Understanding mechanisms of Ketamine-induced human urinary tract damage

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Abstract

Ketamine is a phencyclidine derivative N-Methyl D-Aspartate (NMDA) receptor antagonist used as an anaesthetic and analgesic. A florid non-bacterial cystitis condition known as ketamine-induced cystitis (KIC) has recently been reported in abusers and prescription users of ketamine. Whilst the aetiological agent in patients withKIC is known, the mechanism of disease development is unknown.

The aims of this study were to investigate the potential mechanisms of development of KIC and to establish potential links between KIC and other chronic non-bacterial cystitis conditions; interstitial cystitis (IC) and eosinophilic cystitis (EC).

These aims were pursued under the following objectives;

1. Investigate toxicity of ketamine and its primary metabolite nor-ketamine on normal human urothelial (NHU) cell cultures.
2. Describe human urothelial tissue damage caused by ketamine.
3. Assess a possible relationship between ketamine exposure and development of KIC.

The main findings were that;

Ketamine and nor-ketamine were equally toxic to NHU cells in a concentration dependent manner.

A specific NMDA receptor antagonist (MK-801) did not show any urothelial toxicity on its own but unexpectedly increased toxicity of ketamine when used in combination.

Immunolabelling of the three individual KIC tissue sections demonstrated epithelial denudation and stromal inflammation with oedematous blood vessels.

Results from a telephone questionnaire did not show any obvious relationship between prescription use of ketamine and development of KIC.

Literature review suggested considerable similarities between the reported symptoms and clinical investigation findings of KIC to mainly EC and to some extent to IC.

In conclusion, the study indicated that ketamine might cause human urinary tract damage by impairing the barrier function of urothelium, inducing inflammation and resultant development of KIC.

These experiments should form the basis for future research in to understanding the mechanisms of development of KIC and other difficult to treat chronic uropathies such as EC and IC.

Contents

Figures

Tables

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Author's declaration

The author would like to confirm that the work submitted is his own and that appropriate credit has been given to others where due.

1   Introduction

1.1 Ketamine-induced cystitis (KIC)

1.1.1 History of Ketamine

Ketamine is a phencyclidine derivative, first synthesised by Stevens in 1962 at Parke-Davies laboratories (now part of Pfizer) and first used in humans by Corssen and Domino in 1965 [1]. In 1970, the United States Food and Drug Administration (FDA) approved ketamine for human use, and since then it has been used in the induction and maintenance of anaesthesia [2]. The first wide scale use of ketamine was on American soldiers in the Vietnam War. Recently, it is also increasingly used for the management of chronic pain ([3-5] and reviewed by [6]) and is under research to develop newer faster acting anti-depressants (reviewed by [7]). It is also used in the veterinary medicine and is commonly applied as a horse tranquilliser.

Ketamine is fast becoming a popular recreational drug due to its easy availability and low prices. Anecdotal reports suggest that it is overtaking Ecstasy as the abused drug of choice on the dance and rave party scene. The main reason for this is that whereas Ecstasy leads to euphoria and other unwanted symptoms, ketamine abuse leads to a state of calm, cloudiness of memory and amnesia in association with an out of body experience known as the K-hole phenomenon. In this state, drug abusers have reported feelings of travelling through a light tunnel, near death experiences and speaking to God.

On the streets, ketamine is known by the names of 'Special K', 'Kit Kat', 'Cat Valium', 'Vitamin K' or just 'K'. The routes of abuse include ingestion, inhalation and intravenous injections.

Ketamine is an illegal drug in the UK and was labelled as a Class C drug on 1st January 2006. Due to the illegal nature of ketamine, the true number of drug abusers using it may never be known (reviewed by [8]).

1.1.2 Pharmacology of Ketamine

Ketamine is used in clinical practice for its N-Methyl D-Aspartate (NMDA) receptor antagonist effect on neuronal cells which produces anaesthesia. It does not adversely affect the patient's cardiovascular status during induction and is therefore the anaesthetic of choice in patients with haemodynamic instability.

In addition to the NMDA receptor, it has promiscuous effects on multiple other binding sites including non-NMDA glutaminergic, monoaminergic, opioid, nicotinic and muscarinic cholinergic receptors (reviewed by [9]). Whereas the anaesthetic dose of ketamine is 0.5–2 mg/kg, recreational users report development of tolerance with continued use and admit to using up to 20 g/day (reviewed by [8]), and anecdotal reports of using up to 28g/day. This signifies the development of tolerance in recreational users abusing it in high doses.

Ketamine is water soluble, has a pKa of 7.5 and a fat solubility 10 times that of thiopentone. It is metabolised by cytochrome p450 in the liver in to nor-ketamine (primary metabolite) and dehydronor-ketamine (secondary metabolite). Its redistribution half-life is 4.68 minutes. The excretory route is urine with an elimination half-life of 2.17 hours.

1.1.3 General signs/symptoms from ketamine use

Like all other anaesthetics, ketamine affects almost every system in the body. Its effects on different human physiological systems include:

- **Cardiovascular:** tachycardia, hypertension
- **Respiratory:** bronchodilation
- **Neurological:** hallucinations, amnesia, mental clouding, loss of memory, impaired attention, out of body experiences (K-hole effect)
- **Gastrointestinal:** nausea
- **Physical features:** paralysed feeling, numbness
- **Muscles:** dystonia

Although some of these effects by ketamine are unwanted and bothering for patients in clinical practice, it still has wide applications in the induction

of anaesthesia, intensive care, sedation, paediatrics, bipolar disorders and to relieve bronchospasm in patients with asthma and chronic obstructive airway disease.

1.1.4 Urological signs/symptoms from ketamine abuse/use

Ketamine has been used for recreational purposes for many decades [10]. Although the World Health Organisation (WHO) Expert Committee meeting on Drug Dependence held in Geneva in 2003 raised concerns about the use of ketamine as a recreational drug and recommended a critical review [10], it was only around 2007 that reports of human urothelium damage from its abuse started appearing in literature [11]. Since then, more than 20 articles (case series, case reports, review and letters to the editors) have been published in the literature related to ketamine-induced cystitis (KIC) (reviewed by [12]).

These reports from around the world have described development of non-bacterial cystitis like symptoms not only in patients abusing high doses of ketamine [2, 13], but also in patients using it in much lower prescription doses for pain control [3, 14]. In a recently published study of recreational abusers of ketamine, 20.0% of frequent users (> 4 times per week), 6.7% of infrequent users (2 times per month, <4 per week) and 13.3% of ex-users (abstinent from ketamine for a minimum of 84 days), spontaneously cited cystitis or bladder problems as being an experience or concern for them [15].

At what time bladder symptoms typically start after ketamine abuse is unknown, but the time period may be from one month to several years [13]. Symptoms show a relationship dependent on dose and duration of abuse and this may signify that whereas the urinary tract recovers after initial injury, continuous abuse leads to permanent irreversible scarring/damage.

The urinary signs and symptoms due to ketamine abuse/use reported in literature include:

1.   Dysuria [2, 13, 16-18]

2. Suprapubic pain [3, 18]
3. Nocturia [18]
4. Urinary frequency [2, 13, 16-18]
5. Urinary urgency [2, 13, 16, 17]
6. Urge incontinence [2, 16, 18]
7. Haematuria [2, 13, 16-18]

There are differing reports in the literature about the course of symptoms following abstinence from ketamine. Some authors have suggested that abstaining from ketamine improves the symptoms [3, 13, 14, 17], whereas others suggest that illicit ketamine use may lead to severe urinary tract problems that can be irreversible even after ketamine abuse has ceased [19]. There is another group of clinicians who have reported improvement in some, worsening in some and stable symptoms in others after abstinence [18, 20, 21].

1.1.5 Aetiology of ketamine-induced cystitis (KIC)

Although the causative agent in patients with KIC is known, the aetiopathological mechanism of disease development remains unknown. A number of different theories have been proposed by Chu et al. [2] including:

1. Direct effect of ketamine or its metabolites on bladder interstitial cells.
2. Microvascular changes in the bladder and possibly kidney by ketamine and/or its metabolites.
3. Indirect effect of ketamine by causing an autoimmune reaction against the bladder urothelium and submucosa due to circulating ketamine or urinary ketamine and its metabolites.
4. Bacteriuria; Bacterial infection as the cause of KIC is unlikely as all patients in the study by Chu et al. had abacterial cystitis on presentation. The positive bacterial culture in the subsequent urine tests of two patients signified concomitant bacterial UTI instead of a genuine cause of the initial urinary symptoms. None of the patients in

the study showed any improvement in ketamine-related bladder symptoms after treatment with antibiotics.

1.1.6 Clinical investigation findings in KIC

Literature reports of different investigation findings in patients with KIC include:

- **Radiological investigations:**
1. Hydronephrosis; in one study of 11 patients, 5 had bilateral hydronephrosis, 4 had no hydronephrosis and in 2 patients the renal effects were unknown [13]. In another study of 59 patients, 26 had bilateral and 4 had unilateral hydronephrosis [2]. In a third study, 14% of patients with history of ketamine abuse presented with renal impairment and hydronephrosis [18]. The first 2 studies reported a higher incidence of upper urinary tract changes from ketamine abuse than reported in the third study. Of note is the fact that the first two studies (Tsai et al. [13] and Chu et al. [2]) were published from the South East Asia (Taiwan and Hong Kong respectively), whereas the third study (Mason et al. [18]) is from the UK. This might signify either racial differences in ketamine effects on the urinary tract or different combinational effects due to different cultures (e.g. drug, alcohol, diet or environmental factors) or differences in the drug supply with different additives in different parts of the world.
2. Small capacity bladder
3. Papillary necrosis [2]
- **Urodynamics:**
   Small capacity bladder [2, 13]
- **Cystoscopy:**
   One or more of epithelial inflammation, neovascularisation, petechial (pinpoint) haemorrhages and erythematous bladder mucosa [2].
- **Histology:**
1. Denuded epithelium [2, 16, 17]

2. Infiltration of tissues with granulocytes, predominantly eosinophils [13, 16, 18]

3. Infiltration of tissues predominantly by lymphocytes and a variable number of eosinophils [2].

4. Infiltration of tissues with mast cells [13]

5. Infiltration of tissues with granulation tissue [2, 17]

6. Urothelial atypia (nuclear enlargement and disorganisation) [16, 18]

- **Immunohistochemistry:**

1. High expression of p53 [16, 18]

2. High expression of Ki67 [16, 18]

3. Absence of CK20 expression [16, 18]. This means that although reactive atypia can be found in KIC samples, the negative CK20 rules out the possibility of carcinoma in situ [22].

1.1.7 Management of KIC

Patients with KIC are difficult to manage. Uncooperative behaviour and missed clinical appointments is common among drug abusers and the same trend is followed by patients abusing ketamine. Unfortunately, there is no definite treatment therapy other than symptomatic treatment for patients who do seek medical attention.

Abstaining from ketamine is the first step, which is not always easy. This is because although patients with KIC have troublesome urinary symptoms, they might continue abusing ketamine as the most effective way of suppressing their symptoms.

There is no definite medical therapy in place but different clinicians have tried different medications including anti-cholinergics and protamine sulphate with limited temporary success.

Surgical interventions include cystodistension initially. This intervention either does not relieve symptoms or does so temporarily. Unfortunately, the ultimate treatment option for these patients with permanent bladder damage is either bladder augmentation or urinary diversion procedures in the form of ileal conduit or ureterotomy with or without cystectomy. If the

patients continue to abuse ketamine after surgical intervention, they risk damaging their reservoir and upper renal tracts.

1.2 Non-bacterial cystitis

Non-bacterial cystitis is a permanent diagnostic dilemma for clinicians and includes interstitial cystitis (IC) and eosinophilic cystitis (EC), among other conditions.

1.2.1 Interstitial Cystitis

Interstitial Cystitis (IC) is a debilitating inflammatory disease of the urinary bladder which presents with lower urinary tract symptoms (LUTS) and, is marked by episodes of flare-ups and remissions. IC is sometimes used synonymously with the term Painful Bladder Syndrome (PBS). Whilst PBS is a condition of any bladder pain for which an apparent cause is not found, IC reflects a subset of PBS and includes patients who meet a strict diagnostic criterion established by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). In a recent meeting by the Bladder Pain Syndrome committee of the International Consultation on incontinence, it was suggested that IC should be called Bladder Pain Syndrome (BPS), as the term is more descriptive of the clinical condition (reviewed by [23]).

There are no definite figures of prevalence for interstitial cystitis and different population groups are reported to experience different incidence rates. In a review by Kelada et al. [24], the authors suggest that the heterogeneous data on the prevalence and incidence of IC is due to a lack of uniform diagnostic criteria and universal markers.

There are different questionnaires in use to screen for IC, including the O'Leary-Sant symptom and problem index; the pain, urgency, frequency symptom scale; and the University of Wisconsin IC scale. While these questionnaires help to distinguish IC from other urinary tract pathologies, they do not demonstrate sufficient specificity to serve as the sole diagnostic indicator [25]. Differences in interpretation of these

questionnaire results may be another reason for varying prevalence data. Despite this, almost all studies agree that IC is more prevalent in females.

1.2.1.1 Aetiology of IC

Despite extensive research of IC, no single aetiological cause has been identified. There are multiple theories to explain development of IC with limited evidence for each and they include:

1. Compromised urothelial barrier ([26] and reviewed by [24, 27-29]).

2. Microbiological ([30] and reviewed by [24, 27, 28, 31, 32]); although one of the NIDDK diagnostic criteria for IC is the absence of positive urine cultures. Hanno et al. (reviewed in [33]) suggested that the reason for this may be that the causative bacteria in IC are fastidious or present in small numbers. However, there are studies in literature which did not find any infectious agents in patients with IC [34, 35].

3. Increased number of mast cells in the bladder wall (reviewed by [27, 28, 36]); upon activation these degranulate and release pre-formed mediators of inflammation such as histamine, heparin and tryptase.

4. Neurogenic inflammation (reviewed by [24, 31, 32]); according to this theory, there are increased number of mast cells at nerve endings in the bladder of patients with IC. When triggered these mast cells release various vasoactive substances causing inflammation of nerve endings in the bladder and symptoms of IC.

5. Genetic susceptibility to the development of IC [37].

6. Abnormal cell signalling pathways (reviewed by [38]); according to this theory, there is altered cell signalling in patients with IC resulting in altered cell proliferation, differentiation, and tight junction protein expression and assembly.

7. Anti-proliferative factor (reviewed by [38]); according to this theory, bladder epithelial cells in patients with IC produce an anti-proliferative factor which possibly causes thinning of bladder epithelium, altered production of growth factors and increased paracellular permeability with decreased tight junction formation.

8. Allergic disorder ([39] and reviewed by [27]).

9. Hormonal factors (reviewed by [24]); it was postulated that the fact that most IC patients are women, suggests that the process involving mast cells may be hormonally influenced by oestrogens [40].

10. Immunological factors or auto-immunity (reviewed by [24, 27, 31, 32]); the basis for this theory is the increased association of IC with certain chronic immunological diseases such as SLE, Scleroderma, Fibromyalgia and Sjogren's syndrome ([41] and reviewed by [28]). The patients with IC may have auto-antibodies against mucosal or muscle cells, or various other connective tissue components of the bladder [41, 42]. Also, there is unexplained association with chronic disease or pain syndromes such as irritable bowel syndrome, skin sensitivity, vulvodynia, fibromyalgia, migraine, endometriosis and chronic fatigue syndrome [43].

11. Bladder microvascular damage resulting in decreased microvascular density in the suburothelium [44].

12. Toxic urine components ([26] and reviewed by [28]).

13. Unidentified agents (reviewed by [24, 31]).

1.2.1.2 Signs/Symptoms of IC

The common signs/symptoms reported in literature in patients with IC include:

1. Bladder/suprapubic pain ([39, 45] and reviewed by [23, 24, 27, 28, 31, 32, 46]). Patients usually have a characteristic pain which is associated with bladder filling and relieves with voiding.

2. Chronic pelvic pain (reviewed by [27]).

3. Dyspareunia (reviewed by [27]).

4. Urinary frequency ([39, 45] and reviewed by [23, 24, 27, 28, 31, 32, 46]).

5. Nocturia ([45] and reviewed by [24, 31, 32]).

6. Urinary urgency ([45] and reviewed by [23, 24, 27, 28, 31, 32, 39, 46]).

1.2.1.3 Clinical Investigation Findings in IC

- **Radiological investigations:**

There are no specific radiological findings in patients with IC but long standing IC may lead to small capacity bladder with upper renal tracts involvement.

- **Urodynamics:**

The role of urodynamics in patients with IC is one of excluding other diseases. This is because patients with IC do not have any specific features demonstrated by urodynamics.

- **Cystoscopy:**

Other than features of inflammation, cystoscopy in patients with IC may demonstrate the 2 classical features;

1. Glomerulations (upon hydrodistension, pinpoint haemorrhages or fissures appear in bladder called glomerulations, found more frequently than Hunner's ulcer).

2. Hunner's ulcer (found less frequently) at bladder dome (reviewed by [27]).

- **Histology:**

There are no definitive diagnostic characteristics or markers for this disease (reviewed by [24]) and the role of histopathology in the diagnosis of IC is primarily one of excluding other possible pathologies (reviewed by [23, 24]).

Rosamilia et al. [47] reviewed the literature and found epithelial denudation or ulceration, mononuclear inflammation, oedema, congestion, haemorrhages, and mast cell activation to be the most common findings in the histopathology of IC patients. However in the same paper the authors suggest that these pathological changes were not universal or specific; with 55% of IC subjects in one study having histology that was normal and indistinguishable from control subjects (doctoral thesis referred to in [47]). The histopathological findings most frequently described in the literature in patients with IC are;

1. Mucosal ulceration/absent mucosa ([48, 49] and reviewed by [50]).

2. Submucosal oedema ([44, 51] and reviewed by [50]).

3. Vasodilatation ([51, 52] and reviewed by [50]).

4. Mucosal haemorrhage [49].

5. Lymphocytic and plasma cell infiltrate [49, 52].

6. Submucosal inflammation [48].

7. Granulation tissue infiltration [49].

8. Increase in mast cells (reviewed by [47]).

The notable thing about histopathological findings related to IC is that almost all of these features possibly reflect chronic tissue damage rather than a specific disease process i.e. late stage damage and, reaction to loss of urothelial barrier and chronic exposure to urine.

- **Immunohistochemistry:**

1. From a study published by Southgate et al. [53], the results of immunohistochemistry studies performed on areas of full thickness urothelial samples from patients with IC and urothelium from patients with other benign conditions (idiopathic detrusor over-activity and stress urinary incontinence) indicated no gross difference, with the possible exception of Claudin 5 down-regulation in one IC specimen.

2. There is also little evidence of proliferation being driven in situ in IC specimens, as seen from the lack of Ki67 positive cells by immunohistochemistry [53].

1.2.1.4 Management of IC

Management of patients with IC is an on-going challenge for clinicians. As mentioned before, most of these patients are diagnosed late after excluding other diseases due to the lack of objective specific biomarkers. This means that most of these patients have irreversible bladder damage by the time of diagnosis and are quite difficult to manage.

The conservative treatment includes advising patients about life style changes including fluid management. Medical treatment mainly includes analgesics and anti-cholinergics. If all else fails, surgery in the form of bladder augmentation or urinary diversion procedures is offered.

1.2.2 Eosinophilic Cystitis (EC)

Eosinophilic cystitis (EC) is a relatively uncommon inflammatory disease of the urinary tract. It is characterised by the presence of troublesome lower urinary tract symptoms and eosinophilic infiltration of involved urinary bladder tissue. Although patients with EC present with non-bacterial cystitis symptoms similar to IC, it is a distinct clinical disease.

In a review article in 2009, Ebel Sepulveda et al. reported that there are less than 200 reported cases of EC in the world [54]. EC has been reported at all ages (5 days to 87 years), with 21% of cases occurring in children in one series [55]. There are differing literature reports about its incidence among adult males and females. Popescu et al. [56] suggested in their case series and literature review that EC is more common in females (in adult population) and that the equal sex incidence reported by Itano et al. [57] and Van Den Ouden et al. [55] actually reflects study group composition rather than actual incidence rates. In children, boys are affected more often than girls [55].

1.2.2.1 Aetiology of EC

Although different theories have been proposed to explain the aetiopathology, there is no conclusive evidence for a single cause. These include:

1. Allergy due to food [58].
2. Allergy to iodine, anaesthetic ointments, condom antigens or contraceptive jellies [59].
3. Allergy to chromated catgut suture [60].
4. Immunological factors (reviewed by [54]).
5. Parasitic infestation of bladder [61-63].
6. Medications including salicylazosulfapyridine, tranilast, coumadin, intravesical thiotepa and mytomycin ([64-66] and reviewed by [67, 68]).
7. Prostatic hyperplasia, and surgeries or tumours of the bladder and prostate ([55] and reviewed by [69-71]).

1.2.2.2 Signs/Symptoms of EC

The following signs and symptoms due to EC have been reported in the literature;

1. Suprapubic/abdominal pain ([72] and reviewed by [55, 56]).

2. Dysuria (reviewed by [55, 56]).

3. Macroscopic/microscopic haematuria ([72] and reviewed by [55, 56]).

4. Urinary frequency ([72] and reviewed by [55, 56]).

5. Urinary urgency ([72] and reviewed by [56]).

6. Urge incontinence (reviewed by [56]).

7. Less commonly; urinary retention (reviewed by [55, 56]), gastrointestinal symptoms such as vomiting, diarrhea, tenesmus (feeling of incomplete evacuation) and bloody stools, fever, skin rash, pneumaturia and nocturnal enuresis (reviewed by [56]).

1.2.2.3 Clinical investigation findings in EC

- **Radiological investigations:**

The radiological investigations might show;

1. Bladder tumour associated with EC [72].

2. Less commonly upper renal tract involvement (in 1 out of 8 patients reported by Lin et al. [73] and in 1 out of 17 cases reported by Itano et al. [57]).

- **Urodynamics:**

The role of urodynamics in patients with EC (similar to IC) is to exclude other diagnosis.

- **Cystoscopy:**

1. In most cases, cystoscopy shows oedematous bladder mucosa ([73] and reviewed by [56]).

2. It might also show bladder tumour associated with EC [72].

- **Histology:**

1. Bladder biopsy of involved tissue in EC characeriscally demonstrates eosinophilic infiltration ([72] and reviewed by [74, 75]). Lower urinary

tract symptoms with histology demonstrating eosinophilic infiltration of the bladder tissue are the gold standard for diagnosing EC.

2.   Mast cells and plasma cells in chronic phase [76].

- **Immunohistochemistry:**

There are no literature reports to date of immunohistochemistry studies on EC tissue.

1.2.2.4 Management of EC

There is no definitive treatment regime for patients with EC. Eosinophilic infiltration of the biopsy specimen is one of the main diagnostic criterion and eosinophils are known to be associated with allergy. The conservative treatment strategy is therefore based on avoiding exposure to any allergens and treatment with antihistamines. Steroids and antibiotics have been tried as a medical treatment with limited success. Surgical options include removal of bladder tumour which may be the cause of symptoms as proposed by Verhagen et al. [72], bladder augmentation procedures or urinary diversion procedures.

1.3 Discussion

The gap in knowledge about the aetiology of IC and EC has meant that no effective research models have been developed and neither can we develop any specific biomarkers for clinical diagnosis. This has eventually translated in to the inability to develop any effective treatment regimens for either condition.

Most models to study IC have been based on inducing acute urothelial damage by treatment with drugs such as cyclophosphamide [77]. In a recent article by Altuntas et al., authors have described a method of inducing autoimmune cystitis in an animal (mice) model over a period of weeks by immunization of mice with recombinant mouse uroplakin II (rmUPK2) [78]. This is different and a better IC experimental animal model than the previous ones based on drug-induced cystitis in that cyclophosphamide induces cystitis within hours and is not as such

representative of the disease as IC is a chronic inflammatory condition which develops overs months to years.

Ketamine causes urinary tract damage in patients over months to years and the course of development of KIC is much similar to IC. Clinical presentation in patients with KIC, IC and EC is with lower urinary tract symptoms. KIC as the cause of patient's symptoms is only indicative once a drug history of ketamine use/abuse is given by the patient.

Another condition where a history of ketamine use/abuse is of utmost importance is in patients with suspected carcinoma in situ (CIS) of the bladder. CIS is a flat, non-invasive but high grade cancerous lesion which is confined to the superficial lining of the bladder. Unlike most other CIS, bladder CIS is fairly aggressive and urine samples in almost 90% of the patients demonstrate malignant cells. Patients with this condition present with LUTS similar to the patients with KIC. Oxley et. al. conducted an immunohistological study on 10 KIC biopsies and reported that patients with both KIC and CIS demonstrated nuclear enlargement and disorganisation but the patients with KIC were negative for CK20 staining suggesting reactive atypia only and no CIS [16]. Whether chronic inflammation from ketamine use/abuse would eventually lead to the development of bladder cancer or not is currently unknown as KIC is a relatively new condition and no long term follow-up studies exist.

The clinical and investigation similarities between KIC, IC and EC may mean that the mechanisms behind development of these conditions are similar in some respect as well. If this is true, it will further mean that understanding the mechanism of disease development in one illness may give an insight in to the mechanisms of development of disease in the other condition. KIC can be used as a model for this purpose and although the mechanisms of disease development in KIC are unknown at present, the benefit in using KIC as a research model will be that the causative agent is known.

There are established procedures to grow normal human urothelial (NHU) cells in vitro [79] and these can be used to investigate the possible

mechanisms of disease development in patients with KIC. The advantage in using NHU cells will be that they are not compromised by the in vitro propagation and retain the capacity to contribute to a functional epithelium [79]. This method will therefore provide an adequate model to conduct the research project as an in vivo study involving patients may not be possible due to the ethical constraints associated with knowingly giving someone ketamine to cause urinary tract damage.

1.4 Aims

1. **Primary aim** of the research was to examine potential mechanisms behind development of ketamine-induced human urinary tract damage.
2. **Secondary aim** of the research was to investigate potential links between ketamine-induced cystitis, interstitial cystitis and eosinophilic cystitis.

1.5 Objectives

1. To establish the toxic doses of ketamine for normal human urothelial (NHU) cells grown in culture.
2. To establish whether ketamine or its primary metabolite nor-ketamine is the main toxic agent.
3. To establish whether there is a dose response relationship between ketamine and NHU cell damage.
4. To assess whether the NMDA antagonist effect of ketamine is responsible for human urothelial cell toxicity.
5. To describe urothelial damage caused by ketamine by assessing proliferation, differentiation and inflammatory changes in human KIC tissue.
6. To investigate clinically a possible relationship between analgesic ketamine use and development of KIC symptoms.

2   Materials and Methods

The research on samples from ketamine users was approved by the Northern and Yorkshire research ethics committee (REC reference number 10/H0903/43).

It was a multicentre study and appropriate Research and Development (R&D) approval and material transfer agreement (MTA) was additionally obtained at each site before transporting the tissue to the Jack Birch Unit of Molecular Carcinogenesis, Department of Biology, University of York where all the experiments were carried out.

The NHS trusts participating in this study were;

1.   James Cook University Hospital, Middlesbrough (R&D reference No. 2010081)

2.   Southmead Hospital, Bristol (R&D reference No. 2537)

All the tissue samples were taken after full informed patient consent. Samples were encoded with a research number and patient confidentiality was maintained at all stages of the research.

All cell biology experiments were performed on normal human urothelial (NHU) cells isolated from normal (non-KIC) tissues under a different REC approval and grown as finite cell lines in culture by seeding in to Primaria tissue culture flasks using established procedures [79]. Cells were seeded in known numbers in 96-well plates, treated with different concentrations of drugs (ketamine, nor-ketamine and/or MK-801) as required for the experiment and plates were kept in a humidified incubator at 37ºC in 5% CO_2 in air. Cell biomass was assessed using Alamar blue (AB) assay. Ketamine used for the experiments was racemic, stable and had >99% purity as tested by thin layer chromatography. The purity of nor-ketamine was 100% and that of MK-801 was >99% as tested by high performance liquid chromatography (HPLC). Both nor-ketamine and MK-801 were stable at room temperature. Hydrogen-1 nuclear magnetic resonance (1H NMR) testing showed that both nor-ketamine and MK-801 were consistent with their structures (information from the manufacturer's websites; Sigma-Aldrich and Tocris).

All immunohistochemistry experiments were performed on formalin-fixed and paraffin wax-embedded tissues, and included control (positive and negative) slides.

Unless otherwise stated, all the cell biology and immunohistochemistry experiments were performed using the protocols detailed below.

2.1 Cell Biology

2.1.1 Passaging cells

1. Medium was carefully aspirated from the flask containing the adherent cell culture and 10ml of pre-warmed sterile EDTA (0.1% (w/v) in PBS) was added. The flask was placed in an incubator at 37°C until cells had rounded.

2. EDTA was aspirated off and one ml of TV was added to the flask, and rocked gently to ensure that the surface was covered. The flask was then placed back in the incubator at 37°C until cells had become mobile/lifted (flask was tapped outside the incubator if required to add mobilising/lifting of cells).

3. TI was prepared by adding 10ml of sterile KSFMC to one aliquot of TI and added to the flask. The solution was gently passed through the pipette several times to ensure a single cell suspension.

4. The suspension was then transferred in to a universal tube and centrifuged at 1500rpm for four minutes.

5. After centrifugation, supernatant was removed and the pellet was flicked to suspend.

6. KSFMC was added by gently pipetting up and down. Cells from one 100% confluent T75 flask were passaged in to two to three T75 flasks as per requirement for the experiment.

7. Flasks were then kept in a humidified incubator at 37°C and 5% CO_2 in air, in a tray cleaned with 70% ethanol. Medium was changed every two to three days.

2.1.2 Freezing cells

1. Cells were passaged up to step five as described above in section 2.1.1.
2. Freezing mix was added by gently pipetting up and down.
3. Cryo vials were labelled using a JBU number, date, cell line and passage number.
4. One ml of cell suspension was placed in each cryo vial.
5. The cells were then frozen by keeping them in the isopropanol box at -80°C overnight and then transferring to liquid nitrogen dewar for storage.
6. Cells from one 95-100% confluent T75 flask were frozen down in to three cryo vials.

2.1.3 Bringing cells up from freezing

1. The required cells were located from the initial stock reference book.
2. 10ml of pre-warmed KSFMC was placed in a universal container.
3. One T75 flask was labelled with date, cell type (NHU cells), passage number (one + the passage number at which cells were frozen) and my initials.
4. Cryovial was removed from the liquid nitrogen dewar and thawed under warm water to defrost.
5. One ml cell suspension from the vial was transferred to the universal container and the vial was rinsed with medium to ensure all cells were removed.
6. The universal was centrifuged at 1500rpm for four minutes.
7. The supernatant was carefully removed and the pellet was flicked to suspend.
8. Five ml of KSFMC was added by gently pipetting up and down.
9. Cells were transferred in to the T75 flask and further 10ml of KSFMC was added to the flask to make a total volume of 15ml.

10. The flask was placed in a humidified incubator at $37^\circ C$ and 5% CO_2 in air, in a tray cleaned with 70% ethanol. Medium was changed every two to three days.

2.1.4 Cell counting using the improved Neubauer haemocytometer

1. Cells were passaged up to step five as described above in section 2.1.1.
2. After centrifugation, supernatant was removed and the pellet was flicked to suspend.
3. 10ml of KSFMC was added by gently pipetting up and down to ensure good cell suspension in the medium.
4. Improved Neubauer haemocytometer and a cover slip were cleaned with 70% ethanol and dried. Upper surface of the haemocytometer was moistened by breathing to produce a film of water vapour and cover slip was pressed into the haemocytometer.
5. A drop of cell suspension was placed against the edge of the cover slip in the middle of the chamber using a pipette.
6. The haemocytometer was placed under a microscope and focussed on the grid.
7. Total number of cells in the four outer squares were counted and divided by four. The resulting number represented the number of cells in 0.0001ml medium

2.1.5 Preparing 96-well plates

1. Once cell number/ml was established as described above in section 2.1.4, the cell suspension was further diluted using KSFMC to obtain optimum cell concentration for the experiment.
2. 0.2ml of the pre-determined cell concentration was put in each well of a 96-well plate with 0.2ml sterile water in the wells around them to keep cells hydrated and plates were placed in a humidified incubator at $37^\circ C$ and 5% CO_2 in air for further experiments as planned.

2.1.6 Cell biomass assay

1. Alamar blue (AB) dye was prepared by diluting the dye 1:10 ratio with KSFMC (e.g. 10ml alamar blue dye prepared by mixing one ml AB with nine ml KSFMC).

2. To check cell viability, an appropriate quantity of AB dye was prepared.

3. 96-well plates were removed from the incubator and medium in the plates was removed by turning them upside down and shaking.

4. 0.2ml of prepared AB dye was put in each well with cells and also in one column without cells for reference.

5. 0.2ml sterile water was put in the wells around the AB treated wells to keep cells hydrated and plates were placed in a humidified incubator at $37^{\circ}C$ and 5% CO_2 in air.

6. The plates were then read after four hours using the ascent software previously calibrated to establish number of viable cells using the equation below [80];

1^{-21} x $a^{3\times8886}$ = b (where a = Alamar blue reduction and b = cell number).

2.2 Immunohistochemistry

2.2.1 Formalin fixation of tissues for paraffin wax embedding

1. Tissue samples were dissected to appropriate size in a Petri dish using a fresh scalpel (if needed).

2. Samples were placed into 10% (v/v) formalin in a labelled universal tube ensuring the samples were fully immersed with at least a three cm depth of formalin above the sample and incubated at ambient temperature for:

Sample Size	Fixation Period
<1cm^3	18 hours
1-3cm^3	48 - 72 hours

3. After fixation, formalin was replaced with the same volume of fresh 70% ethanol and the fixed tissue was stored until processed into paraffin wax.

2.2.2 Paraffin wax embedding of tissue specimens

1. Embedding cassettes were appropriately labelled and tissue was placed in them.

2. The cassettes were submerged in fresh 70% ethanol for 10 minutes on a shaker.

3. Cassettes were placed in absolute ethanol for 10 minutes on the shaker and repeated a further two times.

4. The cassettes were moved in to fresh isopropanol for 10 minutes on the shaker and repeated once.

5. The cassettes were put in fresh xylene for 10 minutes and repeated a further three times. Cassettes were taken out of xylene and excess xylene was removed by blotting thoroughly with a tissue.

6. The cassettes were put in four wax pots for 15 minutes each in the incubator at 55°C.

7. Tissue samples were then embedded in paraffin wax in the cassettes using moulds and left on the cool table to set.

8. Once the tissue had set in the wax, it was stored in a cool dry place.

2.2.3 Sectioning of paraffin wax-embedded tissue

1. Cassettes were left on cool table for about 30 minutes to 1 hour before sectioning.

2. Glass slides were labelled with appropriate tissue details.

3. A Leica RM2135 Microtome was used to cut tissue sections of five micron thickness

4. Sections were put in pre-warmed tissue flotation bath.

5. Sections were picked up on to the glass slides and left to air dry overnight.

2.2.4 Haematoxylin and Eosin staining of tissue sections

1. Labelled glass slides with tissue sections were placed on a pre-warmed slide heater for one hour and then air dried for 10 minutes.

2. Sections were de-waxed by placing slides sequentially in to; two xylene pots for 10 minutes each, two xylene pots for one minute each, three pots containing absolute ethanol for one minute each and finally a pot containing 70% ethanol for one minute.

3. The slides were washed under running tap water for one minute and placed in haematoxylin for one minute.

4. Slide rack was placed under running tap water for one minute and placed in Scott's tap water for one minute.

5. The slides were washed in running tap water for one minute and placed in eosin for 30 seconds.

6. The slides were washed in running tap water for the last time for one minute.

7. The tissue sections were re-hydrated by placing slides sequentially in to; a pot containing 70% ethanol for one minute, three pots containing absolute ethanol for one minute each and finally in to two pots containing xylene for one minute each.

8. The slides were then placed in to two pots of histoclear for 15 seconds each.

9. The tissue sections were mounted in DPX using cover slips and left to air dry overnight.

2.2.5 Antigen retrieval methods for immunoperoxidase experiments

Microwave (Citric Acid)

1. Slides were placed in 350ml of 10mM Citric Acid Buffer pH 6.0 in a pyrex dish and glass marbles were placed on each end of glass slide to keep it in place.

2. The dish was covered with cling film and holes were punched in the film to let steam out.

3. The dish was placed in a microwave for 13 minutes at full power and then placed on ice to cool down when finished.

Trypsinisation

1. 100ml of 0.1%CaCl$_2$ pH 7.8 was warmed to 37°C in the microwave.

2. 0.1g of trypsin was added to it and mixed thoroughly to dissolve the trypsin completely.

3. Slides were incubated in this 0.1% trypsin solution (w/v) in an incubator at 37°C for 10 minutes.

Trypsin and Microwave

1. 100ml of 0.1%CaCl$_2$ pH 7.8 was warmed to 37°C in the microwave.

2. 0.1g of trypsin was added to it and mixed thoroughly to dissolve the trypsin completely.

3. Slides were incubated in this 0.1% trypsin solution (w/v) in an incubator at 37°C for one minute and then microwaved in 10mM Citric Acid Buffer pH 6.0 for 13 minutes as described above.

2.2.6 Immunoperoxidase on Paraffin sections

1. Tissue sections were de-waxed as described above in section 2.2.4.

2. The slides were washed under running tap water for one minute and placed in100ml of 3% H$_2$O$_2$ for 10 minutes.

3. The slides were washed under running tap water for 10 minutes.

4. Depending on the manufacturer's recommendations for the antibody used, one of the antigen retrieval methods as described in section 2.2.5 was used.

5. The slides were washed in running tap water for one minute.

6. The slides were then placed in Shandon Sequenza using coverplates and the tissue sections were incubated at ambient temperature sequentially in;

a) 100μl of Avidin for 10 minutes.

b) Washed twice with TBS.

c) 100μl of Biotin for 10 minutes.

d) Washed twice with TBS.

e) 100µl of appropriate serum (normal rabbit serum for mouse primary antibody and normal goat serum for rabbit primary antibody) diluted 1:10 with TBS for five minutes.

f) 100µl of primary antibody at a predetermined dilution in TBS applied to positive slides and 100µl of TBS to negative slides for one hour.

g) Washed three times with TBS.

h) 100µl of biotinylated secondary antibody at a predetermined dilution in TBS dependant on the primary antibody (biotinylated rabbit anti mouse secondary antibody for mouse primary antibody and biotinylated goat anti rabbit secondary antibody for rabbit primary antibody) for 30 minutes.

i) Washed twice with TBS.

j) StrepAB/HRP complex prepared by mixing one drop of A and one drop of B (from Vectastain elite ABC kit) with 2.5ml TBS atleast 30 minutes before applying and 100µl applied to each slide for 30 minutes.

k) Washed twice with TBS and once with distilled water.

l) DAB solution prepared by mixing sigma fast DAB tablets with five ml of TBS thoroughly and 200µl of DAB solution applied to each slide for 15 minutes.

m) Slides were washed twice with distilled water and immediately placed in the staining rack.

7. Counterstaining was done in Mayer's haematoxylin solution (Sigma) for 10 seconds.

8. The slides were washed for one minute in running tap water.

9. Tissue sections were then re-hydrated and mounted in DPX as described above in section 2.2.4.

Visualization of low-expression targets (e.g. NGFR) was enhanced by use of the catalytic signal amplification (CSA) kit as recommended by the manufacturer (Dako).

2.2.7 Alcian Blue – Periodic Acid Schiff (AB-PAS) with diastase digestion staining

1. Tissue sections were de-waxed as described above in section 2.2.4.
2. The slides were washed under running tap water for one minute.
3. The slide rack was placed in Alcian Blue solution for 10 minutes.
4. The slides were washed in running tap water for one minute first and then rinsed once in distilled water.
5. One gram of diastase was dissolved thoroughly in 100ml of deionised (ELGA) water in a glass slide rack and slides were placed in it.
6. The glass slide rack was placed in a microwave at 600 watts for 25 seconds and washed in running tap water for five minutes.
7. The slides were immersed in Periodic Acid solution for five minutes at ambient temperature and rinsed in three changes of distilled water.
8. The slides were immersed in Schiff's reagent for 15 minutes at ambient temperature and washed in running tap water for five minutes.
9. The slides were counterstained in haematoxylin solution for 90 seconds and washed in running tap water for one minute to remove excess haematoxylin.
10. The tissue sections were re-hydrated and mounted in DPX as described above in section 2.2.4.

3 Establishing in vitro toxicity of ketamine

3.1 Introduction

Ketamine-induced cystitis (KIC) is a relatively new condition and although the causative agent in patients with KIC is known, the pathological mechanism of disease development remains unknown. Chu et al. suggested that it may either be from ketamine or its metabolites via either a direct effect or microvascular damage or an autoimmune reaction [2]. The lack of knowledge about mechanism of KIC development means that no effective treatment strategies can be developed for these patients to either block or treat the urinary side effects.

As patients using therapeutic ketamine may be at risk of urinary tract damage as well, one solution can be to take them off ketamine and try them on other treatments which may not be as effective. A better approach will be to keep these patients on ketamine and at the same time try to understand and ameliorate the mechanisms of human urinary tract damage by this drug so that they may long benefit from their symptoms relief without suffering the urinary side effects.

Mechanisms of KIC development are not understood at present but may be via one or both of the following pathways;

1. Direct interaction of ketamine and its metabolite nor-ketamine with the urothelium causing damage to the urothelial barrier function and consequent development of KIC. This hypothesis is based on the fact that both ketamine and nor-ketamine are excreted by urine and have a long duration (ketamine 5 days, nor-ketamine 6 days) of direct contact with urothelium after ingestion [81].

2. Receptor mediated urothelial cell toxicity. This hypothesis is based on the reports of neuronal toxicity in animal cell cultures via NMDA receptor antagonist effect [82, 83].

To test the above hypotheses, an in vitro experimental approach was undertaken.

3.2 Aim

To investigate toxicity of ketamine and its primary metabolite nor-ketamine on normal human urothelial (NHU) cell cultures.

3.3 Objectives

Objective 1: While the accepted human anaesthetic dose of ketamine is 0.5-2 mg/kg, there is no general consensus in literature to suggest a concentration where there will be definite urinary tract damage. Compared to the high concentrations of ketamine abused (up to 20 g/day) (reviewed by [8]), there are literature reports of human urothelium damage from ketamine use as an analgesic in much lower concentrations (up to 1 g/day) [3, 14]. The **first objective** therefore was to establish the toxic concentrations of ketamine for NHU cells.

Objective 2: Once ingested, ketamine is metabolised by the liver to nor-ketamine (primary metabolite) and is excreted in urine [81]. After ingesting ketamine, it can be found in urine for up to 5 days and as nor-ketamine for up to 6 days [81]. As suggested by Chu et al., the development of KIC may be via different mechanisms involving ketamine and/or its metabolites [2]. It is therefore important to establish whether nor-ketamine is toxic to NHU cells as it may contribute to the effects of ketamine. Furthermore, as nor-ketamine stays longer in urine as compared to ketamine, it is possible that if toxic, it causes more damage to NHU cells. The **second objective** therefore was to establish whether ketamine or nor-ketamine is the main toxic agent.

Objective 3: Another interesting observation to emerge from the literature review is that although ketamine abuse is on the rise (reviewed by [8, 12]), the number of patients presenting with KIC is not. The reason for this may be that as it is the party goers who are increasingly using ketamine (reviewed by [8]) and they only use it occasionally, the high

regenerative and proliferative ability of urothelium helps it repair itself between doses after the initial damage. This means that although occasional abusers of ketamine get temporary symptoms, they usually resolve as the urothelium recovers. However if the ketamine insult to human urothelium is regular, it might not have the chance to repair itself. The damage would eventually become permanent and the symptoms irreversible. The **third objective** therefore was to establish whether daily ketamine use is more toxic to NHU cells as compared to a single dose.

Objective 4: Although ketamine is used for its NMDA receptor antagonist properties in clinical practice, it is reported to have promiscuous effects on multiple other receptor sites as well (reviewed by [9]). Literature reports in animal models suggest that the NMDA antagonist activity of ketamine is responsible for neuronal toxicity [82, 83]. It is therefore possible that either the same receptor or any of the other receptors ketamine acts on are responsible for human urothelial toxicity as well. The **fourth objective** therefore was to examine whether the NMDA antagonist effect of ketamine is responsible for human urothelial cell toxicity.

3.4 Experimental approach

To plot a standard curve for future cell biology experiments, two T75 flasks of 100% confluent NHU cells (Cell line Y1071, P3) were harvested and re-suspended in KSFMC to make a final cell concentration of 50×10^4 cells/ml. Different cell concentration solutions including 1.25×10^4 cells/ml, 2.5×10^4 cells/ml, 3.75×10^4 cells/ml, 5×10^4 cells/ml, 12.5×10^4 cells/ml, 25×10^4 cells/ml, 37.5×10^4 cells/ml and 50×10^4 cells/ml were prepared. 0.2ml of each cell concentration solution was seeded in to six wells of a column of one 96-well plate. 0.2ml of sterile water was placed in the wells around the wells with cells to keep them hydrated and the plate was placed in a humidified incubator at 37°C and 5% CO_2 in air for 90 minutes. Cells were then treated with Alamar blue dye and the plate was placed back in the incubator with sterile water in the wells around Alamar blue

treated cells to keep them hydrated. The plate was removed after four hours from the incubator and read using Ascent software. The results were then charted on a graph (figure 3.1) to generate a standard formula for future cell biomass assay using Alamar blue.
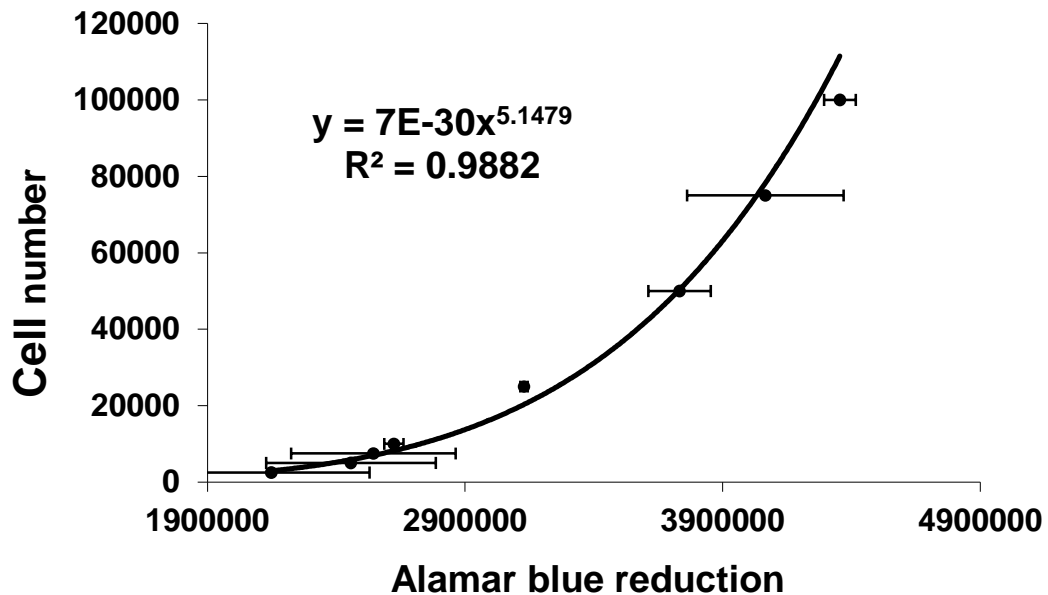
Figure 3.1: Alamar blue reduction by different numbers of NHU cells (n=6, Error bars = Standard Deviation) plotted on a graph to generate a standard formula for future cell biomass assay experiments using Alamar blue. Formula: $y = 7E\text{-}30x^{5.1479}$, R^2 value of the chart 0.9882.

All further experiments were performed after seeding NHU cells in known numbers in 96-well plates, treating with different drugs as appropriate for the experiment and assessing cell viability by using Alamar blue cell biomass assay (as described in Chapter 2). The results from the Alamar blue assay were converted in to cell numbers using the above formula. Statistical analysis was done using the InStat3 software. An Unpaired t-test was used to statistically analyse two sets of cell numbers and one way ANOVA test was used for analysing more than two sets of cell numbers.

P values of <0.001 were considered as extremely significant (***),

<0.01as highly significant (**), <0.05 as significant (*) and >0.05 as not significant (ns).

Objective 1: Experiments were performed to establish the in vitro toxic concentrations of ketamine for NHU cells.

Experiment 1: Two T75 flasks of 80% confluent NHU cells (cell line Y1071, P4) were harvested and re-suspended in KSFMC to achieve a final cell concentration of 2×10^4 cells/ml. Cells were seeded in to four 96-well plates to prepare one control and three plates for ketamine treatment. For the control plate, cells were seeded in to six wells of one column. For the three 96-well plates to be used for establishing toxic concentrations of ketamine, cells were seeded in to six wells of 11 columns in each of the three plates.

Alamar blue assay was performed on the control plate next day (day 0) and NHU cells in the other three plates were treated with different doses of ketamine ranging from 0 to 10mM. Alamar blue assay of ketamine treated 96-well plates was performed on days one, two and five.

Experiment 2: For establishing toxicity of ketamine at concentrations below 1.0mM, a second experiment was performed. One T75 flask of NHU cells (cell line Y588, P4) was harvested and re-suspended in KSFMC to achieve a final cell concentration of 2×10^4 cells/ml. Cells were seeded in to five 96-well plates to prepare one control and four plates for ketamine treatment.

For the control plate, cells were seeded in to six wells of one column. For the four 96-well plates to be used for examining toxicity of ketamine below 1mM doses, cells were seeded in to six wells of six columns in each of the four plates.

Alamar blue assay was performed on the control plate next day (day 0) and the other four plates were treated with different doses of ketamine ranging from 0 to 1.2mM. Alamar blue assay of ketamine treated 96-well plates was performed on days two, five, six and seven with medium change in the remaining three plates on day two.

Objective 2: To compare the toxicity of nor-ketamine and ketamine, two T75 flasks of 60% confluent NHU cells (cell line Y1072, P4) were harvested and re-suspended in KSFMC to achieve a final cell concentration of $3x10^4$ cells/ml. Cells were seeded in to nine 96-well plates to prepare one control and eight plates for ketamine and nor-ketamine experiment.

For the control plate, cells were seeded in to six wells of one column. For the eight 96-well plates to be used for establishing toxicity difference of ketamine and nor-ketamine, cells were seeded in to six wells of six columns in each of the nine plates.

Alamar blue assay of the control plate was performed after 48 hours (day 0). The other eight 96-well plates were divided in to two sets of four and the NHU cells in those plates were treated with different doses of ketamine and nor-ketamine which were same for both. Alamar blue assay was performed on one plate from each set on days three, four, five and seven with medium change in the remaining two plates from each set on day four.

Objective 3: To assess whether daily ketamine use was more toxic to NHU cells than a single dose, three T75 flasks of 70% confluent NHU cells (cell line Y1072, P4) were harvested and re-suspended in KSFMC to achieve a final cell concentration of $3x10^4$ cells/ml. Cells were seeded in to 10 x 96-well plates to prepare one control and nine plates for ketamine treatment.

For the control plate, cells were seeded in to eight wells of one column. For the nine 96-well plates to be used for daily versus single ketamine treatment experiment, cells were seeded in to eight wells of five columns in each of the nine plates.

Alamar blue assay of the control plate was performed after 72 hours (day 0). The other nine 96-well plates were treated with different concentrations of ketamine ranging from 0 to 1.2mM. One plate was selected at random to perform Alamar blue assay next day and results used as day one for

data analysis. The other eight plates were divided in to two sets of four. One set of plates were labelled as single treatment and had no further medium change. Four plates in the other set were labelled as daily treatment and had medium changed daily. Alamar blue assay was performed on one plate from each set on days two, three, four and five. Highest and lowest cell numbers from each column were removed from all 96-well plate results to reduce standard deviation. Day three results were not included in the final analysis due to erroneous nature of the data.

Objective 4: Using MK-801 as a specific NMDA receptor antagonist, work by colleagues done at the Jack Birch Unit of Molecular Carcinogenesis at the University of York showed that NMDA antagonist effect of ketamine was not responsible for NHU cell damage [84]. However, correspondence from Pfizer indicated that although MK-801 (specific NMDA antagonist) itself had no effect on urothelial cells in animal cultures, it enhanced the toxic effect of ketamine when used in combination.

To test the above in NHU cells, the following experiment was performed. Four T75 flasks of 60-70% confluent NHU cells (cell line Y1072, P5) were harvested and re-suspended in KSFMC to achieve a final cell concentration of $3x10^4$ cells/ml. Cells were seeded in to seven 96-well plates to prepare one control and six plates for ketamine and MK-801 treatment.

For the control plate, cells were seeded in to eight wells of one column. For the six 96-well plates to be used for ketamine and MK-801 experiment, cells were seeded in to eight wells of 10 columns in each of the six plates. Alamar blue assay of the control plate was performed after 72 hours (day 0). In the other six plates, cells in one column had no drug treatment (control column), cells in one column were treated with MK-801 at a concentration of 100μM, cells in four columns were treated with ketamine alone in doses of 0.4, 0.6, 0.8 and 1.0mM, and cells in the other four

columns were treated with a combination of ketamine (doses of 0.4, 0.6, 0.8 and 1.0mM) and MK-801 at 100µM concentration.

Alamar blue assay was performed on treatment plates on days one, two, four, seven, eight and nine with medium change in the remaining plates on days two, four and seven.

Highest and lowest cell numbers from each column were removed from all 96-well plate results to reduce standard deviation.

3.5 Results

Objective 1 (Experiments 1 and 2): Treatment of known number of NHU cells seeded in 96-well plates with different concentrations of ketamine showed that it was cytostatic and had a concentration dependent-effect which increased as the dose of ketamine was increased. Although all concentrations of ketamine were cytostatic, there was no statistically significant difference in toxicity between different concentrations above 1mM (figures 3.2, 3.3 and 3.4).
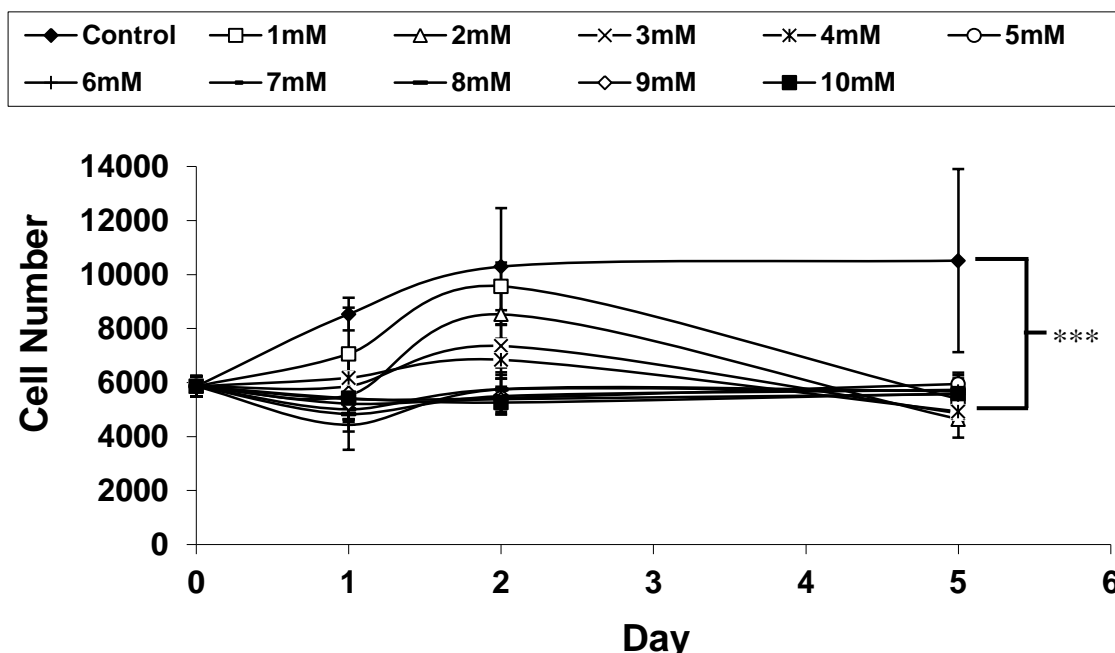
Figure 3.2: Experiment 1; NHU cells treated with 0 - 10mM ketamine showed that it was cytostatic at all concentrations (day 5, n=5-6, p<0.001, Error bars = Standard Deviation). Although there was some increase in cell number at lower doses (1 - 4mM) initially, the cell growth stopped eventually at all concentrations by day 5. Statistical analysis shown is between control and 1mM ketamine concentration at day 5.
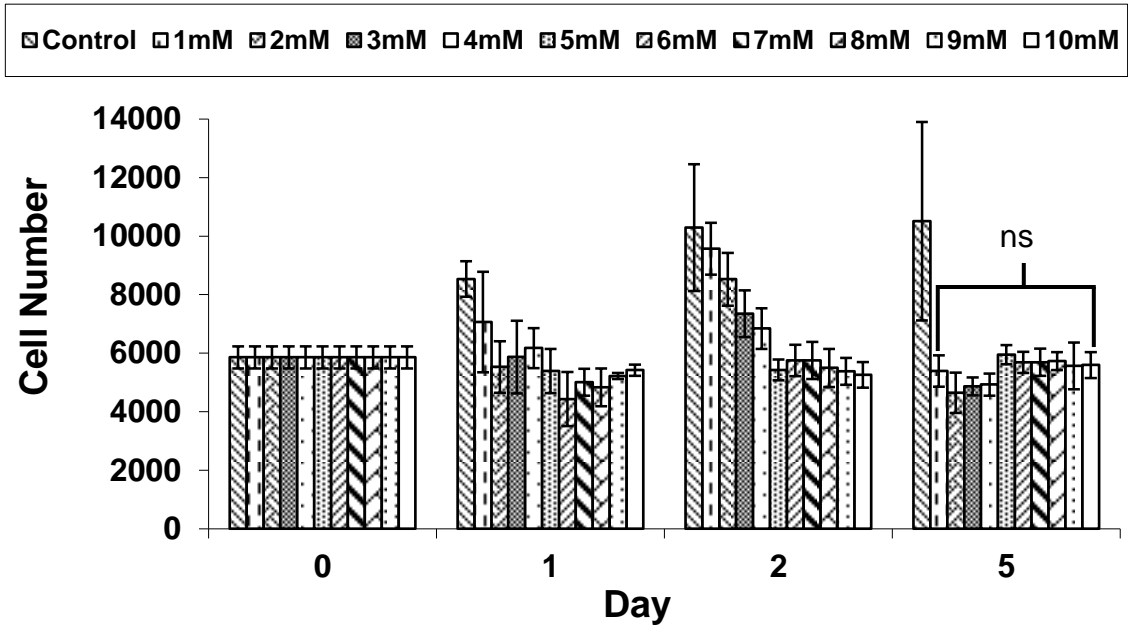
Figure 3.3 (data extracted from figure 3.2): Experiment 1; NHU cells treated with 0 - 10mM ketamine showed that ketamine was cytostatic at all concentrations when compared to control (day 5, n=5-6, $p<0.001$, Error bars = Standard Deviation) but the toxicity difference between different ketamine concentrations (1-10mM) was statistically not significant (day 5, n=5-6, $p>0.05$, Error bars = Standard Deviation).
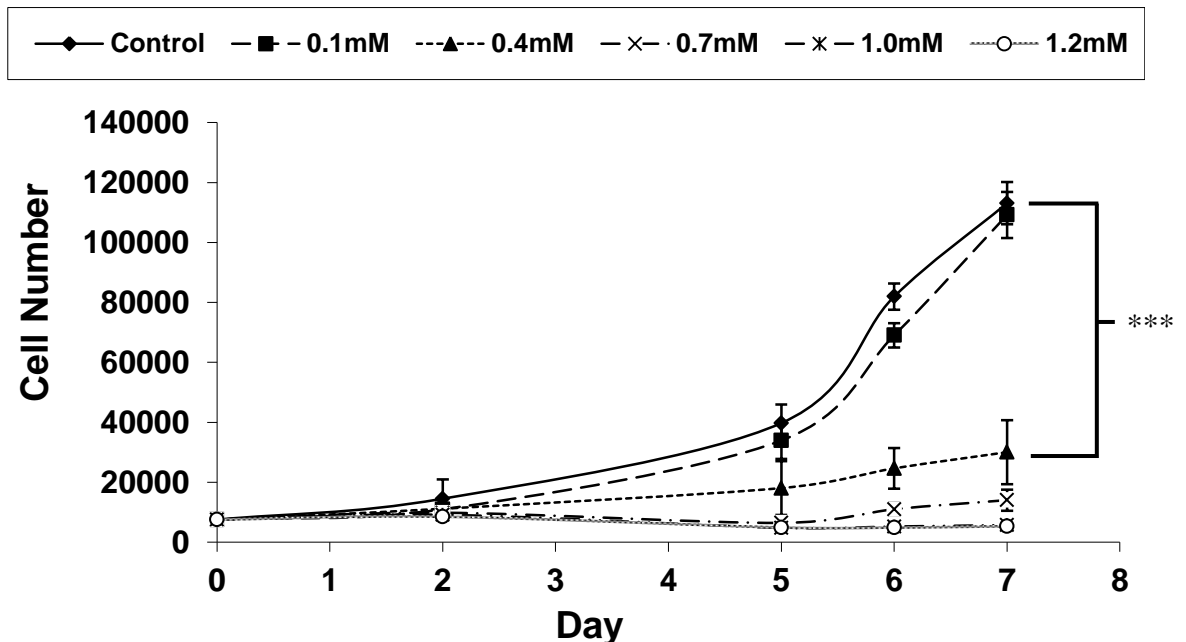
Figure 3.4: Experiment 2; NHU cells treated with 0 - 1.2mM ketamine showed that it was toxic to NHU cells at concentrations of 0.4mM and above from day 6 onwards (n=6, $p<0.001$, Error bars = Standard Deviation). Statistical analysis shown is between control and 0.4mM ketamine concentration at day 7. 0.1mM ketamine concentration did not show NHU cell toxicity but this result may be limited by the duration of experiment (7 days).

Objective 2: When assessed at equal concentrations in vitro, both ketamine (figure 3.5) and nor-ketamine (figure 3.6) were cytostatic/cytotoxic to NHU cells. Any difference in toxicity between ketamine and nor-ketamine at different doses above 1mM was statistically not significant over a 7 day time course (figure 3.7).
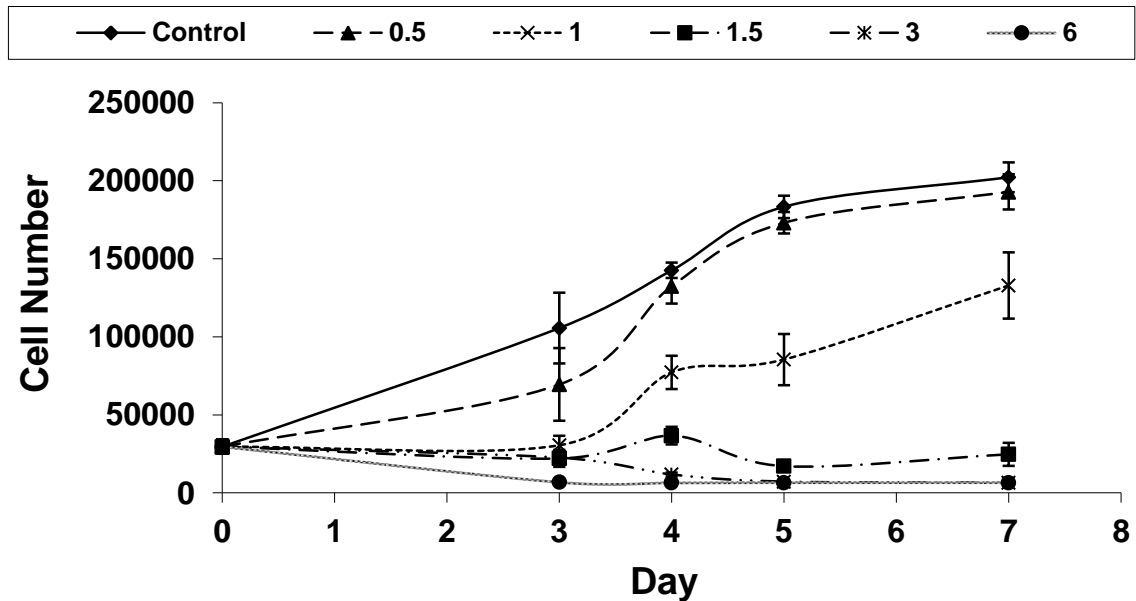
Figure 3.5: NHU cells treated with 0.5 – 6mM ketamine showed statistically significant toxicity difference to control at concentrations of 1mM and above (n=6, p<0.001, Error bars = Standard Deviation).
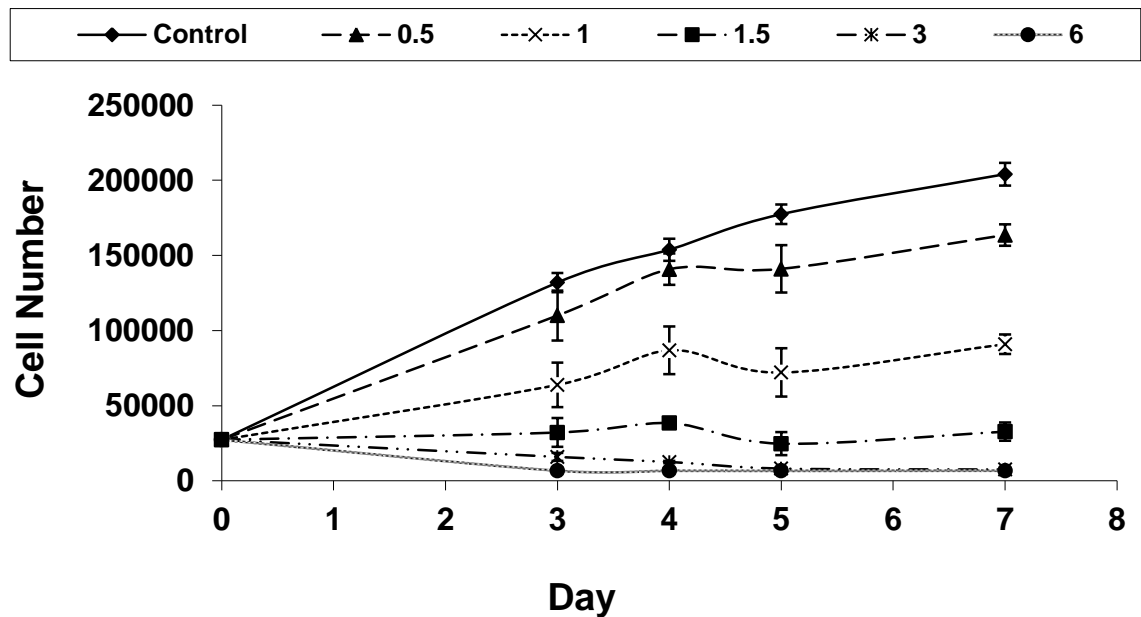
Figure 3.6: NHU cells treated with 0.5 – 6mM nor-ketamine showed statistically significant toxicity difference to control at concentrations of 0.5mM and above (n=6, p<0.001, Error bars = Standard Deviation).
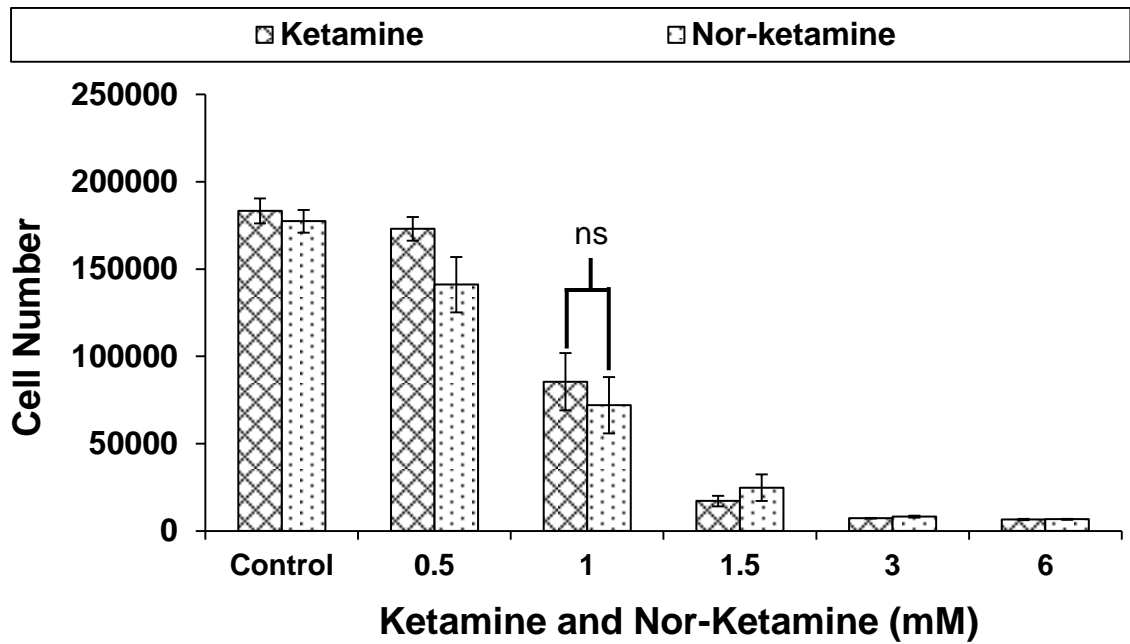
Figure 3.7 (data extracted from figures 3.5 and 3.6): Comparison of toxicity induced in NHU cell cultures by ketamine or nor-ketamine treatment at day 5 (representative data) showed no statistically significant differences (p>0.05) apart from the 0.5mM treatment (p<0.001) (n=6, Error bars = Standard Deviation).

Objective 3: The experiment showed that daily treatment of ketamine was more toxic to NHU cells at concentrations of 0.8mM and above when compared to a single dose at the same concentrations (figures 3.8, 3.9, 3.10, 3.11, 3.12 and 3.13). In clinical practice this is significant as patients abusing/using ketamine daily might be at a higher risk to develop KIC.
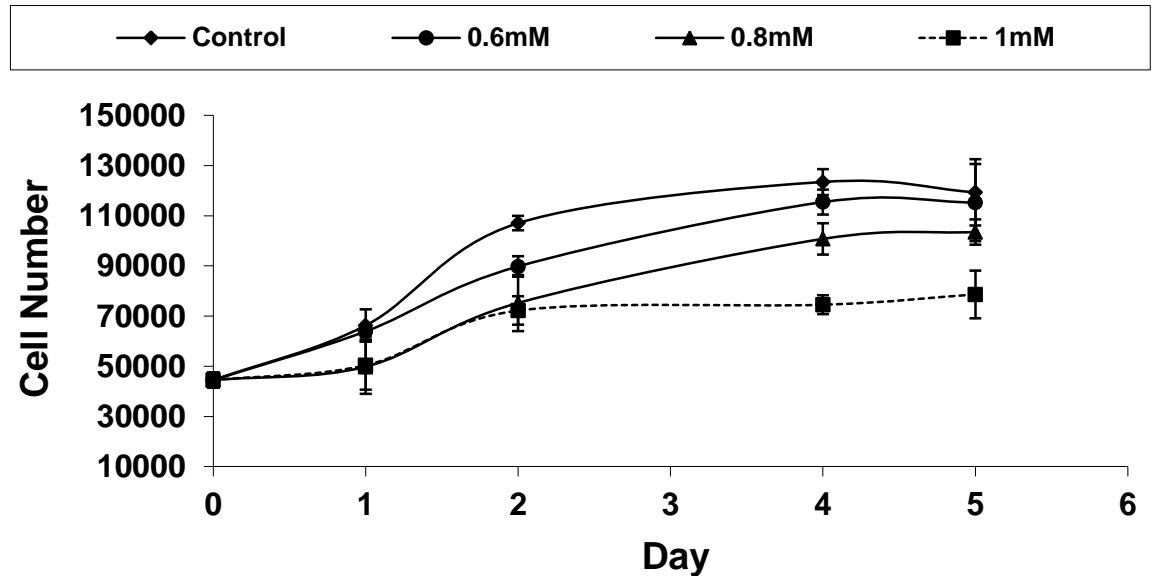
Figure 3.8: Single ketamine treatment of NHU cells with 0.6 – 1mM ketamine showed that it was toxic at concentrations of 1mM and above when compared to control from day 4 onwards (n=6, Error bars = Standard Deviation).
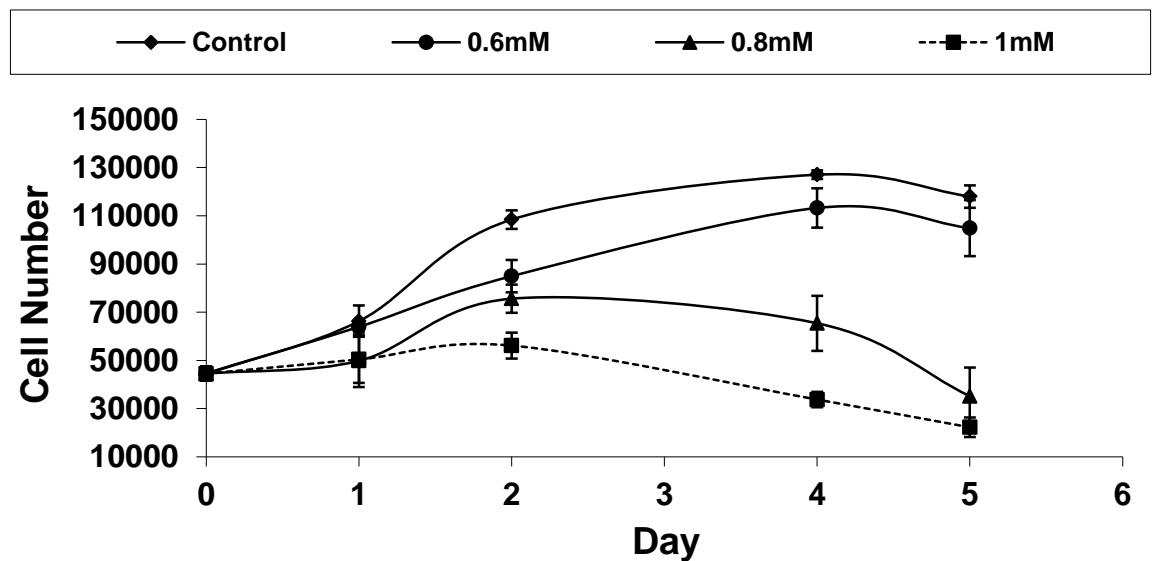
Figure 3.9: Daily ketamine treatment of NHU cells with 0.6 – 1mM ketamine showed that toxicity was increased if ketamine was given daily. This was evident at concentrations of 0.8mM and above from day 4 onwards (n=6, Error bars = Standard Deviation).
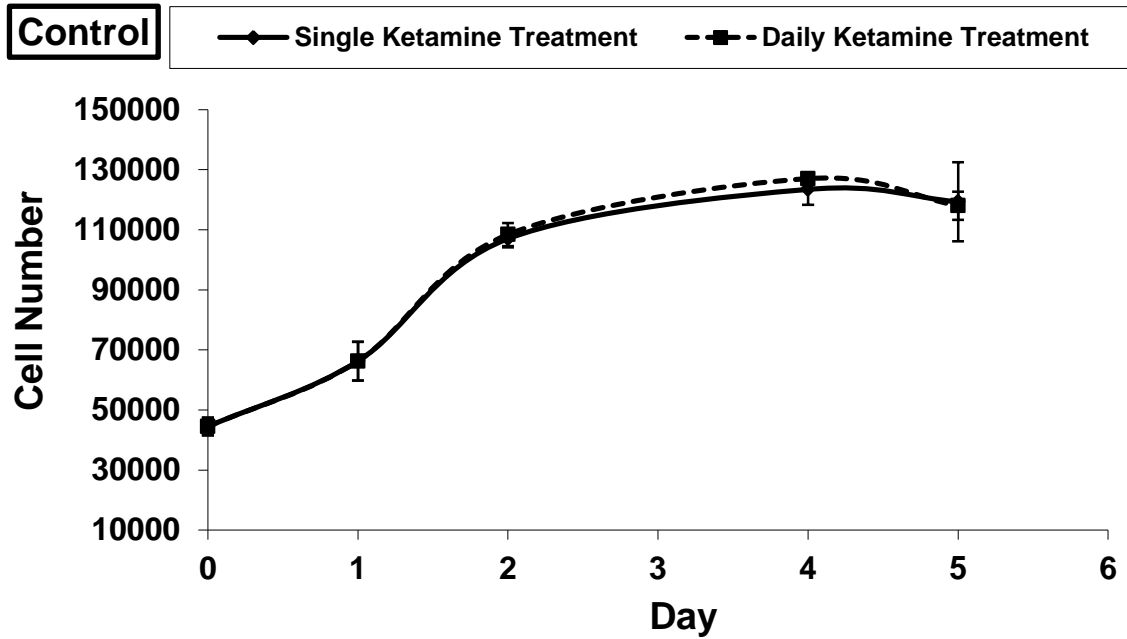
Figure 3.10 (data extracted from figures 3.8 and 3.9): Comparison of control column (no ketamine treatment) of NHU cells from both sets of plates showing that the growth of cells is not affected by the change of medium daily (n=6, p=0.1401, Error bars = Standard Deviation).
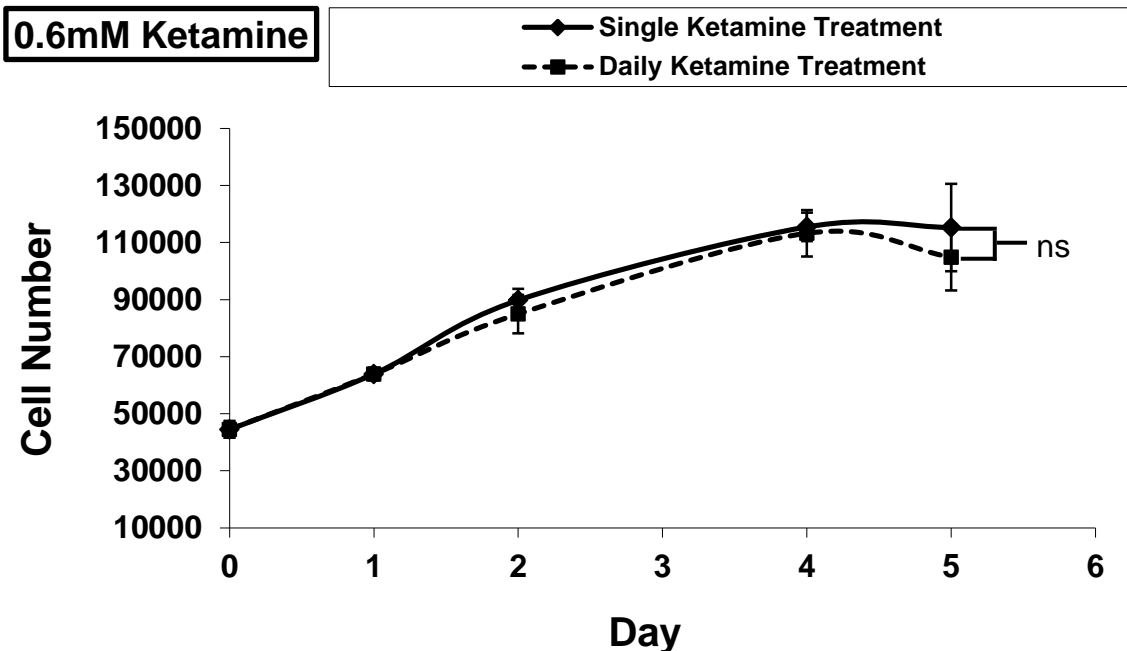
Figure 3.11 (data extracted from figures 3.8 and 3.9): Ketamine did not show a difference in toxicity in either daily or single ketamine dose treated NHU cells at a concentration of 0.6mM (n=6, p=0.5858, Error bars = Standard Deviation).
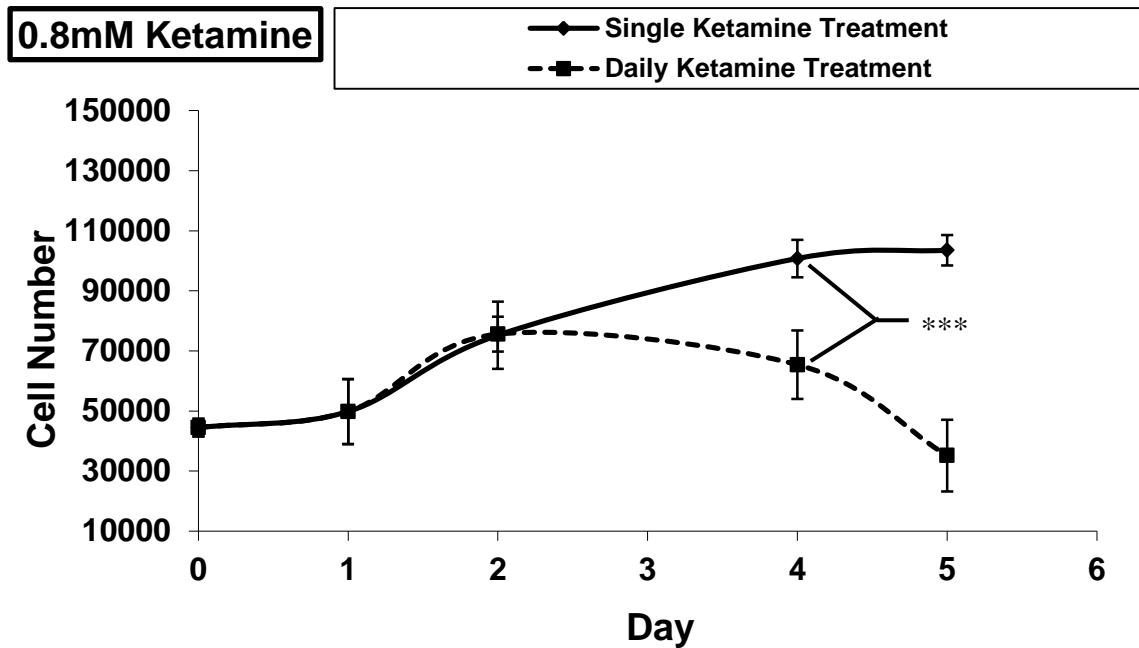
0.8mM Ketamine

Figure 3.12 (data extracted from figures 3.8 and 3.9): Ketamine was more toxic to NHU cells at doses of 0.8mM and above when given daily as compared to a single dose from day 4 onwards (n=6, p <0.0001, Error bars = Standard Deviation).

1.0mM Ketamine
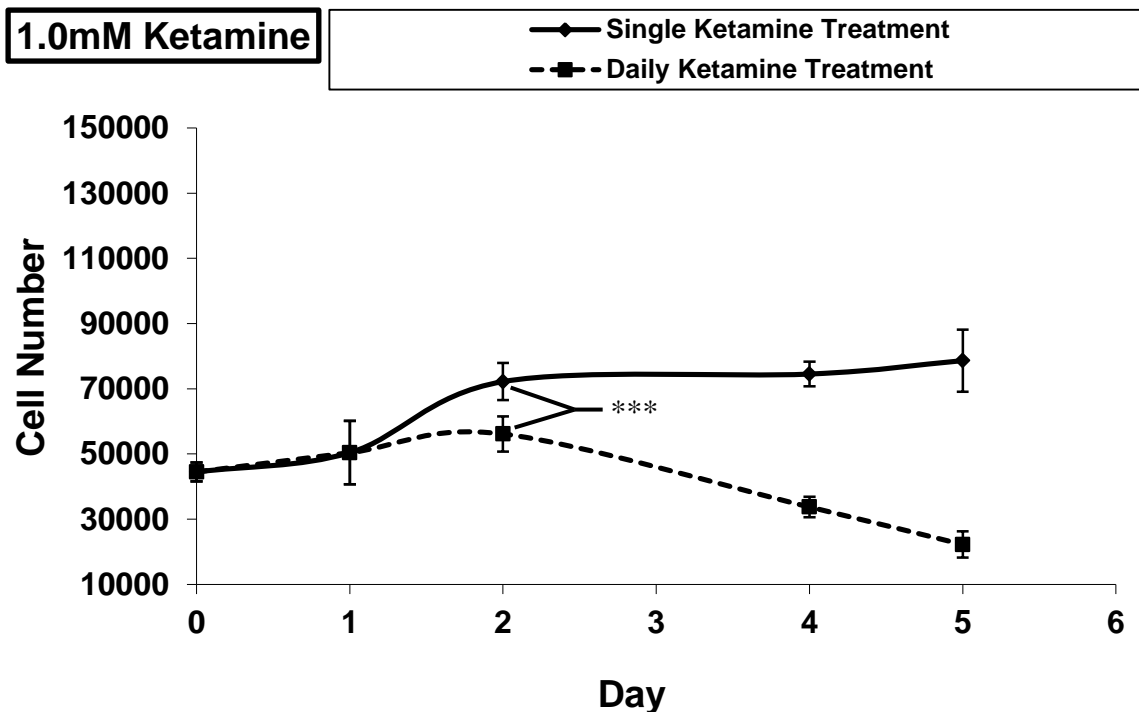
Figure 3.13 (data extracted from figures 3.8 and 3.9): Increasing ketamine concentration in the treatment solution showed that the toxicity difference between daily ketamine treatment and single ketamine treatment was apparent earlier (as compared to 0.8mM concentration) from day 2 onwards (n=6, p=0.0005, Error bars = Standard Deviation).

Objective 4: Using MK-801 as a specific NMDA receptor antagonist, the experiment showed that although MK-801 does not cause any toxicity of NHU cells on its own, it enhances the toxicity of ketamine when used in combination (figures 3.14, 3.15 and 3.16).
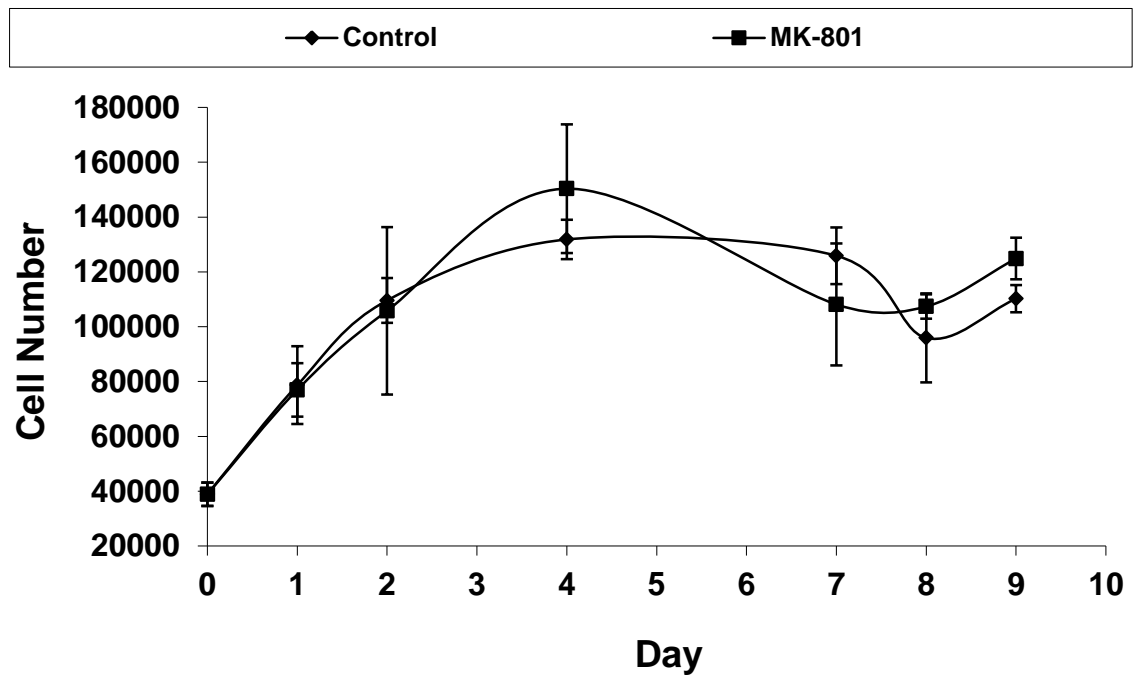
Figure 3.14: NHU cells treated with MK-801 showed same growth as the cells in control column (no treatment)(n=4-6, p=0.1063, Error bars = Standard Deviation).
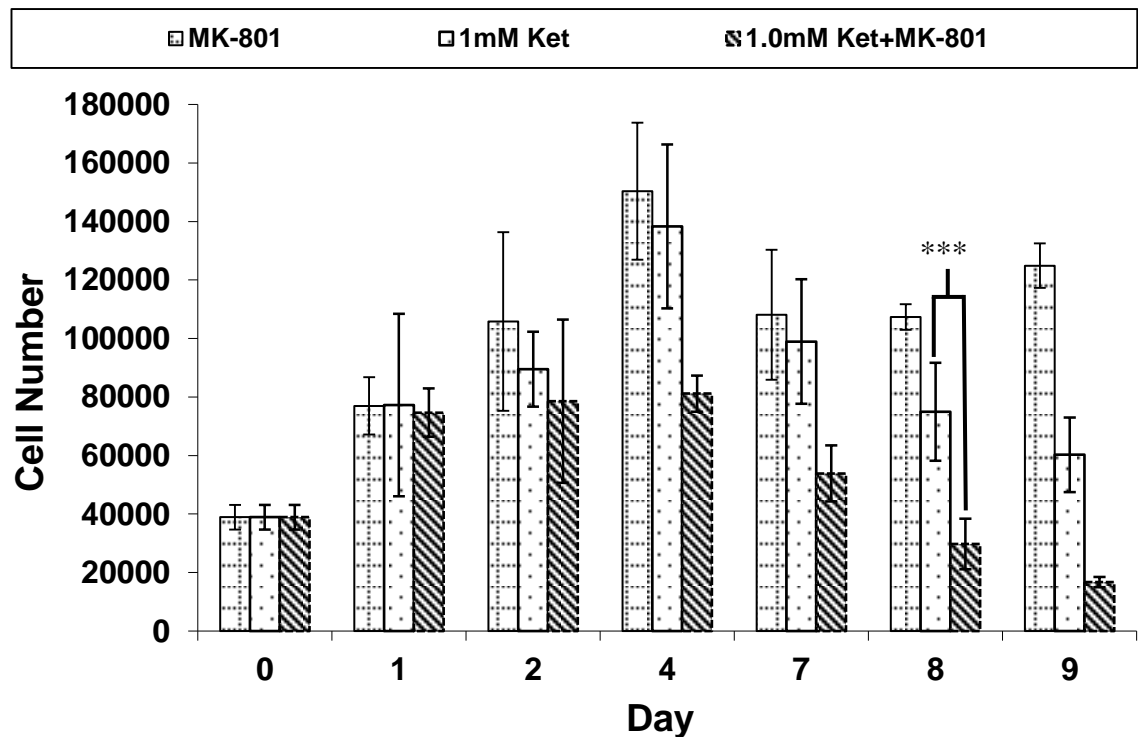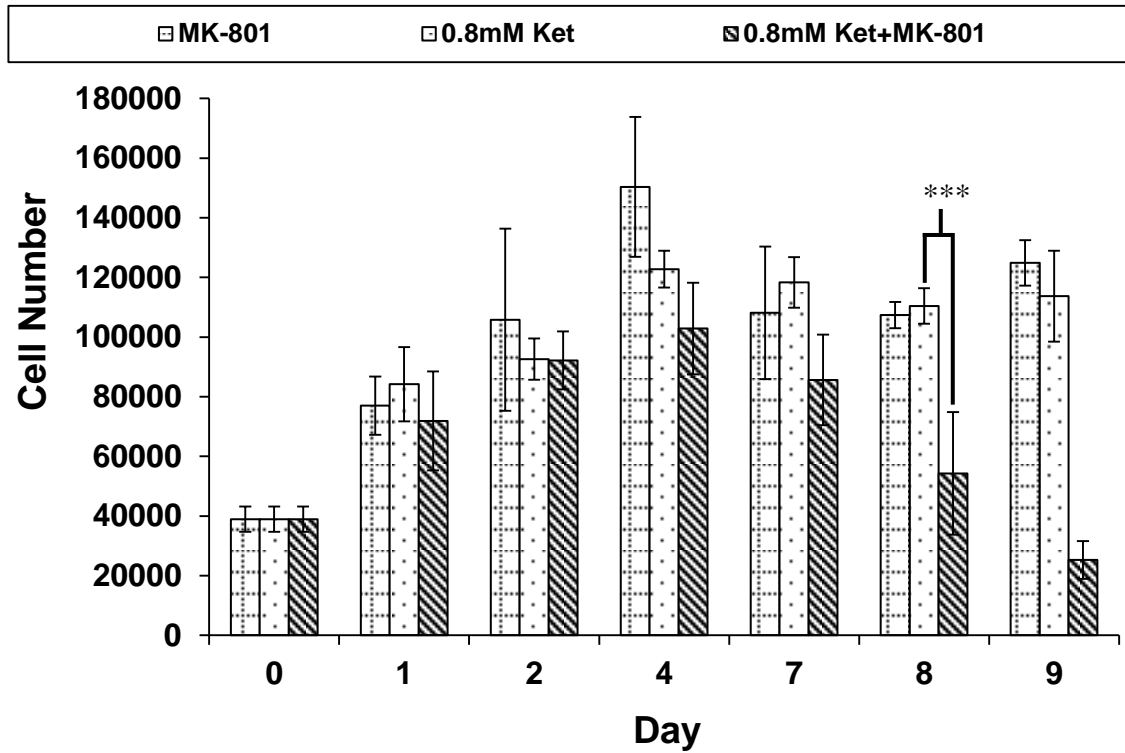
Figures 3.15 and 3.16: (cells treated with MK-801 only did not show any statistically significant difference in growth pattren from the cells with no treatment [figure 3.14], number of cells treated with MK-801 alone were therefore used as control for figures 3.15 and 3.16); NHU cells treated with a combination of ketamine and MK-801 showed more toxicity than ketamine alone (n=6, p<0.001, Error bars = Standard Deviation).

3.6 Discussion

The toxicity difference between different concentrations of ketamine might explain why patients develop symptoms at different times after ketamine abuse/use. This might also be contributed by the individual differences in patient's susceptibility to develop KIC due to internal body's defence mechanisms.

Ketamine was toxic to NHU cells at concentrations of 0.4mM and above. 1mM of ketamine solution used for the experiments was equivalent to a clinical dose of 0.27419mg/ml/day (0.4mM = 0.11mg/ml/day). Literature reports suggested that patients using ketamine for pain relief and as a recreational drug developed symptoms of KIC at doses from 200mg/day to 20g/day respectively [3, 85]. When the experimental and clinical doses were converted, they seemed to be comparable (above clinical doses approximately equivalent to 0.0024mg/ml/day to 0.248mg/ml/day for a patient with body weight of 70kg and assuming that 1g = 1ml).

As nor-ketamine was equally toxic to NHU cells and stays longer than ketamine in the urine [81], it is possible that it may cause more damage to the NHU cells. This will be important information for any future therapies aimed at blocking the urinary effects of ketamine.

Although recreational ketamine use is increasing (reviewed by [8, 12]), the number of patients presenting with KIC is not. This may be due to under-reporting of the problem but is more likely due to people using it occasionally at parties etc. This gives urothelium time to recover from damage before next dose and therefore users experience only temporary symptoms after each occasional dose. The above hypothesis was tested by the experiments which showed that daily ketamine use was more toxic to NHU cells as compared to a single dose.

Ketamine is primarily used in clinical practice for its NMDA receptor antagonist properties and has shown toxicity via this receptor in neuronal cell systems in animal models [82, 83]. Although, ketamine has a highly promiscuous receptor binding profile, it is possible that same mechanism is responsible for causing urothelial toxicity. The experiment showed some

interesting results; MK-801, which is a specific NMDA receptor antagonist, did not cause urothelial damage on its own but enhanced the toxic effects of ketamine when used in combination. This may be due to either ketamine inducing expression of NMDA receptor in urothelial cells on which MK-801 acts or due to a different receptor pathway responsible for urothelial toxicity of ketamine and MK-801 induces increased expression of that receptor.

Although all the above experiments were performed following strict protocols, there was some variation in the results in objective 1, experiment 2 (figure 3.4) and objective 2 (figure 3.5). Ketamine showed statistically significant toxicity at doses of 0.4mM in the first experiment but no toxicity at a higher dose of 0.5mM in the second experiment. This might be due to donor variability as the cell lines used in the two experiments were different. It is unlikely to be due to variations in the prepared ketamine concentrations used to treat NHU cells as ketamine is a stable drug and the ketamine solutions were prepared fresh each time using the same protocols.

3.7 Conclusions

1. Ketamine is toxic to NHU cells and the toxicity increases as the concentration of ketamine is increased.
2. Toxic effects of ketamine are a result of both itself and its primary metabolite (nor-ketamine).
3. Daily ketamine use is more toxic to NHU cells than a single dose.
4. In vitro toxic effects of ketamine on NHU cells are enhanced by specific NMDA antagonist MK-801 despite no direct toxicity.

4 Characterisation of ketamine affected human urothelium

4.1 Introduction

As the awareness increases among the medical community worldwide about development of ketamine-induced cystitis (KIC) in abusers and prescription users of ketamine, increasing number of literature reports are being published about this condition. Most of these reports though describe the clinical presentation and management of KIC patients and limited reports are about the characterisation of ketamine affected human urothelium. Oxley et al. [16] performed immunohistochemistry (IHC) experiments to establish the expression of Ki67, p53 and CK20 antibodies in 10 KIC samples. In their cohort, 9/10 patients had high p53 immunoreactivity and 7/10 patients had moderate to high levels of Ki67 reactivity, but all were negative for CK 20. They concluded that ketamine could lead to reactive urothelial changes that could mimic carcinoma in situ (CIS).

Although, this might be useful information in the context of distinguishing KIC from bladder CIS, it unfortunately did not help to understand aetiopathological mechanisms of disease development in patients with KIC and therefore a more detailed IHC study was needed.

4.2 Aim

To describe human urothelial tissue damage caused by ketamine.

4.3 Objectives

Objective 1: To assess proliferation potential in human KIC tissue.

Objective 2: To assess differentiation potential in human KIC tissue.

Objective 3: To assess inflammatory changes in human KIC tissue.

4.4 Experimental approach

All antibody staining was performed using protocol 2.2.6 described in the materials and methods chapter. Anti-human NGFR antibody staining was kindly performed by Dr F Wezel. CD3 antibody staining using H_2O_2 prepared in distilled water was unsuccessful, and therefore was performed with H_2O_2 prepared in PBS to increase the staining of antigenic sites (http://www.ihcworld.com/_technical_tips/peroxidase_tips.html, website accessed 22/06/2011).

The immunohistochemistry experiments were performed in the following order;

1. H&E staining of normal human urothelial (NHU) tissue was performed first to understand the appearance of normal tissue.

2. H&E staining was performed on 11 KIC tissue blocks from three separate patients (KIC1, KIC2 and KIC3) and based on the results three individual KIC tissue blocks (KIC1a, KIC2b and KIC3d) expected to provide most information during IHC experiments were selected (one from each patient).

3. Based on the information obtained from H&E staining of KIC tissues and advice from Dr J Stahlschmidt (Histopathologist at the St James's University Hospital, Leeds), an initial antibody panel was agreed with the researchers at the Jack Birch Unit (JBU) of Molecular Carcinogenesis, Department of Biology, University of York.

4. The antibodies were first tested on control tissue to check their working condition. Negative sections were included for all antibodies.

5. The antibody panel was then performed on the three individual KIC tissues selected after initial H&E staining. Positive and negative control tissues were included for each antibody. Positive labelling index was determined for some antibodies by selecting urothelial or stromal areas at five random sites in the section and calculating the percentage by counting the positive cells against the total number of cells using a x60-oil objective.

6. Table 1 below shows the selected antibody panel;

Antibody	Host	Localise	Antigen Retrieval Method	Dilution (optimised)
Ki67	Mouse	Cells in cell cycle	Microwave (citric acid)	1:100
UPK3a	Mouse	Differentiation and Superficial cells	Microwave (citric acid)	1:40
CK20.8	Mouse	Superficial cells	Trypsin	1:200
CK13	Mouse	Basal and Intermediate cells	Trypsin & Microwave	1:100
CK5	Rabbit	Basal cells	Microwave (citric acid)	1:100
CK14	Mouse	Squamous Metaplasia	Trypsin & Microwave	1:100
CD34	Mouse	Number and size of blood vessels	Trypsin	1:50
CD3	Mouse	T-cells	Microwave (citric acid)	1:25
CD68	Mouse	Macrophages and Monocytes	Microwave (citric acid)	1:50
Mast Cell Tryptase	Mouse	Mast cells	Microwave (citric acid)	1:300
CD117	Rabbit	cKit positive cells	Microwave (citric acid)	1:600
Anti-human NGFR	Mouse	Neural fibrosis and inflammation	Microwave (citric acid)	1:100

Table 4.1: Initial antibody panel for the three inidividual KIC tissue sections based on the H&E staining results.

4.5 Results

4.5.1 Normal Urothelium
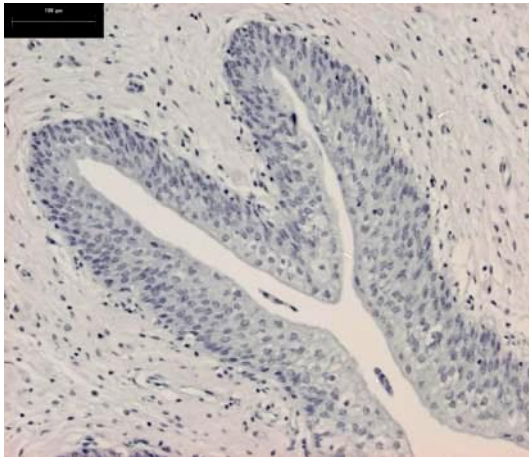
Figure 4.1: H&E staining of a section of normal human ureter demonstrating a transitional epithelium with three layers (Superficial, Intermediate and basal) and underlying submucosa, scale bar 100μm.

4.5.2 H&E staining of KIC tissue

H&E staining of the three individual KIC tissues selected to perform the initial antibody panel showed denudation of epithelium in two individual sections (KIC1a and KIC3d) and oedematous vessels in the lamina propria of two individual sections (KIC2b and KIC3d). A rich cellular infiltrate likely to be immune in nature was also present in the lamina propria of all three individual KIC tissue sections.
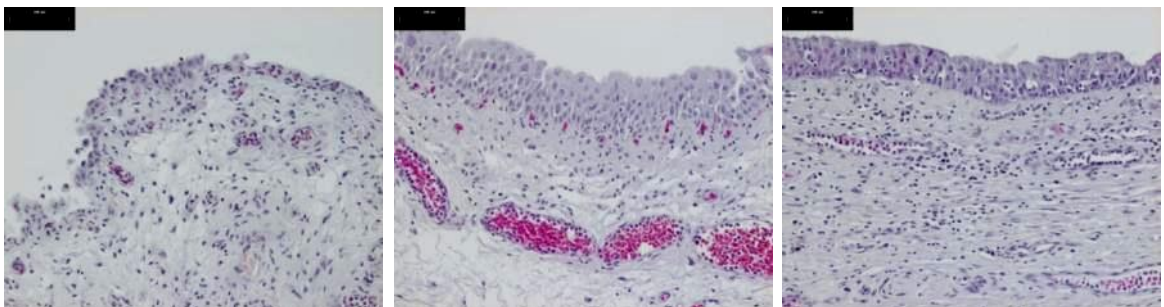
(KIC1a)   **(KIC2b)**   **(KIC3d)**

Figure 4.2: H&E staining of 11 KIC tissue blocks from three individual patients was performed first. Three most informative blocks for future Immunolabelling experiments were selected. H&E staining of these blocks demonstrated tissue damage in the form of epithelial denudation and inflammation of the tissue evident by oedematous blood vessels in the lamina propria. Scale bar 100μm.

In addition, H&E staining of tissue sections from one of the blocks (KIC3b) from patient KIC3 demonstrated intestinal (columnar) metaplasia (scale bar 100µm).
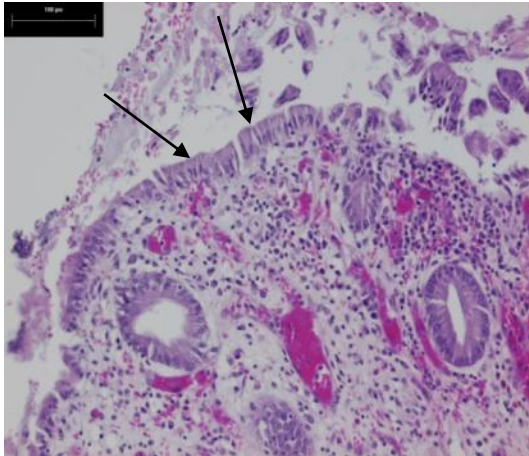
(KIC3b)

Figure 4.3: Colonic metaplasia on H&E staining in one (KIC3b) out of four tissue blocks from one individual patient (KIC3), scale bar 100µm.

4.5.3 Antibody panel for KIC tissues

The antibody panel was checked on control (normal) tissue to confirm its working condition. Negative controls were included for all antibodies.

Proliferation marker:

Ki67
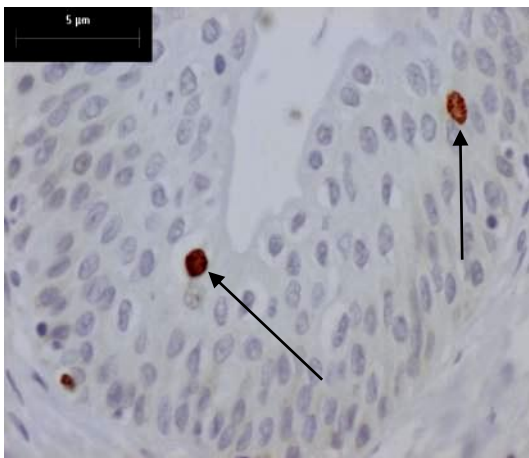
Figure 4.4: Section of human ureter with occasional Ki67 positive proliferating cells in urothelium, scale bar 5µm.

Differentiation markers:

UPK3a
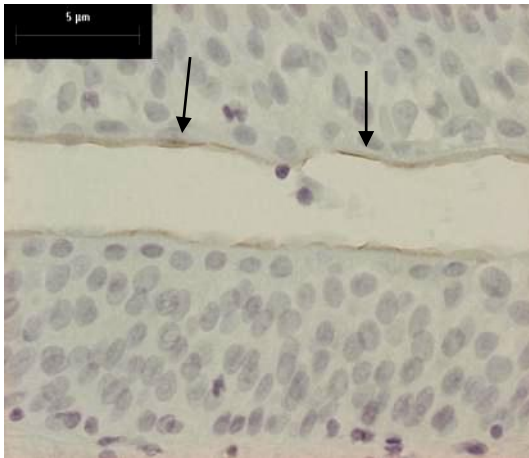
Figure 4.5: Section of human ureter with UPK3a positive cells in superficial layer of urothelium, scale bar 5µm.

CK20.8
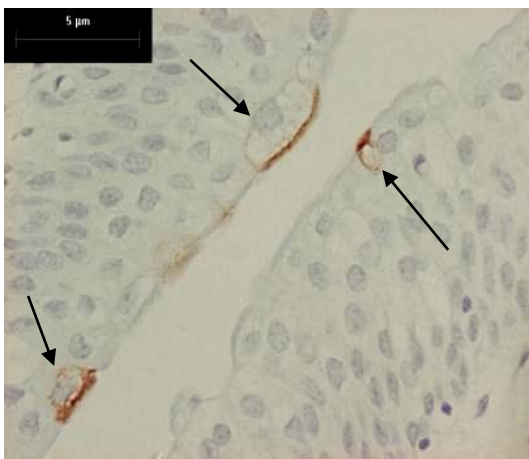
Figure 4.6: Section of human ureter with CK20.8 positive cells in superficial layer of urothelium, scale bar 5µm.

CK13
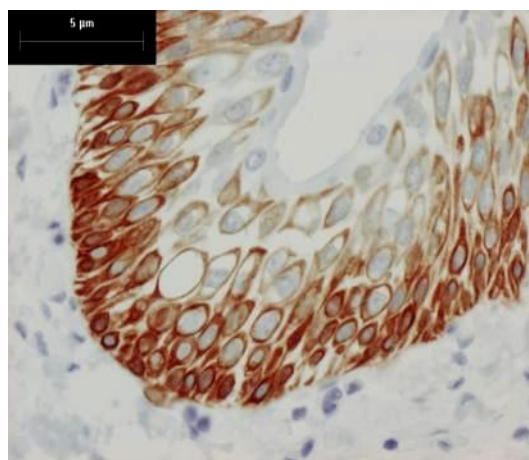
Figure 4.7: Section of human ureter with CK13 positive cells in basal and intermediate layers of urothelium, scale bar 5µm.

CK5
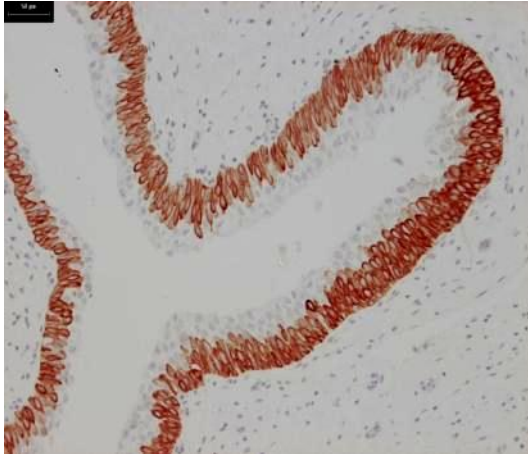
Figure 4.8: Section of human ureter with CK5 positive cells in basal layer of urothelium, scale bar 50µm.

Metaplasia Markers:

CK14
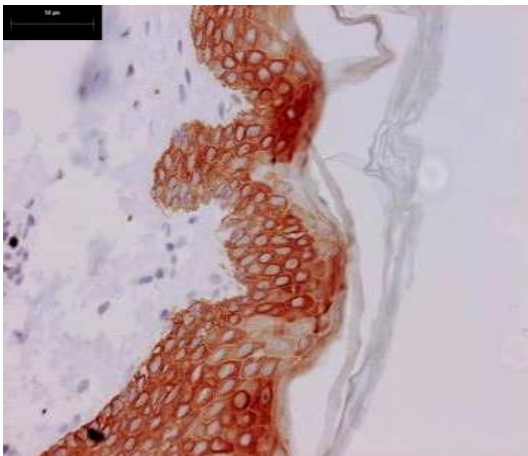
Figure 4.9: Section of human skin with CK14 positive Squamous epithelium, scale bar 50µm.

AB-PAS with diastase digestion
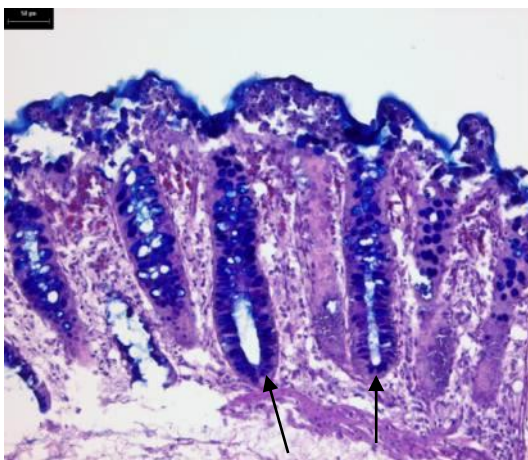
Figure 4.10: Section of human colon with mucus producing Alcian blue positive glands, scale bar 50µm.

Inflammation markers:

CD3
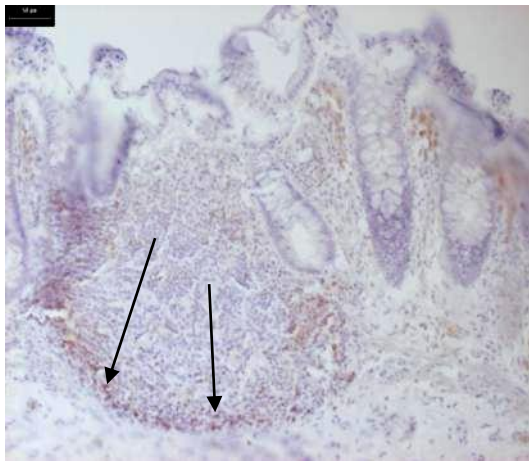
Figure 4.11: Section of human colon with CD3 positive T-cells in a lymph node in the submucosa, scale bar 50µm.

CD34
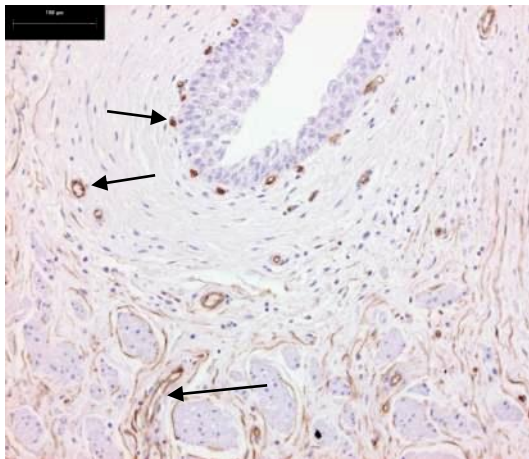
Figure 4.12: Section of human ureter with CD34 positive blood vessels in submucosa and positive stromal fibres, scale bar 100µm.

CD68
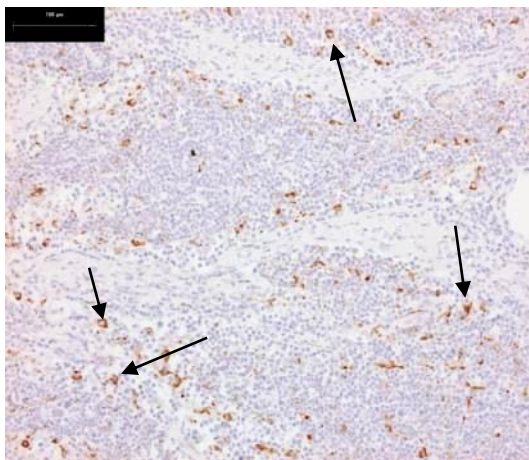
Figure 4.13: Section of human lymph node with CD68 positive macrophages and monocytes, scale bar 100µm.

Mast Cell Tryptase
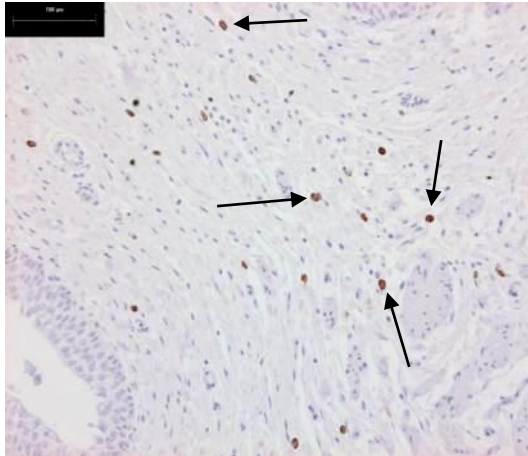
Figure 4.14: Section of human ureter with Mast Cell Tryptase positive mast cells in the submucosa, scale bar 100µm.

CD117
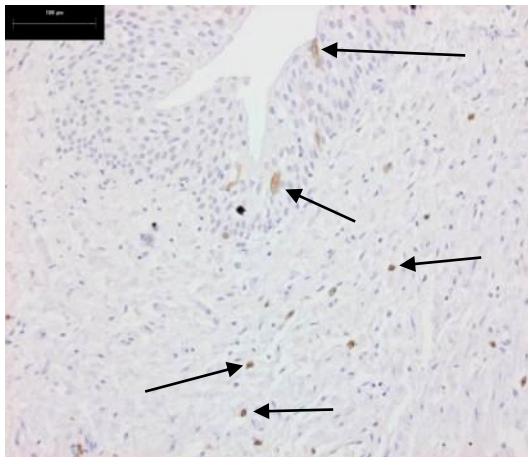
Figure 4.15: Section of human ureter with CD117 positive cells in the urothelium and positive mast cells in the submucosa, scale bar 100µm.

Anti-Human NGFR
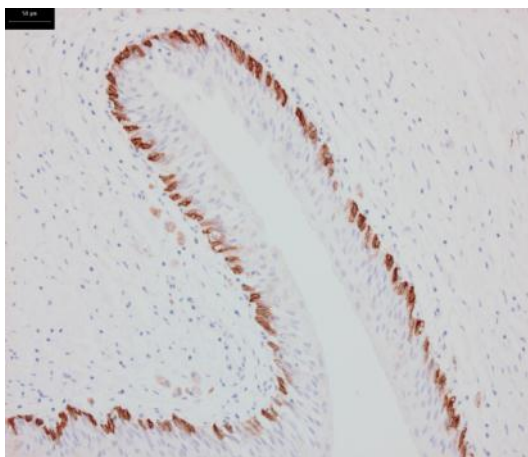
Figure 4.16: Section of human ureter with Anti-human NGFR positive cells in the basal layer of urothelium, scale bar 50µm.

4.5.3.1 Negative control sections for antibody panel

Human ureter
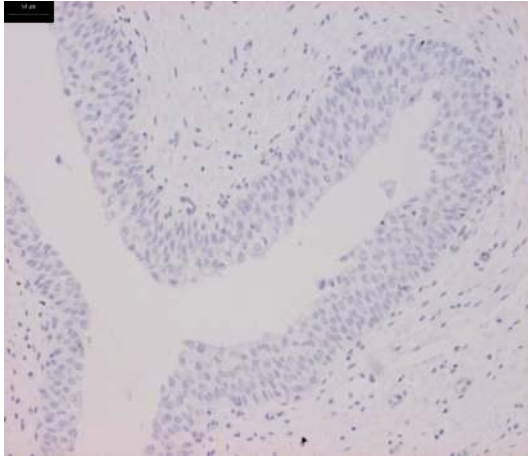
Figure 4.17: One of the human ureter sections used as a negative control for antibodies Ki67, UPK3a, Mast Cell Tryptase, CK13, Anti-human NGFR, CK20.8, CD34, CD68, CD117 and CK5, scale bar 50 µm.

AB-PAS on human ureter
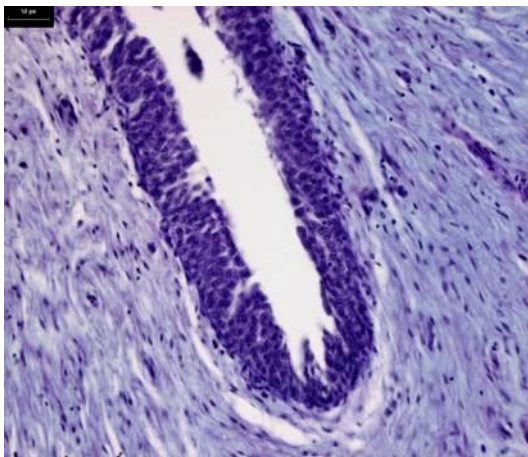
Figure 4.18: Negative control section of human ureter for AB-PAS with diastase digestion staining, scale bar 50µm.
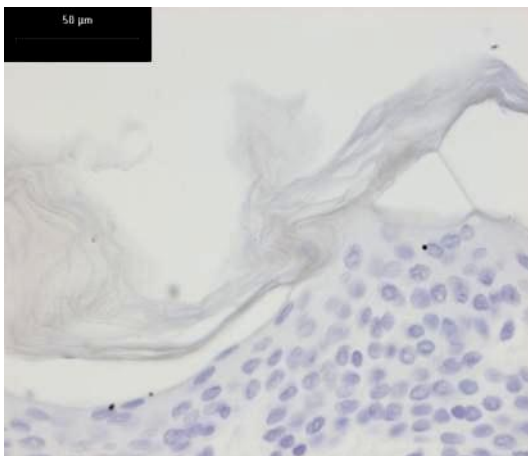
Human skin

Figure 4.19: Negative control section of human skin for CK14, scale bar 50µm.
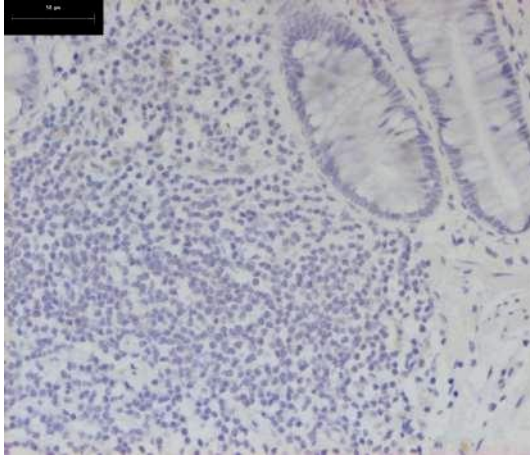
Human colon

Figure 4.20: Negative control section of human colon for CD3 antibody, scale bar 50µm.

4.5.4 Immunoperoxidase staining of KIC tissue sections with antibodies from the panel

4.5.4.1 Ki67

Ki67 was used as a marker of proliferation in the urothelium. The immunolabelling showed that proliferation (and therefore tissue turnover) was increased in two (KIC1a and KIC3d) of the three individual KIC sections (increased Ki67 expression in urothelium). Although section KIC1a had largely denuded urothelium reduced to a simple epithelial layer, the remaining urothelial cells showed increased proliferation (positive labelling index 7/23, 30.43% as compared to 12/85, 14.12% in positive control section). Positive labelling index of Ki67 positive cells in the urothelium of section KIC3d was 31/110, 28.18% as compared to 12/85, 14.12% in positive control tissue.

(KIC1a)   **(KIC2b)**   **(KIC3d)**

(E)   **(F)**
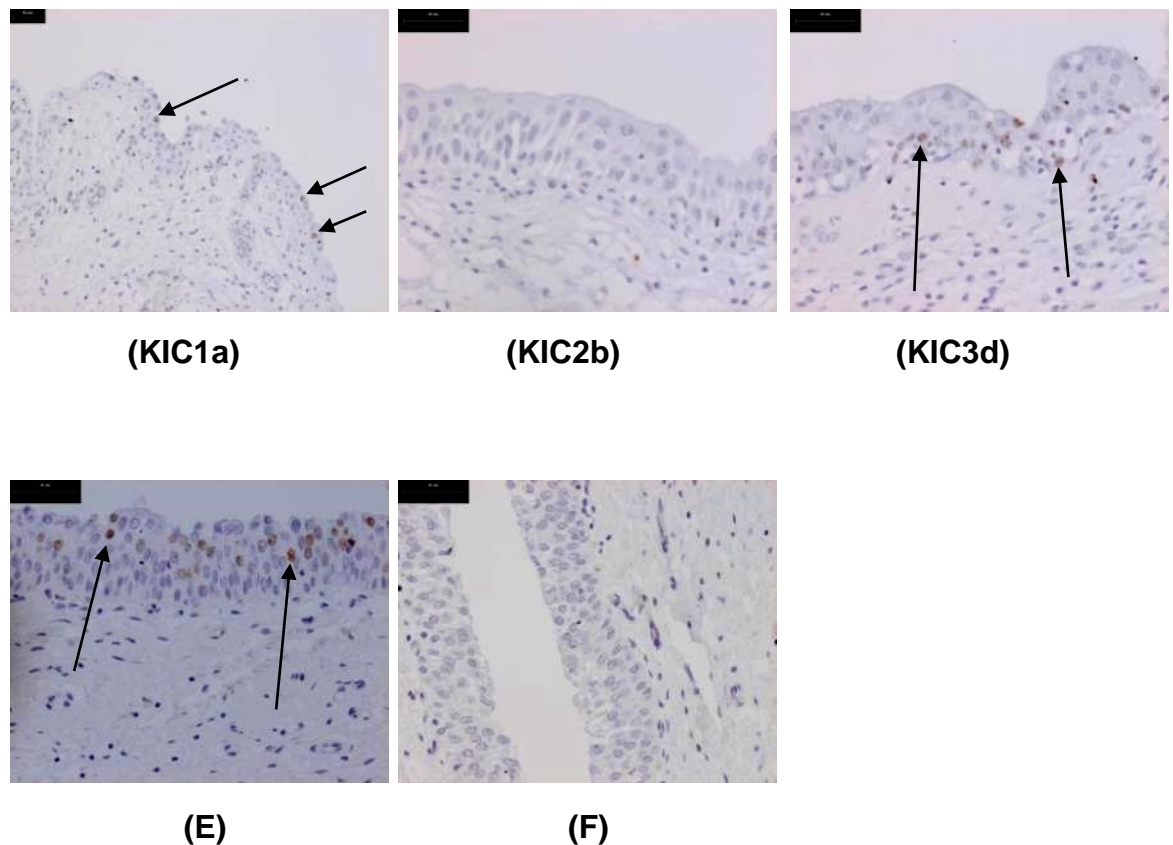
Figure 4.21: Immunoperoxidase labelling of the three individual KIC tissue sections with Ki67 showed increased proliferation in the urothelium of two sections (KIC1a and KIC3d) and negative in one section (KIC2b). A section of normal human ureter (E) was included as a positive control and one as a negative control (F). Scale bar 50µm.

4.5.4.2 UPK3a

The apical surface of the superficial cell membrane of urothelium is covered with multiple thickened plaques of asymmetric unit membrane, known collectively as uroplakins [53]. These integral transmembrane proteins are responsible for maintaining transcellular permeability [53]. UPK3a was selected for the experiments to assess presence of uroplakin along the superficial edge of the urothelium and therefore differentiation. The labelling showed that UPK3a was positive only in one of the 3 individual KIC sections (KIC2b).

(KIC1a)   **(KIC2b)**   **(KIC3d)**

(E)   **(F)**
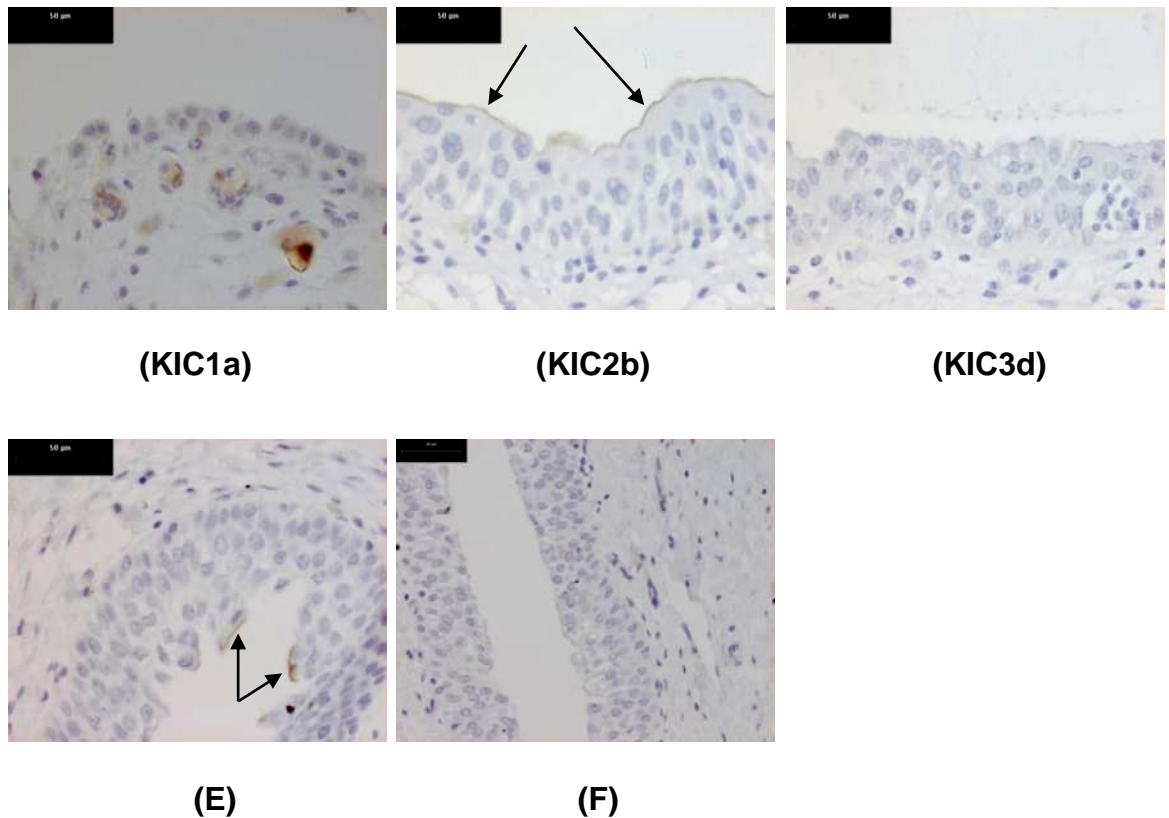
Figure 4.22: Immunoperoxidase labelling of the three individual KIC tissue sections with UPK3a showed presence of the protein in one (KIC2b) and absence in two sections (KIC1a and KIC3d). A section of normal human ureter (E) was included as a positive control and one as a negative control (F). Scale bar 50µm.

4.5.4.3 CK20.8

Cytokeratins (CK) are a group of proteins found only in the epithelial cells, with different CK types expressed differentially by different epithelial types and differentiation stages. CK20.8 was selected for the experiments to assess presence of superficial layer of urothelium.

The labelling showed positive expression in one section (KIC2b) but negative in the other two sections (KIC 1a and KIC3d). This further enforces the results of UPK3a antibody which showed absence of superficial cells in urothelium in sections KIC1a and KIC3d.

(KIC1a)   (KIC2b)   (KIC3d)

(E)   (F)
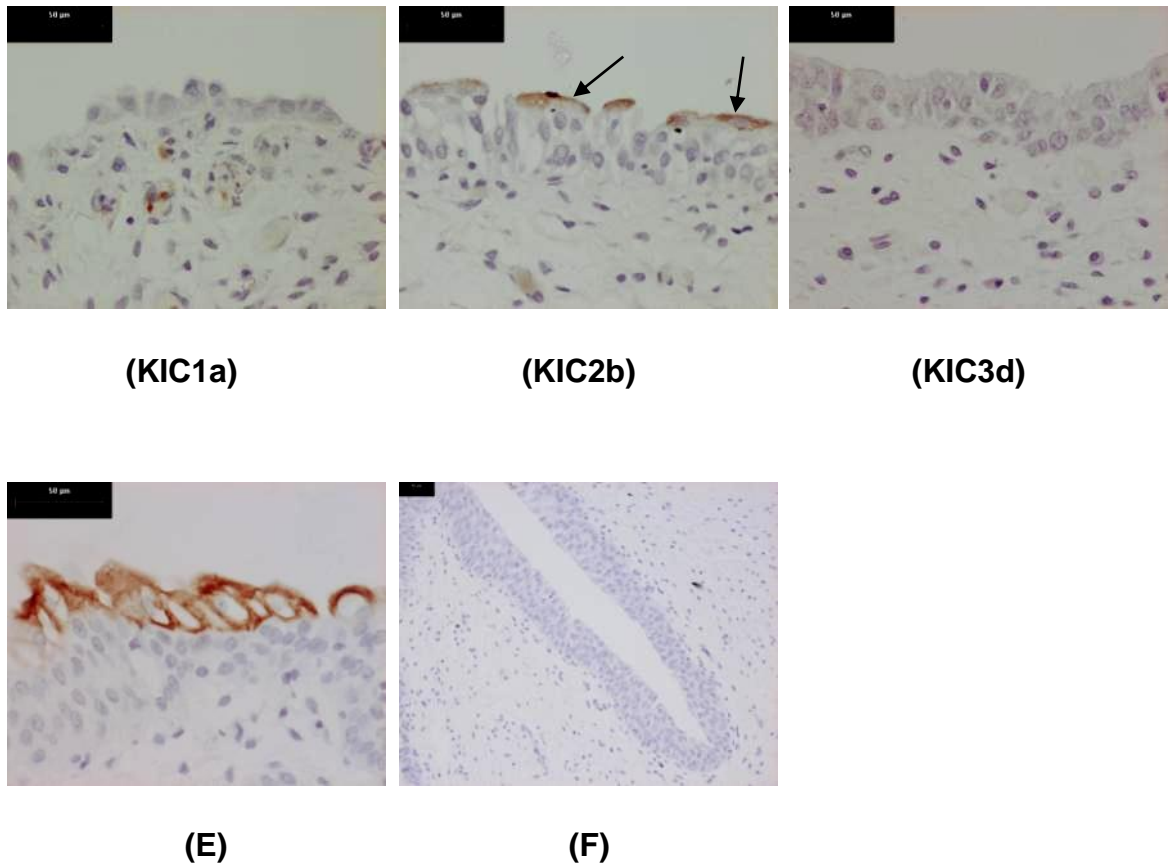
Figure 4.23: Immunoperoxidase labelling of the three individual KIC tissue sections with CK20.8 showed positive labelling in one (KIC2b) and negative labelling in two sections (KIC1a and KIC3d). This signifies loss of superficial cells in the urothelium in sections KIC1a and KIC3d. A section of normal human ureter (E) was included as a positive control and one as a negative control (F). Scale bar 50µm.

4.5.4.4 CK13

As the superficial layer of urothelial cells was absent in two (KIC1a and KIC3d) of the three individual sections, the next step was to identify whether the remaining cells were from intermediate and basal layers, and CK13 was selected for this purpose.

CK13 was expressed by all three individual KIC sections demonstrating that all had intermediate and basal urothelial cells.

(KIC1a)   **(KIC2b)**   **(KIC3d)**

(E)   **(F)**
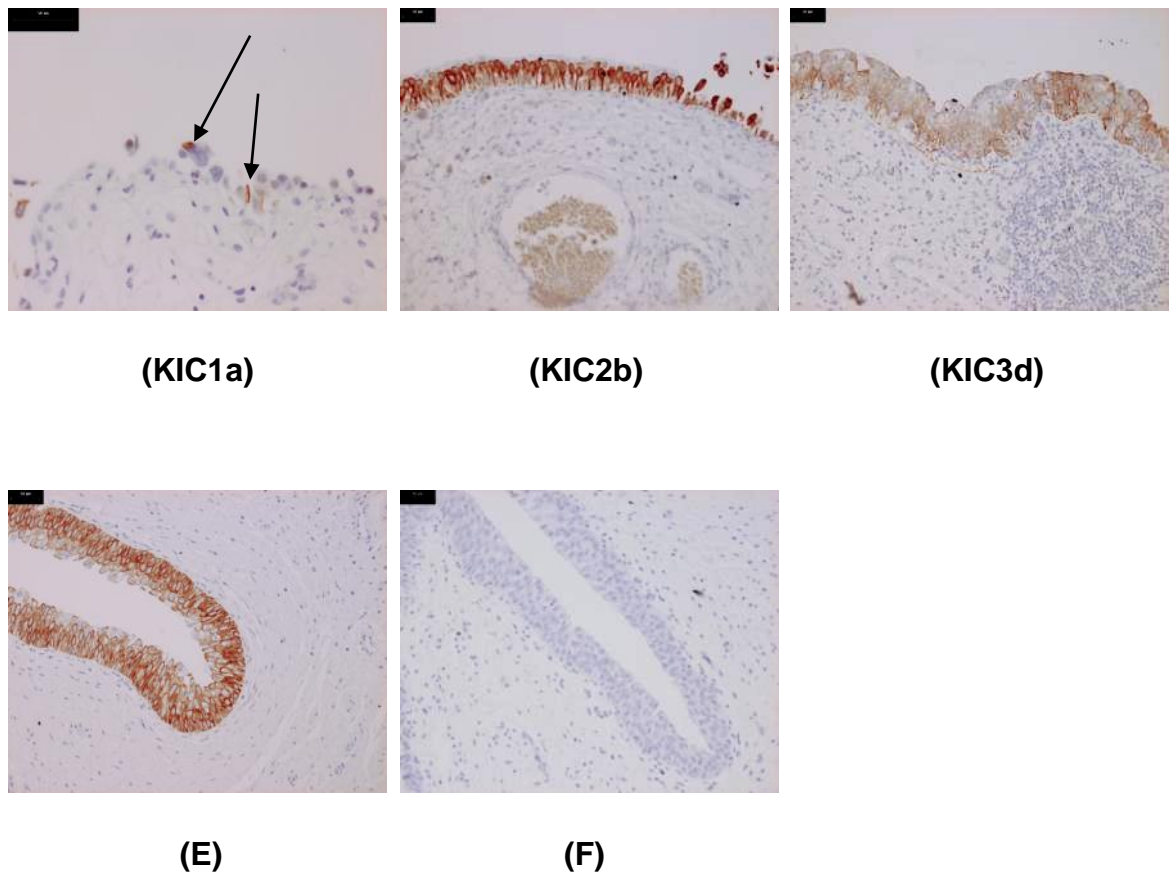
Figure 4.24: Immunoperoxidase labelling of the three individual KIC tissue sections with CK13 showed positive labelling in all three sections. This demonstrated that although superficial cells were absent in sections KIC1a and KIC3d (UPK3a and CK20.8 negative), they still had cells of an intermediate and/or basal urothelial phenotype. A section of normal human ureter (E) was included as a positive control and one as a negative control (F). Scale bar 50μm.

4.5.4.5 CK5

Although CK13 confirmed presence of intermediate and basal layer of cells in urothelium, it could not distinguish between the two types of cells and CK5 was therefore selected which is preferentially expressed by cells in the basal layer of urothelium only.

The labelling showed that the urothelial cells in section KIC1a were mainly basal in nature while those in KIC2b and KIC3d were both basal and intermediate in nature.

(KIC1a)   (KIC2b)   (KIC3d)

(E)   (F)

Figure 4.25: Immunoperoxidase labelling of the three individual KIC tissue sections with CK5 showed positive labelling in all three sections. CK13 (previous page) and CK5 positive cells in urothelium in all three sections demonstrated that the cells were from both basal and intermediate layers. A section of normal human ureter (E) was included as a positive control and one as a negative control (F). Scale bar 50µm.

4.5.4.6 CK14

When exposed to stress, the urothelium can sometimes undergo metaplastic changes which is a process of change of epithelium from one form to another to withstand the changing environment pressures. Chronic infections of urothelium can lead to metaplasia of the urothelial cells from normal transitional epithelium to squamous epithelium.

CK14 was therefore selected to examine the possibility of change of urothelium in KIC patients to squamous type under the stress from ketamine.

The experiment showed absence of CK14 expression in all 3 individual KIC sections.

(KIC1a)   **(KIC2b)**   **(KIC3d)**

(E)   **(F)**

Figure 4.26: Immunoperoxidase labelling of the three individual KIC tissue sections with CK14 showed absence of labelling in all three sections. A section of normal human skin (E) was included as a positive control and one as a negative control (F). Scale bar 50µm.

4.5.4.7 AB-PAS with diastase digestion

Surface of urothelium is protected by a glycocalyx or GAG layer. In certain chronic bladder uropathies such as painful bladder syndrome/IC, it is hypothesised that damage to this layer leads to development of symptoms [24]. Not only the tissue under stress can have metaplasia but also hyperplasia (increased number of normally found cells) of the tissue. It is therefore possible that chronic exposure to ketamine in patients with KIC may lead to an increase in the number of mucus producing glands as a protective mechanism and Alcian blue was selected to indicate any mucus.

The experiment showed that there was no increase in mucus producing glands in any of the three individual KIC tissue sections.

(KIC1a)   **(KIC2b)**   **(KIC3d)**

(E)   **(F)**
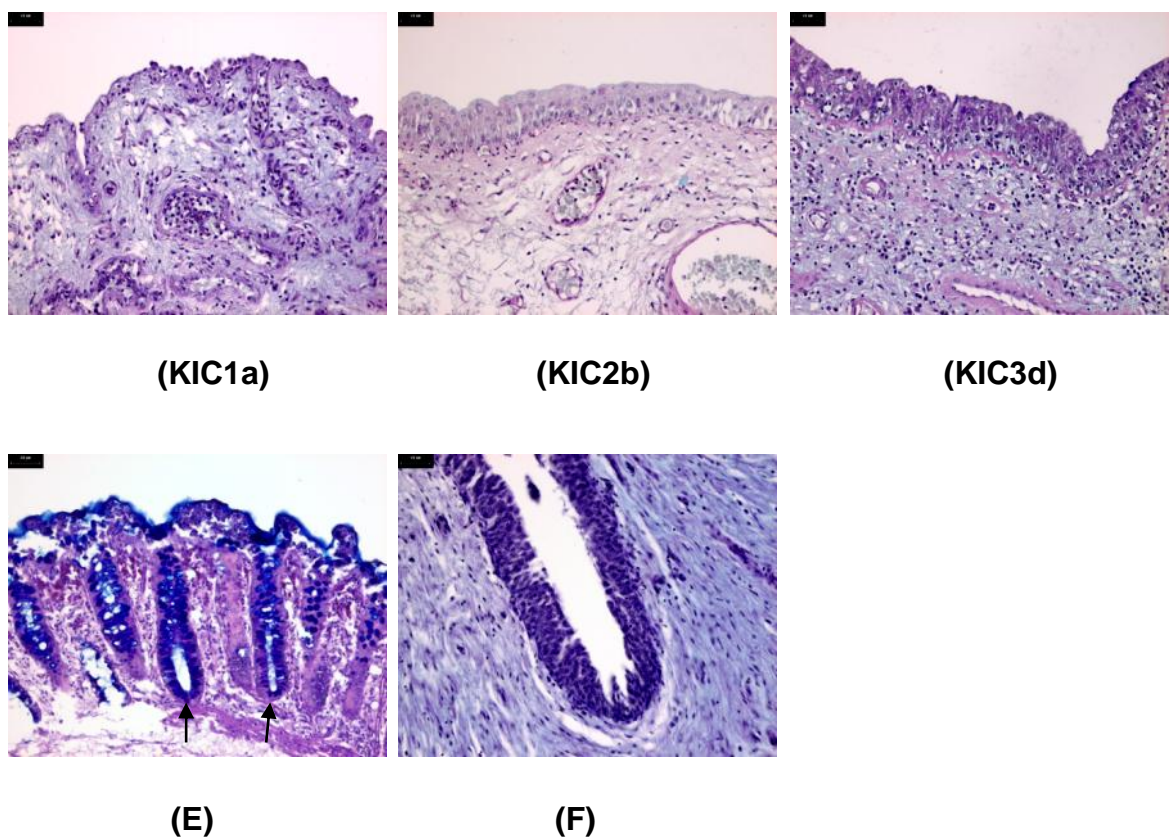
Figure 4.27: Alcian blue staining of the three individual KIC tissue sections showed no increased in mucus in any of the sections. A section of normal human colon (E) was included as a positive control and a section of normal human ureter (F) was included to show the appearance of normal urothelium after AB-PAS with diastase digestion staining. Scale bar 50µm.

4.5.4.8 CD34

H&E staining of the three individual KIC tissue sections showed oedematous vessels. CD34 was selected as the antibody to evaluate the presence of blood vessels.

The antibody labelling demonstrated oedematous blood vessels in sections KIC1a and KIC3d, and normal blood vessels pattern in section KIC2b.

(KIC1a)   **(KIC2b)**   **(KIC3d)**

(E)   **(F)**
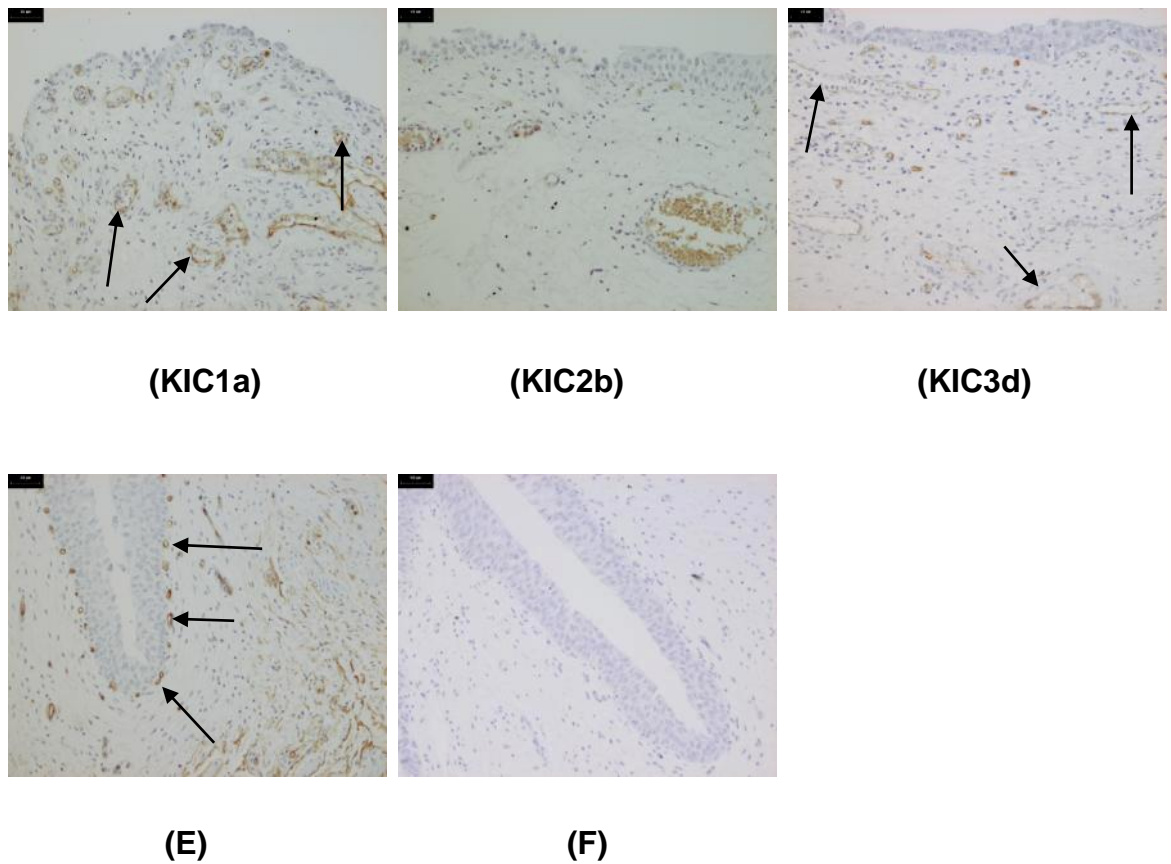
Figure 4.28: Immunoperoxidase labelling of the three individual KIC tissue sections with CD34 showed increased number of oedematous blood vessels in sections KIC1a and KIC3d. A section of normal human ureter (E) was included as a positive control and to demonstrate the normal distribution of blood vessels, and a section of normal human ureter (F) was included as a negative control. Scale bar 50µm.

4.5.4.9 CD3

To evaluate the rich cellular infiltrate in the lamina propria, CD3 was used as the antigen to assess whether these cells were T-cells.

The labelling showed absence of CD3 staining in one (section KIC1a) and CD3 positive T-cells infiltrate in the other two sections (KIC2b and KIC3d). In addition, the number of T-cells in urothelium in section KIC3d was also increased (positive labelling index 53/120, 44.17% as compared to 12/113, 10.62% in the urothelium of control tissue).

(KIC1a)   **(KIC2b)**   **(KIC3d)**

(E)   **(F)**   **(G)**
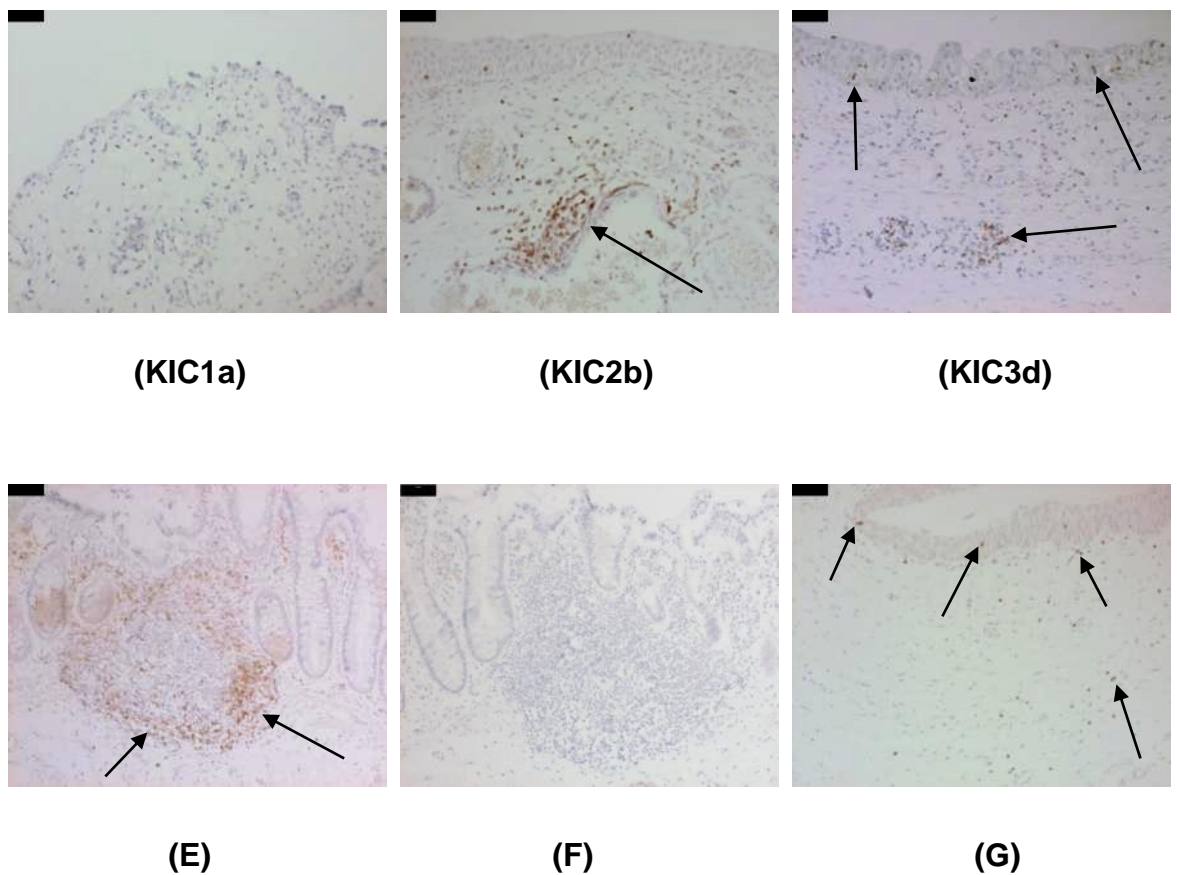
Figure 4.29: Immunoperoxidase labelling of the three individual KIC tissue sections with CD3 showed T-cell rich stromal infiltrate in sections KIC2b and KIC3d, and increased number of T-cells in the urothelium in section KIC3d. A section of normal human colon (E) was included as a positive control and one (F) as a negative control. A positive section of normal human ureter (G) was also included to demonstrate the number of T-cells in a normal urothelial tissue. Scale bar 100µm.

4.5.4.10 CD68

The next step was to assess whether any of the other inflammatory cells were infiltrating the KIC tissue. For assessing the presence of macrophages (and monocytes), CD68 was used as the antibody marker. The experiment showed that all sections had a few positive cells mainly associated with blood vessels in the stroma. In addition, sections KIC2b and KIC3d also had some CD68 positive cells in the urothelium.

(KIC1a)   **(KIC2b)**   **(KIC3d)**

(E)   **(F)**
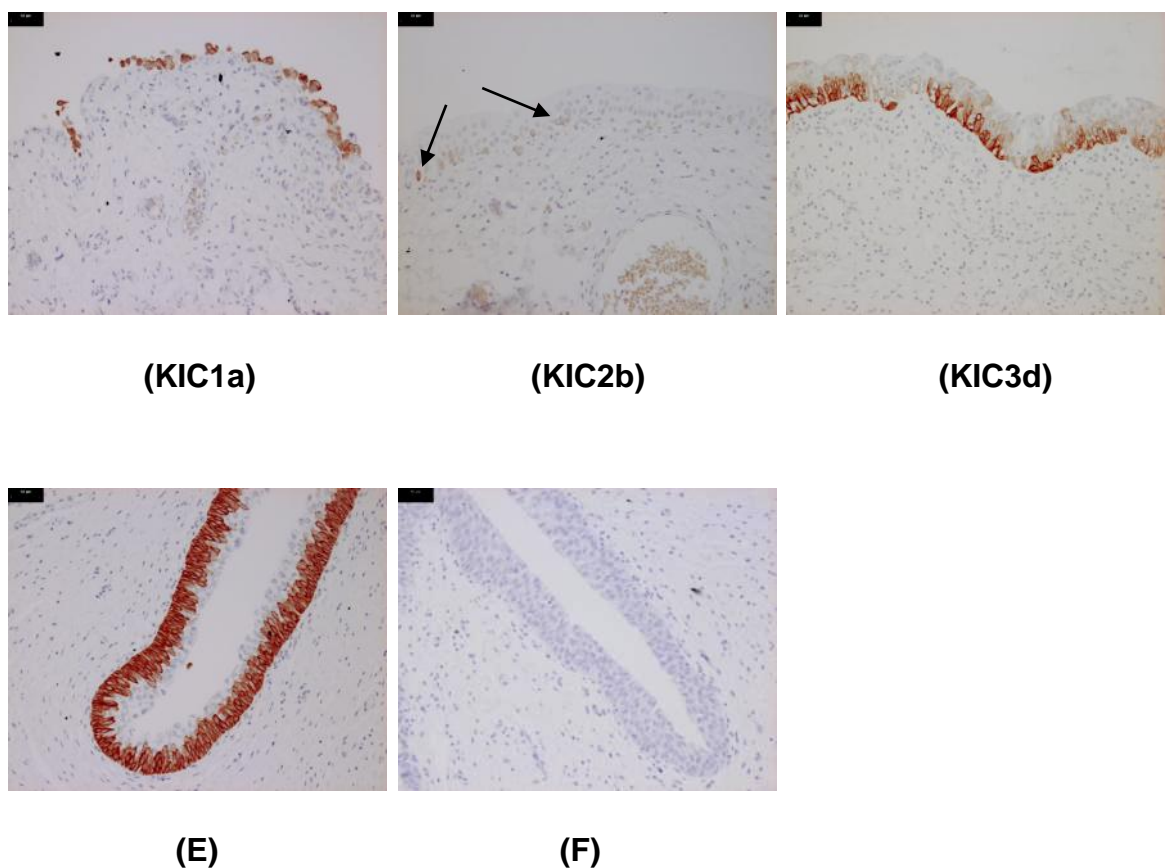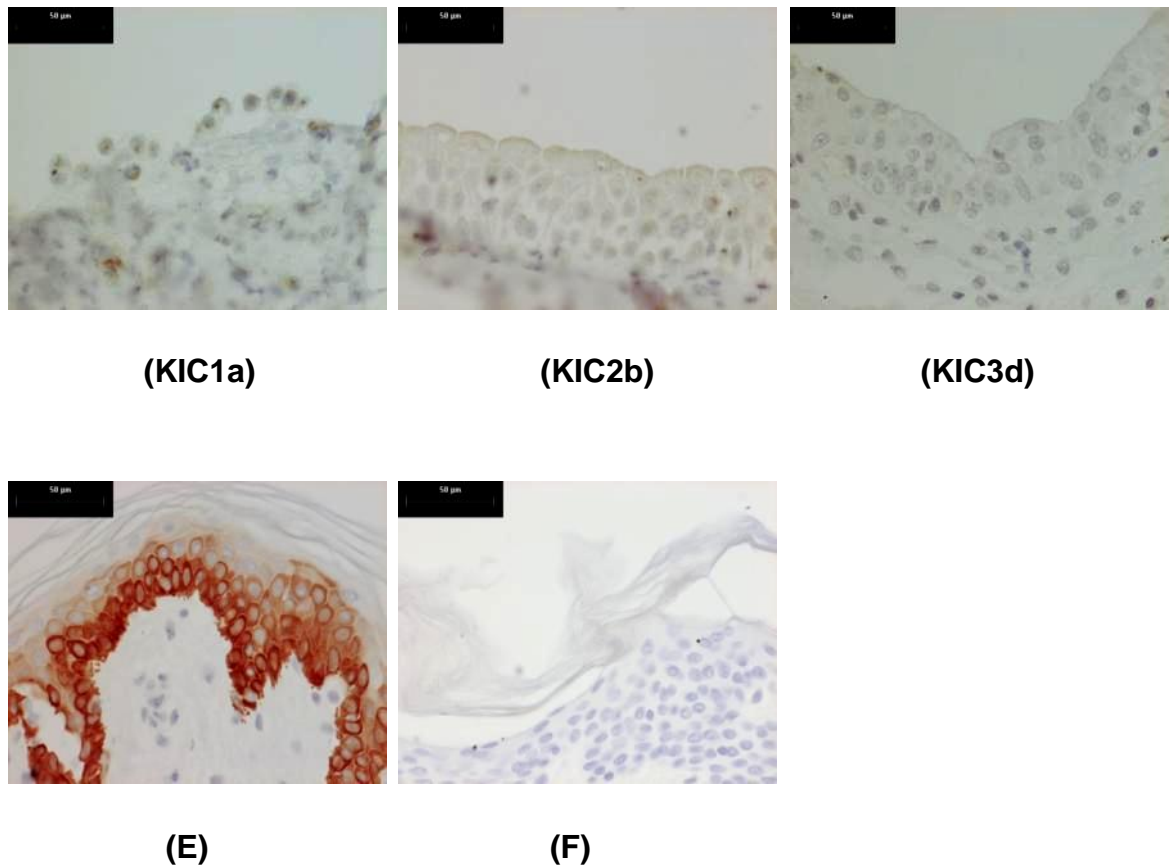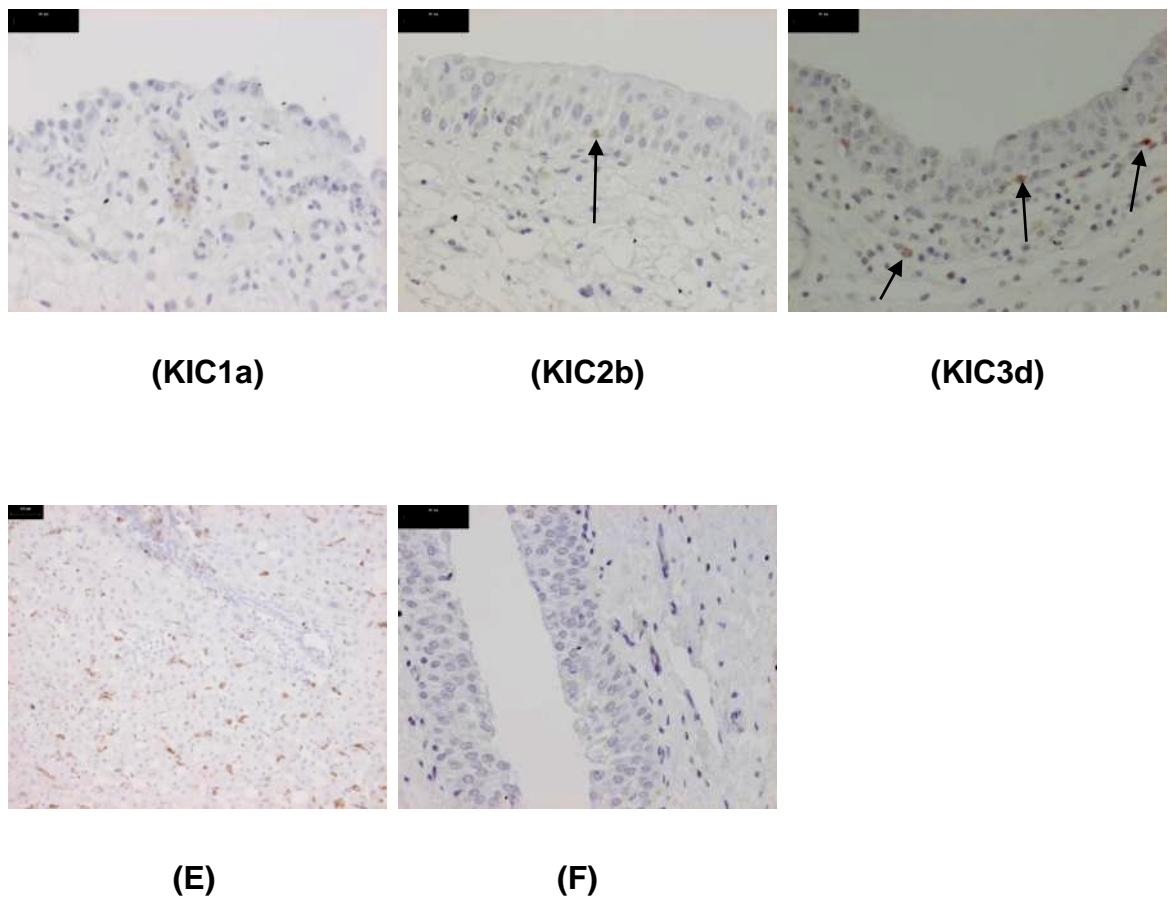
Figure 4.30: Immunoperoxidase labelling of the three individual KIC tissue sections with CD68 showed few positive cells in the stroma of all sections and some positive cells in the urothelium of sections KIC2b and KIC3d. A section of normal human liver (E) was included as a positive control and a section of normal human ureter (F) as a negative control. Scale bar 50μm.

4.5.4.11 Mast Cell Tryptase

Ketamine might be causing an allergic reaction to urothelium and mast cells play an important role in this aspect. To assess the number of mast cells in the three individual KIC tissues, mast cell tryptase was selected as the antibody marker.

The labelling showed that although mast cells were present in all three individual KIC tissue sections, they were not increased in number. Positive labelling index; section KIC1a 9/57, 15.79%, section KIC2b 5/42, 11.9% and section KIC3d 9/54, 16.67% as compared to the positive labelling index of mast cells in the stroma of positive control; 11/50, 22%.

(KIC1a)   **(KIC2b)**   **(KIC3d)**

(E)   **(F)**
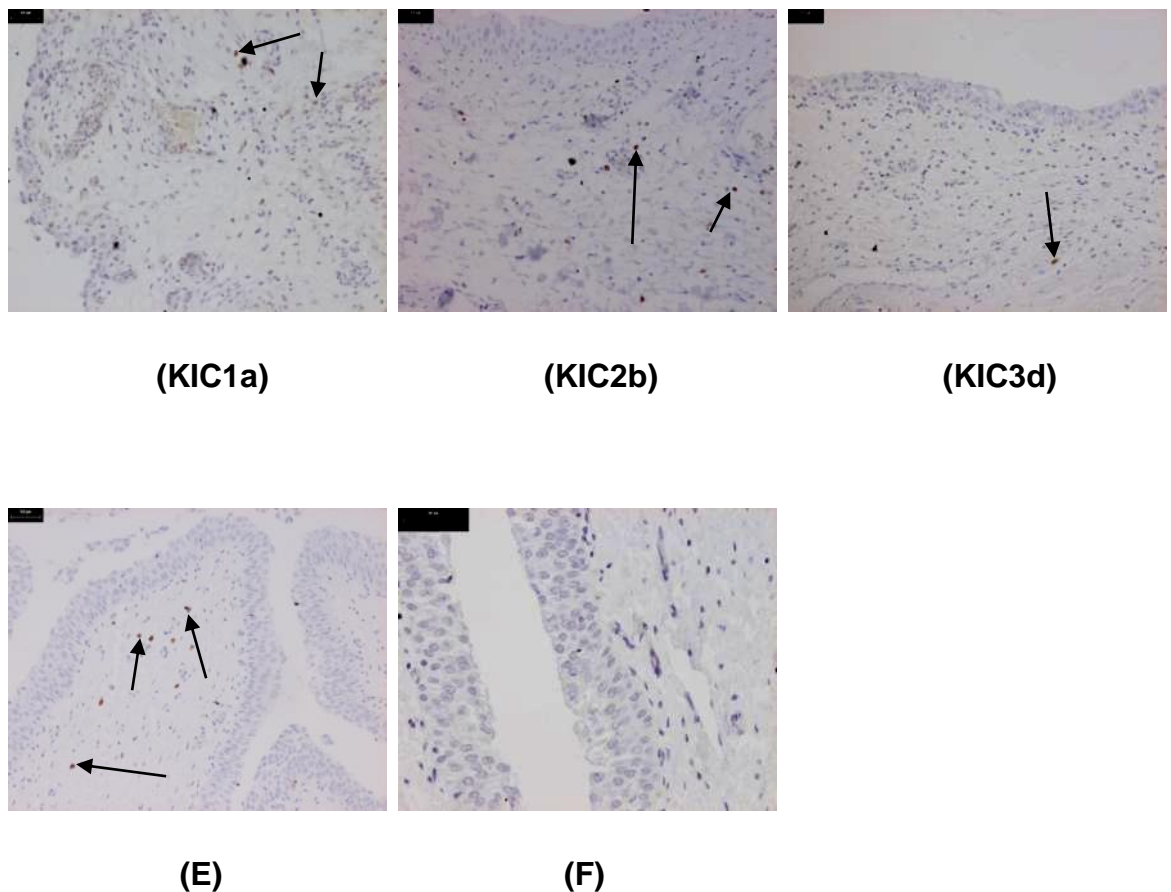
Figure 4.31: Immunoperoxidase labelling of the three individual KIC tissue sections with mast cell tryptase showed no increase in the number of mast cells in the stroma of either of them. A section of normal human ureter (E) was included as a positive control and one (F) as a negative control. Scale bar 50µm.

4.5.4.12 CD117

The proto-oncogene CD117 (c-kit) is a non-specific transmembrane receptor for SCF (stem cell factor) and is often associated with stem/ progenitor cells in various organs such as prostate, cardiac, bone marrow or hematopoietic stem cells [80]. It can also be found in more differentiated cell types such as mast cells [86].

The potential role of these cells in vivo might range from a less differentiated progenitor cell type to a more differentiated cell with specific functions, such as mechano-sensation or neuroendocrine function [80]. Immunoperoxidase labelling using CD117 was therefore performed to assess whether there was increase in the expression of these cells in the KIC tissue. The labelling showed CD117 positive mast cells in the stroma of all three individual KIC tissue sections and no positive cells in the urothelium of any of the sections.

(KIC1a)   (KIC2b)   (KIC3d)

(E)   (F)
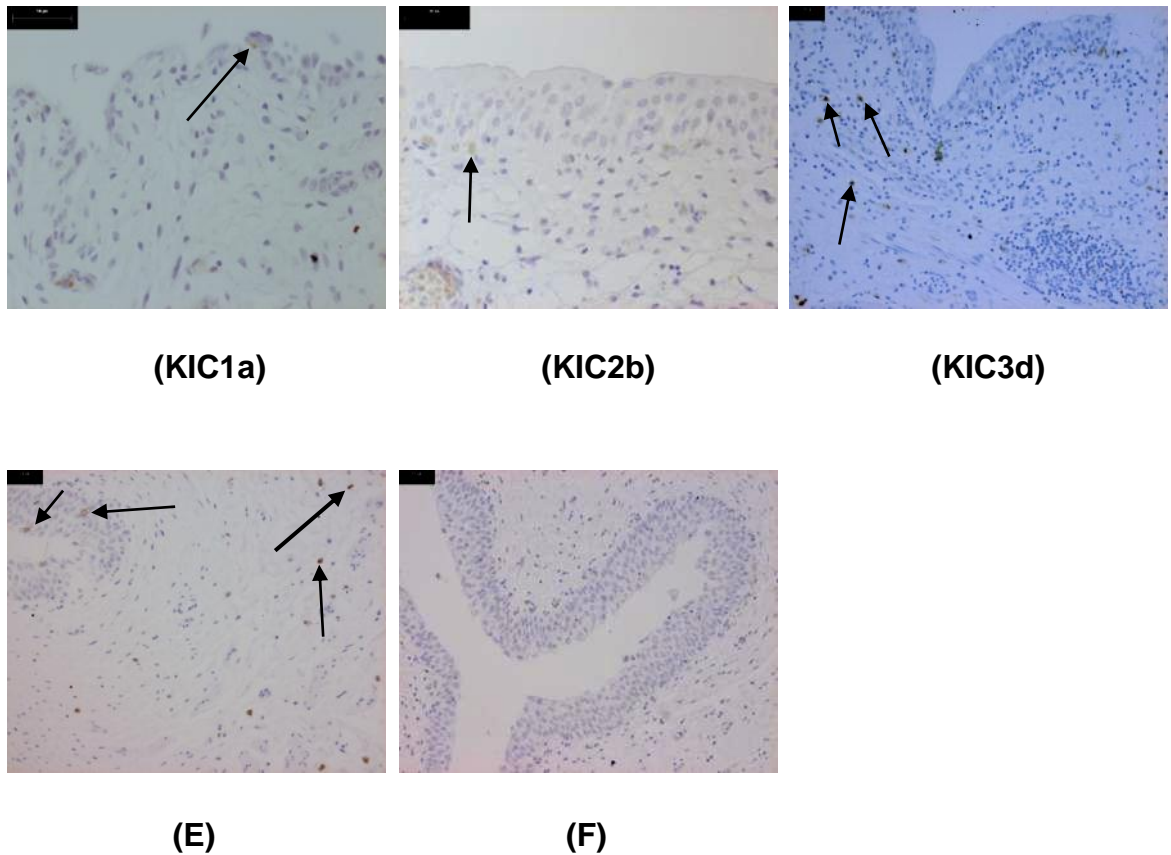
Figure 4.32: Immunoperoxidase labelling of the three individual KIC tissue sections with CD117 showed no increase in expression of the antigen in any of them. A section of normal human ureter (E) was included as a positive control and one (F) as a negative control. Scale bar 50µm.

4.5.4.13 Anti-human NGFR

NGFR is a cell membrane receptor protein whose expression has been demonstrated in urothelium from human neuropathic bladder patients [87] and also in animal models where urothelium was damaged e.g. by cyclophosphamide instillation [88, 89]. It is also expressed by the basal layer of urothelium in the upper urinary tract (renal pelvis and ureter) [80]. In a normal human ureter section (E), anti-human NGFR was only expressed by the basal layer of urothelial cells. In the KIC tissues though, in addition to the positive basal urothelial cells in sections KIC2b and KIC3d, major expression was also found in the stromal cells of all three individual KIC sections. These cells in the stroma could be either myocytes or neurites and further antibody labelling will be needed to establish their exact nature.

(KIC1a)   (KIC2b)   (KIC3d)

(E)   (F)
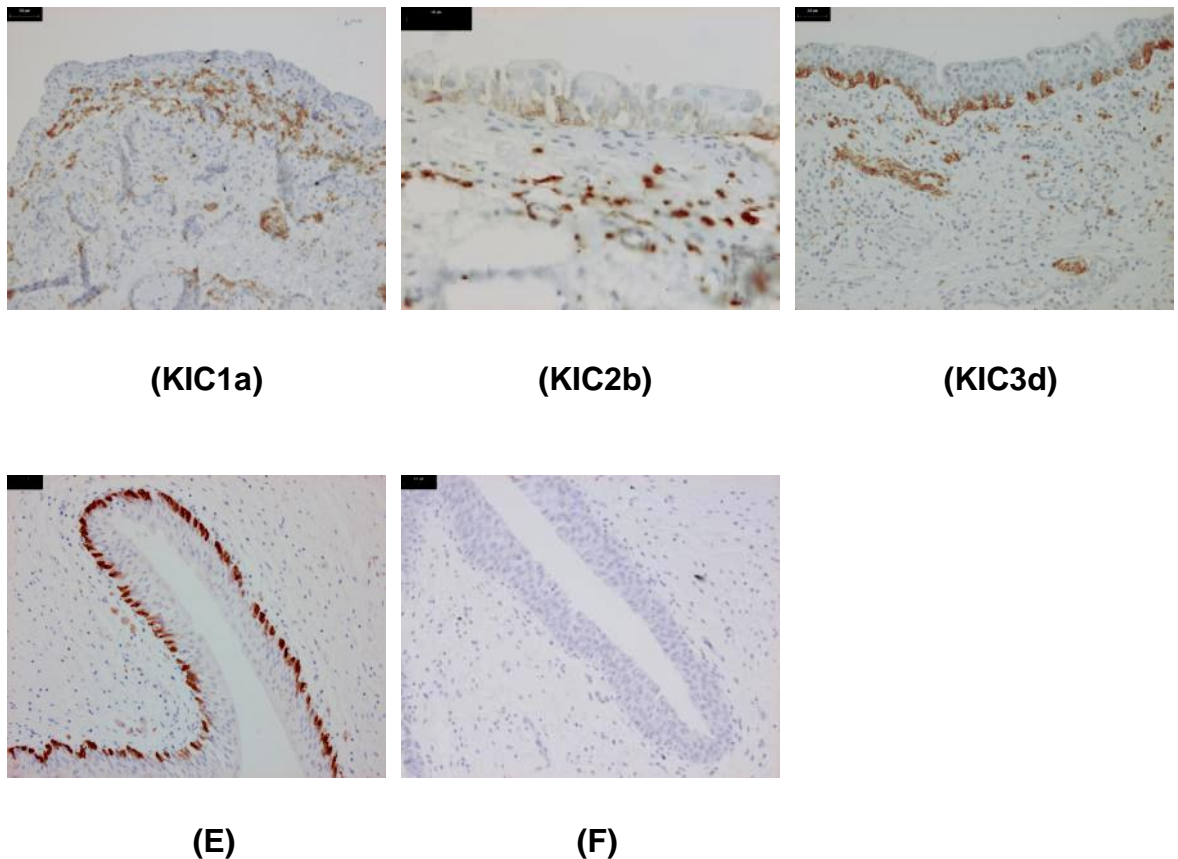
Figure 4.33: Immunoperoxidase labelling of the three individual KIC tissue sections with anti-human NGFR showed positive cells in the basal layer of sections KIC2b and KIC3d, and increased expression of the antibody in the submucosa of all three individual KIC tissue sections. A section of normal human ureter (E) was included as a positive control and one (F) as a negative control. Scale bar 50µm.

4.6 Discussion

The human urinary tract is covered from the renal pelvis to the proximal urethra by transitional epithelium (urothelium). It consists of three layers of cells i.e. basal, intermediate and superficial. Transitional epithelium is a highly specialised structure in that it not only forms a tight barrier preventing urine and toxic agents passing in to the underlying tissues but also can contract and expand in response to the volume of urine in the bladder. Urothelium is mitotically quiescent in normal circumstances but becomes highly proliferative in response to injury.

Ketamine causes damage to the human urinary tract and can lead to the development of a florid non-bacterial cystitis condition known as ketamine-induced cystitis (KIC). This study was aimed at identifying changes caused by ketamine in human urothelium so that it might help us understand possible mechanisms of development of KIC.

In this multicentre study, several difficulties were experienced in collecting a reasonable number of KIC tissues to perform the immunohistochemistry experiments. The main problem encountered was obtaining Research and Development (R&D) approval at multiple sites to transport tissue to the University of York for experiments. Although, R&D approval was gained at two sites and 20 individual KIC tissue blocks were eventually available, some of the tissues were significantly small in size. This meant that not all antibodies from the panel could be performed on each of the blocks without some useful initial information first. As there are no detailed immunohistological studies on KIC published in literature so far, no previous guidance was available from that aspect. It was therefore decided (between the scientists at the Jack Birch Unit of Molecular Carcinogenesis, University of York and a Histopathologist at the St James's University Hospital, Leeds) to select an initial antibody panel based on H&E results from the three individual KIC tissue sections selected. The agreed antibody panel was performed on these three individual KIC tissue sections and showed some interesting results;

1. H&E staining showed inflamed and apparently dysplastic tissue with loss of cohesion in epithelium. **Clinical history in these patients is of utmost importance as without that it would be very easy to diagnose a carcinoma in situ (CIS) on the basis of histology alone.**

2. Absence of superficial cells in the two (KIC1a and KIC3d) individual KIC tissue sections was associated with increased proliferation in the remaining urothelial cells. An interesting point here is that proliferation was absent in section KIC2b which had superficial cells and therefore intact barrier function. **Urothelial damage by ketamine may therefore induce proliferation in cells in the basal and intermediate layers to try and re-store the barrier function of urothelium.**

3. Human urothelium is known to undergo metaplasia in response to chronic infection or inflammation and transforms from a normal transitional to a metaplastic squamous epithelium to withstand the rigours of the environment. However, there was no evidence of urothelial metaplasia in any of the three individual KIC tissue sections, as one might expect in response to the chronic inflammation caused by ketamine.

4. Inflammation as evidenced by oedematous blood vessels was present in two (KIC1a and KIC3d) of the three individual KIC tissue sections. Sections KIC1a and KIC3d also had absent superficial layer of urothelium and therefore the barrier function was lost leaving stroma exposed to the harmful effects of toxic urine components including ketamine and its metabolites. **This may represent one of the mechanisms by which ketamine induces inflammation in the urothelial tissue and the resultant development of KIC symptoms.**

5. The rich cellular infiltrate in the stroma of two (KIC2b and KIC3d) of the three individual KIC tissue sections and in the urothelium in one of the individual sections (KIC3d) was mainly T-cells. **Amongst other inflammatory adaptive responses of a tissue, T-cells are also associated with type IV delayed hypersensitivity reaction and this may represent another mechanism by which ketamine abuse/use leads to**

the development of KIC. This is further supported by the clinical evidence that patients presenting with KIC symptoms have usually abused/used ketamine for a while before permanent urinary tract damage happens.

This was an interesting finding as ketamine managed to induce inflammatory changes (increased number of T-cells in the stroma) in the one individual KIC tissue section (KIC2b) with superficial cells and intact barrier function. **This might mean that action of ketamine on urothelium is two fold: direct toxic effect on the superficial cells and a receptor mediated effect to cause damage in the deeper tissue.**

There was further evidence of inflammation as indicated by the increased anti-human NGFR labelling in the submucosa of all three individual KIC tissue sections and might signify possible neural fibrosis and inflammation. The positive cells in the stroma could be either neurites or myocytes and further antibody labelling would be needed to establish their exact nature.

Some of the other cells (macrophages, monocytes and mast cells) which are well known to take part in inflammatory and allergic reactions did not seem to participate towards the development of KIC. There was also no evidence of a mechano-sensitive cell population in the urothelium which if present might have become active in response to the urothelial injury caused by ketamine.

4.7 Conclusions

The results obtained from this pilot study have helped to understand the urothelial tissue damage caused by ketamine and indicated the possible pathways which might lead to the development of KIC although establishing a definite aetiopathological mechanism of development of KIC at this stage is not possible. This is the first detailed immunohistological study of the human KIC tissue and based on these results, future experiments will be performed aimed at expanding on the information gained already.

5 Relationship between dose, duration and frequency of ketamine use and development of ketamine-induced cystitis (KIC)

5.1 Introduction

Ketamine is a phencyclidine derivative NMDA receptor antagonist used in clinical practice for anaesthesia (reviewed by [90]), analgesia ([3-5] and reviewed by [6]) and is under research to develop next generation of fast acting anti-depressants (reviewed by [7]).

Ketamine has been a target of recreational drug users for many decades [10] and although literature reports ([91] and reviewed by [12]) and anecdotal experience suggests that ketamine abuse is on the rise, the number of patients presenting to Urology clinics is not. This may be due to one of the following reasons;

1) The pattern of use; occasional (weekend/parties) use which allows bladder time to recover and it is only with sustained use that irreversible scarring and damage is likely to occur.

2) Non-association by drug abusers of the problem of non-bacterial cystitis with ketamine abuse.

3) Under reporting of the problem as ketamine is an illegal drug.

KIC has also been reported in patients using analgesic ketamine for weeks to months and the symptoms either improved or completely resolved in all patients following stopping or reducing the dose [3, 14]. However, we have recently reported a case of a patient who used analgesic ketamine for longer (three years) than the above reports and developed bladder symptoms which persisted even after stopping the drug and resulted in surgical treatment [92]. Also, the in vitro experiments showed that ketamine toxicity to normal human urothelial (NHU) cells increased as the concentration of the drug was increased and also by treating them daily with ketamine. It is therefore possible that development

of KIC is related to the dose, duration or frequency of ketamine used and the aim of this study was to test this hypothesis.

5.2 Materials and Methods

Recreational drug users can be a difficult patient group to manage in the clinic due to their un-cooperative behaviour and unwillingness to attend for clinical appointments, the same is the case with ketamine abusers. Furthermore, it is often quite difficult to establish the accurate exposure of drug of abuse from recreational drug users. Ketamine is increasingly used as a third line analgesic for chronic pain treatment [14] and this cohort of patients was identified as a useful group to investigate the above hypothesis.

The study was carried out at the James Cook University Hospital (JCUH), Middlesbrough and was approved as an amendment to the original research project by the ethics and, the research and development (R&D) committees.

All patients dispensed ketamine for pain relief from the pharmacy department at the James Cook University Hospital (JCUH), Middlesbrough between April 2007 and March 2011 were included in this study. These patients were assessed using a telephone questionnaire (figure 5.1) and managed using the management flow chart (figure 5.2) approved by the ethics and R&D committees.

Screening questionnaire about urinary tract symptoms in patients prescribed ketamine for pain relief

Patient number ....

Age ....   Sex ....

Dose of ketamine ....

When started ....

Reason why ketamine prescribed ....

Taking any other medications ....

Urinary symptoms:

How often are you passing urine during the day ....

Are you passing urine more frequently during the day since started ketamine ....

How many times do you have to wake up at night to pass urine ....

Are you waking up more often at night to pass urine since started ketamine ....

Do you have any pain on passing urine ....

Have you developed any abdominal pain since started ketamine ....

Are you able to hold urine when you have the sensation to pass it ....

Have you ever leaked urine if not able to get to toilet in time ....

Have you ever seen blood in your urine ....

If patient found to have any urinary symptoms:

Would you be willing for us to investigate your urinary symptoms further ....

Figure 5.1: The telephone questionnaire used to investigate patients using analgesic ketamine for any urinary tract symptoms.

KIC questionnaire management flow chart

Introduce myself

↓

Explain reasons for questionnaire

↓

Explain that results are linked anonymous

↓

No ← Ask if they will like to participate? → Yes

No ← Ok to ask questions now?

Thank them for their time and finish the conversation.

Arrange for alternate time to ring.

Yes

Does patient want us to investigate this further? ← Yes ← Does patient has urinary symptoms?

No

No

Thank them for their co-operation and finish the conversation.

Thank them for their co-operation and finish the conversation.

Yes

↓

Ask their permission to write to their GP to formally refer the patient to us. Answer any questions they have and thank them for their co-operation.

↓

Once referral letter received, give patient urgent appointment in clinic to further investigate.

Figure 5.2: The management flow chart for any patients found to have urinary symptoms following the KIC questionnaire.

5.3 Results

A total of 61 patients were identified who were dispensed ketamine from pharmacy department at the JCUH, Middlesbrough between April 2007 and March 2011. More than one month (arbitrary value) duration of use of ketamine was considered as significant and any patients who used it for less than four weeks were excluded. In total, 32 of the 61 patients had to be excluded due to various reasons (figure 5.3).
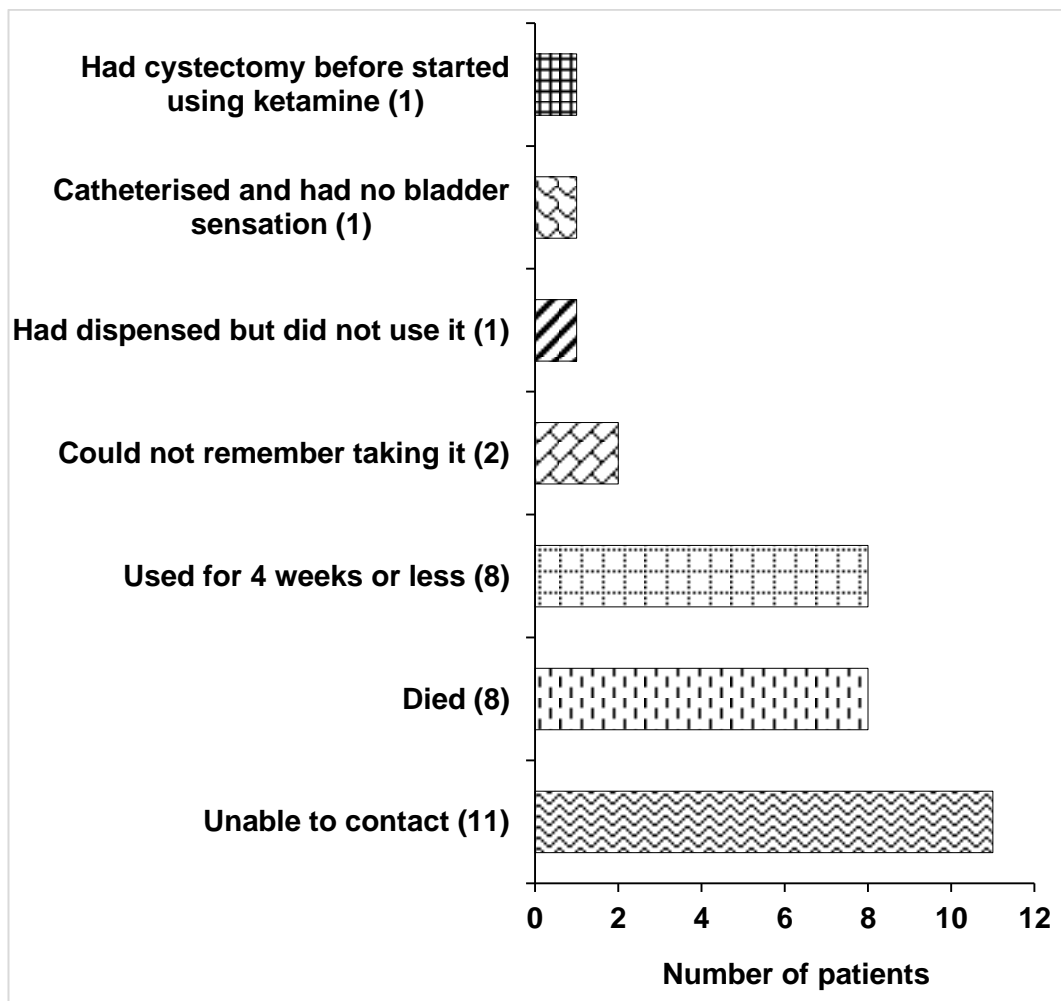
Figure 5.3: 32 patients excluded from the study and the reasons for their exclusion.

A total of 29 patients were included in the study who were either currently taking ketamine, or had taken it in the past for four weeks or more and had now stopped taking it due to either the side effects or no pain relief. 17 patients were males and 12 females, age ranged from 41 – 79 years with a mean age of 57.7 years. All patients were using ketamine in the liquid form and 1ml of the dispensed solution contained 10mg.

Six of the 29 patients were found to have urinary symptoms significant enough to offer further investigations. Four of these six patients were females and two males, age ranged from 42 – 67 years with mean age of 56.1 years. The duration of use ranged from ten months – eight years and the dose from 40mg/day – 375mg/day (table 5.1). One or more of the following urinary tract symptoms were reported by these six patients after analgesic ketamine use; daytime frequency, nocturia, abdominal pain and urinary incontinence (figure 5.4).

Age	Sex	Duration of use	Dose	Further investigations
53	F	5 years	7.5ml 5 times a day	Yes
55	F	5 years	2.5ml 6 times a day	Yes
67	F	8 years	2.5 – 5ml QDS/PRN	Yes
42	F	1 year	1-2ml QDS	No (patient choice)
58	M	10 months – 1 year	5ml QDS	Yes
62	M	2 years	10ml BD – TDS	Yes

Table 5.1: Demographics, dose, duration and frequency of ketamine use in the patients found to have significant urinary tract symptoms possibly from analgesic ketamine use.
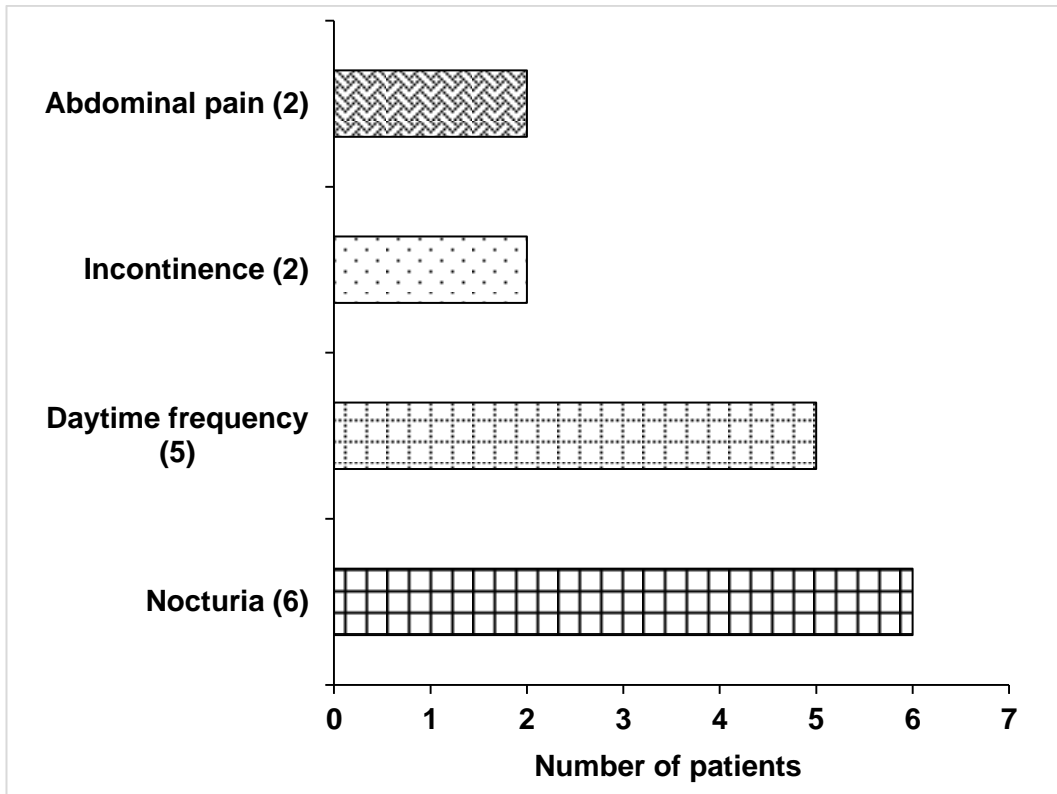
Figure 5.4: Frequency of different urinary tract symptoms in the six patients with possible urinary tract damage from analgesic ketamine use. Note that the symptoms most frequently happening are daytime frequency and nocturia which may be due to bladder overactivity in response to the damage induced by ketamine.

Of the six patients with symptoms, one had recently stopped taking ketamine and did not want any further investigations at present to see if stopping the drug will improve their symptoms. Five patients agreed to further investigations of their urinary symptoms. Three of these five patients have already been seen in the clinic and appropriate investigations arranged. The rest will be seen in clinic in the near future for further management.

23 of the 29 patients had no urinary symptoms. 15 of them were males and 8 females, age ranged from 41 – 79 years with mean age of 58.1 years. The duration of use ranged from two months – six years and the

dose from 25mg/day – 600mg/day. Five of the patients who had used analgesic ketamine in the past but had now stopped taking it, could not remember the dose (table 5.2).

Age	Sex	Duration of use	Dose
45	F	3-4 years	5ml QDS
66	F	5-6 months	2ml QDS
48	F	3-4 months	2ml QDS
68	F	2 years	2.5ml BD – TDS PRN
77	F	4 months	2.5ml TDS
79	F	2 years	2.5ml 6 times a day
58	F	4 years	2.5ml QDS
48	F	2 years	Could not remember
59	M	4 years	10ml 6 times a day
51	M	1 year	5ml TDS
60	M	2 months	5ml TDS
64	M	6 months	15ml TDS PRN
65	M	4 years	5ml OD
47	M	2 years	2.5ml QDS
47	M	6 years	5ml QDS
41	M	1 year	2.5ml 6 times a day
61	M	6 years	5ml QDS – 6 times a day
51	M	1 year	2.5ml OD
69	M	2 1/2 years	5ml QDS
66	M	2 months	Could not remember
41	M	2 months	Could not remember
58	M	3-4 months	Could not remember
68	M	6 months	Could not remember

Table 5.2: Demographics, dose, duration and frequency of ketamine use in the patients with no urinary tract symptoms after analgesic ketamine use.

5.4 Discussion

Ketamine abuse can lead to the development of florid non-bacterial cystitis symptoms which can be so severe that some patients have required surgical treatment in the form of cystectomy and neobladder formation or urinary diversion ([2, 93] and reviewed by [8, 12]). Although the anaesthetic dose of ketamine is 0.5 – 2mg/kg, continued use leads to the development of tolerance and recreational users have reported abusing high doses (up to 20 g/day) (reviewed by [8]). Interestingly, in the previously reported cases, prescription users of analgesic ketamine developed urinary tract symptoms at much lower doses, from 200mg/day to 1g/day [3, 14, 92].

There is no definite evidence in literature to suggest when the symptoms start from ketamine abuse but may be years (anecdotal reports). On the other hand, analgesic ketamine use appears to have led to the development of KIC after use for weeks to months [3, 14]. Furthermore, the in vitro experiments suggest that ketamine is toxic to NHU cells at all concentrations and the toxicity increases following daily use as compared to a single use (results, chapter 3).

This raises three interesting questions;

1. Is development of KIC dependent on the dose of ketamine used?
2. Is development of KIC dependent on the duration of ketamine used?
3. Is development of KIC dependent on the frequency of ketamine used?

Due to the small number of ketamine abusers who have presented to us so far and the relative difficulty to establish the accurate exposure of ketamine from this cohort, patients using analgesic ketamine were identified to conduct this study.

The results showed that a relatively small number of patients (6/29, 20.7%) using analgesic ketamine experienced either development of or deterioration in their lower urinary tract symptoms. There was no clear association between the dose, duration or frequency of analgesic ketamine used to the development of KIC.

Patients using much higher doses (up to 600mg/day) did not report any urinary tract symptoms when compared to patients with symptoms of KIC (up to 375mg/day).

Although three of the patients who developed urinary symptoms following analgesic ketamine use had used it for 5 years or more, the rest of them had used it for ten months to two years and still developed the symptoms. Patients who did not report any significant urinary symptoms had also used it for a considerable duration (up to six years).

Patients in both groups (with and without urinary symptoms) reported comparable frequency of ketamine use and there was no clear association to the development of KIC.

5.5 Conclusions

This study had some limitations;

1. There was no age matched control group of patients with same medical illnesses and using same medications as patients taking ketamine.

2. No investigations have been done so far to confirm the presence of KIC in the six patients who reported urinary symptoms after using ketamine for pain relief.

Within limitations of the study, it is possible that there may be a group of patients who are vulnerable to urinary tract damage from ketamine even at low prescription doses. However, before drawing any definite conclusions, patients reporting lower urinary tract symptoms after analgesic ketamine use will be seen in the clinic and investigated appropriately.

6 Discussion/Conclusions and future direction of research

Other than being used as an anaesthetic for over 40 years [1, 2], ketamine is also increasingly used as an analgesic in chronic pain patients [4-6] and three literature reports so far have described the development of urinary tract damage in patients using ketamine for analgesia [3, 14, 92]. Ketamine is also under investigation to develop the next generation of rapid acting anti-depressants (reviewed by [7]). Time is of essence in patients with depression as they are most vulnerable to self-harm in the initial stages. The current conventional anti-depressant therapies take at least 4-6 weeks to exert their effects and some of these patients may not have that much time. Research in patients with depression treated with ketamine has shown improvement in their symptoms in hours to days [94-96]. This makes ketamine sound like an ideal anti-depressant but the problem will be the urinary tract symptoms these patients may develop from long term prescription ketamine use and that may in turn lead to the re-development of depression symptoms. As no long term trials have been done so far on patients using ketamine for treatment of depression, there are no literature reports of urinary tract damage either.

With ketamine gaining popularity among clinicians to treat patients with chronic pain and depression, and among recreational drug users as the drug of choice to abuse, inevitably more and more patients are going to present to the Urology departments with KIC. It is therefore of utmost importance to understand KIC as a condition so that effective treatment strategies can be developed for these patients. This research project is the first step in that direction.

6.1 Possible mechanisms of development of ketamine-induced cystitis

Although the Urology community worldwide is becoming increasingly aware of ketamine-induced cystitis (KIC) as a condition and there are

more than 20 literature reports on the condition so far, the mechanism of development of KIC is unknown. Chu et. al. [2] suggested different theories but none is proven experimentally.

The first aim of this research project therefore was to provide some experimental evidence about the possible mechanisms of development of KIC. A two pronged experimental approach was undertaken for this purpose and included cell biology experiments (exposure of normal human urothelial cells to different drugs) and immunohistochemistry experiments (immunolabelling of the KIC tissue with different antibodies). Although these are initial experiments but based on the preliminary results from the above experiments, the contributory factors and the possible mechanisms of development of KIC in the ketamine users and abusers might be as below.

6.1.1 Cell Biology experiments

In vitro cell biology experiments demonstrated that ketamine was toxic to normal human urothelial (NHU) cells and daily treatment was more damaging as compared to a single exposure.

Nor-ketamine (a primary metabolite of ketamine) demonstrated equal toxicity to ketamine of NHU cells. The mechanism of action of ketamine on NHU cells may therefore be a combination of itself and its primary metabolite nor-ketamine.

Ketamine has a highly promiscuous receptor binding profile (reviewed by [9]) and it is possible that one of these receptors is responsible for the action of ketamine on NHU cells and therefore development of KIC. In this respect, NMDA receptor seemed to be the obvious target for urothelial ketamine effects based on the reports of neuronal toxicity via the same receptor pathway in animal models [82, 83], but the work presented here did not support this.

6.1.2 Immunohistochemistry experiments

The absence of superficial layer of urothelium in two of the three individual KIC tissue sections used for immunohistochemistry experiments signified that the barrier function was lost. This was further evidenced by increased proliferation rate in the remaining urothelial cells in the same two individual KIC tissue sections and might represent an attempt by the tissue to re-generate superficial cells to try and re-store the barrier function.

Loss of barrier function left the remaining cells vulnerable to toxic urinary components particularly ketamine and its primary metabolite nor-ketamine, and therefore development of inflammation. This was evidenced by oedematous blood vessels, increased T-cells and anti-human NGFR antibody expression in the KIC tissues. Inflammation in bladder tissue could then lead to the development of lower urinary tract symptoms (LUTS) which perhaps be temporary in occasional ketamine users but continuous use might eventually lead to permanent scarring and damage, and development of KIC.

6.2 Is there a possible link between KIC and other chronic non-bacterial cystitis conditions i.e. interstitial cystitis (IC) and eosinophilic cystitis (EC)?

Interstitial cystitis (IC) has been puzzling scientists and clinicians for many decades. It is particularly difficult to diagnose IC in the initial stages of the disease not only clinically, but also on the basis of investigations as patients may not have developed the classical signs of disease (i.e. bladder glommerulations, hunner's ulcer). Although the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) has devised a criterion for the diagnosis of IC but it is used mainly for research purposes. As there are no objective biomarkers to diagnose IC in clinical practice, it is essentially a diagnosis of exclusion. Most patients with LUTS are initially diagnosed with other conditions such as overactive bladder, recurrent urinary tract infection, prostatitis or chronic pelvic pain syndrome. One unfortunate consequence of this is that the clinician/researcher has been

limited from developing new hypotheses based on the actual disease beginnings and instead has been channelled into focusing on patients with late-phase disease and all the secondary effects of the disease process [97].

The difficulty in developing objective specific biomarkers for IC may be becasue it is not a single disease but a collection of subset of conditions with different pathologies the endpoint of whom all is bladder damage and development of LUTS. We therefore may need more than one biomarker to diagnose the specific subtypes of IC for the development and delivery of more targeted therapy.

Eosinophilic cystitis (EC) was first described in 1960 [98] and although EC is classed as a separate clinical diagnosis from IC, it is not possible to separate IC from EC confidently on the basis of clinical presentation alone. This raises the possibility that the aetiopathological mechanism of disease development in EC may be similar to IC in some respect and that EC may represent a subset of IC for whom we have found a diagnostic criterion (LUTS and eosinophilic infiltration of involved bladder tissue) but there are still subsets of IC which we do not know of.

Clinical presentation of KIC patients is with non-bacterial cystitis similar to IC and EC. From the literature review and our clinical experience, it is rather difficult to distinguish these conditions on the basis of clinical presentation alone. The common presenting signs/symptoms in these diseases include one or more of dysuria, suprapubic pain, nocturia, urinary frequency and urgency ([2, 3, 13, 16-18, 39, 45, 56, 72] and reviewed by [23, 24, 27, 28, 31, 32, 46, 55, 56]). In addition, urge incontinence and haematuria in patients with KIC and EC have also been reported ([2, 13, 16-18, 72] and reviewed by [55, 56]).

A small contracted bladder at late stage of disease is evident on radiological investigations as well as urodynamics in all three conditions. Additionally, upper tract involvement has been described in the literature in both KIC [2, 13, 18] and less commonly in EC [57, 73]. But from our

clinical experience, it is possible that long standing IC can also affect the upper renal tracts.

Cystoscopy in all cases shows varying degrees of bladder inflammation with one or more of epithelial inflammation, neovascularisation, petechial haemorrhages and erythematous and/or oedematous bladder mucosa but additionally might also show glommerulations or Hunner's ulcer in cases of IC ([2, 73] and reviewed by [28]).

Although all biopsies in non-bacterial cystitis demonstrate infiltration of involved tissue by non-specific inflammatory cells as a result of chronic inflammation ([2, 13, 16, 17, 48, 49, 52, 76] and reviewed by [47]), eosinophils have been reported additionally in cases of KIC and EC ([16, 18, 72] and reviewed by [74, 75]). It may be possible to further differentiate KIC and EC on the basis of histology as KIC biopsies have been reported to additionally demonstrate urothelial atypia [16, 18].

The only immunohistochemistry studies we found in literature on non-bacterial cystitis were those on IC and KIC patients. Increased expression of Ki67 (proliferation marker) has been demonstrated in KIC specimens [16, 18] but absent in IC specimens in one study [53] and down regulated in another study [99].

6.2.1 Conclusions

From literature review it is evident that there are considerable similarities between the clinical presentation and investigation findings of KIC mainly to EC but also to IC. It is therefore possible that the mechanisms behind development of all these conditions have some similarities as well and that KIC may be a good model to study both EC and IC, once mechanisms behind development of KIC are understood.

KIC is a relatively new condition with the first literature report only published in 2007 [11]. The advantage in using KIC as a research model to understand EC and IC will be that although the mechanism of disease development is currently unknown, the causative agent is known and therefore effective research models can be developed.

As mentioned before, IC may represent an endpoint of different diseases with multiple aetiologies and EC may be a part of this spectrum of currently separately recognised illnesses. Therefore, it may not be possible to use the KIC model to understand all conditions in IC but it is expected that it will certainly be useful in a subset of patients as the symptomatic endpoint in all of them is troublesome lower urinary tract symptoms.

6.3 Implications of the research

Due to the increasing use of ketamine by both clinicians and recreational drug abusers, all medical practitioners need to be aware of ketamine-induced cystitis (KIC) as a disease.

The main advances in knowledge this research project has made are that;

1.   Both ketamine and its metabolite nor-ketamine are toxic to normal human urothelial cells.

2.   Daily use of ketamine may be more toxic to normal human urothelial cells than occasional use.

3.   Despite above findings, not everyone using analgesic ketamine seems to be at the risk of developing KIC.

4.   The mechanism of action of ketamine on normal human urothelial cells may be two fold; direct toxic effect and via a receptor mediated pathway.

5.   Ketamine possibly leads to the development of symptoms of KIC by causing inflammation in the normal human urothelial cells.

6.   Drug history of ketamine use/abuse should be sought from every patient presenting with lower urinary tract symptoms and before diagnosing them with non-bacterial cystitis.

6.4 Future direction of research

The experiments so far have provided some valuable initial information and on the basis of these results, future direction of research will be as below:

1. Based on the initial immunohistochemistry experiments results and advice/discussion with Dr J Stahlschmidt, immunolabelling with the following antibodies will be further performed in the current three representative individual KIC tissues;

    a) AB-PAS with diastase digestion on the one tissue block (KIC3b) from patient KIC3 showing columnar metaplasia to assess if there is mucus glandular metaplasia.

    b) Calponin as a myocyte marker and, NFP and S-100 as neuronal cell markers to assess and differentiate between the anti-human NGFR positive stromal population.

    c) Bcl-2 to assess apoptosis in the KIC tissue.

    d) Cd1a to assess the presence of Langerhans cells (antigen presenting cells to other cells such as T-cells).

    Once all the above antibodies are completed, the results will be assessed and a final antibody panel will be decided to perform on the remaining KIC tissues in future work to explore the possible mechanisms of development of KIC.

2. NHU cells form a tight barrier and the transurothelial electrical resistance (TER) is one of the highest recorded for any tissue [79]. Ketamine may be causing its effects by damage to this urinary barrier and measurement of TER of ketamine affected NHU cells may be a useful avenue to explore the above. It will be interesting to see at what concentrations of ketamine the NHU cells lose their TER and therefore their barrier function. This in turn may be useful to validate the toxic doses of ketamine established by the cell biology experiments.

3. Experiments to establish the urothelial receptor possibly responsible for the action of ketamine.

7 Appendix

7.1 Suppliers

Abcam   330 Cambridge Science Park

Cambridge, UK

CB4 0FL

AbD Serotec   Endeavour House

Langford Lane

Kidlington, UK

OX5 1GE

Adobe Ltd   3 Roundwood Avenue

Stockley Park

Uxbridge, UK

UB11 1AY

AnalaR   Unit 15

The Birches, Willard Way

Imberhorne Industrial Estate

East Grinstead

West Sussex, UK

RH19 1XZ

BD   The Danby Building
(Becton, Dickinson   Edmund Halley Road
and company)   Oxford Science Park,

Oxford, England

OX4 4DQ

BDH   BDH Laboratory supplies

Poole, UK

BH15 1TD

Bio-Rad   Bio-Rad Laboratories Ltd

Bio-Rad House

Maxted Road

Hemel Hempstead

Hertfordshire, UK

HP2 7DX

Co-Star Ltd.   40 Portman Square

5th Floor

London, UK

W1H 6LT

Cymbus   Units J&K

Eagle Close

Chandlers Ford

Hampshire, UK

SO53 4NF

DAKO   Dako UK Ltd

Cambridge House

St Thomas Place, Ely

Cambridgeshire, UK

CB7 4EX

Fisher Scientific   Bishop Meadow Road

Loughborough

Leicestershire, UK

LE11 5RG

Hewlett-Packard	Amen Corner
	Cain Road
	Bracknell
	Berkshire, UK
	RG12 1HN
Invitrogen	3 Fountain Drive
	Inchinnan Business Park
	Paisley, UK
	PA4 9RF
Leica	Balliol Business Park West
	Benton Lane
	Newcastle upon Tyne, UK
	NE12 8EW
Merck	Boulevard Industrial Park
	Padge Road
	Beeston
	Nottingham, UK
	NG9 2JR
NIKON	Nikon Precision Europe GmbH
	Stadium House, Alderstone Road
	Livingston, West Lothian, Scotland
	EH54 7DN
Novocastra	Davy Avenue Knowlhill
	Milton Keynes, UK
	MK5 8LB

Olympus	Olympus UK Ltd
	KeyMed House, Stock Road
	Southend-on-Sea, Essex, UK
	SS2 5QH
PALL Corporation	Europa House
	Havant Street
	Portsmouth, Hampshire, UK
	PO1 3PD
Pfizer	Walto Oaks
	Tadworth
	Surrey, UK
	KT20 7NS
Progen	A division of G H Zeal Ltd
	8 Deer Park Road, Merton
	London, UK
	SW19 3UU
Raymond A Lamb Ltd.	Unit 4/5, Park View
	Eastbourne
	East Sussex, UK
	BN23 6QE
Rockland Immunohistochemicals	Lorne Laboratories Limited
	Unit 1 Danehill
	Cutbush Park Industrial Estate
	Lower Earley
	Reading, UK
	RG6 4UT

SARSTEDT Ltd.	68, Boston Road
	Beaumont Leys
	Leicester, UK
	LE4 1AW
Scientific Laboratories Supplies Ltd.	Wilford industrial estate
	Ruddington Lane
	Wilford, Nottingham, England
	NG11 7EP
Sempermed	Semperit Technische Produkte
	Gesellschaft m.b.H.
	Modecenterstraße 22 | A-1031
	Vienna
Sigma-Aldrich Co. Ltd	The Old Brickyard
	New Road
	Gillingham, Dorset, UK
	SP8 4XT
Sigma Imaging Ltd	13 Little Mundells
	Welwyn Garden City
	Hertfordshire, UK
	AL7 1EW
STARLAB Ltd.	Unit 4 Tanners Drive
	Blakelands
	Milton Keynes, UK
	MK14 5NA

Sterilin Limited	Parkway
	Pen-y-Fan Industrial Estate
	Newport, UK
	NP11 3EF
TOCRIS	Tocris House
	IO Centre
	Moorend Farm Avenue
	Bristol, UK
	BS11 0QL
VECTOR	3 Accent Park
	Bakewell Road
	Orton Southgate
	Peterborough, UK
	PE2 6XS
World Precision Instruments	Astonbury Farm Business Centre
	Aston, Stevenage
	Herts, UK
	SG2 7EG

7.2 Recipes

7.2.1 Cell Biology

1) **KSFMC:** 0.5 ml of CT + 25mg BPE + 500µl (2.5µg) EGF + 500ml bottle of KSFM. Stored at 4^oC.

2) **CT:** 1mg Cholera Toxin Powder-Sigma C8052 + 33.3ml KSFM. Stored in dark at 4^oC.

3) **Stripper Medium:** 500ml HBSS (without Ca^{2+} and Mg^{2+}) Gibco 14170-088 containing 10mM HEPES Buffer + 1ml Trasylol (500,000KIU) + 50ml 1% EDTA. Stored at 4^oC.

4) **Transport Medium:** 500ml HBSS containing 140mg/l $CaCl_2$ and 100mg/l $MgCl_2$ + 10mM HEPES Buffer + 1ml Trasylol (500,000KIU). Stored at 4^oC.

5) **TI:** 100mg Trypsin Inhibitor Sigma T6522 + 5ml PBS. Solution filter sterilised with 0.2µm filter. Stored at -20^oC.

6) **TV:** 20ml Trypsin-Sigma T4549 + 4ml 1% EDTA + 176ml HBSS (without Ca^{2+} and Mg^{2+}). Stored at -20^oC.

7) **0.1% EDTA:** 1g EDTA Disodium salt (BDH 100935V) + 1000ml of PBS (made from 10x DPBS (Gibco 14200-067) in Elga distilled water (ddH_2O)). Stored at ambient temperature.

8) **Freezing Mix:** 10%DMSO + 10% Serum + KSFMC

9) **AB:** 1 part AB dye + 9 parts KSFMC (1:10)

10) **Ketamine:** LOT No. 016K1629, Catalogue No. K2753-5G, Formula weight 274019g/mol, purchased from Sigma-Aldrich.

11) **10mM Ketamine solution:** 27.4mg Ketamine + 10ml KSFMC (filtered using 0.2µm filter before use).

12) **1mM Ketamine solution:** 2.7mg + 1ml KSFMC (filtered using 0.2µm filter before use).

13) **Nor-Ketamine:** Formula weight 260.16, CAS No. 35211-10-0, kind gift from Pfizer.

14) **10mM Nor-Ketamine solution:** 26.5mg Nor-Ketamine + 10ml KSFMC (filtered using 0.2µm filter before use).

15) **1mM Nor Ketamine solution:** 2.6mg Nor-Ketamine + 1ml KSFMC (filtered using 0.2μm filter before use).

16) **MK-801 maleate:** Formula weight 337.37, CAS No. 77086-22-7, purchased from TOCRIS.

17) **100mM MK-801 maleate solution in DMSO:** 33.7mg MK-801 + 1ml DMSO.

18) **100μM MK-801 maleate solution in KSFMC:** 1μl of 100mM MK-801 maleate in DMSO solution + 1ml of KSFMC.

7.2.2 Histology

1) **10mM Citric Acid:** 1.47g Citric Acid powder + 700ml of distilled water + pellets of NaOH to adjust pH to 6.0.

2) **100ml 3% H_2O_2 in distilled water:** 10ml 30% H_2O_2 + 90ml distilled water.

3) **100ml 3% H_2O_2 in PBS:** 10ml 30% H_2O_2 + 90ml PBS.

4) **TBS Buffer:** 25ml 2M Tris pH 7.6 (242.28g/L) solution + 50ml 3M NaCl (175.32g/L) solution, made up to 1L with distilled water.

5) **Strep AB/HRP complex:** 1 drop solution A + 1 drop solution B +2.5ml TBS.

6) **DAB solution:** 2 Sigma Fast DAB tablets (one from silver and other from gold wrapper) + 5ml distilled water.

7) **Scott's Tap Water:** 100ml 20% $MgSO_4$ + 50 ml 7% $NaHCO_3$ + 850ml distilled water.

8) **0.1% Calcium Chloride:** 1g CaCl2 + 900ml distilled water + NaOH pellets to adjust pH to 7.8.

9) **0.1% Trypsin solution:** Pre-warm 100ml 0.1% CaCl2 pH 7.8 to 37^oC + 0.1g Trypsin.

10) **Haematoxylin:** Solution 1 (3g Haematoxylin + 20ml absolute ethanol) + Solution 2 (0.3g Sodium Iodate + 1g Citric Acid + 50g Chloral Hydrate + 50g Aluminium Potassium Sulphate + 850ml distilled water) + 120ml Glycerol. Stored in dark, filtered before use.

7.3 Abbreviations

AB	Alamar Blue
AB-PAS	Alcian Blue-Periodic Acid Schiff staining
AB-PAS-D	AB-PAS with diastase digestion
BPE	Bovine Pituitary extract
CaCl$_2$	Calcium chloride
CIS	Carcinoma in situ
CK	Cytokeratin
CT	Cholera toxin
DAB	3,3-Diaminobenzidine
DMSO	Dimethylsulfoxide
EC	Eosinophilic cystitis
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal Growth Factor
HBSS	Hank's balanced salt solution
HEPES	N-2 Hydroxyethyl piperazine-N-2 ethane sulphonic acid
HLA-DR	Human Leukocyte Antigen-DR
H&E	Haematoxylin and Eosin
H$_2$O$_2$	Hydrogen Peroxide
IC	Interstitial cystitis

IHC	Immunohistochemistry
KIC	Ketamine-induced cystitis
KSFM	Keratinocyte serum free medium
KSFMC	KSFM + BPE + EGF + CT
LUTS	Lower urinary tract symptoms
NHU	Normal human urothelial
PBS	Phosphate buffered saline
TBS	Tris buffered saline
TI	Trypsin inhibitor
TV	Trypsin Versene
UPK	Uroplakin

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