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STUDIES ON THE PATHOGENESIS OF

INTERSTITIAL CYSTITIS

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SUMMARY

Interstitial cystitis (IC) is a condition which predominantly affects women causing symptoms which mimic urinary tract infection. In IC, cystourethroscopy both excludes other causes and reveals the positive findings of Hunner's ulcer and low bladder capacity (classic or severe IC) or glomerulations which are petechial haemorrhages seen on bladder emptying (early IC). Routinely obtained (post bladder hydrodistention) biopsies from IC subjects showed greater degrees of epithelial denudation and submucosal oedema, congestion and ectasia, and inflammatory infiltrate but no difference in the degree of submucosal haemorrhage compared with those from control subjects. Nonetheless the histology of IC was indistinguishable from control subjects more than 50% of the time.

Given the female to male preponderance of 10 to 1 in IC, an immunohistochemical study of oestrogen (ER) and progesterone receptor (PR) expression in the IC bladder submucosa was performed. There was no difference found between the female IC or control subjects in either ER or PR staining cells. However the control males (taken from cystectomy for bladder cancer specimens) had greater numbers of both ER and PR staining cells.

In view of the diagnostic importance of glomerulations, the bladder microvascular density in IC and control women was assessed by CD34immunohistochemistry and computer assisted image analysis. The blood vessel count and proportional area made up by blood vessels in the bladder suburothelium was reduced in the IC group. A possible explanation for these findings may have been increased submucosal oedema in the IC group causing an apparent reduction of microvascular density.

Therefore a comparison of histology and microvascular density of bladder biopsies taken before and after hydrodistention was performed.

A major key finding of this study is that biopsies taken post hydrodistention showed a strong trend toward more submucosal oedema and a reduced microvascular density as compared with the biopsies taken before hydrodistention. This observation was only present in the IC group of patients. In fact in the undistended bladder the subepithelial microvascular density is *increased* in IC presumably due to inflammation. The changes of epithelial denudation and submucosal inflammation were least altered by the hydrodistention process.

There is therefore evidence of bladder inflammation in at least a subset of women with IC. Kinins are potent inflammatory mediators and a second major key finding of this study was a five fold increased increment in bradykinin-(1-8) peptide urinary excretion but no increase in kallidin excretion in the IC group. This finding may be due to increased transudation through an inflamed bladder wall and was not accompanied by an increase in the urinary excretion of substance P or its metabolites.

Bradykinin-(1-8) is known to act on bradykinin type 1 receptors which are thought to be induced in models of inflammation. A trend toward increased number of cells staining for bradykinin type 1 receptors in the IC bladder was demonstrated and some of these cells appear to be a subpopulation of B lymphocytes.

These studies support the role of abnormal epithelial function and inflammation in the pathogenesis of IC.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other institutuion and contains no material previously published or written by another person except where due reference is made in the text of the thesis.

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Rosamilia A, Cann L, Dwyer PL, Scurry J and Rogers P. Bladder microvasculature in women with interstitial cystitis. J Urol. 1999; 161: 1865-1870.

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BOOK CHAPTERS

Rosamilia A and Dwyer PL. Sensory disorders of the lower urinary tract. In: Stanton SL and Dwyer PL. Eds, Urinary tract infection in the female. Martin Dunitz Publishers London. 2000.

CHAPTER ONE

PART 1. LITERATURE REVIEW

PART 2. OBJECTIVES OF THE THESIS

PART 1. LITERATURE REVIEW

1.1. PURPOSE OF REVIEW

The purpose of this review is to provide information on the clinical condition of interstitial cystitis and the various etiological theories which have been proposed.

1.2 INTRODUCTION

Guy Hunner, a gynaecologist working in Howard Kelly's Department at the Johns Hopkins Hospital in Baltimore, USA first described the condition of interstitial cystitis (IC) in 1914. In 1918, Hunner stated "the cause of this type of bladder inflammation remains a mystery. There is no evidence from the history of these patients that the condition began as an infection in the urine". More than eighty years on, our understanding of the basic pathophysiology of this condition is still "a mystery". The diagnostic criteria for interstitial cystitis are controversial and often ill defined, the treatment is frequently unsuccessful and there is a lack of objective

and subjective tests to predict or monitor response to treatment (Messing, 1999).

Interstitial cystitis (IC) is a predominantly female condition with the presenting symptoms of frequency of micturition and pain. Women with interstitial cystitis (IC) are frequently not diagnosed for many years despite multiple attendances to clinicians including gynaecologists (Ratner et al, 1994). The reasons for this are many. The condition of IC is not well understood, the pain of IC is poorly localised and there is no single diagnostic test. Urinary frequency, urgency and bladder pain in the absence of urinary tract infection are the cardinal symptoms of IC. The pain may be described as pelvic suprapubic, vaginal or perineal and could therefore mimic the pain associated with gynaecological conditions such as endometriosis or pelvic infection. The cystoscopic appearance of the bladder wall during filling is frequently normal so the characteristic appearance of petechial haemorrhages may not be seen unless the bladder is viewed during or after it is distended and emptied.

Delay in diagnosis and lack of empathy from their medical practitioners has caused considerable frustration amongst IC sufferers and has been an important factor in the formation of self-help organisations around the world. The Interstitial Cystitis Association (ICA) was founded in the United States in 1984. Since the establishment of this very effective patient advocacy group, the ICA has stimulated and helped to fund increased interest, awareness and research activity in the pathogenesis and treatment of this condition (Ratner et al, 1994).

1.3 PREVALENCE

Estimates of the prevalence of IC in the USA range from 30 to 500 cases / 100,000.

The lower estimate was derived from a postal survey of urologists in 1987 and the higher from self-report of a previous diagnosis of IC in the 1989 National Household Interview Survey (Jones & Nyberg, 1997). Bade and colleagues (1995) calculated the prevalence of interstitial cystitis in the Netherlands to be 8 to 16/100,000 women. This discrepancy illustrates the problem in prevalence estimates which are created by reliance on differing or variable diagnostic criteria (for example, emphasis on mastocytosis in Netherlands study) in addition to the possibility of reduced actual incidence or reduced awareness of the condition. In 1987, a calculation based on an estimate of 44,000 patients with interstitial cystitis in the United States amounted to a minimum financial cost of 428 million dollars per year due to medical care and lost income. The quality of life of women with interstitial cystitis has been said to be worse than women with end stage renal disease (Held et al, 1990).

1.4 DEFINITION

A consensus definition of IC was agreed upon at workshops held by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIDDK), National Institutes of Health, Bethesda, Maryland, USA and reported in 1988 for the purposes of research and clinical trials (Gillenwater & Wein, 1988; Hanno, 1994). Since the establishment of the Interstitial Cystitis Data Base in 1993 and publication of its interim analysis in 1997, it has been recognised that while the NIDDK criteria are useful for the purpose of research, a significant number of patients whom experienced clinicians would classify as very likely having IC may be missed (Hanno, 1994; Simon et al, 1997).

The NIDDK criteria include the urodynamic findings of no bladder instability, a

capacity less than 350 mls with urgency at less than 150mls and cystoscopic evidence of a classic Hunner's ulcer or glomerulations (pin-point petechial hemorrhages). Hydrodistention should be performed to a height of 80 to 100 cm water pressure for 1 to 2 minutes and there must be at least 10 glomerulations per quadrant with 3 quadrants affected. Exclusion criteria (Table 1) according to this definition are age younger than 18 years, symptom duration less than 9 months, nocturia fewer than one and frequency fewer than eight, the presence of urinary or genital tract infection, tumour, radiation effect or cyclophosphamide cystitis (Gillenwater & Wein, 1988). There have been reports of misdiagnosis of IC in patients with non-bacterial cystitis due to tiaprofenic acid (O'Neill, 1994).

1.5 CLINICAL DIAGNOSIS

The criteria for the diagnosis of interstitial cystitis are

1. Symptoms of frequency, urgency and pain

2. Findings of a low bladder capacity on urinary diary or urodynamic assessment

3. Characteristic cystoscopic appearance

1.5.1 SYMPTOMS AND SIGNS

The variable symptomatology of this condition is well demonstrated by an epidemiological study by Koziol (1994) who questioned 500 patients with IC and 171 controls. In the IC group almost 90 % were women and 94 % Caucasian. The average age at the time of first symptoms was 42 years with 30 % younger than 30 years at the onset of symptoms. In comparison with control patients, IC sufferers more commonly had a history of allergy, arthritis, sinusitis, irritable bowel

syndrome, hayfever and asthma. There was a significantly higher proportion ($p<0.00001$) of female IC patients (mean age 51 years) having had a hysterectomy compared with female controls (mean age 45 years). The main symptoms in women with interstitial cystitis were frequency (92 %), urgency (92 %) and pelvic pain (70 %). Over half the women described dyspareunia and being awakened at night by pain. Over 55 % reported daily or constant pain and nearly 57 % described the pain as severe or excruciating. The pain was described as vaginal in 60% and lower abdominal in 60%. Among the IC sufferers surveyed pain was exacerbated by spicy foods, alcoholic, acidic, carbonated or caffeinated beverages in 50 %, by sexual intercourse (50 %), stress (60 %) and exercise (40 %). Only 10 % of IC patients rated leisure activities, travel and sleep to be unaffected by their condition. Family relationships and responsibilities were said to be adversely affected in 70%. There did not appear to be significant worsening of symptoms when analysed over a 5 to 15 year period. (Koziol, 1994)

Physical examination of the patient with IC is generally unrevealing apart from the possible presence of vaginal or suprapubic tenderness (Hanno, 1994).

1.5.2 URINARY DIARY

A urinary diary is an important part of the initial and ongoing assessment. Small frequent volumes of less than 150mls are characteristic. Women with sensory bladder disorders often decrease their fluid intake so that the severity of their frequency and urgency symptoms may be masked on routine questioning. Frequency is defined as more than 7 micturitions per day and nocturnal frequency as arousal from sleep more than once at night (Stanton, 1984). Koziol (1994) found that women

with IC charted a mean number of 21 voids in 24 hours with a mean interval of 2.6 hours between voids overnight. Parsons (1994) found the mean number of voids was 16 per day and 4.7 per night and a mean voided volume of 106mls.

1.5.3 CYSTOSCOPIC APPEARANCE

Cystourethroscopy should be performed under general or regional anaesthesia. The most common diagnostic finding in IC are glomerulations (Messing & Stamey, 1978), generalised petechiae which ooze fresh blood often in cascades (Figure 1a) when the bladder is being emptied following distention. On reinspection of the bladder these glomerulations coalesce to become splotchy haemorrhages (Figure 1b) Local trauma due to contact of the cystoscope with the bladder wall does not constitute glomerulations. As the bladder is emptied the fluid becomes increasingly blood stained which produces the characteristic terminal haematuria. Failure of bladder visualisation during emptying or failure to perform a second inspection after the first distension could be an important reason why the diagnosis of IC is missed. The use of the camera during cystoscopy has been invaluable in allowing the bladder to be easily visualised during emptying.

Messing and Stamey (1978) classified IC as 'early' or 'classic' disease. Patients with early IC had an anaesthetic capacity greater than 450mls; normal first distention apart from slightly prominent vascularity; glomerulations and haemorrhages. Classic IC patients had a bladder capacity less than 450mls; a Hunner's ulcer; fissures (Figure 1c) or linear scars seen as capacity was reached.

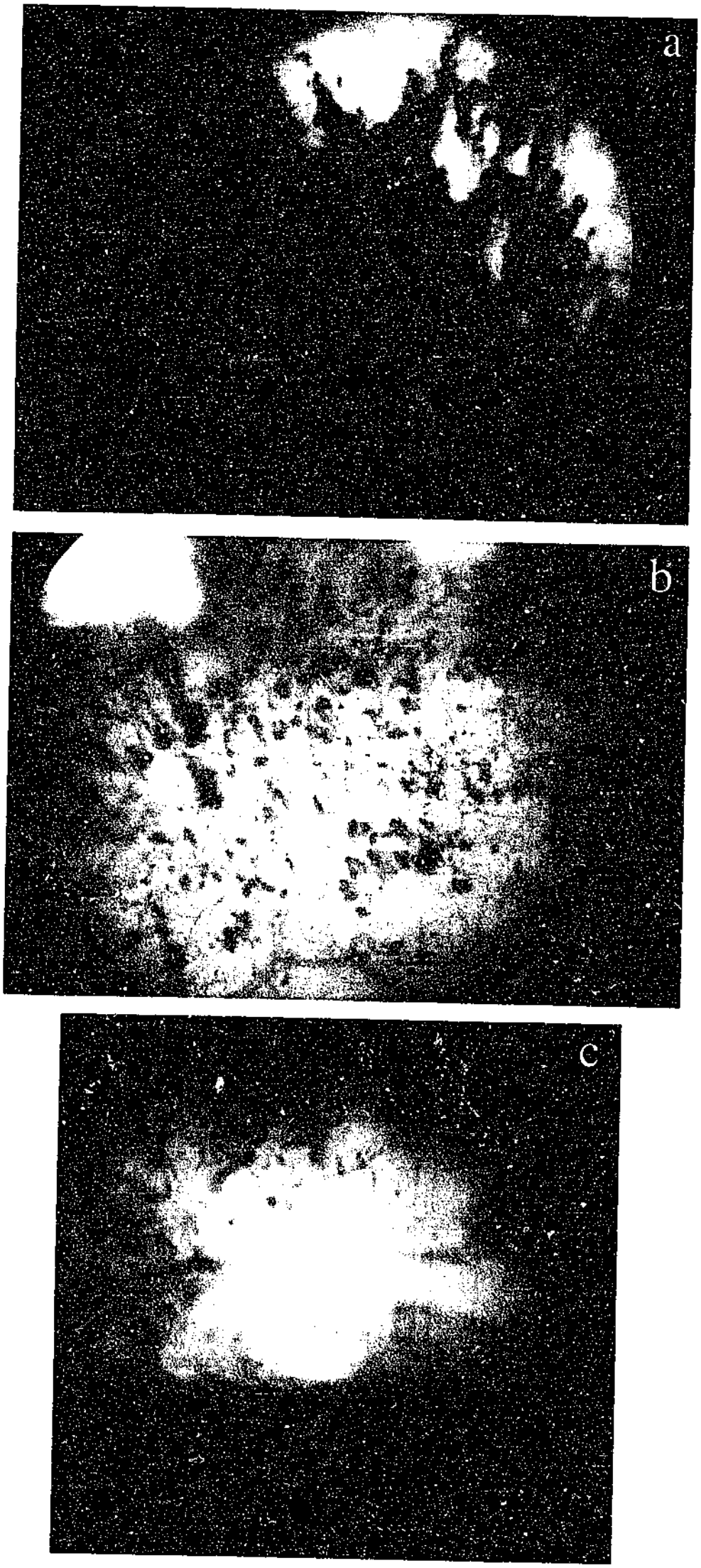


Figure 1(a) Cascade bleeding; **1(b)** Glomerulations; **1(c)** Fissuring seen at cystoscopy and hydrodistention in interstitial cystitis

Hunner in 1918, himself stated that as a rule careful examination would reveal usually small superficial ulcers. Varying descriptions were given for the appearance of the ulcers. He described linear ulcers 1 to 2 mm in width and 0.5 to 2 cm in length which suggests that they may be mucosal ruptures. Two or three minute ulcers may be found in a group and they may be surrounded by a small red area of oedema or a zone of radially converging vessels.

This is an uncommon finding with Hunner reporting 25 cases over a period of eighteen years experience. Johansson and Fall (1990) described 64 "ulcer IC" cases over 13 years and state that Hunner's ulceration is "single or multiple patches of reddened bladder mucosa... with small vessels radiating to a central pale scar, fibrin deposit or coagulum. Central rupture at this site occurs during bladder distention... After mucosal rupture, oozing of blood from the mucosal margins and the ulcer bottom occurs, and this is the so-called elusive ulcer of Hunner."

Parsons (1994) describes a Hunner's ulcer as a velvety red patch rather than a true ulcer and found it present in 8% of his IC patients. He makes the point that the "elusive" ulcer of interstitial cystitis may be quite rare and is possibly over-reported as more than 75% of 'ulcers' previously diagnosed in his series of patients were in fact old biopsy sites.

1.6 HISTOLOGICAL FINDINGS

Urine microscopy, culture and cytology in addition to bladder biopsy of the worst affected area should be performed to exclude infection or carcinoma in situ (Messing, 1992). Utz and Zincke (1974) found that 1% of women initially diagnosed

as IC were subsequently found to have carcinoma in situ. Messing and Stamey (1978) reported a perforation rate of approximately 10% using a resectoscope to obtain biopsies 1 to 2 cm long. To minimize the risk of perforation the cold cup biopsy should be taken following hydrodistension and the bladder may be drained by urethral catheterisation. The use of water or glycine as distension solution allows the use of diathermy for haemostasis (Parsons, 1994; Wein & Broderick, 1994).

The histological findings of the bladder biopsy in women with interstitial cystitis are not consistent. Johansson and Fall (1994) reported that the most usual histologic findings in classic (ulcer) disease were the presence of an ulcer (96%), granulation tissue (89%), mucosal hemorrhage (86%), monocytic infiltration (100%) and an increased number of mast cells in the lamina propria and detrusor muscle. In contrast, the histologic findings in non ulcer interstitial cystitis may include submucosal hemorrhage (89%), mucosal ruptures (83%) and mild inflammatory lymphocytic infiltrate (20%). Lynes and colleagues (1990) reported that the only statistically significant differences in bladder biopsy histology between IC patients and controls were denuded epithelium (present in 7/22 IC and 0/10 controls) and prominent submucosal inflammation (10/22 and 1/10 respectively). However submucosal oedema and vascular ectasia was found in a similar proportion of IC and control patients and marked changes occurred in both groups after bladder distention suggesting that these changes were secondary to the distention rather than primary pathology. In summary, routine light microscopy histological findings in IC are variable and may even be reported as within normal limits (Messing & Stamey, 1978; Johansson & Fall, 1994; Lynes et al, 1990). The role of special staining methods and electron microscopy will be discussed in the etiology section.

1.7 NON-SPECIFICITY OF CYSTOSCOPIC FINDINGS

Some women with frequency, urgency and bladder discomfort have a reduced functional bladder capacity on their urinary diary and urodynamic assessment but no abnormality on cystoscopy. It is unclear whether this is early IC (Frazer et al, 1990) or a different condition (idiopathic sensory urgency). Parsons (1994) comments that the diagnosis of IC is much more likely if continuous symptoms are present for longer than 6 months. Most women with frequency/urgency symptoms in the absence of cystoscopic findings of IC generally respond to simple treatment with bladder retraining, anticholinergic drugs or bladder hydrodistention (Parsons, 1994).

In contrast, glomerulations are seen occasionally in women who do not have symptoms of frequency or pain. Glomerulations have been described in women with intrinsic urethral sphincter deficiency which prevents the bladder filling to capacity and supports the view that glomerulations may be a response to distention of a chronically underfilled bladder. (Erickson, 1995) Waxman and colleagues (1998) found mucosal changes characteristic of IC following hydrodistention (mean volume of 890 ml) in 9 of 20 women with no irritative bladder symptoms. In this current series, 2 of 40 women who had no urge symptoms and urodynamic bladder capacity of 500mls, had glomerulations following hydrodistention (to a median capacity of 600mls) for suprapubic catheterisation after stress incontinence or prolapse surgery. The cystoscopic finding of glomerulations may be a response to distention of a chronically underfilled bladder or perhaps overdistention and it is certainly not specific to IC. Therefore the cystoscopic appearance in isolation does not make the diagnosis (Hanno, 1994).

1.8 ETIOLOGY

1.8.1 INFECTION

One of the earliest theories regarding the etiology of IC was infection. According to Wilkins (1989), the commonest urinary organisms cultured in IC are Gardnerella or lactobacilli. These are common urethral commensals and of uncertain significance. Using polymerase chain reaction cloning and sequencing techniques on bladder biopsy material, Domingue and colleagues (1995) have reported the presence of DNA derived from Gram-negative bacteria and cultured a novel non-bacterial filterable form in IC patients. However the presence of bacterial DNA in IC bladder biopsies was not confirmed in subsequent studies which also used molecular biologic techniques (Haarala et al, 1996; Keay et al, 1998). Haarala and colleagues (1999) cultured obligatory anaerobic organisms in 5 out of 14 subjects with IC but in none of the 5 controls or 5 subjects with urethral syndrome. One subject responded dramatically to metronidazole therapy. An explanation is that these changes in bacterial flora may be secondary to therapeutic interventions used in the IC group. Helicobacter pylori, only recently found to be the cause of peptic ulceration is not increased in interstitial cystitis (English et al, 1998). A recent placebo controlled pilot study of the use of sequential oral antibiotics (doxycycline, erythromycin, metronidazole, clindamycin, amoxicillin and ciprafloxacin for 3 weeks each) in IC was only associated with a two fold improvement in pain and urgenccy as well as a two fold incidence of adverse effects in the antibiotic group. (Warren et al, 2000) Although it is speculated that infection may play a role as the initial trigger of IC, various studies including light microscopy and culture, electron microscopy (EM) studies, serology and molecular biologic techniques have not to date consistently

isolated a micro-organism or viral agent in IC (Duncan & Schaeffer, 1997).

1.8.2 ABNORMAL EPITHELIAL BARRIER

Parsons and colleagues (1994) have proposed that a defective glycosaminoglycan (GAG) mucus layer in IC alters bladder permeability. They found IC patients had larger defects in the mucus layer of the bladder, different percentages of specific carbohydrates in the bladder mucus layer and increased passage of radiolabelled urea and sodium through the urothelium. Others have found that IC patients have a decrease in type IV collagen staining in the urothelial basement membrane (Wilson et al, 1995) and a deficit of urothelial chondroitin sulfate proteoglycans (Hurst et al, 1996). Urinary hyaluronic acid, a nonsulfated glycosaminoglycan located in the bladder subepithelium has been shown to be increased in IC (Erickson et al, 1998), suggesting leakage across an abnormally permeable epithelium. However, increased bladder permeability can also be demonstrated in chemical and infective cystitis. Also, no increased permeability was demonstrated in IC patients to the radionuclide, 99m Tc-DTPA which is used in vesicoureteral reflux imaging (Ruggieri et al, 1994) and electron microscopy studies have not confirmed the presence of ultrastructural defects in the urothelium (Dixon et al, 1986; Nickel et al, 1993). It remains unclear whether the GAG layer abnormality in interstitial cystitis is the cause or effect of the process of interstitial cystitis or a nonspecific finding of inflammation.

Further evidence of a GAG layer defect was reported by Byrne and colleagues (1999) who demonstrated a significant reduction of urinary excretion of GP51, a glycoprotein component of the urothelial mucin layer in women with IC.

Nitrofurantoin was implicated in causing destruction of the GAG layer (Gillespie et al, 1985). Many patients with IC had been treated with nitrofurantoin empirically for infection. However, no change in rabbit bladder mucin, bacterial adherence or bladder ultrastructure after acute or chronic nitrofurantoin therapy was observed (Ruggieri et al, 1994).

Parsons and colleagues (1998) found that intravesical instillation of potassium causes increased pain and urgency in 75% of subjects with IC and infective cystitis compared with 4% of controls, presumably due to a loss of the protective GAG layer. It has been suggested that a positive potassium leak test may identify a subset of IC patients more likely to benefit from combined heparinoid and antidepressant therapy (Teichman & Nielsen-Omeis, 1999) although this may simply reflect disease severity (Hanno, 1999). Intravesical potassium was found to have a positive and negative predictive value for the diagnosis of IC of only 66% and 46% respectively (Chambers et al, 1999); the value of this test requires further clarification.

Recently, the urine of patients with IC has been shown to inhibit bladder epithelial proliferation (Keay et al, 1999) which may result in a damaged epithelium. These authors have gone on to show that bladder epithelial cells from IC patients produce the same antiproliferative factor (APF) as that purified from the urine. This factor specifically inhibited heparin-binding epidermal growth factor (HB-EGF) production by bladder epithelial cells. (Keay et al, 2000). Serum levels of HB-EGF were also lower in the IC group implying a systemic disorder. The cause of these changes are not as yet known.

1.8.3 AUTO-IMMUNE DISEASE

Epidemiological surveys have shown an association between IC and auto-immune diseases such as systemic lupus erythematosus (Alagiri et al, 1997), Sjogren's syndrome (Van de Merwe et al, 1993), and disorders such as asthma, food allergy and fibromyalgia which have an immunological component (Ratliff et al, 1995). Mattila (1982) demonstrated an increased deposition of immunoglobulin and complement components in the bladder vasculature of IC patients. Expression of HLA-DR (Liebert et al, 1995) and urinary levels of interleukin 6 (Lotz et al, 1994) were reported to be increased in IC patients although the latter finding was not confirmed using PCR techniques (Liebert et al, 1993). However no difference has been found in levels of expression of interleukin 1b, (Martins et al, 1994) 1, 2, gamma interferon, the degree of lymphocyte activation, (Miller et al, 1992) complement C3 or eosinophilic cation protein (Steinert et al, 1994). No consistent evidence for an immunological mechanism in interstitial cystitis has been found (Ratliff et al, 1995) but research using experimental and animal models of IC such as the naturally occurring feline interstitial cystitis in cats (Buffington et al, 1997, Buffington et al, 1999) may contribute further to an understanding of its etiology.

Anti-inflammatory agents, immunosuppressives and steroids have not provided a consistent or predictable therapeutic response. However intravesical bacille Calmette-Guerin (BCG), an immune modulator and an established treatment for bladder carcinoma in situ, has been used successfully in the treatment of IC (Peters et al, 1997, Peters et al, 1998). As further evidence for an immunological role in IC, Peters and colleagues (1999) found that urinary interleukin-2, -6 and-8 were elevated in IC subjects but not in controls or IC subjects in remission after BCG therapy.

1.8.4 NEUROGENIC INFLAMMATION AND THE ROLE OF MAST CELLS

Neurogenic inflammation has been proposed to be one of the important pathophysiological processes occurring in IC resulting in mast cell activation, sensory nerve fibre proliferation, and altered or increased sensory neuropeptide and inflammatory mediator expression (Steers & Tuttle, 1997; Elbadawi, 1997). This pathway involves sensitization of nociceptive pathways due to nerve growth factor (NGF) with activation of neurons in the CNS and silent C-fibres in the periphery (Steers, 2000). Molecular events in response to inflammation including increased NGF have been linked to spontaneous burst firing of nerves and a lowering of thresholds for activation. This is what underlies allodynia (non-painful stimuli causing pain) which in the bladder is analogous to non-painful intravesical contents such as potassium causing discomfort, and hyperaesthesia which in the bladder is analogous to low volume filling causing pain. Changes in primary afferents lead to plasticity in second order neurons and elimination of the painful focus may fail to deactivate these neurones in the CNS leading to a behavioural correlate such as phantom pain. The abnormal focus of neural activity shifts into the central nervous system. Animal models for IC are consistent with this (Steers, 2000).

1.8.4.1 Mast cells

Mast cells are triggered by neuropeptides to release inflammatory mediators (Theoharides & Sant, 1997) some of which are implicated in neuronal proliferation (Hohenfellner et al, 1992). An increased bladder mast cell count occurs in some interstitial cystitis patients (Sant & Theoharides, 1994). Preliminary studies have demonstrated the presence of estrogen and progesterone receptors in mast cells (Pang

et al, 1995) which is of interest given the female preponderance of the condition. Activated mast cells are in close proximity to bladder nerve fibres (Letourneau et al, 1996) and proliferation of nerve fibres including those which release sensory neuropeptides such as substance P has been reported in women with IC (Hohenfellner et al, 1992; Christmas & Rode, 1991; Pang et al, 1995).

1.8.4.2 Nerve growth factor

Nerve growth factor (NGF) is responsible for nerve fibre growth and function and is speculated to be a key player linking inflammation to altered pain signalling (Steers & Tuttle, 1997). NGF is expressed widely at the cellular level including in mast cells and can activate mast cells to degranulate and proliferate (Nilsson et al, 1997). NGF receptor expression is increased by inflammation and in preliminary studies levels of NGF and other neurotrophins were reported to be elevated in the bladder and urine of subjects with IC (Lowe et al, 1997; Okragly et al, 1999).

1.8.4.3 Kallikrein-kinin system

Kinins act as vasodilators, increase vascular permeability, contract smooth muscle, release neurotransmitters, degranulate mast cells and are potent pain producing substances (Dray & Perkins, 1993). Tissue and plasma kallikrein are the enzymes that activate kinins; urinary kallikrein was elevated in women with IC and this correlated with the patients' pain score (Zuraw et al, 1994) Kinins may be important in the inititiation and maintenance of chronic inflammatory pain in IC as part of the neurogenic inflammatory process described above

1.8.4.4 Nitric oxide

Nitric oxide synthase (NOS) converts L-arginine to nitric oxide and is present in some bladder sensory nerves, in neutrophils and macrophages. Nitric oxide is involved in inflammation and bladder tone regulation and is a second messenger of bradykinin. Urinary nitric oxide synthase activity is decreased in IC patients compared with increased levels in urinary tract infection (Smith et al, 1996). This finding is the basis for the therapeutic use of L-arginine in IC which has been associated with an improvement in pain intensity, pain frequency and urgency (Korting et al, 1999).

1.8.5 VASCULAR CHANGES

Abnormalities in bladder blood flow have also been reported. Two independent studies using Doppler have found a reduction in bladder blood flow with bladder filling in IC as compared with control subjects in whom blood flow increased with bladder filling (Irwin & Galloway, 1997; Pontari et al, 1999). Messing (1999) commented that if not due to artefact, "relative ischaemia could explain the classic symptoms of interstitial cystitis and other features generally believed to be part of the syndrome, including a mucosal barrier leak, neuronal irritation and the hemorrhagic appearance accompanying bladder distention".

1.8.6 REFLEX SYMPATHETIC DYSTROPHY

Reflex sympathetic dystrophy is a sympathetically mediated pain condition which occurs following injury (or overuse) to bone or soft tissues. This neuropathic

condition is associated with autonomic (e.g. increased sweating and vasomotor abnormalities) and dystrophic changes (e.g. soft tissue and bone atrophy, joint contractures). The initial injury is postulated to cause abnormal neuronal healing with incomplete neuronal repair and regeneration. This results in increased afferent nerve activity to the dorsal horn which increases motor and sympathetic efferent activity. This in turn increases the sensitivity of peripheral sensory receptors and end organ hypersensitivity, and decreases tissue perfusion.

Interstitial cystitis has many of the hallmarks of a neuropathic condition; frequency and urgency (bladder overuse), vascular ischaemia and bladder contracture. There is an increase in urinary norepinephrine in IC, the levels remaining elevated whether patients were treated or untreated (Stein et al, 1999). The possible initial injury is unknown, but may be infection, overuse (frequency) or pelvic surgery. Hunner (1918) stated, "it may be that the frequency of voiding and straining bring about hypertrophy and hypersensitiveness of the sphincter region and inflammation of the urethral tissues''.

1.9 TREATMENT

The multitude of therapeutic modalities used for treating interstitial cystitis is a result of its poorly understood pathogenesis, less than optimal response to treatment regimens and paucity of controlled trials.

1.9.1. GENERAL PRINCIPLES

The following general principles should be observed;

1) A thorough investigative work-up to establish the diagnosis and exclude other systemic and gynaecological conditions.

2) Honest and extensive explanation of the nature and treatment of the condition. Women with IC should understand that IC is not a malignant or premalignant condition. It is usually not progressive and that although there is no magic cure there are many options available to relieve symptoms. The condition may remit either spontaneously or after treatment but relapses are common.

3) Provision of information and reading material can be made available through the Interstitial Cystitis Association. If a self help group exists locally the patient should be encouraged to attend and in this way begin active participation in her own care. Acidic, alcoholic or carbonated beverages, spicy foods, coffee, tea and chocolate have been reported to increase pain in up to 50% of IC sufferers (Koziol, 1994). Therefore dietary modification has much anecdotal support. Urinary alkalinisers may be helpful. The combination of an acid restricted diet and urinary alkalinization for 3 months resulted in 46% response rate however no long-term data is available. (Whitmore, 1994). Calcium glycerophosphate (Prelief) in a non-randomised study caused a decrease in symptom severity after foods such as pizza, coffee, acidic fruits and juices in 40% of responders (Bologna et al, 2000)

4) Easy toilet access and support from family and colleagues are important considerations. Stress-reduction techniques may include hypnosis, relaxation therapy, meditation, massage therapy and regular exercise (Whitmore, 1994).

5) Instruction in bladder retraining so patients gradually extend the time interval between voids, increasing their awake bladder capacity. A common observation with treatment strategies for IC is that pain is the first symptom to be improved and frequency the last; therefore bladder retraining needs to be used in association with other treatment regimens (Parsons, 1994).

6) In IC as with most other chronic pain syndromes, psychopathology is a common sequel and sympathetic support from the clinician and in some cases antidepressants is an important aspect of patient care. More than 50% of women living with IC report varying levels of anxiety and depression (Koziol, 1994). Historically IC was thought to be entirely psychosomatic :"...A pathway for the discharge of unconscious hatreds" (Ratner et al, 1994). However there is no evidence to support this notion. It is hoped that the NIDDK IC database project continues to provide the framework for epidemiological research including the psychological aspects of the disease.

1.9.2 MEDICAL TREATMENT

1.9.2.1 Standard oral therapies

The placebo effect in women with interstitial cystitis for oral or intra-vesical therapy is of the order of 20 to 30% and must be taken into account when assessing the effect of drug therapy (Hanno & Wein, 1994). Moreover, a 50% incidence of temporary remission independent of therapy with an average duration of 8 months has been reported (Jones & Nyberg, 1997). Anticholinergic, non-steroidal anti-inflammatory agents and steroids are of limited value in the treatment of IC (Hanno & Wein, 1994)

Antidepressants may be an important adjunct to other therapies in order to help combat the chronic pain and sleep deprivation which is often part of this condition. Amitryptyline was shown in an uncontrolled trial to result in symptomatic improvement in about half the patients treated. About one quarter of the study group could not tolerate the side effects of fatigue or weakness (Hanno et al, 1989). The dose of amitrypyline starts at 25mg three times daily, increasing to 75mg four times daily, if tolerated.

Pentosanpolysulfate sodium (PPS or Elmiron) is a heparin analogue which is taken orally at a usual dose of 100 mg three times per day. Its proposed mechanism of action was to replenish the defective glycosaminoglycan layer but a recent report of inhibition of mast cell histamine secretion and intracellular calcium ion by pentosan polysulfate suggests more than one mode of action (Chiang et al, 2000). A controlled clinical study performed by Parsons of patients with severe disease showed symptomatic improvement in 32% compared with 16% for placebo. (Parsons et al, 1993) No significant objective improvement had been demonstrated by Holm-Bentzen and colleagues (1987). A long-term, open-label study of the efficacy and safety of Elmiron found that 46% dropped out within the first 3 months (participants were required to pay for medication), and 62% of patients receiving therapy for 6 to 35 months found an overall improvement in symptoms. Adverse events were uncommon (<4%) and included reversible alopecia, diarrhea, nausea, rash and dyspepsia. (Hanno, 1997) Elmiron was approved for the treatment of IC by the US Food and Drug Administration in 1996.

1.9.2.2 Standard intravesical therapies

Dimethyl sulfoxide (DMSO) is the drug most widely used for interstitial cystitis either as single therapy or in combination with heparin, steroids and/or local anesthetic agents. Until 1996, DMSO was the only drug, oral or intravesical, approved by the Food and Drug Administration (FDA) for use in IC. It is a chemical solvent with anti-inflammatory, analgesic, muscle relaxant, mast cell inhibition and collagen dissolution properties (Sant & LaRock, 1994). After current infection is excluded, intravesical instillation can be performed with a small urethral catheter lubricated with 2% lignocaine gel. The treatment regimens vary but once or twice weekly instillations for 4 to 8 treatments is commonly used. Subsequent instillations depend on the response and severity of symptoms. DMSO is teratogenic in animals and should be avoided in pregnancy (Sant & LaRock, 1994). A response rate to DMSO of 50% to 90% has been reported. The relapse rate is up to 40% although 75% of relapsers respond to further instillations. Reported side effects are a garlic-like breath odour and taste and transient bladder spasm and irritability due to a chemical cystitis in 10%. Most studies of intravesical DMSO have been uncontrolled but Perez-Marrero and colleagues (1988) demonstrated both subjective and objective improvement over placebo. DMSO is cheap, has relatively few side effects and self-instillation can be taught if required.

Sodium oxychlorosene (chlorpactin) acts as a detergent on the bladder mucosa. Chlorpactin is used under general or regional anesthesia as the instillation is painful. Vesicoureteric reflux must be excluded pretreatment. As with all other instillations it should not be performed at the time of biopsy (Sant & LaRock, 1994). Chlorpactin has usually been used if DMSO has failed and is also administered via a Foley catheter. The bladder is filled with chlorpactin solution to half bladder capacity and

then emptied. This is repeated until the total volume of the prepared 0.4% chlorpactin solution is used and is followed by copious saline irrigation to the bladder, vaginal introitus and perineum. The perineum and vulva should be protected from contact as chlorpactin can cause caustic burns. Repeated instillations may be required as it is impossible to predict individual response or number of treatments required. Symptoms may worsen for a few days or even occasionally weeks post instillation. (Sant & LaRock, 1994). Messing and Stamey (1978) reported 72% improvement for at least 6 months with one third of their successes only requiring one instillation.

Heparin has been used as an intravesical instillation for IC. Parsons (1994) describes a regimen of 10,000 units in 10ml sterile water administered by self-catheterisation for 1 hour 3 times per week for a trial of at least 6 months. Initial monitoring is required to exclude the formation of antiplatelet antibodies. An uncontrolled study found more than 50% had significant benefit (Parsons, 1994). A diminished relapse rate was demonstrated in patients using intravesical heparin monthly for twelve months following a course of DMSO (Perez-Marrero et al, 1993) .

1.9.3 OTHER THERAPIES INCLUDING NEUROMODULATION

Varying beneficial effect with oral L-arginine, a nitric oxide synthase inhibitor has been reported. Smith and colleagues, 1997 showed double the response rate compared with placebo but Cartledge and colleagues, 2000 more recently found no significant diffrence between L-arginine and placebo. Intravesical TICE ^{R} Bacille Calmette-Guerin (BCG) (Peters et al, 1997; Peters et al, 1998) and intravesical pentosan polysulfate sodium. (Bade et al, 1997) are also undergoing further

evaluation. Peeker and colleagues, 2000 (b) compared intravesical BCG with DMSO and failed to demonstrate any benefit from the BCG treatment Open label trials of the oral histamine antagonists hydroxyzine (Theoharides & Sant, 1997), cimetidine (Sehadri et al, 1994), as well as the leukotriene D4 receptor antagonist, montelukast [Singulair] (Bouchelouche et al, 2000) and intravesical hyaluronic acid (Morales et al, 1997) have also demonstrated some efficacy and the results of randomised trials are awaited. More recently, a new immunoregulator, Suplatast Tosilate (IPD-1151T) has been used in a small series of IC subjects with beneficial results (Ueda et al, 2000).

Transcutaneous electrical nerve stimulation (TENS) has been reported to have a beneficial result in 54% of patients with classic ulcer IC and in 26% of patients with nonulcer IC with follow up ranging between 9 months and 17 years. (Fall & Lindstrom, 1994) Permanent sacral stimulation has also been used in IC. This involves an initial peripheral nerve evaluation followed by major surgery with a re-operation rate of 20 to 30%, and is generally the step prior to major urologic surgery. A series of 17 IC patients underwent insertion of a unilateral sacral foramen electrode (Interstim) with 14 having a marked improvement and 2 requiring re-operation (Caraballo et al, 2000)

Recently, the Interstitial Cystitis Data Base cohort study reported the types and frequency of treatments reported at baseline entry to the study between 1993 and 1997 (largely prior to the introduction of PPS). A total of 183 different types of therapies were recorded. The five most commonly used therapies were cystoscopy and hydrodistention, amitryptyline, phenazopyridine, special diet and intravesical heparin. (Rovner et al, 2000) Treatments reported with time were even more

heterogenous (Propert et al, 2000)

1.9.4 Surgical Treatment

Bladder hydrodistention is part of the diagnostic procedure and does provide short-term symptomatic relief in 20% to 30% of patients with IC (Hanno, 1994). Some patients do well for several months. There is no standard method of performing hydrodistension: usually the bladder is filled by passive gravity at 60 to 100 cm water pressure (vertical height above bladder) for 2 to 6 minutes under general or regional anesthesia (Hanno, 1994). Most patients wake with pain which may require analgesics but which improves over the next few weeks. Parsons (1994) suggests the instillation of 10ml 2% lignocaine following hydrodistension or bladder biopsy for analgesia. Iontophoresis has been utilised in the treatment of IC and involves the creation of an electric field which ionizes drugs such as lignocaine or steroids and thereby enhances their absorption into the bladder wall. Once the bladder is anaesthetised by iontophoresis with intravesical lignocaine, hydrodistention is performed in the awake unsedated patient (Griffith & Gurpinar, 1995; Rosamilia et al, 1997). Treatment of visible ulcers by resection or laser vaporization has been described (Malloy & Shanberg, 1994). Both methods result in short-term improvement but the relapse rate is over 50%. Bowel damage has been described with laser therapy. Local injection of alcohol, hydrocortisone, heparin or subtrigonal phenol has been unsatisfactory (Irwin & Galloway, 1994).

Major open surgery should be reserved for the small percentage of women with severe symptoms who have not responded to conservative therapies. Diversion

procedures alone have not been adequate (Worth & Turner-Warwick, 1973). Denervation procedures such as supratrigonal cytolysis result in short term beneficial effects (Worth & Turner-Warwick, 1973; L ch & Raz, 1983) however 4 year follow-up revealed that many patients had experienced recurrence of their pelvic pain requiring further surgery (Leach & Raz, 1983). Augmentation cystoplasty without excision would seem unlikely to be successful although beneficial results have been described (Smith et al, 1977). Supratrigonal cystectomy and substitution cystoplasty is thought to produce most benefit in those with small capacity, contracted bladders. Afterwards, clean intermittent self catheterisation may be necessary. Reported series have cure rates of 58% to 100% (Irwin & Galloway, 1994).

The remaining option is total cystourethrectomy and urinary diversion (continent or ileal conduit) and may be a better alternative especially in those with significant urethral pain or hypersensitivity (Irwin & Galloway, 1994). Lotenfoe and colleagues (1995) reported an overall clinical success rate of 73% with cystectomy, urethrectomy and formation of a continent colonic urinary reservoir. Best results were obtained with a team approach involving psychological evaluation and pain localization techniques (differential epidural studies) and in older patients with small capacity bladders (88% success in 17 patients with capacity <400ml) compared with 20% in 5 patients with capacity >400ml (Lotenfoe et al, 1995). Even after major surgery the prognosis is guarded as persistent pelvic pain has been reported after cystourethrectomy (Baskins & Tanagho, 1992). Finally, it should be remembered that surgery is a powerful placebo (Johnson, 1994) and therefore in the absence of controlled trials, the reporting of surgical success rates requires adequate length of

follow up.

1.10. CONCLUSION

IC is a disabling condition with sufferers frequently having multiple failed treatments including even radical surgery and a quality of life less than that of end stage renal disease. (Held et al, 1990) A number of etiological theories have been based on clinical, laboratory and pathological bladder changes but these may be largely a reflection of end organ effect rather than the primary cause. Further research is needed to clarify the underlying pathophysiological processes in IC; the initial trigger and subsequent interactions between sensory neuronal activity, inflammatory and immune mediators, neuropeptides, vascular changes and increased epithelial permeabilility. What is certain is that clinical management will only advance significantly once we better understand the disease process.

PART 2. OBJECTIVES OF THE THESIS

The aim of this thesis is to use histological, immunohistochemical and urinary peptide assay to study the pathogenesis of interstitial cystitis. This thesis aims to investigate bladder biopsy histology, microvasculature, steroid receptor status and the role of the kallikrein-kinin system as major mediators of inflammation in interstitial cystitis. The specific aims of the thesis were as follows:

1) To describe the histology of IC in terms of epithelial denudation and the submucosal changes of oedema, congestion and ectasia, inflammatory infiltrate and haemorrhage
2) To quantify any differences in oestrogen and progesterone receptor expression as assessed by immunohistochemistry in the IC bladder submucosa as compared with that of control women and men.
3) To quantify and compare bladder microvascular density in IC and control women.
4) To determine if the histology and microvasculature findings represented a primary process or were secondary to the hydrodistention and biopsy procedure.
5) To investigate the role of the kallikrein kinin system by measurement of urinary levels of kinin peptides, active and total kallikrein, and neutral endopeptidase in women with interstitial cystitis
7) To determine whether activation of the kallikrein kinin system in interstitial cystitis was associated with increased release of substance P into urine by measuring urinary excretion rates of substance P and its metabolites in these same subjects.

8) To determine using immunohistochemistry if there was an increase in bradykinin type 1 receptors in the IC bladder and if so, to demonstrate whether the positively staining cells may belong to a population of inflammatory cells.

TABLE 1 *EXCLUSION CRITERIA FOR INTERSTITIAL CYSTITIS*

1.Bladder capacity of greater than 350 cc on awake cystometry using either a gas or liquid filling medium.

2.Absence of an intense urge to void with the bladder filled to 100 cc of gas or 150 cc of water during cystometry, using a fill rate of 30 to 100 cc/min.

3. The demonstration of phasic involuntary bladder contractions on cystometry using the fill rate described above.

4.Duration of symptoms of less than 9 months.

5. Absence of nocturia.

6.Symptoms relieved by antimicrobials, urinary antiseptics, anticholinergics,or antispasmodics.

7. A frequency of urination, while awake, of less than eight times per day.

8. A diagnosis of bacterial cystitis or prostatitis within a 3 month period.

9.Bladder or ureteral calculi.

10.Active genital herpes

11. Uterine, cervical, vaginal, or urethral cancer.

12. Urethral diverticulum

13. Cyclophosphamide or any type of chemical cystitis.

14. Tuberculous cystitis

15. Radiation cystitis

16. Benign or malignant bladder tumours.

17. Vaginitis.

18. Age less than 18 years.

Reference; Hanno(1994)

CHAPTER TWO

PART 1. ESTABLISHMENT OF CLINICAL DATABASE

PART 2. CLINICAL METHODOLOGY

The laboratory methodology will not be included in this chapter but detailed in the methods section of the first relevant chapter.

PART 1. ESTABLISHMENT OF CLINICAL DATABASE

2.1 CLINICAL SUBJECTS

The 113 subjects included in this clinical database were women recruited from the Urogynaecology units at the Mercy Hospital for Women (MHW) and the Royal Women's Hospital (RWH). Institutional ethical approval was obtained prior to recruitment. Female subjects were recruited for the initial histology study (Chapter 3) and also included subjects involved in the microvasculature (Chapter 5) and steroid study (chapter 4) in addition to the urinary kinin study (Chapter 7) between April 1995 and December 1996. The subjects with IC consisted of women with symptoms of frequency, urgency and often pain who met NIDDK criteria (Gillenwater & Wein, 1987). Control subjects were women who were undergoing stress incontinence or prolapse surgery and had no evidence of bladder hypersensitivity or instability during prior urodynamic assessment. Women with symptoms, signs, a voiding diary

and a urodynamics assessement suggestive of IC but did not show glomerulations at cystoscopy are included for completeness and are represented as the hypersensitive bladder group.

The clinical database is shown entirely in Appendix 1 and consists of demographic, past and current clinical information obtained from each subject when available. Demographic information includes age, race, parity and menopausal status; past history includes prior surgery and prior treatment for the IC subjects. Current clinical information included 3 day voiding diary, urine microscopy and culture, urodynamic assessment results and cystoscopic findings. Each subject underwent multichannel subtracted urodynamics including flowmetry, urethral pressure profilometry, medium fill cystometry and a pressure voiding study. The urodynamics assessment served a number of functions. The control subjects each had a cystometric capacity of at least 500mls with no evidence of detrusor instability (either systolic contractions or low compliance). The IC subjects had an awake cystometric capacity equal to or less than 350ml with no evidence of systolic contractions.

2.2 SUMMARY OF CLINICAL CHARACTERISTICS

2.2.1 IC SUBJECTS

Forty four subjects with IC were recruited for the clinical study. In 30 subjects the diagnosis of IC was made during the current diagnostic work-up. Fourteen subjects were previously known to have IC. Their median age was 43 years with a range between 19 and 80 years. (Table 2.1) The median parity was 2 with a range between

0 and 6 children. Data was missing on the menopausal status of 1 subject. Fifty one percent of the 43 women were premenopausal, 40% were postmenopausal and 9% were on hormone replacement therapy. The details of prior surgery were not available for three IC subjects. Of the remainder 46% had a prior cystoscopy, 15% an abdominal hysterectomy; 20% a vaginal repair. The voiding diary data showed a median of 12 voids in 24 hours with a range between 7 and 26. (Table 2.1) The maximum voided volume was also obtained from the diary and ranged from 99 to 450ml with a median of 280ml voided. The urodynamic (UD) capacity ranged from 99 to 380ml with a median of 251ml. The cystoscopic capacity ranged from 250 to 1000ml with a median of 600ml.

Of the 44 subjects with IC, 8 women had a cystoscopic capacity under general anaesthesia of 400mls or less, and for the remainder of this thesis they will be referred to as having severe IC. Their median age was 61 years with all being postmenopausal; 3 of these subjects were on HRT. Median parity was 3 (range 0-6). Their voiding diary showed a median number of 15 voids in 24 hours (range 10-22) and maximum voided volume of 235ml (range 200-300). The urodynamic (UD) capacity ranged from 100 to 289ml with a median of 203ml. The cystoscopic capacity ranged from 250 to 400ml with a median of 360ml.

2.2.2 Control Subjects

Fifty-seven control subjects were recruited. The median age was 51 years with a range between 30 and 80 years. (Table 2.1) The median parity was 3 with a range of 1 to 6 children. Menopausal status data was missing in 6 subjects. Thirty-seven percent of the 51 women were premenopausal, 27% were postmenopausal and 35%

on hormone replacement. Data regarding surgical past history was unavailable in 7 women. Of the remainder 34% had a prior abdominal hysterectomy and 38% had a vaginal repair and 20% had prior stress incontinence surgery. A proportion had a combination of surgery. The median number of voids in 24 hours was 7 with a range between 5 and 13. The maximum volume voided shown in the diary ranged between 120 and 900ml with a median of 600ml. The median urodynamic capacity was 500ml with a range between 426 and 620ml. The median cystoscopic capacity was 600ml with a range between 500 and 1000ml.

2.2.3 HYPERSENSITIVE BLADDER SUBJECTS

Twelve subjects were recruited who had symptoms of frequency and urgency but did not meet the diagnostic criteria for interstitial cystitis. Their median age was 66 years with a range between 29 and 76 years. (Table 2.1) The median parity was 2 with a range between 0 and 3 children. Data regarding menopausal status was missing in 3 subjects; of the remainder 17% were premenopausal, 50% were postmenopausal and 30% were on hormone replacement therapy. The median number of voids in 24 hours was 9.5 with a range between 8 and 16. The median maximum voided volume was 390ml with a range between 350 and 402ml. The median urodynamic capacity was 249ml with the range between 200 and 395ml. The median cystoscopic capacity was 600ml with a range between 400 and 1000ml.

2.3 DISCUSSION OF CLINICAL DATABASE

Epidemiological studies of IC have described the demographic characteristics of IC patients. The majority are white, middle-aged and female. Oravisto and colleagues in

1975 found a 10;1 female/male ratio, with probably 100% Caucasian subjects. Held and colleagues in 1990 and Koziol and others in 1993 found a similar ratio of 9;1 and 99% and 94% Caucasian respectively. Koziol (1994) described a postal survey sent to IC patients attending their clinic along with a mailing list held by the IC; the survey had a 67% response rate. He also compared this group with a female control group attending the Urology Clinic for other reasons. He found the mean age of the IC group to be 54 years overall; the control group had a mean age of 45 years. The mean age of Hanno's study group was 50 years and of the interstitial cystitis Database (ICDB) was 43 years (Nigro et al, 1997)). The median age of IC patients in this series was 43 years. Five (11%) were aged less than 30 years. The age stated does not represent the age of onset of symptoms which may be significantly younger as delay in diagnosis for up to 7 years has been reported (Held et al, 1990). The control group is comprised of a specific clinical group who attend largely for the surgical treatment of stress incontinence. Their median age was 51 years. None was aged less than 30 years.

Parity has not been described in previous epidemiological series. None of the control group was nulliparous however 6 of the 44 (14%) IC group were nulliparous. Two reasons for this difference are clear; the first is the younger age of the IC group and the second is that parity (or vaginal delivery) is a risk factor for stress incontinence.

Koziol and colleagues (1993) reported a significantly higher incidence of hysterectomy (44%) in IC patients compared with 18% in controls. De Juana and Everett in 1977 reported a 25% and Holm-Bentzen and others in 1987, a 30% incidence of hysterectomy in their series of IC patients. In this series the incidence of abdominal hysterectomy in the control group was greater than the IC group (34%

versus 15%). The incidence of vaginal repair was also greater (38% versus 20%). The younger age of the IC group may be one reason for this as most hysterectomies are performed in women over the age of 40 years. In addition there has been reported a possible association between hysterectomy and stress incontinence. The indications for vaginal repair have included prolapse and stress incontinence both of which are more common in the control group. In addition age is also a risk factor for prolapse.

Associations with other conditions such as food or drug sensitivity (25 to 38%), arthritis (34%), sinusitis (34%), hay fever (25%), irritable bowel syndrome (27%) have been reported (Koziol et al, 1993). The percentage of patients who are diabetic has been reported as varying between 2.7% (Koziol et al, 1993) and 5.9% (Jones et al, 1994).

Most series of IC patients which are derived from the United States have reported a lower proportion of African-Americans than expected but this may be a reflection of a difference in access to care as blacks had a longer time between onset of symptoms to diagnosis compared with whites. This series of patients based in Melbourne, Australia was predominantly Caucasian; 2 (5%) of the IC and 1 (8%) of the hypersensitive group were Asian and 1(2%) of the control group was American Indian.

The identification of a severe IC subgroup was an attempt to make this varied clinical group of IC a more homogenous group. Epidemiological and clinical studies suggest that these may be two groups with the (early) milder form having near/normal or normal cystoscopic capacity and glomerulations occurring in younger subjects as described by Messing and Stamey in 1978. The severe IC group in this

study correspond to the "classic IC" which was originally described by Hunner and subsequent authors. These subjects are said to have Hunner's ulcer and low capacity bladder.

As described in Section 1.5.3 Hunner's ulcer is relatively rare and its appearance variably interpreted. It is therefore difficult to define a subgroup of patients with classic or severe IC by the presence of an ulcer. However cystoscopic capacity is an easy and objective criterion which has been found to correlate well with the presence of classic IC. Messing and Stamey (1978) found that all but 1 of the classic disease subjects had capacities less than 450ml whereas all of the early IC type had capacities of greater than 450ml. There were no early IC subjects in the seies by Johansson and Fall (1990) with a capacity less than 450ml. In the current series cystoscopic capacity of 400 ml or less was chosen as an objective marker of severe disease.

PART 2. CLINICAL METHODOLOGY

2.4 CYSTOSCOPY AND BIOPSY TECHNIQUE

Cystourethroscopy was standardised as much as possible for all subjects. A cystoscopy is performed routinely at the end of all stress incontinence and prolapse surgery. This is generally performed using a 70 degree telescope to enable visualisation of ureteric patency. Water was used as the irrigation fluid at a height of 80 to 100cm for the hydrodistension. Capacity was reached and in the control subjects only a suprapubic catheter was inserted under cystoscopic vision. The water

was released and measured and at second look, one to three cold-cup forceps biopsies were taken. For the purposes of this study, after ureteric patency was confirmed in the control subjects, a 30 degree telescope was substituted through which biopsies were able to be taken under vision.

The camera allowed continuous inspection of the bladder epithelium during both filling and release. In the IC subjects the biopsies were taken of the area with maximum glomerulation or fissuring. A photographic or videotape record of the cystoscopic appearance was obtained if the facility was available. At the time of urodynamic assessment all subjects had a screening urinalysis and at the time of cystoscopy a catheter specimen of urine was sent for microscopy and culture. Current urinary tract infection excluded entry into the study; 1 control and 1 IC subject were excluded for this reason

These cold-cup forceps biopsies were obtained in both control and IC subjects after a standardised hydrodistention, release and second look. They were immediately placed into formalin and entered the routine processing of the histopathology laboratories at the two institutions (93 at MHW, 15 at RWH and 3 at other laboratories).

2.5 URINE COLLECTION

A urine collection protocol described in detail in Chapter 7 and 8 was devised for the bradykinin and Substance P study. The collection needed to be of free flowing urine (to minimise activation of the study peptides within the bladder itself) and 4

collections were taken with 2 after a fixed period of catheter clamping in order to ascertain the bladder contribution to the peptide levels.

2.6 STATISTICS

Data are presented in summarised table or figure form showing median, 25^{th}, 75^{th} and range or mean +/- standard error of the mean (SEM) in each chapter and raw data (tabled in Access, Microsoft) is shown in Appendices I to V. The Mann-Whitney test was used for the majority of the statistical analysis on SPSS, version 10.0 unless otherwise stated in the relevant chapter. A p value <0.05 was considered to be significant.

Table 2.1: Values showing main demographic and clinical characteristics for control, IC and hypersensitive (HSB) subjects showing median and (range)

	Control n=57	All IC n=44	Severe IC n=8	HSB n=12
Age (years)	51 (30-80)	43 (19-80)	61(49-76)	66 (29-76)
Parity	3 (1-6)	2 (0-6)	3 (0-6)	2 (0-3)
Voids/24 hrs	7 (5-13)	12 (7-26)	15 (10-22)	10 (8-16)
Max void (ml)	600 (120-900)	280 (99-450)	235 (200-300)	390 (350-402)
UD capacity (ml)	500 (426-620)	251 (99-380)	203 (100-289)	249 (200-395)
Cyst capacity(ml)	600 (500-1000)	600 (250-1000)	360 (250-400)	600(400-1000)

CHAPTER THREE

HISTOPATHOLOLOGY OF INTERSTITIAL CYSTITIS

3.1. INTRODUCTION

The diagnosis of IC is made on clinical criteria and the exclusion of other causes of cystitis such as infection, radiation or drugs. IC has no pathognomonic histological appearance although bladder biopsy can be performed to exclude other conditions such as carcinoma in situ and eosinophilic cystitis which may have similar clinical features.

The histological appearance of the bladder in interstitial cystitis has been described by a number of authors. Lynes and colleagues (1990) described the pathological findings in 22 IC and 10 control subjects and found an increase in denuded epithelium or prominent submucosal inflammation between IC compared to control groups. Johansson and Fall (1990) studied 64 subjects with classic IC, 44 with non ulcer IC and 20 control subjects. The majority of the classic IC group had mucosal ulcerations and haemorrhage, granulation tissue and mononuclear infiltrate. Of the early IC subjects, 90% had mucosal ruptures and 60% had focal submucosal haemorrhages with minimal inflammatory infiltrate.

Messing and Stamey (1978) described non-specific submucosal oedema and vasodilatation as the main finding in 19 early and 19 classic IC patients. Mattila (1982) found normal bladder biopsy histology in 20 of 47 IC patients with 18/47 (38%) showing lymphocytic and plasma cell infiltrate and 9/47 (19%) showing thickened telangiectatic vessels.

A possible criticism of previous studies was that whereas IC biopsies were usually obtained after hydrodistention, control biopsies were typically fewer in number and were not obtained under the same conditions as the IC biopsies. In addition, the assessment may not have been blinded and the control group may have been derived from a heterogenous group of women with different diagnoses.

The aim of this study was to describe, in a rigorous manner, the histological appearance of bladder biopsies from subjects with the clinical diagnosis of interstitial cystitis and compare this with a similar number of control subjects. The control group was to be well-defined; the conditions of the cystoscopy, hydrodistention and biopsy were as standardised as possible and the assessors of the biopsies blinded to the identity and diagnosis of the subjects. As a result, the sensitivity and specificity of abnormal histological findings in interstitial cystitis could be assessed.

3.2 MATERIALS AND METHODS

3.2.1 BIOPSY COLLECTION AND LABORATORY METHODOLOGY

Control, IC and hypersensitive bladder subjects were as defined in the clinical profile (Chapter 2). Standardised hydrodistention and cystoscopy with cold-cup forcep biopsy was performed as described in Chapter 2. The bladder biopsy was placed directly into 10% buffered formalin for fixation.

In the histology laboratory the biopsy was measured in mm, submitted whole and placed in a biopsy pad and then into a cassette. Tissue processing was performed by the staff of the histology laboratories of the Mercy Hospital for Women or the Royal Women's Hospital. The tissue processor used was the Leica Jung TP 1050; software version 3.10. The overnight run was 13 hours and 36 minutes duration and is shown in Table 3.1. The tissue was paraffin embedded using a Leica Histoembedder. Sections were cut at 3 um using the E. Leitz Wetzlar microtome; they were transferred to a 45° C water bath and then placed centrally on slides. The sections were dried off by allowing to drain in a vertical position for 5 minutes followed by placing the slides on a hot plate at 60 C for 15 minutes. The sections were stained using the Shandon Varistainer 24-4 on the haematoxylin and eosin program as shown in Table 3.2.

3.2.2 HISTOLOGICAL ASSESSMENT

The histological assessment was based on the previously cited study of Lynes (1990) which scored the submucosal appearance of interstitial cystitis using four parameters; oedema, congestion and ectasia, inflammation and fibrosis. In the current study there

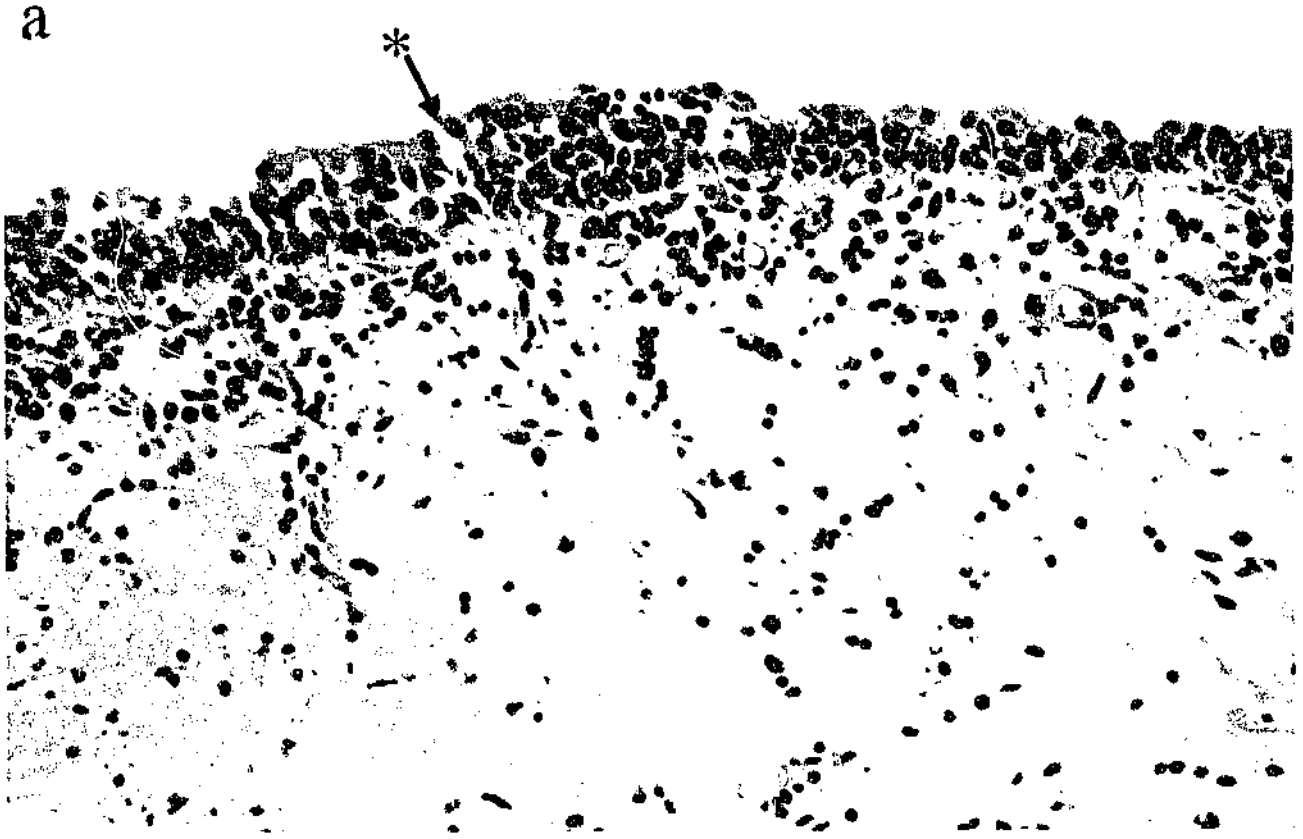
was no evidence of fibrosis in any of the haematoxylin and eosin stained sections and therefore this parameter was not assessed. Instead, haemorrhage was evident and this was scored for each subject. Therefore a score between 0 (none) and 3 (maximal) was given for each of the submocosal findings of oedema, congestion and ectasia, inflammation and haemorrhage. The score for congestion/ectasia represented the mean for these two features. These scores were added to give the composite abnormal histology score which had a possible range between 0 and 12. The appearance of the epithelium was also assessed and scored 1, 2 or 3 which corresponded to intact, partially denuded and completely denuded epithelium respectively. A summary of this semi-quantitative scoring system is shown in Table 3.3. The assessment was performed by Dr J Scurry, consultant histopathologist and Dr A Rosamilia without knowledge of the name or diagnosis of the subject in order to minimise bias. Each subject had at least two or three sections from which the final score was derived. The scoring was repeated (again blinded) for 30 cases in order to confirm consistency.

3.3 RESULTS

Histological assessment was performed on bladder biopsies of 82 women; 35 control, 36 IC and 11 women with hypersensitive bladder (Appendix II). Figure 3.1(a) is a section from the bladder biopsy of a woman with interstitial cystitis showing disruption, oedema and lymphocyte exocytosis in the epithelium, a band of lymphocytes and capillary congestion beneath this, and oedema and fresh haemorrhage in the underlying submucosa, compared with normal bladder epithelium and submucosa in a control subject in Figure 3.1(b)

3.3.1 CYSTOSCOPIC CAPACITY

a



b

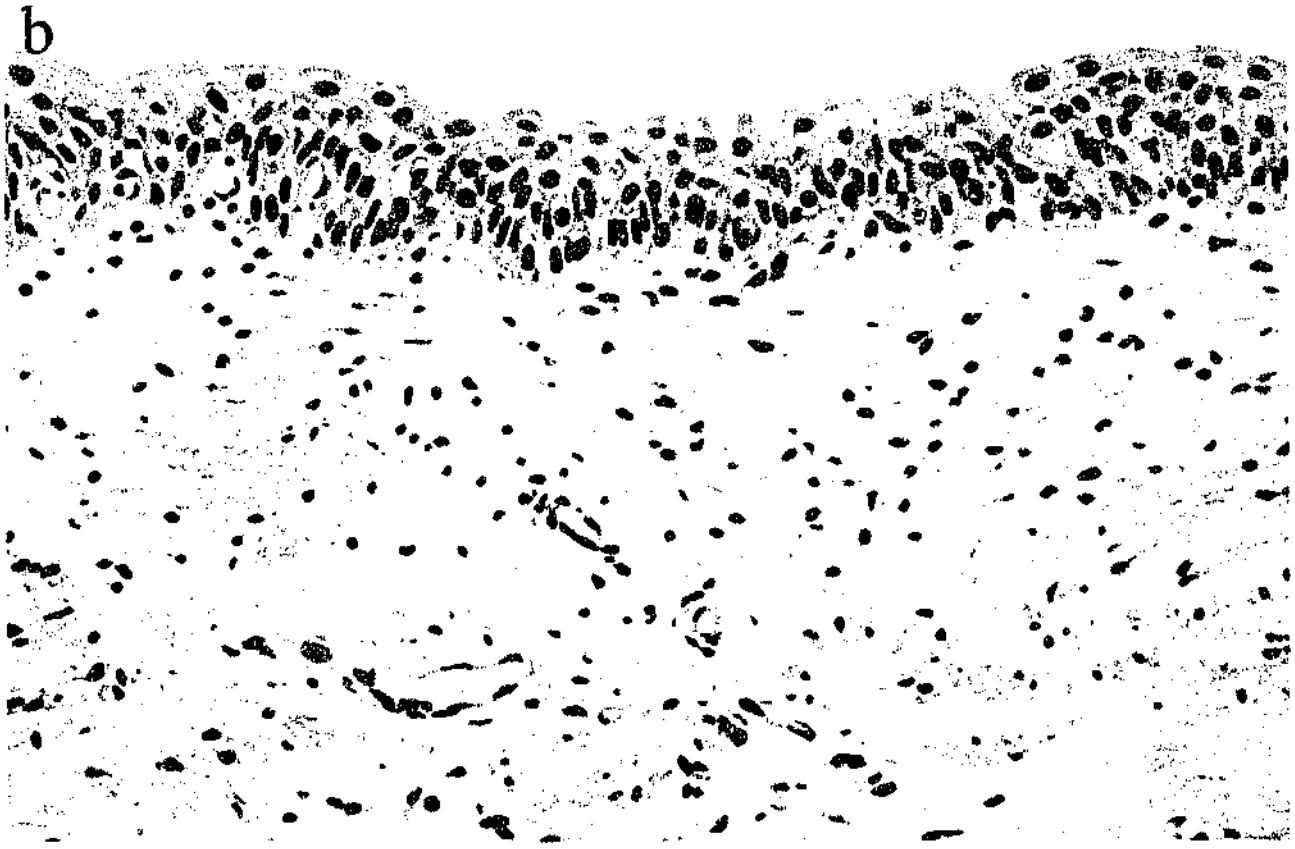


Figure 3.1(a) Bladder biopsy of a woman with interstitial cystitis showing disruption *, oedema and lymphocyte exocytosis in the epithelium, a band of lymphocytes and capillary congestion beneath this, and oedema and fresh haemorrhage in the underlying submucosa, compared with **3.1 (b)** Control H & E x200.

Of the 36 IC subjects, six subjects had cystoscopic capacities of 400mls or less. These 6 subjects will be defined as the group with severe IC for the remainder of this chapter. The cystoscopic capacities are shown in Table 3.4. There was a significant difference between the cystoscopic capacity in the control and the interstitial cystitis groups ($p<0.01$) but no difference between either control or IC and the hypersensitive group.

3.3.2 EPITHELIAL DENUDATION

Two subjects with IC and 1 subject with hypersensitive bladder were unable to be assessed in terms of epithelial denudation. There was a difference ($p=0.01$) in scores of epithelial denudation between control and IC groups (Figure 3.2). The severe IC group had higher scores for epithelial denudation compared with other subjects with IC ($p=0.02$). There was no significant difference between the hypersensitive bladder group and other groups (Table 3.5).

3.3.3 SUBMUCOSAL OEDEMA

There was a difference ($p=0.002$) in scores of submucosal oedema between control and IC groups (Figure 3.3). The submucosal oedema scores for the severe IC group were not significantly different from either other IC subjects or controls. There was no significant difference between the hypersensitive bladder group and IC but a trend ($p=0.05$) toward more oedema than the control subjects (Table 3.6).

3.3.4 SUBMUCOSAL CONGESTION AND ECTASIA

There was a significant difference (p=0.009) in scores of submucosal congestion and ectasia between control and IC groups (Figure 3.4). The severe IC group had a trend to higher scores for submucosal congestion and ectasia compared with other subjects with IC (p=0.07). There was no significant difference between the hypersensitive bladder group and other groups (Table 3.7).

3.3.5 SUBMUCOSAL INFLAMMATORY INFILTRATE

There was a difference (p=0.02) in scores of submucosal inflammatory infiltrate between control and IC groups (Figure 3.5). The severe IC group had a trend toward higher scores submucosal inflammatory infiltrate compared with other subjects with IC (p=0.05). There was a difference (p=0.04) between the hypersensitive bladder group and controls only (Table 3.8).

3.3.6 SUBMUCOSAL HAEMORRHAGE

There was no significant difference in submucosal haemorrhage between control, all women with IC, the severe subgroup of IC and hypersensitive bladder groups (Table 3.9). Figure 3.6 is a bar graph showing the degree of submucosal haemorrhage in bladder biopsies from all women with IC and control subjects.

3.4.7 COMPOSITE ABNORMAL SUBMUCOSAL HISTOLOGY SCORE

When the scores for oedema, congestion and ectasia, inflammation and haemorrhage were added to give a composite abnormal submucosal histology score, there was a

difference between control and IC groups ($p<0.01$) as shown in Figure 3.7 and Table 3.10. The composite abnormal submucosal histology scores for the severe IC group were not significantly different from other IC subjects ($p=0.1$). Hypersensitive bladder subjects had different composite abnormal submucosal histology scores from controls ($p=0.03$) but there was no difference between IC and hypersensitive bladder groups.

3.5 DISCUSSION

This study represents a histological comparison in a series of bladder biopsies taken after standardised hydrodistention in a group of female control and interstitial cystitis subjects. There was increased epithelial denudation, submucosal oedema, congestion and ectasia, inflammation and the composite abnormal submucosal histology score between the control and the IC group. However these histological changes are not pathognomonic and are present in less than half of women with IC.

Epithelial denudation may be the initial step to mucosal ulceration, the histological correlate of Hunner's ulcer or classic IC. This study found that completely denuded epithelium never occurred in the control or hypersensitive population but was only found in 21% of IC subjects. It was much more common in severe cases of IC (50%). These findings were in agreement with Lynes and colleagues (1990) who found 32% of IC subjects and no control subjects had denuded epithelium and Messing and Stamey (1978) who described denuded epithelium in 20% of early and 32% of classic patients. Johansson and Fall (1997) stated that " the mucosa was frequently denuded....This occurred in both ulcer and nonulcer patients but almost never in control patients.".

Johannson and Fall(1990) found histological evidence of mucosal ulceration in all of

their subjects with classic or ulcer disease which were defined at cystoscopy as displaying single or multiple patches of reddened bladder mucosa. Mucosal ulceration was not noted in the current study. This difference may be explained by the relative rarity of a Hunner's ulcer. For example, Holm-Bentzen (1989) considers classical interstitial cystitis to be a rare finding; 1 to 5% of IC subjects in her series had the "elusive" ulcer of Hunner. In addition, as discussed in Chapter 1, Parsons (1994), who found 8% of his series to have Hunner's ulcer, claims that the "elusive" ulcer of interstitial cystitis may be quite rare and possibly over-reported as more than 75% of 'ulcers' previously diagnosed in his series of patients were in fact old biopsy sites. In view of the varied descriptions of Hunner's ulcer and lack of agreement as to what it constitutes, it was decided to use the objective measurement of the cystoscopic capacity to define a "severe" subgroup of IC.

Submucosal oedema could result from epithelial dysfunction particularly after hydrodistention. Lesser degrees occur commonly in controls. Any degree of submucosal oedema was found in 40% of controls, 73% of all IC and 67% of severe IC. However, submucosal oedema of a moderate degree (score of 2) was found in only 3% (1) of controls, 13% of early IC and 50% of severe IC subjects. Johansson and Fall (1997) state "another fairly consistent finding is the presence of relatively marked oedema of the lamina propria...[in IC]". Messing and Stamey (1978) described "oedema plus dilated vessels" in up to 90% of early IC and 75% of classic disease subjects but they had no control subjects and did not score the degree of oedema present. In contrast Lynes and colleagues (1990) found similar grades of submucosal oedema in control and IC patients commenting that the finding was more common in post hydraulic distention biopsies. Submucosal oedema is difficult to assess even in a qualitiative manner; its appearance varies with the thickness of the cut section since it is essentially an

interpretation of tissue lucency. Despite standardising the cutting of biopsy sections, there would be variations in section thickness as both IC and control specimens were collected over a period of 2 years. However, this variation would be random as the biopsies were all subjected to the same process.

Moderate or marked submucosal congestion and/or ectasia (scores of 2 or 3) was present in only 3% (1) control subject. 10% of early IC and 50% of severe IC subjects. Any degree of congestion and/or ectasia was present in 60% of controls, 50% of early IC and 83% of severe IC subjects. Messing and Stamey (1978) and Johansson and Fall (1997) grouped ectasia and dilated venules together with oedema in their studies as described in the previous paragraph. Lynes and colleagues (1990) found similar grades of submucosal vascular ectasia in IC and control subjects and commented that this finding also may be consequent upon hydrodistension.

Submucosal inflammation plays an important role in the pathogenesis of a subset of IC patients, especially those with classic or severe disease. This study found that moderate to severe (scores 2 and 3) submucosal inflammatory infiltrate of predominantly mononuclear cells was found in 6% (2) of controls,13% of early IC and 50% of severe IC subjects. Johansson and Fall (1990) found that 91% of their classic and only 9% of their nonulcer IC group had either Grade 2 or 3 (possible scores 0-3) mononuclear infiltrate. In contrast Messing and Stamey (1978) found approximately the same number (37%) of both early and classic IC subjects had submucosal mononuclear cell infiltrate. Lynes and colleagues (1990) found prominent submucosal inflammation (Grade 3 or 4) in 45% of IC and 10% of control subjects. A recent comparison did not find a statistical correlation between the severity of cystoscopic findings and histological evidence of inflammation in 63 subjects with IC (13 with capacity less than 400ml) but these

authors stated that they did have a subgroup of older patients with more severe cystoscopic abnormalities and histological inflammation (Denson et al, 2000)

Submucosal haemorrhage is most likely a result of cystoscopic biopsy trauma. Submucosal haemorrhage was found to occur in 67% of all IC, whether early or severe, and 54% of control subjects. Johansson and Fall (1990) found that sixty percent of their early IC group demonstrated focal submucosal haemorrhages which were usually associated with mucosal rupture. They stated "The majority displayed suburothelial haemorrhage corresponding with glomerulations, and the haemorrhage was generally limited." Their association of submucosal haemorrhage with glomerulations is not confirmed in the current study as submucosal haemorrhage was not significantly increased in the IC group. This discrepancy may be due to sampling error as cold-cup biopsies rather than transurethral resected specimens were used in our study. Also the crushing as a result of the biopsy may cause greater haemorrhage (in both IC and controls) than any suburothelial haemorrhage due to mucosal rupture which is believed to represent glomerulations. Messing and Stamey (1978) found haemorrhage in only 25 to 30% of both early and classic IC groups.

Fibrosis is an infrequent finding in IC having been found predominantly in cystectomy specimens. No evidence of fibrosis was found in this study and was present in only 8% (5) of the 64 classic subjects and none of the nonulcer group in Johansson and Fall's series. Messing and Stamey (1978) found 5% (1) of both early and classic IC groups had submucosal fibrosis. They found evidence of fibrosis in the muscular layer of some IC subjects identified only after large segments of bladder had been excised and examined histologically. These same patients frequently had normal appearing muscularis on previously performed transurethral biopsies.

Abnormal composite submucosal histology score was arbitrarily defined as the sum of the individual submucosal scores and was increased in the IC subjects. Lynes and colleagues (1990) in their study defined abnormal histology as at least one of the three histologic features of denuded epithelium, ulceration or prominent submucosal inflammation and observed this in 12 of the 22 IC subjects (in those with either small capacity bladder <400ml or pyuria) and 1 control who had Grade 3 inflammation. Six IC and no control subjects of the current series met their criteria of abnormal histology. Of the six, 3 had a cystoscopic capacity of 400ml or less.

In this series there were differences in the degree of oedema, inflammatory infiltrate, submucosal congestion and composite submucosal histology score between IC and control subjects overall. Scores of 4 or greater for the composite histology score were obtained by 44% of the IC group compared with 3% (1) of the control subjects. One control subject (No 59) scored 2 for submucosal congestion/ectasia, inflammation and haemorrhage and therefore 6 for composite abnormal histology score. Her medical notes were reviewed and although at the time of this biopsy, urine infection was not documented she had subsequent repeated urine infections followed by a cystoscopy and bladder biopsy 18 months later reported as demonstrating mild nonspecific cystitis.

A composite submucosal histology score of 4 or greater has a sensitivity of only 44% to detect IC (67% for severe IC) and a specificity of 97%. The positive predictive value for a composite submucosal score of 4 or greater to predict IC is 94%. Conversely, this study confirms that in more than half (55%) of IC subjects studied, the histology was normal and indistinguishable from control subjects (composite submucosal histology scores of 3 or less).

The role of bladder biopsy in IC remains largely a research tool with significant limitations. By necessity, only a small often superficial area of the bladder is obtained with the cold-cup forceps for assessment and there may be significant sampling errors as illustrated in the discussion on fibrosis. Biopsies should only be performed with cold-cup biopsy forceps as there is an inherent risk of bladder perforation. Messing and Stamey (1978) reported that in their series 10% (2 of 19) of the early and 16% (3 of 19) of the classic subjects suffered a perforation as a 1 to 2 cm bladder strip was resected. Johansson and Fall (1990) also obtained biopsies with the use of the resectoscope and reported postoperative bleeding requiring irrigation in 7%(7 of 108) and perforation (1 requiring laparotomy) in 6%(6 of 108) of their series. The latter were using complete resection as a therapeutic measure.

Abnormal histology of the bladder biopsy specimen can be useful in supporting a diagnosis of IC and according to the literature is useful in excluding eosinophilic cystitis and carcinoma in situ. These two differential diagnoses are rarely found. During a clinical experience comprising of the order of 100 female IC subjects all of whom had a bladder biopsy, two cases of eosinophilic cystitis and no case of carcinoma in situ have been diagnosed.

The NIDDK appear to be justified in not including biopsy as part of the diagnostic work-up. The histology in IC, as defined by NIDDK criteria, is abnormal less than half the time. However the histology is abnormal in 67% of IC subjects who have a cystoscopic capacity of 400mls or less. The histological findings may be varied in IC because IC is not a well-defined condition and may have a number of causes. This study lends support to the concept of 2 types of IC; severe or classic IC with a reduced

cystoscopic capacity; and in 67% of cases an abnormal submucosal histology (score 4 or greater), and IC with a normal or near normal cystoscopic capacity of whom 40% had abnormal histology.

The pattern of abnormal histology when it occurs supports a theory of pathogenesis involving epithelial dysfunction and leakage allowing submucosal oedema and ectasia. In addition, there is histological evidence supporting a significant role for the inflammatory process occurring in a subset of 20 to 40% of IC subjects. Potentially, bladder biopsy may predict those individuals who by virtue of having histological evidence of inflammation may respond to treatments which target inflammatory mediators.

Also included in the tables are the subjects with frequency/urgency symptoms who did not meet the NIDDK criteria for IC (did not demonstrate glomerulations) and were named "hypersensitive bladder." This last group was included for completeness but will not be discussed at length. The diagnostic relevance of glomerulations in IC has been questioned and it is of interest to note that for the most part the histology of this hypersensitive group fits somewhere between control and IC groups. Submucosal inflammation and the composite submucosal histology score was increased in this group compared with controls and one possible explanation is that this group includes women with other causes of chronic cystitis. On the other hand, it is possible that the women in the hypersensitive group could be part of an IC continuum histologically as well as in a clinical sense and have a very early and mild form of IC.

In conclusion the usefulness of bladder biopsy has been difficult to assess in the past with discrepant results partly due to a lack of consensus regarding the definition of IC

and because the condition is one most likely of multiple etiologies. This study has attempted to remove some of the confounding factors present in older series by utilising an equal number of controls, standardising the method by which the biopsy is obtained in both IC and control subjects, and performing a blinded assessment in order to minimize bias. In approximately half the IC subjects, the bladder biopsy histology was indistinguishable from control subjects. In the half where the histology was significantly different to controls, those differences were in the degree of epithelial denudation, submucosal congestion, oedema and inflammatory infiltrate. Those IC subjects with a low cystoscpic capacity tended to have more severe changes. Some of these changes, for example epithelial denudation and submucosal oedema, may be due to greater vulnerability of the IC bladder to the physical trauma of hydrodistention.

Table 3.1 Tissue processing protocol

Reagent	Station	Time (hr:min)	Temp	P/V	Drain (sec)	Stir
Formalin	1	0:30	37	P/V	120	ON
Formalin	2	0:30	37	P/V	120	ON
70% Ethanol	3	0:30	37	P/V	120	ON
90% Ethanol	4	1:00	37	P/V	120	ON
100% Ethanol	5	1:00	37	P/V	120	ON
100% Ethanol	6	1:00	37	P/V	120	ON
50%Ethanol/ 50% Xylene	7	1:00	37	P/V	120	ON
100% Ethanol	8	1:00	37	P/V	120	ON
Xylene	9	1:30	37	P/V	120	ON
Xylene	10	1;30	37	P/V	120	ON
Wax	Left	1:30	62	P/V	120	ON
Wax	Middle	2:00	62	P/V	120	ON

- *P/V refers to alternate evacuation and pressurisation of the retort.
- Drain time refers to the time it takes for the retort to be fully drained.

Table 3.2 Haematoxylin and eosin staining protocol

Step	Reagent	Immersion period(Min:sec)
1	Xylene	3:00
2	Xylene	2:00
3	Abs. Alcohol	1:00
4	Abs. Alcohol	0:30
5	95 % Alcohol	0:30
6	Water rinse	1:00
7	Instant haematoxylin	3:00
8	Water rinse	0:30
9	Acid alcohol	0:50
10	Water rinse	0:30
11	Scott's water	0:30
12	Water rinse	0:30
13	Eosin-Y-Aqueous	2:30
14	95% Alcohol	0:30
15	100% Alcohol	1:00
16	100% Alcohol	1:00
17	Xylene	1:00
18	Xylene	1:00

Figure 3.2 Epithelial denudation score in bladder biopsies from women with interstitial cystitis and control women

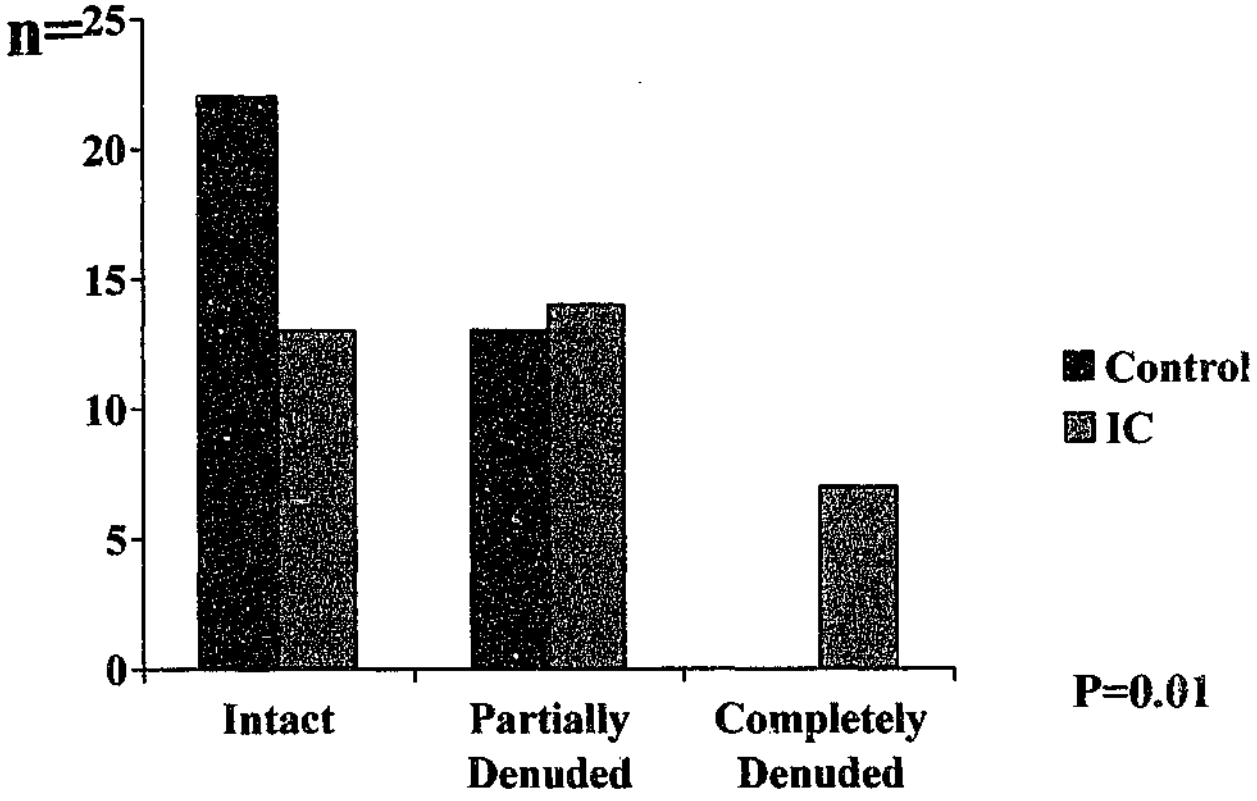


Figure 3.3 Submucosal oedema score in bladder biopsies from women with interstitial cystitis and control women

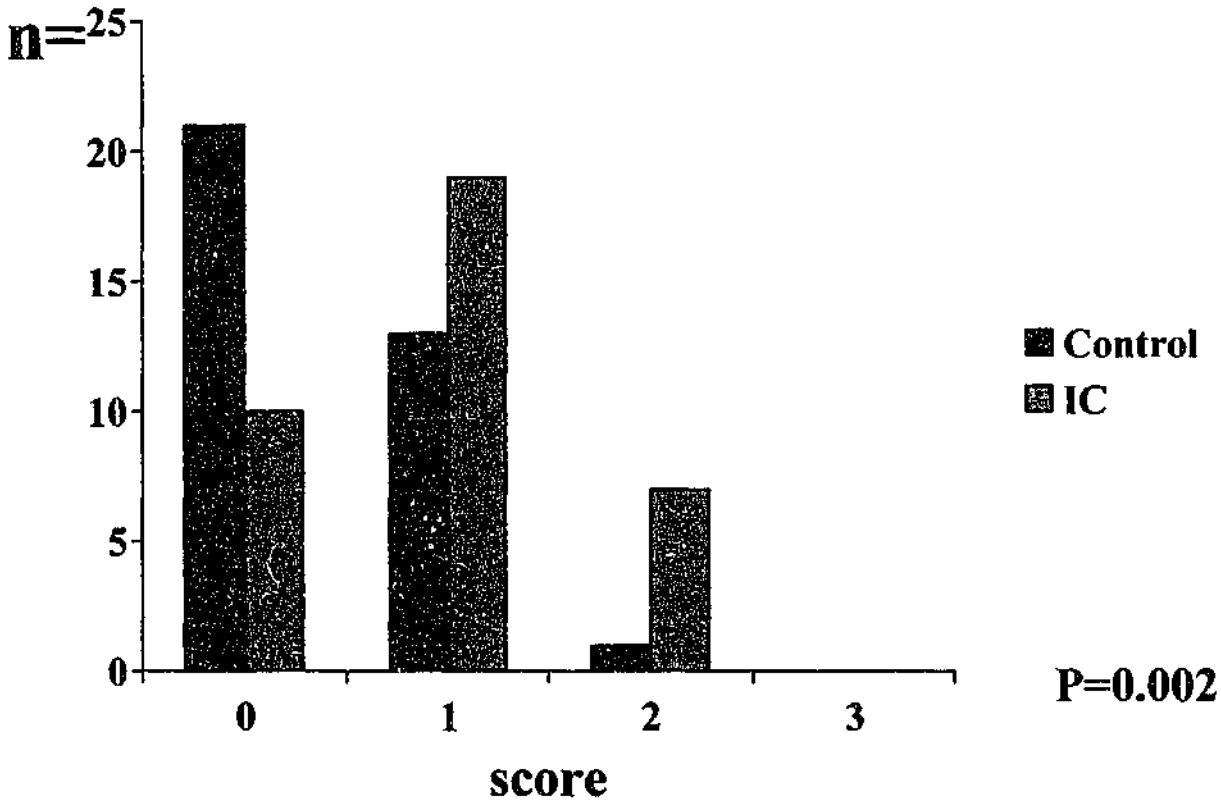


Figure 3.4 Submucosal congestion/ectasia score in bladder biopsies from women with interstitial cystitis and control women

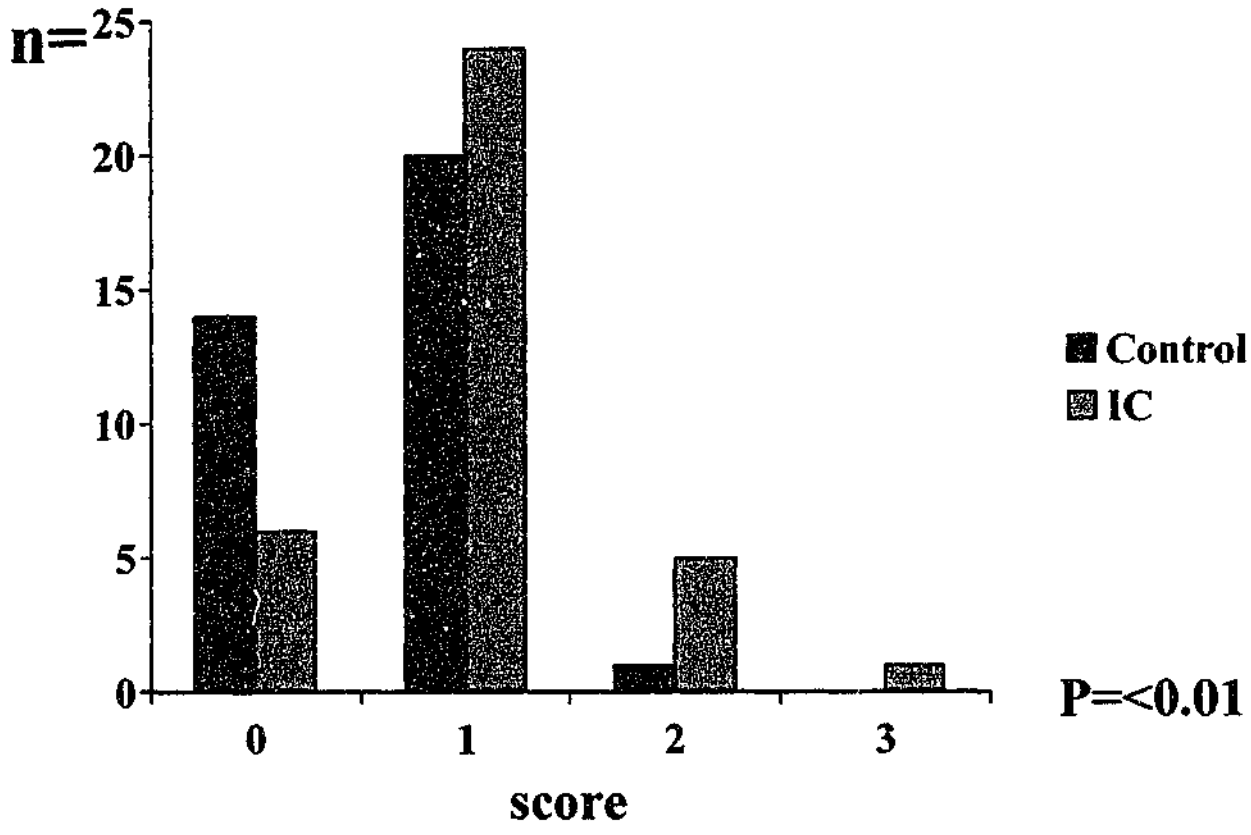


Figure 3.5 Submucosal inflammation score in bladder biopsies from women with interstitial cystitis and control women

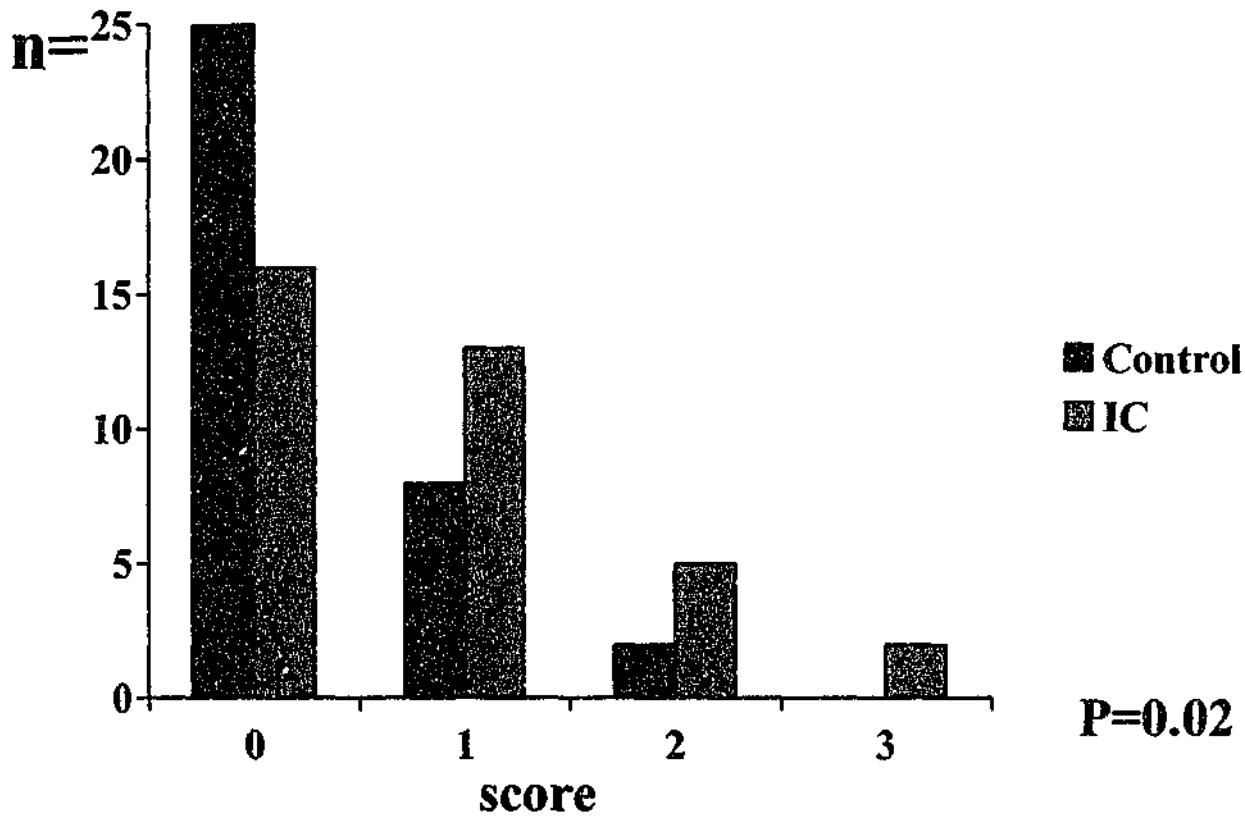


Figure 3.6 Submucosal haemorhage score in bladder biopsies from women with interstitial cystitis and control women

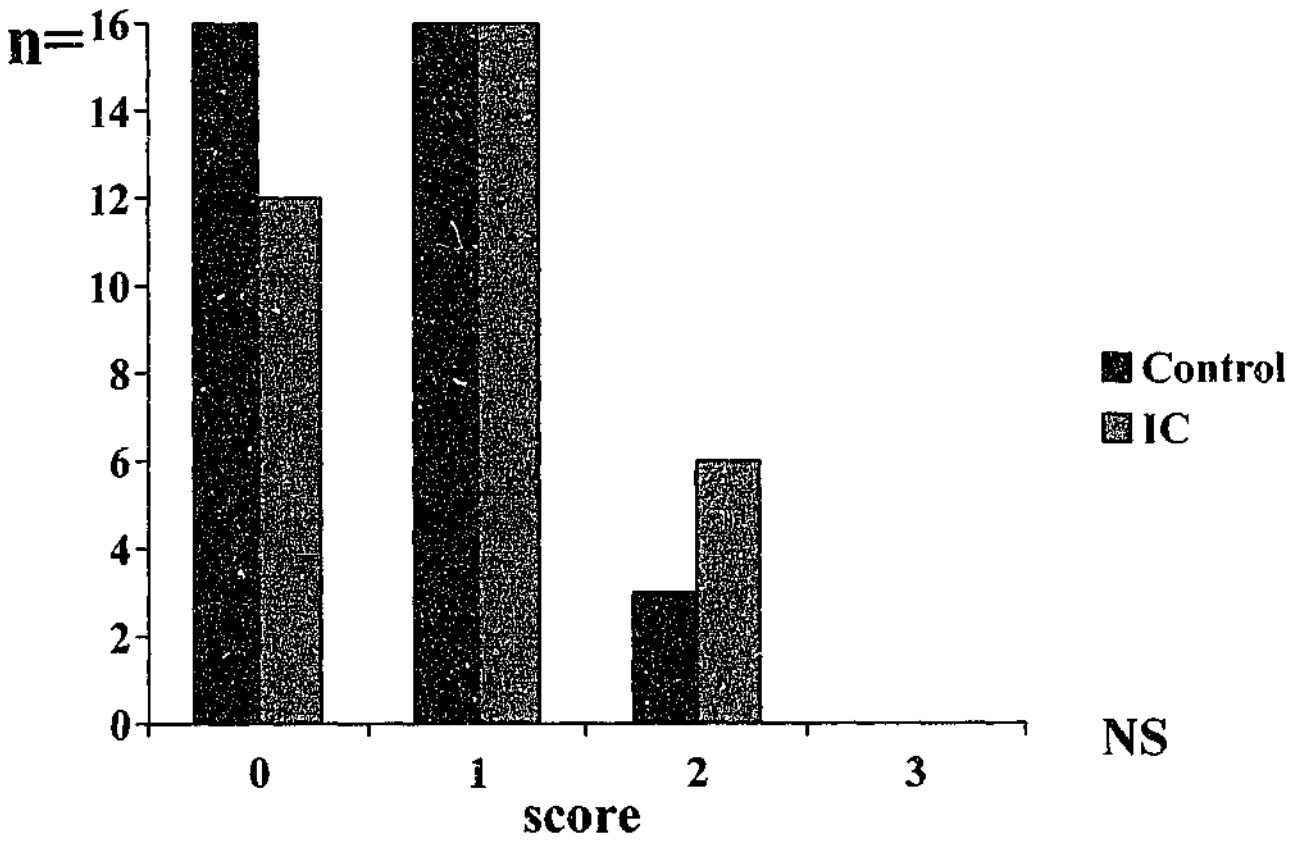


Figure 3.7 Composite abnormal histology score in bladder biopsies from women with interstitial cystitis and control women

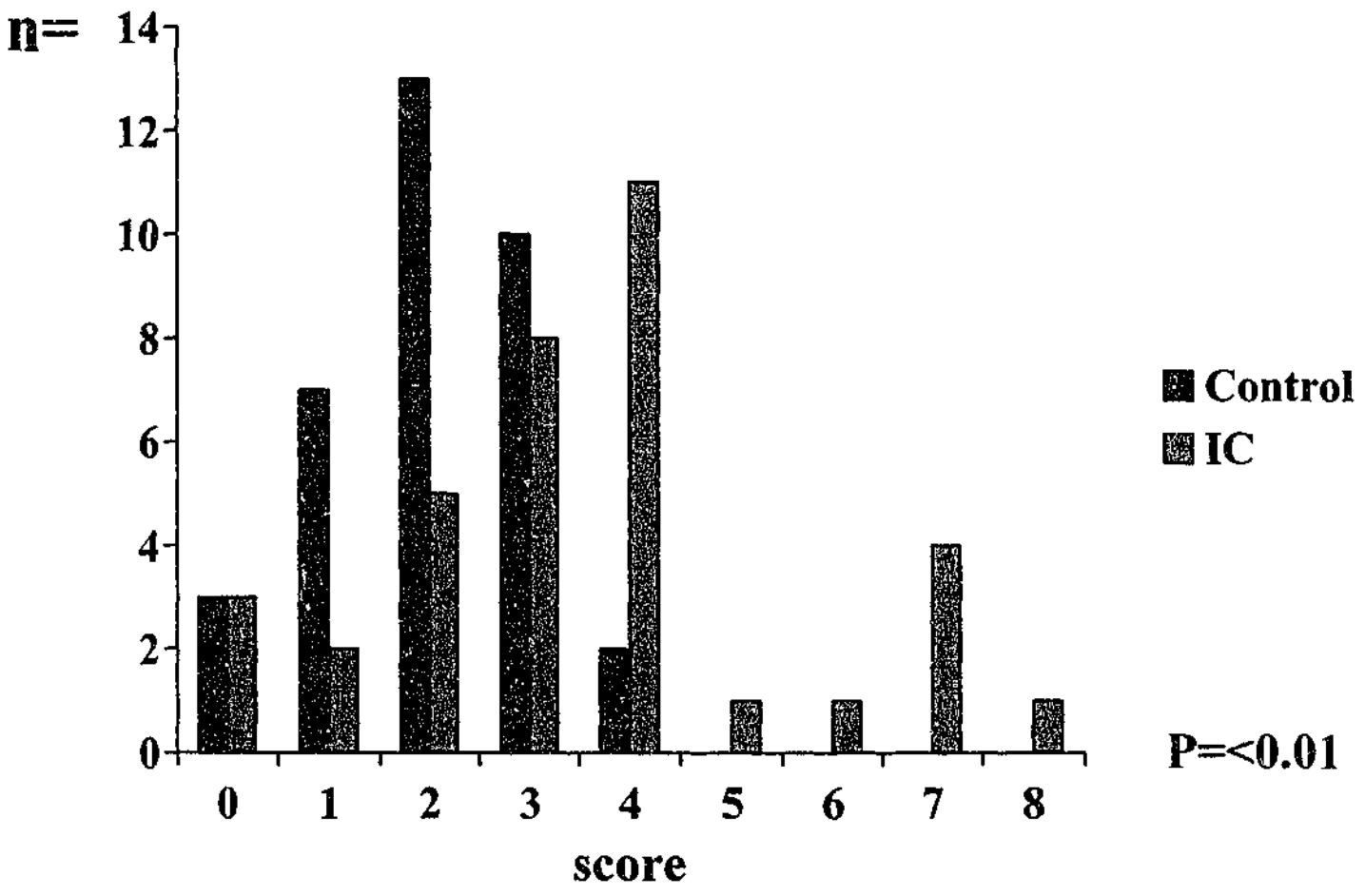


Table 3.3 Bladder biopsy histological parameter and the range of possible scores

Epithelial denudation	1-3
Submucosal oedema	0-3
Submucosal congestion/ectasia	0-3
Submucosal inflammation	0-3
Submucosal haemorrhage	0-3
Submucosal composite abnormal histology score	0-12

Table 3.4 Cystoscopic capacity of IC, control and hypersensitive bladder (HSB) subjects (ml)

	Control	all IC	Hypersensitive
	n=35	n= 36	n=11
Minimum	500	250	520
25^{th} centile	600	450	540
Median	600	550	625
75^{th} centile	720	600	675
Maximum	1000	1000	680

Table 3.5 Epithelial denudation score (1 to 3) for control, interstitial cystitis and hypersensitive bladder subjects

Score	Control n=35	all IC n= 34	severe IC n=6	Hypersensitive n=10
1	22 (63%)	13 (38%)	1 (17%)	6 (60%)
2	13 (37%)	14 (41%)	2 (33%)	4 (40%)
3	0	7 (21%)	3 (50%)	0

Table 3.6 Submucosal oedema score (0 to 3) for control, interstitial cystitis and hypersensitive bladder subjects

Score	Control n= 35	all IC n=36	severe IC n=6	Hypersensitive n=11
0	21 (60%)	10 (28%)	2 (33%)	4 (36%)
1	13 (37%)	19 (53%)	1 (17%)	5 (56%)
2	1 (3%)	7 (i9%)	3 (50%)	2 (18%)
3	0	0	0	0

Table 3.7 Submucosal congestion and ectasia score (0 to 3) for control, interstitial cystitis and hypersensitive bladder subjects

Score	Control	all IC	severe IC	Hypersensitive
	n= 35	n=36	n=6	n=11
0	14 (40%)	6 (17%)	1 (17%)	2 (18%)
1	20 (57%)	24 (67%)	2 (33%)	9 (82%)
2	1 (3%)	5 (14%)	2 (33%)	0
3	0	1 (3%)	1 (17%)	0

Table 3.8 Submucosal inflammatory infiltrate score (0 to 3) for control, interstitial cystitis and hypersensitive bladder subjects

Score	Control	all IC	severe IC	Hypersensitive
	n=35	n=36	n=6	n=11
0	25 (71%)	16 (44%)	1 (17%)	4 (36%)
1	8 (23%)	13 (36%)	2 (33%)	7 (46%)
2	2 (6%)	5 (14%)	2 (33%)	0
3	0	2 (6%)	1 (17%)	0

Table 3.9 Submucosal haemorrhage score (0 to 3) for control, interstitial cystitis and hypersensitive bladder subjects

Score	Control	all IC	severe IC	Hypersensitive
	n= 35	n=36	n=6	n=11
0	16 (46%)	12 (33%)	2 (33%)	4 (36%)
1	16 (46%)	16 (44%)	3 (50%)	7 (64%)
2	3 (9%)	6 (17%)	1 (17%)	0
3	0	0	0	0

Table 3.10 Composite abnormal histology score (possible range 0 to 12) for control, interstitial cystitis and hypersensitive bladder subjects

	Control n=35	all IC n= 36	severe IC n= 6	Hypersensitive n=11
0	4 (11%)	3 (8%)	0	0
1	10 (29%)	6 (17%)	1 (17%)	2 (18%)
2	13 (37%)	3 (8%)	0	4 (36%)
3	7 (20 %)	8 (23%)	1 (17%)	0
4	0	9 (25%)	1 (17%)	5 (46%)
5	0	1 (3%)	0	0
6	1 (3%)	2 (6%)	0	0
7	0	3 (8%)	2 (33%)	0
8	0	1 (3%)	1 (17%)	0

CHAPTER FOUR

OESTROGEN AND PROGESTERONE RECEPTOR STATUS IN BLADDER SUBMUCOSA OF WOMEN WITH INTERSTITIAL CYSTITIS AND CONTROL SUBJECTS

4.1 INTRODUCTION

Interstitial cystitis predominantly affects women rather than men with a ratio of 10:1. Hormonal factors may influence symptomatology with anecdotal reports of premenstrual exacerbation and improvement during pregnancy. Oestrogen and progesterone receptors have been described in the lower urinary tract in both men and women (Iosif et al, 1981; Batra and Iosif, 1987). Wolf et al (1991) found that the trigone and posterior part of the bladder neck were receptor positive for both oestrogen and progesterone whereas the bladder neck and dome were negative in all cases.

There is only one previous report of the assessment of oestrogen and progesterone receptors in the IC bladder. In a small series, Pang and colleagues, (1995) analysed bladder biopsies from 6 women with IC and 4 female control subjects and found by immunohistochemistry and image analysis that the IC group demonstrated a greater number of pixels of oestrogen receptor staining bladder mast cells (2700 pixels) than the control group (2000 pixels). In this study thirty per cent of mast cells were also positive

for progesterone receptor. These are immunohistochemical studies of steroid receptor binding; studies with evidence of endocrine function in IC have not been performed.

In 1996 a second oestrogen receptor, ERβ was cloned from rat ovary and prostate (Kuiper et al, 1996). Subsequently human ERβ cDNA encoding a 530 amino acid protein was reported (Ogawa et al, 1998) with further reports from others of a number of different isoforms (Warner et al, 1999). ERβ receptor expression was demonstrated by immunohistochemistry in multiple rat tissues including bladder (Saunders et al, 1997) In rat bladder tissue moderate expression of ERβ but no ERα expression was demonstrated using RT-PCR (Kuiper et al,1997).

In this study the aim was to

i) quantitatively assess any difference in oestrogen and progesterone receptor expression in the submucosa of bladder biopsies in a cohort of women with IC and control subjects; these latter controls including both control women and men

ii) perform a pilot assessment of the feasibility of a ERβ immunohistochemical study on the same cohort

4.2 METHODS

4.2.1 BIOPSY COLLECTION

Bladder biopsies were randomly chosen from 26 of the IC and 28 of the female control subject tissue bank described in Chapter 2. In the IC group, bladder biopsies were obtained from different sites within the bladder, often from the area with maximum glomerulations. In the control group biopsies were also from different bladder locations usually the posterior and lateral bladder walls. There were no biopsies in either control

or IC subject from the trigonal area. In the male control group biopsy material was archival tissue from 5 men having cystectomy for bladder carcinoma. The location of the bladder biopsy section analysed in the male group was not known apart from its distant location from the site of carcinoma.

4.2.2 IMMUNOHISTOCHEMISTRY

5um sections were cut and placed onto APES coated slides and the tissue allowed to adhere by incubation at 37°C overnight. The slides were initially incubated at 56° C for 60 minutes. The sections were dewaxed and rehydrated. For antigen retrieval the sections were placed in a boiling solution of 0.01M sodium citrate over a bunsen burner for 10 minutes. The burner was then turned off and the slides remained in the hot citrate solution for a further 20 minutes. They were incubated in 3% peroxide in methanol for 10 minutes at 37°C to block endogenous peroxidase followed by incubation with anti-oestrogen receptor antibody (ER Mouse Monoclonal Antibody NCL-ER-6F11 IgG1 conc 50ug/ml, Novacastra Lab, Newcastle upon Tyne, UK) diluted 1 in 200 in PBS/1%BSA or anti-progesterone receptor antibody (PR Mouse Monoclonal Antibody NCL-PGR IgG1 conc 30ug/ml, Novacastra Lab, Newcastle upon Tyne, UK) diluted 1 in 500 in PBS/1%BSA overnight at 4°C. For the ERβ pilot study the PA1.1-310 antibody (Affinity Bioreagents Inc) was used. The slides were then incubated with biotinylated rabbit anti-mouse IG (Dako LSAB+ kit) for 15 minutes at room temperature (RT), followed by 15 minute incubation with HRP-Streptavidin (Dako LSAB+ kit). PBS washes followed each step. The slides were developed using a DAKO kit for 5 minutes at RT. The slides were washed in distilled water and coverslipped using Depex. The negative controls were treated in an identical manner but normal rabbit serum was

substituted for the primary antibody. Endometrial tissue was used as the positive control.

A double stain technique was employed in an attempt to identify the cell type which was staining positive for steroid receptor. Actin, alpha smooth muscle (Clone 1A4) is a mouse anti-human monoclonal antibody (M0851) which reacts with the alpha-smooth muscle isoform of actin present in smooth muscle cells of vessels, pericytes and some stromal cells in intestine, ovary and testis. It does not react with actin from fibroblasts or striated muscle. The above protocol was followed until the progesterone receptor antibody incubation. This was followed by biotinylated rabbit anti-mouse 1:100 (Zymed, California USA) antibody for 30 minutes at RT and HRP-Streptavidin 1:400 (Zymed, USA) for 10 minutes, and the slides were developed using AEC kit (Zymed, USA) for 5 minutes as per manufacturer's instructions. The slides were washed with distilled water and incubated with double stain enhancer (Zymed, USA) for 30 minutes at RT. The slides were incubated with Smooth Muscle Actin (Dako, USA) 1:400 for 60 minutes at RT followed by biotinylated rabbit anti-mouse 1:100 (Zymed, California USA) for 30 minutes at RT and then APStreptavidin (Zymed, USA) 1:200 for 10 minutes at RT. The slides were washed with PBS after each step, developed with AP Blue (Zymed, USA) for 30 minutes at RT and mounted in aqueous mountant (Faramount (Dako, USA).

4.2.3 Quantitative assessment of Immunostaining

The bladder epithelium was not assessed in this study. The bladder submucosa was analysed using a rectangular sampling window of size $0.02mm^2$. Four scores were obtained for each section at a high intensity setting of the image analyser (Analytical

Imaging Station version 3.0 rev 1.4, Imaging Research Inc.) and four scores at a low intensity setting. The high intensity score (intensity setting; 702 to 902) which corresponds with the lower number of stained cells was the count of the very intensely stained cells only. The low intensity scores (intensity setting; 502 to 902) which had more cells was the count of all the stained cells including the intensely stained cells. A combined total of each of the four low and high intensity scores was derived to give the number of oestrogen and progesterone receptor positive cells in an area corresponding to 0.08 mm^2.

4.3 RESULTS

4.3.1 AGE

The median age for the female control group was 51 years with a range between 30 and 80 years; the IC group had a median age of 43 years with a range between 19 and 80 years. The male control subjects were aged between 52 and 75 years (52,62,65,75,75).

4.3.2 MENOPAUSAL STATUS

The menopausal status was known in 25 of the female controls and all of the IC subjects and is shown in Table 4.1. There were more postmenopausal subjects in the IC group ($p<0.01$). Menopausal status was assessed as an independent variable and there was no significant difference in the number of oestrogen or progesterone receptor staining submucosal cells between premenopausal and postmenopausal subjects overall.

4.3.3 OESTROGEN RECEPTOR STAINING

Oestrogen receptor immunohistochemistry was performed on 26 subjects with IC, 26 female control subjects and 5 control male subjects. Thirteen of 26 female control subjects and 12 of 26 IC subjects had no evidence of oestrogen receptor staining submucosal cells in the bladder biopsy. Eight of 26 control subjects and 7 of 26 IC subjects had ten or more positive staining submucosal cells / 0.08 mm^2. The positive staining cells were individual stromal cells; they were not vascular or epithelial cells but may represent fibroblasts or inflammatory cells. Approximately half of the positively staining cells stained maximally and half stained moderately in both the IC and control groups. There was a greater variation within the control than the IC group.

Table 4.2 shows the median values and the range of cells staining positive for oestrogen receptor in the submucosa of female control and interstitial cystitis subjects and male control subjects. Figure 4.1. a, b, c and d are representative sections showing oestrogen receptor staining cells in the submucosa of bladder biopsies from female control, interstitial cystitis subject, control male and a negative antibody control respectively. There was no significant difference in oestrogen receptor positive submucosal cells in the bladder biopsies of control women or women with interstitial cystitis at the low intensity setting ($p=0.58$) but a trend toward increased number of cells in the control group at high intensity settings ($p=0.08$). There was no difference in oestrogen receptor positive submucosal cells in the bladder biopsies of control females or males at both low ($p=0.14$) and high ($p=0.28$) intensity settings. The control males had a greater number of oestrogen receptor positive submucosal cells compared with the interstitial cystitis group at both low ($p=0.04$) and high ($p=0.08$) intensity settings.

4.3.4 PROGESTERONE RECEPTOR STAINING

Progesterone receptor immunohistochemistry was performed on bladder biopsies of 24 subjects with IC, 28 control women and 5 control male subjects. Approximately 20 % (5 of 24) of the IC group and half (14 of 28) of the female control group had no evidence of progesterone receptor staining submucosal cells at the low intensity setting with 58 % (14 of 24) of the IC group and 68% (19 of 28) of the control group showing zero staining at the high intensity settings. At the low intensity setting, 50 % (12 of 24) of the IC group and 36 % (8 of 28) of the control group had ten or more positive staining submucosal cells in an area of 0.08 mm^2 with only 13 % of the IC (3 of 24) and the control (4 of 28) groups showing ten or more positive staining submucosal cells / 0.08 mm^2 at the high intensity setting. In contrast, at both low and high intensity settings all of the male bladder biopsies had greater than 20 progesterone positive submucosal cells / 0.08 mm^2. As with the oestrogen receptor study, the positive staining cells were individual stromal cells which may represent fibroblasts or inflammatory cells.

Table 4.3. shows the median values and the range of cells in an area of 0.08 mm^2 staining positive for the progesterone receptor in the submucosa of bladder biopsies from control female and male subjects and interstitial cystitis subjects. Figure 4.2.a, b, c and d are representative sections of progesterone receptor staining submucosal cells in bladder biopsies from female control, interstitial cystitis subject, control male subject and a negative antibody control respectively. There was no difference in submucosal cells staining positively for progesterone receptor between female control and IC subjects at low intensity ($p=0.13$) or high intensity ($p=0.6$) settings. There was a significantly increased number of progesterone receptor positive submucosal cells in

bladder biopsies derived from male subjects as compared with either female controls ($p<0.01$) or female IC subjects ($p<0.01$).

4.3.5 PR/SMOOTH MUSCLE ACTIN DOUBLE STAINING

Figure 4.3(a to c) show a bladder biopsy from a male control, female control and a negative control with progesterone being a nuclear stain (red) and smooth muscle actin staining blue both in the cytoplasm of cells which are progesterone antibody positive and stromal fibrillary staining. This appearance is consistent with stromal smooth muscle cell showing progesterone staining.

4.3.6 ER BETA RECEPTOR RT-PCR

ERβ receptor expression was present in both a control and IC bladder biopsy (performed by Simon Chu, Prince Henry's Institute of Medical Research) and this provided the rationale for attempting the ERβ immunohistochemistry.

4.3.7 BLADDER ERβ RECEPTOR IMMUNOHISTOCHEMISTRY

A pilot study of ERβ immunohistochemistry in IC and control bladder biopsies was planned using the polyclonal anti-ERβ antibody 1-310 (Affinity Bioreagents Inc, USA). Attempts were made to stain potential positive control tissue such as human endometrium and rat bladder but unfortunately the staining was non-specific. Further attempts to improve specificity were unsuccessful and the study was discontinued.

4.4 DISCUSSION

This is the first study of bladder submucosal oestrogen and progesterone receptors in interstitial cystitis apart from a small study which dealt with the relationship between steroid receptors and mast cells. The current study demonstrated no difference in the number of submucosal cells staining positively for the oestrogen or the progesterone receptor between IC and female controls. The bladder epithelium was not assessed. Bladder submucosal cells positive for oestrogen receptor were present in approximately half of the control and interstitial cystitis subjects but generally with weak to moderate staining. Eighty per cent of the IC and half of the control group demonstrated bladder submucosal cells positive for progesterone receptor with 14 % of both control and IC groups showing intense staining cells. Male controls showed a trend toward a greater number of oestrogen receptor positive submucosal cells and a significantly greater number of progesterone receptor positive submucosal cells compared with female controls or those with IC.

Steroid receptor studies of bladder tissue have demonstrated variable results. Kvist and Albrectsen (1994) did not find evidence of oestrogen receptors in a series of bladder transitional cell tumours in both women and men. Bodker et al., (1995) were unable to demonstrate oestrogen receptors in the bladder by immunohistochemical or biochemical means in 31 males with haematuria, prostatic hyperplasia or bladder malignancy.

Noronha and Rao, (1986) measured steroid binding capacity by a charcoal adsorption technique and found oestrogen receptors in two of five bladder tumour specimens (both advanced tumours;1 female;1 male) and progesterone receptors in 3 of 5 (2 advanced, one Grade 2; 2 female;1 male) bladder tumour specimens.

Oestrogen receptor staining was detected by Kaufmann et al., 1998 in 18 % of 88 invasive and 97 non-invasive bladder carcinomas using conventional immunohistochemistry and in 34 % using the tyramide staining amplification technique. They were more often detectable in higher grade and invasive carcinomas and there was no association with the age or sex of the patient.

Pacchioni et al., (1991) analysed bladder biopsies taken from the trigone and the lateral bladder wall in 15 women having staging for gynaecological malignancy and 15 women with "pseudomembranous trigonitis". They found oestrogen receptor (ER) positive cells only in the trigone in half of each group but none in the lateral bladder wall. Progesterone receptor (PR) positive cells were found in 11 of 14 trigonal and 7 of 14 lateral bladder wall biopsies from the gynaecology staging group in addition to 11 of 15 trigonal and 2 of 15 lateral bladder wall biopsies from the trigonitis group. No significant differences in ER or PR staining was associated with age or menopausal status. The areas which stained positive for ER were those where squamous metaplasia occurred in the trigone. In the staging group, PR positivity did not have any association with ER positivity or squamous metaplasia. In the trigonitis group, there was a significant difference in PR content between the trigone and the lateral bladder wall but no association between PR positivity and squamous metaplasia. The ER positive cells were distributed throughout the full thickness but especially in the superficial layers of metaplastic squamous epithelium whereas the PR positive cells were observed in the basal layers.

One could speculate that the trigonitis group has some clinical overlap with IC. Pacchioni and colleagues (1990) describe their subjects as complaining of recurrent

abacterial cystitis with repeatedly negative urine cultures and no benefit from antibiotics, with trigonal hyperaemia or white epithelium at cystoscopy and histological evidence of oedema or trigonal squamous metaplasia. The finding of trigonal squamous metaplasia is of doubtful pathological or diagnostic importance. It is commonly found in asymptomatic women and is thought to be due to the effect of oestrogen on trigonal squamous epithelium. Fourteen of the 15 trigonitis subjects were aged 52 or less thus likely to have significant circulating levels of oestrogen resulting in the appearance of squamous metaplasia. It is possible that the trigonitis group are similar to the hypersensitive bladder group described in Chapter 2 and 3. It may even include IC subjects as the trigonitis group did not undergo hydrodistention in order to exclude IC.

In the current study, there is no evidence of a difference in the number of oestrogen (ERα) or progesterone staining submucosal cells in bladder biopsies from women with IC as compared with controls. The analysis was only of submucosal cells and did not study the urothelial or deeper detrusor muscle layers. The template size chosen allowed a representative sample of the whole section to be analysed. The analysis was performed in a blinded manner and two intensity settings were chosen which allowed for both intensely staining and moderately staining cells to be included in the assessment.

Compared with endometrium, breast and vagina, the components of the lower urinary tract not derived from the urogenital sinus are relatively oestrogen insensitive. The trigone and urethra which have stratified squamous epithelium do show a clinical response to oestrogen deficiency and/or replacement. In this study, there was no uniformity in the location of the bladder biopsies in the females apart from an avoidance of the trigone. It is possible that there may be other bladder location and individual variations accounting for the very large range in steroid staining cells in both female

controls and IC individuals. As discussed in Chapter 3 the reliance on bladder cold-cup biopsies by necessity means that only a tiny area of the bladder is sampled and assessed. For both oestrogen and progesterone receptor the variation was greater within the control group. The male specimens were archival cystectomy tissue (not post hydrodistention cold-cup biopsies) and the site was unknown but they were located in the presence of a bladder tumour. The higher level of progesterone staining cells in the male bladder was interesting and may be due to a low circulating progesterone level; may have been stimulated by the concurrent bladder carcinoma.or due to the difference between tissue obtained at cystectomy versus after hydrodistention. It in fact may reflect a real male/female difference for example with males having a greater submucosal cellularity with consequently more of these stromal cells positive for progesterone.

The lack of success with ERβ immunohistochemistry was disappointing but may be due to the fact that at the time of the pilot study (1997-8) the polyclonal antibody employed was relatively recently developed. Taylor and Al-Azzawi, (2000) recently reported immunohistochemical expression of ERβ receptor in multiple human tissues including male bladder epithelial cells and smooth muscle cells. Hodges and colleagues, (2000) employed a technique of relative quantification of ERα and ERβ mRNA in vascular smooth muscle "because reliable monoclonal antibodies for human ERβ are not commercially available".

The human progesterone receptor is expressed as two isoforms, PRA and PRB (Horwitz and Alexander, 1983); PRA lacks 164 amino acids from the N-terminus. In vitro studies suggest the different isoforms are also functionally different and their relative expression may determine the type of functional responses to progesterone and oestrogen.(Vegeto et al, 1993) Mote and colleagues, 1999 demonstrated that PRA and

PRB reside in the same cell in the human endomterium using immunofluorescent histochemistry. Future receptor studies could include ERβ and differential PRA and PRB immunohistochemistry.

The very commonly anecdotally reported premenstrual symptom exacerbation in IC is not explained by any difference in bladder submucosal oestrogen and progesterone receptors as there were no differences. This finding is consistent with the clinically observed lack of response of IC symptoms to hormonal manipulation. However, future studies utilising reliable specific isoform antibodies for both oestrogen and progesterone receptors may show a difference in expression throughout male and female bladder tissue and IC bladder.

TABLE 4.1. MENOPAUSAL STATUS OF CONTROL AND IC SUBJECTS

	Control n= 25 , (%)	Interstitial cystitis n= 26 , (%)
Premenopausal	7 (28%)	14 (54%)
Postmenopausal with no HRT	6 (24%)	10 (38%)
Postmenopausal with HRT	12 (48%)	2 (8%)

Table 4.2. Oestrogen receptor staining submucosal cells/0.08 mm^2 in bladder biopsies from control women and men, and women with interstitial cystitis

	Control females median (range)	Interstitial cystitis median (range)	Control males median (range)
Low intensity Setting	0.5 (0-65)	1 (0-29)	17 (0-31)
High intensity setting	0 (0-27)	0 (0-10)	3 (0-9)

Table 4.3. Progestrogene receptor staining submucosal cells/0.08 mm^2 in bladder biopsies from control women and men, and women with interstitial cystitis

	Control females median (range)	Interstitial cystitis median (range)	Control males median (range)
Low intensity setting	0 (0-109)	10.5 (0-87)	36 (32-79)
High intensity setting	0 (0-58)	0 (0-34)	22 (21-28)

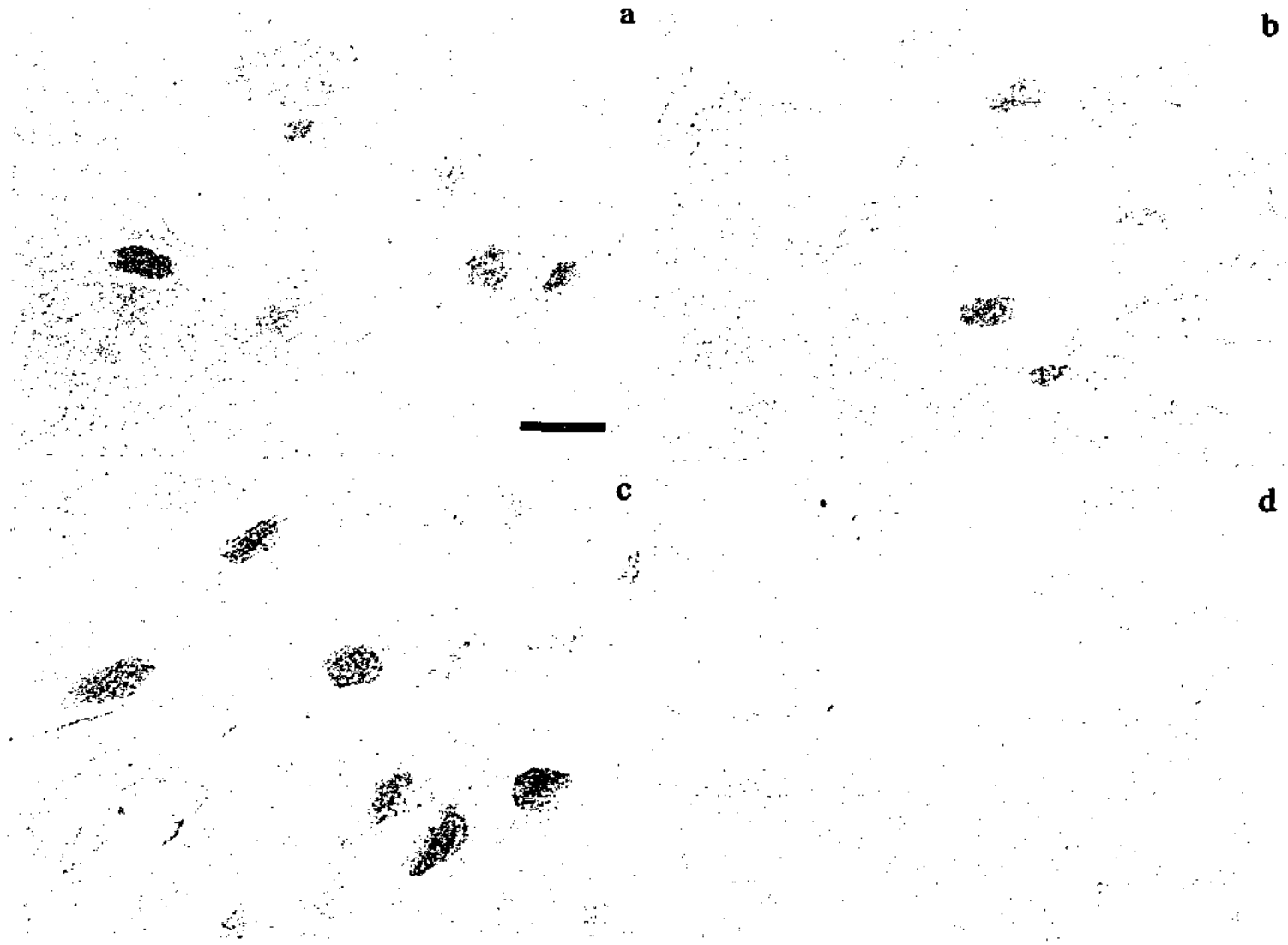


Figure 4.1 Oestrogen receptor immunostaining in bladder biopsy submucosa

(a) Female control subject (b) Female interstitial cystitis subject

(c) Male control subject (d) Negative control

Scale bar=10um

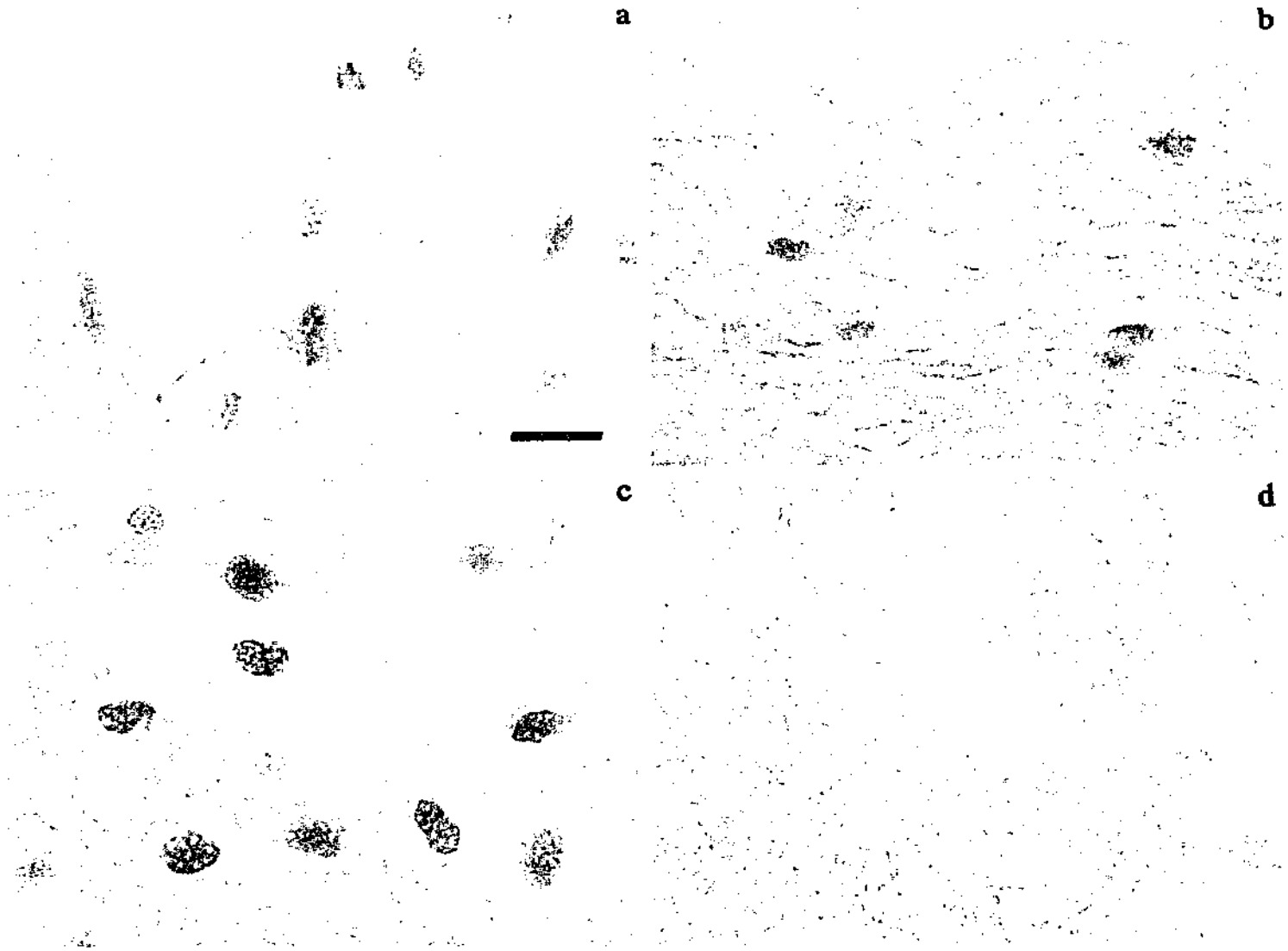


Figure 4.2 Progesterone receptor immunostaining in bladder biopsy submucosa

(a) Female control subject (b) Female interstitial cystitis subject

(c) Male control subject (d) Negative control

Scale bar=10um

Figure 4.3 Double immunostaining with progesterone receptor (red/brown) and smooth muscle actin (blue) in bladder biopsies

(a) Male control subject

(b) Female control subject

(c) Negative control

Scale Bar=10um

CHAPTER FIVE

BLADDER MICROVASCULATURE IN WOMEN WITH INTERSTITIAL CYSTITIS

5.1 INTRODUCTION

The main clinical criteria for the diagnosis of interstitial cystitis (IC) are the symptoms of urinary frequency, urgency and pain, a low functional bladder capacity and the characteristic cystoscopic appearance of glomerulations, fissuring or ulceration in the absence of other causes of cystitis. (Gillenwater & Wein, 1987) Although it is recognised that they are not pathognomonic of IC, the petechial haemorrhages or glomerulations on emptying accompanied by terminal haematuria may be the only abnormal finding at cystoscopy as seen in Figure 1b.(Messing & Stamey, 1978). Despite the diagnostic importance of glomerulations, there has been no quantitative investigation of the bladder microvasculature in IC.

There is evidence that the bladder microvasculature is altered or possibly damaged in IC. Histological and immunohistochemical studies of IC have reported qualitative descriptions of features such as dilated venules (Holm-Bentzen & Lose, 1987), suburothelial haemorrhage (Johansson & Fall, 1990) and vasculitis (Johansson & Fall,

1990: Matilla,1992). In addition electron microscopy studies of IC have described a highly thickened basement membrane of the subepithelial vessels (Elbadawi 1997) and evidence of blood vessel damage such as bloated endothelial cells with focal degeneration. (Collan et al, 1976)

The aim of this study is to use immunohistochemical techniques to compare the bladder microvasculature in women with early and severe IC to that in controls. The microvascular density in the suburotheliium in addition to the deeper submucosa will be studied, and endothelial cell proliferation rates will also be assessed.

5.2 MATERIALS AND METHODS

5.2.1 SUBJECTS, CLINICAL ASSESSMENT, BIOPSY COLLECTION.

The subjects were twenty-six females with IC (mean age 48 +/- 16 years) who were diagnosed using National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIDDK) criteria. (Gillenwater & Wein, 1987) Seventeen subjects were newly diagnosed and a further 5 had undergone single (4) or multiple (1) hydrodistension previously. Prior oral medications included anticholinergics (4), prednisolone (2), antihistamine (2). One subject had been treated with two courses of intra-vesical dimethyl sulfoxide (12 instillations per course) one and two years previously. The twenty-six female controls (mean age 52 +/- 11 years) were patients undergoing stress incontinence and/or prolapse surgery. They had a clinical history, physical examination and urinary diary consistent with stress incontinence and/or prolapse. The control subjects had all undergone urodynamic assessment which demonstrated no sensory urgency at less than 350ml, a capacity of 500ml and no evidence of detrusor instability.

Each subject (IC and control) underwent a standardised hydrodistention for 2-6 minutes at 80cm water, emptying to measure capacity, and second inspection followed by cold cup forceps biopsy. In IC patients the biopsy was taken at the site of maximum glomerulation, patch or fissure. Control subjects underwent this procedure at the end of their surgery. Six of the IC subjects were classified as "severe" based on the cystoscopic findings of bladder capacity < 400ml and the presence of patches or fissures.

5.2.2 IMMUNOHISTOCHEMISTRY.

Bladder biopsies were processed in 10% buffered formalin and embedded in paraffin wax. Five micron thick sections were used for immunohistochemistry which for the immediate suburothelial microvasculature involved the following protocol; dewaxing and rehydrating the sections followed by 3% hydrogen peroxide in methanol for 10 minutes. The tissue was then incubated with the primary antibody against endothelial cell antigen, anti-CD34 (Clone Qbend 10 Serotec, Australian Laboratory Services, Melbourne, Vic) at a dilution of 1 in 50 for 1 hour followed by the addition of biotinylated secondary antibody for 15 minutes and then streptavidin peroxidase for 15minutes (DAKO kit, DAKO, Denmark).

The chromogen, diaminobenzidine (DAB) was added for 5-10 minutes. All steps were followed by washes with phosphate buffered saline and were performed at room temperature. The sections were mounted in Clearmount mounting solution (Zymed, San Francisco, California, USA) or Depex. mounting medium (B.D.H. Laboratory Supplies, Poole, UK). Negative controls were performed by using normal rabbit serum instead of the primary antibody.

The differences for the deeper submucosal microvasculature immunohistochemical protocol were; pepsin pre-treatment for 30 to 40 minutes and anti-CD31 (antibody to platelet endothelial cell adhesion molecule or PECAM) incubation at a dilution of 1 in 50 for 3 hours at 37^{O}C or overnight at 4°C.

Endothelial cell proliferation was assessed using a double stain technique. Antibody to proliferating cell nuclear antigen (Nova Castra, Newcastle upon Tyne, UK) was used for the initial reaction in a peroxidase based Zymed kit (San Francisco, USA). Three amino 9 ethyl carbazole (AEC) was the red chromogen. The subsequent step was incubation with anti-CD34 using the alkaline phosphatase (AP) based Zymed kit (San Francisco, USA) with AP-Blue as the blue chromogen.

5.2.3 BLOOD VESSEL AND CELL NUCLEI ESTIMATION.

Computer assisted image analysis (Analytical Imaging Station version 3.0 rev 1.4, Imaging Research Inc.) was used in addition to manual counting for endothelial cell estimation (Rogers et al, 1993; Orre et al, 1998). Analysis was performed independent of patient clinical diagnosis. The suburothelial section was imaged and each image was scanned using a 0.005 mm^2 template (Figure 5.1). Between 5 and 10 computer generated readings of the image were obtained depending on the amount of suburothelium. This process was repeated once on a different area of the same section to generate a total analysed image area of 0.05 to 0.1mm^2. The deeper submucosal blood vessel count was performed by manual counting on two different sections within an area scanned by a template of 0.01mm^2 dimension. Suburothelial endothelial cell

proliferation was assessed using a 0.005mm^2 template; 4 readings were manually counted per image and the process repeated to generate a total analysed area of 0.04mm^2. The proliferative index for each section was then obtained by dividing the number of proliferating nuclei by the total number of endothelial cell nuclei (Goodger & Rogers, 1993).

5.2.4 HISTOLOGICAL ASSESSMENT

Independent of the immunohistochemical study a blinded histological assessment of the corresponding haematoxylin and eosin (H & E) stained sections was performed as described in Chapter 3. This was based on a histological study of IC bladder biopsy specimens performed by Lynes et al (1990) which graded oedema, ectasia, inflammation and fibrosis between 0 and 4. There was no evidence of fibrosis in our study however there was evidence of haemorrhage. Accordingly, the four features assessed on light microscopy in this study were submucosal oedema, haemorrhage, vascular ectasia and congestion, and inflammatory infiltrate, and each was graded 0 to 3. The four scores were added to give the composite submucosal abnormal histology score.

5.2.5 STATISTICAL ANALYSIS

The CD-34 data for blood vessel count were subjected to a 1-way analysis of variance (ANOVA) with Fisher's pairwise comparison for subgroup analysis. The CD-31 and CD-34 proportional area data underwent logarithmic transformation followed by 1-way ANOVA; the untransformed data was also evaluated by Kruskal-Wallis ANOVA on Ranks. One-way ANOVA was done to compare both proliferating and total endothelial

suburothelial vessels compared with the control bladder. It is possible that more sparsely spaced capillaries may not accommodate to distention and result in rupture and the consequent appearance of petechial haemorrhages (glomerulations). There was a trend toward fewer suburothelial blood vessels in the clinically more severe group and in the group with the more abnormal histological grade. Microvascular density and routine light microscopy histological features were independently assessed but were nonetheless related.

In the current series, utilising the submucosal histology grading as described above, a score of 4 or greater has a sensitivity of only 48% but a specificity of 100% for IC. Overall in this series, IC was associated with histological changes evident on routine pathological assessment in less than 50% of cases.

Previous qualitative descriptions of blood vessel changes in IC have been of features such as vasculitis. Johansson & Fall in 1990 noted "vasculitis", which was defined as "abundant neutrophils in dilated venules and small veins involving the wall of the vein and extending into the surrounding lamina propria" in one third of the biopsies with classical disease and less than one quarter with nonulcer disease. Mattila in 1982 found that 70% of IC patients and no control patient had immune deposits in bladder vessel walls. Elbadawi in 1997 found evidence of blood vessel damage in his ultrastructural study of IC which involved the capillaries and venules in the suburothelium and the interstitial septa of the muscularis. He described bloated endothelial cells with focal degeneration and fragmentation as the most striking feature.

The findings of this study do not support the observation made by Collan and colleagues in 1976. Their study which was qualitative rather than quantitative described

the subepithelial tissue in IC as "rich in capillaries and very much like granulation tissue". In addition, our study did not confirm the findings of Deen and Ball in 1994 of closer abutment of subepithelial capillaries in two cases of (uncharacterised) cystitis in their study of archival bladder specimens.

In the present study we were unable to use antibody to CD34 for all immunohistochemical staining of blood vessels due to elevated background staining of stromal cells in the deeper submucosa. For this reason CD31 was used in the deeper submucosa. CD31 did not stain the suburothelial capillaries as well as CD34 had done. CD-34 may be a better endothelial cell marker than CD-31 or the vascular beds of the suburothelium (capillaries) and the deeper submucosa (venules and arterioles) may be different in their antigen expression.

Risau (1997) proposed that the vasculature exists as a consequence of a balance between pro-angiogenic and anti-angiogenic factors. If this is correct, our finding of decreased suburothelial vascular density suggests that the balance between pro-angiogenic and anti-angiogenic forces is altered in women with interstitial cystitis. This change is vascular density may be due to a decrease in pro-angiogenic factors, such as vascular endothelial growth factor or fibroblast growth factor, or an increase in anti-angiogenic factors, such as angiostatin or endostation (Risau, 1997). It is currently unclear whether this change in vascular density is a major contributing factor to interstitial cystitis or more of a response by the vasculature to other factors that caused the initial problem.

An alternative explanation for decreased suburothelial microvascular density in interstitial cystitis is that it is an artifactual result of submucosal edema in the interstitial

cystitis group. Oedema may be difficult to assess histologically. It is generally visualized as lucency in the stroma, and it is also affected by such factors as the cut thickness of the section.

An early theory regarding etiology associated interstitial cystitis with bladder ischemia. Irwin and Galloway (1997) found decreased bladder perfusion at capacity on laser Doppler flowmetry in interstitial cystitis cases compared to controls. When the bladder was filled to capacity, overall blood flow increased a mean of 7.6 laser Doppler flowmetry units in the control group and only 3.4 in the interstitial cystitis group. They suggested that the finding of a failed increase in bladder perfusion with distention may explain the pain that develops with increasing volume in interstitial cystitis. In addition, the underlying process in interstitial cystitis may be neurovascular and mediated by prolonged inappropriate sympathetic efferent activity.

A state of relative vascular insufficiency may be a secondary phenomenon in interstitial cystitis rather than the primary cause. However, decreased blood supply to the urothelium has important implications, such as reduced epithelial barrier function and increased permeability or increased susceptibility to infection. It may contribute to the epithelial thinning or denudation in some interstitial cystitis biopsies, which are usually obtained after hydrodistention. Decreased microvascular density may compromise the supply of oxygen and nutrients to the urothelium and, thereby, contribute to pain in interstitial cystitis.

To the author's knowledge, this is the first quantitative estimate of the microvascular density of the suburothelium and the deeper submucosa, and endothelial cell proliferation in the suburothelium of bladder biopsies from women with interstitial

cystitis and control subjects with stress incontinence or prolapse. A decreased suburothelial microvascular density in women with IC was found and this correlated with both clinical disease severity and degree of histological change. If confirmed and not found to be due to increased oedema, this finding may be responsible for the glomerulations seen after hydrodistension and contribute to the symptomatology and altered epithelial functioning in IC.

Table 5.1 (a) Blood vessel count and (b) proportional area of blood vessel wall in the suburothelium (CD34 stain) in bladder biopsies from IC and control subjects.

(a)

Group	No. Pts	No. obs	Blood vessel count/.005mm²			Significance
			Median	25%	75%	
Severe IC	5	66	2.5	1.4	4.5	
IC	17	206	3.0	2.4	3.7	
Control	14	169	3.8	3.2	4.8	p=0.01

(b)

Group	No Pts.	No. obs	Proportional area of blood vessel wall/.005mm²			Significance
			Median	25%	75%	
Severe IC	6	77	0.10	0.05	0.12	
IC	18	234	0.12	0.07	0.18	
Control	27	373	0.15	0.10	0.21	p=0.03

Table 5.2 Blood vessel count in the deeper submucosa (CD31 stain) in bladder biopsies from IC and control subjects.

Group	No. Pts.	No. obs	Blood Vessel count/0.01mm²		
			Median	25%	75%
IC inc severe	18	71	10.0	8.0	14.0
Control	14	56	10.0	7.0	13.0

No statistical significance

Table 5.3(a) Proliferating and total numbers of endothelial cell nuclei and (b) proliferative index in the suburothelium (PCNA/CD34) in bladder biopsies from IC and control subjects.

(a)

GROUP	NO. PTS	Number of Cell Nuclei / 0.04mm²					
		TOTAL ENDOTHELIAL			PROLIFERATING		
		Median	25%	75%	Median	25%	75%
IC inc severe	11	26.0	15.5	33.0	2.0	1.0	9.0
Control	14	31.0	23.8	40.0	5.0	2.0	9.8

No statistical significance

(b)

PROLIFERATIVE INDEX

Group	No.Pts	Mean	SE
IC inc severe	11	0.21	0.05
Control	14	0.21	0.10

No statistical significance.

Table 5.4 Histological assessment of IC and control bladder biopsies

(a) Grading of vascular congestion/ectasia (Score:0-3)

Group	No.	Median	25%	75%
Severe IC	6	1	1	2
IC	15	1	0	1
Control	23	0.5	0	1

(control vs all IC; $p<0.01$)

Table 5.5 Composite submucosal histology score comprising degree of oedema, haemorrhage, vascular ectasia and congestion, and inflammatory infiltrate; each graded 0 to 3. (Actual score range: 0-7.5)

Group	n	Median	25%	75%
Severe IC	6	5	4	7
IC	15	3	1.5	4
Control	23	1.5	1	2

(control vs all IC;<0.01)

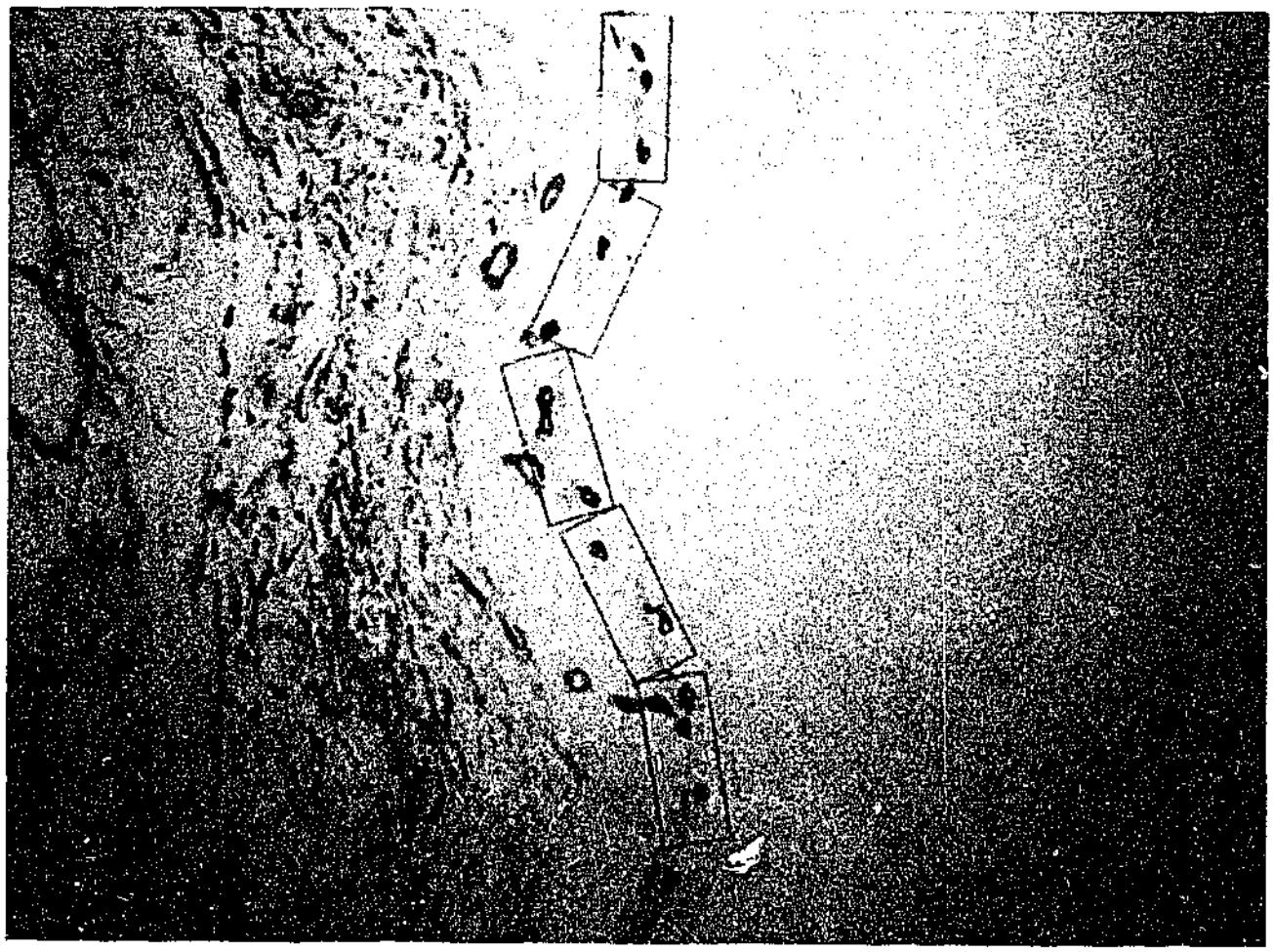


Figure 5.1 Counting the number of CD-34 stained suburothelial blood vessels/template using the computer assisted image analysis system.

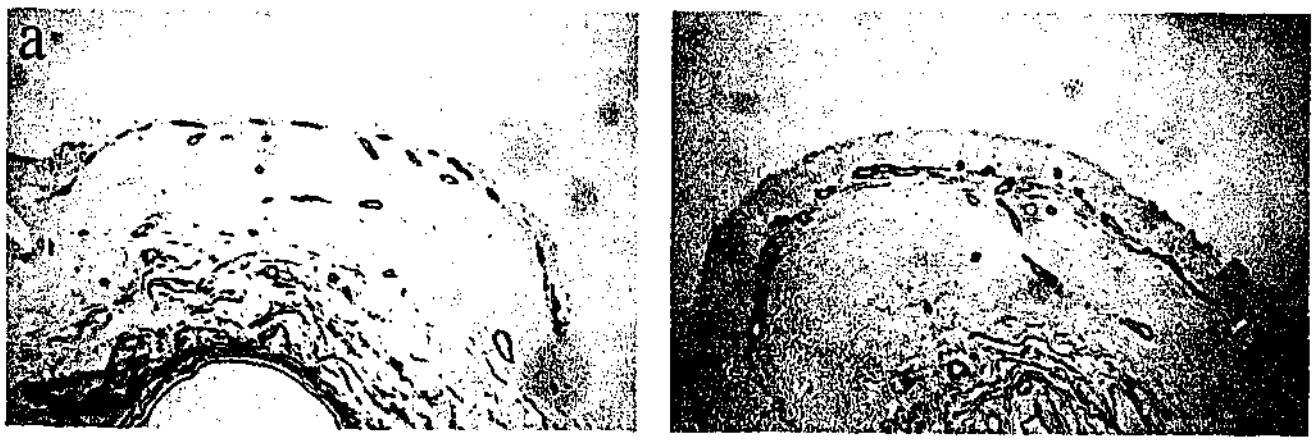


Figure 5.2(a) CD-34 stained IC bladder biopsy showing denuded epithelium and widely spaced suburothelial capillaries (x40) **5.2(b)** CD-34 stained control bladder biopsy showing normal epithelium and closely abutting sub urothelial capillaries (x40)

nuclei in the IC and control groups. Data are expressed as median and quartile ranges and represented graphically as box plots. Regression analysis was used to compare the distribution of abnormal histology score, degree of vascular congestion and ectasia and the blood vessel proportional area data in the IC and control groups. Differences were considered significant at $p < 0.05$.

5.3 RESULTS

Table 5.1(a) and (b) lists the results, including the number of patients in each category, the number of observations (obs) made per category, the blood vessel count and the proportional area of blood vessel wall in the suburothelium. Figure 5.2(a) and (b) are representative CD-34 immunostained bladder biopsies from a patient with IC and a control female subject, respectively. In bladder biopsies from women with IC there was a lower blood vessel count (p=0.01) and lower proportion of the total image made up by blood vessel wall (p=0.03) in the suburothelium (CD34 stain) than controls as illustrated by the box plots in Figure 5.3 (a) and (b). In addition, this trend of fewer suburothelial blood vessels became more obvious with the clinically more severe IC although formal sub-group analysis showed no difference between the IC and severe IC groups.

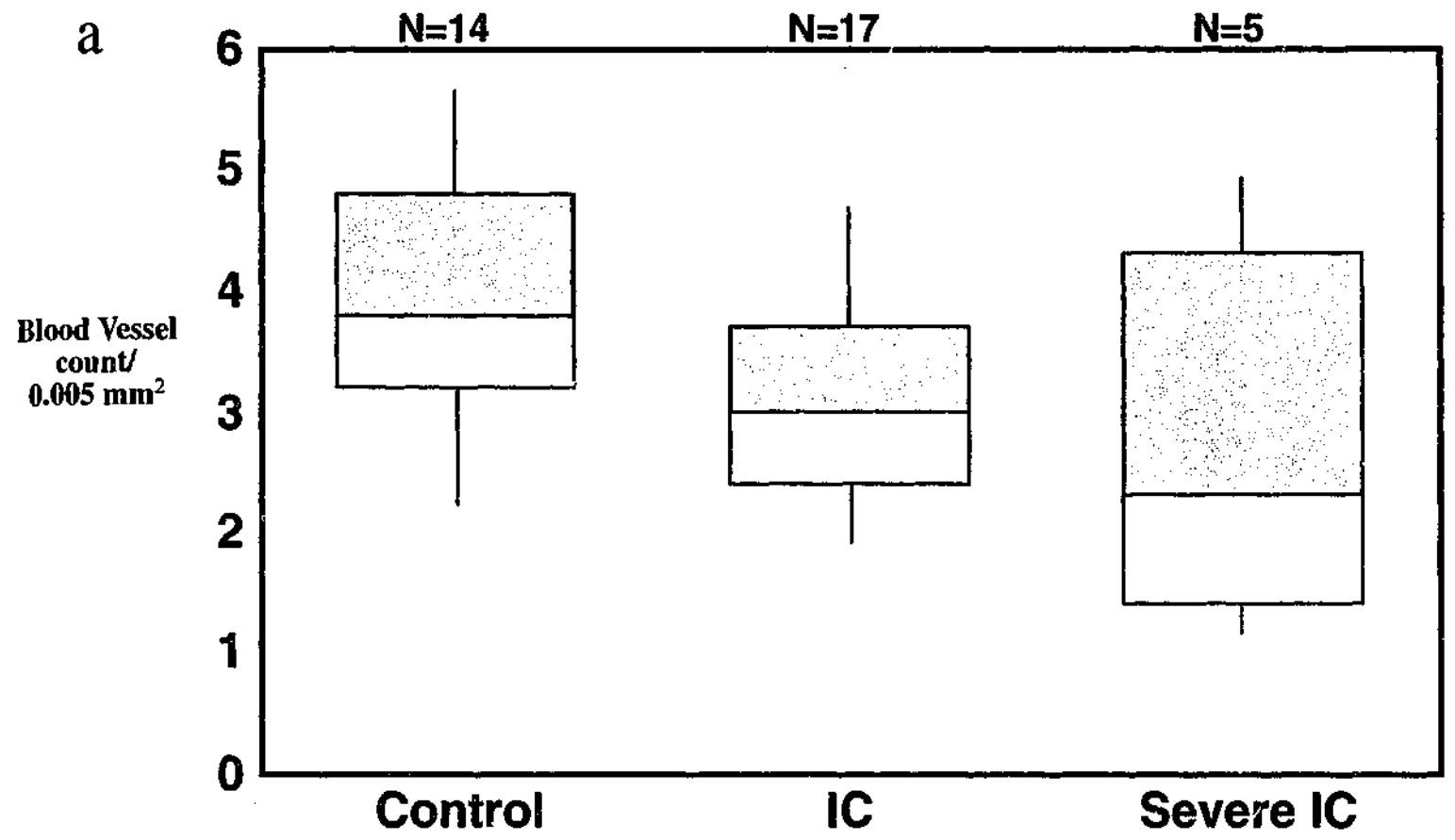
Table 5.2 graphs the blood vessel count of the CD-31 immunostained deeper submucosal vessels in IC and control subjects with Figure 5.4 showing the staining in a control subject. There was no difference in blood vessel count in the deeper layer (CD31 stain) of the submucosa (p=0.25) between control and IC subjects. Figure 5.5 demonstrates the PCNA/CD-34 double stain with proliferating cells staining red with AEC chromogen and endothelial cells blue with AP-blue chromogen. Table 5.3(a) demonstrates a trend toward fewer absolute numbers of suburothelial total and

proliferating endothelial cell nuclei in the IC group but this did not reach statistical significance (p=0.09 for total endothelial cell nuclei). There was no difference in the proportion of proliferating endothelial cells (proliferative index) in the suburothelium as shown in Table 5.3(b).

Figures 5.6 (a) and (b) are representative sections of the H & E stained bladder biopsies of severe IC and a control with histology scores of 7 and 0 respectively (scores derived as described in Section 5.2.4). Table 5.4 and Figure 5.7 summarise the histology results showing the degree of vascular ectasia and congestion and the distribution of the composite lamina propria score between the different groups. Regression analysis demonstrated that the distribution of IC was different to controls with respect to both the abnormal histology score and degree of vascular congestion and ectasia (both analyses; chi-square; $p<0.01$). However an abnormal histology score of 4 or greater had a sensitivity of only 48% for IC. An accumulated analysis of variance combining the composite histology score and the proportional blood vessel wall data was performed. The proportional area made up by blood vessel wall was significantly different between the three arbitrarily defined histology grades of (0-3) low, (4) moderate and (5-8) high ($p<0.05$). Conversely, if an adjustment is made for low, moderate or high histology score, the proportion of blood vessel area was not different between IC and control groups. In other words, the severity of histological change was related to the observed blood vessel changes.

5.4 DISCUSSION

The present study, a quantitative estimate of the bladder microvasculature as demonstrated immunohistochemically found that the IC bladder has a lower density of



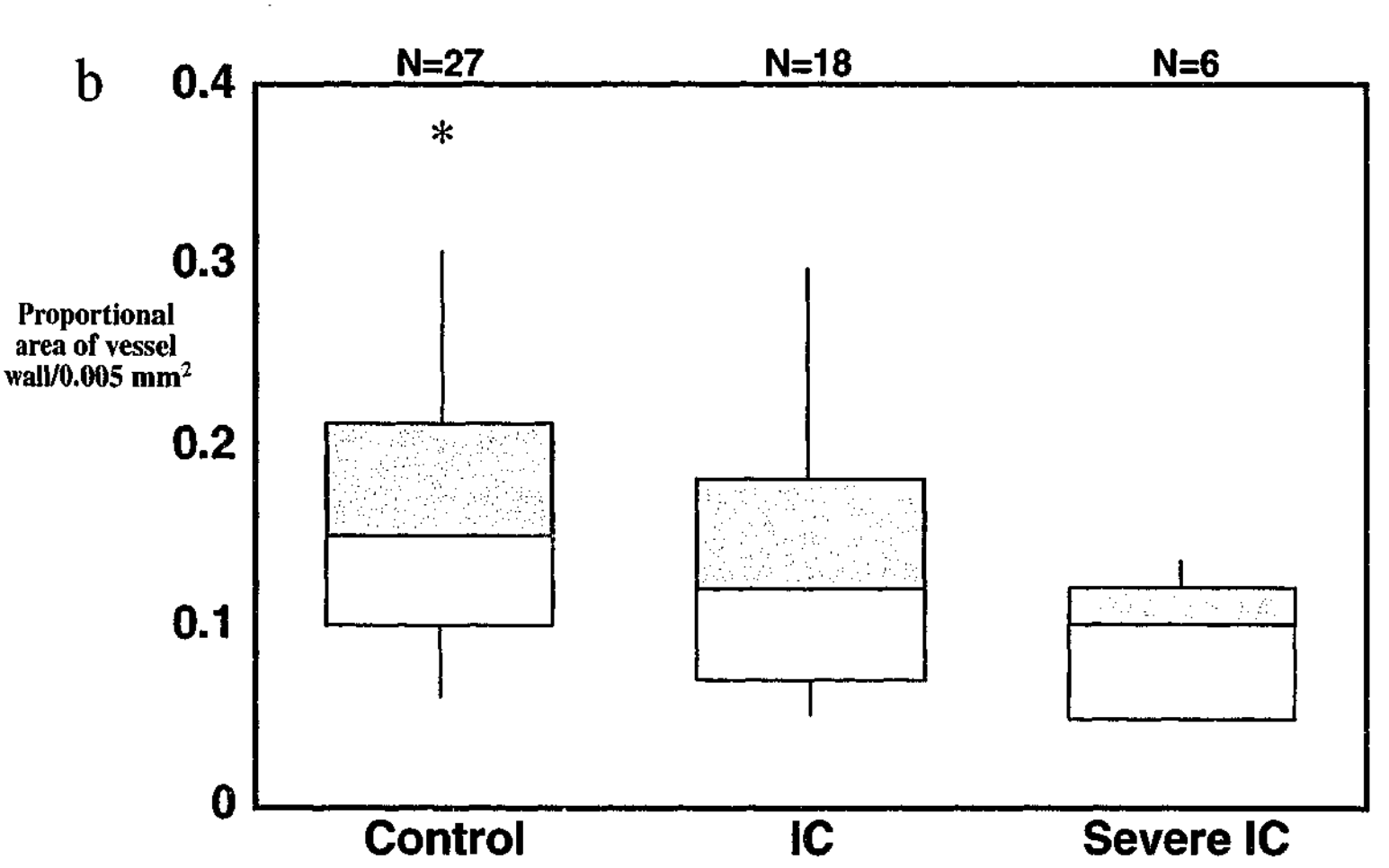


Figure 5.3 Comparison of median (25 to 75 interquartile range) of bladder suburothelial (a) blood vessel count/0.005 mm^2 and (b) proportional area of blood vessel wall/0.005mm^2. Data are present as box plot displaying 25th (lower margin of box), 50th (line within box) and 75th (upper margin of box) percentiles.

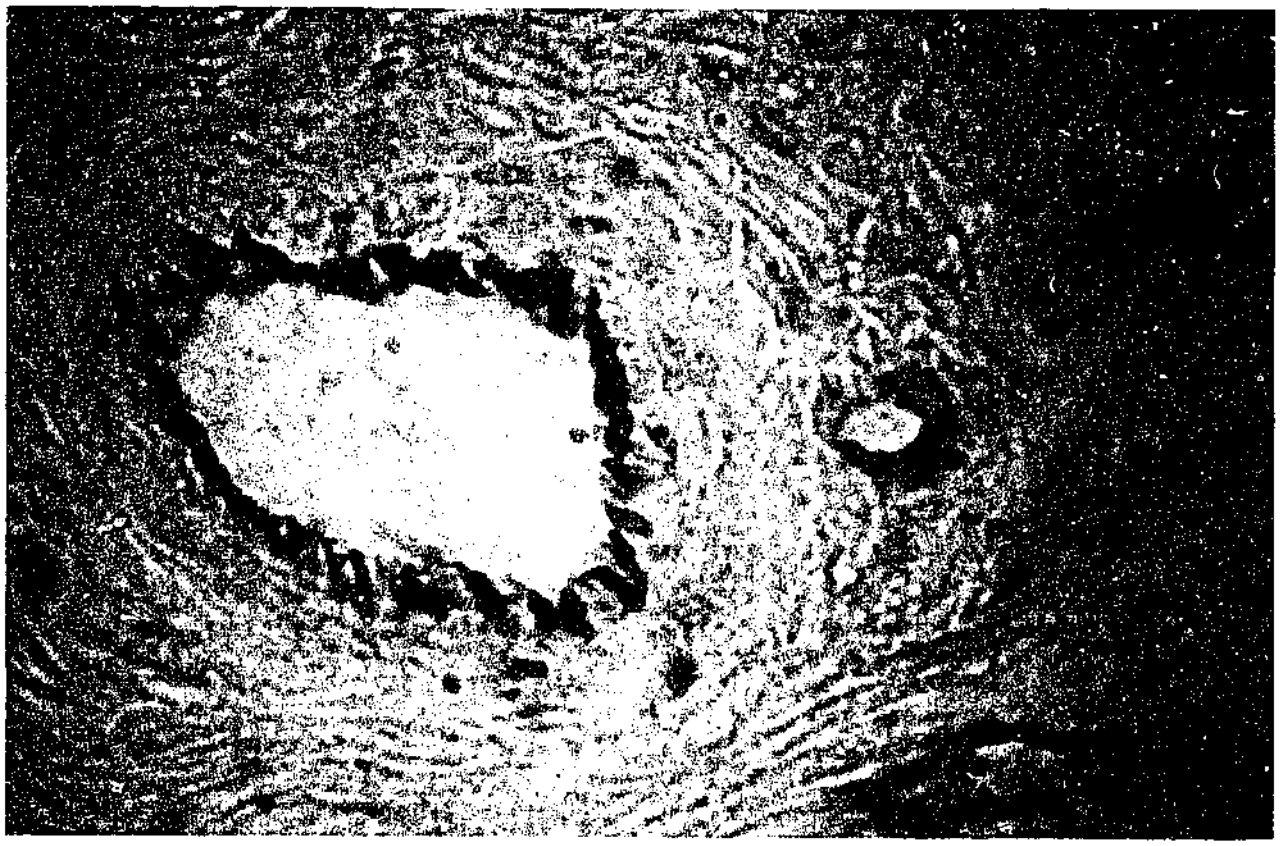


Figure 5.4 CD-31 stained control bladder biopsy showing medium sized blood vessels in deeper submucosa (x1000)

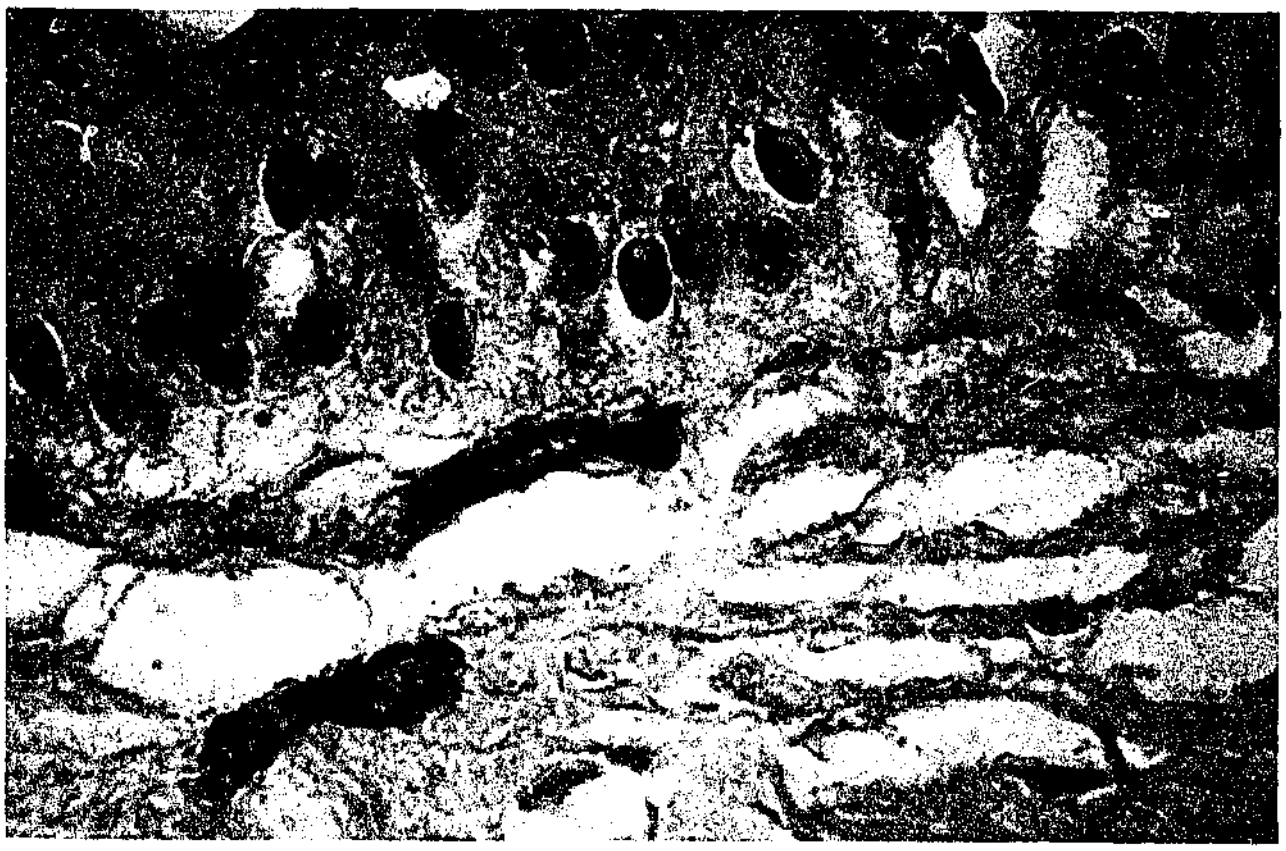


Figure 5.5 PCNA/CD-34stained IC bladder biopsy showing proliferating cells staining red/brown and endothelial cells staining blue (x1000)

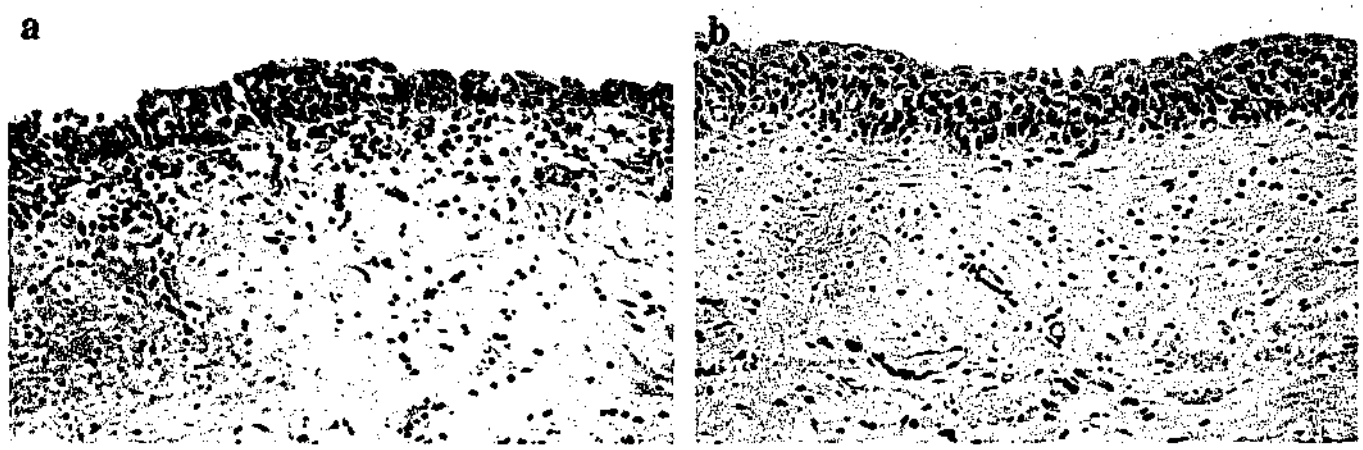


Figure 5.6(a) IC bladder biopsy with composite abnormal histology score of 7
(b) Control bladder biopsy with score of 0. H&E, (x200).

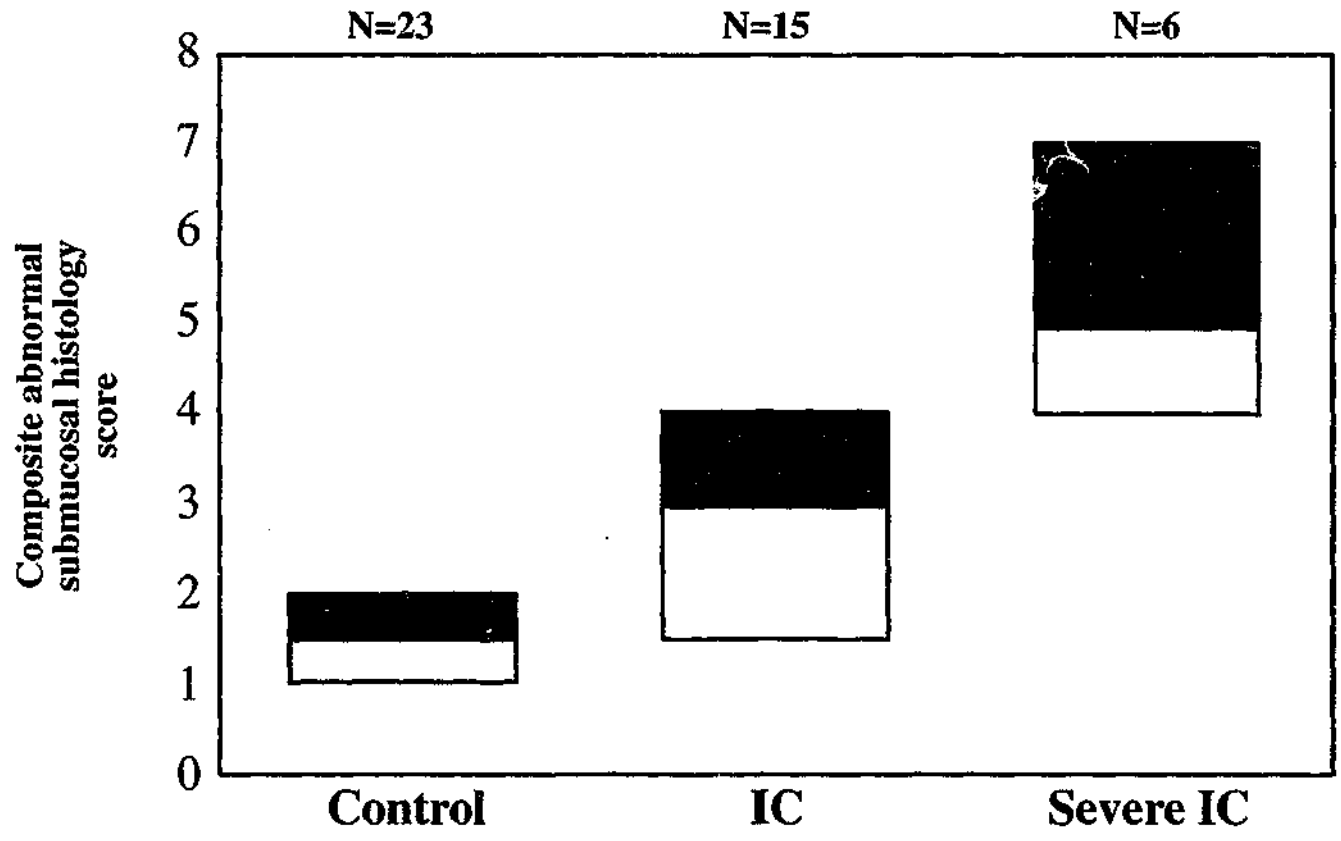


Figure 5.7 The composite abnormal submucosal histology score; grading 0-3 of each of the features; oedema, haemorrhage, inflammatory infiltrate and vascular congestion and ectasia. Data are presented as box plot displaying 25th (lower margin of box), 50th (line within the box) and 75th (upper margin of box) percentiles.

CHAPTER SIX

HISTOLOGICAL AND MICROVASCULAR DENSITY ASSESSMENT OF BLADDER BIOPSIES TAKEN BEFORE AND AFTER HYDRODISTENTION IN WOMEN WITH INTERSTITIAL CYSTITIS AND CONTROL WOMEN

6.1 INTRODUCTION

The value of histological assessment in IC is controversial. In chapter 3 histological changes in bladder biopsies from control and IC subjects after standardised hydrodistention were assessed. In the biopsies of women with IC there was an increased incidence of epithelial denudation, submucosal oedema, congestion/ectasia and inflammatory infiltrate compared with controls. These changes were present in approximately half the IC population whereas histology was normal in the other half.

Prior published studies have demonstrated changes such as denuded epithelium or prominent submucosal inflammation (Lynes et al, 1990), mucosal ulcerations and haemorrhage, granulation tissue and mononuclear infiltrate (Johansson & Fall, 1990) or submucosal oedema and vasodilatation as the main finding (Messing & Stamey, 1978). Lynes and colleagues (1990) state in their discussion that "there is a marked difference in oedema and vascular ectasia between pre- and post hydraulic distention in both control and IC patients" which they assert is due to the distention process. However

evidence for this statement is not described in their study.

In Chapter 5 a reduced suburothelial microvascular density in the IC compared with the control group was demonstrated. One possible explanation for this was that it was an artefactual result of submucosal oedema in the interstitial cystitis group. Submucosal oedema was increased in the IC groups but it was unclear to what extent this was a primary finding or one secondary to hydrodistention. If hydrodistention were to alter subepithelial microvascular density more so in IC than controls, it would be important to assess the microvasculature in the undistended bladder to try to determine whether the previously described reduction was a primary phenomenon or secondary to the distention process.

In this study the aims were to:

1. determine the effect of hydrodistention on normal and IC bladder histology;
2. confirm the histological differences described in Chapter 3 between control and IC subject bladder biopsies;
3. confirm the suburothelial microvascular density assessment as described in Chapter 5 in control and IC subjects;
4. compare the suburothelial microvascular density in pre and post hydrodistention states in IC and control subjects.

6.2 MATERIALS AND METHODS

6.2.1 SUBJECTS

Thirteen women with IC as defined by NIDDK criteria (Gillenwater & Wein, 1988) and 11 control subjects were recruited. Controls were females who were undergoing stress incontinence surgery and had a bladder capacity of 500ml with no evidence of sensory urgency or bladder instability on prior urodynamics assessment. These subjects (both IC and control) were recruited after completion of the clinical database and were a different population to those recruited for the histological and immunohistochemical studies described in Chapters 3 to 5 with the exception of 1 IC subject. This subject had initially been included in the clinical database and the studies described in Chapter 3 and Chapter 5. She had returned requiring a therapeutic hydrodistention and was enrolled in the current pre and post hydrodistention study.

6.2.2 BIOPSY COLLECTION

In each case (IC or control) two cold-cup forceps bladder biopsies were taken. In detail, the cystoscope was gently placed into the bladder under vision, the bladder emptied and the initial biopsy taken with approximately 50ml water instillation. The biopsy was placed into a specimen container with 10% buffered formalin and labelled pre-distention biopsy. The water hydrodistention was performed at a height of 80 to 100 cm water for two minutes to bladder capacity followed by release and inspection. The second biopsy was taken and labelled post-distention biopsy. In 3 of the control subjects the cystoscopy, hydrodistention and biopsy was performed prior to the colposuspension and in the remaining 8, it was performed after the surgery. Cystoscopy is routinely performed following pelvic surgery to exclude urinary tract injury.

6.2.3 HISTOLOGICAL ASSESSMENT

The histological assessment was based on a previous study (described in Chapter 3) comparing the histology of routinely obtained (post hydrodistention) biopsies. The sections were assessed by Dr J Scurry and Dr A Rosamilia in a random and blinded manner; the identity, diagnosis or timing of the biopsy was not known at the time of assessment. The features of epithelial denudation, submucosal oedema, congestion and ectasia, inflammatory infiltrate and haemorrhage were assessed and scored between 0 and 3. The composite abnormal score was derived from the sum of the individual submucosal scores as detailed in Chapter 3.

6.2.4 IMMUNOHISTOCHEMISTRY

Bladder biopsies were processed in 10% buffered formalin and embedded in paraffin wax. Five micron thick sections were cut and the sections dewaxed and rehydrated followed by incubation in 3% hydrogen peroxide in methanol for 10 minutes. The tissue was then incubated with the primary antibody against endothelial cell antigen, anti-CD34 (Clone Qbend 10 Serotec, Oxford, UK) at a dilution of 1 in 50 for 1 hour followed by the addition of biotinylated secondary antibody for 15min and then streptavidin peroxidase for 15min (DAKO kit, DAKO, Denmark). The chromogen, diaminobenzidine (DAB) was added for 5-10 minutes. All steps were followed by washes with phosphate buffered saline and were performed at room temperature. The sections were mounted in Clearmount mounting solution (Zymed, San Francisco, California, USA) or Depex. mounting medium (B.D.H. Laboratory Supplies, Poole, UK). Negative controls were performed by using normal rabbit serum instead of the primary antibody.

6.2.5 BLOOD VESSEL ESTIMATION

Computer assisted image analysis (Analytical Imaging Station version 3.0 rev 1.4, Imaging Research Inc.) was used in addition to manual counting for endothelial cell estimation as described in Chapter 5. Analysis was performed blinded to subject identity, clinical diagnosis and relationship of biopsy to hydrodistention. The suburothelial section was imaged and each image was scanned using a $0.005mm^2$ template. Five computer generated readings of the image were obtained for each pre and post hydrodistention section and this was repeated in a different section to generate a total of ten readings corresponding to a total analysed image area of $0.05mm^2$

6.2.6 STATISTICAL ANALYSIS

Statistical analysis was performed by SPPS version 10.0 using the Mann-Whitney test for non-parametric data for the interstitial cystitis versus control comparisons. The paired comparisons between pre and post scores for each individual were made using the Wilcoxon signed ranks test. Significance was at $p<0.05$.

6.3 RESULTS

The results are tabled in full and shown in Appendix IV. Figure 6.1a is a photomicrograph of an IC subject showing submucosal oedema and haemorrhage. The same subject has had a second biopsy following hydrodistention and Figure 6.1b shows epithelial denudation and greater degree of submucosal oedema, congestion and haemorrhage. Figure 6.1c and d show biopsy histology findings from a control subject before and after hydrodistention, respectively. The cystoscopic capacity under general anaesthesia was decreased ($p<0.01$) in the IC group (Table 6.1).

6.3.1 EPITHELIAL DENUDATION

Epithelial denudation did not differ significantly between control and IC subject groups in both the pre-hydrodistention biopsy (p=0.48) and the post-hydrodistention biopsy (p=0.15). Figure 6.2 suggests some tendency towards more epithelial denudation in the IC group in both pre- and post-hydrodistention biopsies.

6.3.2 SUBMUCOSAL OEDEMA

Submucosal oedema did not differ significantly between control and IC subject_groups in the pre-hydrodistention biopsy (p=0.76) but a trend toward greater oedema in the IC group occurred after hydrodistention (p=0.09) as shown in Figure 6.3.

6.3.3 SUBMUCOSAL CONGESTION AND ECTASIA

Submucosal congestion and ectasia were increased in the control group as compared with the IC group in the pre-hydrodistention biopsy (p=0.03) and as represented in Figure 6.4 was not significantly different in the post-hydrodistention biopsy (p=0.51).

6.3.4 SUBMUCOSAL INFLAMMATORY INFILTRATE

Submucosal inflammatory infiltrate did not differ significantly between control and IC subject groups in both the pre-hydrodistention biopsy (p=0.12) and the post-hydrodistention biopsy (p=0.3) although Figure 6.5 suggests a tendency toward greater inflammatory infitrate in the IC group.

6.3.5 SUBMUCOSAL HAEMORRHAGE

The characteristic of submucosal haemorrhage did not differ between control and IC subject groups in both the pre-hydrodistention biopsy (p=0.78) and the post-hydrodistention biopsy (p=0.85) as shown in Figure 6.6.

6.3.6 COMPOSITE ABNORMAL SUBMUCOSAL SCORE

The total of the submucosal scores for oedema, congestion and ectasia, inflammatory infiltrate and haemorrhage were added to give the composite abnormal submucosal score. This score is represented as a bar graph in Figure 6.7 and did not differ between control and IC subject groups in both the pre-hydrodistention biopsy (p=0.98) and the post-hydrodistention biopsy (p=0.26).

6.3.7 SUBGROUP ANALYSIS OF SEVERE AND EARLY IC

In Chapter 3 a subgroup of severe IC was arbitrarily defined as those with a cystoscopic capacity of 400ml or less. Of the 13 IC subjects in the present study, 5 had severe IC according to this definition. This group was compared with the control group and the remainder of the IC subjects (cystoscopic capacity >400ml). In comparison with the

control group, the severe IC subjects had increased submucosal oedema (p=0.008). They also showed a tendency to increased epithelial denudation (p=0.08), inflammation (p=0.09) and composite abnormal score (p=0.05) which occurred only in the post-hydrodistention state. Inflammatory infiltration was increased in the pre-distention biopsy also (p=0.02).

The only difference between the control subjects and the early IC group was increased congestion pre-distention in the control group (p=0.02) as also described in Section 6.3. There were no differences between the severe IC and the early IC subjects apart from a trend toward increased submucosal oedema in the severe IC group both pre- and post-distention (p=0.06 and p=0.05), respectively.

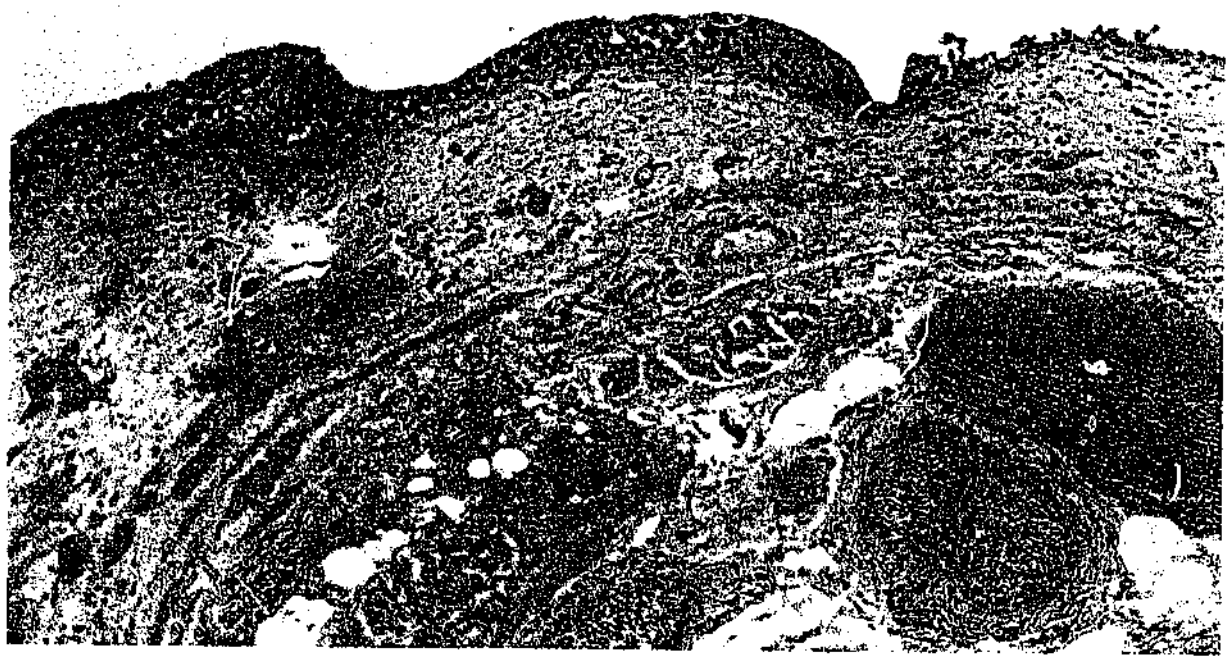
6.3.8 COMPARISON OF THE HISTOLOGICAL CHARACTERISTICS BETWEEN THE PRE- AND POST HYDRODISTENTION BIOPSIES WITHIN THE IC AND CONTROL GROUPS

Within the control group paired comparisons revealed no difference between the pre and post-hydrodistention biopsy scores for any of the histological characteristics described (Table 6.2).

Within the IC group the differences did not reach statistical significance however there was a definite trend toward a worsening of post versus pre–hydrodistention scores for oedema and total abnormal composite score (Table 6.3).

IC Pre

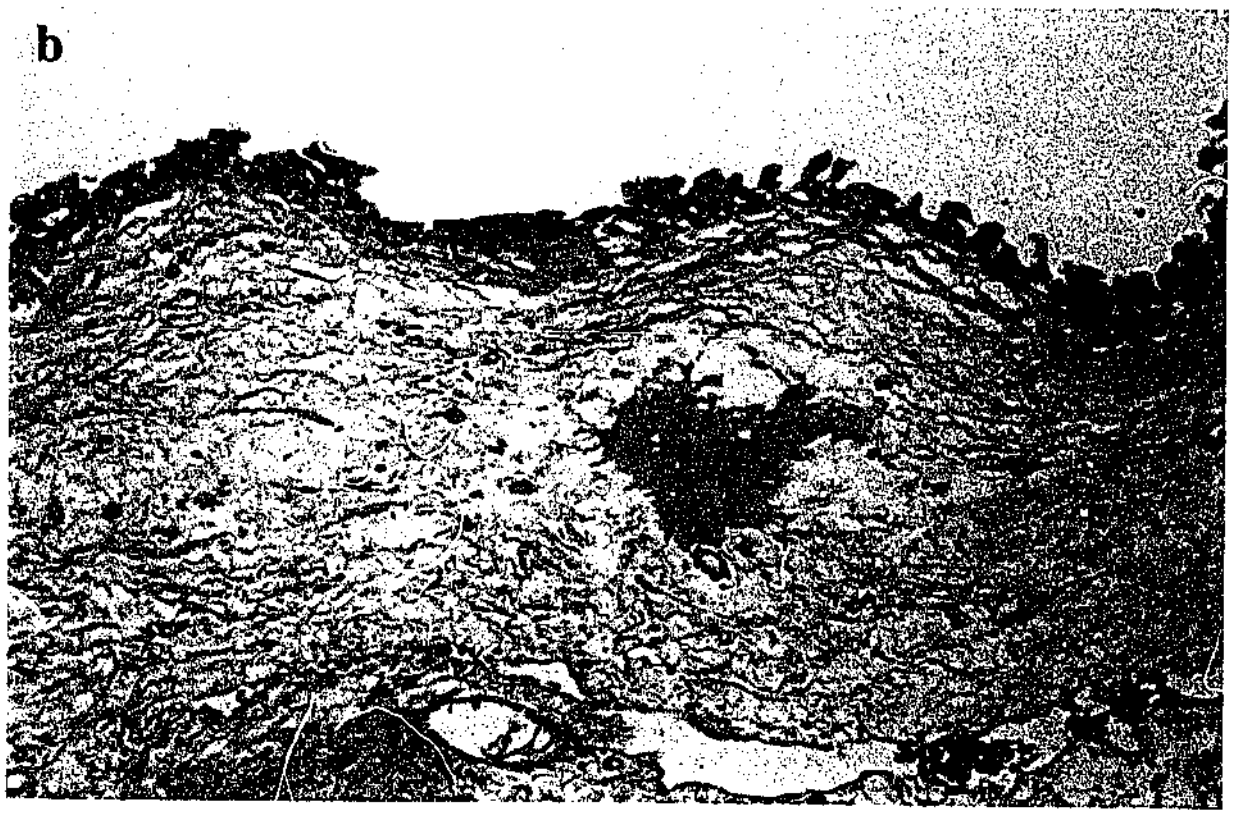
a



x200

IC Post

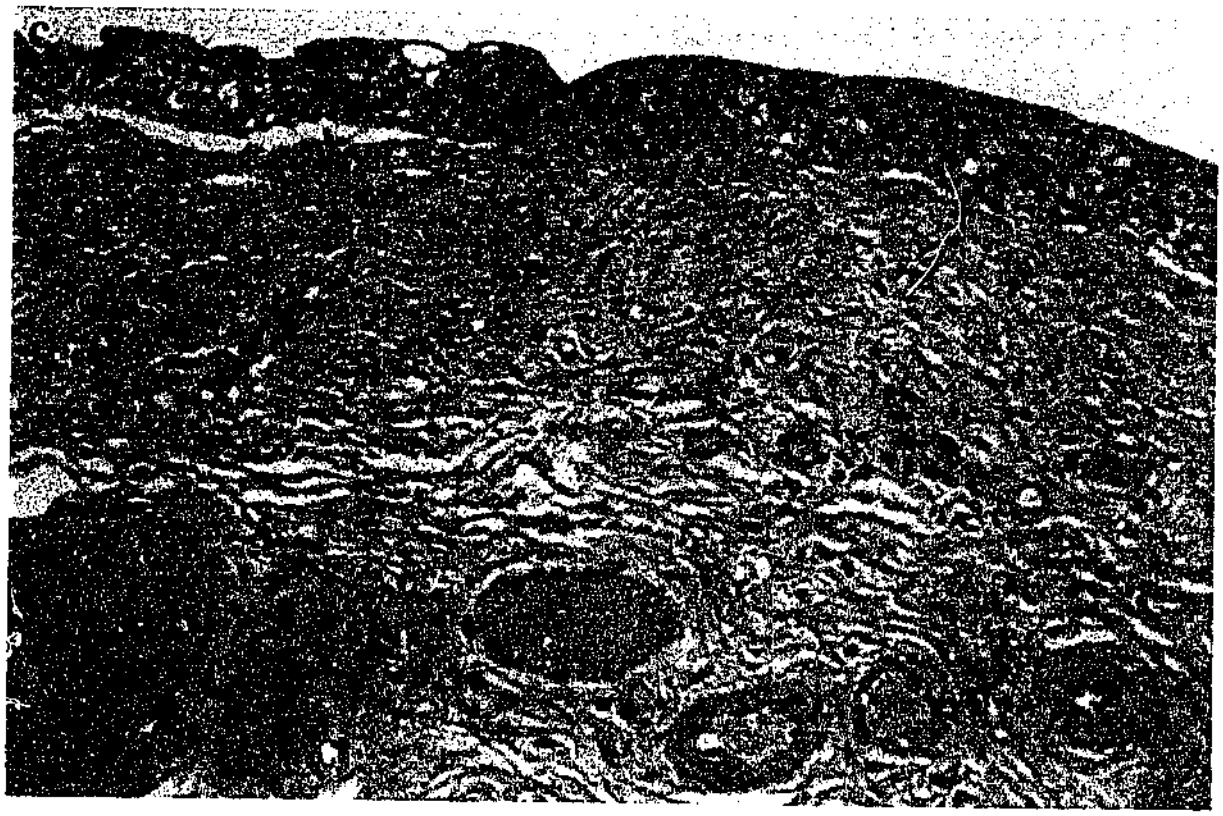
b



x200

Figure 6.1 Photomicrographs of bladder biopsy histology in interstitial cystitis (**a**) pre & (**b**) post hydrodistention. H&E

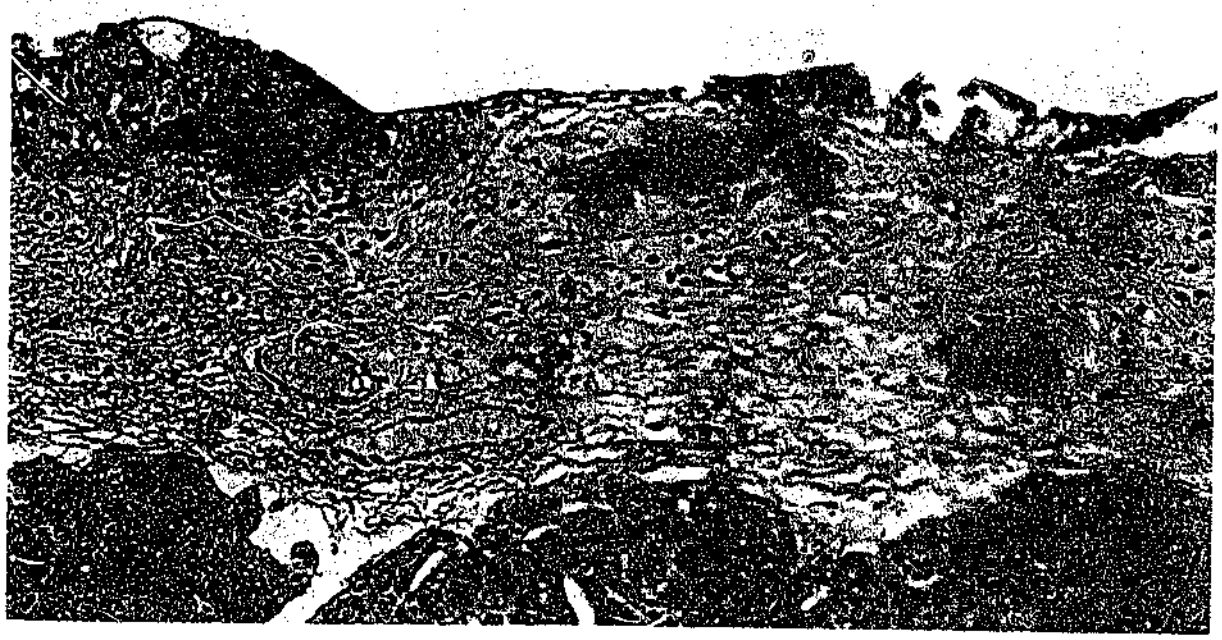
Control Pre



x200

Control Post

d



x200

Figure 6.1 Photomicrographs of bladder biopsy histology in control subject (**c**) pre & (**d**) post hydrodistention. H&E

Fig 6.2 Epithelial denudation in pre and post hydrodistention bladder biopsies from women with IC and controls.

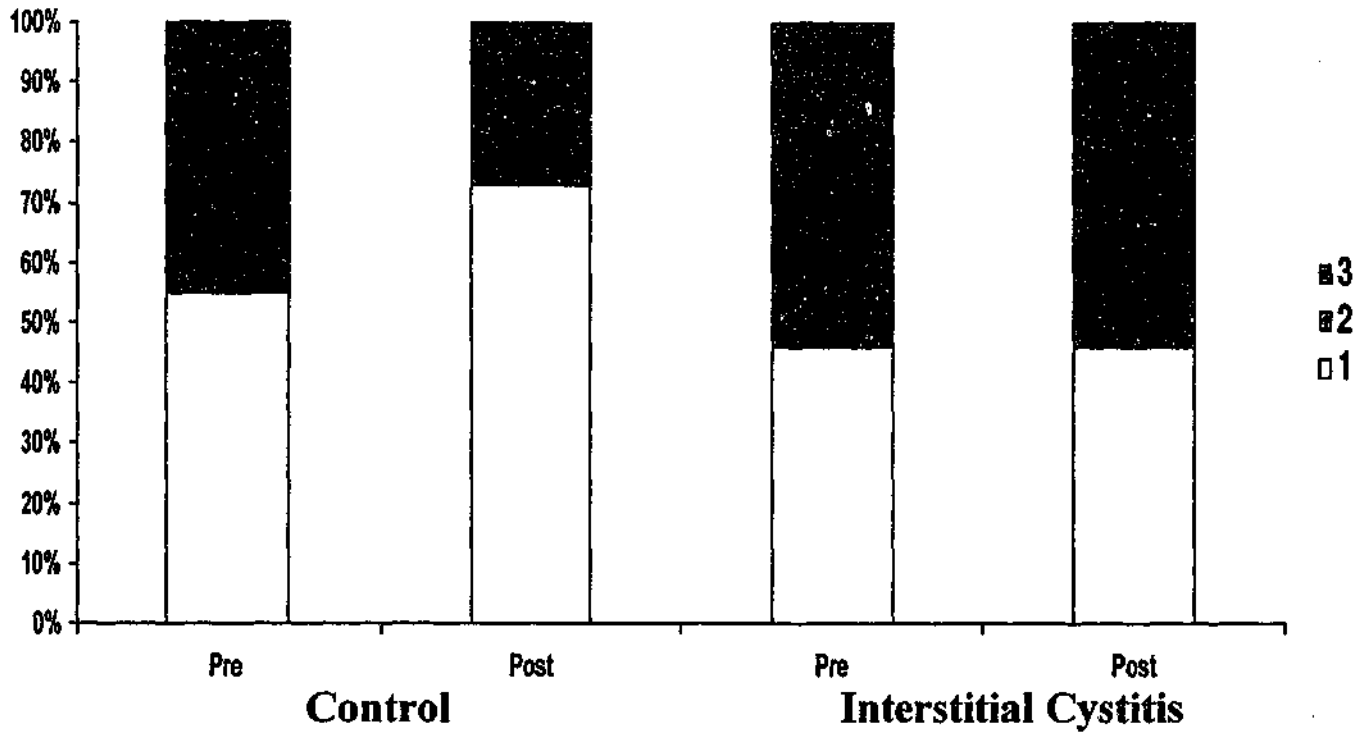


Fig 6.3 Submucosal oedema in pre and post hydrodistention bladder biopsies from women with IC and controls.

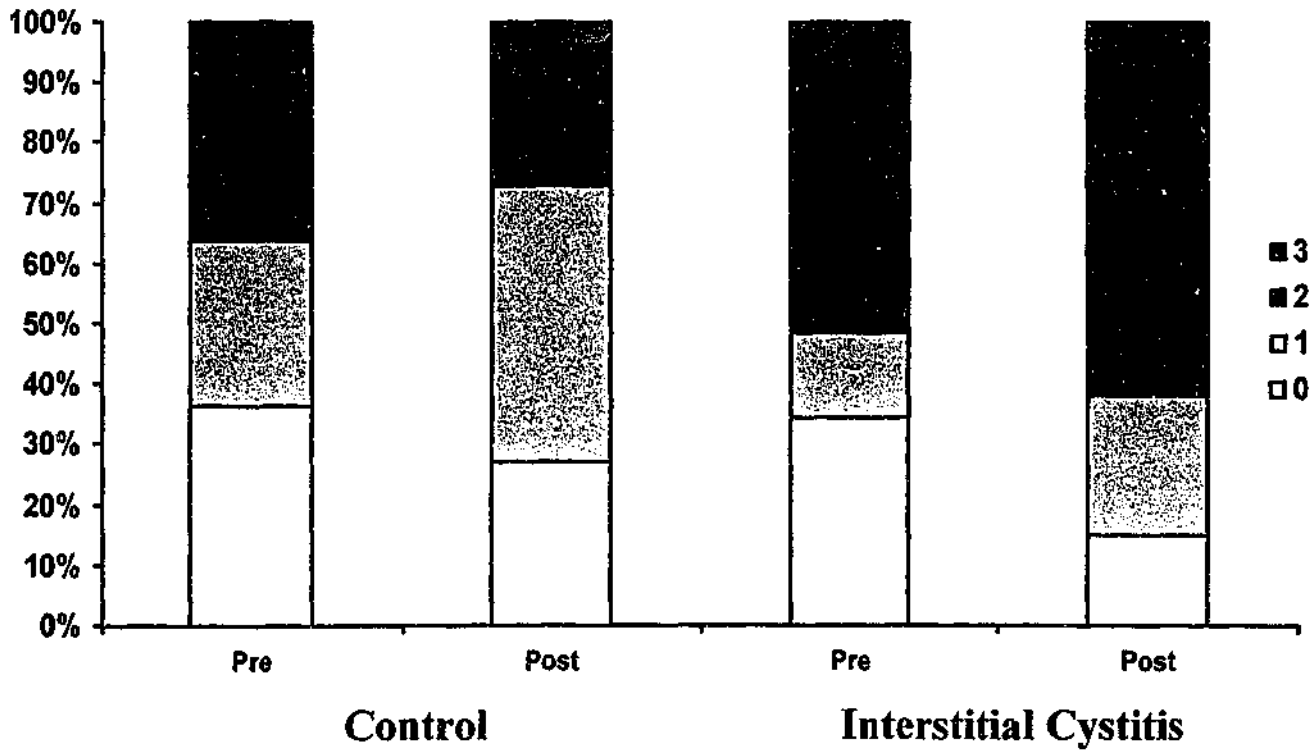


Fig 6.4 Submucosal congestion/ectasia in pre and post hydrodistention bladder biopsies from women with IC and controls.

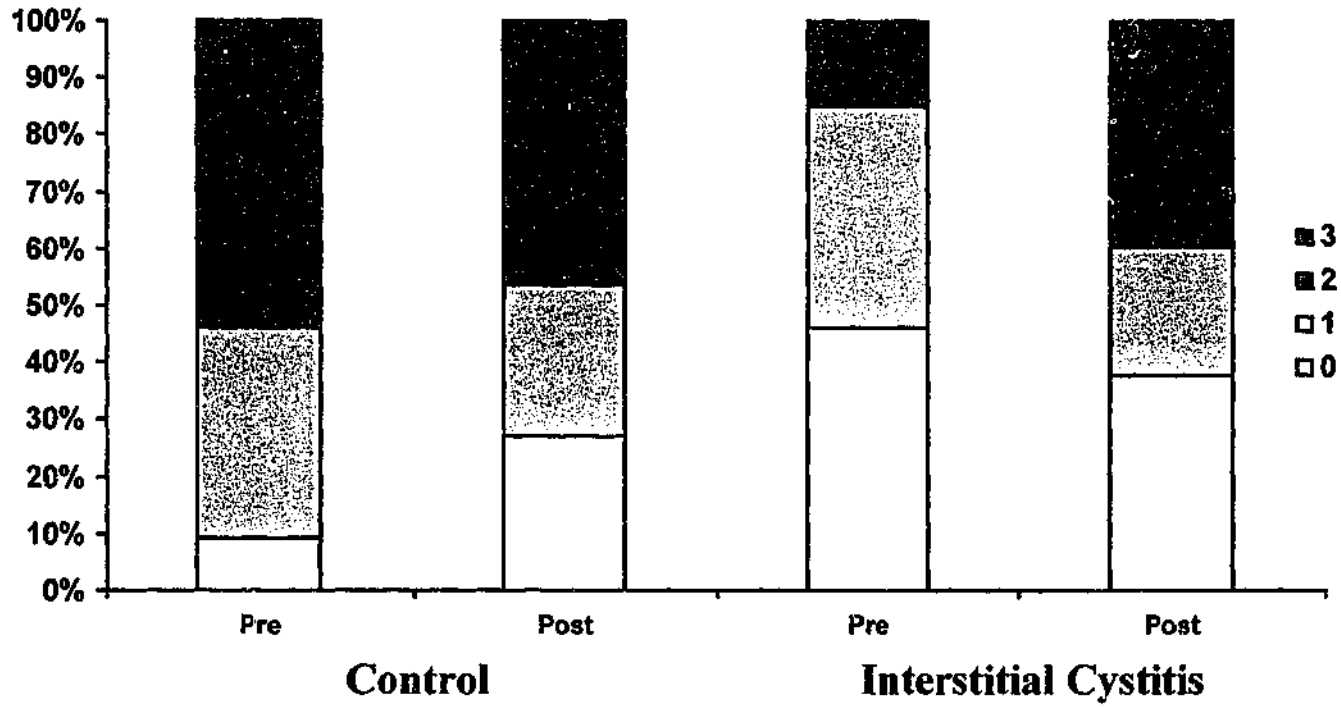


Fig 6.5 Submucosal inflammation in pre and post hydrodistention bladder biopsies from women with IC and controls.

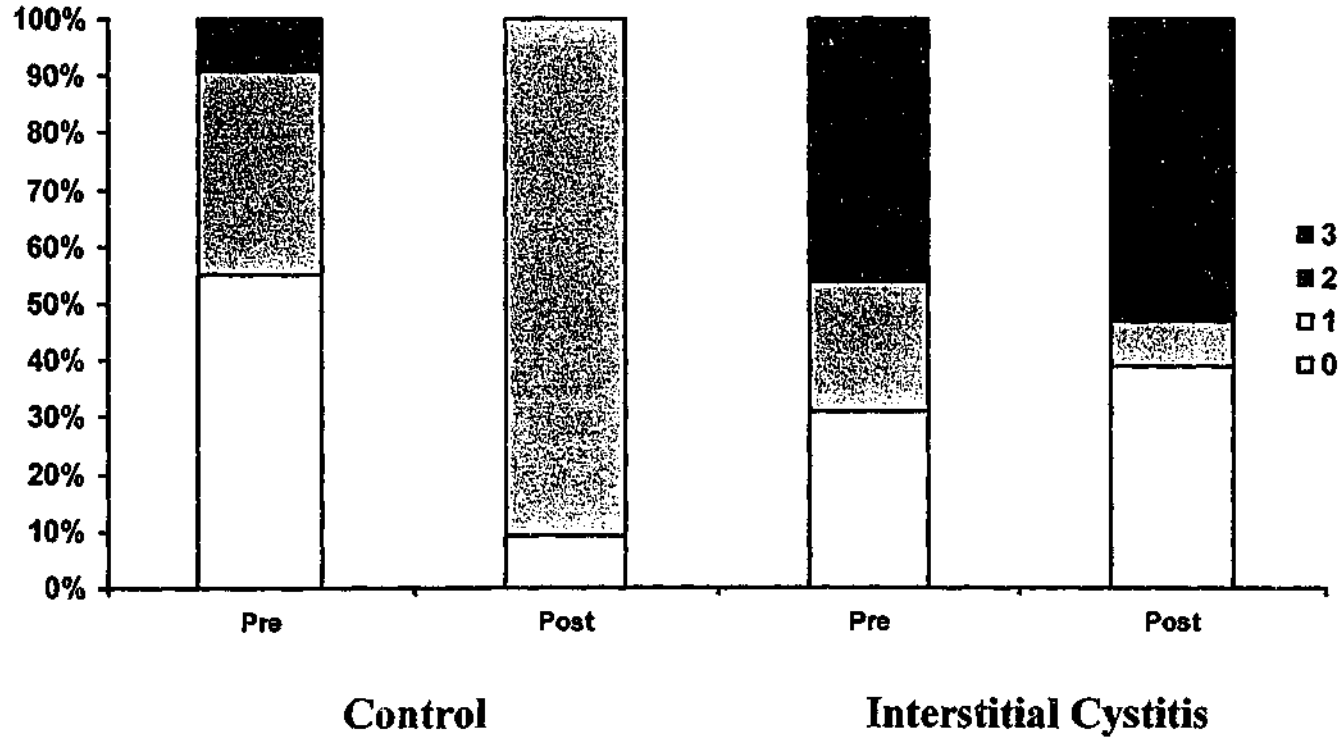


Fig 6.6 Submucosal haemorrhage in pre and post hydrodistention bladder biopsies from women with IC and controls.

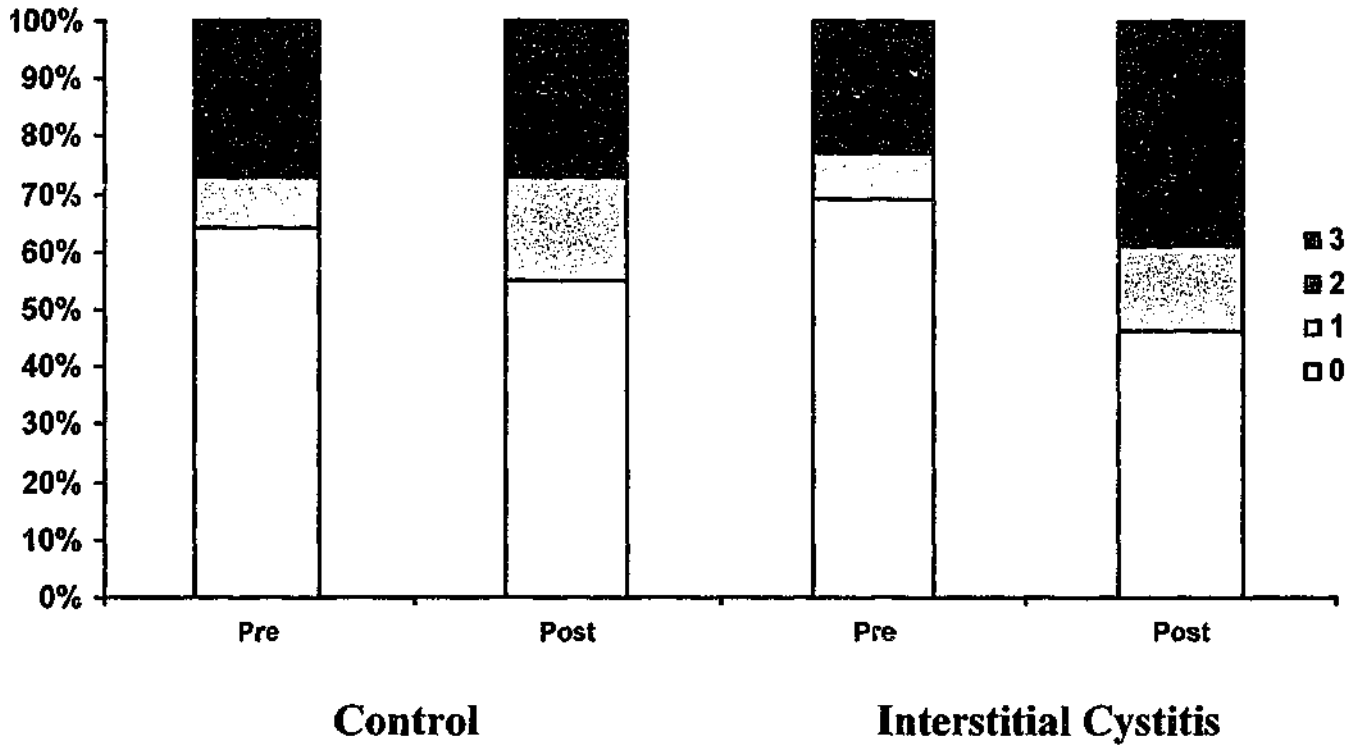
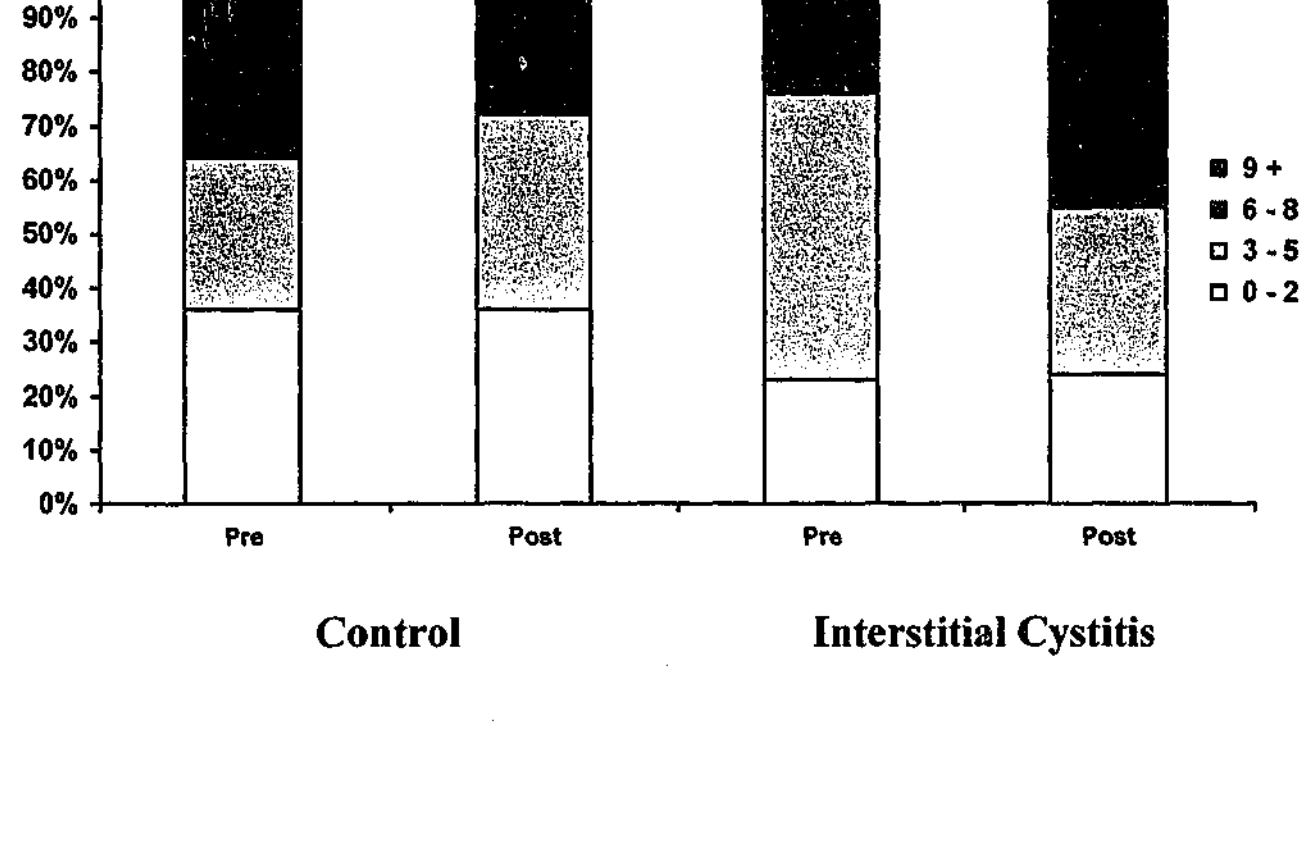


Fig 6.7 Composite abnormal submucosal histology score in pre and post hydrodistention bladder biopsies from women with IC and controls.



6.3.9 MICROVASCULAR DENSITY ESTIMATION

In comparing the pre-hydrodistention biopsies there was a tendency to *increased* microvascular density in the interstitial cystitis group as compared with control subjects (p=0.05). Figures 6.8 a and b are photomicrographs of CD-34 stained suburothelial blood vessels in an early IC subject before and after hydrodistention. Figures 6.8 c and d show CD-34 stained suburothelial blood vessels in a control subject (same subject as Figures 6.1c and d before and after hydrodistention. This trend to higher blood vessel density in the empty bladder was apparent in the early IC group (cystoscopic capacity > 400ml) but not in the severe IC subgroup. There was no significant difference in the suburothelial microvascular density between control and IC groups in the post-hydrodistention biopsies (p=0.12).

A comparison of the pre- and post-hydrodistention biopsies in the control group showed no difference in microvascular density (p=0.4). In the IC group there was a reduction in blood vessel density in the post-hydrodistention compared with the pre-hydrodistention biopsy (p=0.02)

6.4 *DISCUSSION*

This is the first report to the author's knowledge of an histological assessment of bladder biopsies taken before and after hydrodistention. This comparison of histological characteristics between control and IC showed a trend towards increasing epithelial denudation, oedema and inflammatory infiltrate in the post-hydrodistention biopsies which did not reach significance. Submucosal congestion and ectasia and composite abnormal score in the post hydrodistention state did not differ between the control and

subjects had scores of 2 or 3 for congestion/ectasia. This discrepancy may be due to differences in scoring between the two studies since there is an overall increase in scores for congestion in both control and IC groups. It is the control group which has had a disproportionate increase in higher congestion scores and it is behaving as a different control population to that of Chapter 3. There are no other published series of bladder biopsy in the undistended state with which to compare the pre-distention results. It is possible that in the undistended state the blood vessels are congested and hydrodistention in producing a physical stretch effect causes blood vessel emptying. A further explanation is that the surgery in the control subjects preceding the cystoscopy which involved bladder reflection and catheterisation caused increased congestion however this did not occur in the Chapter 3 controls. The IC subjects did not have prior surgery or catheterisation.

The most interesting and novel finding of this study was the difference by which the control and IC bladder histology altered after hydrodistention. The histological findings in biopsies from control subjects were not altered significantly by the hydrodistention process which is contrary to the discussion comments made by Lynes and colleagues (1990). However in the IC subjects the post-distention biopsies showed a strong trend toward increasing oedema and total abnormal composite score. This is further evidence to support the concept of a more permeable epithelium in IC which allows the hydrostatic pressure of a bladder hydrodistention to cause increased submucosal oedema, congestion and haemorrhage. The findings of epithelial denudation and inflammation in the IC group were those less altered by the distention process

To the author's knowledge this is the first study of the suburothelial microvasculature in the normal and IC bladder before and after hydrodistention. The suburothelial

microvascular density study also suffered from a lack of power and on first impression gives dissimilar results to those of Chapter 5.

The post hydrodistention data of the current study was compared with the blood vessel data of Chapter 5 (no pre hydrodistention biopsies were taken in Chapter 5). The control groups had similar suburothelial microvasculature density measured as number of vessels/0.005mm^2 (median: Chapter 5; 3.7 versus Chapter 6; 3.4). However, the IC groups in the two chapters varied (median: Chapter 5; 3.0 versus Chapter 6; 4.0). This difference was largely due to the early IC subgroup (median: 4.4) as compared with the severe IC subjects (median: 3.6).

There are no prior studies with which to compare the pre-distention microvascular results. There was an increased microvascular density in the pre-hydrodistention biopsy in the IC group. As it was present prior to hydrodistention, this finding must be a primary phenomenon. This increase may be due to the associated inflammation seen in some cases of IC although the higher microvascular density occurred in the early IC group where histological analysis suggested that inflammation is not prominent. Prior to hydrodistention the severe IC group had a median density of 3.6 (2.7 to 4.7), the early group a median of 4.4 (range 2.8 to 5.4) and controls a median of 3.5 (range 2.4 to 4.3). Clearly the IC group in Chapter 5 are behaving as a different population in terms of microvascular density to those of Chapter 6.

Given that classic IC tends to occur in older women another possible explanation was that the early IC group were a younger group, and that youth may somehow be associated with greater bladder subepithelial microvascular density. The median age for the severe group was 59 years, for the early group was 44 years and the control group

54 years. However within each group, including the control group there was no correlation between age and microvascular density.

It is clear that in the IC group, hydrodistention has the effect of reducing microvascular density most likely by increasing submucosal oedema. This effect does not occur in control subjects despite undergoing the same standardised cystoscopy, hydrodistention and biopsy process. The hydrodistention effect is the reason why in Chapter 5, the IC group has a significantly decreased bladder subepithelial microvascular density and also the reason for the finding in the current study that, compared with controls, the IC group had an increased microvascular density pre-distention and no difference post-distention.

A criticism of this study is its small sample size and the intention is to extend this study with numbers approximating those of Chapter 3 and 5. Despite the sample size problem, the paired comparison have still provided an insight into the reason for some of the histological and microvascular findings reported earlier in this thesis and in the published literature. All prior reported studies in interstitial cystitis have analysed biopsies taken after hydrodistention. The practical reason for this is that is during and after the hydrodistention where the diagnosis of IC is made and other causes of urinary frequency, urgency and pain are excluded. However it is clear that bladder hydrodistention has an effect on histological findings of submucosal oedema, congestion and possibly haemorrhage and little evidence of effect on inflammation and epithelial denudation in interstitial cystitis. The consequence of these findings, particularly, increased oedema means that blood vessels are spread apart and microvascular density is reduced. This finding was seen in the subepithelial small blood vessels. In Chapter 5, the deeper submucosal blood vessel density was no different to controls, suggesting that the increased oedema although assessed for the whole of the

submucosa did not cause the deeper submucosal vessels to be spread apart. Perhaps this is due to relatively less oedema in the deeper submucosa or as a result of larger or different population of vessels.

This study provides evidence for epithelial dysfunction allowing increased submucosal oedema after hydrodistention causing a reduction in microvascular density in interstitial cystitis which may obscure differences from control subjects. The subepithelial microvasculature is in fact increased in IC prior to hydrodistention.

By demonstrating changes due to hydrodistention in the IC bladder biopsy, this study makes a strong case for reassessing all previously published reports on histological changes in IC (and indeed in other conditions such as detrusor instability). Some previously reported changes, for example neuronal counts may also be affected by the hydrodistention process. Future studies may need to be performed using biopsies taken from the undistended bladder.

Table 6.1 Cystoscopic capacity in IC and control subjects (ml)

	Control	IC
	n=11	n=13
Minimum	600	180
25th centile	750	350
Median	825	410
75th centile	925	556
Maximum	1000	650

Table 6.2 The p value for histological findings in control subjects before and after hydrodistention

Pre versus post	Epithelial denudation	Submucosal oedema	Submucosal congestion/ ectasia	Submucosal inflammation	Submucosal haemorrhage	Total score
p value	0.32	0.61	0.75	0.53	0.4	0.92

Table 6.3 The p value for histological findings in IC subjects before and after hydrodistention

Pre versus post	Epithelial denudation	Submucosal oedema	Submucosal congestion/ ectasia	Submucosal inflammation	Submucosal haemorrhage	Total score
p value	1.00	0.06	0.13	0.41	0.10	0.06

Table 6.4 Mean blood vessel count/0.005 mm^2 in the suburothelium (CD34 stain) of biopsies from IC and control subjects pre and post bladder hydrodistention

Control	Pre	Post distention		IC	Pre	Post distention
1	3.5	2.4		1	4.7	4.4
2	4.1	4.9		2	4.0	3.5
3	4.0	3.1		3	4.5	4.2
4	4.3	3.4		4	4.9	4.2
5	3.5	2.8		5	3.5	2.8
6	3.3	4.0		6	4.1	4.1
7	3.6	3.4		7	5.4	4.0
8	3.4	2.9		8	3.7	3.4
9	2.8	3.8		9	3.6	3.6
10	3.5	3.3		10	2.7	2.9
				11	3.5	3.3
				12	4.1	4.5
				13	5.1	4.6

Table 6.5 Summary of data; Blood vessel count/0.005 mm^2 in the suburothelium (CD34 stain) of biopsies from IC and control subjects pre and post bladder hydrodistention

	Control		Interstitial cystitis	
	Pre distention Bv /0.005mm^2	Post distention Bv /0.005mm^2	Pre distention Bv /0.005mm^2	Post distention Bv/0.005mm^2
Median (range)	3.5 (2.4-4.3)	3.4 (2.4-4.9)	4.1 (2.7-5.4)	4.0 (2.8-4.6)
centiles				
25	3.4	2.9	3.6	3.4
75	4.0	3.9	4.8	4.3

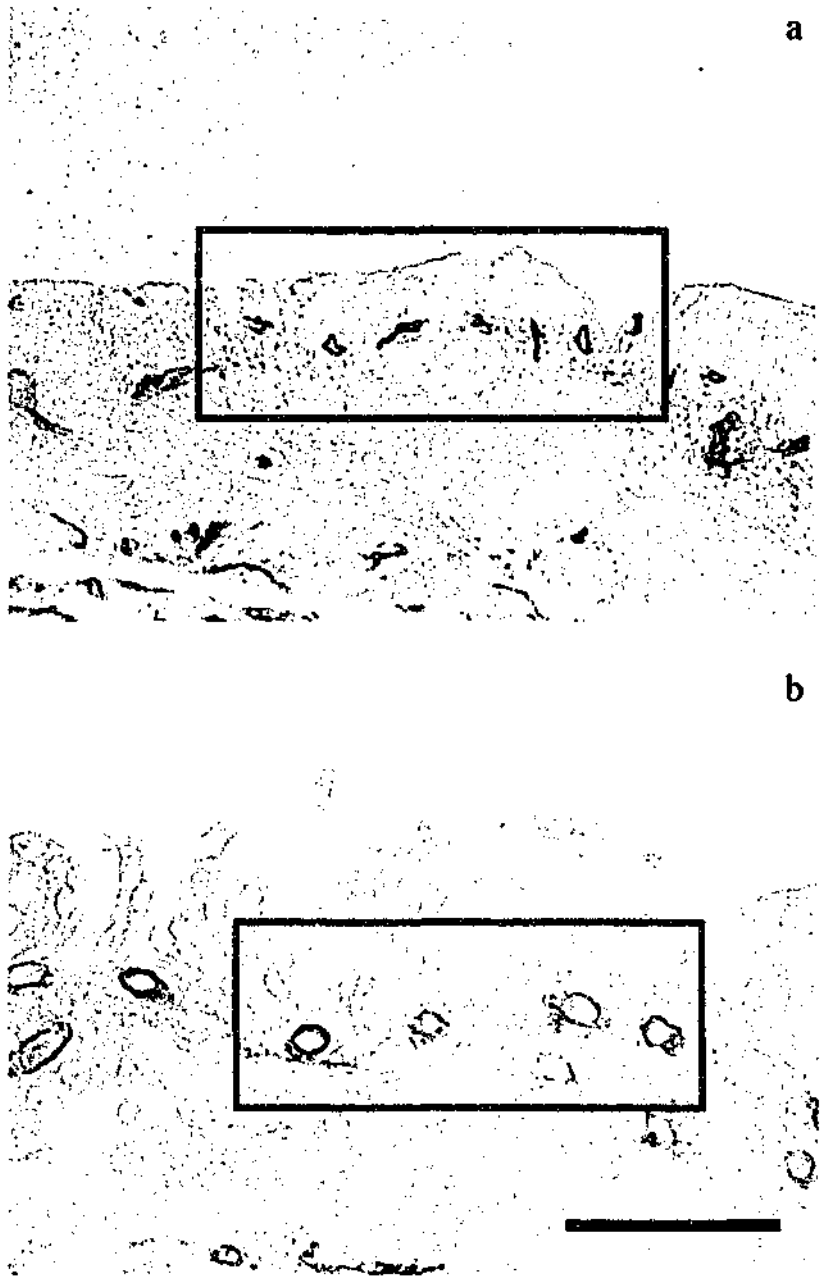


Figure 6.8 CD34 immunostaining of subepithelial blood vessels in bladder biopsies from interstitial cystitis subject

(a) Pre distention biopsy

(b) Post distention biopsy

Scale bar = 50um

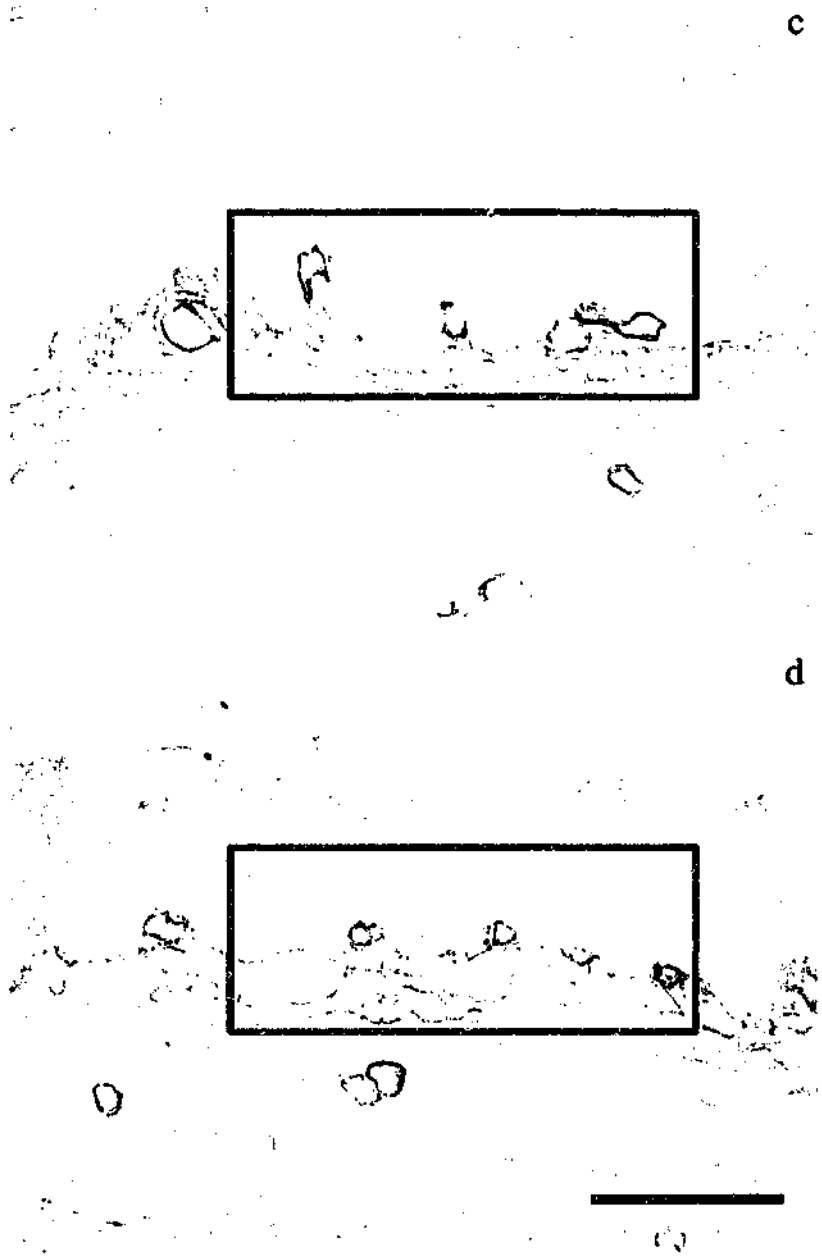


Figure 6.8 CD34 immunostaining of subepithelial blood vessels in bladder biopsies from control subject

(c) Pre distention biopsy

(d) Post distention biopsy

Scale bar = 50um

CHAPTER SEVEN

URINARY EXCRETION OF KALLIKREIN, BRADYKININ AND NEUTRAL ENDOPEPTIDASE

7.1 INTRODUCTION

Interstitial cystitis (IC) is characterised by urinary frequency, urgency and often pelvic or suprapubic pain in addition to the cystoscopic features of mucosal tearing or fissuring at bladder capacity and/or glomerulations (petechial haemorrhages seen at bladder emptying) in the absence of other causes such as radiation, drug-induced or infective cystitis (Hanno, 1994). The underlying cause of interstitial cystitis is unknown however many theories regarding pathogenesis have been proposed. These include a defective or functionally deficient glycosaminoglycan (GAG) layer, infection, auto-immunity, mast cell involvement and most recently the concept of neurogenic inflammation. In a subset of patients with interstitial cystitis, the inflammatory process is clearly involved, as evidenced by histological changes of inflammatory cell infiltrate in the bladder submucosal layer as described in Chapters 3 and 6 and by a number of authors including Johansson and Fall (1990) and Lynes et al (1990).

Kinins are potent mediators of inflammation. Bradykinin, a nonapeptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is generated in plasma and tissues in response to injury and

infection. Its effects include the stimulation and sensitisation of nociceptive afferent neurones, vasodilatation, increased vascular permeability and enhanced fluid secretion from epithelia. There is increased bradykinin in injured tissue and exogenously administered bradykinin has a potent algesic effect (Bhoola et al,1992).

There are two types of kinin receptors, type 1 (B1) and type 2 (B2) receptors. The B2 receptor normally predominates, whereas the B1 receptor is induced following tissue injury (Bhoola et al, 1992) The number of kinin peptides that can act through these receptors is complex.

Current understanding of kinin peptide formation in man is that tissue (glandular) kallikrein forms kallidin [Lys^0-BK-(1-9), KBK-(1-9)] from low molecular weight kininogen (LMWK) whereas plasma kallikrein forms bradykinin [BK-(1-9)] from high molecular weight kininogen known as HMWK (Bhoola et al, 1992). Moreover, a proportion of HMWK is hydroxylated on $proline^3$ (Hyp^3) of the BK-(1-9) sequence (Regoli et al, 1989), and both hydroxylated and non-hydroxylated kinins are derived from HMWK (Kato and Enjyoji, 1992) Hydroxylated kinins have similar biological activity to non-hydroxylated kinins (Kato et al, 1988).

The kininase I metabolites of BK-(1-9) and KBK-(1-9) are bradykinin-(1-8) [BK-(1-8)] and Lys^0-bradykinin-(1-8) [KBK-(1-8)], which are also bioactive, whereas the kininase II metabolites bradykinin-(1-7) [BK-(1-7)] and Lys^0-bradykinin-(1-7) [KBK-(1-7)] are inactive.. Whereas BK-(1-9) and KBK-(1-9) are more potent on B2 receptors, BK-(1-8) and KBK-(1-8) are more potent on B1 receptors (Kato et al, 1988).

The aim of the present study was to investigate the role of the kallikrein-kinin system in interstitial cystitis by measuring levels of kinins, active and total kallikrein, and neutral endopeptidase (NEP) in the urine of IC subjects and control subjects with stress incontinence and normal bladder function. High performance liquid chromatography (HPLC)-based radioimmunoassays (RIA) was utilised to measure hydroxylated and non-hydroxylated BK-(1-9), and KBK-(1-9), and their metabolites. Urine levels of NEP were also measured because this is a major kininase of urine (Ura et al, 1987), cleaving BK-(1-9) and KBK-(1-9) to produce the inactive metabolites BK-(1-7) and KBK-(1-7), respectively.

7.2 MATERIALS AND METHODS

7.2.1 SUBJECTS

The 13 subjects with IC were women diagnosed using NIDDK criteria (Gillenwater and Wein, 1988). Nine subjects had been diagnosed at cystoscopy between 8 months and 6 years previously; one subject two months previously and one subject one month previously. They were either patients who were about to start new therapy or volunteers from the IC support group which met regularly at the Mercy Hospital for Women, Melbourne. A further four women had a history and urinary diary suggestive of IC; two were diagnosed cystoscopically subsequent to the urodynamic assessment and urine collection and the remaining two patients were diagnosed as hypersensitive bladder with no evidence of IC and their urine collections were not included in the study.

The 12 control patients were recruited from women attending the Mercy Hospital for Women, Urogynaecology Unit for management of urinary incontinence. They had a

clinical history, physical examination and urinary diary consistent with pure stress incontinence. To prevent confounders urine collections were performed before urodynamic assessment. Inclusion criteria for the control subjects were no sensory urgency < 350mls, capacity of 500mls and no evidence of detrusor instability. In three further cases, the assessment did not confirm these criteria and the urine collections of those patients were not included in the study. The urine collections of a further control subject were discarded due to the macroscopic appearance of blood in the first specimen possibly due to catheter trauma. None of the women was hypertensive or receiving angiotensin converting enzyme inhibitor therapy.

7.2.2 URINARY DIARY, PAIN SCORE AND URODYNAMIC ASSESSMENT

All subjects were asked to complete a 3-day urinary diary. Mean maximum voided volumes were 614ml (range 350 to 1000) for controls and 265ml (range 200 to 350) for IC subjects. Average voided volumes were 285ml (range 112 to 400) for controls and 114ml (72 to 141) for IC subjects.

A full urodynamic assessment was performed at the time of urine collection in all of the control subjects with stress incontinence. 10 of the 13 interstitial cystitis subjects had a urodynamic assessment however this was not usually performed at the time of urine collection but was derived from the assessment performed at their initial diagnostic work-up. Briefly, each patient voided in private; the urinary flow rate and volume voided were measured. A urethral catheter was inserted to measure the residual volume, followed by the urine collections for the current study. Where the urodynamic assessment followed, subtracted dual channel cystometry at a filling rate of 100ml / min using dual and single sensor 7 French diameter catheters was then performed. Bladder

stability and compliance was noted. Urethral pressure profiles were obtained; stress and provocation were used to assess continence. Pressure-flow studies concluded the assessment.

Urodynamically assessed capacities were 535ml (range 500 to 620) for control subjects and 279ml(range 100 to 334) for IC subjects. IC patients were asked to give a pain score (range 0 to 10) on the day of urine collection (mean score 5, range 1 to 8).

7.2.3 CYSTOSCOPY, SEVERITY SCORE

All 13 interstitial cystitis subjects had undergone at least one cystoscopy between 3 years before and 2 weeks after the study urine collection. Nine of the 12 control subjects went on to have stress incontinence surgery with cystoscopy and hydrodistension to capacity performed for suprapubic catheterisation. Cystoscopy and hydrodistention was performed by filling the bladder by passive gravity at 80 to 100cm water pressure (vertical height above bladder) for 2 or more minutes under general or regional anaesthesia. Initial thorough inspection of the urothelium was followed by measurement of capacity, (insertion of suprapubic catheter under vision in the control subjects) and visualisation with the aid of the camera during bladder emptying. A second inspection was then performed. During emptying and 2nd inspection the 4 quadrants of the bladder were photographed where possible and the degree of severity of the cystoscopic appearance was arbitrarily graded 0 to 4. The scoring system was 0 -no or less than 10 glomerulations per quadrant, 1 (mild) -10 to 20 per quadrant in 3 out of 4 quadrants (2 subjects), 2 (moderate) – greater than 20 glomerulations per quadrant but less than 50% surface involvement, and capacity greater than 500ml (5 subjects), 3- (severe) – greater than 50% surface involvement with glomerulations, generalised cascade bleeding and

capacity greater than 500ml (3 subjects), and 4- fissures, ulcer or linear tearing present on initial inspection with and capacity 500ml or less (3 subjects).

7.2.4 URINE COLLECTION PROTOCOL

On arrival at the clinic, subjects were asked to drink 500-1000mL water (~5-10ml/kg). They were free to void at any time if they desired but after 30 minutes each subject was asked to void to completion. A 7 French urethral catheter was passed to drain residual urine and positioned to ensure a free flow of urine. The catheter was left in position throughout the urine collection protocol. There were 4 collection periods labelled A to D. After a free flow of urine was established, collection A (approximately 10ml) was performed. To assess the contribution of the bladder wall to urine composition, the effect of 2-minute bladder stasis on urine composition was determined. The catheter was clamped for 2 minutes, and urine which had accumulated in the bladder was collected after release of the clamp (collection B). The bladder was instilled with 100mL of water via the catheter; then after 1 minute of distension, the bladder was emptied and free urine flow re-established. The third collection was performed (period C), the catheter clamped again for 2 minutes, and on its release, the final collection performed (period D). Urine flow rate was determined from a timed urine collection at the end of the urine collection protocol. If the urine flow rate was less than 4ml per minute, the urine collections for that subject were abandoned. In addition the samples were discarded (1 subject) if there was any evidence of macroscopic blood in the urine presumably due to catheter trauma.

7.2.5 SAMPLE PREPARATION

For each collection, 1ml. urine for kinin peptide measurement was immediately added to 10ml. 4mol/L guanidine thiocyanate, 1% trifluoroacetic acid (GTC/TFA) in order to inhibit all peptidase activity. Two samples of 5ml. urine were immediately snap frozen in dry ice and stored at -80°C for measurement of kallikrein and neutral endopeptidase levels. A further urine sample was sent for microscopy and culture. None of the control or IC subjects had evidence of urinary tract infection. None of the control subjects but 4 of the IC subjects had urine microscopy showing between 10 and 29 x 10^6/l red blood cells.

7.2.6 EXTRACTION AND HPLC OF KININ AND KALLIDIN PEPTIDES

The HPLC and radioimmunoassay for the kinin peptides and the assay for neutral endopeptidase was performed in the laboratory of Dr DJ Campbell at Saint Vincent's Institute of Medical Research

The GTC/TFA urine samples were stored at 4°C and extracted within 1-2 hours of collection. Samples were initially extracted on Sep-Pak C_{18} cartridges (Waters Chromatography Division, Milford, Mass.). The Sep-Pak cartridges were pretreated sequentially with 3ml. methanol, 10ml. 1% TFA in water, 10ml. methanol:TFA:water (80:1:19, v/v/v), then 10ml. 1% TFA in water. After sample loading, the Sep-Pak cartridge was washed with 10ml. 0.5% sodium chloride:0.5% TFA in water, then 2ml. 1% TFA in water, and eluted with 6ml. methanol:TFA:water (80:1:19). The sample was taken to dryness in a vacuum centrifuge, then resuspended in 2ml. TFA:acetonitrile:water (0.1:2:97.9) in preparation for further purification on a cation exchange Isolute PRS cartridge (International Sorbent Technology Ltd., Mid Glamorgan, UK). The PRS cartridge was pretreated with 2ml acetonitrile, 10ml

TFA:acetonitrile:water (0.1:2:97.9), 5 ml TFA:acetonitrile:water (0.1:2:97.9) containing 1mol/L sodium chloride, and 10ml. TFA:acetonitrile:water (0.1:2:97.9). After sample loading, the PRS cartridge was washed with 5ml. TFA:acetonitrile:water (0.1:2:97.9), then eluted with 5ml. TFA:acetonitrile:water (0.1:2:97.9) containing 1mol/L sodium chloride. A second C_{18} Sep-Pak procedure was required to remove sodium chloride from the sample. The sample was eluted from the PRS cartridge directly onto a C_{18} Sep-Pak cartridge pretreated as described above, then washed with 10ml. 0.1% TFA in water and eluted with 6ml. methanol:TFA:water (80:1:19). The extract was evaporated to dryness in a vacuum centrifuge, then acetylated and treated with piperidine as described by Campbell et al (1995) before HPLC.

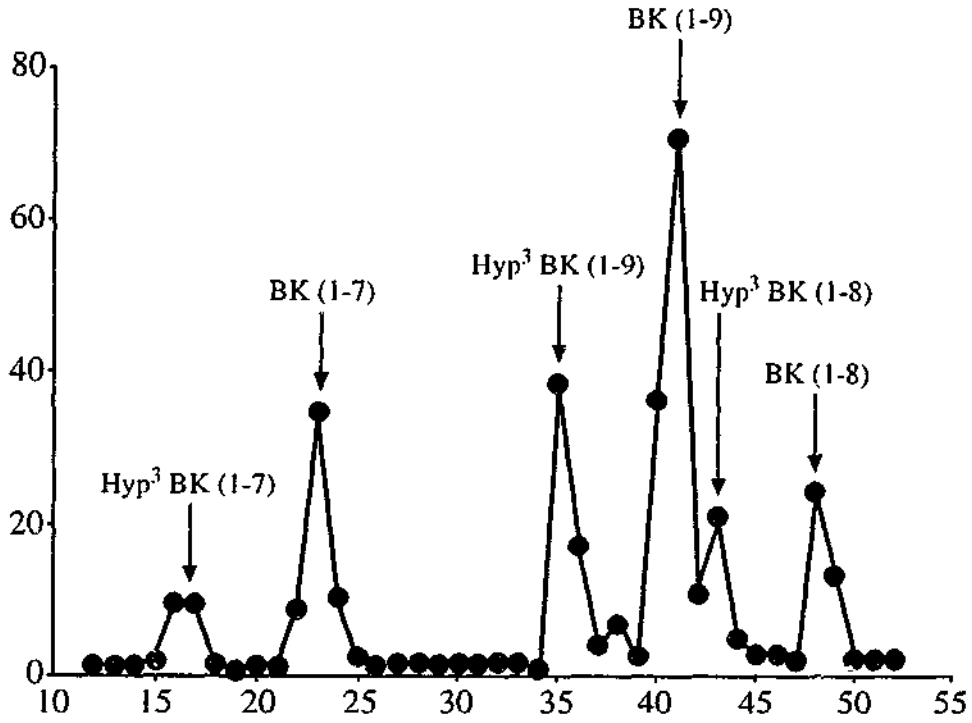
All peptides were separated on a 100 x 4.6mm Brownlee RP-18 Spheri-5 column preceded by a 15 x 3.2mm RP-18 guard column (Applied Biosystems, Inc., Foster City, Calif). Solvent A was 0.1% TFA and 0.15mol/L sodium chloride in water; solvent B was 0.1% TFA and 90% acetonitrile in water. Peptides were eluted with a linearly increasing gradient of 18-38% solvent B over 30min. Figure 7.1. is a line graph show HPLC elution positions of acetylated angiotensin, bradykinin, and kallidin peptides in representative urine extracts. Immunoreactivities were not corrected for peptide recovery, or cross-reactivity with antisera. The flow rate was 1mL/min, and 0.5min fractions were collected into 10 x 75mm borosilicate glass tubes containing 50μL of 5mg/mL protease-free bovine serum albumin (Miles Inc., Diagnostics Division, Kankakee, Ill) in water. HPLC fractions were evaporated to dryness before radioimmunoassay.

7.2.7 PEPTIDE RADIOIMMUNOASSAY

Acetylated kinin peptides were measured with two different antisera. Acetylated bradykinin peptides were measured with antibody B24, an N-terminal directed antibody which enables the measurement of the acetylated forms of bradykinin-(1-7), bradykinin-(1-8), bradykinin-(1-9) as described in Campbell et al (1995) and Campbell et al (1993). Antibody B24 cross-reacted with hydroxylated bradykinin peptides, allowing measurement of both hydroxylated and non-hydroxylated bradykinin peptides. Kallidin peptides were measured with antibody K10, raised in a rabbit immunized with α, ε-acetyl-Lys^0-Hyp^3-Lys^9-BK-(1-9) conjugated via the *C*-terminal lysine residue to bovine thyroglobulin with glutaraldehyde. This is an N-terminal directed antibody which enables the measurement of the acetylated forms of KBK-(1-7), KBK-(1-8), KBK-(1-9), and the corresponding hydroxylated peptides. Tracer for this RIA was ^{125}I-acetyl-Lys^0-Hyp^3-Tyr^8-BK-(1-9). Antibody K10 had similar cross-reactivities for kallidin and Hyp^3-kallidin peptides. The cross-reactivities, recoveries, and minimum detectable amounts are shown in Table 7.1. Data were corrected for antibody cross-reactivity and peptide recovery.

7.2.8 MEASUREMENT OF KALLIKREIN AND NEUTRAL ENDOPEPTIDASE

The kallikrein assay was performed in the Biochemistry Laboratory of Monash Medical Centre under the supervision of Dr James Doery. Urinary kallikrein activity was measured using H-D-Val-Leu-Arg-*p*-nitroaniline (S2266) as substrate and purified porcine pancreatic kallikrein as standard (Spragg, 1988; Zuraw et al, 1994). The reaction was performed with a Cobas-Bio (Roche Diagnostics, Nutley, NJ) microcentrifugal analyser. Nonspecific amidolytic activity was determined by adding aprotinin to each sample. The specific amidolytic activity was determined as the



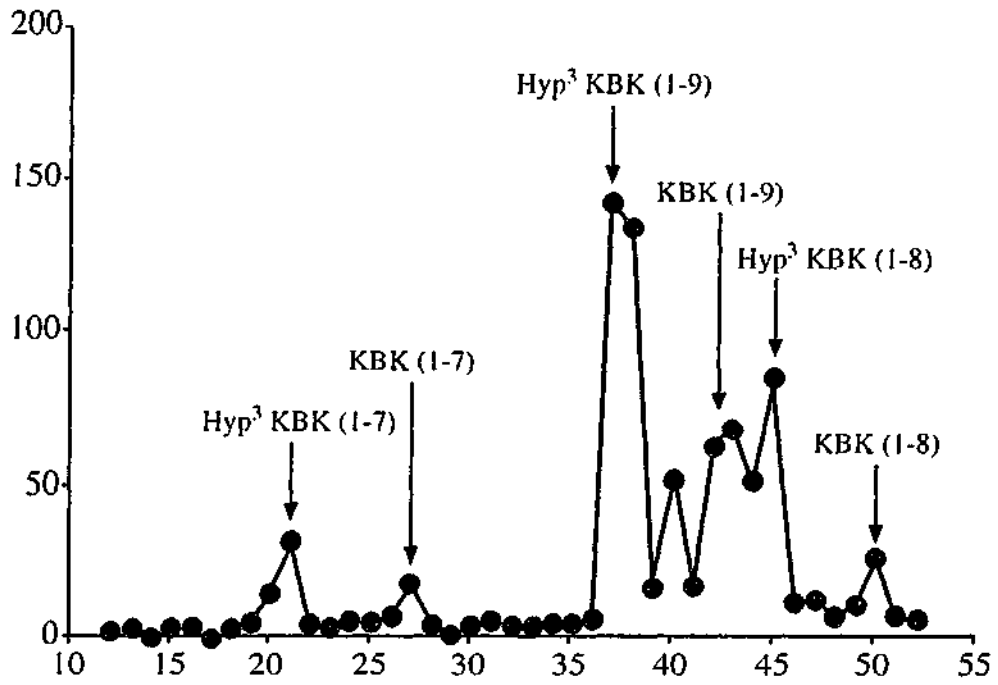


Fig 7.1 High-performance liquid Chromatography showing elution of bradykinin and kallidin peptides

difference in enzyme activity with and without aprotinin. Urinary kallikrein activity was expressed in units/L of standard porcine pancreatic kallikrein, where 1 unit hydrolyses 1μmol of *N*α-benzoyl-L-arginine ethyl ester to N-benzoyl-L-arginine and ethanol per min at pH 8.7 at 25°C. To measure total kallikrein activity, urine was first incubated with trypsin to convert prokallikrein to kallikrein, and the reaction was terminated by the addition of soybean trypsin inhibitor. Neutral endopeptidase enzymatic activity was measured as described by Yandle and colleagues, (1995) with succinyl-Ala-Ala-Phe-amidomethylcoumarin as substrate; further incubation with aminopeptidase M released free amidomethylcoumarin which was measured fluorometrically.

7.2.9 Statistical Methods

Absolute data were analysed using a mixed model with repeated measures on collection period. All data are expressed as mean ± SEM. Comparisons were performed for the individual collection periods with Bonferroni correction for multiple comparisons. Increments in kinin peptide excretion during bladder stasis were analysed by the unpaired t test. Logarithmic transformation of the data was performed when required to obtain similar variances between groups. All tests were two-tailed. Differences were considered significant at $P < 0.05$.

7.3 RESULTS

7.3.1 CHARACTERISTICS OF SUBJECTS

The characteristics of the subjects are summarised in Table 7.2. Control and interstitial cystitis subjects were of similar age, with similar proportions post-menopausal. Of the 13 subjects with interstitial cystitis, 5 had previous treatment with dimethyl sulfoxide, and 3 had prior treatment with chlorpactin. Of the 12 control subjects, 2 were using hormone replacement therapy and 1 was receiving tamoxifen therapy. None of the 13 interstitial cystitis subjects was receiving hormone replacement or tamoxifen therapy.

In comparison with control subjects, interstitial cystitis subjects had smaller self reported maximal and average voided volumes, and increased frequency during the day and night. Interstitial cystitis subjects had reduced urgency volume and capacity as determined by urodynamics, although the pressure rise was not statistically significantly different from that of control subjects (Table 7.2).

7.3.2 URINE FLOW RATES AND CREATININE CONCENTRATION

Urine flow rates were similar for interstitial cystitis and control subjects (Table 7.2). However, control subjects had higher urine creatinine concentrations during the initial collection period, and urinary creatinine concentrations were similar for the two groups during later collection periods (Table 7.3).

7.3.3 URINARY EXCRETION OF KININ PEPTIDES

HPLC-based RIA enabled the measurement of 6 bradykinin peptides and 6 kallidin peptides (Figure 7.1). The sum of the urinary excretion rates of non-hydroxylated and hydroxylated bradykinin peptides showed no difference between interstitial cystitis and control subjects, and a similar result was obtained for the sum of the excretion rates of the kallidin peptides. However, analysis of the excretion rates of the individual kinin peptides showed increased urinary excretion rates of BK-(1-8) and Hyp^3-BK-(1-8) in interstitial cystitis subjects for both collections B and D (Table 7.3).

Increments in urinary kinin excretion rates during bladder stasis were calculated for both periods of stasis, before and after bladder distension with 100mL water. The initial stasis period (difference between periods A and B) was not associated with any increase in increment in kinin excretion rate in interstitial cystitis subjects, in comparison with control subjects. However, interstitial cystitis subjects showed higher increments in bradykinin excretion rates during the second stasis period (difference between periods C and D), indicating a contribution of the bladder wall to urine kinin peptide levels. Summation of non-hydroxylated and hydroxylated bradykinin peptides (1-7), (1-8), and (1-9) showed a higher increment in interstitial cystitis than in control subjects (657 ± 148 fmol/min, mean ± SEM, vs 121 ± 157, $P < 0.02$). Moreover, analysis of the (1-7), (1-8), and (1-9) peptides separately showed greater increments for all peptides (Figure 7.2). The difference in increment was statistically significant for the (1-8) peptides ($P = 0.01$), and of borderline statistical significance for the (1-7) ($P = 0.06$), and (1-9) ($P = 0.09$) peptides (Figure. 7.2). By contrast, there were no differences between interstitial cystitis and control subjects in either the increment in total urinary kallidin peptide excretion or the increment in urinary excretion of individual kallidin peptides (Figure 7.3).

7.3.4 URINARY EXCRETION OF ACTIVE KALLIKREIN, TOTAL KALLIKREIN, AND NEUTRAL ENDOPEPTIDASE

There were no differences between interstitial cystitis and control subjects in urinary excretion rates of total kallikrein (Figure 7.4). However, interstitial cystitis subjects tended to have higher excretion rates of active kallikrein, the increase being statistically significant for collection period D. By contrast, interstitial cystitis subjects had lower urinary neutral endopeptidase excretion rates than control subjects in collection period A, although neutral endopeptidase excretion rates were similar for interstitial cystitis and control subjects during later collection periods (Figure 7.4).

7.4. DISCUSSION

This is the first report of urinary kinin excretion in subjects with interstitial cystitis. Kinins are potent inflammatory peptides and, therefore, are potential mediators of the inflammation of interstitial cystitis. Given that plasma and glandular kallikrein may contribute differently to kinin generation in the bladder wall, it was important to measure bradykinin and kallidin peptides separately in the present study. The finding of increased increment in the bradykinin peptide excretion during bladder stasis suggests that there is increased bradykinin formation and/or decreased bradykinin degradation in the bladder wall of subjects with interstitial cystitis, which may affect the pathogenesis and symptomatology of this condition.

The reduced neutral endopeptidase excretion rate in the IC subjects during the initial collection period suggests decreased kinin degradation in the urine or the bladder wall.

BK-(1-8) and Hyp^3-BK-(1-8) peptides are B1 receptor agonists. Expression of the B1 receptor is often associated with tissue injury (Bhoola et al, 1992).

Urinary kallikrein, an enzyme produced in the kidney, is glandular kallikrein, which produces kallidin peptides from low and high molecular weight kininogen, thus accounting for the predominance of kallidin peptides in urine. The much higher renal excretion of kallidin peptides made it unlikely that a contribution of the bladder wall to urinary kallidin peptide levels could have been detected. Moreover, several factors contribute to variability in urinary kallikrein levels, including potassium excretion and family history of essential hypertension (Clements 1994; Hunt et al, 1993; Berry et al, 1988). None of the subjects in our study had a history of hypertension. The failure to detect increased kallidin peptide excretion does not exclude a role for kallidin peptides in interstitial cystitis. Bradykinin peptides may be produced either directly by cleavage of high molecular weight kininogen by plasma kallikrein or indirectly by aminopeptidase cleavage of kallidin peptides. In the present study the mechanism of the increase in bradykinin peptide levels was not able to be determined, and either or both increased plasma kallikrein or aminopeptidase activity may have been the cause.

Additionally, reduced urinary neutral endopeptidase levels in IC may have contributed to the higher increment in bradykinin peptide levels during bladder stasis. The increased increment in urine bradykinin peptide levels in IC was only seen after bladder distention with 100ml of water. Bladder distention may have activated the kallikrein kinin system in the bladder wall. Moreover this small volume of distention may have flushed out the bladder, removing any artefact due to previous water intake, frequency of micturition, and completeness of bladder emptying. The data illustrate the difficulty associated with study of bladder pathology by analysis of urine composition.

Zuraw and colleagues (1994) reported increased urinary excretion of active glandular kallikrein in subjects with interstitial cystitis measured on random voided urine samples. However, the significance of this finding is uncertain given that the kidney is the main source of urinary kallikrein, and neither kallikrein nor kallidin peptide excretion was increased in the IC subjects of the current study.

The control subjects were similar in age and menopausal status to the interstitial cystitis subjects, and all controls underwnt a full urodynamic assessment. Of the control subjects 7 maintained a urinary diary, and 9 underwent cystoscopy and stress incontinence surgery after this study. The control subjects with urinary stress incontinence were not asymptomatic women but did upon questioning they did not have bladder pain or frequency; and their urinary diaries and urodynamic assessments showed uncomplicated stress incontinence. These women demonstrated an awake bladder capacity of at least 500ml with no detrusor instability or hypersensitivity and thus, they were an appropriate control group to compare with women with interstitial cystitis.

The current study provides direct evidence of activation of the kallikrein kinin system in interstitial cystitis by kinin peptides and their metabolites. This activation of the kallikrein kinin system may have a primary role in the pathogenesis of interstitial cystitis or may be a consequence of plasma transudation through inflamed tissue. Increased local kinin levels have the potential to contribute to the pathology and symptomatology of interstitial cystitis, and the findings suggest a potential therapeutic role for kallikrein inhibitors and kinin receptor antagonists.

In conclusion, an increased increment was found in urinary bradykinin but not kallidin peptide excretion after 2 minutes of bladder stasis following bladder distention associated with a reduced neutral endopeptidase excretion rate in subjects with interstitial cystitis. These data provide evidence of increased kinin formation and/or reduced kinin degradation in the bladder wall of subjects with interstitial cystitis, which may affect the pathogenesis of this condition, possibly via B1 receptor activation.

Table 7.1. Cross-reactivities, recoveries and minimum detectable amounts for RIA of kinin peptides in HPLC fractions.

Peptide	Cross-reactivity (fmol/mL)	Recovery (%)	Minimum detectable (%)
Bradykinin peptides			
BK-(1-7)	79	85 ± 8	1
BK-(1-8)	94	63 ± 8	1
BK-(1-9)	100	32 ± 8	2
Hyp^3-BK-(1-7)	22	64 ± 10	4
Hyp^3-BK-(1-8)	31	53 ± 14	3
Hyp^3-BK-(1-9)	20.5	58 ± 14	4
Kallidin peptides			
KBK-(1-7)	82	61 ± 11	2
KBK-(1-8)	88	42 ± 10	3
KBK-(1-9)	149	23 ± 6	3
Hyp^3-KBK-(1-7)	95	60 ± 7	2
Hyp^3-KBK-(1-8)	91	63 ± 9	2
Hyp^3-KBK-(1-9)	85.5	39 ± 8	3

Minimum detectable amounts refer to measurement of bradykinin peptides using antibody B24, and kallidin peptides using antibody K10, in 1 ml urine. For recoveries and endogenous levels, data shown as means ± SD, n=6-7. Recoveries were determined by adding standard peptides to 1 mL urine, and represent recoveries through the whole assay procedure including Sep-Pak extraction, ether extraction, acetylation, piperidine treatment, and HPLC.

Table 7.2. Characteristics of control subjects and subjects with interstitial cystitis.

Parameter	Subjects		P
	Control	Interstitial cystitis	
n	12	13	
Age	50 (35-71)	54 (21-80)	NS
Duration of symptoms (years)		5 (1-7)	
Time since diagnosis (years)		2 (0-7)	
Pain score (scale of 0-10)		5 (1-8)	
Number postmenopausal:Yes	5	8	
No	7	5	
Self reported: Diary (n = 7)	(n = 13)		
Maximum voided volume (mL)	633 (350-1000)	265 (200-350)	0.002
Average voided volume (mL)	285 (112-400)	114 (72-141)	0.007
Frequency per day	6 (4-8)	11 (6-15)	0.003
Frequency per night	1 (0-2)	3 (0-6)	0.046
Frequency per 24 hours	7 (6-9)	13 (8-19)	0.002
Urodynamics:	(n =12)	(n = 10)	
Urgency volume (mL)	444 (286-507)	204 (30-336)	0.002
Capacity (mL)	535 (500-620)	279 (100-394)	0.001
Pressure rise (cm H2O)	3.6(0-9)	9.8(1-20)	NS
Residual volume(ml)	10(0-30)	21(0-80)	
Flow rate at capacity(ml/sec)25(12-50)	23(0-40)		
Cystoscopy	n=8	(n = 13)	
Capacity	740(500-1000)	550 (350-900)	
Macroscopic disease score	0	2.5 (1-4)	
Urine flow rate	11 (5-21)	11 (4-17)	NS

Data shown as mean ± range. NS, not statistically significant.

Table 7.3. Urine creatinine levels and kinin excretion rates in control subjects and subjects with interstitial cystitis

Peptide	Collection period Subject			
	A	B	C	D
Creatinine (mmol/L)				
Control	1.4 ± 0.4	1.4 ± 0.3	1.0 ± 0.1	0.9 ± 0.1
IC	0.8 ± 0.1*	1.0 ± 0.2	0.9 ± 0.1	1.0 ± 0.1
BK-(1-7) excretion rate (fmol/min)				
Control	135 ± 38	126 ± 32	81 ± 9	108 ± 16
IC	356 ± 192	218 ± 67	141 ± 47	213 ± 62
Hyp^3-BK-(1-7) excretion rate (fmol/min)				
Control	217 ± 53	157 ± 38	253 ± 48	201 ± 31
IC	462 ± 212	231 ± 50	150 ± 35	178 ± 38
BK-(1-8) excretion rate (fmol/min)				
Control	62 ± 27	43 ± 11	28 ± 5	38 ± 6
IC	85 ± 27	108 ± 41	51 ± 26	98 ± 33*
Hyp^3-BK-(1-8) excretion rate (fmol/min)				
Control	216 ± 96	152 ± 56	137 ± 25	149 ± 34
IC	520 ± 223	416 ± 123*	288 ± 145	421 ± 154*
BK-(1-9) excretion rate (fmol/min)				
Control	439 ± 164	359 ± 91	216 ± 29	291 ± 54
IC	1138 ± 503	863 ± 339	591 ± 269	797 ± 282
Hyp^3-BK-(1-9) excretion rate (fmol/min)				
Control	593 ± 222	473 ± 130	366 ± 60	415 ± 85
IC	1643 ± 884	781 ± 243	581 ± 225	752 ± 24
KBK-(1-7) excretion rate (fmol/min)				
Control	196 ± 98	198 ± 127	136 ± 31	123 ± 32
IC	439 ± 139	244 ± 77	279 ± 96	252 ± 89
Hyp^3-KBK-(1-7) excretion rate (fmol/min)				
Control	276 ± 96	281 ± 118	194 ± 41	174 ± 35
IC	623 ± 208	304 ± 93	307 ± 94	241 ± 59
KBK-(1-8) excretion rate (fmol/min)				
Control	816 ± 283	744 ± 122	476 ± 88	644 ± 116
IC	659 ± 163	724 ± 178	536 ± 208	717 ± 188
Hyp^3-KBK-(1-8) excretion rate (fmol/min)				
Control	1868 ± 762	1714 ± 459	991 ± 102	1144 ± 158
IC	1668 ± 646	1734 ± 440	952 ± 218	1328 ± 287
KBK-(1-9) excretion rate (fmol/min)				
Control	4187 ± 1960	3657 ± 1334	2025 ± 309	2405 ± 347
IC	4505 ± 1935	3886 ± 1091	3478 ± 1622	4177 ± 147
Hyp^3-KBK-(1-9) excretion rate (fmol/min)				
Control	6930 ± 2496	6307 ± 1895	4348 ± 506	4482 ± 584
IC	8550 ± 3682	7375 ± 1743	4936 ± 1442	6105 ± 141

Data shown as mean ± SEM, n = 10-13. *, $P < 0.05$, in comparison with controls

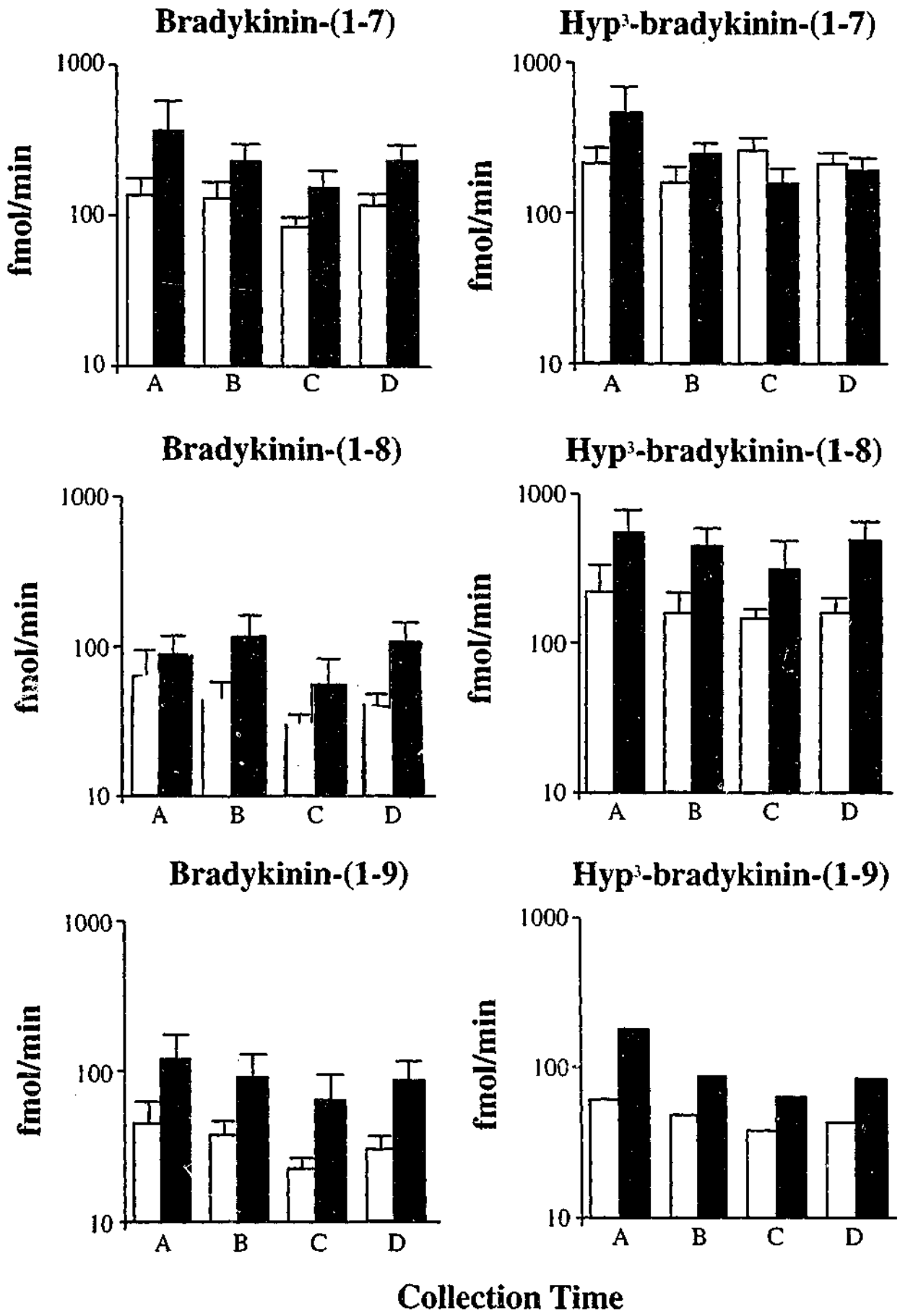


Fig 7.2 Urinary bradykinin peptide excretion rate (mean ± SEM) during 4 collection periods A to D for 10 control subjects (open bars) and 13 subjects with interstitial cystitis (closed bars).

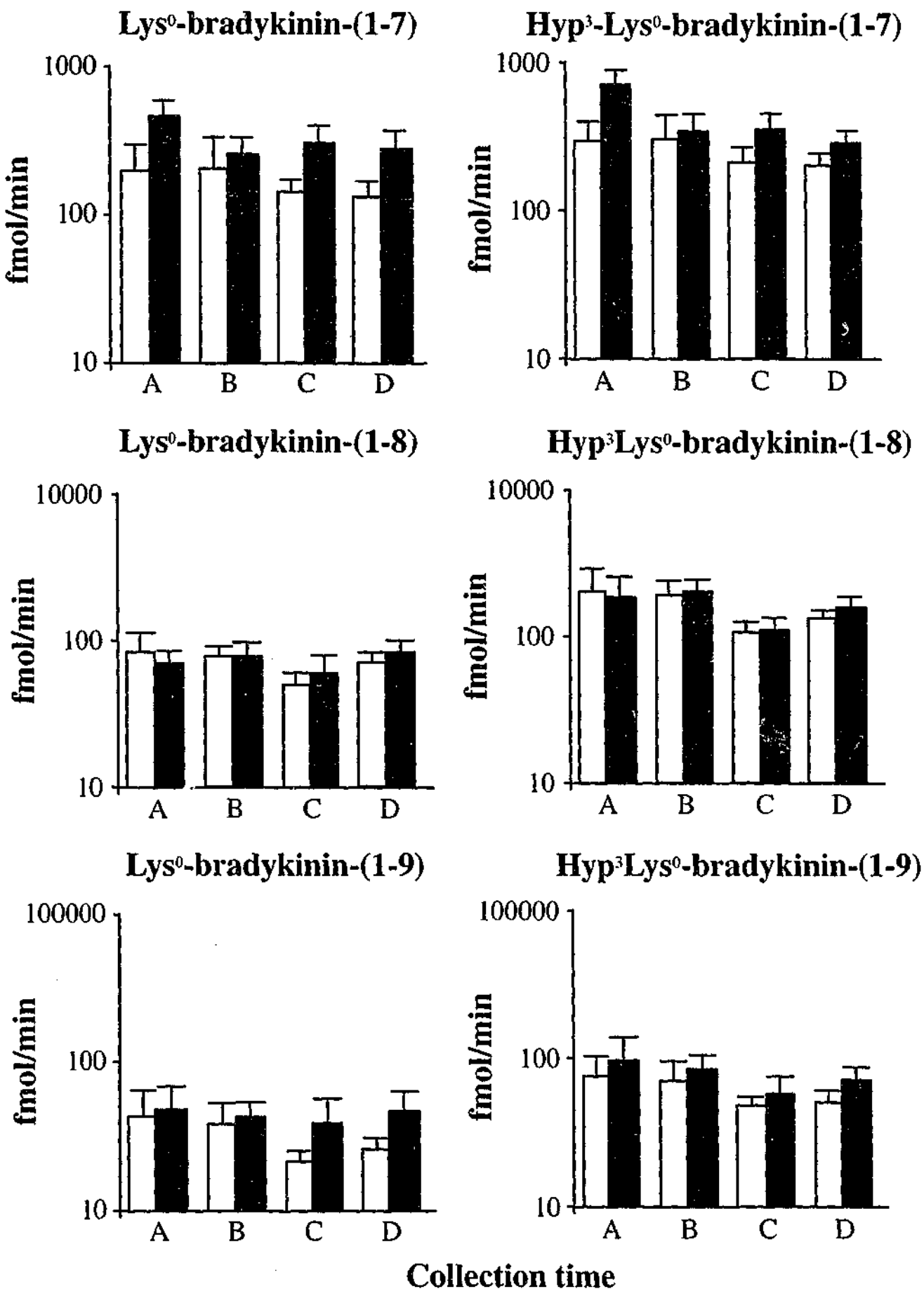


Fig 7.3 Urinary kallidin (Lys⁰-bradykinin) peptide excretion rate (mean ± SEM) during 4 collection periods A to D for 10 control subjects (open bars) and 13 subjects with interstitial cystitis (closed bars).

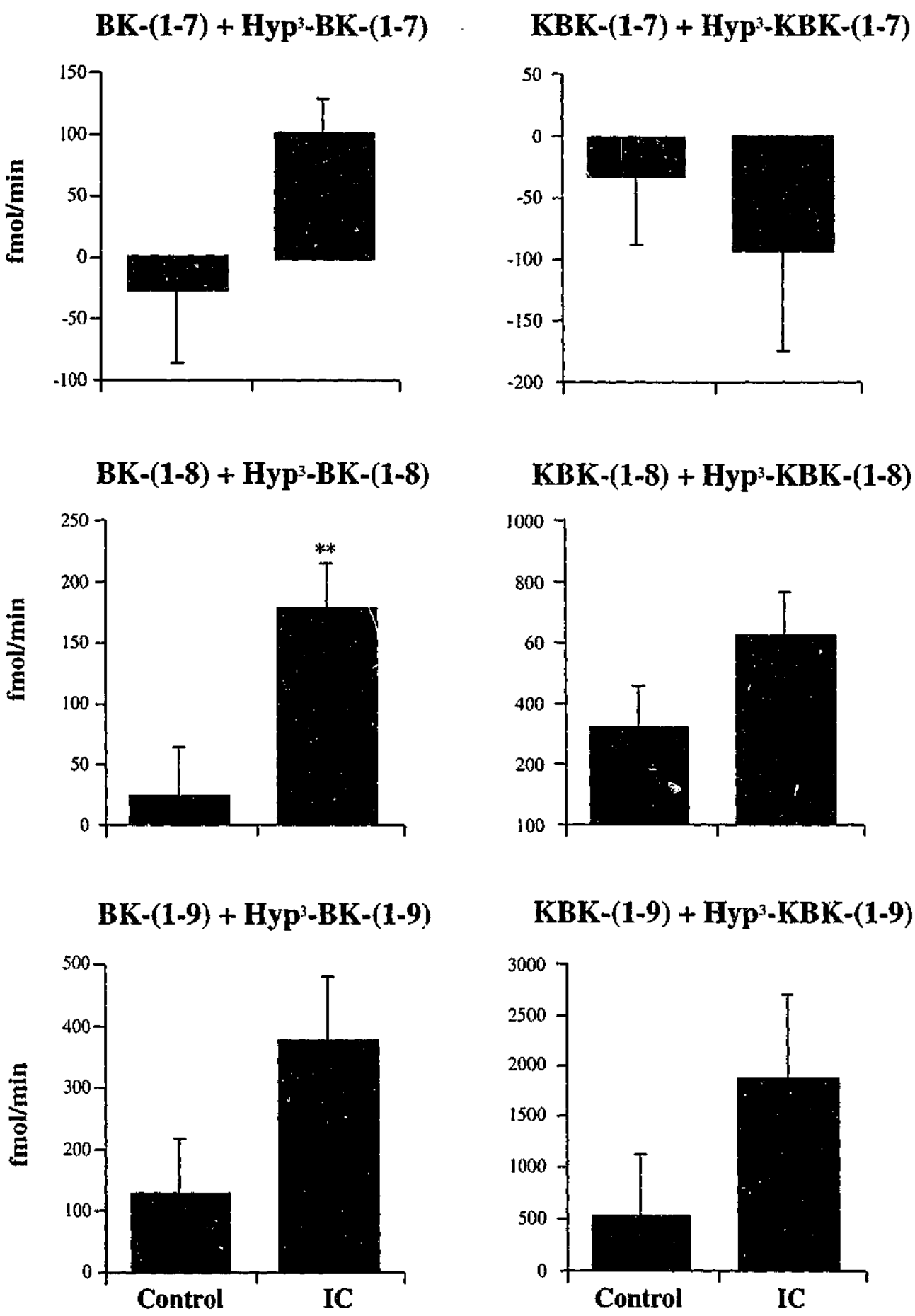


Figure 7.4 Increment (mean ± SEM) in urine bradykinin (BK) & kallidin (KBK) peptide excretion rate during 2 minute bladder stasis after 100ml distention (difference between collection periods C & D) for 10 control subjects and 13 patients with interstitial cystitis (IC). Each column represents sum of non hydroxylated and hydroxylated peptides.

** p= 0.01, compared to controls

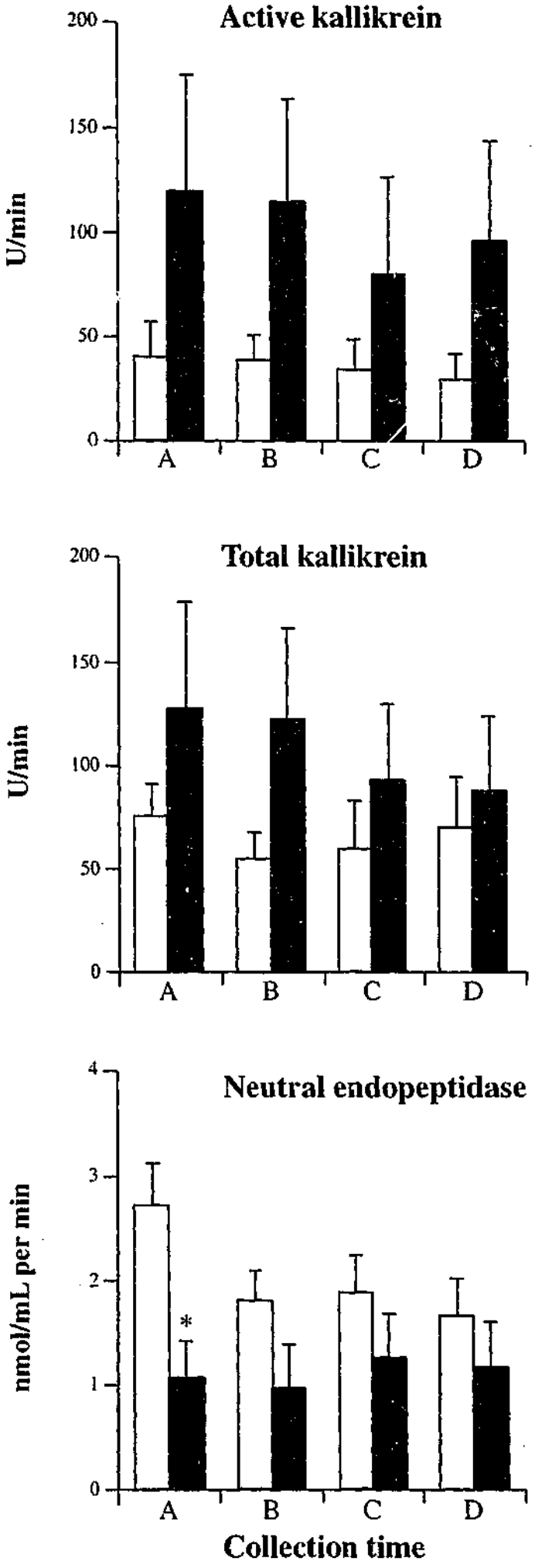


Fig 7.5 Urine excretion (mean ± SEM) rates of active tissue kallikrein, total tissue kallikrein and neutral endopeptidase for 10 control subjects (open bars) and 13 patients with interstitial cystitis (closed bars).
* $p < 0.05$ compared to control subjects

CHAPTER EIGHT

URINARY EXCRETION OF SUBSTANCE P AND ITS METABOLITES

.8.1. INTRODUCTION

In Chapter 7, evidence was provided for increased bradykinin (BK) peptide formation in the bladder wall of interstitial cystitis subjects, where it may contribute to the pathology and symptomatology of this condition. The undecapeptide tachykinin, substance P (SP), is another potent inflammatory peptide that has been implicated in the pathogenesis of interstitial cystitis. SP is released from certain sensory nerves as a chemical neurotransmitter or modulator (Otsuka and Yoshioka, 1993). SP is a potent pain-producing substance that also produces endothelium-dependent, nitric oxide-mediated vasodilatation and increased vascular permeability (Otsuka and Yoshioka, 1993). SP-containing nerves are present in normal and interstitial cystitis bladder (Pang et al, 1995; Hohenfellner et al, 1992).

Both SP and BK produce plasma extravasation in the bladder (Giuliani et al, 1993; Figini et al, 1997) and are implicated in the pathogenesis of experimental models of bladder inflammation (Giuliani et al, 1993; Hammond et al,. 2000; Ahluwalia et al, 1994). Moreover, genetic models demonstrate an important role for SP and neurokinin 1 receptors (SP receptors) in pain and inflammatory processes (Iversen, 1998).

Several studies showed that the inflammatory actions of BK might be due to stimulation of SP release from sensory nerve endings (Figini et al, 1997; Kopp et al,1997; Emanueli et al, 1998). Given the potential for SP to contribute to the

pathogenesis of interstitial cystitis, it was decided to examine whether the increase in BK peptide release observed from the bladder wall of subjects with interstitial cystitis was accompanied by increased SP release. A high performance liquid chromatography (HPLC)-based radioimmunoassay (RIA) for SP and its metabolites in urine was utilised, and the levels of SP and its metabolites were measured in the urine of interstitial cystitis subjects and a control group of women with stress incontinence.

8.2. MATERIALS AND METHODS

8.2.1. SUBJECTS, URINE COLLECTION PROTOCOL

This study was performed in the same patient group in whom was demonstrated increased BK release from the bladder wall in interstitial cystitis (Chapter 7). At the time the previous study was performed, duplicate urine samples were collected and extracted as described for the kinin assay. Of the 13 patients and 12 controls from the original study, samples were available from all 13 patients and 10 controls for SP assay. Demographic and clinical data including urinary diary, pain score, urodynamics, and the results of cystoscopy and bladder hydrodistention for patients and control subjects were reported in Chapter 7. The urine collection protocol is described in Chapter 7.

8.2.2. SAMPLE PREPARATION, EXTRACTION AND HPLC OF SP PEPTIDES

For each collection, 1mL urine for SP peptide measurement was immediately added to 10mL 4 M guanidine thiocyanate, 1% trifluoroacetic acid in water (GTC/TFA) in

order to inhibit all peptidase activity. The GTC/TFA urine samples were stored at 4°C and extracted within 1-2 hours of collection. Samples were initially extracted on Sep-Pak C_{18} cartridges (Waters Chromatography Division, Milford, MA) The extract was acetylated and treated with piperidine, then oxidized with 10% formic acid in hydrogen peroxide before HPLC. (Campbell et al, 1995).

8.2.3. PEPTIDE RADIOIMMUNOASSAY

Acetylated SP peptides were measured with antibody SP1, raised in a rabbit immunized with acetyl-Arg-Pro-Lys(acetyl)-Pro-Gln-Gln-Tyr-Lys [acetyl-[Tyr^7-Lys^8]-SP-(1-8)] conjugated via the carboxy-terminal lysine residue to bovine thyroglobulin with glutaraldehyde. This is an amino-terminal directed antibody that enables the measurement of the acetylated forms of SP-(1-7), SP-(1-8), SP-(1-9), SP-(1-10), and SP-(1-11). Tracer for this RIA was ^{125}I-acetyl-[Tyr^7-Lys^8]-SP-(1-8) and the standard for the assay was acetyl-SP-(1-11). The RIA had a sensitivity of less than 0.25 fmol/tube, a within assay coefficient of variation of 9%, and a between assay coefficient of variation of 26%. Antibody cross-reactivities for acetylated peptides were 113% for SP-(1-7), 100% for SP-(1-8), 92% for SP-(1-9), 136% for SP-(1-10), and 100% for SP-(1-11). HPLC achieved excellent separation of 5 fractions or more between SP and its metabolites. Recoveries (mean ± SD, n = 7) through the extraction and HPLC were 19.4 ± 4.4% for SP-(1-7), 28.9 ± 3.7% for SP-(1-8), 34.5 ± 7.9% for SP-(1-9), 24.3 ± 3.4% for SP-(1-10), and 31.3 ± 4.5% for SP-(1-11). Data were corrected for antibody cross-reactivity and peptide recovery.

8.2.4. STATISTICAL ANALYSIS

Absolute data were analysed by analysis of variance with repeated measures on collection period (Statview, SAS Institute Inc., Cary, NC). Changes in SP peptide excretion rate during bladder stasis were analysed by paired t test. Logarithmic transformation of data was performed when required to obtain similar variances between groups. All tests were two-tailed. Differences were considered significant at $p < 0.05$.

8.3. RESULTS

Urine flow rates during urine collection after water load were similar for IC subjects (11mL/min, range 5-21mL/min) and control subjects (11mL/min, range 4-17mL/min). There were no differences between IC and control subjects in excretion rates of individual SP peptides (Figure 8.1). The data shown in Figure 8.1 demonstrated metabolism of SP-(1-11) to SP-(1-8) during the 2 minute bladder stasis in both patients and control subjects. When data for the two periods of bladder stasis (comparison of collections A and B, and collections C and D) were combined for both patients and control subjects, there was a fall in SP-(1-11) excretion rate from 30 ± 6 to 14 ± 2 fmol/min (P = 0.03), and an increase in SP-(1-8) excretion rate from 8.9 ± 1.4 to 16.9 ± 3.0 fmol/min (P = 0.01). There were no changes in the excretion rates of SP-(1-7), SP-(1-9), or SP-(1-10).

8.4. DISCUSSION

This is the first report of the use of chromatography-based RIA to specifically measure SP-(1-11) and several of its metabolites in urine. Also reported for the first

time is the rapid metabolism of SP-(1-11) in urine. There was no difference between IC subjects and controls in urinary excretion of individual SP peptides. Moreover, there was evidence for rapid metabolism of urine SP-(1-11), with decreases in SP-(1-11) excretion rate, and increases in SP-(1-8) excretion rate during 2 minutes bladder stasis. Neutral endopeptidase plays a major role in the metabolism of SP-(1-11), the main metabolites being SP-(1-6), SP-(1-7), and SP-(1-9) (Otsuka and Yoshioka, 1993; Skidgel et al, 1984), and the detection of SP-(1-7) and SP-(1-9) in urine was consistent with SP-(1-11) metabolism by neutral endopeptidase. In Chapter 7 decreased neutral endopeptidase activity was found in urine of interstitial cystitis patients during the first collection period but not during the subsequent collection periods, raising the possibility that impaired SP-(1-11) metabolism may result in increased SP-(1-11) levels in urine of interstitial cystitis subjects. However, the present study found no difference between IC and control subjects in the decline in SP-(1-11) excretion rate during bladder stasis, indicating similar rates of urinary SP-(1-11) metabolism in IC and control subjects.

Chen et al, 1999 in a preliminary report, found increased levels of immunoreactive SP in urine of subjects with interstitial cystitis. These workers measure immunoreactive SP in a single voided urine specimen without precaution to minimize SP metabolism while urine was resident in the bladder. Thus, the higher immunoreactive SP levels in subjects with interstitial cystitis reported by Chen et al, (1999) may reflect less metabolism of SP as a consequence of increased urinary frequency in these subjects.

Several lines of evidence suggest a role for SP in the pathogenesis of interstitial cystitis, possibly involving stimulation of mast cell degranulation. Pang and

colleagues in 1995, reported increased numbers of SP-containing nerve fibres in the submucosa of interstitial cystitis patients. By contrast, Hohenfellner and co-workers in 1992, in a larger series of IC patients, did not find increased numbers of SP-containing nerve fibres in interstitial cystitis. Bladder mast cells are not only increased but highly activated in interstitial cystitis (Theoharides et al, 1995). The increased numbers of mast cells in the interstitial cystitis bladder, the proximity of SP-containing neurons to mast cells (Pang et al, 1995), and the stimulation of mast cell degranulation by SP (Otsuka and Yoshioka, 1993; Theoharides et al, 1995), have led to the hypothesis that SP may stimulate inflammation in the bladder wall by stimulating mast cell degranulation (Theoharides et al, 1998). However, Saban et al, 2000 recently showed that SP receptor gene knockout mice have increased numbers of mast cells in the bladder that degranulate in response to antigen challenge, but these mice are protected from inflammation, suggesting that SP mediates the inflammatory consequences of mast cell degranulation.

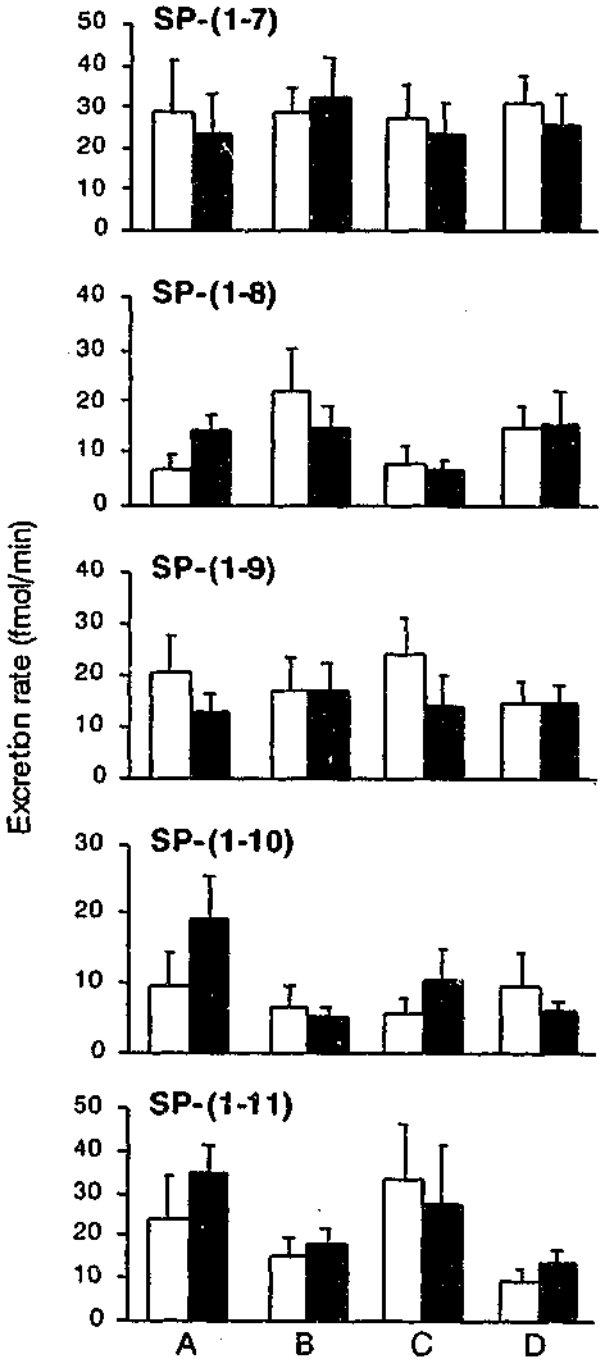
There is no information about the relative contribution of the kidney and the bladder wall to SP in urine. The collection of rapidly flowing catheter urine before and after bladder stasis in the current study was designed to reveal a contribution of the bladder wall to urine SP levels. This protocol was successful in revealing a contribution of the bladder wall to urine BK levels in interstitial cystitis (Chapter 7). It may be argued that the failure to detect increased SP in urine of interstitial cystitis patients was due to the failure of SP to diffuse from the bladder wall into urine, or to its rapid metabolism in urine. However, Saban and colleagues in 1997, demonstrated spontaneous release of both SP and BK from isolated guinea pig bladder mucosa, and showed that BK stimulated SP release in this model. Moreover, the collection of free-flowing urine in the current study during a water diuresis minimised the

potential for urinary metabolism of SP before it was added to the potent denaturant GTC/TFA. The data indicated similar rates of SP metabolism in urine of interstitial cystitis and control subjects. BK and SP are both metabolised by neutral endopeptidase, the predominant kininase in urine. The finding of increased BK levels in urine of interstitial cystitis subjects after bladder stasis indicated that increased SP release from the bladder wall of interstitial cystitis subjects should have been detectable if it had occurred. The evidence for rapid metabolism of urine SP during bladder stasis raised the possibility of similarly rapid metabolism of urine BK, and indicated that the previous study (Chapter 7) probably underestimated the release of BK peptides from the bladder wall during bladder stasis.

The failure to obtain evidence for increased SP release from the bladder wall does not exclude a role for this peptide in the pathogenesis of interstitial cystitis. Receptors for SP are localised in the endothelium of arterial blood vessels of the detrusor muscle and lamina propria, and in small vessels in the bladder subepithelium (Burcher et al, 2000). Marchand and colleagues in 1998, reported increased expression of SP receptor mRNA in bladder of subjects with interstitial cystitis, raising the possibility that increased SP receptor levels may amplify the actions of SP in this condition.

In conclusion, urine levels of SP and its metabolites were not found to be increased in interstitial cystitis. These data provide evidence that the increased BK peptide release from the bladder wall of interstitial cystitis subjects is not associated with increased SP release, and indicate that the inflammatory actions of BK are not mediated by increased SP release from sensory nerves in the bladder of these subjects.

Figure 8.1 Bar graphs showing the excretion rates of substa ... P and its metabolites, SP-(1-7) to SP-(1-11) in control (empty bars) and interstitial cystitis (solid bars) subjects for collections A to D.



CHAPTER NINE

BLADDER BIOPSY BRADYKININ-1 RECEPTOR EXPRESSION IN WOMEN WITH INTERSTITIAL CYSTITIS

9.1 INTRODUCTION

An increase in the urinary levels of the bradykinin-(1-8) peptide in women with IC was described in Chapter 7. This peptide is known to act solely on the bradykinin-B1 receptor which has been shown to be induced or up regulated in animal models of inflammation.

The members of the tissue kallikrein-kinin system are widely distributed in the urinary tract. Apart from the extensively reported expression in the kidney (Figueroa et al, 1988; Iwai et al, 1988), components of the kallikrein-kinin system have been localised to the lower urinary tract in a number of animal studies. Immunohistochemical studies in the rat bladder have demonstrated kallikrein-like substance in the lamina propria (Orfila et al, 1993). Autoradiographic studies indicate that the density of bradykinin receptors in the guinea pig bladder is maximal in the lamina propria especially in the immediate suburothelial layer (Manning & Snyder, 1986).

Kinin receptors are presently characterised as type 1 (B1) and type 2 (B2). The B2 receptors mediate most of the actions of kinins. Both rat and human B2 receptor genes have been cloned. The level of human B2 receptor density is highest in the kidney although detectable levels are found in heart, lung, brain, uterus and testis. B1 receptors are normally absent, but expression in smooth muscle cells and fibroblasts becomes evident in pathological states, particularly in inflammation or after exposure of tissue to noxious stimuli (Regoli et al, 1981). It has been proposed that de novo synthesis of B1 receptors occurs in some tissues as a result of inflammation or tissue trauma and that the B1 receptor is implicated in the chronic inflammatory and pain producing responses to kinins (Bathon and Proud, 1991).

The normal urothelium is described as impermeable to substances which are physiologically present in the urine (Lewis, 1986). The barrier function of the urothelium is thought to be due to the transitional epithelium in addition to the mucus (glycosoaminoglycan) layer covering the apical cells of the urothelium. Urinary kinins are unlikely to diffuse across the urothelium as they are of relatively large size and the mucosal layer has powerful kinin degrading activity (Maggi et al, 1989). However urothelial permeability is increased in circumstances of inflammation or injury so that urinary kinins may potentially gain access to target structures in the urinary tract which may express bradykinin receptors. In addition the inflammatory process may activate the cascade leading to activation of kallikrein and production of bradykinin in the bladder itself (Bathon and Proud, 1991).

In this study the aim was to

1. confirm B1 receptor expression and document its localisation in bladder biopsies from female controls and to determine whether it is increased in bladder biopsies of women with interstitial cystitis
2. identify the cell population demonstrating bradykinin-1 receptor positivity with the use of double stain immunohistochemistry

9.2 METHODS

9.2.1 SUBJECTS, BIOPSY COLLECTION

Interstitial cystitis and control subjects were derived from the original series of subjects described in Chapter 2 in addition to subjects recruited after the histology study described in Chapter 3. Biopsy collection and processing was as described in Chapters 2 and 3.

9.2.2 BRADYKININ-1 RECEPTOR IMMUNOHISTOCHEMISTRY

Sections were cut at 5μm, placed onto APES coated slides and incubated at 37°C overnight. The sections were dewaxed and rehydrated. The slides were placed into a boiling solution of 0.01M sodium citrate buffer (pH 6.0) for 10 min followed by a further 20 minutes with the Bunsen burner turned off. The slides were rinsed in distilled water followed by incubation in 3% hydrogen peroxide in methanol at room temperature for 10 minutes. The slides were washed with PBS and incubated overnight with primary antibody at 4°C (1:2000 in 1% BSA/PBS), a BK1 receptor polyclonal

antibody raised against a BK1 receptor peptide in rabbits (gift from Merck Labs. USA). Normal rabbit Ig (Santa Cruz 2027) was used as the negative control.

The use of umbilical vein as positive control (Sardi et al, 1997) was attempted but not successful due to the presence of increased background staining.

The sections were washed with PBS and incubated with biotinylated anti-mouse/goat/rabbit Dako LSAB+ kit for 15 min at RT (Dako Corporation, Carpinteria, CA). The sections were washed with PBS and incubated with HRP-Streptavidin Dako LSAB+ kit for 15 minutes at RT (Dako Corporation, Carpinteria, CA). The sections were washed with PBS and developed using DAB Dako LSAB+ kit for 5 min at RT (Dako Corporation, Carpinteria,CA). Finally the slides were washed with distilled water, dehydrated and mounted in Depex.

9.2.3 BRADYKININ-1 RECEPTOR LEUCOCYTE DOUBLE STAIN IMMUNOHISTOCHEMISTRY

The above bradykinin-1 receptor staining method was followed but the sections not mounted. The slides were washed with PBS and incubated with Double stain enhancer (Zymed, San Francisco, CA) for 45 minutes followed by a further PBS wash. The sections were then incubated with the anti-leucocyte antibody at 37°C for 60 minutes. The anti-leucocyte antibodies with the corresponding concentrations used for incubation and the corresponding leucocyte population is shown in Table 9.1.

All of the leucocyte antibodies were obtained from Dako Corporation, Carpinteria California. CD20 (clone L26) is a mouse anti-human monoclonal antibody (M0755)

directed against an antigen present on the majority of B-cells. The antibody reacts with the majority of B-cells present in peripheral blood and lymphoid tissue. There is no reactivity with other haematopoietic cells, normal cells and malignant non-lymphoid cells. CD68 (clone KP1) is a mouse anti-human monoclonal antibody (M0814) directed against macrophages in a wide variety of human tissues. Mast cell tryptase (clone AA1) is a mouse anti-human monoclonal antibody (M7052) directed against mast cells and can differentiate between mast cells and basophils. Neutrophil elastase (clone NP57) is a mouse anti-human monoclonal antibody (M0752) labelling neutrophilic granulocytes and their precursors.

Instead of double stain technique with B1 receptor antibody, serial sections were performed with CD3 (clone UCHT1), a mouse anti-human monoclonal antibody (M0835), a pan T-cell marker which reacts with normal and neoplastic T-cells.

The slides were then washed with PBS and incubated with biotinylated anti-mouse/goat/rabbit Dako LSAB+Alkaline Phosphotase kit (Dako Corporation Carpinteria CA) at RT for 15 minutes followed by a further wash with PBS. Incubation with Streptavidin-AP Dako LSAB+ Alkaline Phosphatase kit (Dako Corporation, Carpinteria, CA) at RT for 15 minutes followed. A further wash with PBS occurred and the slides then developed using Dako New Fuschin Substrate System (Dako Corporation, Carpinteria, CA) at RT for 5 minutes. The slides were then washed with distilled water and mounted with aqueous mountant.

9.2.4 HISTOLOGICAL ASSESSMENT

The assessment was performed by Dr J Scurry and Dr A Rosamilia. The slides were assessed and scored in a blinded manner so that the identity and diagnosis was not

known. The scoring ranged from a minimum of 0 (no staining) and a maximum of 3 (maximal staining).

9.2.5 PREABSORPTION EXPERIMENT

Two peptides - a control #3 peptide (which was used to immunise the animals) and a specific #5 peptide synthesized from a region of the BK1 receptor – were obtained from Merck Labs (USA). The peptide antibody solutions (both specific and control peptides) were incubated at 4C overnight at a concentration of 25ug/ml and then used in the standard BK1 receptor staining protocol as the primary antibody. The experiment was repeated using peptide concentrations of 6.25ug/ml and 100ug/ml.

9.2.6 STATISTICS

Mann-Whitney test (two-tailed) was used for the statistical analysis of the bradykinin-1 receptor result using SPSS version 10.1. Correlation was assessed using Pearson two tailed test. Significance was at the $p<0.05$ level.

9.3 RESULTS

9.3.1 COMPARISON BETWEEN IC AND CONTROL GROUPS

Bladder biopsies from 28 subjects with interstitial cystitis and 27 control subjects were assessed. One of the IC group and one of the control subjects were excluded because of current urinary tract infection. Therefore results from 27 subjects with IC and 26 controls were analysed and are listed in Appendix V. Table 9.2 and Figure 9.1(a) and

(b) demonstrate the range of scores for the bradykinin-1 receptor results for IC and control subjects respectively. There was a trend toward a greater number of biopsies with bradykinin 1 receptor staining in the interstitial cystitis as compared with the control group (p=0.09).

Cystoscopic capacity was known in 44 of the subjects and in 3 of the IC group the cystoscopic capacity was 400 ml or less (classified as severe in Chapter 3). Two of these subjects had scores of 3 for BK1staining; the other had a score of 0. Forty two of the subjects had been recruited for the histology study described in Chapter 3 and therefore had scores for submucosal inflammation available for comparison. There was no correlation between the BK1 receptor staining scores and the scores for inflammation.

Close inspection of the staining suggested cytoplasmic staining in stromal cells (superficial fibroblasts), inflammatory cells (possibly lymphocytes) and to a variable degree in epithelial cells. The endothelium, the deep submucosal cells, detrusor mast cells and erythrocytes were consistently negative. Figure 9.2 (a to d) shows bradykinin-1 receptor immunostaining in interstital cystitis and control subjects and the negative immunostain control for each.

9.3.2 BRADYKININ 1 AND LEUCOCYTE RECEPTOR DOUBLE STAIN

Results of the bradykinin-1 receptor and leucocyte double stain immunohistochemistry (serial section for CD3) in biopsies from interstitial cystitis subjects are illustrated in Figures 9.3 to 9.5.

Figure 9.3 is a bradykinin-1 receptor/ CD20 double stain which shows some cells staining for BK1 receptor which appear to be different to those staining positive for CD20 although there are a few cells which may be staining positive for both. Therefore a subpopulation of B cells may express the B1 receptor. However the BK1 staining cells appear distinct from cells staining positive for the macrophage (Figures 9.3c and d) and neutrophil (Figures 9.4a and b). There may have been some mast cells which also stained for BK1 receptor but the majority are differently staining cells (Figures 9.4c and d) and the T lymphocytes are a distinct population also (Figure 9.5).

9.3.3 PREABSORPTION EXPERIMENT

Two pre-absorption experiments were performed. In the first experiment, there were problems with the BK1 antibody, alone, not staining although preabsorption with the control peptide #3 produced staining which was similar to that seen previously with the BK1antibody. Preabsorption with the specific BK1 receptor peptide #5 produced heavy background staining all over the section with no apparent reduction in 'specific' BK1 staining. However, it was difficult to interpret this experiment as the non-treated or non pre-absorbed control did not work.

In the second experiment, substitution with a fresh batch of BK1 antibody produced the same staining as seen previously and reported above in sections 9.3.1 and 9.3.2 but incubation with the #3 control peptide did not reduce this staining and neither did pre-absorption with the specific peptide #5 suggesting that the staining observed was not specific.

9.4. DISCUSSION

This study showed a trend toward increased numbers of cells demonstrating positive staining for bradykinin-1 receptor (p=0.09) in the bladder submucosa of subjects with interstitial cystitis as compared with female control subjects. It is consistent with the only other bradykinin1 receptor immunohistochemical study performed in IC. Ruggieri and colleagues in 1997 reported expression of bradykinin 1 receptor subtype in 9 of 20 interstitial cystitis bladder biopsies but none of the 7 cadaveric bladder and 4 human aorta specimens. The appearance they describe was of moderate to intense staining in distinct thread-like cellular areas that did not stain in the normal rabbit serum control slides. However, a control antibody preabsorption experiment was not described by Ruggieri and colleagues, 1997.

The positive staining cells in the current study may have been stromal cells and/or a population of leucocytes. In order to try to identify the positive staining cell type, a double stain immunohistochemical technique was employed and cells staining as macrophages, neutrophils and T lymphocytes were seen to be distinct or different from the bradykinin-1 receptor positive cell population. There may have been some cells in the B lymphocyte population and to a lesser extent in the mast cell population which were also positive for BK 1 antibody although clearly there were also cells which were quite distinct. The double stain immunohistochemistry study suggests that some of the positive BK1 cells could be a subpopulation of B cells.

Expression of the B1 receptor is often associated with tissue injury or inflammation. Schneck and colleagues, (1994) demonstrated the presence of inducible B1 receptors on rabbit aorta smooth muscle cells in culture that couple to phospholipase C activation.

Very few immunohistochemical studies of bradykinin 1 receptor have been described in human tissue. Bhoola and colleagues in 1997 found immunohistochemical evidence of B1 receptor in regenerating epithelial cells in mucosal biopsies from subjects with gastritis but not in control subjects. Raidoo and colleagues in 1997 found immunohistochemical evidence of intense staining for B1 receptors in the endothelial cells, on foamy macrophages, fibroblasts and smooth muscle cells in atheromatous plaques of many large arteries such as the coronary and vertebral arteries.

Roslan and colleagues, (1995) used concentration response curves for B1 agonists (desArg BK and des ArgKD) and antagonists (desArg Leu BK and desArgHOE140) to demonstrate that B1 receptors are not present in normal rat bladders. However a definite induction of B1 responses during in vitro incubation and following bladder inflammation has been demonstrated. Lecci and colleagues (1999) demonstrated that stimulation of bladder B1 receptors evokes local, tonic-type contractions with superimposed high amplitude contractions (micturition reflex contractions) and these motor responses are magnified in the inflamed (cyclophosphamide-induced) rat model. Using the same model, Belichard et al, (1999) demonstrated induction of B1 receptors which occurred maximally at 48 hours and was associated with an increase in mRNA expression of the gene coding for B1 receptor. Trevisani and colleagues, (1999) demonstrated in vitro expression in mouse urinary bladder with a time-dependent induction of B1 receptors.

Recently Wotherspoon and Winter (2000)reported immunohistochemical evidence for B1 receptor expression in the rat sensory nervous system, specifically in the dorsal horn of the spinal cord where primary afferents terminate and in peripheral nerve terminals in

the bladder. This distribution of B1 receptors in sensory neural pathways suggests a role in hyperalgesia.

Inflammation may not always be present in IC as shown in Chapter 3 where inflammatory infitrate on H & E sections was found in a subset of approximately 30 % and the submucosal inflammation scores did not correlate with BK1 scores. However, the sample size may require much greater numbers to reach significance and may be better confined to the group of severe IC where 50% have moderate to severe inflammation. There were only 3 subjects with severe IC included in the current study.

Preabsorption of the BK1 antibody with the specific BK1 receptor peptide gave results that were of concern and cast doubt on the specificity of the positive staining. It is not clear why this occurred but perhaps may be a reflection of the length of time the peptides were stored in the freezer (~ 4 years) and that degradation may have occurred to the small peptides. A lack of effect of pre-absorption with specific antigens sometimes occurs if the concentration of the antigen is not sufficient and the experiment was repeated with 100ug/ml of antigen which should have been sufficient to block the antibody. Clearly these studies will need to be repeated with fresh antibody and antigen stocks.

The issue of which type of stromal cell the BK1 receptor staining occurs has not been entirely addressed. A double stain with smooth muscle actin could be a first step and in fact this was planned but the BK1 antibody began to produce inconsistent staining and was no longer used. Further studies could consist of repeating the immunohistochemistry with a new BK1 antibody, repeating the preabsorption study and

performing a double stain with smooth muscle actin as there is evidence in the literature of BK I receptor being located in smooth muscle cells and fibroblasts.

There are limitations of such a study. There are the documented technical problems with the polyclonal antibody, its inconsistency and apparent lack of specificity. In addition the scoring is quite crude and only semi-quantitative at best and there are the same problems of possible sampling error in drawing general conclusions from a small biopsy as discussed in Chapter 3.

Nevertheless, it is possible that the BK1 receptor staining observed in some of the control and IC bladders is real as the findings are consistent with the previous study of Ruggieri et al (1997). Clearly, further experiments are warranted to definitively substantiate the involvement of the BK1 receptor in IC. If confirmed, the finding of induced bradykinin 1 receptor cells in IC would provide further evidence for an inflammatory pathogenesis, and a role for kinins in the bladder and the nervous system in producing the symptoms of pain, urgency, frequency and hyperalgesia.

Table 9.1. Primary antibodies used for Bradykinin-1 receptor double stain with the corresponding leucocyte

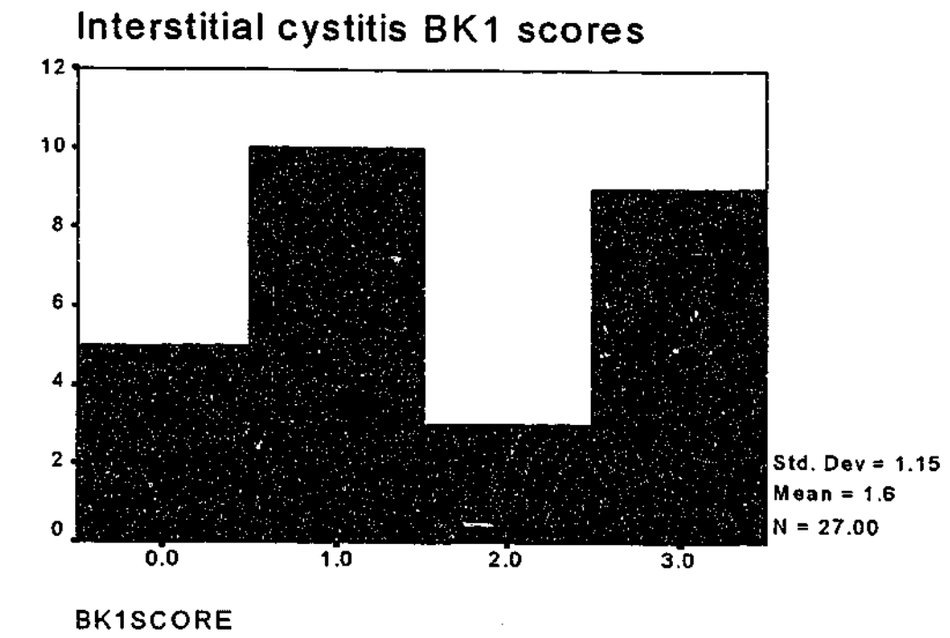
Primary Antibody	Concentration	Leucocyte
CD20 Code No M0755	1:50 in PBS	B cells
CD68 Code No M0814	1:500 in PBS	Macrophage
Mast cell tryptase Code No M7052	1:250 in PBS	Mast cell
Neutrophil elastase Code No M752	1:50 in PBS	Neutrophil
CD3	1:100 in PBS	T cells

Table 9.2 Scoring for BK1 receptor staining intensity in bladder biopsies from subjects with interstitial cystitis and female controls

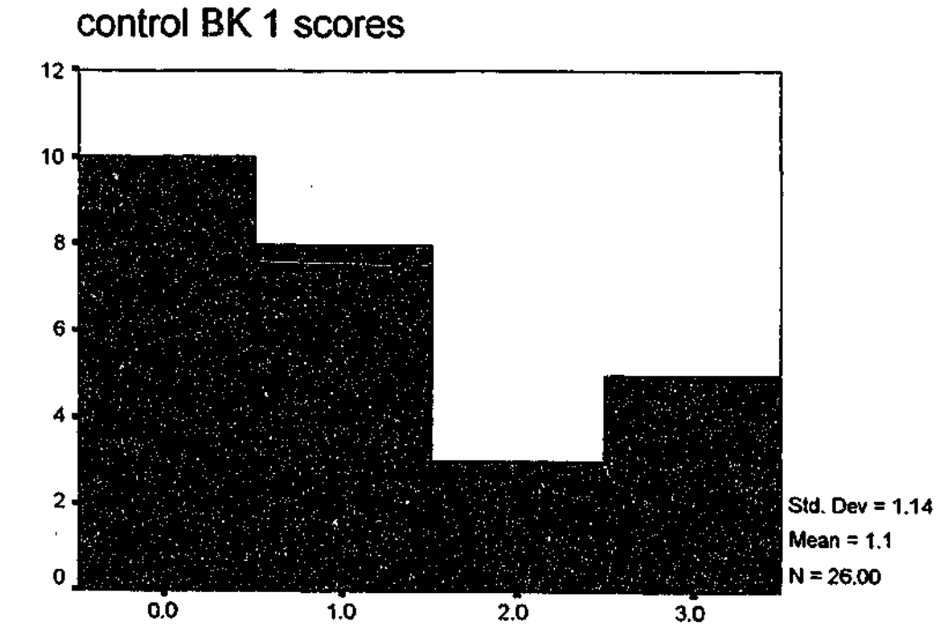
	Control	IC
	N=25	n=27
25th centile	0	1
50th centile	1	1
75th centile	2	3
Range	0-3	0-3

Figure 9.1(a) Bar graphs showing bradykinin-1 receptor antibody staining intensity score (0 to 3) in bladder biopsies from interstitial cystitis subjects and (b) control subjects

(a)



(b)



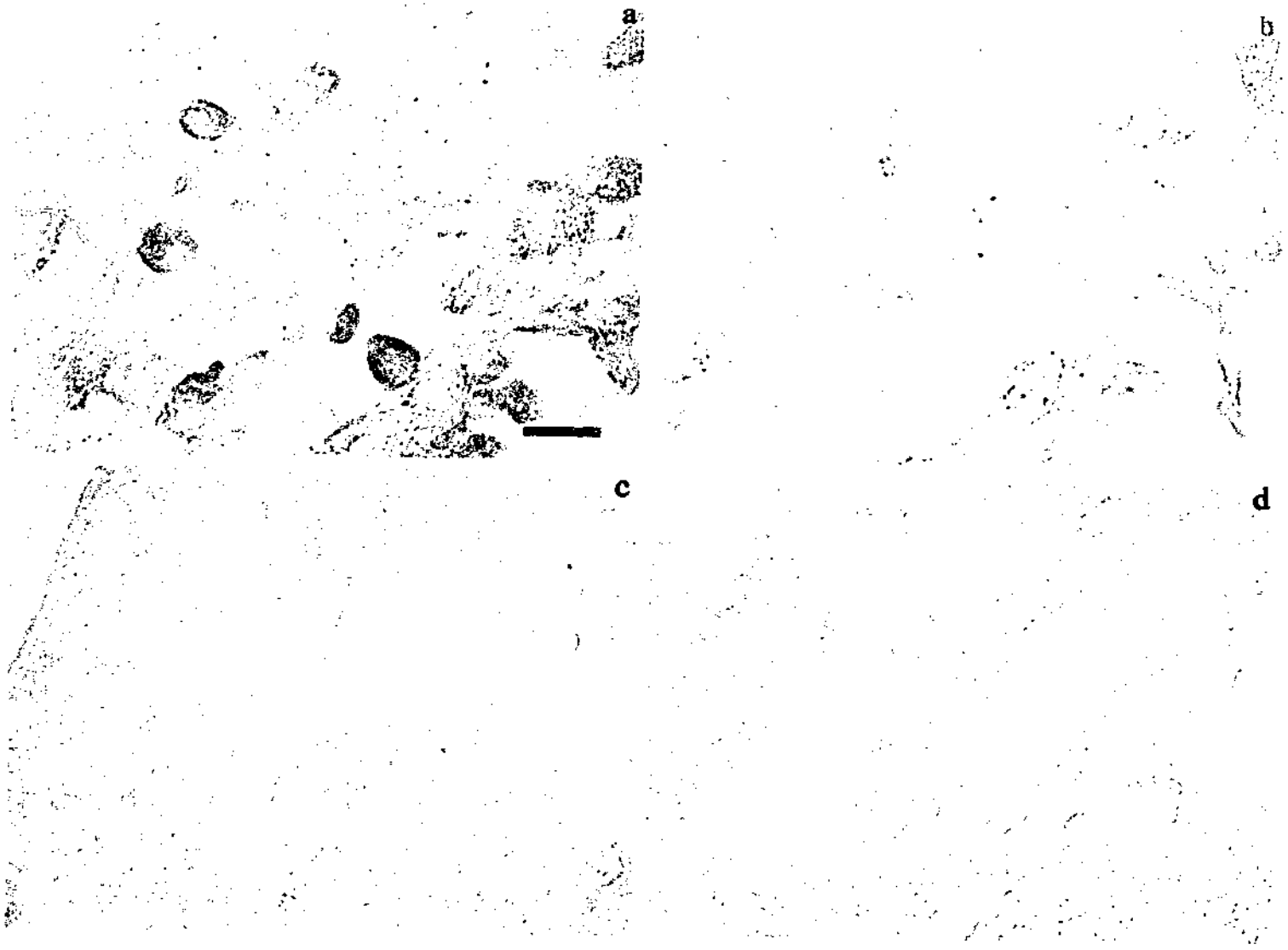


FIGURE 9.2 Bradykinin type1 (BK1) receptor immunostaining in bladder biopsy submucosa

(a) BK1 receptor immunostaining interstitial cystitis subject

(b) Negative control interstitial cystitis subject

(c) BK1 receptor immunostaining control subject

(d) Negative control - control subject

Scale Bar=10um

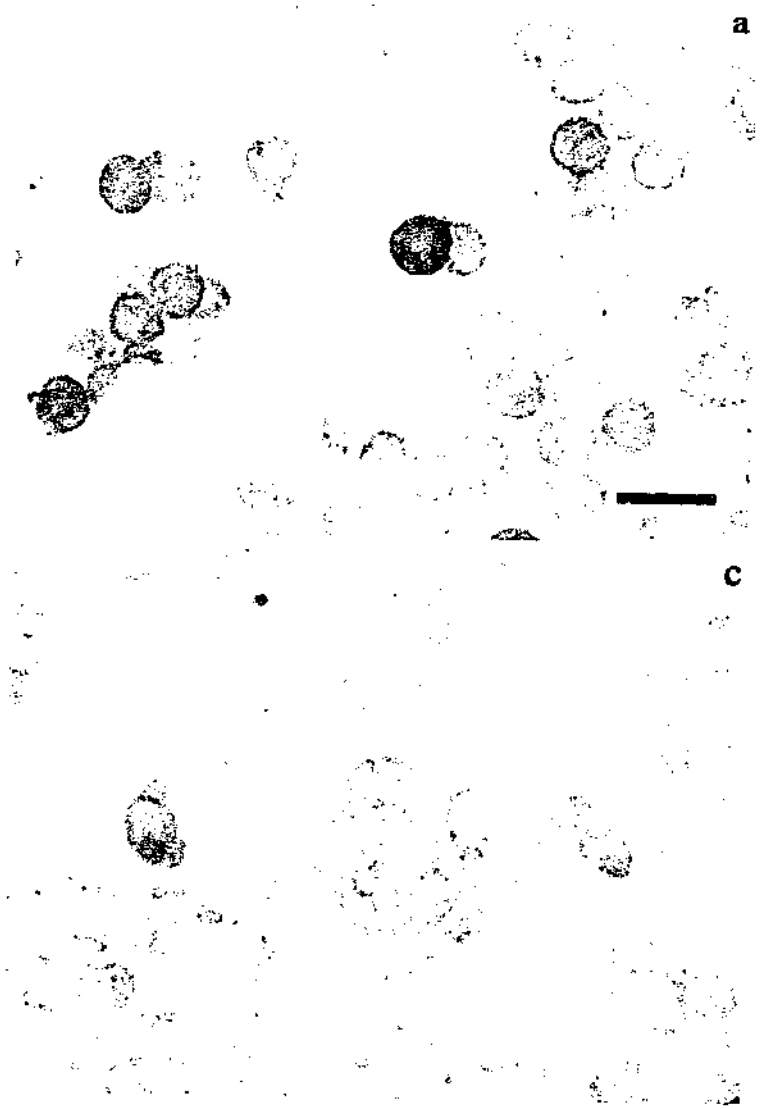


Figure 9.3 Double immunostaining with leucocyte antibodies (pink staining) and BK1 receptor Ab (brown staining) in bladder biopsies of interstitial cystitis patients

(a) CD20 (pink), BK1 receptor (brown)

(b) Negative control for slide (a)

(c) CD68 (pink), BK1 receptor (brown)

(d) Negative control for slide (c)

Scale bar=10um

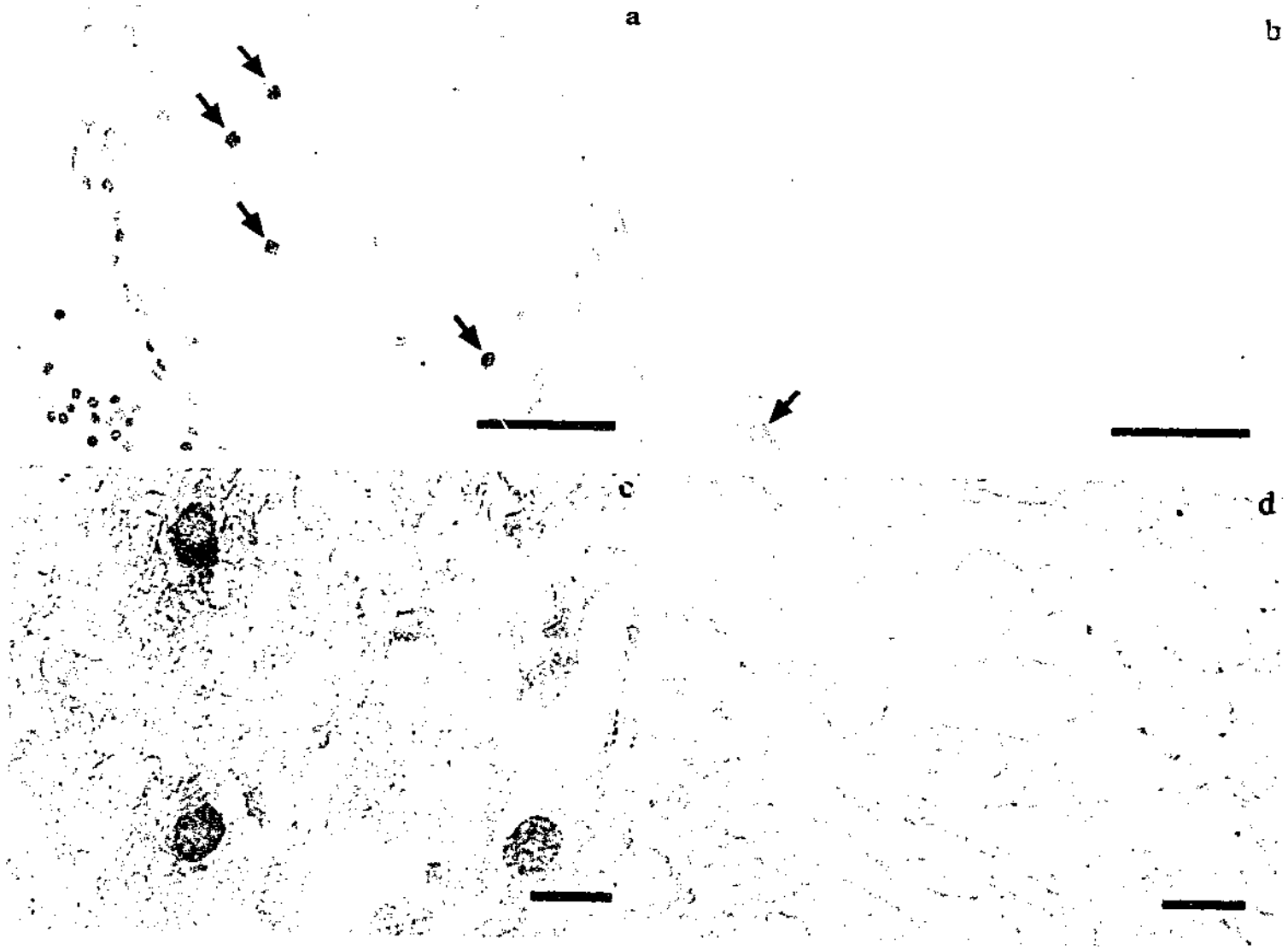


Figure 9.4 Double immunostaining with leucocyte antibodies and BK1 receptor antibody in bladder biopsies

(a) Neutrophil elastase (brown), nuclei (blue)

Scale bar=50um

(b) BK1 receptor (brown) serial section to (a)

Scale bar=50um

(c) Mast cell tryptase (pink), BK1 (brown) IC subject

Scale bar=10um

(d) Negative control for slide (c)

Scale bar=10um

Figure 9.5 Single immunostaining of serial sections with leucocyte antibody (CD3) and BK1 receptor antibody in bladder biopsy

(a) CD3 (brown)

(b) BK1 receptor (brown) serial sections to (a)

Scale bar=50um

CHAPTER 10

GENERAL DISCUSSION

Interstitial cystitis is a condition predominantly affecting women characterised by a non-specific symptomatology, a poorly described histology, an unknown etiology and theories of pathogenesis including epithelial dysfunction and neurogenic inflammation. This thesis has attempted to better define the histology, and investigate and then progress some theories of the pathogenesis of IC.

These studies led to a number of conclusions:

1) Histological changes seen in bladder biopsies taken after standard hydrodistention were evident in fewer than half of IC subjects. Epithelial denudation, submucosal oedema, vascular congestion and ectasia, and inflammatory infiltration and occurred more commonly in the severe (low bladder capacity) group. A comparison of biopsies taken before and after hydrodistention showed that epithelial denudation and inflammatory infitrate were present primarily and did not alter after hydrodistention. However, oedema and to a lesser extent congestion, were increased by the hydrodistention procedure in the IC group alone.

2) Microvasculature studies showed a reduction of subepithelial blood vessel density in IC compared with controls when routinely obtained post hydrodistention biopsies were analysed. However an increase in blood vessel density in the undistended IC bladder was found with a subsequent decrease in blood vessel density presumably brought about by the oedema of the hydrodistention procedure.

3) Using high-performance liquid chromatography and radiommunoassay, this thesis found a five-fold increased increment in urinary bradykinin but not in kallidin peptide excretion after 2 minutes of bladder stasis following bladder distention. A trend toward a greater number of cells staining for bradykinin-1 receptor in IC was demonstrated and some of these cells may be a subpopulation of lymphocytes.

4) The increased bradykinin peptide release from the bladder wall of interstitial cystitis subjects was not associated with increased substance P release. The inflammatory actions of bradykinin did not appear to be mediated by increased substance P release from sensory nerves in the IC bladder.

5) There was no difference in the number of oestrogen and progesterone receptor positive cells in the bladder submucosa of IC females compared with female control subjects. Male controls had a marked increase in submucosal cells staining for progesterone receptor and a trend toward increased oestrogen receptor positive cells.

Histological analysis of a small tissue biopsy has many limitations. There is artefact created by the biopsy (haemorrhage), and artefact created by the hydrodistention process (oedema). There is the potential for major sampling errors. Cystoscopic changes may not be correctly visualised or accurately interpreted and therefore not biopsied. Biopsy may not be taken of the most affected area. To what extent is the remainder of the bladder histologically abnormal?

Notwithstanding these limitations, histological changes in this thesis provide evidence for possible pathogeneses. Data in Chapter 3 and 6 support a pathogenesis involving

abnormal epithelial fragility and permeability with submucosal inflammation. A putative mechanism for many of the observed changes is as follows: stretching at the time of hydrodistention causes linear fissuring or ruptures of the abnormal epithelium. The hydrostatic pressure forces hydrodistension fluid across the abnormally permeable epithelium in IC producing greater degrees of oedema. This fluid then moves into the vessels and produces the appearance of congestion in the submucosa. Expansion of the subepithelial space by this influx of fluid causes a reduction in the density of the subepithelial microvasculature from the elevated density found in undistended bladders in IC. This reduction in microvascular density is great enough to obscure and may even reverse the relationship between the baseline density in IC and control subjects. When biopsies are taken, tissue crushing causes haemorrhage in many specimens of all subjects.

The increase in subepithelial blood vessels in IC is unlikely to be a primary vascular problem as there is no increase in proliferating endothelium in the subepithelium and the deeper submucosal blood vessels are not affected. It may be related to the inflammatory process but it was not found in the severe IC cases in which inflammation is otherwise implicated.

The histological studies in this thesis have highlighted some of the differences between women with early and severe disease. The early and classic IC groups have many differences. The classic group is older and have more cystoscopic changes along with reduced capacity and Hunner's ulcers including mucosal tearing. Histologically there are differences with epithelial denudation and inflammation reported more often in the low capacity group. Abnormal submucosal histology is seen in two thirds of subjects with low capacity IC compared with only 40 % of early IC. However, due to their

relative rarity, there was available only a small group of severe IC subjects for comparison.

Despite these differences the early group have equivalent type and severity of symptoms. Are the early and classic/severe forms of IC separate entities with two different etiologies or do these differences suggest a spectrum of the same condition with the hypersensitive group as the mildest end of this spectrum? These large number of differences and the natural history which suggests no significant progression from mild to severe IC tends to support the former theory.

This thesis supports a role for inflammation in the pathogenesis of IC. Histological evidence of an inflammatory infiltrate composed mainly of lymphocytes was demonstrated in 50 % of severe IC and 15 to 20 % of the early IC group. The kallikrein-kinin system is a major player in inflammation and there is evidence of a five fold increased increment in the bradykinin-(1-8) peptides in IC. These peptides are bioactive and more active on type 1 rather than type 2 bradykinin receptors. There is also evidence of decreased urinary neutral endopeptidase in IC during the initial collection period. This implies either increased kinin formation and/or reduced kinin degradation in the bladder wall of subjects with interstitial cystitis.. Furthermore, there is preliminary data showing a trend toward an increased number of cells staining for bradykinin 1 receptor in the IC bladder submucosa. Some of these cells appear to be B lymphocytes although many did not stain with any of the leucocyte antibodies used in Chapter 9 and could represent fibroblasts.

Increased bradykinin excretion was not accompanied by increased substance P urinary excretion. The only other study of substance P in IC (Chen et al, 1999) found

increased urinary levels however this was based on a freshly voided sample and may reflect less metabolism of substance P as a consequence of increased urinary frequency in IC subjects. The inflammatory actions of bradykinin do not appear to be mediated by increased substance P release from sensory nerves in the IC bladder. This is of interest given the proposed role of the tachykinin, substance P in the pathogenesis of chronic inflammatory pain. Increased expression of substance P receptor mRNA in bladder of subjects with IC has been demonstrated in one study (Marchand et al, 1998) which implies possible amplification of the actions of substance P in this condition. However, Peeker and colleagues, 2000 found low numbers of substance P immunopositive nerve fibres in IC and controls. Clearly further studies are require to better understand the role of substance P in interstitial cystitis

The oestrogen and progesterone receptor studies are intriguing. The finding of markedly increased levels of progesterone receptor positive cells in the male bladder submucosa was unexpected. Possible explanations include low circulating progesterone levels, the presence of a bladder cancer or due to differences in specimen retrieval; the male bladder tissue was obtained at cystectomy rather than hydrodistention and cold-cup biopsy. The double immunohistochemistry identified the cell as a stromal smooth muscle cell and perhaps there exists a real gender difference in their number. Further studies on receptor positive cells in the bladder epithelium and the use of specific estrogen and progesterone receptor subtypes are required.

This thesis provides evidence for an abnormal bladder epithelium in IC. A completely denuded epithelium was present in 20 % of early and 50 % of severe IC

subjects; it was found to be present to the same degree both before and after hydrodistention and therefore appears to be a real rather than artefactual finding.

The abnormal bladder epithelium may be involved in the kinin findings. Bradykinin peptides, as opposed to kallidin peptides which were not increased, are derived from the action of plasma rather than tissue kallikrein and imply transudation through an inflamed and permeable bladder wall as a possible mechanism. A reduction in neutral endopeptidase which is located in bladder epithelial cells was also seen in the IC group and could be an alternative or additional explanation for the increased bradykinin excretion levels in IC.

The studies on the role of kinins are novel and require confirmation. Further studies would need to include repeating the immunohistochemistry with a new bradykinin-1 receptor antibody and on a larger series of severe IC subjects. The preabsorption experiment is of concern and needs to be repeated. If the increase in bradykinin-1 receptor cells in IC is confirmed to be a real finding, further studies including dual immunohistochemistry with smooth muscle actin could be informative as there is evidence in the literature of the bradykinin-1 receptor being located in smooth muscle cells and fibroblasts.

The study addressing the difference before and after hydrodistention was also novel with the drawback of a small sample size. Recruitment of a larger series is necessary to confirm the findings and this is currently underway. Although previous authors have reported the histology seen in IC, this is the first study to try to disentangle the primary histological findings from the changes caused by the hydrodistention and

biopsy procedure. This is the first step in utilising histological changes to gain an insight into the pathogenesis of IC.

There are many limitations in performing clinical studies in IC. The condition is heterogenous as highlighted by the differences between the early and severe group. The means of making the diagnosis of IC obscures the primary histological changes. The histological assessment is semi-quantitative and empirical. Very small tissue samples are available for assessment. Ultimately the studies deal with pathogenesis but the findings may not be etiological or primary with the initial trigger far removed chronologically from the endpoint where the patient is diagnosed.

The primary cause therefore of the epithelial denudation remains unknown.. So does the trigger for inflammation and the involvement of kinins. Regardless of the primary trigger, the development of specific kinin antagonists which are being assessed in animal models, and, if promising, in the clinical setting, could provide a way of intervening in the inflammatory pathway leading to chronic pain.

APPENDICES

NOTES TO APPENDICES

Appendix I details the clinical information gathered on the initial series of IC and control subjects. Throughout the appendies the legend is as follows;

Diagnosis 1 refers to control, diagnosis 2 refers to IC

Menopause 1 refers to premenopausal , 2 to postmenopausal ,3 to hormone replacement

Previous surgery; 1= Hysterectomy , 2= Vaginal repair, 3= other,

4= hysterectomy and repair, 5= abdominal stress incontinence (si)surgery,

6= repair and si surg, 7= hysterectomy and colposuspension,8=cystoscopy

UD capacity refers to the maximum awake urodynamic capacity

Cysto capacity refers to the cystoscopic capacity under anaesthesia

Voids/24 ho refers to the number of voids in 24 hours as obtained from the urinary diary

Max diary v refers to the maximum volume voided in the urinary diary

Appendix II details the histology database corresponding to Chapter 3

Diagnosis 1 refers ti control,2 refers to IC, 3 refers to hyprersensitve bladder

Capacity refers to cystoscopic capacity

Epi denud refers to the score (1 to 3) obtained for epithelial denudation

Oedema refers to the score (0 to3) obtained for submucosal oedema

Cong/ect refers to the score (0 to 3) obtained for submucosal congestion and ectasia and where the scores were different the result was the mean of the 2 figures

Inflamm refers to the score (0 to 3) obtained for submucosal inflammation

Haem refers to the score (0 to 3) obtained for submucosal haemorrhage

Total score refers to the score (0 to 12) obtained for submucosal composite score

Appendix IIIA and IIIB are the oestrogen and progesterone receptor results,respectively

Diag refers to diagnosis with 1 control; 2 IC; 3 male bladder

For each study 4 individual scores wete taken at low Intensity (sc1lo to sc4lo) and 4 at high intensity settings (sc1hi to sc4hi)

Meno refers to menopausal status as described in Appendix I notes.

Appendix IV refers to the pre and post histology results and follows a similar legend to Appendix II

Appendix V refers to bradykinin 1 (bk 1) receptor results with controls(1) and IC (2) and bk1 scores shown as 0 to 3

Appendixl;Clinical Database

diagnosis	age	parity	menopaus	prev surg	ud capac	cysto cap	voids/24 ho	max diary v	id
1	49	3	1	0	510	900	7	900	1
2	49	2	3	4		800	13	450	2
1	53	2	3	2		550			3
1	53		3	2	450	500	13	120	4
1	68	2	2	2	500	600	13	120	5
3	66	2	3	4	248	650	9	350	6
1	56								7
2	40	2	1	2	250				8
1	58	3	3	1		550			9
1	58	2	3	4	500	610	8		10
1	41	2	2	1	500	600	10		11
3	67	0	2	3	200	1000	16	402	12
3	74	3	3	2	280	400			13
1	44	1	1	2	500	600	7		14
1	31	2	1	3	480	600			15
1	44	3	1	6	467	600			16
1									17
1	67	3	3	6	500	1000	8		18
1	58	2	3	4	484	600	7	600	19
1	48	5	3	7	470	700			20
1	48	2	1	8	516	1000	10	750	21
1	39	2	1	7	510	700	8	530	22
1	52	4	3	7	511	700	6	510	23
1	57	5	2	5	505	600	5	450	24
1	64	6	3	4	500	800			25
1	56	2	2	5	511	720			26
1	47	3	3	5	515	600	10	500	27
1	61	3	3	1	506	600	10	600	28
1	52	4	2	5	500	550	7	600	29
1	63	3	3	6	528	725	5		30
1	44	2	1	3	520	700	11	600	31
1	48		1	0	494	750		750	32
3	52				296	600		350	33
1	71		2			800			34
1	48					700			35
1	50								38
1	39	6	1	5	500	600	5		39
1	80	3	3	4	500	700	5		40
1	50	2	3	6	500	600	9	500	41
1	61	2	2	3	426	600			42
1	60	2	2	3	558	900	6	900	43
2	64	1	2	8		400	17	200	44
2	65	3	2	3	209	450	15	300	45
2	56	1	3	2	100	375			46
2	28	1	1	88	290	600	18		47
2	54	3	2	0	289	400	10		48
2	68	0	2	8	130	450	12	250	49
1	48	3	1	0	496	600	7	550	50
2	78		2	888	315	600	11	220	51
2	40	2	1	8	251	500	26		52
2	40	0	1	38	266	600	11	300	53
2	35	3	1	8	489	800	14		54
2	61	2	2	2	380	700	14	200	55

Appendixl;Clinical Database

diagnosis	age	parity	menopaus	prev surg	ud capac	cysto cap	voids/24 ho	max diary v	id
2	60	4	2	1	221	600	13	250	56
2	38	4	1	3	340	850	12	410	57
2	37	1	1	0	324	600	12	280	58
2	34	3	1	0	216	450	12	250	59
2	69	4	2	1		1000	10		60
2	70	2	2	4	299	600			61
2	62	6	2	88	100	350			62
2	76	1	2	881	140	250	13	200	63
2	65	3	3	2	250	600	15	300	64
2	60	4	2	1	263	500	9	300	66
2	25	1	1	3	358	600	9	340	67
2	43	2	1	0	228	500	10	320	68
2	40		1			600			69
2	43	1	1	0	338				70
2	31	1	1	888	278	650	14		71
2	19	0	1	0		600	12	260	72
2	40	5	1	3	286	500	11	240	73
3	29		1	0	300				74
2	61	0	3	8	288	450	13	210	75
4	38		1	8		500			76
4	55	6	3	88	370	400	11	300	77
4	65	5	2	1					78
1	46	3	1	3	500	800	5		79
3	72		2			600			80
1	58		2	4		600			81
2	41	3	1	3	206	550	12	300	82
1	41	3	1	1	516	600			83
3	59	3	3	1	354	500	10	400	84
3						600			85
3	76	2	2	2	250	680	8	390	87
3	63	1	2	3	324	900			88
3	67		2		395	520			89
1	51	2	3	4	501	600	8	710	90
2	21	0	1	0		600	15	300	91
1	30	3	1	2	470	700			92
2	70	4	2	9		750	9	425	93
2	33	2	1	2	218	800	8		94
2	37	1	1		220	450			95
2	31	0	1	0		600	12	350	96
2	52	2	2	8888888	200	550	21	200	97
2	49	3	2	88888		330	22		98
1	55	2	3	1	470	600			99
1	40	3	1	5	460	850	5		100
2	70		2	88					101
2	48	2	1	8		900	7		102
1	49	2	1	3	501	700		600	103
1	46	4	1	2					104
1	45	3	1						105
1	62		2	5	500				106
1	67	3	2	3	507				107
1	46	2	3	2	503		9	350	108
1	35	3	1	0	620	800	6	675	109
1	54		2						110

Appendixl;Clinical Database

diagnosis	age	parity	menopaus	prev surg	ud capac	cysto cap	voids/24 ho	max diary \	id
1	45	4	3	2	500	700			111
1	54	3	2	3	620		6	625	112
1	71	1	2	1		550			113
1	43	3	1	0	500		6	550	114
3	29		1	0		500			115
2	80	2	2	82		600	16	200	116
2	33	0	1	3		700	11		117

Appendix II;Histology

diagnosis	capacity	epi denud	oedema	cong/ect	inflamm	haem	total score	id
2	600	1	0	0	0	0	0	2
3	520	2	0	1	1	0	2	3
1	800	1	0	0	2	0	2	4
2	750	3	0	2	2	2	6	5
3	680	2	0	1	1	0	2	6
2	400	3	2	2	2	2	8	7
1	600	2	1	1	0	1	3	8
1	600	1	1	1	0	1	3	9
1	900	1	1	1	0	1	3	10
2	600	2	1	0	0	0	1	11
1	700	2	0	1	1	1	3	12
1	550	1	1	1	0	2	3	13
1	600	1	0	1	0	1	2	14
2		1	0	1	0	0	1	15
1	550	1	1	0	0	0	1	16
1	610	1	1	1	0	0	2	17
1	600	1	1	0	0	1	2	18
1	600	1	0	0	0	1	1	19
1	600	2	0	0	0	1	2	20
1	600	1	0	0	1	1	2	21
1		2	1	0	1	1	3	22
1	1000	1	0	1	0	0	1	23
1	700	2	0	0	0	1	1	24
1	600	2	0	1	0	1	2	25
2	375	3	0	3	3	1	7	26
2	600	2	0	1	1	1	3	28
1	550	1	0	0	0	0	0	29
2	500	3	1	0	0	0	1	30
2	600	2	1	1	1	1	4	31
1	600	1	1	1	0	1	3	32
1	800	2	0	0	0	0	0	33
1	720	2	0	1	0	0	1	34
1	600	1	0	1	0	1	2	35
1	700	1	0	0	1	0	1	36
1	600	1	0	0	0	2	2	37
1	725	1	0	1	0	0	1	38
1	550	1	0	0	0	2	2	39
1	700	2	1	0	0	0	1	40
1	600	2	0	0	0	0	0	41
2	800	1	1	1	0	2	4	42
2	400	2	1	0	1	1	3	43
2	900	1	0	2	0	2	4	45
2	700	1	0	0	0	1	1	46
2	600	1	1	1	0	1	3	47
2	350	3	2	2	2	1	7	48
2	500	1	2	1	0	1	4	49
2		1	1	1	0	1	3	50
2	650	1	0	0	1	0	1	51
1	500	1	0	0	0	1	1	52
2	500	2	1	0	1	1	3	53

Appendix II;Histology

diagnosis	capacity	epi denud	oedema	cong/ect	inflamm	haem	total score	id
2		2	1	1	1	2	5	55
2	400	2	0	1	0	0	1	56
2		1	1	1	0	1	3	57
2		2	0	0	0	0	0	58
1	600	2	0	2	2	2	6	59
2			0	1	0	1	2	60
1	800	1	0	1	0	0	1	61
3	600	1	2	1	0	1	4	62
3	650	1	1	1	1	1	4	63
2	550	2	2	2	2	0	6	65
2	250	1	2	1	1	0	4	66
2	500	2	1	1	1	1	4	67
2	600	2	2	1	1	0	4	68
1	800	2	2	0	0	0	2	69
2	600	3	0	0	0	0	0	70
2	600	2	0	1	2	0	3	71
2	1000		1	0	1	0	2	72
2		2	1	0	0	1	2	73
1		1	1	1	0	0	2	74
1		2	1	0	1	0	2	75
1		1	0	0	0	0	0	76

Appendix IIIA;Oestrogen receptor Database

id	diag	sc1lo	sc2lo	sc3lo	sc4lo	sc1hi	sc2hi	sc3hi	sc4hi	age	meno
3	1	0	0	0	0	0	0	0	0	49	3
4	2	0	0	0	0	0	0	0	0	49	3
9	4	0	0	0	0	0	0	0	0	66	3
13	1	0	0	0	0	0	0	0	0	58	3
15	1	0	0	0	0	0	0	0	0	41	2
16	1	0	0	3	9	0	1	1	1	44	1
17	1	1	0	3	1	1	0	1	1	31	1
18	1	0	0	0	0	0	0	0	0	44	1
19	1	0	0	0	0	0	0	0	0	59	2
21	1	0	0	0	0	0	0	0	0	58	3
22	1	0	0	0	0	0	0	0	0	48	3
23	1	0	0	0	0	0	0	0	0	48	1
25	1	0	0	0	1	0	0	0	0	52	3
27	1	0	1	8	4	0	0	1	0	64	3
28	1	0	0	0	0	0	0	0	0	56	2
29	1	0	0	0	0	0	0	0	0	47	3
30	1	1	3	0	2	0	0	0	0	61	3
31	1	0	0	0	0	0	0	0	0	52	2
32	1	4	5	3	4	0	0	0	0	63	3
34	1	10	10	3	5	0	1	0	1	50	0
35	1	8	9	11	17	2	2	5	7	39	1
36	1	8	2	0	0	0	0	0	0	80	3
37	1	6	8	2	3	1	0	0	0	50	3
38	1	0	0	0	0	0	0	0	0	61	2
41	2	0	3	2	0	0	0	0	0	64	2
44	2	0	0	3	0	0	0	0	0	28	1
46	2	0	0	0	0	0	0	0	0	68	2
50	2	0	0	0	0	0	0	0	0	40	1
52	2	3	1	2	3	0	0	1	0	35	1
53	2	0	0	0	0	0	0	0	0	61	2
54	2	8	7	8	6	0	1	0	1	60	2
55	2	1	1	0	0	0	0	0	0	38	1
56	2	0	0	2	2	0	0	0	0	37	1
57	2	7	4	3	0	0	0	0	0	34	1
58	2	2	4	0	0	0	0	0	0	69	2
59	2	2	2	7	8	0	0	0	0	70	2
60	2	0	0	0	0	0	0	0	0	62	2
61	2	0	0	0	0	0	0	0	0	76	2
63	2	0	0	0	0	0	0	0	0	28	1
64	2	0	0	0	0	0	0	0	0	60	2
65	2	0	0	0	0	0	0	0	0	25	1
66	2	0	0	0	0	0	0	0	0	43	1
70	2	0	0	0	0	0	0	0	0	19	1
71	2	0	0	0	0	0	0	0	0	40	1
72	4	0	0	0	0	0	0	0	0	29	1
73	2	0	0	0	0	0	0	0	0	61	3
85	1	0	0	0	0	0	0	0	0	46	1
88	1	2	1	4	2	0	0	0	0	58	2
98	1	12	11	12	5	4	3	1	0	51	3
99	2	0	0	1	4	0	0	0	0	21	1
101	1	16	18	16	15	11	5	8	3	30	1
103	2	3	3	2	2	0	0	0	0	33	1
106	2	6	3	7	5	3	1	4	2	31	1

Appendix IIIA;Oestrogen receptor Database

id	diag	sc1lo	sc2lo	sc3lo	sc4lo	sc1hi	sc2hi	sc3hi	sc4hi	age	meno
107	2	0	0	6	5	0	0	0	0	52	2
1592	3	3	3	5	6	0	2	1	1		
2771	3	4	5	9	4	1	1	1	0		
3351	3	7	3	14	7	2	1	0	1		
3451	3	0	0	0	0	0	0	0	0		
3707	3	1	2	5	7	0	0	0	0		

Appendix IIIB: Progesterone receptor Database

id	diag	sc1low	sc2low	sc3low	sc4low	sc1high	sc2high	sc3high	sc4high
3	1	0	0	0	0	0	0	0	0
4	1	0	0	0	0	0	0	0	0
9	1	0	0	0	0	0	0	0	0
15	1	0	0	0	0	0	0	0	0
16	1	0	0	0	0	0	0	0	0
17	1	0	0	0	0	0	0	0	0
18	1	0	0	0	0	0	0	0	0
19	1	0	0	0	0	0	0	0	0
21	1	0	0	2	1	0	0	0	0
22	1	0	0	0	0	0	0	0	0
23	1	3	3	0	0	2	1	0	0
25	1	0	0	0	0	0	0	0	0
26	1	0	0	1	0	0	0	0	0
27	1	7	4	8	4	2	0	1	1
28	1	0	0	0	0	0	0	0	0
29	1	0	0	0	0	0	0	0	0
30	1	0	0	0	0	0	0	0	0
31	1	7	8	11	8	0	0	0	0
32	1	0	0	0	0	0	0	0	0
34	1	9	11	6	14	2	0	3	3
35	1	28	20	30	31	17	11	14	16
36	1	9	9	3	4	1	0	0	3
37	1	18	13	8	9	6	3	5	6
38	1	0	0	0	0	0	0	0	0
41	2	10	5	4	4	4	4	1	1
44	2	3	5	0	0	0	0	0	0
45	2	0	0	0	0	0	0	0	0
47	1	17	18	6	20	5	5	3	6
50	2	10	6	3	2	6	1	0	0
52	2	0	3	1	0	0	0	0	0
53	2	7	3	2	4	0	0	0	0
54	2	10	7	0	0	0	0	0	0
55	2	0	1	0	0	0	0	0	0
56	2	6	6	1	0	0	0	0	0
57	2	0	0	0	0	0	0	0	0
58	2	8	3	5	7	2	0	1	1
59	2	3	0	5	6	0	0	1	0
60	2	0	1	0	0	0	0	0	0
61	2	0	0	0	0	0	0	0	0
62	2	1	0	1	1	0	0	0	0
63	2	0	0	0	0	0	0	0	0
64	2	0	0	2	2	0	0	0	1
65	2	0	0	0	0	0	0	0	0
67	2	6	4	7	9	1	0	3	2
70	2	8	2	3	5	0	0	1	3
71	2	0	0	4	3	0	0	0	0
85	1	37	21	24	22	9	5	5	10
88	1	3	2	1	0	1	0	0	0
93	1	1	0	0	0	0	0	0	0
98	1	4	0	0	0	0	0	0	0

Appendix !!IB: Progesterone receptor Database

Id	diag	sc1low	sc2low	sc3low	sc4low	sc1high	sc2high	sc3high	sc4high
103	2	4	9	11	8	0	0	0	1
106	2	13	16	16	12	7	12	8	6
107	2	20	12	20	35	13	9	5	7
1592	3	8	7	11	6	8	4	6	4
2771	3	8	6	11	11	3	0	7	11
3351	3	5	5	14	12	5	8	7	3
3451	3	16	14	30	19	8	6	10	4
3707	3	11	27	24	11	5	4	9	4

Appendix IV; pre and post histology database

Id	diag	preepi	postepi	preo	posto	prec	postc	prei	posti	preh	posth	pretot	posttot	cap
1	2	3	3	2	2	0	1	3	2	0	0	5	5	480
2	2	2	1	2	3	2	3	3	3	0	2	6	11	350
3	2	1	1	2	3	1	0	1	2	0	0	4	6	350
5	2	2	2	1	2	0	1	2	2	0	0	3	5	350
6	1	2	1	2	0	2	2	2	0	0	0	6	0	
7	1	2	1	2	1	2	0	0	1	2	0	6	2	1000
8	1	1	1	1	0	1	2	1	1	1	0	4	3	
9	1	2	2	1	2	3	1	0	1	0	0	4	4	
10	2	1	1	0	0	0	0	1	0	0	0	2	0	700
11	1	2	2	3	3	3	3	1	1	2	2	9	9	600
12	2	2	2	2	2	1	2	1	0	2	3	6	7	400
13	1	1	1	0	1	0	0	0	1	0	0	0	1	
14	2	2	2	0	0	1	0	3	2	0	0	4	2	650
15	1	2	1	1	1	2	2	1	1	3	3	7	7	
16	2	1	2	2	2	1	2	2	2	0	1	5	7	200
17	1	1	1	0	2	1	2	0	1	0	2	1	7	800
18	2	1	1	1	2	1	1	0	0	1	2	3	5	
19	1	1	1	2	1	1	1	0	1	0	1	2	4	800
20	2	2	2	2	1	0	3	0	1	3	2	5	7	
21	2	1	1	0	1	0	0	0	0	0	0	0	1	620
22	1	1	1	0	1	3	1	1	1	0	1	4	4	
24	1	1	2	0	0	1	0	0	1	0	0	1	1	
27	2	3	3	3	3	2	2	2	2	2	2	9	9	180
28	2	1	1	0	1	0	0	0	0	0	1	0	3	500

Appendix V; Bradykinin 1 receptor database

Field1	Field2	Field4
patient no	ic or control	bk1 J Scurry
3	1	0
9	1	0.5
15	1	0.5
16	1	0.5
17	1	2
18	1	0
21	1	3
22	1	0
25	1	0
26	1	1.5
27	1	2
28	1	1
29	1	0
30	1	1
31	1	0
32	1	3
34	1	0
36	1	0
37	1	0
38	1	1
41	1	3
44	2	3
46	2	3
48	2	1
50	2	3
51	2	1
52	2	1
55	2	1
56	2	0
57	2	0
58	2	0
59	2	3
60	2	0
61	2	3
62	2	0
64	2	1
65	2	1
66	2	1
67	2	3
70	2	1
71	2	1
73	2	2.5
85	1	3
88	1	1
93	1	2
98	1	1
101	1	3
102	2	2.5
103	2	3

Appendix V; Bradykinin 1 receptor database

Field1	Field2	Field4
104	2	1
106	2	3
107	2	3
108	2	2

APPENDIX VI

REAGENTS; BUFFER RECIPES

REAGENTS

Unless otherwise indicated general chemical reagents, such as MaC1, ethanol etc, were supplied by either BDH (Poole, UK), Ajax Chemicals (Auburn, VIC) or Sigma (Castle Hill, NSW).

BUFFER RECIPES

PBS (Phosphate buffered saline)

Na2HPO4	1.26 g
NaH2PO4	0.26 g
NaCl	7.6 g
Add Distilled water up to	1 litre

Check pH = 7.4

CITRATE BUFFER

tri-Sodium Citrate	2.94 g
citric acid	0.38g
Distilled water→	1 litre

Check pH = 6

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