Chondroitin sulphate: a candidate modulator of urothelial barrier formation and reconstitution following injury

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March 2018

Abstract

Chondroitin sulphate (CS) has been described as contributing to the barrier function of the urothelium by forming a glycosaminoglycan (GAG) layer. It has been suggested that a defect in this layer contributes to chronic inflammatory uropathies, and this forms the basis of medical device therapies purported to replace the GAG layer. A review of the literature questioned the evidence supporting these claims.

This thesis set out to formulate a reproducible damage model using differentiated normal human urothelial (NHU) cells *in vitro*. Techniques employed to introduce damage included physical damage by scratch wounding, chemical damage using ketamine and hypoxia. Barrier formation and damage was assessed by measuring transepithelial electrical resistance (TEER) across differentiated cells.

The role of CS on barrier formation and restitution following injury was assessed by TEER studies. Assessment of the presence of CS on the luminal surface of the urothelium *in situ* was carried out by immunohistochemistry.

Damage by scratch wound resulted in a loss of TEER that repaired within 22 hours. Ketamine had a toxic effect on the cell cultures. Hypoxia produced a reproducible defect in barrier resistance. Cell cultures exposed to CS during differentiation formed a tighter maximum barrier compared to controls however, when CS was added to the medium following hypoxic injury there was no difference to barrier restitution. Immunohistochemical studies failed to demonstrate the presence of CS apart from in the stroma.

It is concluded that CS is not present on the urothelial surface and has no effect on barrier function in the diseased model. In a NHU culture model there is evidence that the maximum barrier is higher with CS. Evidence from this research supports clinical studies which have only demonstrated a placebo effect of CS. Future work should investigate mode of action of CS and other GAGs on NHU cells in culture.

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Acknowledgements

I would like to give thanks to Professor Jennifer Southgate who has supervised me throughout this project. I would also like to thank members of my training advisory panel, Mr Nicholas Smith and Professor Anthony Maraveyas for their help and support.

To my friends of the Jack Birch Unit, your advice and support was greatly appreciated. Special mention to Rosalind Duke, Joanne Pearson, Jennifer Hinley and Simon Baker you make science exciting and fun.

Finally, to my family who have endured me throughout this project, who have picked me up and pushed me to keep going, thank you.

Author Declaration

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1 Introduction

The overall aim of this thesis was to determine whether chondroitin sulphate enhances barrier function of urothelial cells *in vitro*. This introductory chapter provides an overview of the urinary tract, describes benign dysfunctional conditions of the urinary bladder, including bladder pain syndrome, radiation cystitis and recurrent urinary tract infection (rUTI) which all exhibit damage to the urothelium. Current clinical practice and management of these disorders is discussed including the use of chondroitin sulphate and other glycosaminoglycans (GAGs). This chapter will highlight current knowledge and controversies in published literature.

1.1 The human urinary tract

The urinary tract consists of the kidneys, ureters, bladder and urethra. The system functions to produce and store urine until socially acceptable to void. As the contents of urine can be harmful to cells, the urinary tract has evolved a urine-proof inner lining, the urothelium, which segregates the urine and protects tissues from urinary reabsorption or damage. The urothelium is present from the renal pelvis to the proximal urethra (reviewed by (Elbadawi, 1996)).

1.2 The urothelium

The urothelium is a transitional epithelium consisting of basal, intermediate and superficial cells which comprise of 4-6 layers depending on the expanded state of the tissue (Figure 1.1). Superficial luminal cells are highly specialised differentiated cells also known as umbrella cells. They cover multiple intermediate cells creating an umbrella effect (Hicks, 1975; Paul Jost, Goslingt and Dixon, 1989). Umbrella cells are frequently multinucleated and in man, can be identified by the presence of uroplakins (UPK) (discussed below) and cytokeratin (CK) 20 (Hicks, 1965; Harnden *et al.*, 1996). Cytokeratins are intermediate filament proteins which all epithelia contain. The cytokeratins which are specific to the urothelium are CK5, CK7, CK8, CK18, CK19 and CK20 (Southgate, Harnden and Trejdosiewicz, 1999). The specialist umbrella cells are

the main barrier-forming cells and allow for the bladder to expand during filling while protecting underlying cells from noxious stimuli in the urine.

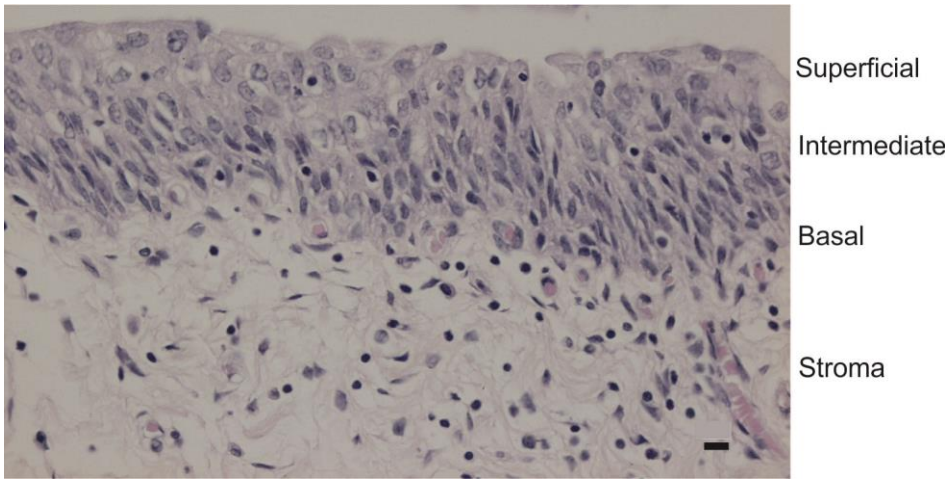


Figure 1.1: Haematoxylin and eosin stain showing layers of the urothelium.
Image taken from human ureter tissue sample Y1837. Three layers of the urothelium are shown; the basal cells connect with the stroma. There are several intermediate cell layers and a superficial layer demonstrating umbrella cells. Small condensed nuclei in the urothelium are infiltrating lymphocytes indicative of mild inflammation. Scale bar =10 µm.

1.2.1 Barrier function

Unlike other epithelia, cell turnover of the urothelium is low, in normal conditions it remains relatively quiescent (Limas, 1993). This aids the primary role of the urothelium in maintaining a tight barrier. A tight barrier is achieved by the complex organisation of tight junctions and surface uroplakins. Tight junctions consisting of occludins, zona occlucins (ZO) and claudins are proteins that structurally link adjacent urothelial cells and control paracellular diffusion (Schneeberger and Lynch, 2004). This is particularly important during the storage stage of the micturition cycle when the urothelium is stretched to accommodate increased fluid. Claudins identified on *in situ* normal ureteric specimens include claudin 3, 4, 5 and 7 as well as ZO1 and occludin proteins (Claire L. Varley *et al.*, 2006). A key component of barrier tightness is claudin 3 (Smith *et al.*, 2015). When there is damage to the urothelium, cells change to a proliferating phenotype in order to repair (Varley *et al.*, 2005).

Uroplakins are urothelium-restricted markers of differentiation. The uroplakins include UPK 1a, 1b, 2, 3a and 3b, organised as specific tetrameric structures in the Golgi and exported to the superficial cell surface where they form characteristic plaques of asymmetric unit membrane (AUM) on the apical membrane of the umbrella cell (Sun *et al.*, 1996). In mouse studies, plaques cover the majority of the surface of the urothelium and are an important feature of urothelial barrier by preventing the transcellular movement of water and molecules (Hu *et al.*, 2002; Min *et al.*, 2003). UPK 1a, 1b and 3a are glycosylated whereas UPK2 has the glycosylated propeptide cleaved during the maturation process (reviewed by (Kątnik-Prastowska, Lis and Matejuk, 2014)). The importance of UPK 3a has been shown in studies of knockout mice whereby ablation of the gene resulted in small plaque formation, further methylene blue penetrated into the urothelial cells of the knockout mice but not the cells of the controls which remained impermeable (Hu *et al.*, 2000).

The urothelium is one of the tightest epithelia of the human body. Barrier function can be measured by transepithelial electrical resistance (TEER) (Powell, 1981),whereby an electrical current is passed across a membrane and Ohm's law is applied to calculate the resistance (reviewed by(Rubenwolf and Southgate, 2011; Srinivasan *et al.*, 2015)). A tight barrier is classified as a reading >500 $\Omega.cm^2$(Cross *et al.*, 2005). An alternative method of assessing barrier is permeability studies, whereby water and solute movement can be assessed across a membrane (Negrete *et al.*, 1996).

1.3 The glycocalyx

Superficial urothelial cells are described as having a dense sugar layer on the luminal surface which is constituted of a complex of glycoproteins and proteoglycans making up the glycocalyx (Figure 1.2) (Cornish *et al.*, 1990). The glycoprotein structures consist of N and O-linked glycoproteins, forming uroplakins and mucins respectively. Glycoproteins consist of a core protein with an oligosaccharide attached whereas proteoglycans consist of a core protein with an attached alternating disaccharide unit which build chains of variable lengths (reviewed by (Montreuil, 1980; Ruoslahti, 1988)).

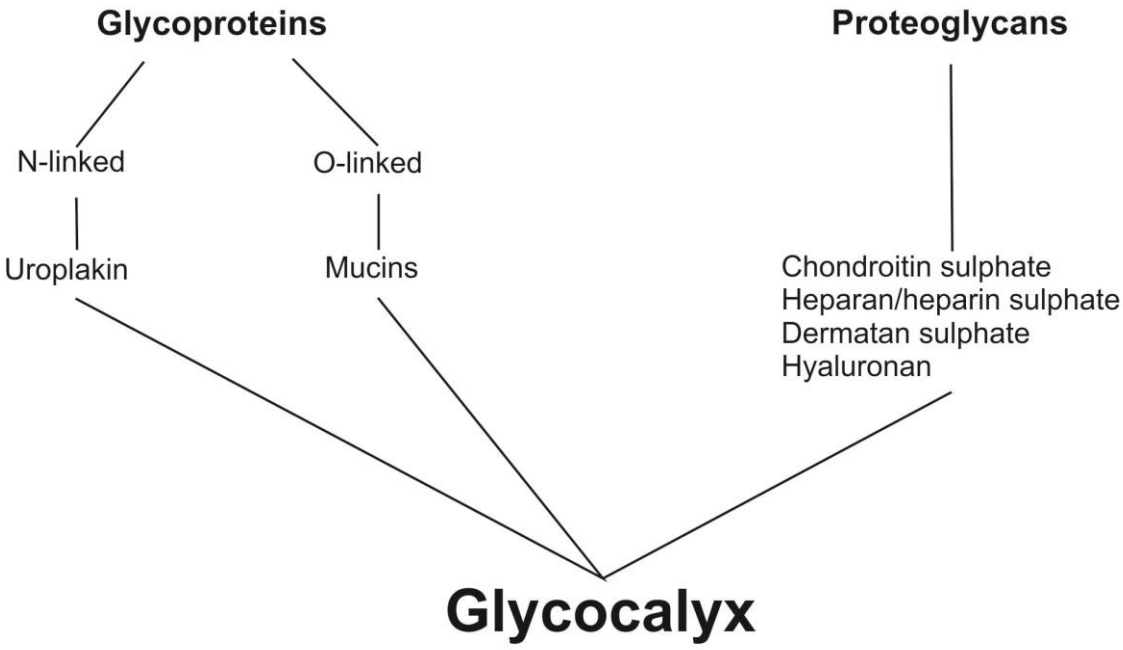


Figure 1.2: Diagrammatic representation of the constituents of the glycocalyx.
The glycocalyx is on the luminal surface of the umbrella cells. Literature describes the components consisting of a complex of glycoproteins and proteoglycans forming a surface barrier to urinary solutes. (Figure adapted from review by (Cornish *et al.*, 1990).

The proteoglycans associated with the glycocalyx are glycosaminoglycans (GAGs). These are repeating disaccharides which are linked to a core protein by a constant trisaccharide unit consisting of two galactose and one O-xylose glycan (linkage region). There are four families of GAGs namely; heparan/heparin sulphate (HS), chondroitin/dermatan sulphates (CS/DS), hyaluronate and keratan sulphate; all bar keratan sulphate have been described within the urinary tract. Each GAG consists of a different alternating disaccharide which bond to form molecules of variable chain lengths. The action of glycosylation of the core protein occurs in the endoplasmic reticulum and in the Golgi apparatus of the cell. When one or more GAGs are attached to the core protein a proteoglycan is formed. Glycosaminoglycans are predominantly sulphated; hyaluronate is the only non-sulphated GAG. Sulphated GAGs carry a negative charge making them hydrophilic therefore an abundance of water molecules coat GAGs (Hurst, 1978; Menter *et al.*, 1979); this has historically been described as adding to the barrier function of urothelial cells (Hurst, 1994). GAGs have been described as predominantly being present on the luminal surface of the urothelium but have also been described as being present to a lesser extent in the intermediate and basal cell layers (Ruoslahti, 1988; Kjellén and Lindahl, 1991; Hurst, 1994; Severino *et al.*, 2012; Lindahl *et al.*, 2015).

The focus of this thesis was to assess CS because this is used in clinical practice to treat inflammatory bladder pathologies. This molecule has the same linkage region as HS but has differing sugar residues. Chondroitin sulphate is composed of alternating units of glucuronic acid and N-acetylgalactosamine (Figure 1.3); these units are further modified by sulphation of the 2-hydroxyl group of the glucuronic acid and the 4 and/or 6- hydroxyl group of the N-acetylgalactosamine. When there is sulphation of the 4-hydroxyl group this molecule is known as chondroitin-4-sulphate and at the 6-hydroxyl group is chondroitin-6-sulphate (Reviewed by (Hurst, 1994; Caterson, 2012)).

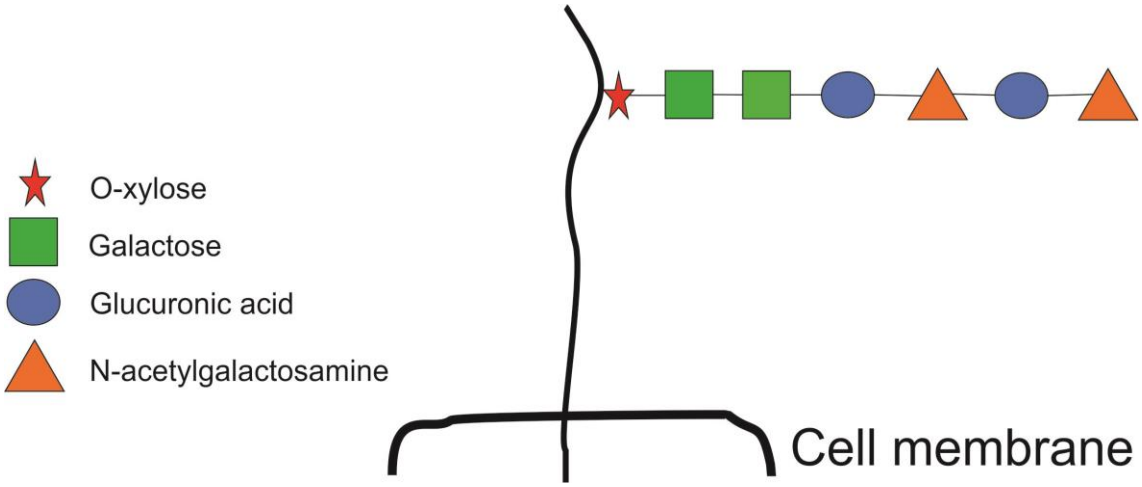


Figure 1.3: Diagrammatic representation of chondroitin sulphate.
Chondroitin sulphate chains consist of alternating disaccharide units of glucuronic acid and N-acetyl galactosamine. Disaccharide units are joined to a core membrane by a linkage region consisting of two galactose molecules and on O-xylose (Kjellén and Lindahl, 1991). Figure adapted from (Caterson, 2012).

1.3.1 Evidence for GAGs on the urothelium

A number of researchers have described the presence of different GAGs within samples of human and animal bladders. When comparing the composition of GAGs between the human and porcine bladders, Janssen *et al*. demonstrated by immunofluorescence there was no difference between the two. This study analysed five human bladders and an undocumented number of porcine bladders, immunofluorescence was used to localise GAGs on both human and porcine bladders, while gel electrophoresis was used predominantly on samples from porcine bladders to quantify amount of GAGs. For electrophoresis experiments three sampling techniques were used including; superficial sampling (scraping with a blunt spatula), deep urothelial sampling (scraping with a microtome blade) and full thickness sampling (included additional human sample). Immunofluorescence using mouse monoclonal antibodies which bind to individual CS, HS and DS chains were used to assess GAG location on human and porcine bladders.

Results described from the immunofluorescence study suggest that DS was found in the connective tissue, HS was predominantly found in the basal urothelial layer with a small amount in superficial layer and CS was found on the luminal surface and within the connective tissue. Analysis of the blot for superficial and deep urothelial sampling reported CS was at higher concentrations than HS (Janssen *et al.*, 2013). The final conclusion was that CS was found on the urothelial surface, with low amounts of HS. Questions are raised from this based on the evidence shown, firstly they fail to convincingly display urothelial labelling of HS on their immunofluorescence slides yet describe it as present in low amounts. Secondly, the results from the full thickness blot of porcine and human bladder show no CS, yet the immunofluorescence display this within the connective tissue. The bands on the blot for superficial and deep urothelial porcine samples do not line up with the bands of the reference range and there is considerable background noise. Thirdly, the antibodies used were chain antibodies, therefore they could have included controls with a digest step to show specificity of labelling; the only control described was where the primary antibody was omitted. Lastly the sampling method is dubious particularly for the samples of superficial and deep urothelium; from the H&E slides it is concerning this method did not just collect samples from the designated areas.

The results from Janssen *et al*. contradict the earlier results from a small study conducted by Hurst *et al*. whereby a single human and bovine bladder were investigated for the presence of GAGs. Each bladder was everted and placed in guanidinium hydrochloride, with proteolysis inhibitors for 1 hour. Proteoglycans were then isolated by ion exchange and gel filtration. Hurst *et al*. concluded that HS accounted for 54% in human and 57% in bovine bladders; CS was the second most prevalent GAG accounting for 29% of the GAGs from both samples. The method of GAG extraction was by denaturing membrane proteins with guanidinium hydrochloride thus releasing the surface GAGs, it is difficult to ascertain if this method would only release surface GAGs or would also release GAGs from the connective tissue (Hurst and Zebrowski, 1994).

In later immunohistochemical studies by Hurst *et al*. using polyclonal antibodies against the core proteins of GAGs, the group reported that the CS core protein decorin and the HS core protein perlecan was present on the luminal surface of the urothelium but syndecan-1 which covalently bonds chondroitin and heparan chains was identified within the intermediate and basal layers. The specimens were samples taken from human bladders which were undergoing bladder biopsy for cancer, the specimens were deemed free from cancer prior to inclusion into the study (Hurst, Moldwin and Mulholland, 2007). Unfortunately they failed to show the immunohistochemistry results or discuss experimental controls.

In the immunohistochemical comparison of normal bladders and those from patients with interstitial cystitis, Slobodov et al. labelled surface CS with a monoclonal antibody, 2B6 (chondroitin-4-sulphate) after chondroitinase digest. This showed immunolabelling throughout the entire urothelium and the stroma. Further they investigated markers of terminal differentiation and tight junction proteins associated with barrier function (uroplakins, ZO1 and cadherin). Uroplakins were labelled on the superficial urothelial cells which demonstrated that umbrella cells were present (Slobodov *et al.*, 2004). The labelling with ZO1 was unexpected, there was dense full thickness labelling of the urothelium, which contradicts the hairline immunolocalisation of ZO1 at the superficial intercellular junctions described by another group (Claire L Varley *et al.*, 2006; Smith *et al.*, 2015).

There have been studies which have not demonstrated a significant presence of GAGs on the urothelial surface. When immunolabelling rabbit bladders with mouse monoclonal antibodies to detect the pre-digested stub of chondroitin and heparan sulphates, Buckley *et al*. found only small amounts of HS on the urothelium. There was intense labelling of the lamina propria and muscle for heparan, chondroitin and hyaluronate, they also showed strong labelling on the luminal surface with several lectins (Buckley *et al.*, 1996).

It is evident from the above studies that there remains controversy surrounding the composition of GAGs within the glycocalyx. There are multiple different species investigated with an array of antibodies, from monoclonal to polyclonal, to those labelling the chain, the digested stub or the core protein. Although all studies have indicated the presence of some GAGs on the luminal surface not all experiments have been well designed.

1.3.2 Role of GAGs in urothelial damage

When the urothelium is damaged the structure of the glycocalyx is altered and GAGs are lost. Intravesical instillation in rodent studies have shown exogenous GAGs to adhere to the urothelial surface. Kyker *et al*. used mouse bladders and damaged them by adding protamine, hydrochloric acid or trypsin. They then added Texas red labelled chondroitin for one hour. After staining, it revealed that the exogenous GAG had coated the urothelial cells of the damaged bladders but not the control where the GAG layer was intact (Kyker, Coffman and Hurst, 2005). In the study, it is not demonstrated the extent of damage that each of these agents produce to the glycocalyx or urothelium.

The functional relevance of loss of GAGs was investigated by Janssen *et al*. using primary cultures of porcine urothelial cells; a link was demonstrated between loss of GAGs and effect on barrier function over a 24 hour period. Following the application of enzymes to digest chondroitin sulphate or heparin sulphate from the urothelial cells TEER readings were taken at baseline and at regular intervals over a 24 hour period to assess barrier repair. When chondroitinase ABC was applied there was a significant decrease in barrier function of 27% +/- 20% within the first hour. Protamine was used as a positive control on the basis that it was assumed to remove all GAGs; barrier

function dropped by 73% +/- 9% in this group. Heparinase III resulted in a minimal reduction of barrier function and was not statistically significant from non-treatment control. Twenty-four hours post treatment the barrier function was fully restored in the chondroitin and protamine group. The authors interpret this study as demonstrating that chondroinase ABC has a significant effect on barrier function however the barrier is repaired after 24 hours. A concerning feature with this study is that there is little detail about the methods applied for differentiation of the urothelial cells or how the digesting enzymes were added; it is also not known whether the cells *in vitro* produce a GAG layer therefore the effects of the enzymes may be due to an effect on other cellular structures causing the barrier to decrease. The specificity of the enzyme preparations is not detailed or controlled therefore these could have had off target effects (Janssen *et al.*, 2013).

There is limited research into the functional efficacy of GAGs on urothelial repair, although research has suggested exogenous GAGs attach to damaged urothelium, the extent of the initial damage is not shown. Further, the role of GAGs in repair is not identified. Only one study has attempted to assess a change in barrier function by digesting GAGs on cultured urothelial cells; however there are concerns with enzyme specificity, and further the study failed to adequately show the presence of GAGs on the urothelial cells after 7 days in culture.

1.4 Benign bladder disorders

Glycosaminoglycans have been used to treat benign bladder conditions. This section will provide an overview of bladder pain syndrome (BPS), radiation cystitis and rUTI. It will discuss the clinical evidence behind the use of GAGs in these conditions. A systematic literature review using the search terms bladder, urothelium and glycosaminoglycans and glycosaminoglycans, cystitis or urinary tract infections or bladder pain syndrome or radiation cystitis was conducted to ensure all relevant literature was reviewed. Searches were limited o clinical trials, reviews and systematic reviews.

1.4.1 Bladder pain syndrome

Much debate has occurred in the last decade regards the definition of bladder-associated chronic pain. Initially, the disorder was termed interstitial cystitis (IC) (PARSONS and PARSONS, 2004). In subsequent years an umbrella term of "painful bladder syndrome" was used and IC became the definition based on cystoscopic findings. Due to ongoing confusion with the terminology in 2008 the European Society for the Study of Interstitial Cystitis (ESSIC) published a consensus on the definition, diagnostic criteria and disease classification. It was decided that BPS should be used to describe chronic (>6 month) pelvic pain, pressure or discomfort perceived to be related to the urinary bladder associated with either a persistent urge to void or frequency (van de Merwe *et al.*, 2008). However this definition is not used by the American Association of Urologists who use the Society for Urodynamics and Female Urology definition:

'An unpleasant sensation (pain, pressure, discomfort) perceived to be related to the urinary bladder, associated with lower urinary tract symptoms of more than six weeks duration, in the absence of infection or other identifiable causes'.

The difference between the two definitions includes the length of symptoms; the American definition includes symptoms for more than six weeks duration, justification for this is that it does not preclude early treatment (Hanno *et al.*, 2014).

The lack of uniform definition has made epidemiological studies difficult. The Cystitis and Overactive Bladder Foundation estimate approximately 400,000 people within the United Kingdom suffer from BPS (Cystitis and Overactive Bladder Foundation, 2018). The RICE study was a large US population based study which interviewed 12,752 women with bladder symptoms within the US. Two definitions of painful bladder were used observing either high sensitivity or high specificity. The results showed that between 3.3 to 7.9 million women 18 years and older suffered with painful bladder syndrome (Berry *et al.*, 2011). Predominantly the condition affects the female population however males are not excluded (Suskind *et al.*, 2013).

Pain is often the most debilitating symptom and has a significant impact on quality of life and work productivity. Symptoms can be present daily or can affect the patient for several days or weeks of the month. Occasionally symptoms can be linked to the menstrual cycle, further there have been links to other functional disorders for example irritable bowel syndrome and fibromyalgia (reviewed by (Bogart, Berry and Clemens, 2007)).

The aetiology of bladder pain syndrome remains unclear. Many theories have been postulated which include a defect in the glycocalyx which allows the passage of solutes within urine, such as potassium and proteases, to move into the cell and trigger an inflammatory reaction. This is highlighted by a positive potassium provocation test (Parsons *et al.*, 1994; Parsons, 1996). The resultant increase in inflammatory markers activates C-nerve fibres and the sensation of pain is perceived. Histologic findings have suggested there is chronic submucosal inflammation with inflammatory infiltrates restricted to the lamina propria (Lynes *et al.*, 1990). Further mast cells have been shown to be of significant importance but only in a subset of bladder pain patients (Sant and Theoharides, 1994). With a damaged urothelium there is loss of tight junction proteins and resultant loss of barrier function (Southgate *et al.*, 2007).

There is also evidence of reduced blood flow to the bladder of patients with BPS, with resultant hypoxic injury but this does not correlate with severity of symptoms (Pontari, Hanno and Ruggieri, 1999). Studies have shown increased upregulation of Hypoxia-inducible factor-1α (HIF1α) when comparing patients with normal bladders to those with BPS (Lee and Lee, 2011, 2014). HIF1α is a transcription factor which is upregulated

in hypoxic conditions to promote angiogenesis by regulating vascular endothelial growth factor (VEGF) transcription. VEGF is a major factor in angiogenesis promoting new blood vessel formation and thus restoring oxygenation and homeostasis (reviewed by (Ziello, Jovin and Huang, 2007)).

Although much research has been performed, the initial insult which leads to the pathological findings of BPS remains unknown; it may be there is an autoimmune element. BPS seems to be associated with a loss of barrier function, but the extent of damage to the urothelium and the causes of that damage are poorly defined. Some authors have suggested that barrier function is mediated at the level of the GAG layer and have used this supposition to promote the therapeutic use of exogenous GAGs however; this explanation is too simplistic and is more likely to be due to multifactorial processes.

1.4.1.1 Treatment options for BPS

Current treatment options are given in a stepwise approach, from conservative measures to invasive procedures. Education and lifestyle changes, including avoidance of food/drink triggers, bladder training exercises and learning coping strategies to manage the pain are first line (reviewed by (Bosch and Bosch, 2014)).

As part of the clinical investigations patients will undergo cystoscopy, most will receive hydro-distension under general anaesthetic. Hydro-distension allows observation of cystoscopic changes to be seen, for example glomerulations and Hunners ulcers (Hunner, 1915; Parsons and Walker, 1996). Should ulcers be present these can be resected. Patients often have improvements in their symptoms following resection. Some patients have symptomatic improvement from hydro-distension, a surgical technique that involves distending the bladder to 80-100cm H_2O for 1-2 minutes (reviewed by (Ens and Garrido, 2015)). In a prospective study of 361 consecutive patients with IC who received hydro-distension and bladder training, there were significant improvements in bladder capacity and voiding episodes at the end of the 72 week follow up period. However, this study failed to highlight the impact regular review and bladder training alone has on improving patient symptoms (Hsieh *et al.*, 2008).

Growing in popularity amongst Urologists is the use of GAGs: synthetically derived sugars which are delivered via a urinary catheter directly into an empty bladder. Treatment regimens involve the patient attending for treatment every week for 4-6 weeks then monthly thereafter. After six months symptoms are assessed, some patients will continue with monthly instillations, others will not require further treatment. This treatment is costly and time consuming. For the patient it involves an invasive procedure, they are advised to hold the solution within the bladder for up to two hours which is often difficult when they have been suffering with urgency and frequency to urinate. Interestingly CS and sodium hyaluronate treatment is recommended by the European Association of Urologists (Engeler *et al.*, 2014) but not the American Association of Urologists who favour heparin (Hanno *et al.*, 2014).

Current GAGs available for intravesical use in the United Kingdom include sodium hyaluronate (Hyacyst and Cystistat 40mg/50mls) chondroitin sulphate (Gepan 2mg/ml) and a combination therapy of sodium hyaluronate and chondroitin sulphate (iAluril 800mg sodium hyaluronate and 2g chondroitin sulphate/50mls) (Cystistat, 2018; Gepan, 2018; iAluril, 2018). These are sold as class III medical devices, therefore suggesting they have no active role on the cells and simply act as a physical barrier-replenishment therapy. None of these products have undergone rigorous clinical trials to assess efficacy. All randomised control trials assessing the differences between GAG therapy and placebo in BPS patients have shown no difference between the treatment arm and placebo (reviewed by (Madersbacher, Van Ophoven and Van Kerrebroeck, 2013)). Side effects are minimal and likely to be associated with urethral catheterisation. Patients are keen to undergo treatment; they receive regular appointments with the clinical team and further advice and support on lifestyle modification.

Surgical techniques vary from minimally invasive to major surgery. Evidence is growing for the use of sacral nerve stimulation to modulate the pain pathways from the bladder although further research is required (reviewed by (Rahnama'i *et al.*, 2018)). This option has been used for patients with idiopathic urinary retention and detrusor overactivity since the 1990s in the UK. It involves placing a lead into the S2-4 foramen and stimulating the sacral nerves to modulate pain or micturition pathways. Precise

mode of action remains unclear however patients do have symptomatic benefit (reviewed by (Hussain and Harrison, 2007)). Other surgical options are more invasive and involve major operations to augment the bladder or simple cystectomy and ileal conduit formation. These procedures come with significant risks and long term complications (Brown *et al.*, 2017).

1.4.2 Radiation cystitis

This disorder arises after radiotherapy to the pelvis as a side effect of treatment for pelvic malignancies, for example prostate cancer, rectal cancer and gynaecological malignancies. Incidence of developing radiation cystitis is between 23-80%. Patients present with symptoms of urgency and frequency to urinate, small volume voids and often haematuria. Mechanism of injury has been described as an inflammatory insult which injures the urothelium, vasculature and detrusor musculature. The resultant vascular injury is believed to contribute to the end stage small fibrotic bladder(reviewed by (Browne *et al.*, 2015)). If haematuria is present a flexible cystoscopy will be performed, this often shows areas of inflammation and telangiectasia often patients will feel discomfort if the bladder is over-filled (reviewed by (Smit and Heyns, 2010)).

1.4.2.1 Treatment options

Depending on which symptoms are most bothersome will guide treatment pathways. If urgency and frequency are predominant then anticholinergic medications and bladder training may suffice. Side effects may inhibit long-term therapy. GAG therapy may be recommended and this may reduce the inflammatory response (Smit and Heyns, 2010). Hyperbaric oxygen has been indicated for recurrent episodes of haematuria (Suzuki *et al.*, 1998).

1.4.3 Recurrent UTI

A third of women who develop an acute urinary tract infection (UTI) will subsequently suffer with recurrent infections. These give symptoms of urgency and frequency to urinate and dysuria. Females are at a higher risk than males due to anatomical differences, further risk factors include diabetes and obesity, and there is also a link to intercourse. Recurrent infections are deemed as two or more infections in a 6 month

interval. This has a detrimental effect on a patient's quality of life, affecting work and social relations. The most common pathogen is *Escherichia coli*, but *Kelbsiella* and *Pseudomonas* are also prevalent. *E.Coli* have fimbriae which attach to the hosts surface and allow invasion. Pathogenic bacteria then multiply and trigger an immune response (guideline review (M. Grabe, R. Bartoletti *et al.*, 2015)) .

1.4.3.1 Treatment options

Conservative measures may be all that is required to break the cycle of infection. Encouraging increased fluid intake and hygiene advice can alleviate the need for recurrent courses of antibiotics. There is little evidence for the use of cranberry juice (reviewed by (Jepson, Williams and Craig, 2012)); post-menopausal women may have a reduction in episodes with the use of topical oestrogens (reviewed by (Perrotta *et al.*, 2008)). Evidence for use of long-term low dose antibiotics is poor and leads to antibiotic resistance. A self-start antibiotic is one method to alleviate this but it requires a proactive patient.

There is growing evidence from clinical trials that GAGs can reduce the number of urinary tract infections (reviewed by (De Vita, Antell and Giordano, 2013)). One study from this meta-analysis provided good evidence that combined GAG treatment iAluril reduced the time to infection, and number of overall infections over a 12 month study period. This study was a double-blind placebo controlled trial results generated give a high level of evidence for the use of combined treatment of iAluril for rUTIs (Damiano *et al.*, 2011).

1.5 Chapter Summary

It is evident from a review of the literature that evidence for a thick 'GAG layer' is dubious, it is possible that GAGs do contribute to the glycocalyx, but potentially not to the extent that has been suggested. It is important to identify if GAGs play a role in urothelial cell biology given the important role they play in treating inflammatory bladder pathologies.

1.6 Project aim and hypothesis

The overall aim of this project was to investigate the effects of exogenous CS on human urothelial cells *in vitro* during barrier formation and restitution following injury in order to gain insight into their efficacy and possible mode of action. The null hypothesis to be tested was the exogenous CS has no effect on urothelial biology.

To investigate this, key experimental project objectives were:

1. To develop a prolonged model of cell injury which would deplete the normal human urothelial cell barrier function for a sustained period of time to be able to assess the role of exogenous CS on barrier repair.

2. To identify which types of CS are present on urothelium *in situ*.

3. Investigate if exogenous CS modulates barrier development in differentiating NHU cells in culture.

4. To assess the role of exogenous CS on barrier restitution following prolonged barrier injury.

2 Materials and Methods

2.1 General

Laboratory work was conducted the Jack Birch Unit of Molecular Carcinogenesis which is located in the Department of Biology at the University of York.

2.2 H_2O and Buffers

Unless otherwise stated where H_2O is discussed in this thesis it refers to ultrapure water, with a purity of 18 MΩ.cm. This was prepared from a circulated deionised water supply using a type 1 ultrapure system (ELGA Veiola PURELAB® Ultra). For tissue culture use, water was autoclaved (121 °C for 20 minutes) in a Priorclave.

Buffers and solution recipes are listed in the appendix (Section7.3). Unless otherwise stated ELGA-purified water was used in preparations.

2.3 Glassware

Glassware for tissue culture was washed and dried in a 60 °C hot air oven. It was subsequently autoclaved (121 °C for 20 minutes) and dried prior to use. Glass Pasteur pipettes (Fisherbrand®) were placed in metal containers (Jencons) and autoclaved. Autoclaved items were marked with tape.

2.4 Plasticware

Plastic 7 ml bijoux containers (Ramboldi 327145), 30 ml universal containers (Greiner bio-one, 201170), 120 ml containers (Sarstedt, 75.9922.420) were all supplied sterile for tissue culture. Pasteur pipettes (Scientific Laboratory Supplies, PIP4204), disposable micro-pipette tips (STAR lab, S1111-6001) and micro-tubes (Sarstedt, 72.699) were supplied unsterile. These were therefore autoclaved (121 °C for 20 minutes) and oven dried prior to use.

Urothelial cell cultures were grown and maintained in Primaria™ flasks and dishes. Primaria™ have modified polystyrene surfaces which incorporate a mixture of anionic

and cationic functional groups; this differs from standard tissue-culture dishes which are negatively charged. Primaria™ 25 cm^2 and 75 cm^2 flasks (Sarstedt, 83.3911.302) were used along with 6 and 96 well plates.

2.5 Tissue Collection

Tissue from ureters and bladders were collected from patients from NHS hospitals (Table 2.1). These were processed following laboratory protocols (Southgate *et al.*, 1994).

Samples were collected aseptically at the time of the operation and placed into transport medium containing HBSS with $Ca^{2+}$and Mg^{2+} (Gibco 24020-091), 10 mM Hepes pH 7.6 (Gibco 15630-056) and 20 kallikrein-inhibiting units (KIU)/ml of aprotinin (Trasylol; Bayer Pharmaceuticals) (Southgate *et al.*, 1994). Samples were stored at 4 ^{o}C before use for up to 3 days.

2.5.1 Ethics

Urothelial cells and tissues for immunohistochemistry (IHC) were used in accordance with pre-existing NHS Research Ethics Committee (REC) project approvals. For IHC experiments, paraffin wax-embedded blocks of urothelial tissues from patients with benign dysfunctional bladder disease obtained with enduring consent were accessed from a REC-approved urothelial research tissue bank (URoBank (Yorkshire and Humber REC 16/YH/0396)). Fresh ureter tissue was obtained as anonymous surgical waste (Leeds East REC 99/095) for use in tissue culture.

2.6 Tissue Culture

2.6.1 General

All cell and tissue culture work was conducted in a BioMat² laminar flow class 2 safety cabinet (Contained Air Solutions Ltd). Cultures were incubated in 95 % humidified HERAcell™ 240 CO_2 incubators. In standard conditions cultures were grown at 37 ^{o}C in 5 % (v/v) CO_2 in air (21 % (v/v) O_2).

Cell suspensions were centrifuged using a Sigma 2-4 centrifuge at 250 g for 4 minutes. Cell cultures were visualised and photographed using an EVOS®XL Core microscope.

2.6.2 Sample arrival and processing

Upon arrival at the laboratory samples were logged and allocated a sequential Y number. Samples were then dissected of fat and connective tissue using sterile autoclaved instruments. A representative section of the tissue was separated and placed into 10 % (v/v) formalin for 48 hours and then transferred to 70 % (v/v) ethanol for later processing for histology. Approximately 1 cm^2 sections (ureters opened) were placed into stripper solution (HBBS without $Ca^{2+}$and Mg^{2+} (Gibco 14170-088), 10 mM Hepes pH 7.6(Gibco 15630-056), 20 kallikrein-inhibiting units (KIU)/ml of aprotinin (Trasylol; Bayer Pharmaceuticals), and 50 ml 1% EDTA (final concentration 0.1%)) (Southgate *et al.*, 1994). Sections were left in stripper medium for 4 hours at 37 °C. The urothelia was removed by running it between the blades of a sterile forcep. Sheets were transferred to a universal container with stripper medium and centrifuged for 4 minutes at 250 g. Medium was aspirated and the pellet agitated. The pellet was then re-suspended in 2 ml of 100 U/ml collagenase for 20 minutes at 37 °C. 3 ml of keratinocyte serum-free medium (Gibco) supplemented with recombinant epidermal growth factor (5 ng/ml), bovine pituitary extract (50 µg/ml) and cholera toxin (30 ng/ml) (with supplements medium is referred to as KSFMc) was added and the samples were centrifuged for 4 minutes at 250 g. The pellet was then agitated, re-suspended in KSFMc and placed into Primaria flasks.

Once isolation had occurred Normal Human Urothelial (NHU) cells become established in Primaria flasks as adherent finite cell lines. NHU cell cultures were maintained in normoxic conditions. Medium was changed every 2-3 days. Within these conditions cells adopt a proliferative phenotype and will continue to divide until they become confluent, where there is then contact inhibition. Considering this, cultures were sub-cultured (passaged) when 80-90 % confluent, to maintain a proliferative phenotype. To passage the cultures, the KSFMc medium was aspirated and 10 ml 0.1 % sterile EDTA was added to a T75 flask, 5 ml was added to a T25 flask. The cultures were placed in a 37 °C incubator for 5-10 minutes until the cells rounded. EDTA was aspirated and 1 ml of trypsin (Sigma, T4549) was added to a T75 and 0.5 ml to a T25

Primaria flask. Flasks were left in the incubator for 1-2 minutes until the cells detached form the substrate. Trypsin activity was neutralised by 100 µl of 20 mg/ml trypsin inhibitor (Sigma, T6522) which was combined with 5 ml KSFMc, the full solution is centrifuged to separate the cells from solution. After aspiration the cells are agitated and re-suspended in KSFMc. When passaging cells were split from one T75 into 2 -4 T75 flasks, this enabled an adequate seeding density. For experiments, NHU cell lines were used between passages 3-6. The cell lines used in experiments are shown in Table 2.1.

Table 2.1: Source of donor cell lines for tissue culture

Sample ID number	Source	Tissue type	Demographics (age/sex)
Y1695	Renal transplant	Ureter	52, F
Y1747	Renal transplant	Ureter	42, F
Y1751	Renal transplant	Ureter	22, F
Y1790	Nephrectomy	Ureter	68, F
Y1798	Nephrectomy	Ureter	n.d., M
Y1833	Renal transplant	Ureter	42, M
Y1837	Renal transplant	Ureter	54, F
Y1847	Renal transplant	Ureter	32, M
Y1858	Renal transplant	Ureter	72, F
Y1863	Renal transplant	Ureter	56, F
Y1889	Nephrectomy	Ureter	62, M
Y1891	Renal transplant	Ureter	27, M
Y1892	Renal transplant	Ureter	50, M
Y1896	Renal transplant	Ureter	53, M
Y1906	Renal transplant	Ureter	66, M
Y1913	Nephrectomy	Ureter	57, M
Y1914	Nephrectomy	Ureter	61, F

n.d. no data

2.6.3 Freezing and thawing of NHU cells

NHU cell cultures were harvested as described (section 2.6.2). Following centrifugation, cells were re-suspended in ice-cold freeze mix (KSFMc with 10 % (v/v) FBS and 10 % (v/v) DMSO (Sigma-Aldrich, D2650)). Cells from a T75 flask were divided between three cryovials; these were placed into a Mr. Frosty™ freeze container and left at -80 ^{o}C overnight. The vials were transferred into liquid nitrogen-containing dewars the following day.

To bring cells up from frozen, vials were removed from the liquid nitrogen-containing dewars and rapidly thawed in a 37 ^{o}C water bath. Vial contents were transferred into a universal container containing 10 ml KSFMc and centrifuged. The pellet was then re-suspended in KSFMc and seeded into Primaria flasks.

2.6.4 ABS/Ca^{2+} Differentiation method

Differentiation of non-differentiated cells was induced using a previously established method (Cross *et al.*, 2005). Medium was switched from KSFMc to KSFMc plus 5 % Adult Bovine Serum (ABS, SeraLab, S-202 (preselected batch)) for 3-5 days. Cells were harvested as previously described, excluding the trypsin inhibitor step because serum contains a natural trypsin inhibitor. Cells were seeded onto permeable membranes 12-well Thincert™ cell culture inserts (Greiner Bio-One, 665640) at 0.5 x10^{6} cells per membrane. These membranes provide a culture surface which is 113.1 mm² with a pore size of 0.4 µm. Cultures were maintained in KSFMc/ABS medium overnight, and then the calcium concentration in the medium was increased to 2 mM (near physiological), taking into consideration that KSFMc contains 0.09 mM Ca^{2+} and ABS contains 2.2 mM Ca^{2+}. Cultures were maintained in medium containing KSFMc/ABS /2 mM [Ca^{2+}] for the remainder of the experiments. 0.5 ml of medium was added to the apical compartment of the membrane insert and 1 ml to the basal compartment. Cultures on permeable membranes were used for transepithelial electrical resistance (TEER) studies, and cell sheets were harvested at set time points (sections 2.6.5 and 2.6.8).

For scratch wound experiments that did not require permeable membranes, cells were seeded onto 12 well plates and each well was maintained in 1 ml of KSFMc. When

confluent, 5 % ABS was added to the medium for 3-5 days, before increasing the calcium concentration to 2 mM. Where drug treatments were trialled, these were added to the medium at appropriate concentrations, with controls maintained in matched non-treated medium.

2.6.5 Measurement of urothelial barrier

Transepithelial electrical resistance (TEER) of cultures seeded onto permeable membranes was measured using chopstick electrodes (World Precision Instruments, STX2) and an Epithelial Voltohmmeter X (World Precision Instruments, EVOMX). TEER is a measurement of electrical resistance across an epithelial membrane which indicates barrier function (Rubenwolf and Southgate, 2011). The chopsticks are first sterilised in Cidex Plus solution (World Precision Instruments, 7364) for 10-15 minutes at ambient temperature and then placed in the same medium as the cultures (either KSFMc/ABS or KSFMc/ABS/Ca^{2+}) for 10 minutes, to wash and equilibrate the probe; this was performed twice. Once stabilised readings were taken and recorded on an Excel spreadsheet.

2.6.6 Scratch wounding and time-lapse videomicroscopy

Scratch wounding was performed using the tip of a 10 µl disposable micro-pipette; the pipette was drawn from one side of the membrane to the other in a single smooth motion. TEER readings were taken pre- and post-wounding, to assess changes in barrier function.

To visualise cell migration post scratch wound, cell cultures grown on a 12 well plate were scratched as above then transferred to the time-lapse videomicroscope. This was set up on an Olympus IX81 microscope with an enclosed humidified environmental chamber (Figure 2.1). Using a mechanised stage images were captured and processed in Cell™ software. Images were taken two hourly, wound area was calculated in Cell™ software by drawing freehand around the wound area for each captured picture and expressed as percentage of wound closed over time.

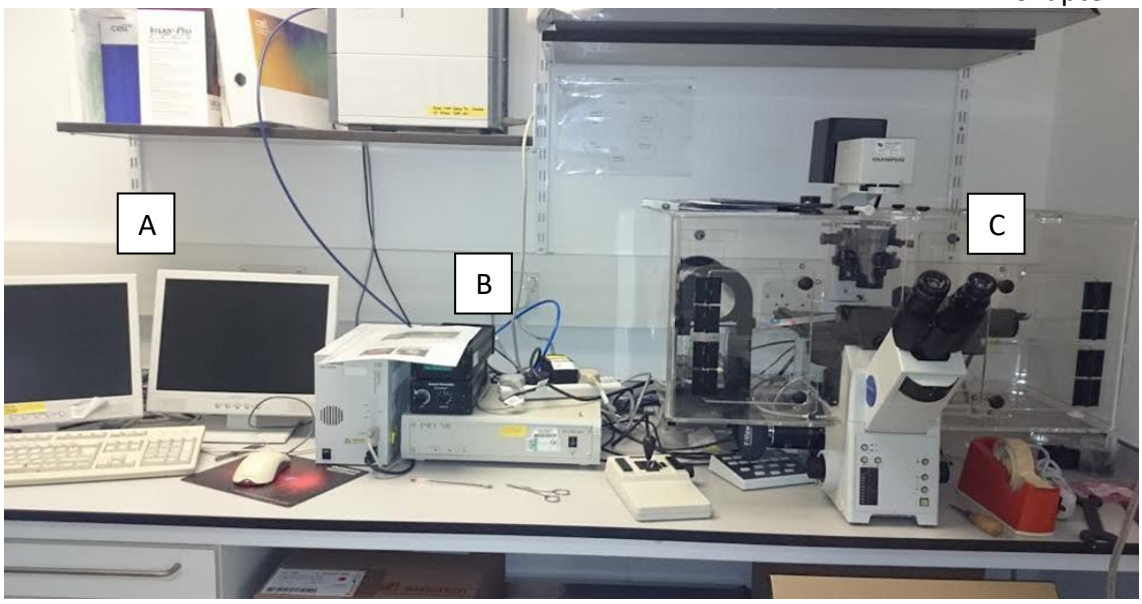


Figure 2.1: Setup for time-lapse videomicroscopy.

A = computer with software to store captured images. B = control boxes for temperature and CO^2 control. C = microscope with enclosed humidified environment.

2.6.7 Hypoxia

Cultures placed in the humidified hypoxic incubator (Galaxy® S Series incubator) were maintained at 37 °C in 5 % (v/v) CO_2 and in hypoxic conditions (2 % (v/v) O_2). Nitrogen was used to generate a hypoxic environment (Figure 2.2).

Figure 2.2: Hypoxic incubator.

A= setup of hypoxic incubator and gas cylinders, the nitrogen cylinder is at the forefront (set at 0.5 bar = 7.25 psi) and CO_2 (0.35 bar = 5 psi) at the rear. B= Incubator display with set parameters.

2.6.8 Harvesting of cell sheets from permeable membranes for immunohistochemistry

Cultures that had been maintained on permeable membranes (Greiner Bio-One, 665640) were harvested as cell sheets by harvesting them from the permeable membranes at different time points during experiments. Lifting was completed using Dispase II 8 units/ml (Roche, 04942078001). Dispase II was made by mixing with Dulbecco's Phosphate Buffered Saline (D-PBS Gibco, 14200-067) to a final concentration of 1 % (w/v) and filtered through a 0.2 µm low protein-binding syringe filter. Dispase was made in batches, aliquoted into 7 ml bijoux containers (Ramboldi 327145), and stored at -4 ^{o}C until required, it was thawed prior to use. Medium was aspirated from the apical and basal surface of the permeable membrane and 500 µl of dispase was applied to the apical surface and 1 ml to the base of the well. The cells were incubated for up to 30 minutes at 37 ^{o}C or until the sheets started to detach. The sheet was floated out into a Petri-dish containing D-PBS and transferred to a Cellsafe+ biopsy capsule (CellPath EBE-0301-02A) which was secured in a System II cassette (CellPath, EAD-0120-03A). The sheet was then fixed in 10 % (v/v) formalin overnight, and later transferred to 70 % (v/v) ethanol until further processing for immunohistochemistry (section 2.7).

2.7 Immunohistochemistry

The process of immunohistochemistry (IHC) involves labelling desired target proteins on tissue samples and cell sheets with antibodies that recognise epitopes specific to the targeted protein. This allows localisation and distribution to be visualised, and gives an indication of abundance.

2.7.1 Embedding

System II cassettes (CellPath, EAD-0120-03A) containing formalin-fixed tissue were placed into fresh 70 % (v/v) ethanol and placed on the orbital shaker (Luckham R100 rotatest shaker) for 10 minutes. 70 % (v/v) ethanol was exchanged for absolute ethanol for a further three washes to dehydrate the samples. Cassettes were then placed in the hood and the ethanol was exchanged for isopropanol (propan-2-ol) for two washes both 10 minutes duration. Cell sheets underwent the above process; however time of washes was reduced to 5 minutes. Four xylene washes were completed followed by emersion in wax in a 60 °C oven (Scientific Laboratory Supplies Ltd). Emersion in wax pots was repeated four times for 15 minutes each. Samples were embedded in wax and left to cool at -12 °C. Blocks were stored at ambient temperature.

2.7.2 Sectioning

Ureter/ bladder tissue samples and cell sheets were placed on the -12 °C cool plate prior to sectioning. 5 µm sections were cut on the microtome (Leica RM2135) floated on a warm bath and captured onto Superfrost® plus, microscope slides (Thermo scientific, J1800AMNZ). The slides were left to dry overnight and baked at 50 °C on the slide dryer for 1 hour prior to further processing.

Slides were dewaxed and re-hydrated in two 10 minute xylene washes, two 1 minute xylene washes; three absolute ethanol washes and one 70 % (v/v) ethanol wash. They were then placed in running tap water for 1 minute to remove excess ethanol. Tissue sections underwent fresh 3 % hydrogen peroxide block for 10 minutes to block any endogenous peroxidase activity in the tissue. A further 10 minute wash under running tap water was then performed.

2.7.3 Antigen retrieval and antibody application

The method of antigen retrieval is dependent on the primary antibody to be used. Antigen retrieval allows the crosslinking of proteins caused by formalin fixation to be broken down, ensuring more antigen sites are available. Depending on antibody, two antigen retrieval methods were used during standard IHC experiments, namely the microwave method (slides microwaved for 13 minutes in 10mM citric acid Buffer pH 6.0) and trypsinisation and microwave (slides incubated for 10 min in 0.1 % (w/v) trypsin buffer solution (TBS) at 37 ^{o}C in a water bath followed by above microwave technique).

Following antigen retrieval, slides were placed in a Shandon Sequenza. Avidin (Vector, sp-2001), was applied for 10 minutes at ambient temperature to block endogenous biotin. The avidin/biotin staining method may result in high non-specific background staining; therefore this can be reduced by pre-treatment with avidin/biotin prior to incubation with a biotinylated antibody. Slides were washed with TBS (see appendix for recipe, section 7.3) three times to remove avidin. Biotin (Vector, sp-2001) was then applied to each slide for 10 minutes at ambient temperature. Excess biotin was washed off twice with TBS and normal serum was applied for 5 minutes. Serum was from the same animal that the secondary antibody is raised. Serum is a blocking buffer which contains antibodies that bind to and neutralise reactive sites in the tissue thus prevents the non-specific binding of secondary conjugated antibodies to the tissue. Primary antibody was then applied and the slides were left overnight (approx. 16 hours) at 4 ^{o}C (Table 2.2). Negative controls which no primary antibody was applied remained in TBS overnight. The slides were washed three times with PBS to remove the primary antibody and an appropriate biotinylated secondary antibody (Table 2.3) was applied for 30 minutes at ambient temperature. The slides were then washed twice with TBS to remove the secondary antibody. Streptavidin-biotinylated/horseradish peroxidase complex (StrepAB/HRP, Vector, PK6100) was applied and left for 30 minutes. Slides were washed and incubated with diaminobenzidine (DAB, SigmaAldrich, D4293) for 10 minutes. DAB detects peroxidase activity and stains brown, this identifies the location of the target protein/epitope. Slides were washed, counterstained with Haemotoxylin for 5-10 seconds, dehydrated

then mounted. The dehydration steps were a reverse of the re-hydration steps but the two 10 minute xylene washes are omitted.

All labelling experiments were designed with negative and positive controls. Negative controls had no primary antibody added; positive controls were the antibody being investigated on a tissue which is known to express the protein. An example of a negative and positive control is shown in Figure 2.3. A further experimental control was included in each experiment. This control was an irrelevant antibody to those being investigated but one known to label on the urothelium. Cell lines used in experiments are shown in Table 2.4.

Table 2.2: List of primary antibodies used for immunohistochemistry

Antigen	Antibody clone or catalogue #	Host	Supplier	Dilution and retrieval method
ZO1	1A12	Mouse (monoclonal)	Life Technologies	1:100 (MW)
ZO3	3704	Rabbit (monoclonal)	Cell signalling	1:200 (MW, trp)
Claudin 3	Ab52231	Rabbit (polyclonal)	ABCAM	1:5000 (MW, trp)
Claudin 4	3E2c1	Mouse (monoclonal)	Zymed	1:500 (MW)
Claudin 5	C43C2	Mouse (monoclonal)	Invitrogen	1:150 (MW)
HIF1α	H1α67	Mouse (monoclonal)	Enzo Life Sciences	1: 300 (MW)

MW-microwave, trp-trypsin. Dilutions previously determined by titration

Table 2.3: List of secondary antibodies

2^o Antibody	Company, code	Dilution
Biotinylated Rabbit anti-Mouse	Dako, E0354	1:100
Biotinylated Goat anti-Rabbit	Dako, E0466	1:600

Dilutions previously determined

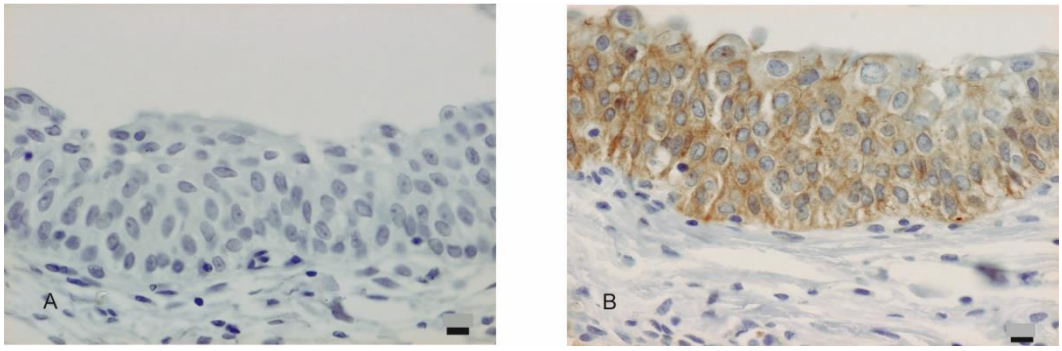


Figure 2.3: Examples of control tissue for IHC experiments.

A: Ureter, negative control (secondary antibody only). B: Ureter labelled with claudin 4 used as experimental control. Ureter was used as a positive control tissue for experiments with cell sheets. Scale bar = 10 µm.

Table 2.4: Source of donor tissue for immunohistochemistry

Sample ID number	Source	Tissue type	Demographics (age/sex)
Y1376	Bladder biopsy	Bladder	30, F
Y1751	Renal transplant	Ureter	22, F
Y1779	Nephrectomy	Ureter	80, F
Y1817	Nephrectomy	Ureter	60, M
Y1837	Renal transplant	Ureter	54, F
Y1851	Renal transplant	Ureter	59, F
Y1858	Renal transplant	Ureter	72, F
Y1891	Renal transplant	Ureter	27, M

2.7.4 Additional procedure for antibody detection of chondroitin sulphate

As discussed in chapter 1 (section 1.3) there are different types of CS depending on where the sulphate is attached. Different antibodies are therefore required to bind to different epitopes and reveal each CS. Three antibodies (7D4, 6C3 and 4C3) bind to the CS chain, whereas 3B3, 2B6 and 1B5 bind to the digested stub (Figure 2.4). Chondroitinase ABC is used to digest the CS chain leaving a stub displaying epitopes for 3B3, 2B6 and 1B5 antibodies to bind. 3B3 antibody has an additional characteristic in that it will bind to a mimitope on the end of an undigested CS chain. A mimitope is structure that is similar to an epitope which the antibody will recognise and bind to.

Chondroitin sulphate antibodies and chondroitinase ABC were supplied by Prof Hughes, Cardiff University (Table 2.5) (Hayes, Hughes and Caterson, 2008). Dehydration and hydrogen peroxide steps were completed as above. Slides were placed in a Shandon Sequenza and chondroitinase ABC diluted in 0.1 M Tris with acetate buffer (Section 7.3) to a 0.1 U/ml concentration was added. The slides were placed in a 37 ^{o}C oven for 30 minutes. After 30 minutes the chondroitinase ABC was replenished and left for a further 30 minutes, to allow for maximum enzyme activity. Following chondroitinase ABC digest avidin and biotin were applied and steps continue as above (section 2.7.3). Controls in these experiments included a positive tissue control from embryonic chick tissue which is known to express CS, a negative control of no antibody treatment and an experimental control, which was a non-specific antibody that is known to bind to the urothelium.

Table 2.5: Chondroitin sulphate antibodies, supplied by Prof C Hughes, Cardiff University

Antibody	Species raised	Dilution	Chondroitinase digest required to reveal epitope
3B3 (stub plus chain mimitope)	Mouse, monoclonal	1:20	Performed both
2B6 (stub epitope)	Mouse, monoclonal	1:20	Yes
1B5 (stub epitope)	Mouse, monoclonal	1:5	Yes
7D4 (chain epitope)	Mouse monoclonal	1:5	No
6C3 (chain epitope)	Mouse, monoclonal	1:5	No
4C3 (chain epitope)	Mouse, monoclonal	1:5	No

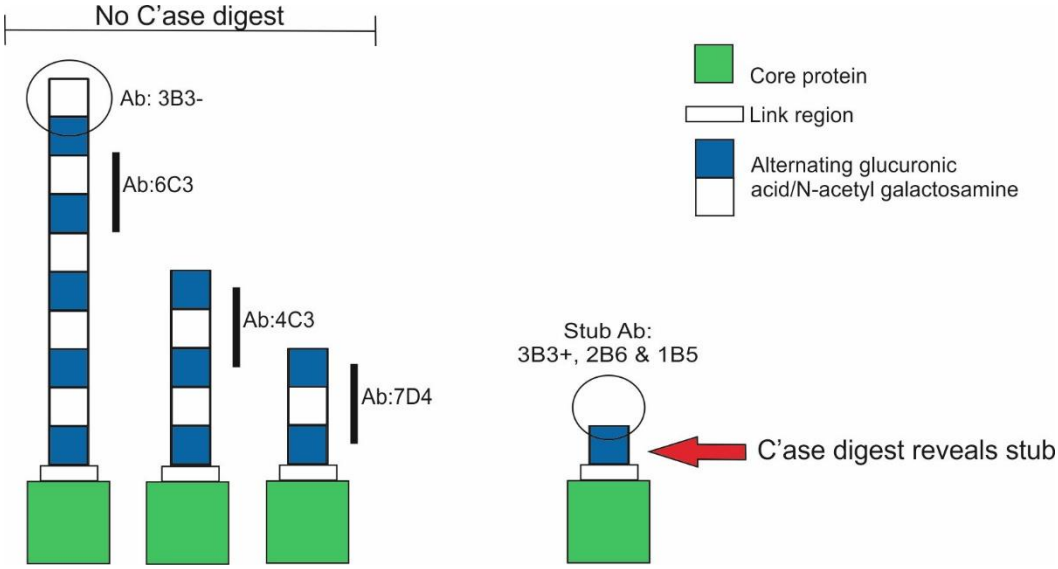


Figure 2.4: Binding sites of CS antibodies along the CS chain and stub.

6C3, 4C3 and 7D4 bind to the CS chain and therefore do not require a chondroitinase ABC digest prior to antibody application. 3B3, 2B6 and 1B5 bind to the CS stub and so require a chondroitinase ABC digest to remove the chain. 3B3 has a mimitope which is seen at the end of an undigested CS chain, as well as the epitope which is revealed after digestion.

2.7.5 Imaging

Slides were viewed on a brightfield microscope (Olympus, BX60) using x10 (air), x20, x40 and x60 (oil) lenses. Images were captured using an Olympus DP50 digital camera in Image-Pro Plus Software (Media Cybernetics, version 4.5.1.29). Editing of images was completed in CorelDRAW (version 8).

3 The development of a chronically-damaged differentiated NHU cell culture model

3.1 Aim and objectives

Studies reviewed in Chapter 1 suggest that the urothelium may be compromised in benign bladder conditions with, for example, loss of tight junction proteins in BPS. Most experimental models study the sequelae of acute damage on bladder dysfunction, but are limited by the regenerative capacity of the urothelium and poorly replicate a chronic (non-repairing) condition. The aim of this chapter was to create a chronically-damaged NHU cell culture model, assessed by monitoring of barrier function recovery.

The experimental objectives were:

1) To monitor the effect of physical damage (scratch wounding) on the restitution of barrier properties in differentiated NHU cell cultures and
2) To determine whether exposure to ketamine or hypoxic conditions provides evidence for chronic barrier damage in differentiated NHU cell cultures.

3.2 Experimental approach

NHU cell cultures were differentiated using the ABS/Ca^{2+} method (Section 2.6.4). Firstly cultures were pre-treated with KSFMc/ABS for 3-5 days. The cultures were then harvested and seeded onto permeable membranes (12-well Thincert™ cell culture inserts) for barrier monitoring by TEER or on 12 well plates for timelapse microscopy.

3.2.1 Normal barrier development

Normal barrier development was assessed by serial TEER measurements every 2-3 days. Medium changes were conducted every 2-3 days.

3.2.2 Scratch wounding

Time to wound closure and percentage area closed was assessed visually using time-lapse videomicroscopy. Differentiated NHU cell cultures were established on 12 well plates and left for 7 days in KSFMc/ABS/Ca^{2+} before scratching with a 10 µl disposable micro pipette in a continuous motion across the well. Images were captured every 2 hours. Wound area was measured initially and then two hourly until the wound had visually closed. Closure was expressed as percentage of wound closed over time. Two biological replicates were used: NHU cell line Y1751 which had 5 experimental replicates and Y1695 which had 6 experimental replicates. See chapter 2 (Table 2.1) for details of NHU cell lines (Y number).

To measure barrier function after scratch injury, cultures were established on permeable membranes. Once the mean TEER reading reached 1000 Ω.cm^2 the culture was scratched across the membrane in a continuous motion with the tip of a 10 µl disposable micro pipette. Immediate post scratch TEER readings were taken to record a change in barrier. TEER readings were monitored every 2-4 hours to assess barrier restitution. Three biological replicates were used: Y1751 had 3 experimental replicates, Y 1747 had 4 experimental replicates and Y1837 had 3 experimental replicates.

3.2.3 Ketamine

Under the premise that ketamine is a damaging agent that would be first encountered by an intact barrier urothelium, an experimental approach was designed where ketamine was applied to differentiated NHU cell cultures. Differentiated NHU cultures were maintained on permeable membranes (12-well Thincert™) until a barrier >1000 Ω.cm^2 was achieved. Ketamine (Sigma, K2753-5G) was diluted in KSFMc/ABS/Ca^{2+} and filtered. An initial concentration of 4 mM and 5 mM was used based on published literature (Baker *et al.*, 2016). The ketamine /KSFMc/ABS/Ca^{2+} solution was placed in the apical compartment only and was replenished at medium changes. There were four experimental replicates in each ketamine group and a parallel control of three experimental replicates which was maintained in KSFMc/ABS/Ca^{2+}. The basal surface remained submerged in KSFMc/ABS/Ca^{2+} in all groups. TEER was monitored every 2-3 days to record the barrier and medium changes took place every 2-3 days. To examine

the capacity for barrier repair, once the TEER had reduced to ≤ 500 Ω.cm², the medium containing ketamine was removed and replaced with KSFMc/ABS/Ca^{2+}. Assessment of barrier function was continued for several days after removal of ketamine.

3.2.4 Hypoxia

The role of hypoxia has been demonstrated in chronic inflammatory bladder conditions, such as BPS (Lee and Lee, 2011), therefore an experimental approach was designed where differentiated NHU cultures were exposed to hypoxia once a barrier was established. Hypoxia has been shown to compromise barrier function of differentiated NHU cell cultures when cells are exposed during proliferation (Radford, 2015).

3.2.4.1 Effect of Hypoxia on Urothelial Barrier

Five NHU cell lines were used to study the effect of hypoxia on barrier formation. Each cell line was seeded as replicates onto permeable membranes (Section 2.6.5) and was maintained in normoxia until a mean barrier of 1000 Ω.cm² had developed. Half the replicates (n=3-5) were transferred into the hypoxic incubator (2 % O_2) and maintained for 7 or 11 days. Parallel controls were maintained in normoxia. Experimental cultures were only removed from hypoxia for TEER readings and medium changes, which took place every 1-3 days.

3.2.4.2 Effect of Hypoxia on Barrier Proteins

Culture sheets were harvested using dispase (Section 2.6.8) and formalin-fixed after 7 days in hypoxia; matched controls from normoxia were taken at the same time point. Additional sheets were harvested at the end of the experiment. After fixation all sheets were kept in 70 % (v/v) ethanol until processing into paraffin wax (section 2.7) and later sectioning for immunohistochemistry assessment. The tight junction proteins Claudin 3 and 4, ZO1 and ZO3 were assessed on two cell culture sheets from two NHU cell lines. To qualify the results two independent assessors who were blinded to the results were asked to review the IHC. They were presented with three pictures of different regions for each experimental sheet at day 7 and were given a matched normoxia control image to compare to (Figure 3.1). They were asked to score the

labelling of each antibody as less, the same or more as -1, 0 and 1, respectively. A comparison between the hypoxia sheets taken on day 7 was also made to the matched sheet at the end of the experiment .Observers were asked to score these as less (-1), the same (0) or more (+1) labelling, respectively.

Cell lines used for hypoxia experiments with associated number of replicates are shown below in Table 3.1.

3.2.5 The Association of Hypoxia in Benign Bladder Disease

To examine any association of hypoxia with benign bladder disorders, immunohistochemistry was used to assess the presence of HIF1α. Bladders from neuropaths, patients with overactive bladders and bladders from patients with stress urinary incontinence were used. This work was performed by Dr Amy Glover (Histology Technician) images were analysed by the candidate.

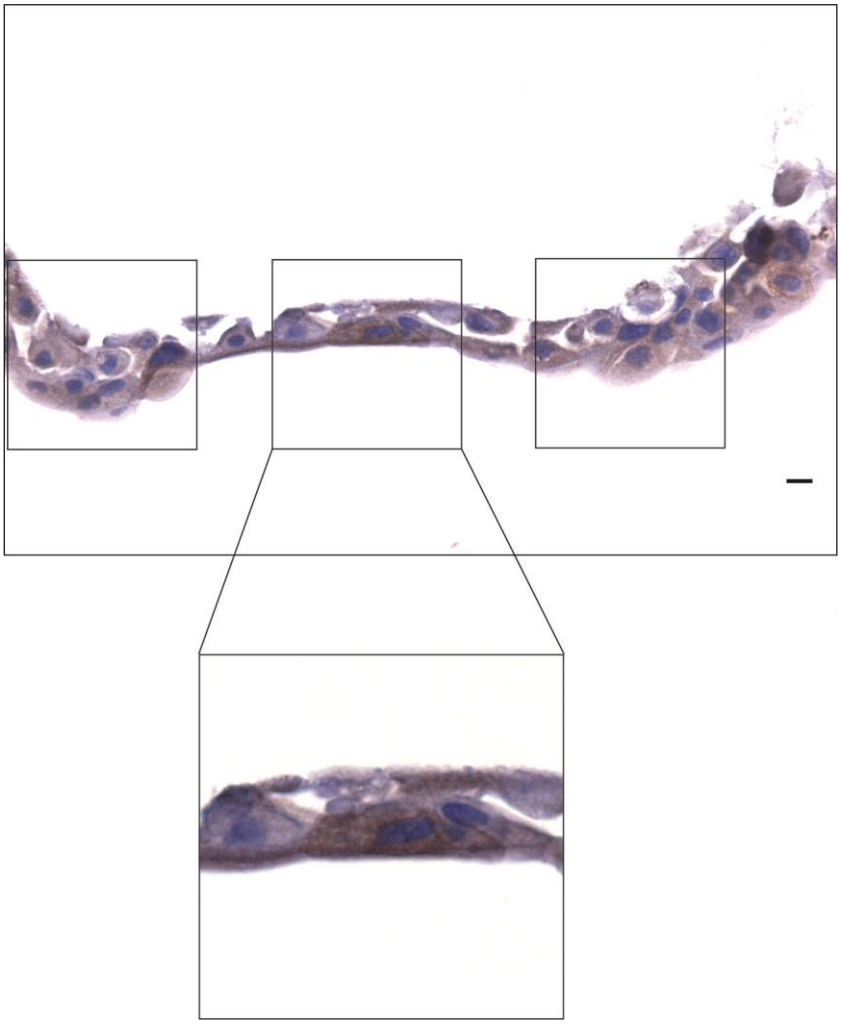


Figure 3.1: Example of how the image of the cell sheet was divided into sections for individual assessors to review.

This diagram displays a representative example of a cell sheet from cell line Y1751 labelled by ZO1 after 7 days in hypoxia. Three individual areas were evenly spaced along the sheet and the images were magnified (central box shown as an example). This image was then used in a PowerPoint presentation for scoring. Assessors were asked to take into consideration the number of cells in each image when scoring the intensity of labelling to control. Scale bar on original image = 10 µm.

Table 3.1: Cell lines used for hypoxia experiments with associated number of replicates.

Cell line reference	TEER 7 Day hypoxia (n)	TEER 11 Day hypoxia (n)	Cell sheets 7 Day hypoxia (n)	End TEER (n) after 7 Day hypoxia
Y1863	Cont:3 Exp:3	Cont:3 Exp:4		Cont:3 Exp:3
Y1751	Cont:5 Exp:3	Cont:5 Exp:3	Cont:1 Exp:1	Cont:5 Exp:3
Y1858	Cont:3 Exp:3			Cont:3 Exp:3
Y1913		Cont:3 Exp:3		
Y1891			Cont:1 Exp:1	Cont:3 Exp:3

3.3 Results

3.3.1 Normal barrier development

In all NHU cell cultures a barrier >1000 Ω.cm² was developed between 2 to 5 days after increasing the calcium concentration to 2 mM. For a representative example (cell line Y1889 (n=10)) a barrier >1000 Ω.cm² was reached between day 2 and 3 and the maximum barrier was achieved on day 10 (Figure 3.2). When comparing the time to maximum barrier for four cell lines this varied from 7-15 days (Table 3.2). Mean maximum barrier for the four cell lines was 4137 +/- 1121 Ω.cm² (mean, SD, n=44).

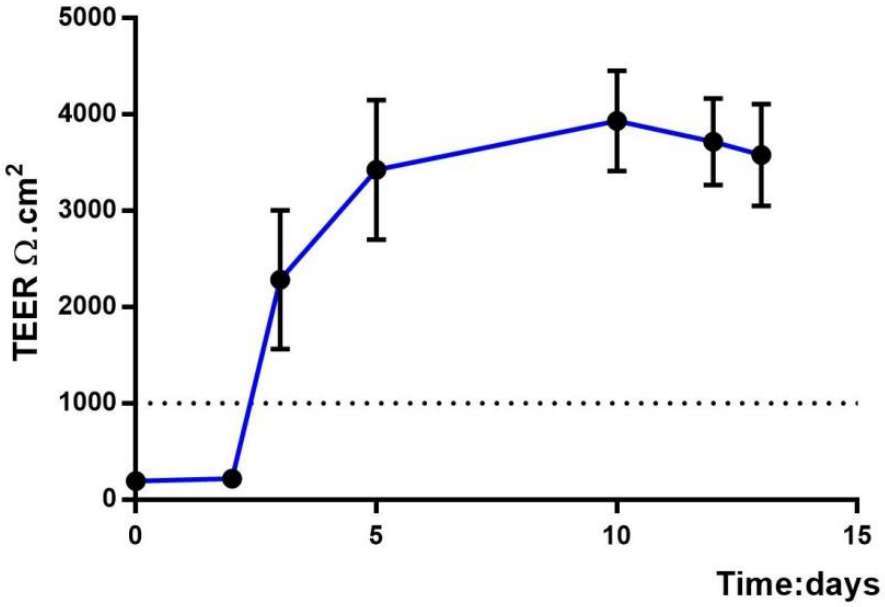


Figure 3.2: Formation of a urothelial barrier.

Representative example of barrier formation and maintenance from donor cell line Y1889. There were 10 experimental replicates. A barrier > 1000 Ω.cm² was achieved between day 2 and 3, this is represented as a dotted line on the graph. Maximum barrier was achieved on day 10. Points indicate mean with SD error bars (n=10).

Table 3.2: Maximum barrier achieved and time to reach maximum barrier of four independent cell lines (biological replicates).

Cell line	Number experimental replicates	Maximum TEER Ω.cm²(mean, SD)	Day maximum barrier achieved
Y1889	10	3935 (522.6)	10
Y1892	11	3140 (461.9)	15
Y1896	12	3713.3 (400)	7
Y1906	11	5778 (680)	7

3.3.2 Scratch wounding

Timelapse videomicroscopy showed that cells begin to migrate and close the wound within two hours post injury. Cell migration was assessed by drawing around the scratch area and working out the percentage of wound closure at two hourly time points (Figure 3.3). At 28 hours in the example shown the wound was 95 % closed, this is consistent with combined normalised data from two independent cell lines, where by 28 hours wounds were 95% (±11.3 SD; n=11) closed. The combined data from the two biological replicates showed that by 14 hours the wound was 45% (±24.7 SD; n=11) closed (Figure 3.4).

Injury and subsequent repair of barrier function as measured by TEER readings in three individual donor cell lines demonstrated that the TEER taken immediately after scratch wounding showed the barrier to reduce to < 600 Ω.cm² in each cell line, and although this is still a tight barrier for epithelial cells this was significantly different from the pre-scratch readings of the urothelial cell cultures. Two cell culture lines had full barrier recovery at 24 hours (Y1837 and Y1847) while one culture line took until 48 hours to have full recovery (Y1751) (Figure 3.5). Combined normalised data showed that there was a statistically significant difference in readings post scratch up to and including readings at 14 hours ($p=0.0141$, one-way ANOVA with Dunnett's comparison). At 22 hours there was no longer a statistically significant difference ($p=0.9896$ one-way ANOVA with Dunnett's post-comparison test) (Figure 3.6).

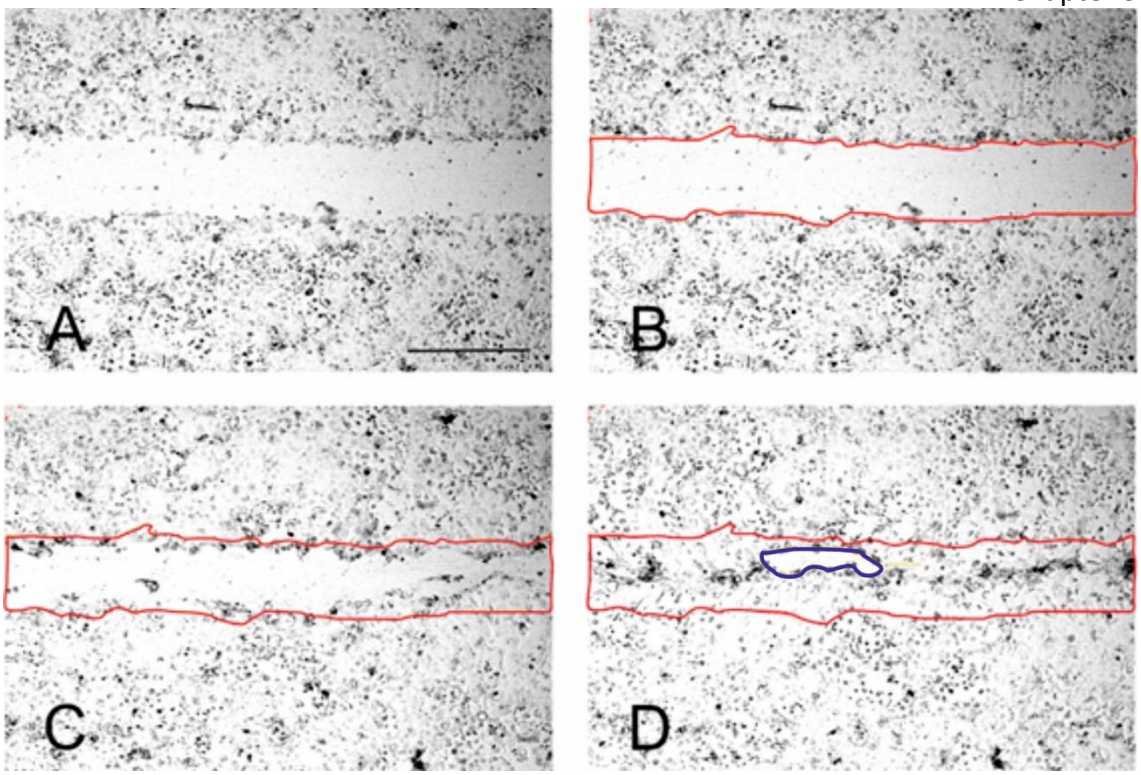


Figure 3.3: Scratch wound with repair

Images from one experimental replicate of cell line Y1695. Wound closure was assessed two hourly by calculating the area of the open wound and subtracting this from the initial scratch wound area (drawn in red). This was then expressed as percentage of wound closure. Scale bar = 500 µm.

A. Scratch wound at zero hours. This image shows the initial scratch wound.
B. Scratch at zero hours with marker (red line) showing area of scratch wound. The wound area was drawn around freehand; the area was then calculated using built in computer software. This was repeated two hourly and percentage of wound closure at each time-point was calculated.
C. Wound at 14 hours, with original scratch wound area marked. At 14 hours the cells had started to migrate to close the wound.
D. Wound at 28 hours, there remained a small area of wound still open (blue line) however there was 95 % wound closure.

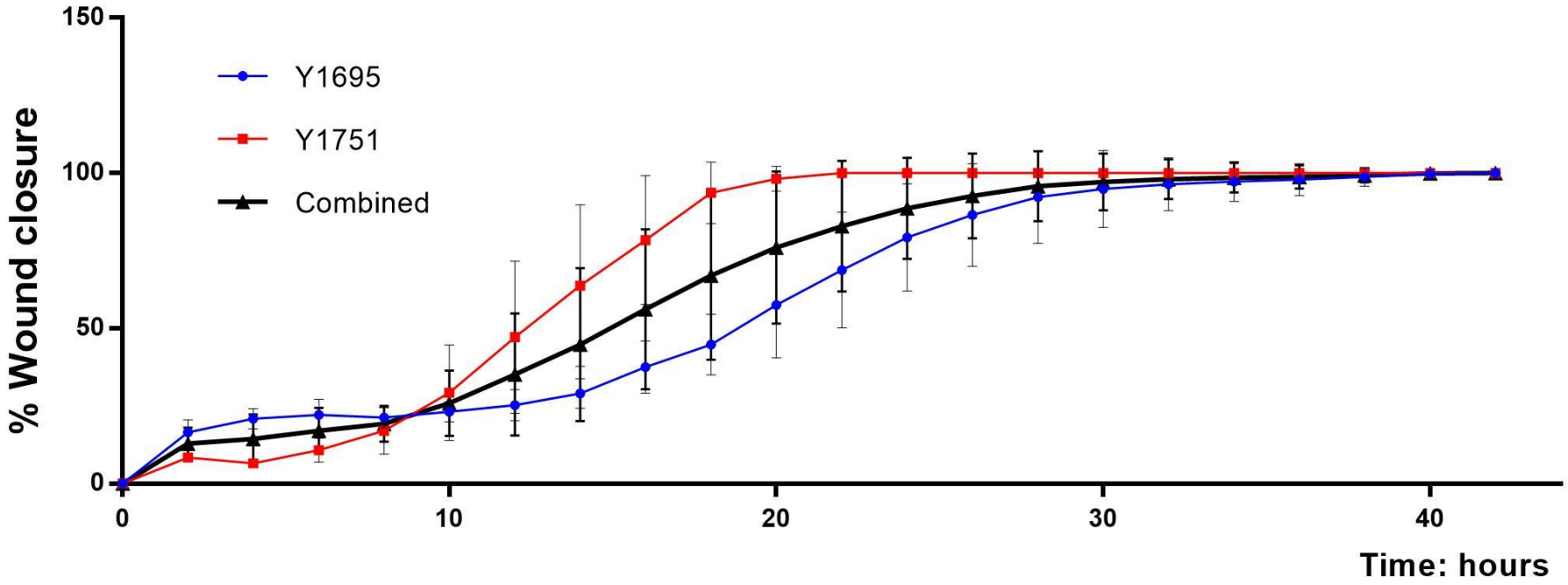


Figure 3.4: Wound closure after scratch of NHU cell cultures.

Percentage of wound closure over time, data for two cell lines (Y1695 and Y1751 (n=6 and 5 respectively)). The wounds of Y1751 closed earlier than Y1695. The black line represents the mean (SD) of combined data. After an initial lag the wound closed exponentially between 10 and 20 hours. Error bars represent SD. The error bars of the combined data are wide between 10-20 hours representing differences within the cell lines however after 24 hours these begin to reduce. After 28 hours combined data show that 95 % of the wounds are closed.

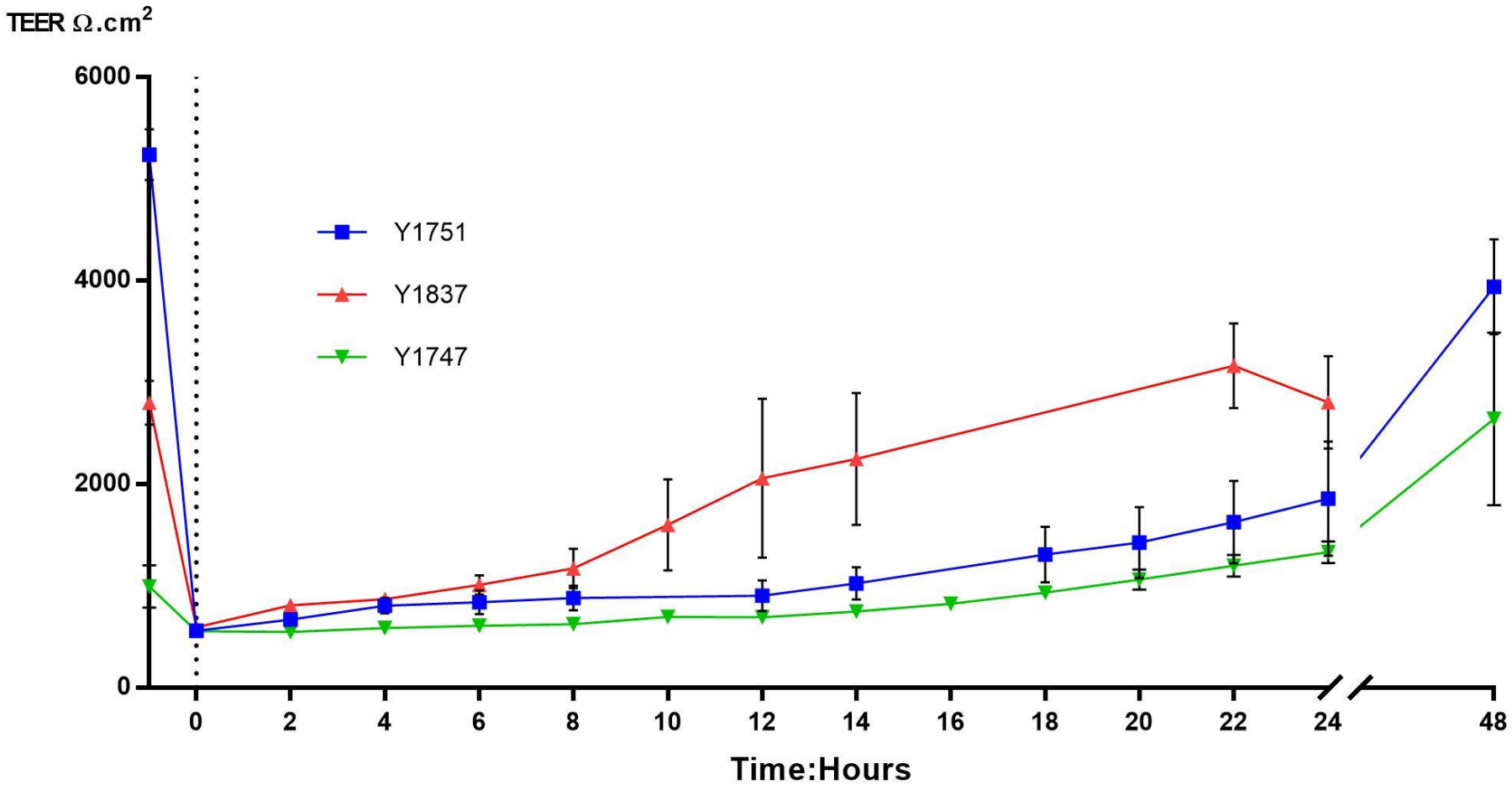


Figure 3.5: Barrier damage and recovery of three independent cell lines after scratch wounding.

Figure 3.5: Barrier damage and recovery of three biological replicates after scratch wounding.

Three independent cell lines with different initial barriers at time 0 showing initial reduction in barrier function (TEER) after scratch wounding with subsequent return of barrier over 24 – 48 hours. Dotted line represents time of scratch. TEER readings were taken regularly over a 24 hour period. There was a degree of variability between cell lines however by 24 hours two cell lines (Y1837 and Y1747) had recovered their barrier and by 48 hours the barrier was recovered in Y1751. Y1751 and Y1837 had n=3 experimental replicates and Y1747 had n=4.

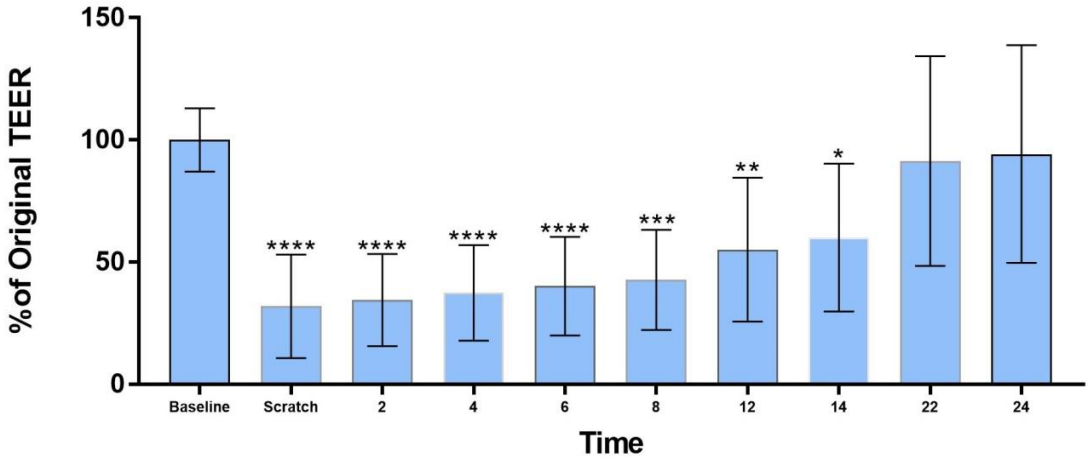


Figure 3.6: Percentage of original TEER after scratch wounding

Combined normalised data for three cell lines (Y1751, Y1837 and Y1747 (n=10 replicates)) showing reduction in barrier post scratch wound and repair over the subsequent 24 hours. Time is measured in hours, baseline was the reading pre-scratch and the scratch reading was taken immediately post scratch. Data was normalised to the individual biological replicate control and combined. There was a statistically significant difference between the baseline reading (pre-scratch) and the interval readings taken to 14 hours. As shown the significance became less over the subsequent hours after scratch wounding until there was no significant difference at 22 hours. Statistical significance was assessed using one-way ANOVA with Dunnett's multiple comparisons test. * $p<0.5$, ** $p<0.05$, *** $p<0.01$ and **** $p<0.001$ indicate statistical significance.

3.3.3 Ketamine

Ketamine was applied to a single differentiated NHU culture line which had reached a mean TEER of >1000 Ω.cm². Initially a concentration of 4 mM and 5 mM ketamine resulted in a tighter barrier to control. When the concentration was increased (4mM to 6 mM and 5 mM to 7mM) the barrier significantly reduced however remained above 1000 Ω.cm². There was a statistically significant difference in both ketamine groups when compared to control (t-test, $P<0.05$). With continued exposure to 7 mM ketamine in group 2 and an increase from 6 to 7 mM in group 1 the barrier reduced to <500 Ω.cm², however when ketamine was removed the barrier was unrecoverable (Figure 3.7).

This experiment was repeated with a second biological replicate using a starting concentration of 6 mM and 7 mM of ketamine. No consistent reduction in barrier could be reached. Due to the longevity and unreproducible nature of using ketamine to cause chronic depletion in barrier no further experiments were conducted. Further, ketamine appeared to be toxic to the cells above a concentration of 6 mM.

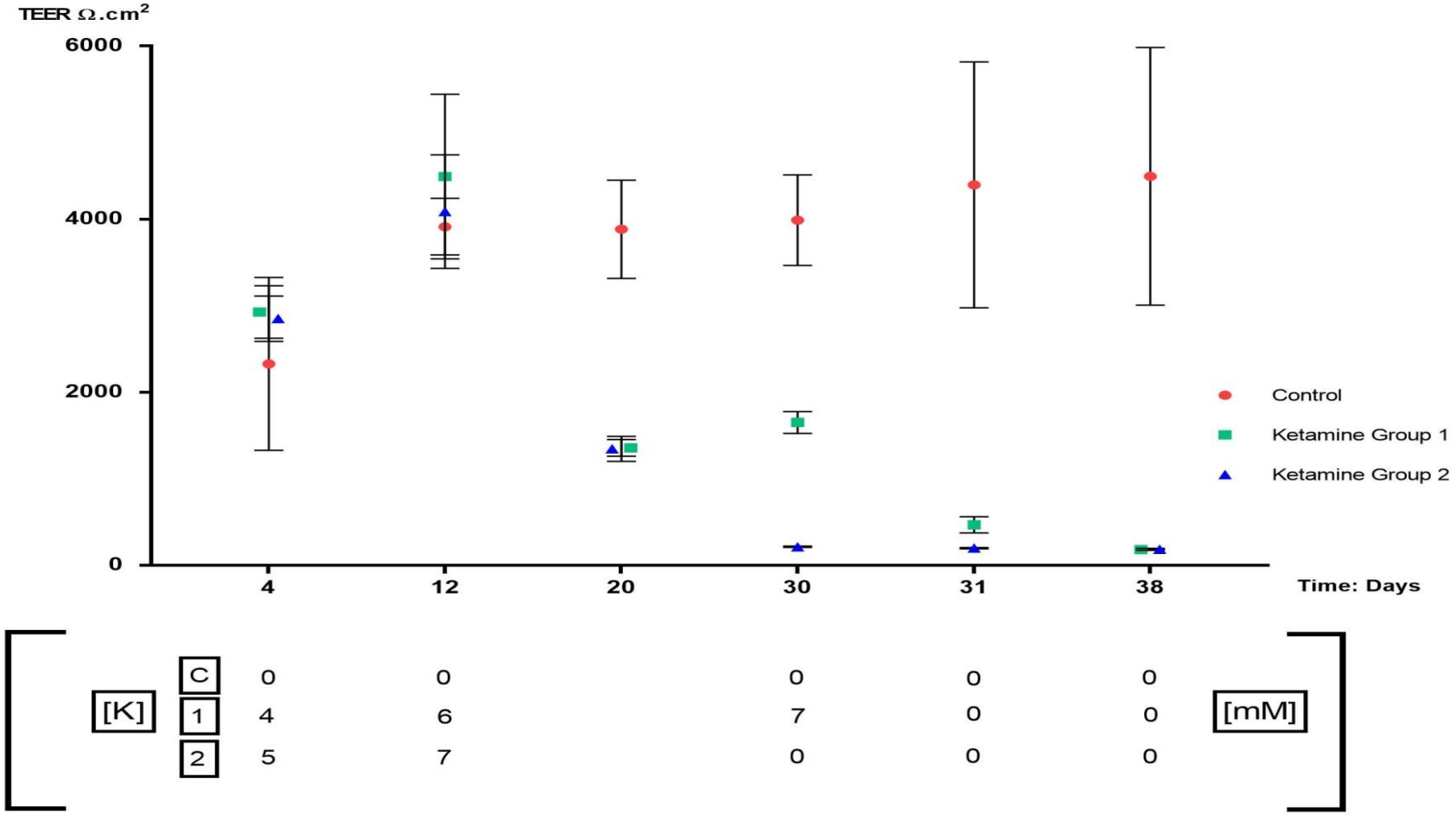


Figure 3.7: The effect of ketamine on barrier function

Figure 3.7: The effect of ketamine on barrier function

This figure demonstrates the effect of ketamine on the barrier function of an individual NHU cell line (Y1858). On day 4, ketamine was added to the medium in the apical compartment of eight experimental replicates. One group of four had 4 mM and the other four replicates had 5 mM ketamine added, parallel control consisting of three replicates remained in KSFMc/ABS/Ca^{2+}. On day 12 the barrier in all groups remained high, therefore the concentration of ketamine in the experimental groups were increased (4 mM to 6 mM and 5 mM to 7 mM). By day 30 the barrier had reduced in the second group (7 mM) and so the medium containing ketamine was removed and the cultures remained submerged in KSFMc/ABS/Ca^{2+}. In group one the barrier remained greater than 500 Ω.cm^2 and so the concentration was increased to 7 mM. After one day in the medium with a higher concentration of ketamine the barrier in group one had reduced to a mean 468.5 ±94.2 (SD; n=4) Ω.cm^2, this was therefore removed and replaced with KSFMc/ABS/Ca^{2+} for the remainder of the experiment. After an extended time in KSFMc/ABS/Ca^{2+} the barrier in both ketamine groups were irreparable.

3.3.4 Hypoxia

3.3.4.1 Effect of hypoxia on Urothelial Barrier

NHU cell cultures were transferred into hypoxia once a mean barrier of 1000 Ω.cm² had been established. For all cell lines the mean TEER reduced while cultures were maintained in hypoxia for 7 days. Matched normoxia control cell culture lines had barriers that continued to tighten. There was variability between cell lines in the minimum TEER reached, however hypoxia produced a predictable reduction when compared to control (Figure 3.8). Normalised data for three cell lines (Y1751, Y1863 and Y1858) showed that there was a statistically significant difference between TEER readings on the day of removal ($p = <0.001$, t-test) from hypoxia and up to four days post return to normoxia ($p=0.0088$, t-test) (Figure 3.9). There was gradual return of barrier in the hypoxic group during the recovery period. To ascertain if an extended exposure time to hypoxia would achieve a chronic damage model, three cell lines (Y1751, Y1863 and Y1913) were exposed to 11 days of hypoxia. On the day of removal from hypoxia there was a statistically significant difference ($p=<0.001$, t-test) between control cultures and cultures exposed to hypoxia, however by day 4 after return to normoxia there was no longer a statistically significant difference (Figure 3.10).

3.3.4.2 Effect of hypoxia on barrier proteins

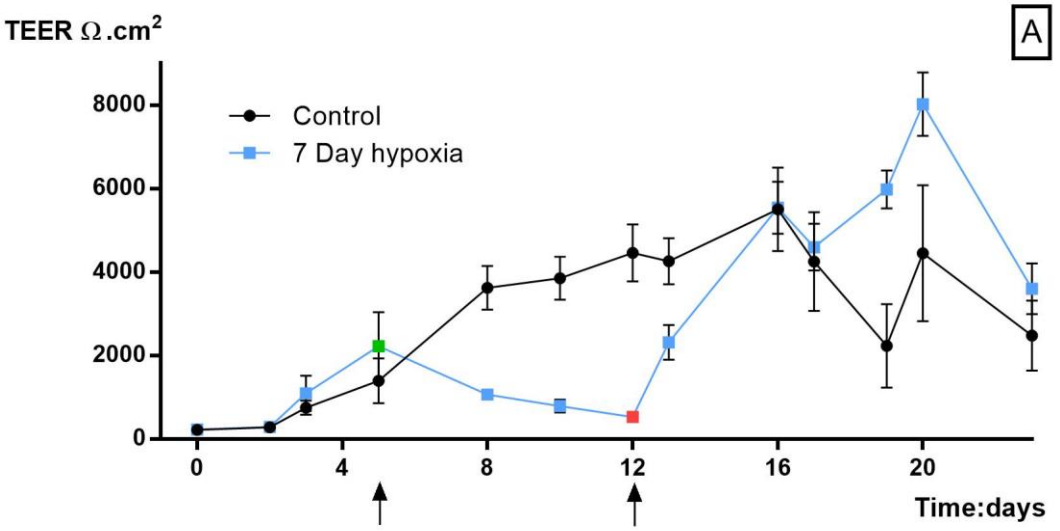
Assessment by the candidate of IHC performed on cell sheets from two individual NHU cell cultures (Y1751 and Y1891) exposed to 7 days of hypoxia showed that there was reduced expression of claudin 3 and 4 (Figure 3.11 and Figure 3.12) upon removal from hypoxia. When the hypoxic sheet taken at day 7 was compared to the hypoxic exposed sheet at the end of the experiment there was increased expression of claudin 3 and 4. This was consistent between the two cell lines. There was a slight reduction in ZO1 at day 7 of hypoxic exposure and increased expression of ZO3 when compared to normoxic controls (Figure 3.13 and Figure 3.14). When the hypoxia-exposed sheet taken at the end of the experiment was compared with the hypoxia sheet taken at the time of removal from hypoxia there was more ZO1 and ZO3 expressed at the end of the experiment.

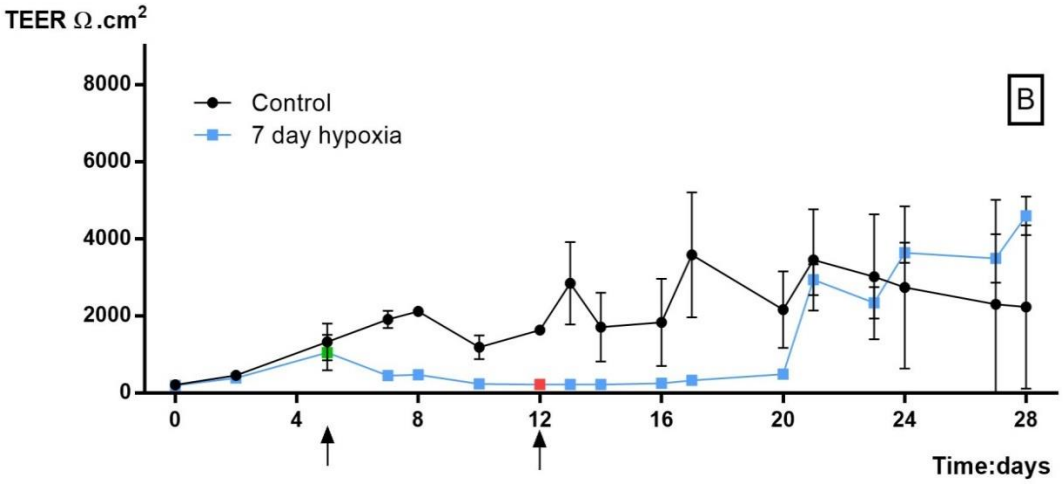
When the IHC images were presented to two blinded assessors, combined analysis showed that objectively there was less claudin 4 expressed after removal from hypoxia, but more ZO1 and ZO3. Claudin 3 was equivocal. However when the hypoxic sheet taken on day 7 was compared with the hypoxic exposed sheet at the end both assessors agreed that there was increased expression of all tight junction proteins (Figure 3.15 and Figure 3.16). This suggests that there could be upregulation of tight junction proteins after damage or downregulation during injury. There was consistency in the results of claudin 3 and ZO3 between the candidate and the observers however there was discrepancy between the results of ZO1 expression. The equivocal result of claudin 3 between blinded assessors highlights further inter-observer error.

TEER readings were assessed on the same day as sheets were taken for each cell culture line and normalised to control. There was a statistically significant difference on the day of removal from hypoxia (Figure 3.17; p=<0.001, t-test) however at the end of the experiment when the final sheets were taken and the TEER readings had returned there was no longer a statistically significant difference (Figure 3.17). There did however appear to be a slightly tighter barrier at the end of the experiment after hypoxic exposure for the two cell lines represented therefore further analysis and normalisation of data from four cell lines (Y1751, Y1891, Y1863 and Y1858) all exposed to 7 days of hypoxia was carried out. Grouped data from these cell lines showed there to be no difference in final TEER reading from control (Figure 3.18).

3.3.4.3 The association of hypoxia in benign bladder disease

Bladder biopsies taken from patients with stress urinary incontinence (SUI), overactive bladder (OAB) and neuropathic bladders (controls) showed labelling for HIF1α. There was some variability between individuals however 7 of the 8 SUI samples and 5 of the 8 OAB samples had nuclear labelling demonstrating the role of hypoxia in benign bladder pathologies (Figure 3.19).


TEER Ω.cm²
A
Control
7 Day hypoxia
8000
6000
4000
2000
0
0
4
8
12
16
20
Time:days


TEER Ω.cm²
B
Control
7 day hypoxia
8000
6000
4000
2000
0
0
4
8
12
16
20
24
28
Time:days

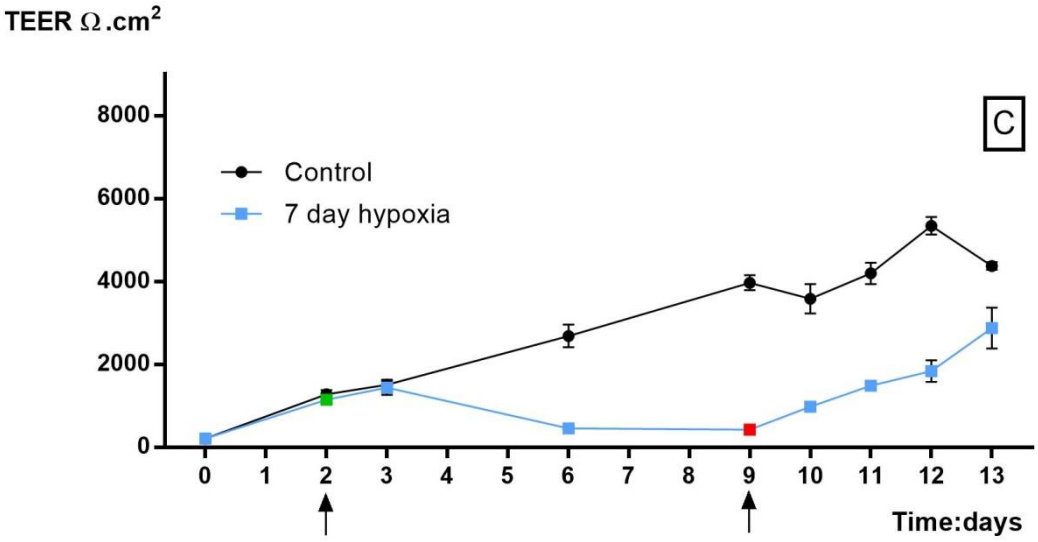


Figure 3.8: Barrier function of NHU cell cultures maintained in hypoxia for 7 days

Graphs A, B and C represent NHU cell lines Y1751, Y1863 and Y1858 respectively. For each cell line, replicate cultures were retained in normoxia (control) or placed in hypoxia once a mean barrier of 1000 Ω.cm² was achieved. Cultures remained in hypoxia for 7 days and were removed for TEER readings and medium changes. During the hypoxic insult the barrier dropped <600 Ω.cm² in all biological replicates, while the barrier of the control cultures continued to tighten. After removal from hypoxia the barrier repaired in all culture lines, time to restitution varied however this was at least four days giving a window for further experiments. N numbers for each biological replicates are shown in Table 3.1. The green point (arrowed) on each graph represents the day the cultures were placed into the hypoxic incubator and the red point (arrowed) indicates time of removal. Error bars = SD.

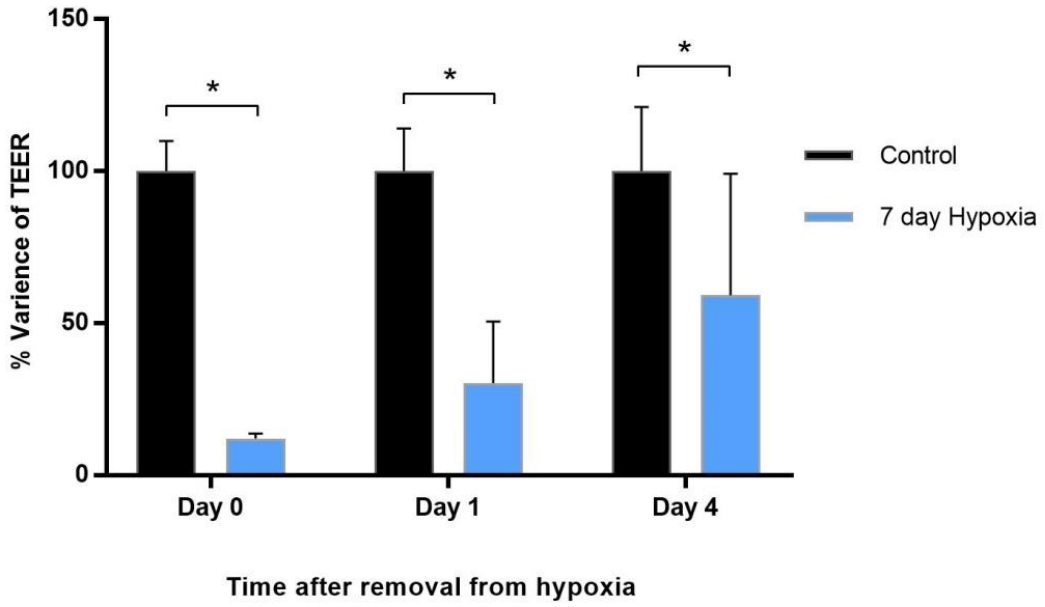


Figure 3.9: Combined normalised data for three cell lines after removal from hypoxia after 7 days of exposure.

This graph compares the combined normalised results for cell culture lines Y1751, Y1863 and Y1858 with control after removal from hypoxia. There remained a statistically significant difference at 4 days (p=0.0088, t-test). Control n = 11, hypoxia n = 9. Error bars= SD.

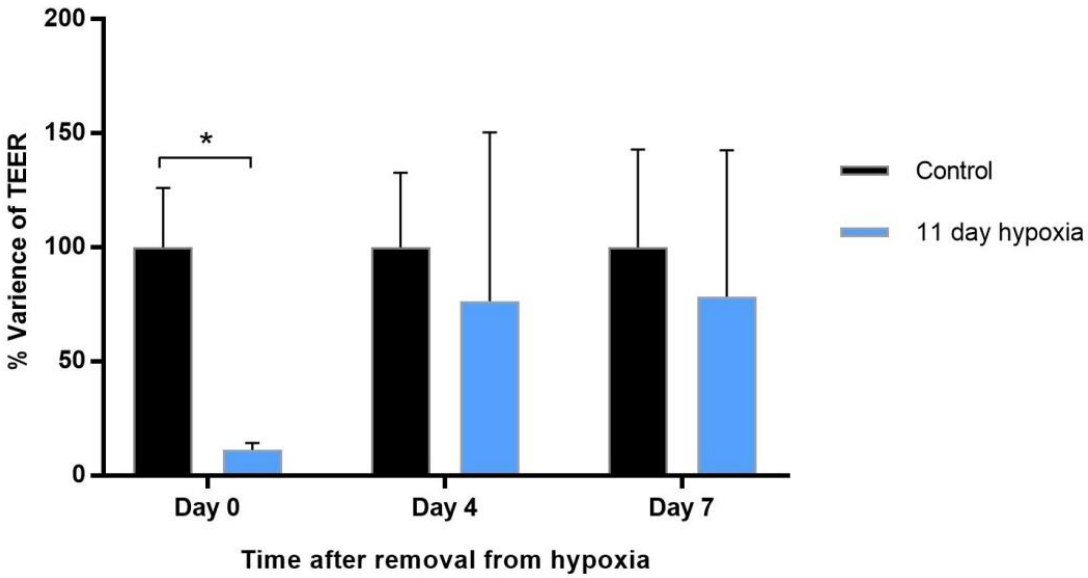


Figure 3.10: Combined normalised data for three cell lines after removal from hypoxia after 11 days of exposure.

Combined normalised data for cell lines Y1751, Y1863 and Y1913 showing that by day 4 after removal from hypoxia the barrier had returned and there was no difference to control cultures. There was a statistically significant difference the day of removal ($p=<0.001$, t-test). Control n= 11, hypoxia n = 10. Error bars = SD.

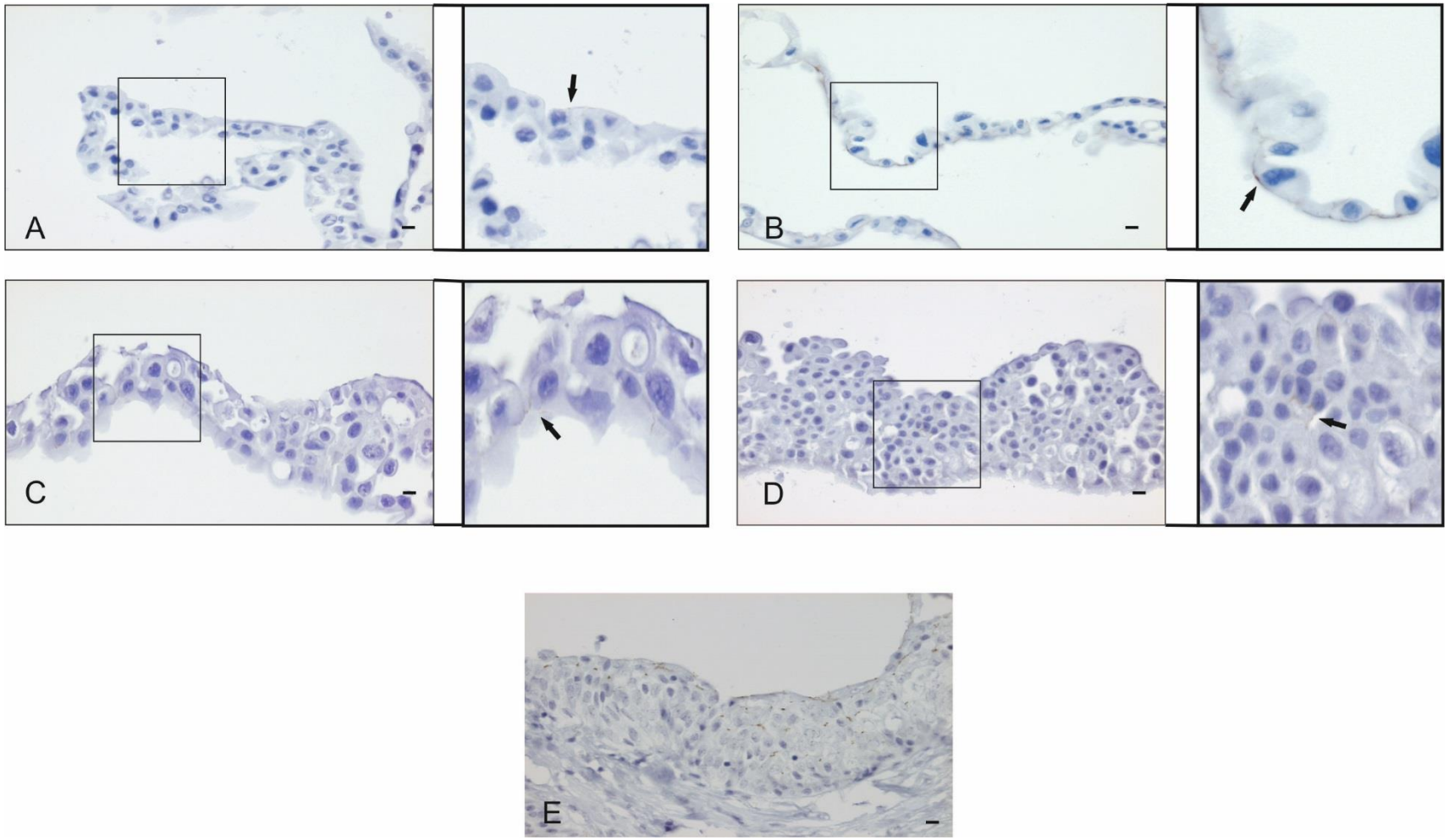


Figure 3.11: Claudin 3 expression on cell sheets with exposure to 7 days of hypoxia

Figure 3.11: Claudin 3 expression on cell sheets with exposure to 7 days of hypoxia

By IHC, Claudin 3 is present as a thin line between adjacent superficial urothelial cells. Image A is of the cells that were exposed to 7 days of hypoxia and were fixed on the day of removal. Image B is the same cell line after return to normoxia at the end of the experiment. C and D are the normoxic controls at each time point (day 7 and end of experiment). There appeared to be reduced expression of claudin 3 at day 7 of hypoxic exposure when compared to control. At the end of the experiment there was upregulation of claudin 3 (B) when compared to the matched hypoxic sheet at day 7 (A). Image E represents a ureter positive control. Scale bar = 10 µm, the image displayed to the right of each sheet in a thicker box shows an area of magnification.

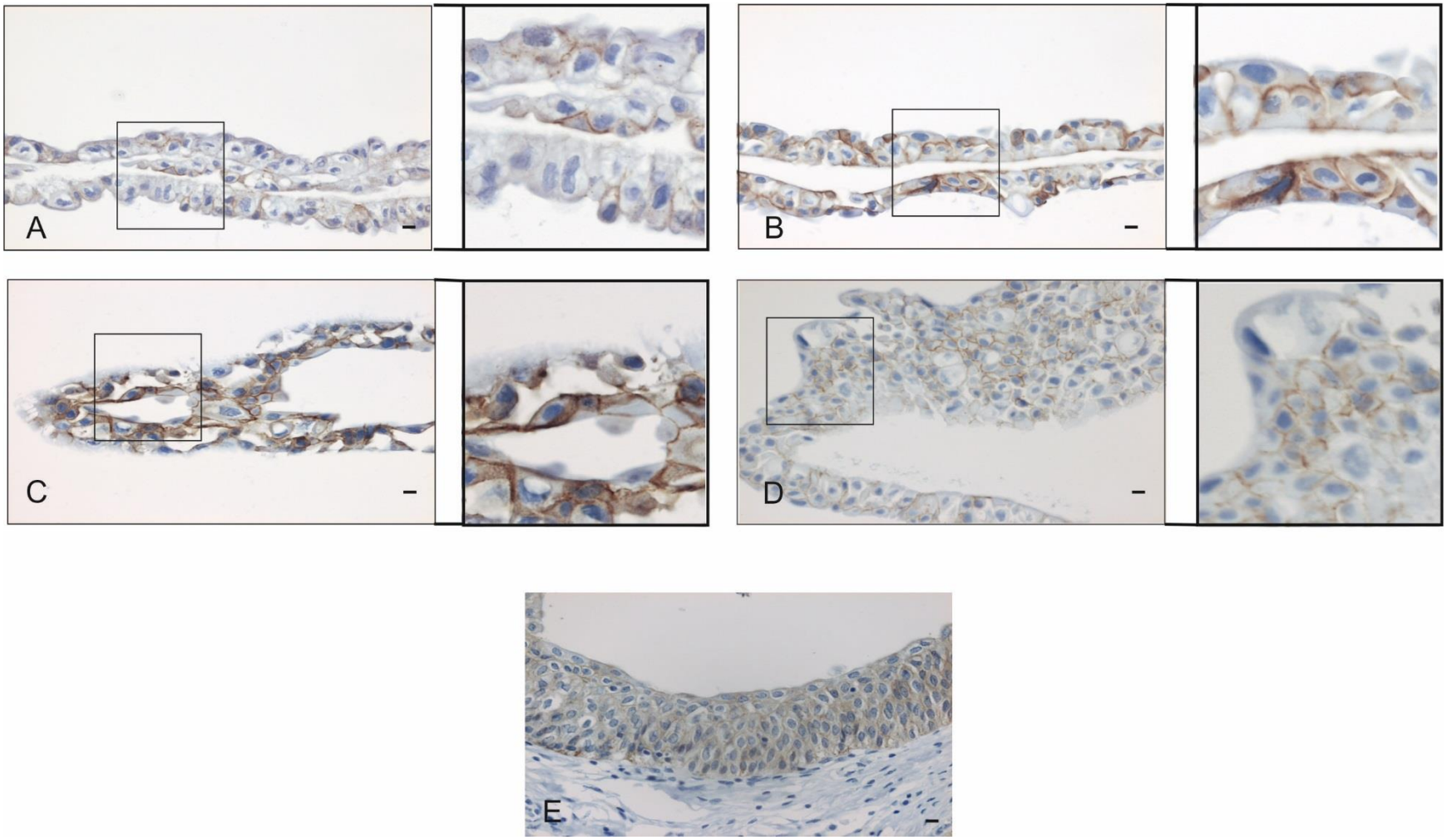


Figure 3.12: Claudin 4 expression on cell sheets with exposure to 7 days of hypoxia

Figure 3.12: Claudin 4 expression on cell sheets with exposure to 7 days of hypoxia

Claudin 4 expression is found at urothelial intercellular membranes. At 7 days of hypoxia (A) there was less claudin 4 expressed when compared to matched normoxic control (C). When returned to normoxia there was increased expression of claudin 4 (B) when compared to the matched day 7 hypoxic sheet (A). After return to normoxia the expression increased (B). Image E represents a ureter positive control. Scale bar = 10 µm, the image displayed to the right of each sheet in a thicker box shows an area of magnification.

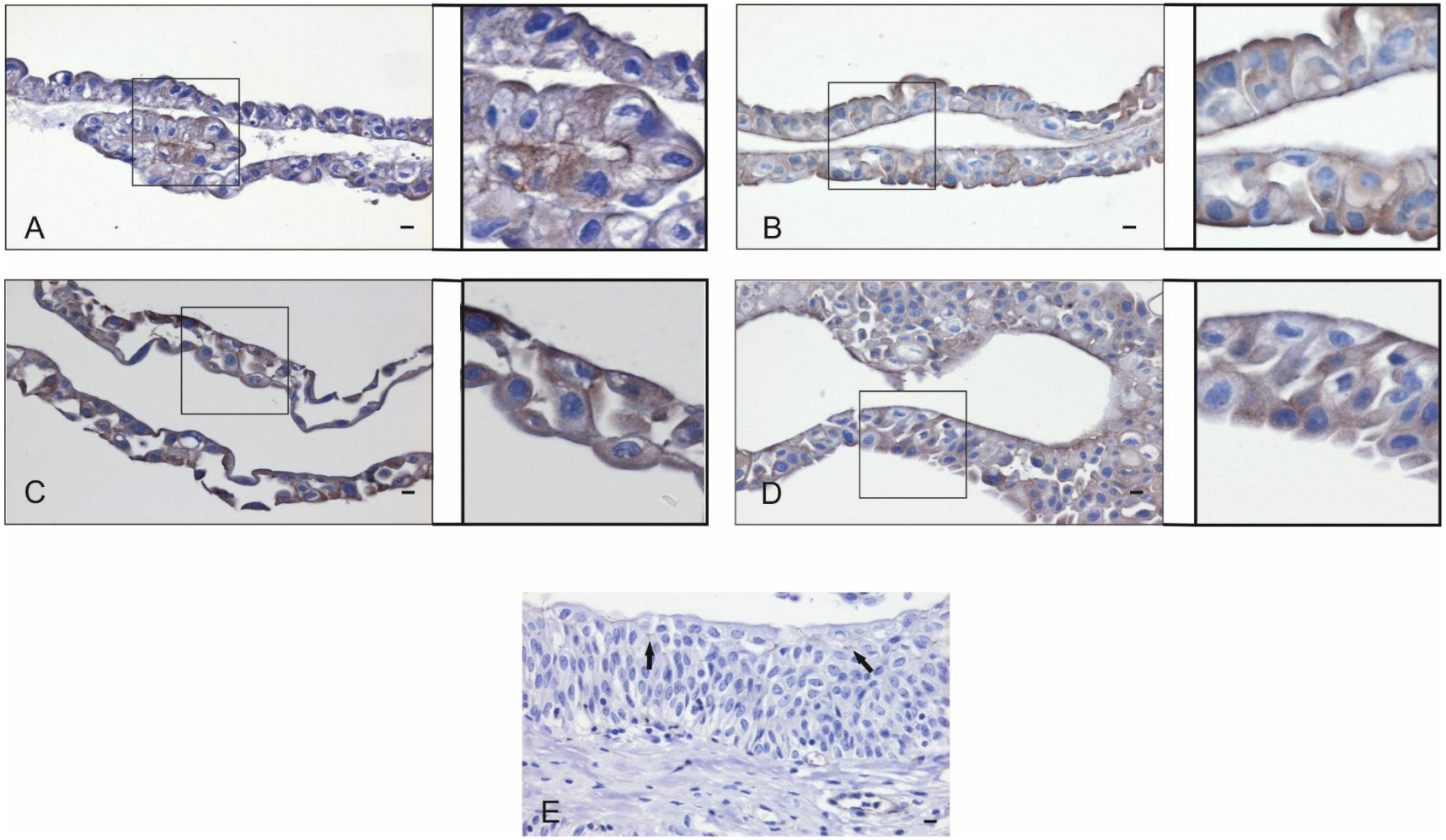


Figure 3.13: ZO1 expression on cell sheets with exposure to 7 days of hypoxia

Figure 3.13: ZO1 expression on cell sheets with exposure to 7 days of hypoxia
ZO1 expression on cell sheets was predominantly around the cell membranes with some cytoplasmic expression. There appeared to be slight reduction of expression between the 7 day hypoxic sheet (A) and the matched normoxic control (C). After return to normoxia at the end of the experiment (B) there did appear to be more intense labelling when compared to the day 7 hypoxic sheet (A). Image E represents a ureter positive control. Scale bar = 10 µm, the image displayed to the right of each sheet in a thicker box shows an area of magnification.

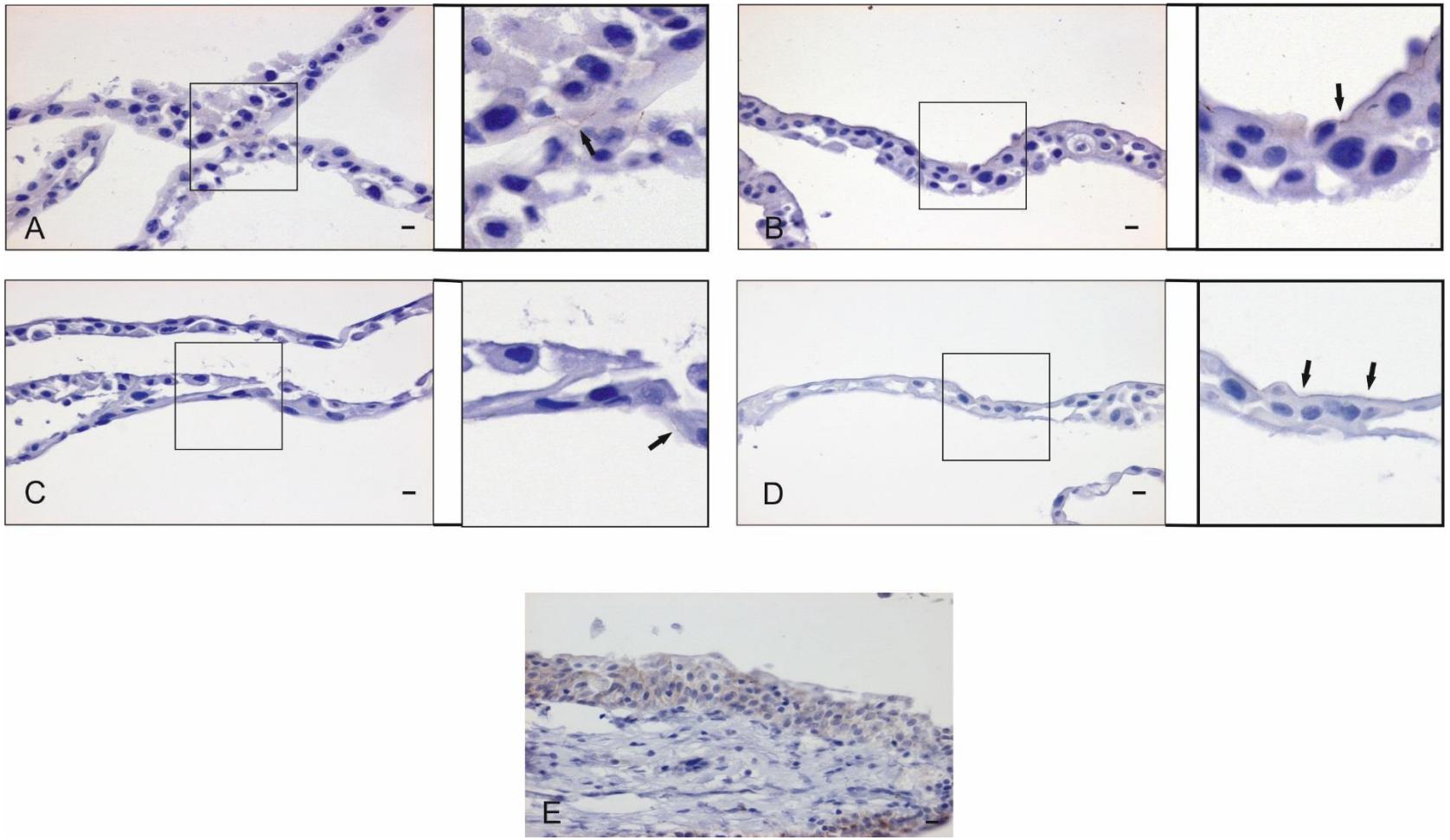


Figure 3.14: ZO3 expression on cell sheets with exposure to 7 days of hypoxia

Figure 3.14: ZO3 expression on cell sheets with exposure to 7 days of hypoxia

ZO3 expression on urothelial cell sheets is shown as a thin line between adjacent cells, with additional finger like projections away from the cells where adjacent cells meet. When comparing ZO3 expression after 7 days of hypoxic exposure (A) to matched normoxic control (C) there appeared to be more intense labelling of the hypoxic sheet. When comparing the 7 day hypoxic cells (A) to the matched hypoxic exposed cells taken at the end of the experiment (B) there appeared to be increased expression of ZO3 in the hypoxic exposed group. Image E represents a ureter positive control. Scale bar = 10 µm, the image displayed to the right of each sheet in a thicker box shows an area of magnification.

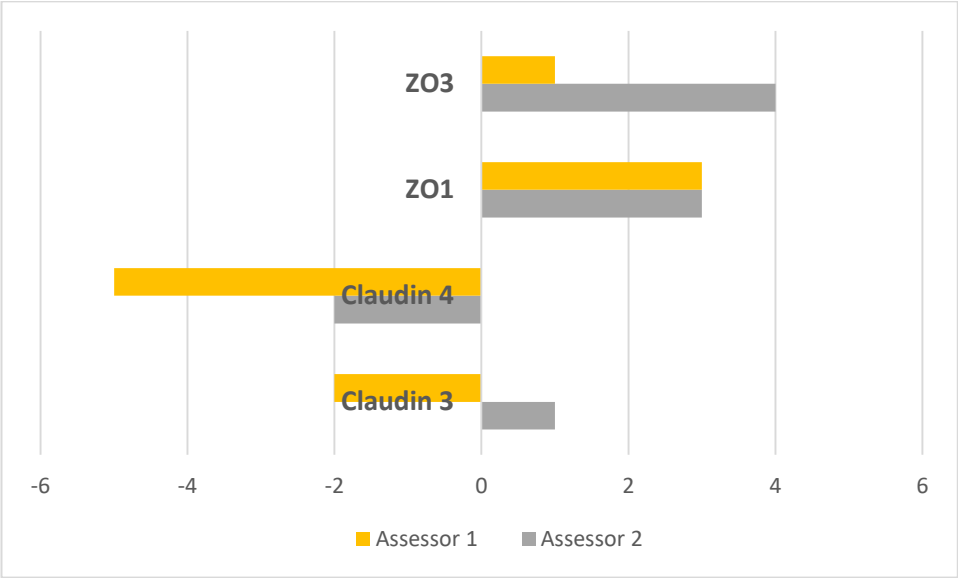


Figure 3.15: Scores from assessors on the labelling of tight junction proteins on the day of removal from hypoxia.

This chart shows the combined score from each assessor on expression of individual tight junction proteins. The expression of claudin 3, 4 and ZO1 and ZO3 on the sheet removed from hypoxia on day 7 was compared to its matched normoxic control taken on the same day. Each number represents the score of three images from each cell line (Y1751 and Y1891) combined. A negative score suggests that they thought there was less expression than control and a positive suggests there was more, zero is no difference. It can be seen that there was discrepancy with claudin 3 expression, however both thought there was less claudin 4 expression and more ZO1 and ZO3 of the 7 day hypoxic sheets.

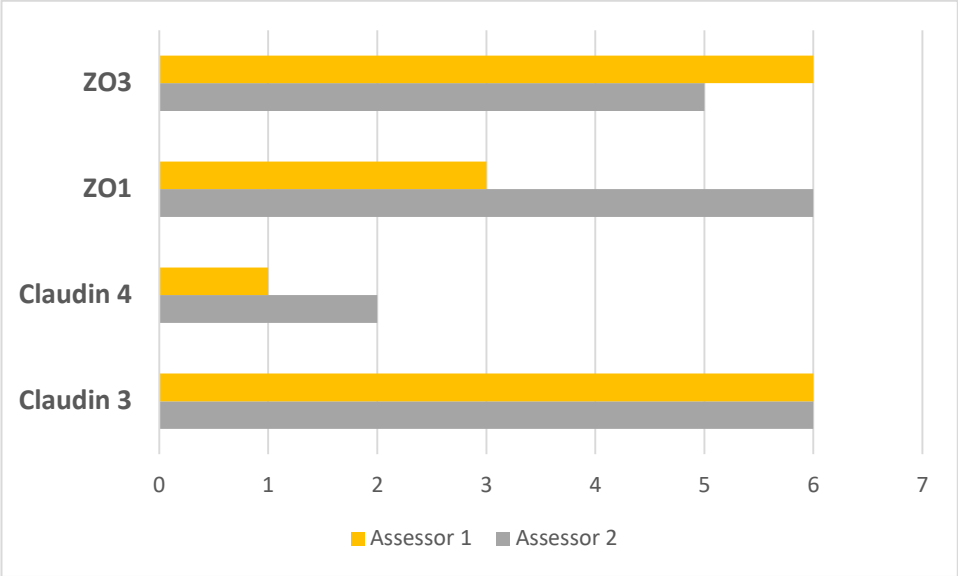


Figure 3.16: Scores from assessors on the labelling of tight junction proteins after hypoxic exposure and return to normoxia.

This graph represents the combined scores of each assessor when they compared the expression of tight junction proteins on cells that were exposed to hypoxia and returned to normoxia with the cells that were exposed and fixed on the day of removal from hypoxia (day 7). There was consistency between results with all samples showing there was a greater expression of the four tight junction proteins upon transfer to normoxia after hypoxic exposure. Each number represents the score of three images from each cell line (Y1751 and Y1891) combined.

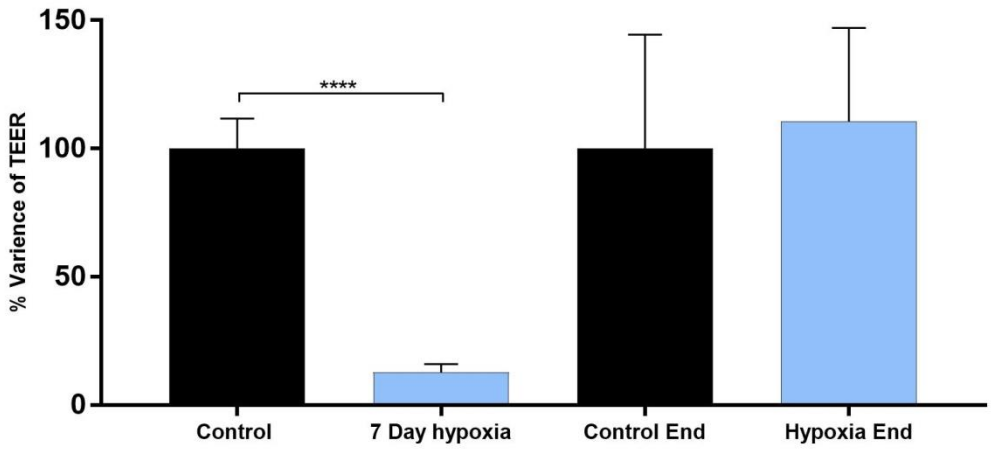


Figure 3.17: Normalised data showing percentage difference of TEER readings from normoxia control at 7 days of hypoxia and final readings for the two cell lines shown in IHC experiment.

This normalised data represents the percentage difference of TEER readings from normoxic control and hypoxic cell cultures when removed from hypoxia on day 7 and at the end of the experiment. The data represents the two cell lines that are shown in the previous cell sheets (Y1751 and Y1891). On the day of removal there was a statistically significant difference between control TEER and hypoxic TEER readings ($p=<0.001$, t-test). At the end of the experiment there was no difference between normoxic controls and hypoxic exposed groups, although the barrier appeared a little tighter in the hypoxic group. See Table 3.1 for number of replicates.

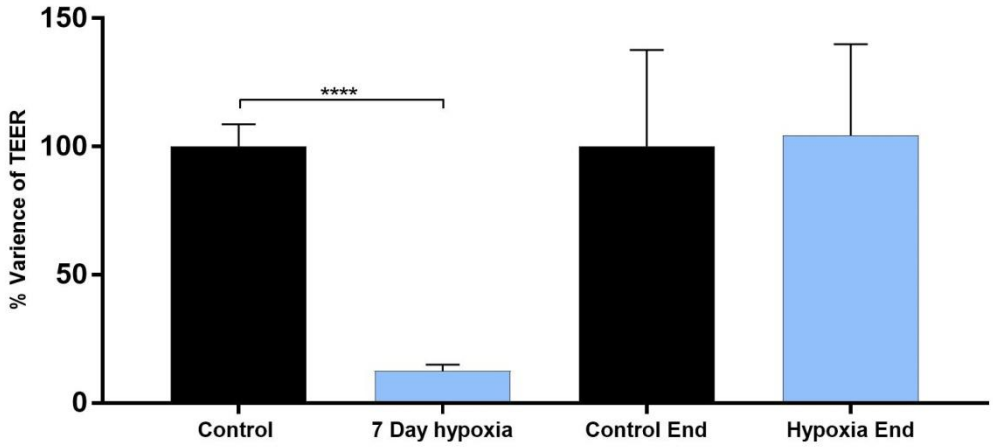


Figure 3.18: Normalised data combined for four cell lines demonstrating the difference between TEER after 7 days in hypoxia and following return to normoxia.

This combined normalised data represents four cell lines (Y1751, Y1891, Y1863 and Y1858). After 7 days of hypoxic exposure there was a statistically significant difference between normoxic control TEER and the hypoxia exposed groups (p=<0.001, t-test). After return to normoxia at the end of each experiment the TEER readings return to the same as normoxic controls. See Table 3.1 for number of replicates.

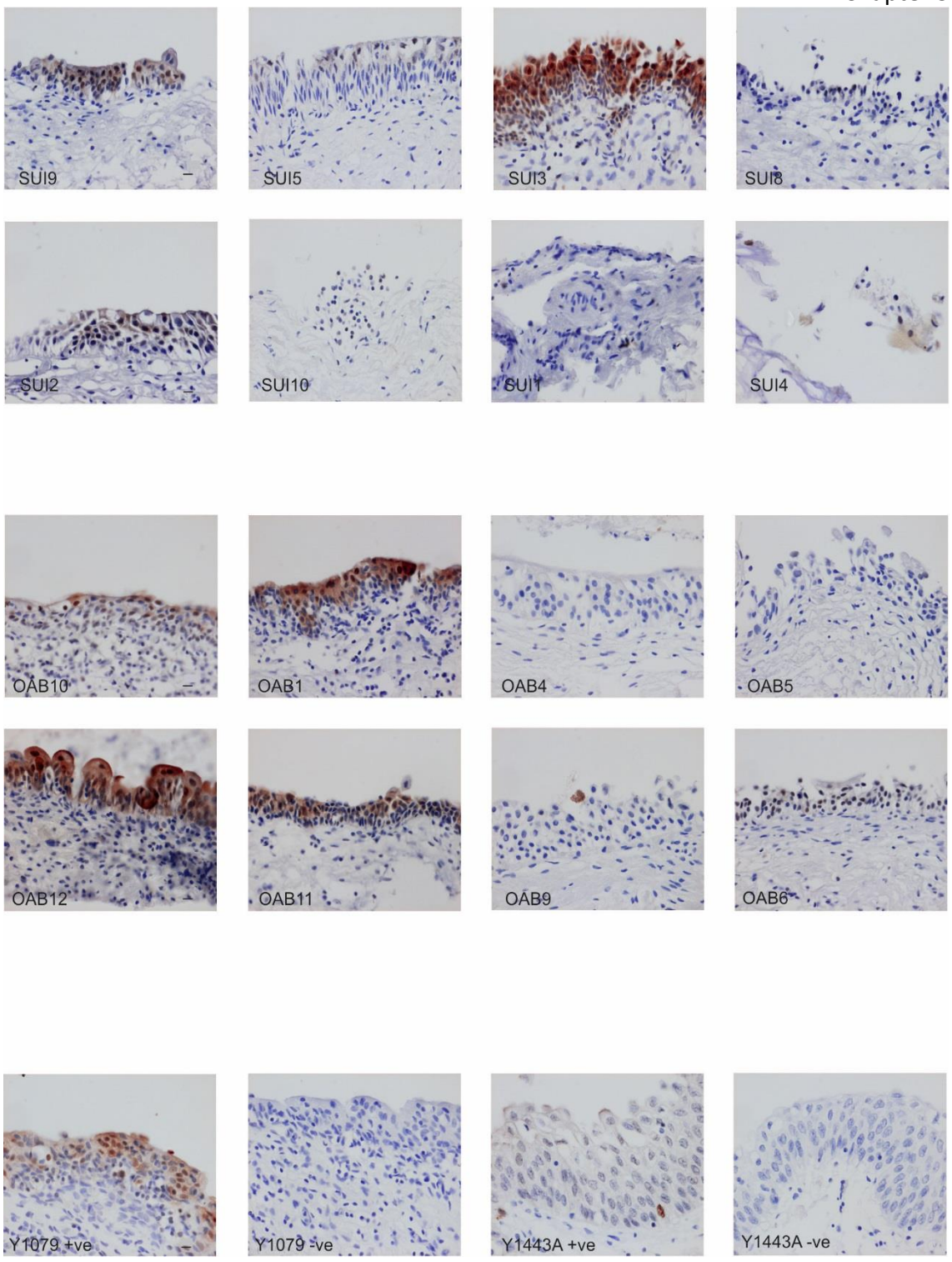


Figure 3.19: HIF1α labelling in benign bladder pathologies

8 SUI and 8 OAB samples demonstrating HIF1α labelling. Neuropathic bladders Y1079 and Y1443 acted as positive control tissue with known labelling of HIF1α. Negative controls were neuropathic bladder specimens with no primary antibody. Scale bar = 10 μm.

3.4 Chapter Summary

In assessing differentiated NHU cell cultures, barrier development, measured as a TEER >1000 Ω.cm² is achieved 2-5 days after the calcium concentration is increased. Maximum barrier is achieved between 7 and 15 days.

Scratch wounding gave a reproducible acute damage model however time to repair was short. By 28 hours cells had migrated across the wounds and combined time-lapse data showed that there was 95 % closure. TEER measurements showed there was still a statistically significant difference at 14 hours post scratch wound in barrier function when compared with pre-scratch readings, however by 22 hours the barrier had repaired, and there was no longer any statistically significant difference.

Ketamine did not give a reproducible model and large concentrations of ketamine were required to compromise the barrier. Once a barrier < 500 Ω.cm² was achieved the barrier was irreparable. High concentrations of ketamine were required to damage the cell cultures which subsequently lead to toxicity.

Hypoxia gave a reproducible damage model. Seven days of hypoxic exposure gave continued barrier depletion for 4 days post exposure, whereas with 11 days of hypoxic exposure the barrier repaired quicker. IHC showed that there is a reduction of claudin 4 with hypoxic exposure, and increased expression of claudin 3, 4, ZO1 and ZO3 post exposure. There was no difference in TEER (barrier function) post hypoxic exposure when compared to control. HIF1α is demonstrated in bladder biopsies from benign disorders demonstrating a potential link between what benign bladder disorders and hypoxia.

4 The effect of chondroitin sulphate on the urothelium

4.1 Aims & objectives

Chondroitin sulphate has been described as a major component of the GAG layer with a role in urinary barrier function. As discussed in Chapter 1, it is used to treat patients with BPS, radiation cystitis and recurrent UTIs, but with no clinical evidence or insight into scientific mechanism. The aim of this chapter was to examine possible modes of action. The objectives were to:

1. Examine the presence of CS on urothelium *in situ* by immunohistochemistry.
2. Assess the effect of exogenous CS on barrier formation in an *in vitro* urothelial barrier-forming cell culture system.
3. Determine if exogenous CS has an effect on barrier restitution, using hypoxia as a model of injury. Hypoxia as a model of injury gives a longer window to examine any treatment effect than scratch wounding.
4. Examine if therapeutic GAGs alter barrier formation.

4.2 Experimental approach

4.2.1 Presence of chondroitin sulphate

An in-house RNA-sequencing dataset representing the transcriptomes of freshly-isolated urothelium (n=3) and NHU cell cultures in non-differentiated (n=3) and differentiated states (n=3) were interrogated for expression of genes encoding enzymes involved in the biosynthesis of CS (Mikami and Kitagawa, 2013) (Figure 4.1).

To demonstrate the presence of CS as a component of the glycocalyx, fixed sections of human ureter and bladder were used for immunohistochemistry labelling with antibodies against CS epitopes (supplied by Prof Hughes, Cardiff University). Six antibodies were used: three bind to the pre-digested stub of the chondroitin sulphate chain (3B3, 2B6 and 1B5) and three bind directly onto the chain (7D4, 6C3, 4C3) as described earlier (section 2.7.4). 3B3 has a known mimitope at the end of the

chondroitin chain therefore sections were examined with and without chondroitinase ABC digest, to assess for specificity. Four ureters and one bladder sample were used. Positive control tissue was a paraffin block of chick embryo; this should label with all chondroitin sulphate antibodies. The chick tissue was provided by Prof Clare Hughes. For the antibodies which required a pre-digest to reveal the epitopes (3B3, 2B6 and 1B5), controls excluded the chondroitinase ABC digest step. Controls for antibodies that did not require a chondroitinase ABC digest (7D4, 6C3, 4C3), a digest was performed. A further methodology control included ZO1 antibody, a tight junction protein that is known to be present in the urothelium.

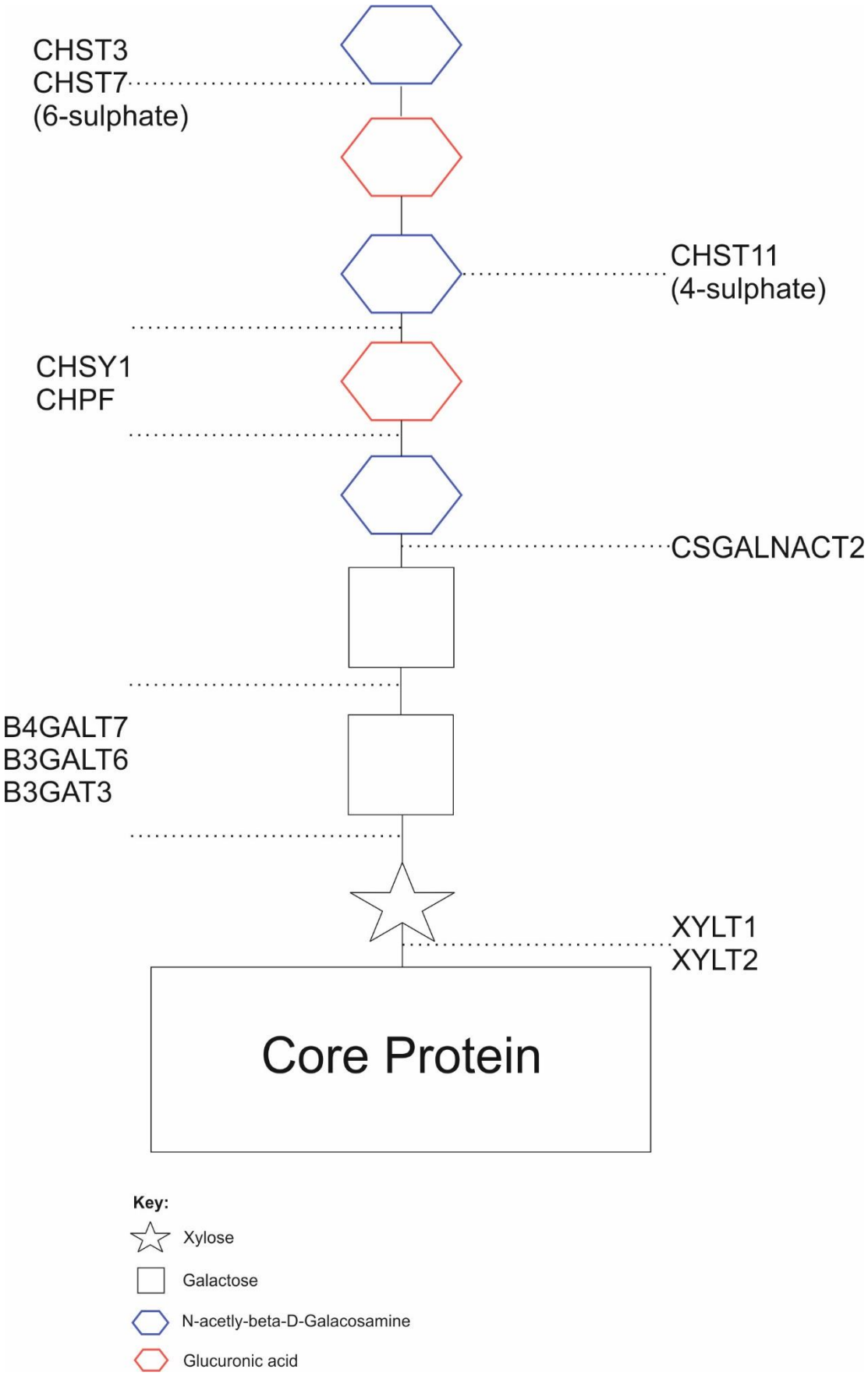


Figure 4.1: Genes that code enzymes that build chondroitin sulphate molecules (adapted from(Mikami and Kitagawa, 2013))**.**

4.2.2 Exogenous Chondroitin sulphate and barrier formation

Clinically it is difficult to estimate the concentration of CS applied to each cm^2 of bladder during treatment as each individual's bladder will have a different capacity. Further, intravesical treatments are held for up to 2 hours rather than a chronic application which gives an added dilution effect by the continued production of urine.

Preliminary studies to assess viability and toxicity of NHU cells to CS were conducted using the Alamar blue assay, which determines changes in population biomass. Concentrations of 50 ng/ml, 100 ng/ml, 200 ng/ml and 400 ng/ml of chondroitin-4-sulphate were tested, with 8 experimental replicates. This concentration range was based on a study that had assessed the effect of heparan sulphates on fibroblast cells (Walkers, Turnbullson and Gallaghers, 1994). Once NHU cells were seeded, chondroitin-4-sulphate was added to the medium at the above concentrations and replenished every 2 days; the assay was read on day 0, 1, 3, 5 and 7. Control cells were maintained in KSFMc.

Exogenous CS was added to differentiating NHU cell cultures in order to assess any effect on barrier formation. The standard protocol (section 2.6.4) was followed, except that chondroitin sulphate was added to the medium at concentration 50 ng/ml during the ABS preconditioning stage and retained in all consequent steps. The concentration was extrapolated from published literature on the lowest dose of heparin sulphate required for effect on fibroblast cells (Walkers, Turnbullson and Gallaghers, 1994) and from the Alamar blue assay above. The medium containing CS was replenished every 2-3 days. Three cell lines were used each with three experimental replicates. TEER readings were taken 1-3 days apart. Controls were maintained in chondroitin sulphate free medium and underwent TEER and medium changes at the same time as the experimental arm.

4.2.3 Barrier restitution following hypoxic injury

To see if CS promoted repair of compromised urothelium, NHU cell cultures were differentiated onto permeable membranes (12-well Thincert™) (section 2.6.4). Once a mean barrier of 1000 $\Omega.cm^2$ was achieved, the cultures were placed into the hypoxic incubator for 7 days and removed only for medium changes and TEER monitoring

every 2-3 days. Day 7 cell cultures were removed from hypoxia and chondroitin 4 or 6 sulphate was added to the medium on both sides of the membrane and the cultures were placed into the normoxic incubator. A low and high concentration of chondroitin 4 sulphate was used (50 ng/ml and 500 ng/ml). Chondroitin-6-sulphate was added at a concentration of 50 ng/ml only. Serial TEER readings were taken over the subsequent days to assess recovery. Three cell lines were used with three experimental replicates in each group. There were two control groups which were not exposed to treatment with CS: one underwent hypoxic injury, and the other was maintained in normoxia throughout.

4.2.4 NHU cell culture response to clinical GAGs

To attempt to mimic the clinical application of GAGs into the bladder, differentiated NHU cell cultures on permeable membranes (12-well Thincert™) (section 2.6.4) were exposed to clinical GAGs. Prior to increasing the calcium concentration, the medium was aspirated from the apical surface only and cells were treated with iAluril (chondroitin sulphate and sodium hyaluronate), Hycyst (sodium hyaluronate) or Cystistat (sodium hyaluronate) (see appendix 7.3.3 for individual treatment composition). 500 µl of each treatment was added to the apical surface of each membrane and left for two hours. The treatments were removed and replaced with KSFMc medium supplemented with 2 mM Ca^{2+} and 5 % ABS. Barrier development was monitored by TEER readings every 1-3 days. One cell line underwent all the above treatments, while a further two cell lines underwent treatment with iAluril alone; in each experiment, 2-4 experimental replicates were included in each arm. Parallel control cultures underwent medium changes alone.

4.3 Results

4.3.1 Presence of chondroitin sulphate

RNA transcriptome data showed that there was a difference between proliferating, differentiated and *in situ* cell phenotypes. No gene had a higher Read per Kilobase of transcipt per million (Rpkm) in the *in situ* group. Statistical difference favouring the proliferating phenotype was shown with XYLT2, CSGALNACT2, CHSY1, CHST11 and CHST3. XYLT2 codes the enzyme that catalyses the addition of xylose to the core protein, CSGALNACT2 codes the enzyme that catalyses the addition of N-acetyl-beta-D-Galacosamine to the link region and CHSY1 codes the enzyme that builds the alternating disaccharide units. CHST11 and CHST3 code the enzymes that add sulphate at 4 and 6 region respectively. This demonstrates at the gene level each step of chondroitin sulphate molecule formation is represented by an in increase in Rpkm in the proliferating phenotype.

CHSY1 showed a statistically higher read in the *in situ* group than the differentiated, this gene codes the enzyme which builds the alternating disaccharide units. CHPF was the only gene that was statistically higher in the differentiated group than both the proliferating and *in situ* groups; this enzyme is also known to catalyse the addition of alternating disaccharide units to the chondroitin sulphate chain. It was expected that if chondroitin sulphate was a major component of the GAG layer then the Rpkm would be significantly higher in the differentiated and *in situ* phenotypes (Figure 4.2).

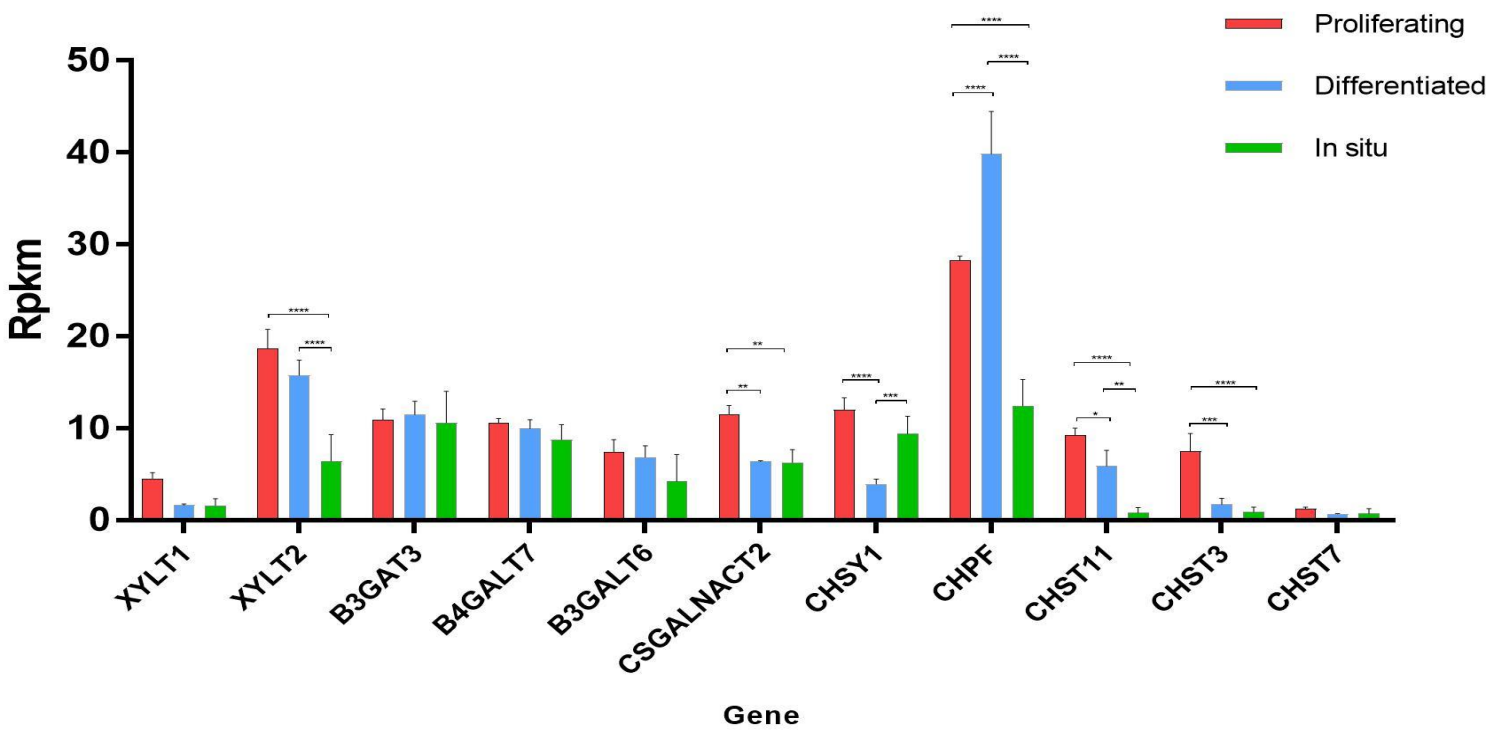


Figure 4.2: RNA transcriptome data for genes that code enzymes that build chondroitin sulphate.

This data shows the Reads Per Kilobase of transcript per Million (Rpkm) mapped reads for the expression of genes encoding enzymes that build CS molecules for three urothelial cell phenotypes: proliferating, differentiated and *in situ* (P0). For each group n=3 biological replicates. * $p<0.5$, ** $p<0.05$, *** $p<0.01$ and **** $p<0.001$ indicate statistical significance (two-way ANOVA, with Tukey's comparison).

4.3.2 Immunodetection of chondroitin sulphate

The chick embryo control tissue revealed appropriate labelling for each antibody. All chain antibodies (4C3, 6C3 and 7D4) labelled throughout the specimen, predominantly in the developing bones. After chondroitinase ABC digest two stub antibodies (2B6, 3B3) also labelled throughout the specimen, but predominantly within the developing bones. For consistency a single area on the tail was chosen to display in the figures. 1B5 antibody labelled only in the body of the chick embryo as shown Figure 4.3.

Methodological control specimens produced varied results. It was expected that the epitopes for antibodies 1B5 and 2B6 which require a chondroitinase ABC digest to be revealed would not show any labelling if the chondroitinase ABC step was omitted. However, 2B6 antibody without chondroitinase ABC digest intensely labelled the urothelium and to a lesser extent the stroma. The chick embryo showed some labelling on the outside of the edges of each vertebra with some further non-specific labelling within the soft tissue (Figure 4.4). This could represent a mimitope detected within the urothelium (Farrugia *et al.*, 2016) or an alternative explanation is that there is non-specific labelling of the urothelium which is not blocked by the IHC process. 1B5 did not label the specimen or the control tissue without chondroitinase ABC digest.

The methodological control specimens for 7D4 antibody with which the tissues underwent chondroitinase ABC digest also produced varied results. It was expected that the digest would remove all chondroitin chains and so 7D4 would not be labelled however, both ureter and the chick embryo labelled 7D4 at the same areas as the non-digested specimens (Figure 4.4). It is known that some of the antibodies have mimitopes the results from 7D4 would suggest that this is what is being observed. An alternative explanation would be that the chondroitinase had not completely digested the disaccharide chain however other controls produced expected reproducible results and the chondroitinase ABC digest was optimised during initial experiments.

In human urinary tract sections, CS was present in abundance within the stroma however there was no or equivocal labelling of the urothelium. Some specimens did label the urothelium however this was inconsistent and there were differences between biological replicates. When compared to methodical controls it is more likely

urothelial labelling was due to a non-specific antibody reaction. From previous research it was expected that a 'thick GAG layer' would be identified on the luminal surface of the urothelium, this was not identified with any of the CS antibodies.

3B3 antibody with chondroitinase ABC digest revealed dense labelling within the stroma and some labelling of the urothelium. Urothelial labelling was inconsistent between samples. Two donors (Y1817 and Y1851) appeared to have CS labelled on the surface but this was not uniform throughout the entire urothelial surface. Without chondroitinase digest there was minimal or no stromal labelling and weak labelling present throughout all layers of the urothelium. The urothelium labelled with varying intensity and no consistent pattern. Overall, these results suggest that the epitope to 3B3 antibody is found within the stroma (Figure 4.5).

2B6 antibody identified dense labelling within the stroma of all specimens. There is a suggestion of labelling of the urothelium on specimens Y1817, Y1851 and to a lesser extent Y1858. No labelling was confined to the luminal surface. Y1851 had more intense labelling on the urothelial surface in comparison to other specimens, but there was labelling present throughout all layers of the urothelium of that specimen (Figure 4.6). When comparing the urothelial labelling of the digested specimens to the non-digested control it is evident that there is less urothelial labelling when a digest is performed suggesting that the labelling of the urothelium in the digested samples is a non-specific occurrence.

There was no labelling with 1B5 antibody in any of the human urinary tract specimens. The chick embryo control specimen was positive in the region of interest (Figure 4.7).

7D4 was heavily labelled within the stroma on all ureter specimens and some labelling was seen within the stroma of the bladder sample. There was some labelling on the luminal surface of the urothelium on Y1817 and Y1851, which extended beyond the umbrella cells to the intermediate cells (Figure 4.8). Given control results it is likely the urothelial labelling is non-specific.

6C3 was not seen within urothelium and weakly labelled within the stroma (Figure 4.9).

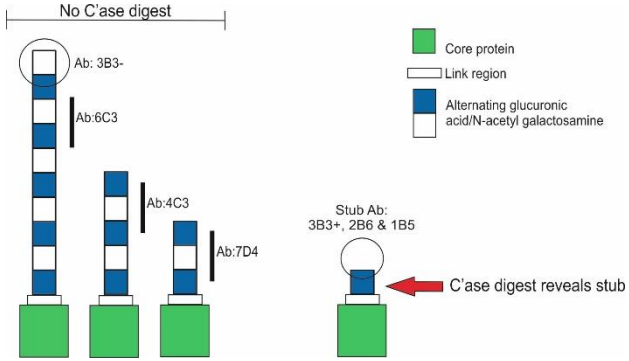
Labelling with 4C3 was varied between donors. Y1817 showed labelling on and between superficial urothelial cells; Y1851 revealed minimal labelling of the urothelium throughout superficial and intermediate layers and all revealed minimal labelling of the stroma (Figure 4.10).

A summary of findings is displayed in Table 4.1.

Table 4.1: Summary of Chondroitin sulphate labelling on Human Urinary Tract Specimens

Antibody	Con/Exp	Summary of findings:	Explanation:
3B3 +	Exp	Intense stromal labelling Inconsistent weak urothelial labelling	The antibody epitope revealed intense stromal labelling which was not present without the C'ase digest. Both +/- C'ase showed weak urothelial labelling, suggesting a non-specific reaction of the Ab to the urothelium.
3B3 -	Exp	Minimal or no stromal labelling Full thickness weak urothelial labelling but inconsistent between specimens	
2B6 +	Exp	Intense stromal labelling Weak urothelial labelling on 3/5 specimens throughout urothelium	Intense stromal labelling with C'ase digest suggests the epitope is present within the stroma. Without digest there was intense labelling of the urothelium and weak stromal labelling, which differed from the results with digest. This may suggest 2B6 – has a mimitope present but more likely represents non-specific labelling.
2B6 -	Con	Weak stromal labelling Intense urothelial labelling	
1B5 +	Exp	No stromal labelling No urothelial labelling	Not present within the stroma or urothelium.
1B5-	Con	No stromal labelling No urothelial labelling	
7D4 +	Con	Intense stromal labelling Weak urothelial labelling	Similar results with and without digest. May suggest there is a mimitope present which is being labelled.
7D4 -	Exp	Intense stromal labelling Weak urothelial labelling	
6C3 +	Con	No stromal labelling No urothelial labelling	Weak stromal labelling in the experimental arm, represents labelling of epitope.
6C3 -	Exp	Weak stromal labelling No urothelial labelling	
4C3 +	Con	No stromal labelling No urothelial labelling	Weak stromal labelling in the experimental arm, represents labelling of epitope.
4C3 -	Exp	Minimal or no stromal labelling Weak urothelial labelling	

Con = control, Exp = experimental



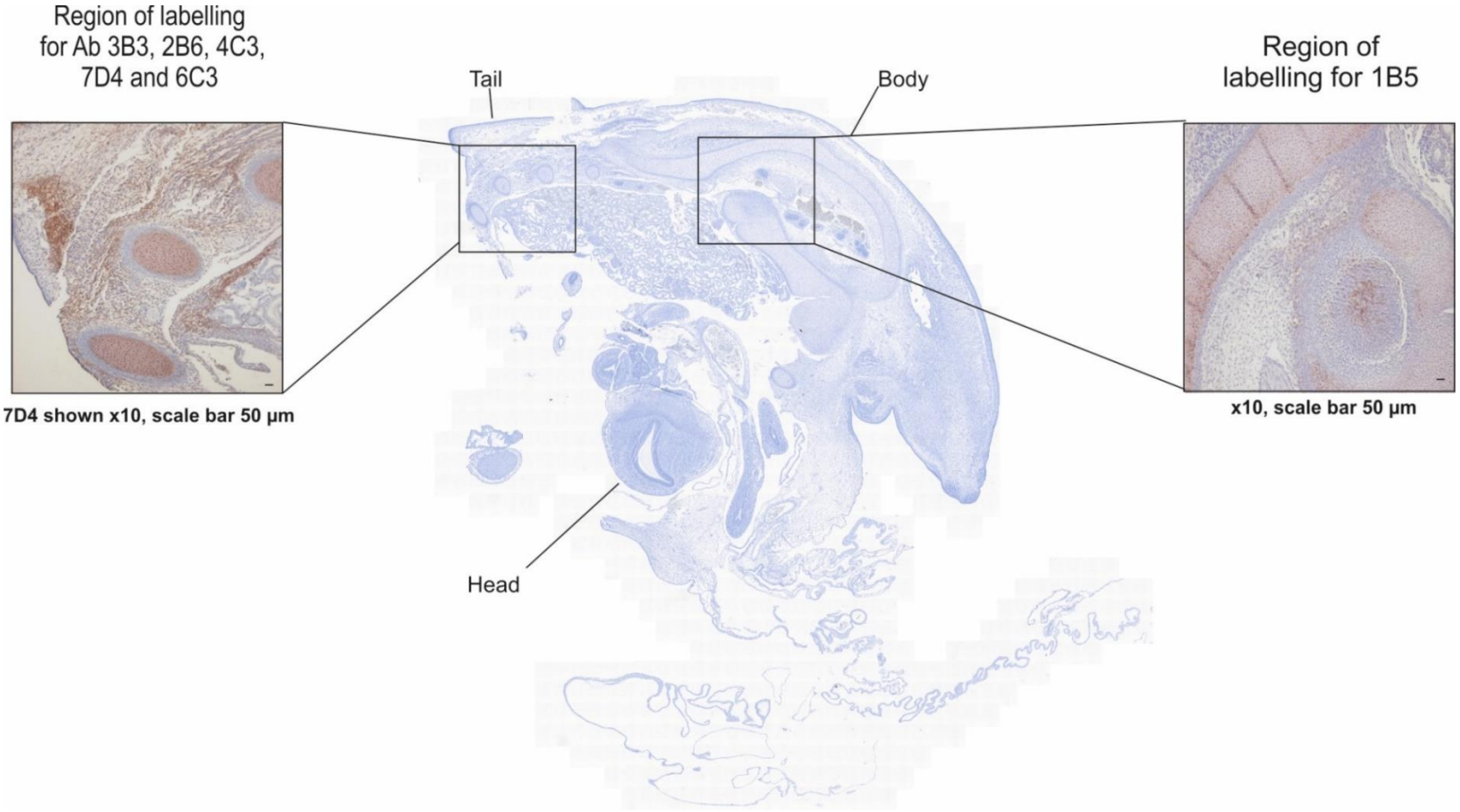


Figure 4.3: Chick embryo with areas of labelling for chondroitin sulphate antibody.

This diagram shows the full image of the chick embryo specimen with regions of interest for chondroitin antibody labelling. Antibodies to 7D4 and 1B5 are shown. Scale bar 50 µm.

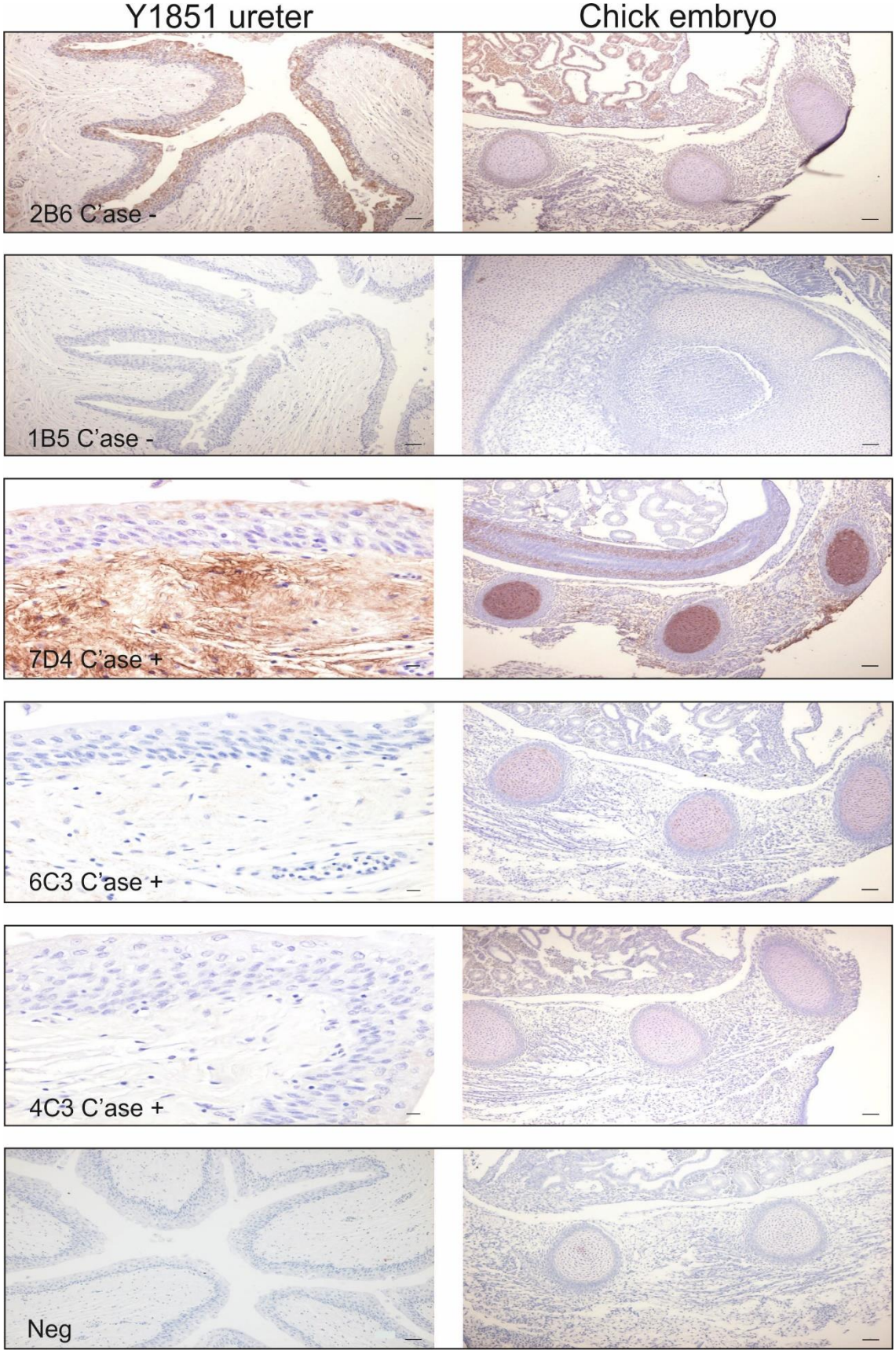


Figure 4.4: Control slides for immunodetection of chondroitin sulphate.

Figure 4.4: Control slides for immunodetection of chondroitin sulphate

2B6 and 1B5 are shown without chondroitinase ABC digest; these antibodies require this step to show the epitope. 7D4, 6C3 and 4C3 underwent chondroitinase digest and then subsequent labelling of epitope, with the digest step it is expected these antibodies will not label. The negative slides underwent chondroitinase digest but no primary antibody.

Unexpectedly, 2B6 antibody was labelled within the urothelium and stroma; further the chick embryo control tissue also showed some labelling of the outside edges of the vertebra and within the soft tissue. 7D4 specimens which underwent chondroitinase digest showed labelling on both urinary tract tissue and control tissue. This may represent a mimitope, but is more likely to represent non-specific labelling which failed to be blocked during the processing of specimens.

Ureter slides for 7D4, 6C3 and 4C3 have scale bar 10 µm, and the remainder of the slides have a 50 µm scale bar.

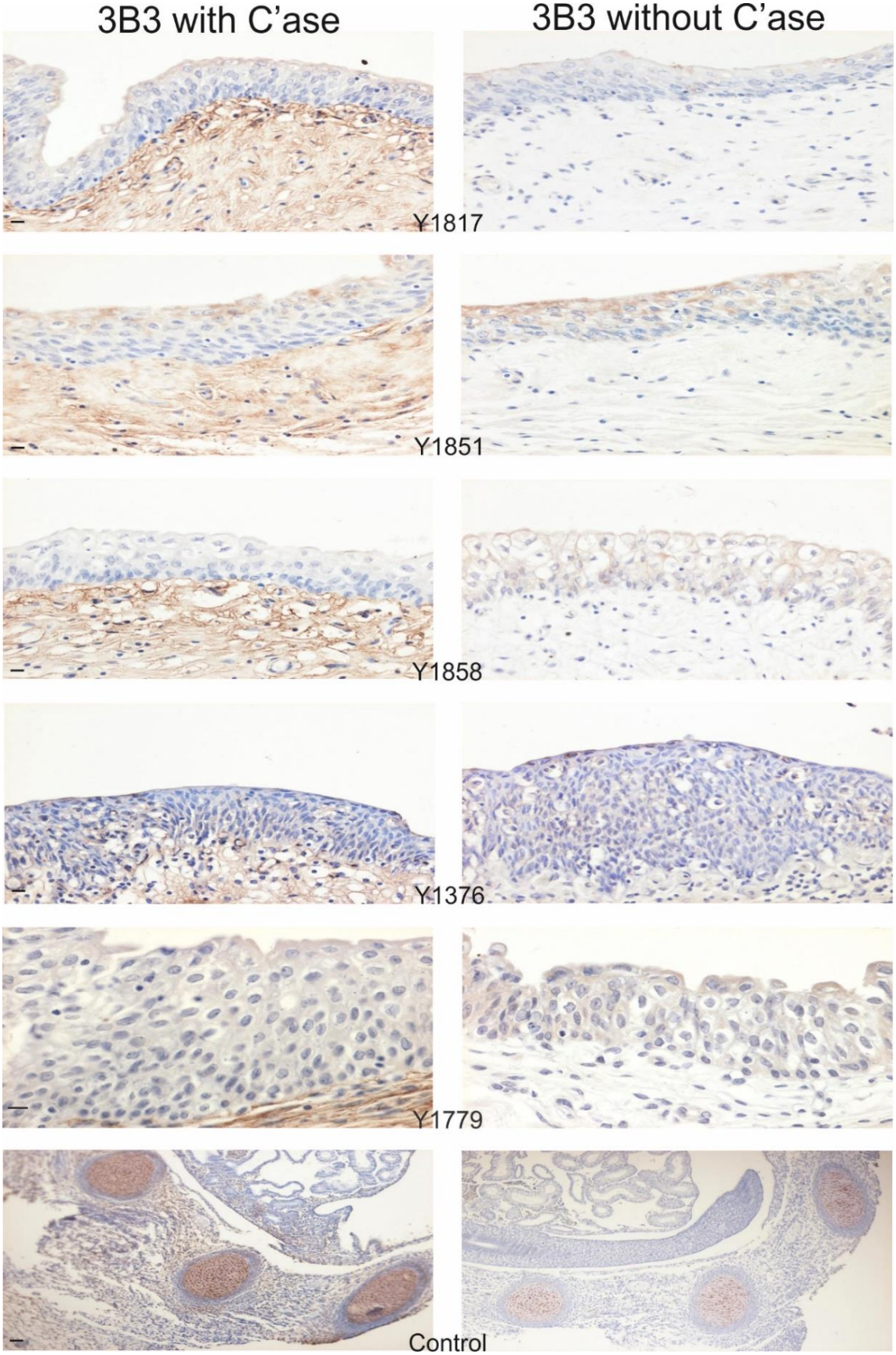


Figure 4.5: Labelling of 3B3 +/- chondroitinase ABC digest.

Figure 4.5: Labelling of 3B3 +/- chondroitinase ABC digest.

With chondroitinase digest there is dense labelling of the stroma in all human urinary tract specimens. Two donors (Y1817 and Y1851) did have diffuse labelling of the superficial urothelial cells; however this was not uniform throughout the entire specimen and there was labelling throughout the intermediate and basal layers. There was labelling of the urothelium in the remainder of the specimens however this was not as intense and spread throughout all layers. Without a chondroitinase digest the mimitope was labelled within the urothelium only. All layers of the urothelium were labelled with no dominance of the superficial cells. Given these findings the labelling of the urothelium of the digested specimens is likely to represent a mimitope or non-specific antibody labelling which was not blocked during processing. Y1817, Y1851, Y1858 and Y1779 are ureter specimens, Y1376 is bladder. Scale bar = 10 µm control tissue scale bar = 50 µm.

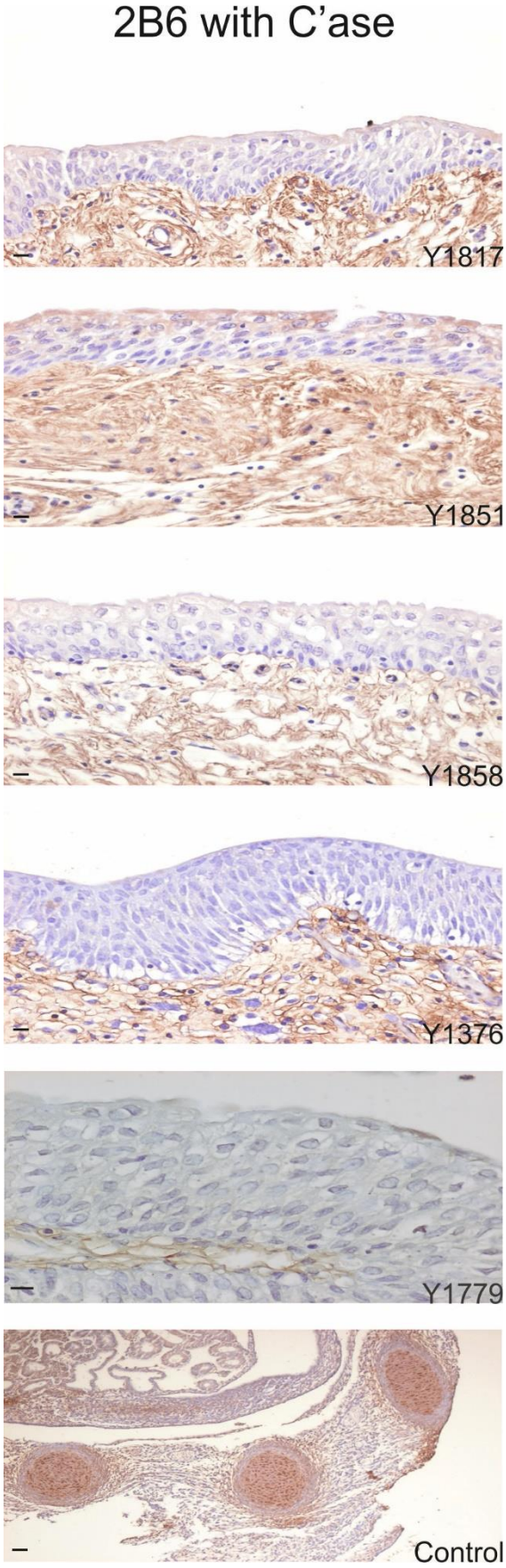


Figure 4.6: Labelling of 2B6 antibody with chondroitinase ABC digest.

Figure 4.6: Labelling of 2B6 antibody with chondroitinase ABC digest.

2B6 antibody revealed dense labelling within the stroma of all urinary tract specimens. There was some diffuse labelling of the urothelium on specimens Y1817, Y1851 and Y1858. This labelling was not confined to the superficial urothelial cells. Scale bar = 10 µm for urinary tract specimens, control tissue scale bar = 50 µm.

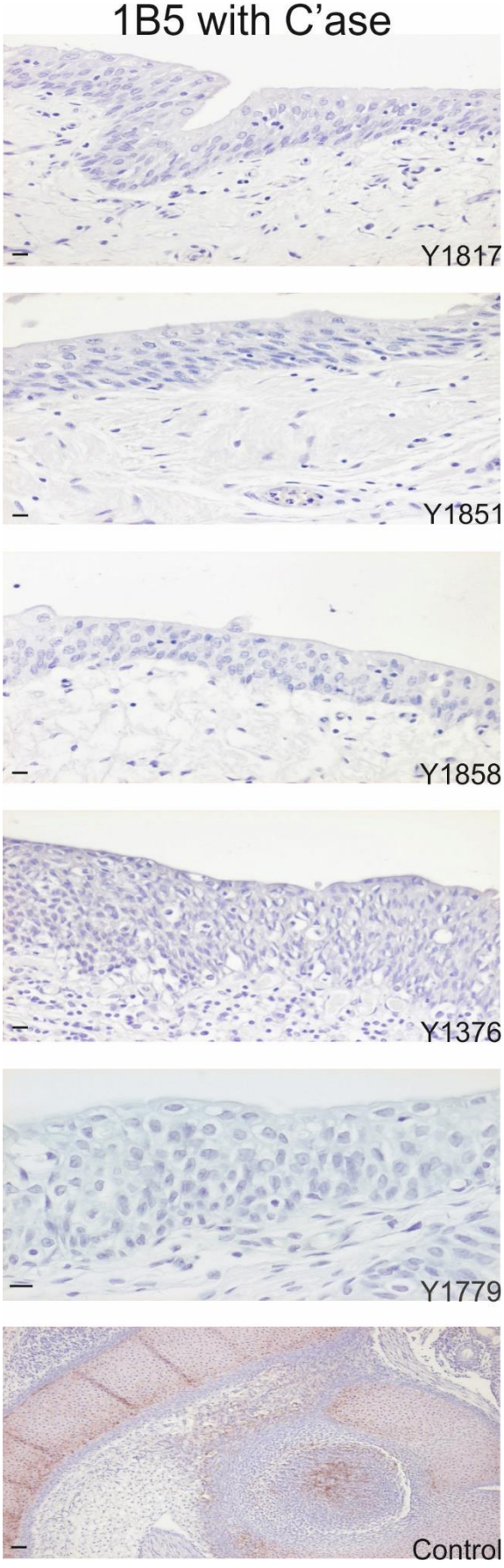


Figure 4.7: Labelling of 1B5 with chondroitinase ABC digest.

Figure 4.7: Labelling of 1B5 with chondroitinase ABC digest.

There was no labelling of 1B5 antibody on any of the human urinary tract specimens. Scale bar = 10 μm for the urinary tract specimens, control tissue scale bar = 50 μm.

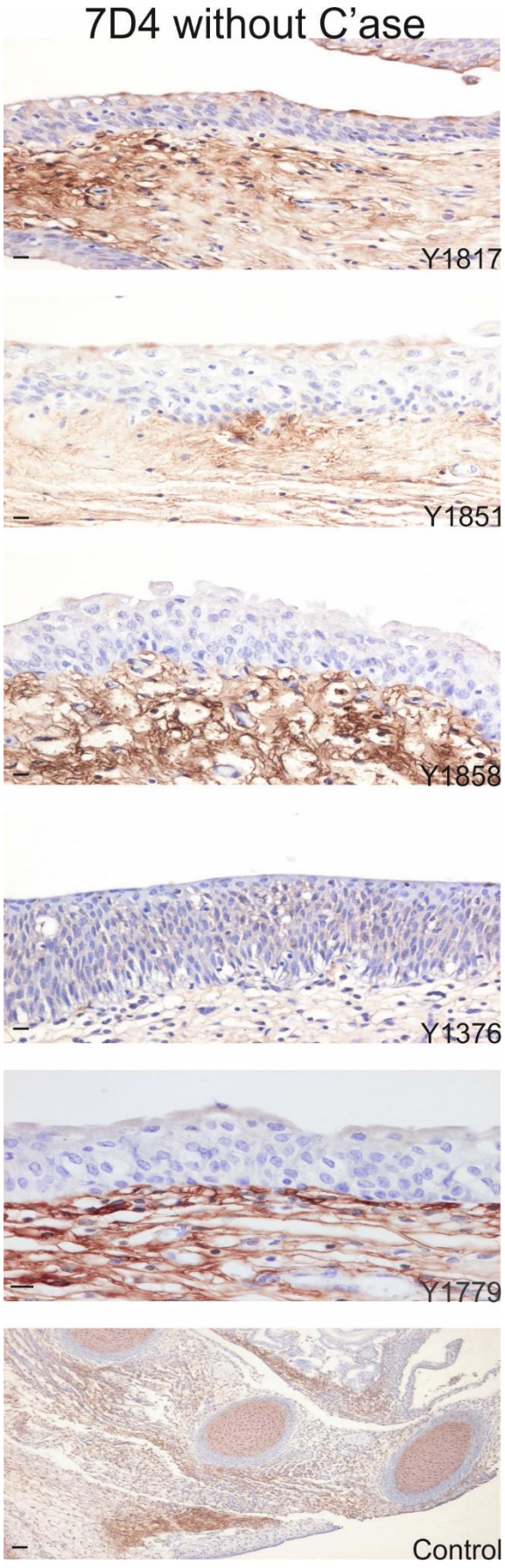


Figure 4.8: Labelling of 7D4 without chondroitinase ABC digest.

Figure 4.8: Labelling of 7D4 without chondroitinase ABC digest.

7D4 chondroitin sulphate chain antibody revealed labelling of the stroma in all urinary tract specimens. There was some labelling of the superficial urothelial cells of specimens Y1817 and Y1851 which extended into the intermediate layer. The remaining urinary tract specimens showed some labelling throughout the all urothelial layers but this was not as dense. Scale bar for urinary tract specimens = 10 μm, control tissue scale bar = 50 μm.

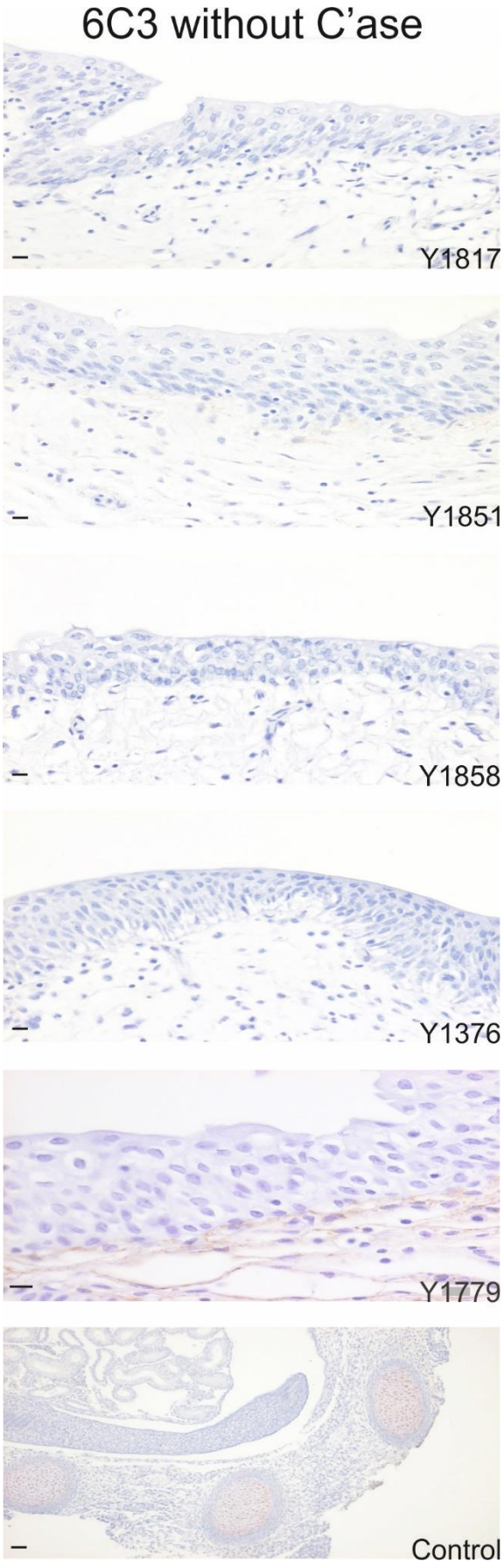


Figure 4.9: Labelling of 6C3 without chondroitinase ABC digest.

Figure 4.9: Labelling of 6C3 without chondroitinase ABC digest.

6C3 chondroitin sulphate chain antibody was not labelled within the urothelium and there was on only mild labelling of the stroma. Human urinary tract specimens scale bar = 10 μm, control tissue scale bar = 50 μm.

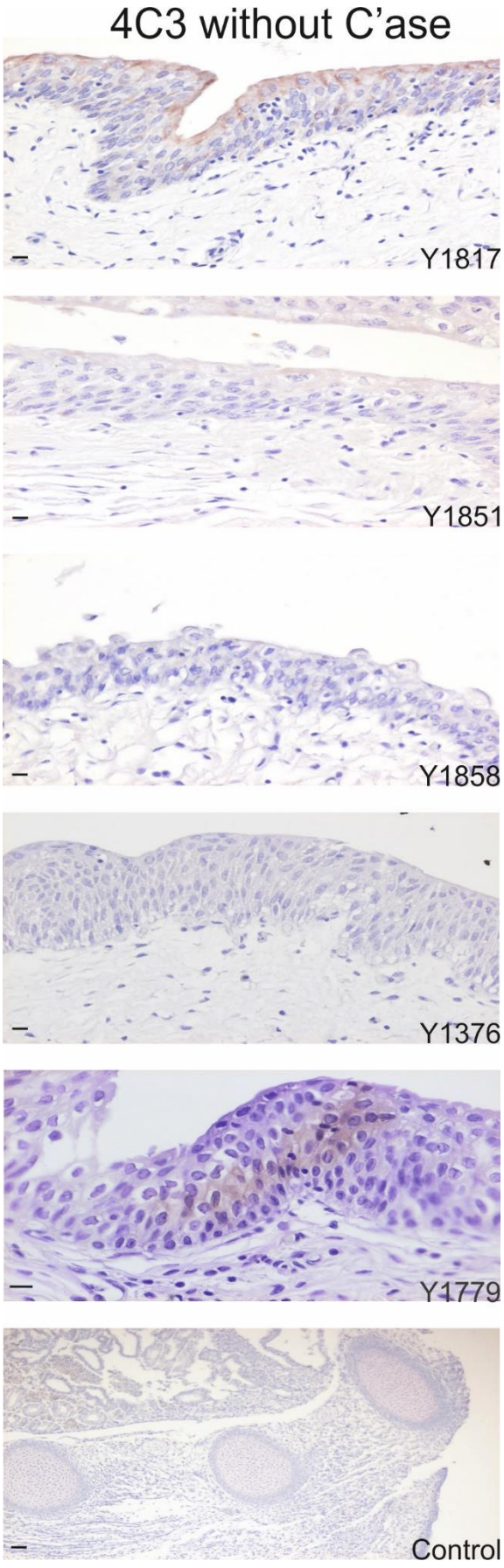


Figure 4.10: Labelling of 4C3 without chondroitinase ABC digest.

Figure 4.10: Labelling of 4C3 without chondroitinase ABC digest.

4C3 chondroitin sulphate chain antibody labelling varied. There was labelling of the urothelial superficial cells on specimen Y1817 and to a lesser extent Y1851 however this extended beyond the superficial cells to the intermediate and basal layers. All human urinary tract specimens revealed minimal labelling of the stroma. Human urinary tract specimens scale bar = 10 µm, control tissue scale bar = 50 µm.

4.3.3 The effect of exogenous Chondroitin Sulphate on barrier formation

Alamar blue assay for assessment of toxicity and dose assessment showed there to be no toxic effects of adding chondroitin-4-sulphate to proliferating cells (Figure 4.11). There was a statistically significant difference in the reduction of Alamar Blue with 400 ng/ml of chondroitin-4-sulphate on day 5 however by day 7 there was no difference ($p=0.0093$, one-way ANVOVA with Dunnett's comparison) (Figure 4.12). After 7 days when compared to non-treated controls, cells treated with chondroitin-4-sulphate were equally metabolically active suggesting no toxic side effects.

For barrier studies with chondroitin 4 and 6 sulphate treatment (50 ng/ml) a tight barrier was formed in each donor cell line (Y1790, Y1751 and Y1837) (individual results shown in Appendix 7.4). Normalised grouped data combining the three cell lines on day 3 showed that there was a significant difference between the barrier of the control (untreated) cultures and chondroitin 6-sulphate ($p=0.0044$, one-way ANOVA with Tukey's comparison) which indicated that chondroitin-6-sulphate promoted barrier formation (Figure 4.13).

When maximum barrier attained was assessed with normalised grouped data there was a significant difference between chondroitin-4-sulphate and control and chondroitin-6-sulphate and control ($p=0.0087$ and $p=0.0403$ respectively, one-way ANOVA with Tukey's comparison) (Figure 4.14). This demonstrates that with the addition of chondroitin sulphate the overall maximum barrier attained was higher; although by the end of the experiment (between days 9-10) there was no longer a significant difference apparent between chondroitin-6-sulphate and control. There was still a significant difference between chondroitin 4-sulphate and control ($p=0.004$, one-way ANOVA with Tukey's comparison) (Figure 4.15).

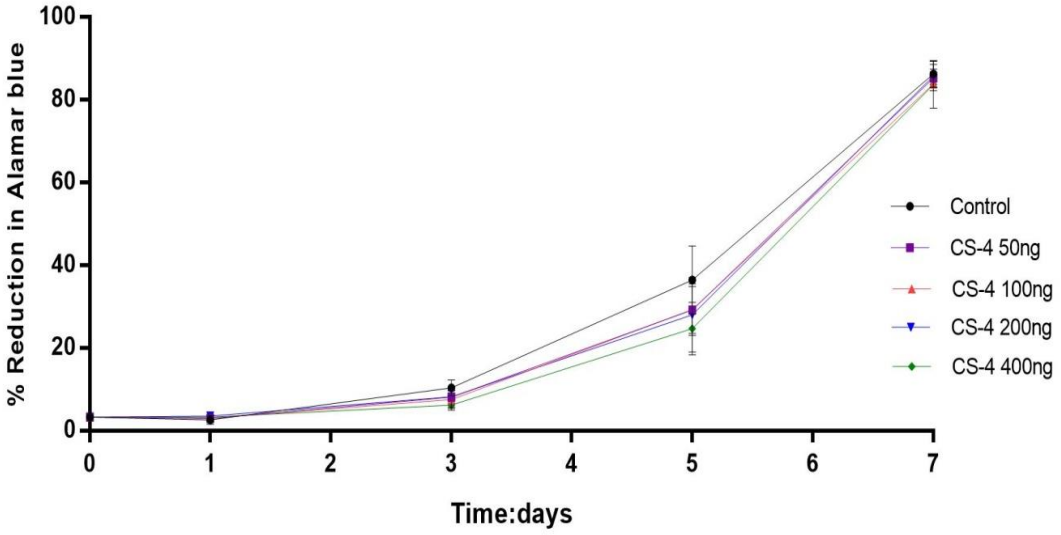


Figure 4.11: Alamar Blue assay for chondroitin-4-sulphate.

This assay demonstrates that there were no toxic effects of adding chondroitin-4-sulphate to proliferating cells. Cells remained metabolically active throughout chondroitin exposure. On day 5 there appeared to be a difference between control and cells exposed to CS-4 500 ng/ml therefore data, which was statistically significant (Figure 4.12, below). On day 7 there was no longer a difference between groups. N=8 in each group, error bars =SD.

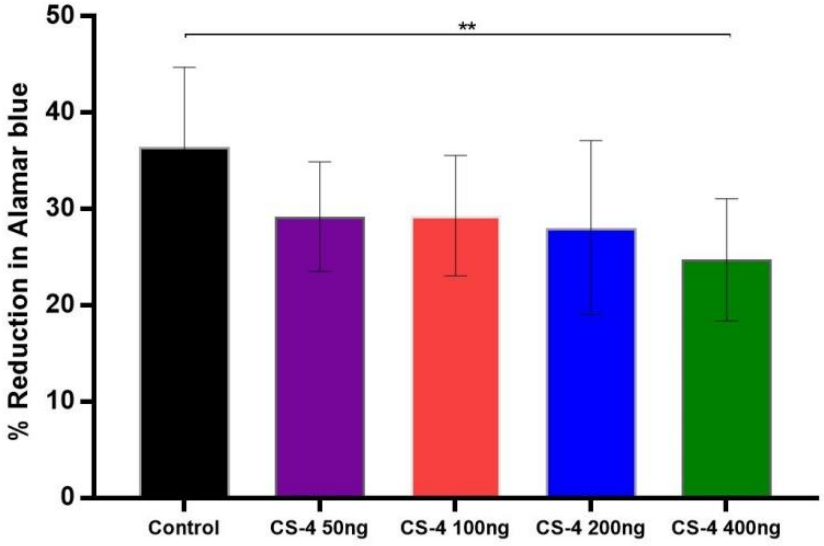


Figure 4.12: Alamar blue assay on day 5 of exposure to chondroitin sulphate. After 5 days of exposure to CS-4 at a concentration of 400 ng/ml there was a statistically significant difference in metabolic activity to control (p=0.0093, one-way ANOVA, Dunnett's comparison). N= 8 in each group, error bars=SD.

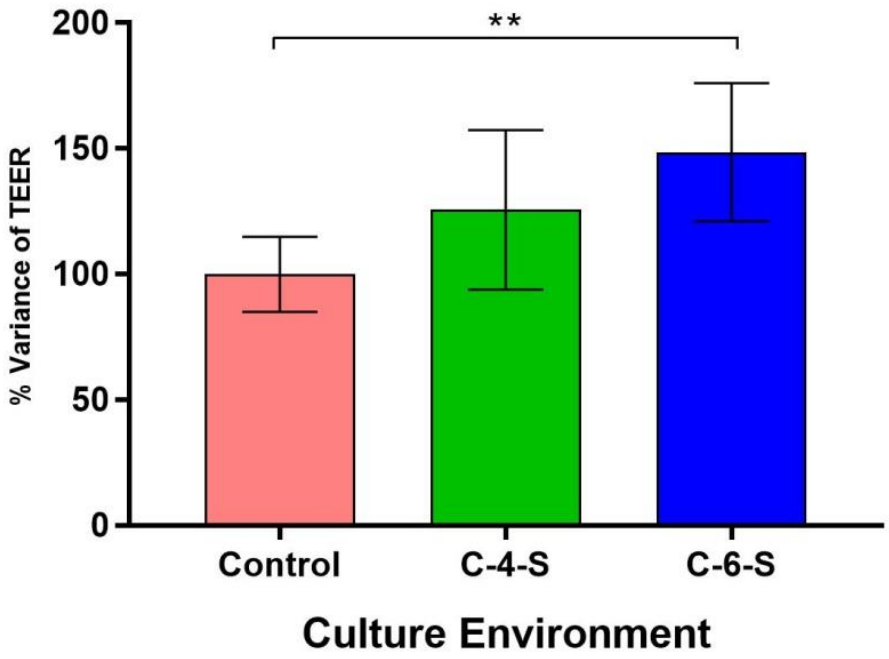


Figure 4.13: Day 3 TEER percentage variance from control.

Normalised combined data for cell lines Y1790, Y1751 and Y1837 showing the effect of the chronic application of chondroitin-4 and 6-sulphate (50 ng/ml) on NHU cell cultures during barrier development. In the chondroitin sulphate treated groups there was a higher overall mean barrier. There was a statistically significant difference between the tightness of the barrier between the control and chondroitin-6-sulphate group, (p=0.0044) one-way ANOVA with Tukey's comparison for analysis. N=9 for control and C-4-S and n=6 for C-6-S. Error bars = SD.

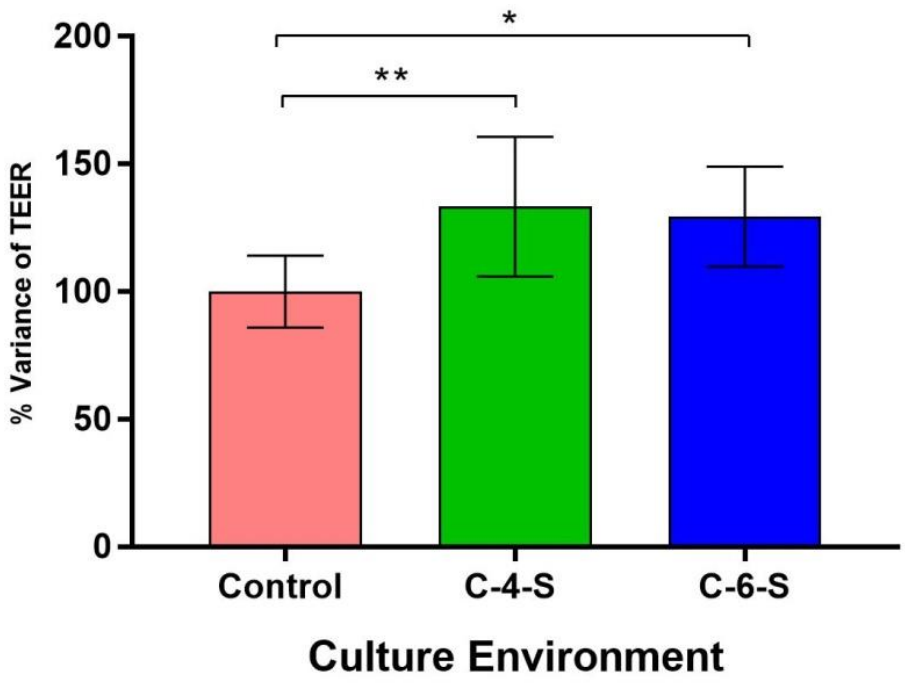


Figure 4.14: Maximum TEER percentage variance from control.

Normalised combined data for cell lines Y1790, Y1751 and Y1837 showing the percentage difference in maximum barrier achieved between control cultures and those treated with chondroitin sulphate. There was a significant difference between the maximum barrier of chondroitin-4-sulphate and control (p=0.0087, one-way ANOVA with Tukey's comparison) and maximum barrier of chondroitin-6-sulphate and control (p=0.0403, one-way ANOVA with Tukey's comparison). There was no difference between the CS groups. N=9 for control and C-4-S and n=6 for C-6-S. Error bars = SD.

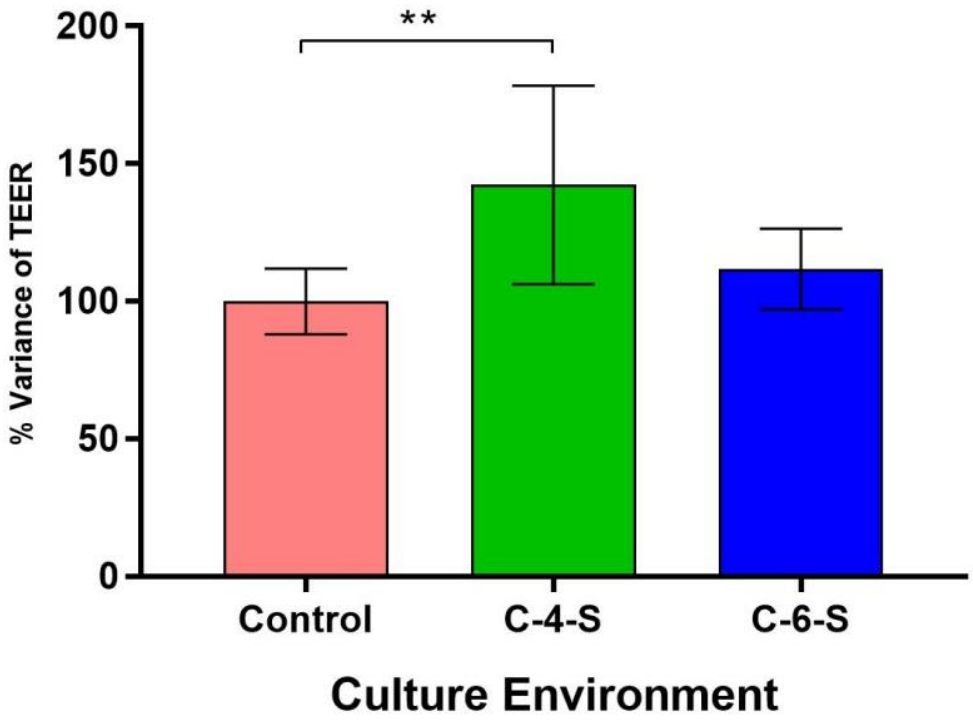


Figure 4.15: End of experiment TEER readings percentage variance from control. Normalised combined data for cell lines Y1790, Y1751 and Y1837. Chondroitin 4-Suphate had the highest TEER at the end of the experiment (day 9-10), which was statistically different from control (p= 0.004, one-way ANOVA with Tukey's comparison). At the end of the experiment the difference between chondroitin-6-sulphate and control was lost. N=9 for control and C-4-S and n=6 for C-6-S. Error bars = SD.

4.3.4 The effect of Chondroitin sulphate on barrier restitution following hypoxic injury

Each individual cell line was placed into hypoxia for 7 days; barrier was monitored. As shown in chapter 3 (section 3.2.4.1) cell cultures had a lower barrier while maintained in hypoxia. After removal from hypoxia barrier restitution began within 24 hours but took between 2-6 days before readings were similar to normoxic control (individual cell culture results shown in Appendix section 7.4).

Normalised combined data used to assess the variance of recovery between treated cultures and hypoxic control demonstrated that 2 days after removal from hypoxia there was no difference between any CS treated groups and control (Figure 4.16). Maximum barrier after hypoxic exposure was also assessed to see if CS groups had a higher overall barrier when compared to control. Although chondroitin-6-sulphate did have a higher overall barrier when compared to the hypoxic control group this was not statistically significant (Figure 4.17).

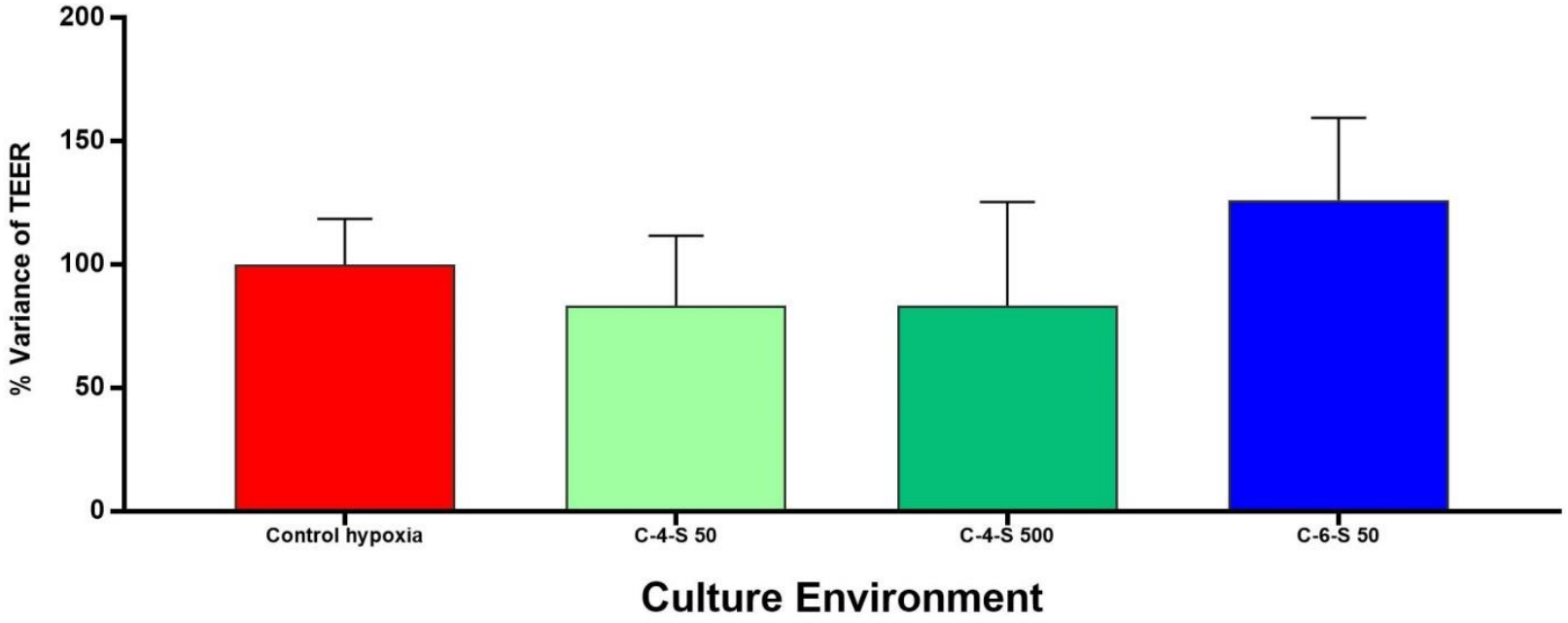


Figure 4.16: Normalised combined data Day 2 post hypoxic exposure and addition of Chondroitin sulphate.

This graph shows the percentage change of TEER readings from hypoxic control for cultures treated with chondroitin sulphate 2 days after removal from hypoxia. Although the barrier from C-6-S was higher it was not statistically significant. Cell lines Y1858, Y1751 and Y1891 are shown. Data normalised to hypoxic control. N=9 in each group. Error bar = SD.

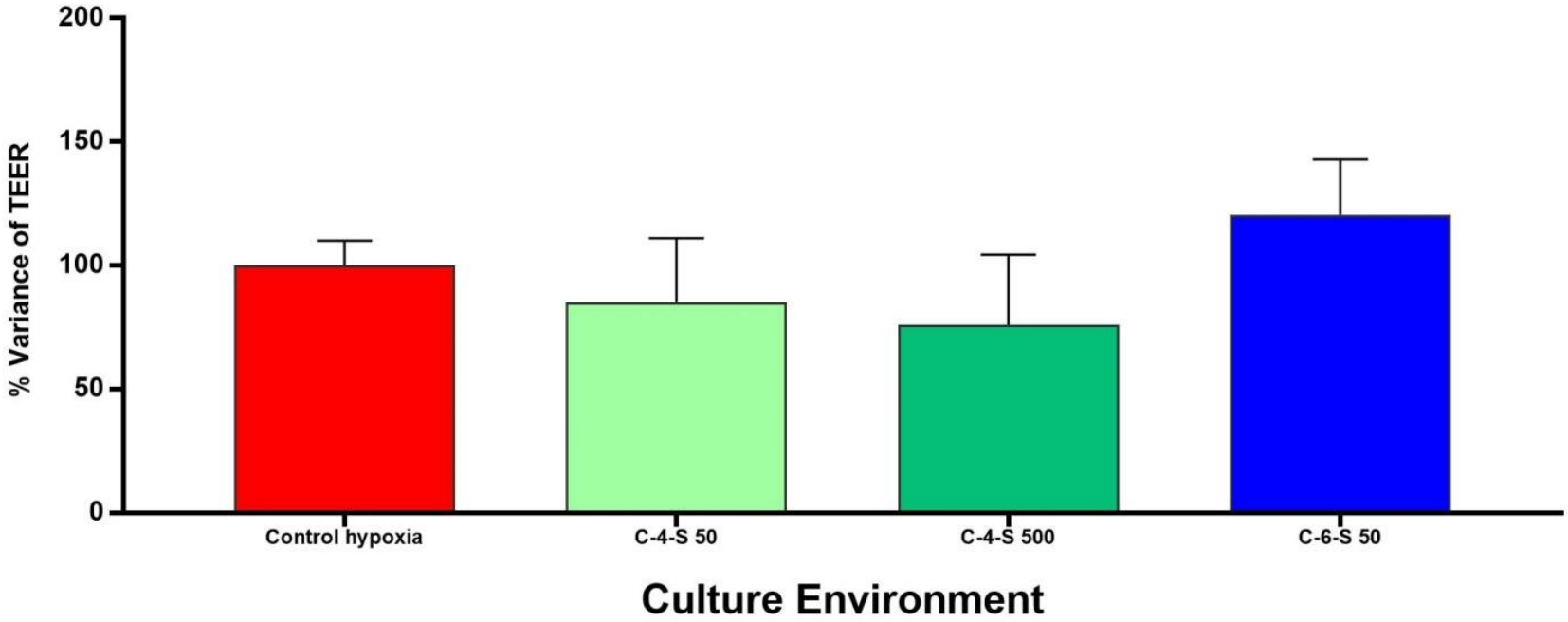


Figure 4.17: Normalised combined data for the variance in maximum TEER of cultures treated with chondroitin sulphate and control after hypoxic injury.

Combined data demonstrating when cultures are returned to normoxia and treated with different concentrations and types of chondroitin sulphate there is no difference between barrier recovery in the treated groups to control cultures. Although there appeared to be a higher barrier in the C-6-S group this was not statistically significant. Barrier had returned in all groups. Cell lines Y1858, Y1751 and Y1891 are shown. Data normalised to hypoxic control. N=9 in each group, error bar = SD.

4.3.5 Effect of clinical GAGs on barrier formation of NHU cell cultures

The initial experiment using cell line Y1747 with Cystistat, Hycyst and iAluril showed that iAluril appeared to have an effect on NHU cell culture barrier formation. The barrier did not reach the same resistance as non-treated controls or the Sodium hyaluronate treated groups (Figure 4.18).

Further experiments using two different biological replicates and with two batches of iAluril confirmed that cells did not form a barrier with the resistance as high as control. The maximum barriers were normalised and compared, this showed a statistically significant difference between control and iAluril groups ($p=0.0031$ and $p=0.008$, batch 1 and 2 respectively, one-way ANOVA with Tukey's comparison) (Figure 4.19).

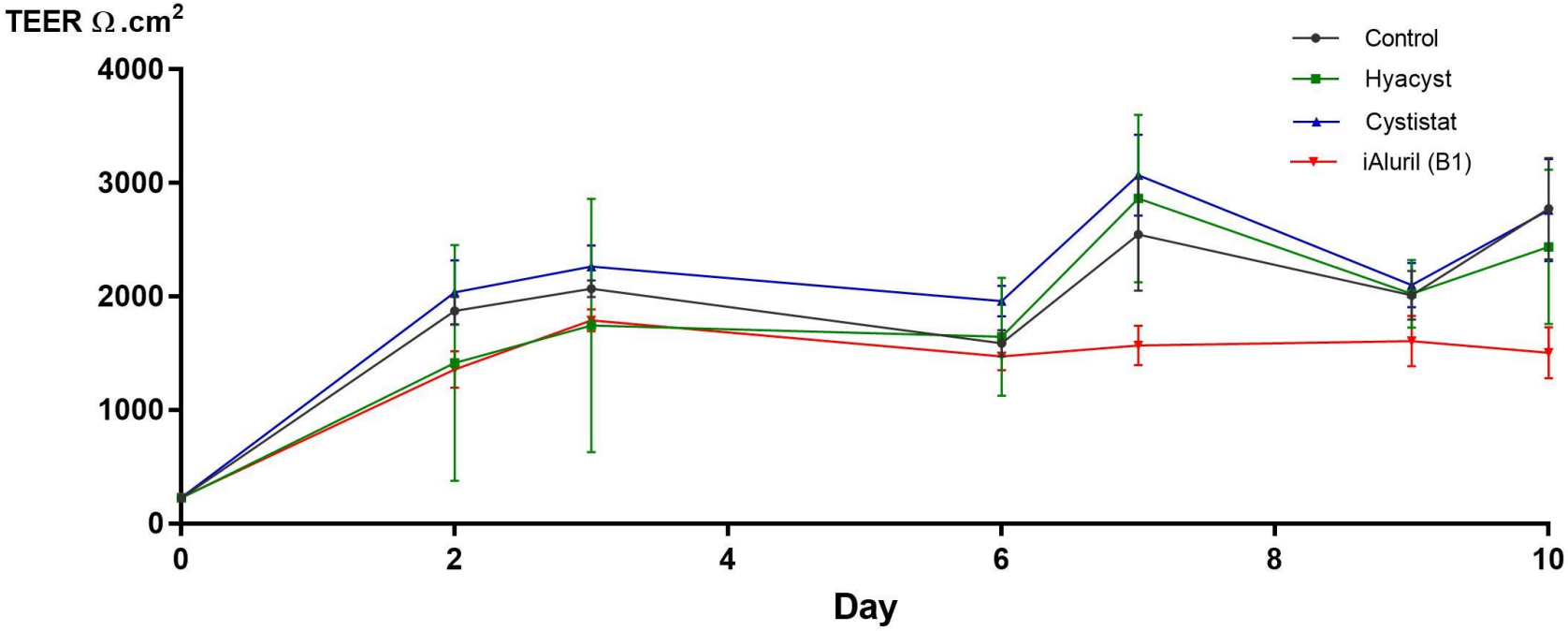


Figure 4.18: Cell line Y1747 treated with clinical GAGs for 2 hours.

1 day after seeding onto semi-permeable membranes, prior to increase in Ca^{2+} concentration experimental replicates were treated with different clinical glycosaminoglycans for 2 hours. iAluril (B1) refers to the first batch received. This figure demonstrates the formation and maintenance of barrier over time. It can be seen that the barrier formation with iAluril is inhibited. N=4 in each experimental group, error bars = SD.

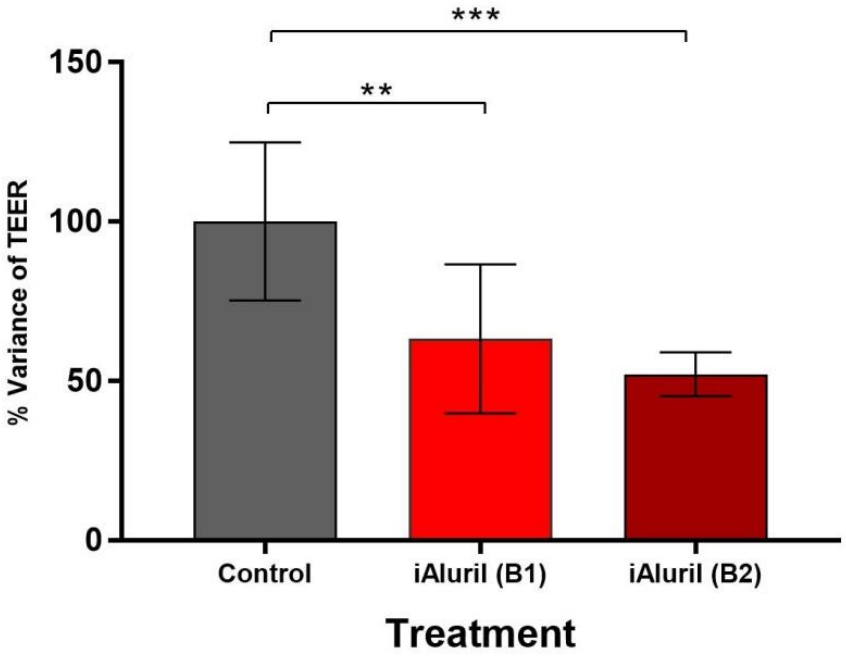


Figure 4.19: Normalised data showing the variance of the maximum barrier achieved between control and iAluril treated cell cultures.

Combined data demonstrates that there was a statistically significant difference between the maximum barrier achieved with both batches of iAluril and control ($p=0.0031$ and $p=0.008$, batch 1 and 2 respectively one-way ANOVA using Tukey's comparison) which favoured control. There was no difference between batches. B1 and B2 refer to the first and second batches. Control n=10, iAluril (B1) n=9, iAluril (B2) n= 6, error bars = SD.

4.3.6 Chapter summary

Immunohistochemistry of human urinary tract specimens did reveal chondroitin sulphate labelling, which was within the stroma. There was some labelling on the luminal surface and between the urothelial cells but when control slides are reviewed it is likely this is non-specific labelling or secondary to a mimitope. There was not a 'thick layer' on the luminal umbrella cells as expected. This is supported by RNA transcriptome data which showed some CS genes had a higher Rpkm predominantly in the proliferating phenotype. It would be expected if CS was a component of the glycocalyx the Rpkm would have been higher in the *in situ* and differentiated phenotype.

The chronic addition of CS to the medium did have an effect on barrier formation. During early development the barrier was tighter earlier with the addition of chondroitin 6-sulphate which was statistically significant. The maximum barrier achieved was higher in cultures that were exposed to chondroitin-4-sulphate and chondroitin-6-sulphate however this effect was not sustained. At the end of the experiment only the barrier with chondroitin-4-sulphate remained statistically significantly higher than control.

After cell cultures were exposed to hypoxia, barrier development appeared to be enhanced by chondroitin-6-sulphate, however combined data showed there was statistically no difference from control in early barrier restitution or maximum barrier post exposure. There was no difference between chondroitin-4-sulphate and control.

Clinical GAGs containing sodium hyaluronate alone did not appear to affect barrier formation; however iAluril containing both sodium hyaluronate and chondroitin sulphate did affect barrier formation causing a depleted barrier. Maximum barrier formed with iAluril treated cell cultures did not reach control values and was statistically different, this was confirmed with two batches of iAluril.

5 Discussion

In the light of controversy over the use of intravesical CS as a bladder therapeutic, the overall aim of this project was to use *in situ* and *in vitro* techniques to provide objective evidence to support or disprove the hypothesis that exogenous CS enhances urothelial barrier function.

Using a well-established urothelial cell culture model (Cross *et al.*, 2005) in which the effect of exogenous CS on barrier formation and restitution following injury could be assessed, the results have shown that continuous application of chondroitin-4 or-6 sulphate did have an effect on the development and overall maximum barrier achieved, although insight into the mode of action was not identified. A secondary aim was the development of a urothelial model representing a chronic non-repairing condition in order to assess the effects of CS on barrier restitution. Different techniques were explored, but hypoxia gave the most reproducible barrier deficient model, where the barrier took four days to recover after return to normoxia. When CS was applied to the hypoxia damaged model there was no difference in barrier restitution between the CS-treated cultures and control, suggesting the effects of CS could be during barrier development rather than repair. When clinical GAGs were applied to the *in vitro* culture system to mimic clinical practice, iAluril containing both sodium hyaluronate and CS supressed barrier formation.

5.1 Presence of chondroitin sulphate

One major controversy that this thesis has addressed is where in the urothelium, endogenous chondroitin sulphate is located. On human urothelial specimens, chondroitin sulphate was located within the stroma with equivocal or no labelling on the urothelium. Chondroitin sulphate is known to be a major component of the extra-cellular matrix throughout the body (Caterson, 2012) however reports from other investigators describe a thick “GAG” layer on the luminal surface of urothelial umbrella cells (Hurst, 1994; Janssen *et al.*, 2013). Early research into the GAG layer used Alcian blue staining to show this layer (Grist and Chakraborty, 1994) however it is well

understood that this stain labels more than GAGs as it will also label some mucopolysaccharides (Scott, 1996) therefore specificity is questionable.

Using well-characterised antibodies which bind to the stub and chains of CS, in combination with careful controls, this study has been unable to confirm a thick layer of CS on the urothelial surface. The findings in this thesis are supported by Buckley *et al.* who observed that in rabbit bladders CS labelling was confined to the lamina propria, with small amounts of HS located within the urothelium (Buckley *et al.*, 1996). This contradicts current thoughts of authors from United States and Canada (Hurst RE/ Parsons CL and Nickel C respectively) who believe that CS makes up a significant proportion of the GAGs on the urothelial surface. It has been estimated that of the GAGs extracted from a human bladder, 29 % were CS (Hurst and Zebrowski, 1994). However, given the methodology it is likely the lamina propria was included in the analysis. Researchers who have used immunohistochemistry to demonstrate CS on the luminal surface have failed to include adequate controls, or an intact urothelium. Further only one CS stub antibody was used, this was the same antibody that has been identified in this thesis as having non-specific urothelial labelling (2B6). (Slobodov *et al.*, 2004; Hurst *et al.*, 2016). The controls presented within this thesis show that non-specific labelling of the urothelium can occur due to incomplete blocking during the immunohistochemistry process. Without adequate controls in the published literature, for example applying a chondroitin antibody without chondroitinase digest when one is required to display the epitope; it raises uncertainty on the validity of their results. Further, there are known mimitopes to some of the antibodies. These are molecular species with binding sites that mimic the epitopes (Caterson, 2012; Farrugia *et al.*, 2016). It is likely the pattern of labelling with 7D4 antibody with chondroitinase ABC digest has identified one such cross-reactive mimitope.

One limitation of this thesis is that only one bladder specimen was used for IHC experiments with CS antibodies and the remainder were ureter samples. The decision to use ureter specimens was made based on the knowledge that ureter urothelium expresses tight junction proteins and the cells in culture form barriers similar to bladder cells. It is difficult to obtain bladder biopsies with an intact urothelium from cold cup biopsies, and larger specimens from simple cystectomies are rare therefore

the decision to use ureter samples was made. It is noted that the function of the bladder is different to the ureter and therefore there may have been structural differences in the glycocalyx which was not observed. The bladder specimen presented did show consistent results of CS antibody labelling, which adds reassurance to the validity of the results. This limitation could be rectified by repeating the experiment on bladder biopsies, noting it may be difficult to obtain an intact urothelium from cold cup biopsies.

The immunohistochemical findings of this study are supported by the RNA transcriptome data which has been presented. It was anticipated that if chondroitin sulphate was a major component of the glycocalyx that the transcript reading would be higher in the *in situ* and differentiated phenotypes, compared to proliferating; this was not found. There is no published literature from urothelial cells to compare these results, however it is known that during brain injury there is up-regulation of CS, with specific upregulation of chondroitin-6-sulphate. RNA data presented by Properzi *et al* specifically identified the CS synthesizing enzyme C6ST1 as being upregulated during brain injury, which may provide an avenue for further assessment for urothelial cell injury (Properzi *et al.*, 2005). The evidence from CS labelling during IHC and the data attained from RNA transcriptome analysis questions current opinions in the published literature and therefore this new finding warrants publication to improve understanding.

5.2 Effect of chondroitin sulphate on a damaged model

Most models of bladder damage that try to mimic chronic disease cause only acute injury where the urothelium is effectively able to mount a repair and barrier function is restored within 24-72 hours. The objective of this thesis was to develop a prolonged damage model. After excluding physical damage by scratch wounding and chemical damage by ketamine a model using hypoxia was established. The hypoxia model was then used to explore the effects of CS on the restitution of a compromised urothelial barrier. The role of hypoxia in patients with bladder pain syndrome has been shown by Lee *et al.* whereby there was upregulation of HIF1α in biopsies of patients with bladder pain when compared to control (Lee and Lee, 2011). By developing a model of damage using hypoxia, a consistent reduction of barrier function of exposed cultures was

achieved. When placed back into normoxia, a functional barrier returned after 4 days which provided a longer treatment window than scratch wounding. Further the morphology of the cells remained comparable with control cell sheets. There was a reduction of claudin 4 of the exposed cell cultures which was restored when placed back into normoxia, indicating an involvement of tight junction components. Tight junction anomalies have been shown in patients with benign bladder disorders (Southgate *et al.*, 2007) therefore hypoxia provided a good damage model.

A limitation of the presented work in this thesis is the inter-observer error in the quantification of changes in tight junction proteins when cell cultures were exposed to hypoxia as highlighted in the results (section 3.2.4.2). The use of hypoxia as a damaged model for differentiated human urothelial cell cultures is a new finding and therefore prior to publication an increase in the sample size of blinded assessors would be required to rectify this.

Although hypoxia did not produce a long-term model of barrier damage there was no damage to urothelial cells during the process. Published models of bladder damage have used hydrochloric acid (Hauser *et al.*, 2009; Engles *et al.*, 2012) and protamine (Kyker, Coffman and Hurst, 2005) to try and represent chronic bladder disease. These methods use large doses of solutes which produce an acute model of damage where there is damage to the urothelial cells. Lavelle *et al* has studied the effect of protamine on Dawley rat urothelial cells and found after exposure there is necrosis and sloughing of the umbrella cells, however barrier function is restored after 72 hours (Lavelle *et al.*, 2002). When protamine has been applied to a porcine cell culture model barrier is repaired after 24 hours (Janssen *et al.*, 2013).

It is suggested that the protamine damaged animal model only removes GAGs however as described above there is evidence that protamine damages the underlying urothelium. The associated damage to the umbrella cells also highlights damage to the glycocalyx as a whole; therefore the important barrier function of uroplakins is lost. When such models are used to assess the effect of exogenous application of CS it cannot therefore be established if the chondroitin is providing benefit or if it is simply the urothelium regenerating. This study used normal human urothelial cells *in vitro* therefore the role of CS on barrier repair after injury would be expected to a true

effect. This study has not demonstrated an effect of CS on barrier restitution following hypoxic injury.

Currently CS is sold as a mechanical device for intravesical use for chronic inflammatory bladder disorders; however there are very few randomised control trials to prove its efficacy. In fact published randomised trials assessing the effect on patients with BPS have shown no difference when compared to placebo (Nickel *et al.*, 2010, 2012). The first trial published by Nickel *et al.* was underpowered and although his second trial took into consideration a power calculation it was still classed as a pilot study and they were not anticipating a significant result based on the power calculation. He reports 38 % of respondents who received CS had moderate or marked improvement in their symptoms, 31 % of the inactive control group also fell within this category, there was no statistically significant difference (Nickel *et al.*, 2012). A further well designed randomised control trial by Damiano *et al.* has assessed the effect of hyaluronic acid-chondroitin sulphate (iAluril) on patients with rUTI and found a significant difference between time to infection and infection episodes per year with iAluril treatment, this also improved quality of life scores (Damiano *et al.*, 2011). It is interesting to note that the majority of research on GAGs within the bladder and their use in benign inflammatory conditions has come from America and Canada yet the American Association of Urologists do not recommend either hyaluronic acid or chondroitin sulphate in the treatment guidelines for BPS patients. Current European guidelines suggest there is a role in management (Engeler *et al.*, 2014; Hanno *et al.*, 2014).

Chondroitin sulphate is used for treatment of other chronic inflammatory disorders with osteoarthritis being predominant. There are more randomised control trials assessing the efficacy of CS therapy in osteoarthritis. However a recent Cochrane review has left doubts on its overall benefits. This review showed only mild improvement in pain and no significant radiological improvements in participants who took chondroitin supplements (Singh *et al.*, 2015). Currently CS supplements are not supplied by an NHS prescription and are not recommended by NICE for the treatment of osteoarthritis (National Institute for Health and Care Excellence, 2014).

5.3 Candidate mode of action

The TEER studies presented in this thesis have shown CS exhibits a positive effect on barrier formation of normal human urothelial cells. This suggests that CS is not acting as a mechanical or physical device as indicated, but may have some biological effect. There is evidence that CS has anti-inflammatory, anti-apoptotic and anti-oxidant effects (Du Souich *et al.*, 2009; Henrotin *et al.*, 2010) as well as facilitating and inhibiting proliferation and differentiation of different types of cells (Properzi *et al.*, 2005; Purushothaman, Sugahara and Faissner, 2011; Girshovich *et al.*, 2012; Hayes *et al.*, 2018). Research into neural cell development has identified the mode of action during proliferation and differentiation to be in the presentation of growth factors namely EGF and FGF. The long chondroitin sulphate chains act as a docking site for the presentation of growth factors to developing cells (Sirko *et al.*, 2007). The role of HS with FGF is more established and has been shown to be an important player in cell signalling (Brickman *et al.*, 1998; Guimond and Turnbull, 1999; Lin, 2004). There is some evidence to suggest that CS acts as a facilitator for HS (Deepa *et al.*, 2002) therefore further research is required to understand the role of heparan in the urothelium.

The significant improvement in barrier formation and maximum barrier of urothelial cultures in this study when supplemented with CS may be secondary to increased presentation of growth factors to the cells, either exogenous in the KSFMc medium, or expressed as autocrine factors. Research has already displayed an EGFR autocrine signalling loop associated with *in vitro* urothelial cell proliferation and migration during wound healing (Varley *et al.*, 2005) and an autocrine TGFβR loop has also been identified (Fleming *et al.*, 2012). It is possible CS is a facilitator for one or other of these, but further research would be required to confirm this hypothesis.

5.4 Conclusion

The evidence presented in this thesis has shown that CS is not a constituent of the glycocalyx as published literature suggests. There remains a myriad of possibilities for the structure of the glycocalyx, further it is likely there are variations between individuals. Superficial urothelial cells are not simply covered by a ‘thick glycosaminoglycan’ layer but a complex of uroplakins (glycoproteins), mucins and

proteoglycans predominantly heparans, making up a glycocalyx which provides a barrier to urinary solutes. The role of GAGs within this structure remains inconclusive: studies which have attempted to damage the GAG layer are likely to have damaged the entire glycocalyx and so conclusions drawn from these studies remain in doubt. It is evident from this research project that CS does have an effect on barrier formation during differentiation of NHU cells and is not simply a device to facilitate restoration of a damaged bladder urothelium. The next step to investigating the mode of action of CS and other GAGs would involve laboratory research assessing the potential link with growth factors. The role of heparan sulphate and the urothelium could also be established by immunohistochemistry. Should a link with growth factors be established then further research to ascertain if the effect could be amplified could be of benefit patients in the future.

It is also apparent from literature review that there is no level one evidence for intravesical use of CS in BPS; however evidence does appear to show there is benefit for patients with recurrent UTIs. To understand if intravesical GAGs have an effect on the structure of the urothelium a clinical study whereby bladder biopsies are taken before and after treatment with matched placebo controls is warranted. Immunohistochemistry could then be performed to assess tight junction proteins, uroplakins and the presence of GAGs.

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7 Appendix

7.1 List of Acronyms

ABS	Adult bovine serum
AUM	Asymmetric unit membrane
BPS	Bladder pain syndrome
CS	Chondroitin sulphate
C-4-S/C-6S	Chondroitin 4 or 6 sulphate
CK	Cytokeratin
DAB	Diaminobenzidine
DMSO	Dimethylsulphoxide
DS	Dermatan sulphate
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
H&E	Haematoxylin and eosin
HIF1α	Hypoxia-inducible factor 1-alpha
HS	Heparan sulphate
IC	Interstitial cystitis
IHC	Immunohistochemistry
KIU	Kallikrein inhibiting unit
KSFMc	Keratinocyte serum-free medium with supplements
NHU	Normal human urothelial
PBS	Phosphate buffered saline
REC	Research ethics committee

RNA	Ribonucleic acid
rUTI	Recurrent urinary tract infection
TEER	Transepithelial electrical resistance
TBS	Tris buffered saline
TGFß	Transforming growth factor beta
UPK	Uroplakin
VEGF	Vascular endothelial growth factor
ZO	Zona occluden

7.2 List of Suppliers

Company	Website
Abcam	www.abcam.com
Bayer Pharmaceuticals	www.bayer.co.uk
Cell Signalling	www.cellsignal.com
CellPath Ltd	www.cellpath.com
Dako	www.agilent.com/en-gb/dako-products
Enzo Life Sciences	www.enzolifesciences.com
Fisher Scientific	www.fishersci.com
Greiner Bio-One Ltd	www.greinerbioone.com
Olympus	www.olympus-lifescience.com
Priorclave	www.priorclave.co.uk
Ramboldi	www.ramboldi.com
Sarstedt	www.sarstedt.com
Scientific Laboratory Supplies	www.scientificlabs.co.uk
Sera Laboratories	www.seralab.co.uk
Sigma-Aldrich	www.sigmaaldrich.com
STAR Lab	www.starlabgroup.com
Thermo Fisher Scientific Inc • Life Technologies – Gibco, Invitrogen, Zymed	http://www.thermofisher.com
World Precision Instruments	www.wpiinc.com

7.3 Solutions and Buffers

7.3.1 Tissue culture:

Transport medium	Hanks' Balanced Salt Solution (HBSS) with $Ca^{2+}$and Mg^{2+} (Gibco 24020-091), 10 mM Hepes pH 7.6 (Gibco 15630-056) and 20 kallikrein-inhibiting units (KIU)/ml of aprotinin (Trasylol; Bayer Pharmaceuticals),
Stripper medium	HBBS without $Ca^{2+}$and Mg^{2+} (Gibco 14170-088), 10 mM Hepes pH 7.6(Gibco 15630-056), 20 kallikrein-inhibiting units (KIU)/ml of aprotinin (Trasylol; Bayer Pharmaceuticals), and 50 ml 1% EDTA
Collagenase	10, 000 U collagenase (Sigma C5138) in 100 ml HBSS with $Ca^{2+}$and Mg^{2+} (Gibco 24020-091)
KSFMc	Keratinocyte serum-free medium (Gibco) supplemented with recombinant epidermal growth factor (5 ng/ml), bovine pituitary extract (50 µg/ml) and cholera toxin (30 ng/ml)
Freeze mix	KSFMc with 10 % (v/v) FBS and 10 % (v/v) DMSO (Sigma-Aldrich, D2650))
Dispase II	Dispase II powder (Roche, 04942078001) dissolved in Dulbecco's Phosphate Buffered Saline (D-PBS Gibco, 14200-067) to a final concentration of 1 % (w/v)

7.3.2 Immunohistochemistry

Citric Acid Buffer	2.2 g citric acid in 1050 ml H2O, sodium hydroxide pellets to achieve pH 6.0
TBS Buffer	3M NaCl (175.32 g/L), 2M Tris (242.28 g/L) with HCL to pH 7.6. To make 1 L of TBS buffer combine 25 ml 2M Tris, 50 ml of 3M NaCl and make up to 1 L with dH2O
Haematoxylin	3 g haematoxylin in 20 ml ethanol.
PBS Buffer	5 PBS tablets (Sigma, P4417) in 1 L ELGA purified water
Chondroitinas ABC	Chondroitinase ABC is diluted in Tris Buffer at pH 8.0. pH adjustment by adding acetic acid in a stepwise manner.

7.3.3 Clinical solutions

Hyacyst	40 mg of sodium hyaluronate in 50 ml sterile buffered saline
Cystistat	40 mg Sodium hyaluronate in 50 ml sterile buffered saline
iAluril	800 mg (1.6 %) Hyaluronic acid sodium salt, 1 g (2 %) sodium chondroitin sulphate, water and calcium chloride in 50 ml solution

7.4 Additional results Chapter 4

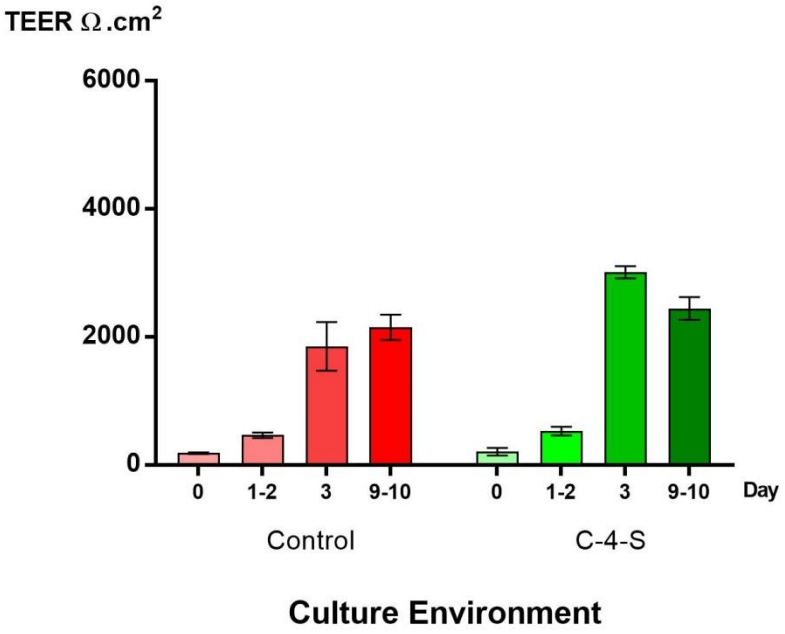

Figure 7.1: Barrier development in cell line Y1790 with application of Chondroitin-4-Sulphate

Chondroitin-4-sulphate (50 ng/ml) was added to the medium of cells during the ABS pre-treatment and continued for the remainder of the experiment. A barrier was formed by day 3 in both the C-4-S and the non-treated control. N= 3 in both experimental arms. Error bars = SD.

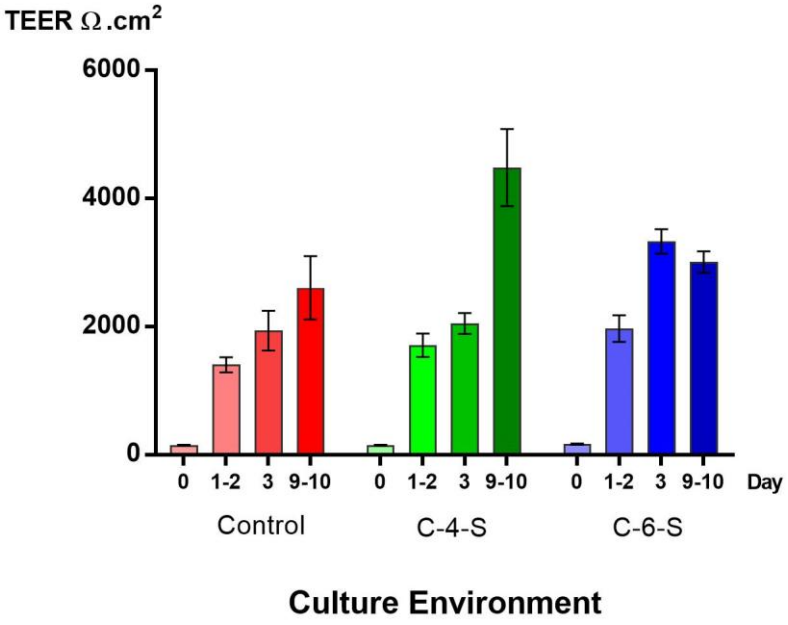

Figure 7.2: Barrier development in cell line Y1751 with the chronic application of Chondroitin 4-Sulphate and Chondroitin-6-Sulphate.

Cultures were exposed to chondroitin 4 and 6 sulphate during the ABS pre-treatment and throughout barrier studies. This cell line demonstrated a tight barrier on day 2 in all arms after the calcium concentration was increased. The end barrier was higher in the chondroitin sulphate treated groups. N=3 in each experimental group. Error bars = SD.

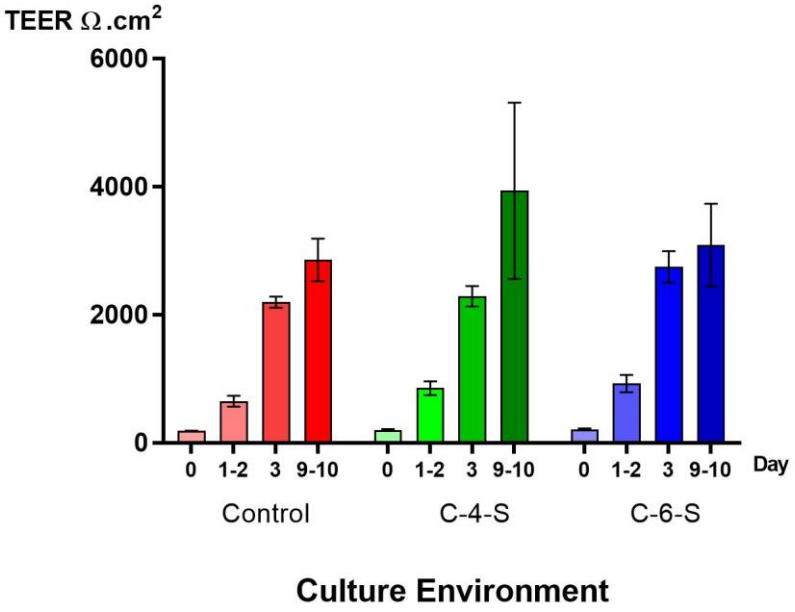

Figure 7.3: Barrier development in cell line Y1837 with the chronic application of Chondroitin-4 and 6-sulphate.

Third cell line with exposure to a chronic application of chondroitin sulphate during barrier development. A tight barrier was formed on day 3 in all arms. The end barrier was tighter in the chondroitin-4-sulphate group. N=3 in each experimental group. Error bars = SD.

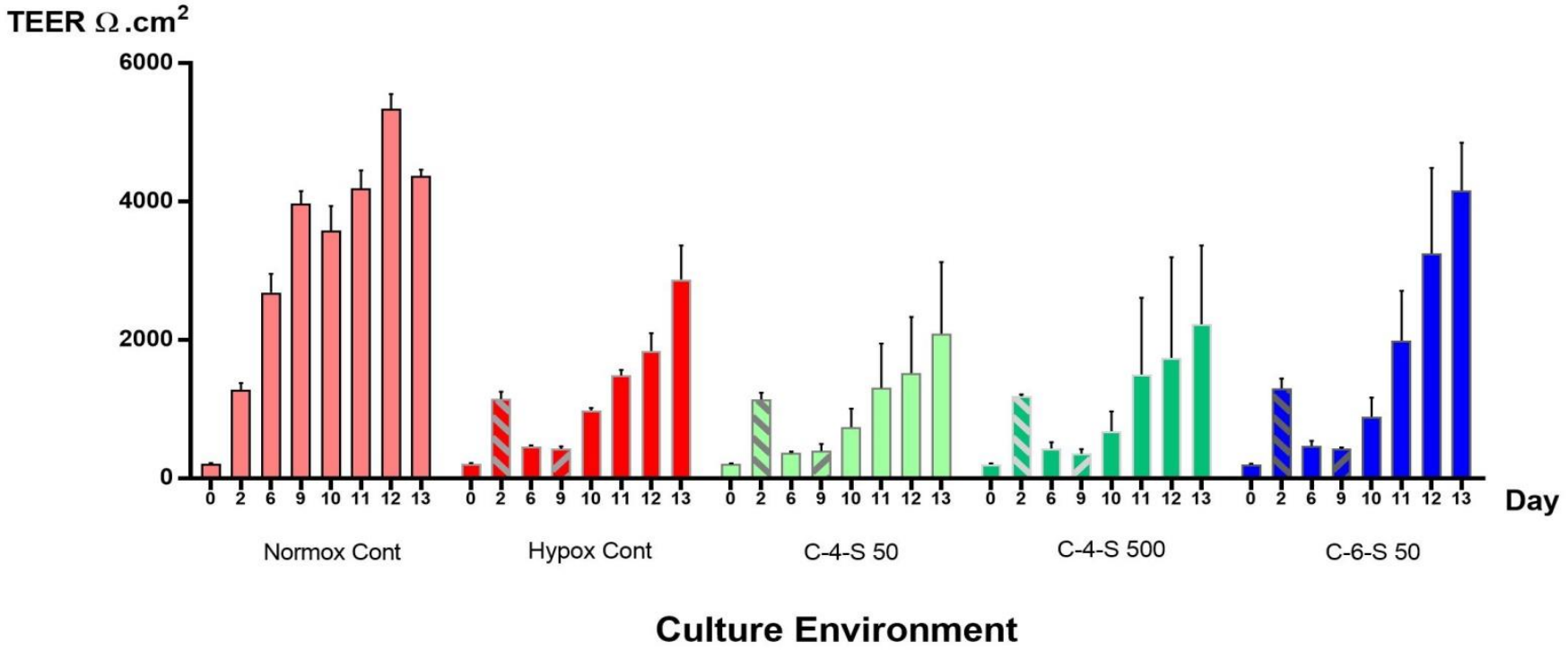

Figure 7.4: The effects of chondroitin sulphate on barrier restitution after hypoxic exposure cell line Y1858.

Cell cultures were placed into hypoxia on day 2 and removed day 9 (shown as hatched bar). Barrier restitution was observed over the subsequent days. C-4-S represents chondroitin-4-sulphate at concentration 50 and 500 ng/ml and C-6-S represents chondroitin-6-sulphate at concentration 50 ng/ml. The barrier appeared to repair earlier with C-6-S than with C-4-S. N=3 in each experimental group. Error bars = SD.

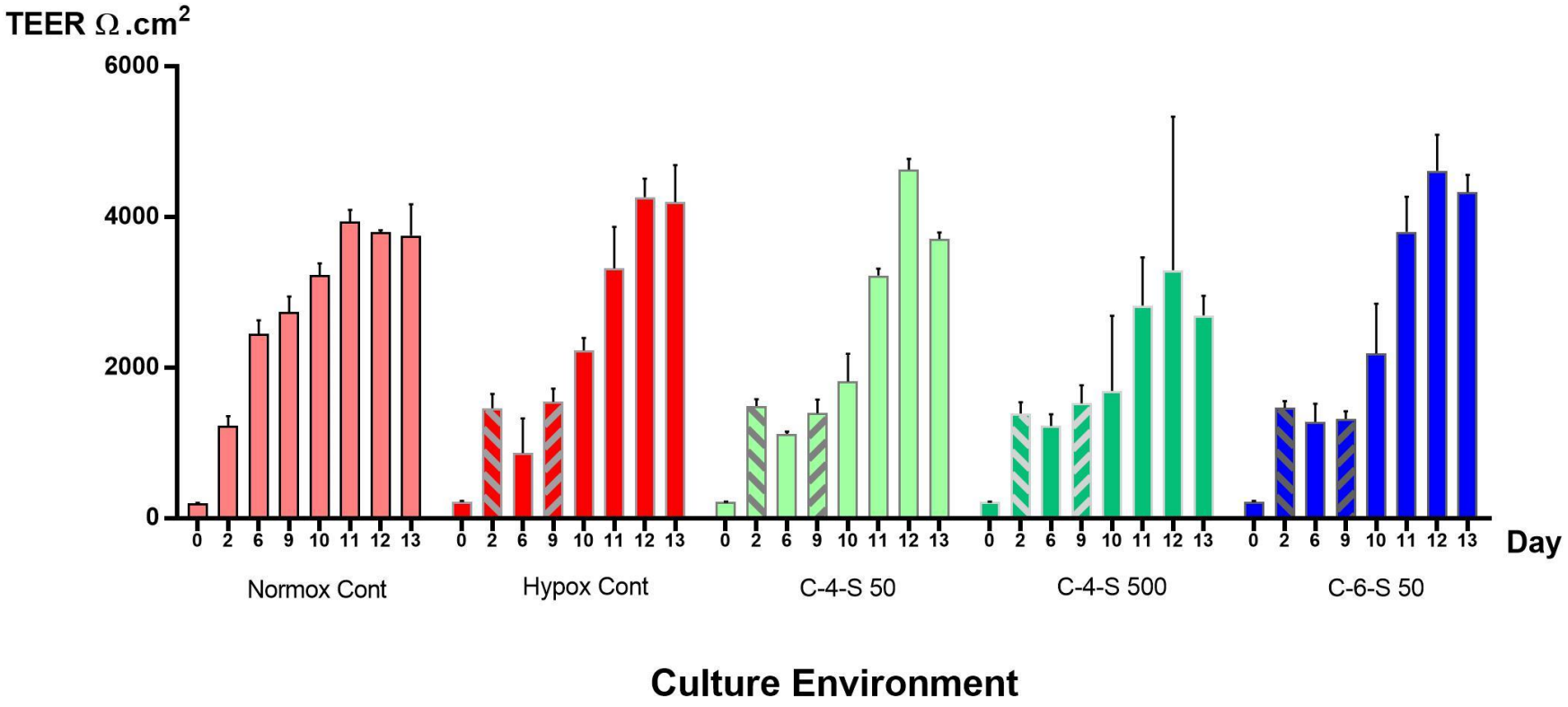

Figure 7.5: Cell line Y1751 showing the effect of chondroitin Sulphate on barrier restitution following hypoxic exposure.

This graph shows that the barrier recovered quickly after return to normoxia (2 days in each arm). There appeared to be no difference between groups. C-4-S represents chondroitin-4-sulphate at concentration 50 and 500 ng/ml and C-6-S represents chondroitin-6-sulphate at concentration 50 ng/ml. N=3 in each group, except normox control where n=2. Hatched bar represents the day into and out of hypoxia. Error bars = SD.

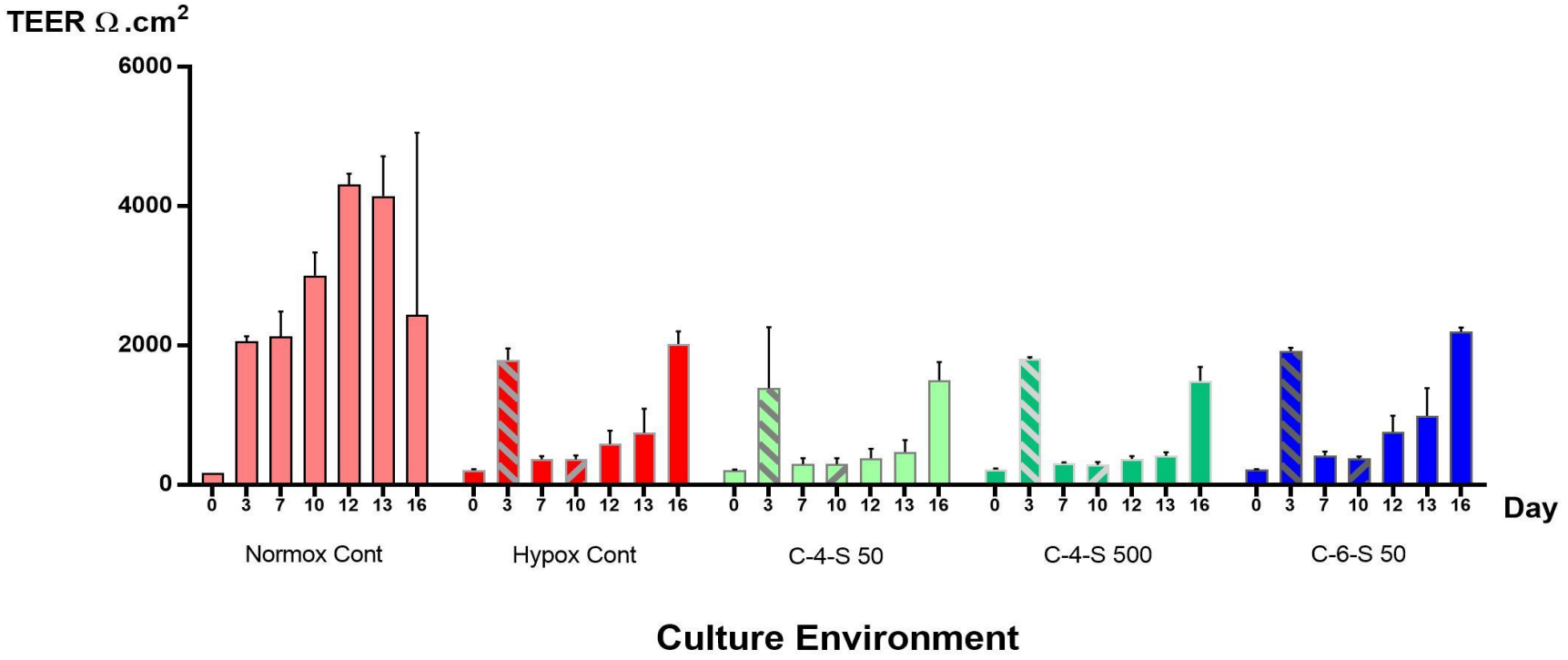

Figure 7.6: Cell line Y1891 showing the effect of chondroitin sulphate on barrier restitution following hypoxic exposure.

This graph shows that C-4-S followed a similar recovery to the hypoxia control when placed back into normoxia, however C-6-S group did appear to recover earlier. C-4-S represents chondroitin-4-sulphate at concentration 50 and 500 ng/ml and C-6-S represents chondroitin-6-sulphate at concentration 50 ng/ml. N=3 in each group, except normoxia control where n=2. Hatched bar represents the day into and out of hypoxia. Error bars = SD.