, and W. C. Mobley. Nerve growth factor signaling, neuroprotection, and neural repair. *Annu Rev Neurosci* **24**: 1217-81 (2001).
289. D. H. Mathews, M. E. Burkard, S. M. Freier, J. R. Wyatt, and D. H. Turner. Predicting oligonucleotide affinity to nucleic acid targets. *Rna* **5**: 1458-69 (1999).
290. M. Scherr, J. J. Rossi, G. Sczakiel, and V. Patzel. RNA accessibility prediction: a theoretical approach is consistent with experimental studies in cell extracts. *Nucleic Acids Res* **28**: 2455-61 (2000).
291. B. Tang, M. Wang, and B. C. Wise. Nerve growth factor mRNA stability is controlled by a cis-acting instability determinant in the 3'-untranslated region. *Brain Res Mol Brain Res* **46**: 118-26 (1997).
292. V. V. Demidov, V. N. Potaman, M. D. Frank-Kamenetskii, M. Egholm, O. Buchard, S. H. Sonnichsen, and P. E. Nielsen. Stability of peptide nucleic acids in human serum and cellular extracts. *Biochem Pharmacol* **48**: 1310-3 (1994).
293. S. E. Hamilton, C. G. Simmons, I. S. Kathiriya, and D. R. Corey. Cellular delivery of peptide nucleic acids and inhibition of human telomerase. *Chem Biol* **6**: 343-51 (1999).
294. M. Pooga, U. Soomets, M. Hallbrink, A. Valkna, K. Saar, K. Rezaei, U. Kahl, J. X. Hao, X. J. Xu, Z. Wiesenfeld-Hallin, T. Hokfelt, T. Bartfai, and U. Langel. Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. *Nat Biotechnol* **16**: 857-61 (1998).
295. H. Knudsen and P. E. Nielsen. Antisense properties of duplex- and triplex-forming PNAs. *Nucleic Acids Res* **24**: 494-500 (1996).
296. K. Kaihatsu, K. E. Huffman, and D. R. Corey. Intracellular uptake and inhibition of gene expression by PNAs and PNA-peptide conjugates. *Biochemistry* **43**: 14340-7 (2004).
297. M. Tyagi, M. Rusnati, M. Presta, and M. Giacca. Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. *J Biol Chem* **276**: 3254-61 (2001).
298. M. M. Poggi, P. A. Johnstone, and R. J. Conner. Glycosaminoglycan content of human bladders. a method of analysis using cold-cup biopsies. *Urol Oncol* **5**: 234-237 (2000).

299. Y. Sun and T. C. Chai. Effects of dimethyl sulphoxide and heparin on stretch-activated ATP release by bladder urothelial cells from patients with interstitial cystitis. *BJU Int* **90**: 381-5 (2002).
300. V. Y. Hu, S. Malley, A. Dattilio, J. B. Folsom, P. Zvara, and M. A. Vizzard. COX-2 and prostanoid expression in micturition pathways after cyclophosphamide-induced cystitis in the rat. *Am J Physiol Regul Integr Comp Physiol* **284**: R574-85 (2003).
301. M. R. Saban, H. Hellmich, N. B. Nguyen, J. Winston, T. G. Hammond, and R. Saban. Time course of LPS-induced gene expression in a mouse model of genitourinary inflammation. *Physiol Genomics* **5**: 147-60 (2001).
302. D. Oddiah, P. Anand, S. B. McMahon, and M. Rattray. Rapid increase of NGF, BDNF and NT-3 mRNAs in inflamed bladder. *Neuroreport* **9**: 1455-8 (1998).
303. V. Bergonzini, S. Delbue, J. Y. Wang, K. Reiss, M. Prisco, S. Amini, K. Khalili, and F. Peruzzi. HIV-Tat promotes cellular proliferation and inhibits NGF-induced differentiation through mechanisms involving Id1 regulation. *Oncogene* **23**: 7701-11 (2004).
304. M. Baykara, T. Erdogru, K. H. Gulkesen, C. F. Sargin, M. Savas, and M. Ates. Does interstitial cystitis urine include possible factors effecting the nociceptive system of the spinal cord? *Urol Int* **71**: 66-72 (2003).
305. D. E. Bjorling, H. E. Jacobsen, J. R. Blum, A. Shih, M. Beckman, Z. Y. Wang, and D. T. Uehling. Intravesical Escherichia coli lipopolysaccharide stimulates an increase in bladder nerve growth factor. *BJU Int* **87**: 697-702 (2001).