UNIVERSITY OF
Southampton

University of Southampton Research Repository

Copyright © and Moral Rights for this thesis and, where applicable, any accompanying data are retained by the author and/or other copyright owners. A copy can be downloaded for personal non-commercial research or study, without prior permission or charge. This thesis and the accompanying data cannot be reproduced or quoted extensively from without first obtaining permission in writing from the copyright holder/s. The content of the thesis and accompanying research data (where applicable) must not be changed in any way or sold commercially in any format or medium without the formal permission of the copyright holder/s.

When referring to this thesis and any accompanying data, full bibliographic details must be given, e.g.

Thesis: Author (Year of Submission) "Full thesis title", University of Southampton, name of the University Faculty or School or Department, PhD Thesis, pagination.

Data: Author (Year) Title. URI [dataset]

UNIVERSITY OF SOUTHAMPTON

FACULTY OF HEALTH SCIENCES

Innovation and Leadership in Health Sciences

Interstitial Cystitis: Aetiology and Therapeutic Potential of Honey

by

Shabana Tabassum Malik

Thesis for the degree of Doctor of Philosophy

June 2017

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF HEALTH SCIENCES

Thesis for the degree of Doctor of Philosophy

INTERSTITIAL CYSTITIS: AETIOLOGY AND THERAPEUTIC POTENTIAL OF HONEY

Shabana Tabassum Malik

Painful bladder syndrome/interstitial cystitis (PBS/IC) is an under-researched urological disorder, and thus, is poorly understood, diagnosed, and managed. This project was designed to elucidate the role of mast cells (MCs) and basophils in PBS/IC, identify any difference in the distribution of MC subtypes (MC_T and MC_{TC}), and assess the potential of honey as a treatment for PBS/IC.

The study was successful in that it identified a difference in the distribution of MCs within the bladder wall, and a difference in distribution of MC subtypes, in comparison to controls. Novel findings from this study included the discovery that a particularly high presence of MC_{TC} in the mucosa may be linked to the presence of ulcers, and thus, identified the patients that may have had the Hunner's Ulcer PBS/IC subtype. It was also found that some patients had a higher amount of MC_{TC} in the lamina propria ($p<0.001$), and this may identify those patients that were also suffering from fibrotic bladder. Immunohistochemistry findings confirmed detrusor mastocytosis in PBS/IC bladder ($p<0.001$), compared to controls. Basophils were not found in PBS/IC bladder tissues. It was also discovered that honey does not bind histamine, and so is stabilising MCs at a molecular level. Histamine release was decreased in explants that had been incubated in 5% or 10% honey, and tissues showed signs of apoptosis in concentrations above 20%.

After further validation, findings could be used to develop much-needed comprehensive diagnostic criteria for the successful identification of different subgroups of PBS/IC sufferers, and for the development of tailored therapeutic options, that would consequently be more effective for the patient. The development of a honey-based intravesical agent, that has the potential to treat the histamine-related inflammation seen in PBS/IC, and other difficult to treat inflammatory disorders, is a promising and exciting future direction for this study.

Table of Contents

INTERSTITIAL CYSTITIS: AETIOLOGY AND THERAPEUTIC POTENTIAL OF HONEY i

Table of Contents i

List of Tables vii

List of Figures ix

Glossary of Terms xvii

DECLARATION OF AUTHORSHIP xix

Conflicts of Interest xxi

Acknowledgements xxiii

Chapter 1: Literature Review 1

1.1 Interstitial cystitis 1

1.1.1 The glycosaminoglycan layer 2

1.2 Inflammation and painful bladder syndrome/interstitial cystitis 4

1.2.1 Inflammation 4

1.2.2 Painful bladder syndrome/interstitial cystitis 6

1.2.3 Mast cells and painful bladder syndrome/interstitial cystitis 6

1.2.4 Basophils in painful bladder syndrome/interstitial cystitis 7

1.3 Current treatment options for painful bladder syndrome/interstitial cystitis 11

1.4 Honey 11

1.4.1 Honey and mast cell degranulation 12

1.5 Literature review search methods 14

1.6 Search results 14

1.6.1 Oral therapies 15

1.6.2 Intravesical therapies 17

1.7 Honey as a therapeutic agent 29

1.8 Discussion 31

Chapter 2: Painful Bladder Syndrome/Interstitial Cystitis 33

2.1 The urinary system 33
2.2 The urinary bladder 34
2.3 The bladder wall 35
2.3.1 The mucosa 36
2.3.2 The lamina propria 36
2.3.3 The detrusor muscle 36
2.3.4 The adventitia 37
2.3.5 The GAG layer 37
2.4 Bladder disorders 38
2.5 Painful bladder syndrome/interstitial cystitis 38
2.5.1 History 38
2.5.2 Classification 41
2.5.3 Symptoms 43
2.5.4 Bladder pathology 44
2.6 Diagnosis 46
2.7 Current therapies 48
2.8 Cost and financial burden 49
2.9 Summary 50
2.10 Hypotheses 50
2.11 Aims 50
Chapter 3: Mast Cells in Painful Bladder Syndrome/Interstitial Cystitis ..53
3.1 Introduction 53
3.2 Immunohistochemistry 54
3.2.1 Background 54
3.2.2 The indirect immunohistochemistry technique 55
3.3 Aim 55
3.4 Materials and methods 56
3.4.1 Participants 56
3.4.2 Bladder tissue preparations 56
3.4.3 Ethical considerations 56
3.4.4 Immunohistochemistry 57

3.5 Outcome measurements 58
3.5.1 Macro development 58
3.5.2 Cell counting 59
3.6 Statistical analysis 59
3.7 Results 59
3.8 Discussion 71
Chapter 4: Basophils in Painful Bladder Syndrome/Interstitial Cystitis .. 77
4.1 Introduction 77
4.2 Basophils 78
4.3 Validity of control tissues 80
4.3.1 Ethical considerations 81
4.3.2 Tonsil 82
4.3.3 Bone marrow 82
4.3.4 Inflamed appendix 83
4.3.5 Sarcoidosis 83
4.3.6 Asthmatic lung 84
4.3.7 Skin disorders 84
4.3.8 Chronic myeloid leukaemia 85
4.4 Dilution optimisation 88
4.5 Painful bladder syndrome/interstitial cystitis 90
4.6 Limitations 91
4.7 Discussion 92
Chapter 5: Honey and Rat Bladder Explants 93
5.1 Introduction 93
5.2 Hypothesis 93
5.3 Aims 94
5.4 Stage I 94
5.4.1 Materials and methods 95
5.4.2 Ethical considerations 98
5.4.3 Optimisation and standardisation 99

5.4.4 Statistical analysis 100
5.4.5 Results 100
5.4.6 Discussion 106
5.5 Stage II: Fluorescent imaging of bladder explants 113
5.5.1 Introduction 113
5.5.2 Materials and methods 114
5.5.3 Fluorescent dyes 117
5.5.4 Protocol development 119
5.5.5 Analysis 120
5.5.6 Results 121
5.5.7 Discussion 127
Chapter 6: General Discussion 131
6.1 Introduction 131
6.2 Summary of key findings 131
6.3 Importance of key findings 133
6.3.1 Mast cells 133
6.3.2 Honey 136
6.3.3 Basophils 137
6.4 Limitations 137
6.5 Future work 139
6.5.1 Painful bladder syndrome/interstitial cystitis 139
6.5.2 Honey and painful bladder syndrome/interstitial cystitis 142
6.5.3 Honey and apoptosis 146
6.6 Conclusion 146
Appendices 149
Appendix A Search Terms 151
Appendix B O'Leary/Sant Voiding and Pain Indices 153
Appendix C Protocol: Immunohistochemistry 154
Appendix D Mast Cell Counting 157
Appendix E Protocol: Bladder Explant 158

Appendix F Cutting Mat 162
Appendix G Randomisation Lists 163
Appendix H Standard Histamine ELISA Procedure 166
List of References 173

List of Tables

Table 1-1: The main mediators released from activated mast cell subtypes, MC_T and MC_{TC}, and basophils. (Adapted from http://what-when-how.com). 9

Table 2-1: The grading categorising that Hand developed to separate PBS/IC subjects in accordance to the main observations during cystoscopy. 40

*Table 2-2: The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) diagnostic criteria for PBS/IC. Note the list of exclusion criteria for a positive diagnosis for PBS/IC to be made. (Adapted from Hanno 2002).*47

Table 2-3: The European Society for the Study of Interstitial Cystitis (ESSIC) list of diseases commonly confused with PBS/IC. (Adapted from van de Merwe et al. 2008). 48

Table 4-1: A summary of the tissues used in the development of the 2D7 mAb immunohistochemistry protocol for the detection of basophils. 90

Table 5-1: Fluorochrome data table showing the excitation and emission wavelengths of Propidium Iodide and Acridine Orange. (Adapted from Microscopy Resource Centre, 2012)....120

List of Figures

Figure 1-1: The bladder with a damaged GAG layer (left) and an undamaged GAG layer (right). (Reproduced from http://gepan-instill.co.uk/medical-conditions/; accessed on 14/05/15). 2

Figure 1-2: Bladder biopsies showing the presence (control; left) and absence (PBS/IC; right) of the GAG layer in human urothelium. Arrow heads indicate loss of structure. (Adapted from www.uracyst.co.uk; accessed on 12/05/2015). 3

Figure 1-3: The main events and mediators in the inflammatory response. Note how each stage overlaps. (Adapted from McCance and Huether 2002). 4

Figure 1-4: A possible model for the pathogenesis of PBS/IC. (Adapted from Evans 2002). 6

Figure 1-5: The differentiation of HPCs into immune cells, such as basophils and mast cells, dependent upon the local surrounding levels of cytokines and chemokines. (Adapted from Fischer and Agrawal 2013). 8

Figure 1-6: Representative image of a mast cell, showing the binding of an antigen to IgE leading to histamine release. 10

Figure 1-7: The cytokines that drive chronic inflammation. (Adapted from Arend 2001). 13

Figure 2-1: An overview of the urinary system. (Reproduced from: Keck Medicine http://keckmedicine.adam.com; accessed on 29/06/17). 33

Figure 2-2: The main layers of the bladder wall from the luminal surface (mucosa) to the deepest layer (adventitia). (Adapted from Birder and Andersson 2013). 35

Figure 2-3: Typical cystoscopic appearance of the classic interstitial cystitis bladder. Arrows indicate Hunner's ulcers/lesions. (Adapted from Lee et al. 2013). 42

Figure 2-4: Normal urothelium compared to the dysfunctional bladder lining, as seen in PBS/IC. Note the lack of a continuous GAG layer in the dysfunction urothelium image. (Adapted from http://perthurologyclinic.com.au/). ... 45

Figure 3-1: Representative images of AA1 positively stained human full thickness bladder wall, taken using light microscopy, and either standard histochemical or immunohistochemical procedures. Scale bar= 50µm. A) GIEMSA stained

full thickness human bladder tissue at x4 magnification. The GIEMSA stain is a contrasting stain that will stain erythrocytes pink, platelets a light pale pink, lymphocyte cytoplasm sky blue, monocyte cytoplasm stains pale blue, and leukocyte nuclear chromatin stains magenta. In this image it can be seen that the structural bladder wall has been stained as pink/red, and the granulocytic cells of interest as blue. B) Positively stained bladder mucosa using the mAb AA1 and toluidine blue, with positively stained mast cells stained brown. Magnification is x60. C) Positively stained bladder lamina propria using the mAb AA1 and toluidine blue, with positively stained mast cells stained brown. Magnification is x60. D) Positively stained bladder detrusor muscle using the mAb AA1 and toluidine blue, with positively stained mast cells stained brown. Magnification is x60. E) Positively stained bladder adventitia using the mAb AA1 and toluidine blue, with positively stained mast cells stained brown. Magnification is x60. 61

Figure 3-2: Representative images of human PBS/IC bladder detrusor tissue at x60 magnification using light microscopy. Human bladder tissue samples have been immunohistochemically stained using toluidine blue and mAb AA1. A) Control bladder tissue immunohistochemically stained using toluidine blue and mAb AA1. Positively stained cells are identified by brown colouring. B) PBS/IC bladder tissue immunohistochemically stained using toluidine blue and mAb AA1. Positively stained mast cells are identified by brown colouring. Scale bar= 50µm. 62

Figure 3-3: Representative images of human PBS/IC bladder detrusor tissue at x60 magnification using light microscopy. Human bladder tissue samples have been immunohistochemically stained using toluidine blue and mAb AA1. A: Detrusor muscle layer positively stained for mast cell tryptase with the AA1 antibody (brown). B: 'Skeleton' left after the image has been successfully processed by ImageJ^{TM}. Scale bar= 50µm. 63

Figure 3-4: Schematic representation of CC1 positively stained mast cells amongst AA1 positively stained mast cells in PBS/IC bladder detrusor tissue. Human PBS/IC bladder detrusor tissue were stained using mAbs and toluidine blue, and imaged at x80 magnification using light microscopy Scale bar= 50µm. A) AA1 positively stained mast cells in the detrusor layer of PBS/IC bladder. B) CC1 positively stained mast cells in the detrusor layer of PBS/IC bladder. C) CC1 positively stained mast cells as detected by

ImageJ™ processing of image (B). D) Image (A) superimposed with Image (C) to show the distribution of CC1 positive mast cells as constituents of AA1 positive mast cells. 64

Figure 3-5: Distribution of AA1 positive and CC1 positive mast cells in PBS/IC bladder tissue (±SEM bars). Both AA1 and CC1 positive mast cells show a similar pattern of distribution, although the presence of AA1 positive mast cells is the greatest overall. Within each layer of the bladder wall, the difference in density of AA1 and CC1 mast cells is statistically significant at p<0.001. Mast cells were identified using the mAbs AA1 and CC1, and a standard immunohistochemical procedure. 66

Figure 3-6: Distribution of CC1 positive mast cells in PBS/IC bladder tissue and controls (±SEM bars). The difference in the density of CC1 positive mast cells between the PBS/IC and control group was statistically significant within the detrusor (p=0.01), and within the lamina layer (p<0.01). 67

Figure 3-7: Distribution of AA1 positive mast cells in PBS/IC bladder tissue and controls (±SEM bars). The difference in the density of AA1 positive mast cells between PBS/IC and control was statistically significant within all layers of the bladder wall (p<0.001). 68

Figure 3-8: Distribution of AA1 positive and CC1 positive mast cells in PBS/IC and control bladder tissue (±SEM bars). Both mast cell subtypes show a similar pattern of distribution in both groups, although the presence of AA1 positive mast cells is the greatest overall. Mast cells were identified using the mAbs AA1 and CC1, and a standard immunohistochemical procedure. 69

Figure 3-9: Distribution of patient diagnosis of PBS/IC in years, grouped by sex. The age at which patients are positively diagnosed with PBS/IC was recorded during standard histochemical analysis of the bladder wall, and combined with routine patient information, this data was correlated in order to identify any relationship between the age of diagnosis and the sex of the individual. PBS/IC males are statistically significantly more like to be successfully diagnosed at a later age when compared to females (p<0.05). 70

Figure 3-10: The mean no. of AA1 and CC1 mast cells within the bladder wall of PBS/IC individuals, when subjects have been separated according to sex. Note, there is a difference in the distribution of mast cell subtypes between

females and males, the most obvious being that males have a higher count of CC1 mast cells in comparison to females. 71

Figure 3-11: Distribution of AA1 positive mast cells in human PBS/IC bladder tissue, imaged at a magnification of x80 using light microscopy. Note that positively stained brown MCs are predominantly localised around transversely cut nerve fibres (as denoted by). Mast cells were identified using the mAbs AA1 and CC1, the toluidine blue stain, and a standard immunohistochemical procedure. Scale bar= 100µm. 73

Figure 4-1: A flowchart depicting the decision process for the selection and inclusion of tissue as a control for the validation of the basophils antibodies. 81

Figure 4-2: A series of immunohistochemically stained chronic myeloid leukaemia skin biopsies, with positive staining for mast cells and basophils. A series of immunohistochemically stained chronic myeloid leukaemia skin biopsies, with positive staining for mast cells and basophils. Tissues have been stained using toluidine blue, and imaged at a magnification of x4 using light microscopy. 2D7 positive identification of basophils in CML tissue; AA1 positive identification of mast cells in CML tissue; TBS image with no positive staining of cells of interest in CML. Scale bar= 500µm. 87

Figure 4-3: An image of nasal polyp, taken at x20 magnification using light microscopy. The tissue has been immunohistochemically stained using the mAb BB1, and toluidine blue. The image shows no positive staining for basophils or any other inflammatory cells, and an area of brown staining that is irregular and considered as an artefact. Scale bar= 100µm. 89

Figure 5-1: Sampling timeline for the tissue culture portion of the study. 95

Figure 5-2: A typical histamine standard curve. (Reproduced from: Oxford Biomedical Research, http://www.oxfordbiomed.com/histamine-eia-kit; accessed on 28/05/15). 98

Figure 5-3: The effect of an increasing concentration of honey on the concentration of histamine release (µg) by rat bladder explant, over the duration of 8 days. Note that in comparison to the 0% control, lower concentrations of honey (5% and 10%) have the greatest effect on the reduction in the release of histamine from explants (p<0.001 and p<0.01 respectively), in comparison to higher honey concentrations (20% and 40%). 101

Figure 5-4: The effect of an increasing concentration of honey on the concentration of histamine release (µg) by rat bladder explant (n=5), over the duration of 8 days (±SD bars). Note that the greatest difference within a group is seen with 0% honey, and the least variance within the 10% honey group...103

Figure 5-5: The effect of honey concentration on the % inhibition of histamine release from rat bladder explants over a period of 8 days (±SD bars). Note that 5% honey has the greatest inhibitory effect on the release of histamine from mast cells, and 40% honey has the weakest inhibitory effect. Additionally, 5% also exhibits the least inter-group variability as shown by the short standard deviation bars....105

Figure 5-6: The effect of an increasing concentration of honey on the concentration of histamine released (µg) by rat bladder explant. It can be seen that as the percentage concentration of honey is increased, there is a reduction in the quantity of histamine release from explants. The greatest reduction in the release of histamine is seen as honey is increased from 0 to 5%. 106

Figure 5-7: Toluidine blue stained rat bladder explants that were incubated for 4 days in either 0%, 5%, 10%, 20%, or 40% honey. Tissues have been stained using a standard histochemistry procedure, and imaged at a magnification of x2 using light microscopy. Notice the gradual loss of structure throughout the entire bladder explant, and the disintegration of the outer epithelial layer, as the honey concentration increases from 10% to 40%. Scale bar= 1mm...112

Figure 5-8: A representative schematic of confocal fluorescence microscopy. Laser light is focused onto a thick specimen by reflection from a dichroic mirror (DM) and the objective lens. The laser excites fluorescence throughout the specimen that passes through the DM and is focused onto the image plane. A pinhole only allows light from the confocal plane of the specimen to reach the photomultiplier tube (PMT). The image is then visualised using appropriate software. (Adapted from Sanderson et al. 2014). ..115

Figure 5-9: Mounting structure used for confocal microscopy. The structure consists of two coverslips secured together with a sticky tape frame, enclosing the tissue specimen of interest in mounting medium. 117

Figure 5-10: Fluorescent staining procedure....119

*Figure 5-11: Rat bladder tissue explant at day 4 in 40% honey. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide, and appear red. Image taken at x20 magnification using confocal microscopy. Note the appearance of bright green spots, which are indicative of clumped chromatin from non-viable cells. The * denotes possible epithelial cells disassociating from the remainder of the explant. Scale bar= 100µm.* 122

Figure 5-12: Rat bladder tissue explant at Day 4 in 0% honey. Rat bladder tissue explant at Day 4 in 0% honey. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide, and appear red. Image taken at x20 magnification using confocal microscopy. Note the absence of any obvious epithelial structure. Scale bar= 100µm. . 123

Figure 5-13: Rat bladder tissue explant at Day 4 in 5% honey. Rat bladder tissue explant at Day 4 in 5% honey. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide and appear red. Image taken at x20 magnification using confocal microscopy. Note the absence of any epithelial structure. Scale bar= 100µm. 124

Figure 5-14: Rat bladder tissue explants in increasing concentrations of honey. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide, and appear red. Images taken at x20 magnification using confocal microscopy. The red staining is indicative of non-viable cells that appear to be undergoing apoptosis. Scale bar= 100µm. 125

Figure 5-15: Signs of toxicity and apoptosis at the epithelial surface of rat bladder tissue. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide (PI), and appear red. Image taken at x20 magnification using confocal microscopy. 1) Blebbing of epithelial cells, 2) Excessive PI staining and loss of clear structure of tissue and cells, and 3) Nuclei are clumped, and these fragments appear brightly stained but are not indicative of viable cells. Scale bar= 100µm. 126

Figure 5-16: Rat bladder tissue explants of different sizes and shapes after incubation in 1.25% honey for 1 hour. Rat bladder tissue explants of different sizes and shapes after incubation in 1.25% honey for 1 hour. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using

Propidium Iodide, and appear red. Images taken at x20 magnification using confocal microscopy Scale bar= 100μm.......................................127

Figure 5-17: An example of how 3D rendering can be used to enhance a poor confocal image. On the left is a bladder explant ring which has been incubated for 1 hour in 10% honey and on the right is the same image after 3D rendering. Note that the 3D rendered image gives a clearer visualisation of the contours of the tissue, and the epithelial layer. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide, and appear red. Images taken at x20 magnification using confocal microscopy. Scale bar= 100μm.......................................130

Figure 6-1: MrgprB2 (the mouse mast cell basic secretagogue receptor) and mediates side- effects of peptidergic and small molecule therapeutic drugs. (Adapted from Grimbaldeston 2015). 144

Figure 6-2: Lidocaine-releasing intravesical system; LiRIS (TARIS Biomedical, Lexington, MA, USA). Ruler= 5 cm. (Adapted from Nickel et al. 2012). 145

Glossary of Terms

AL – Asthmatic Lung

AO – Acridine Orange

BIU – Biomedical Imaging Unit

CML – Chronic Myeloid Leukaemia

CS – Chondroitin Sulphate

CYP – Cyclophosphamide

DMSO – Dimethyl-Sulfoxide

ELISA – Enzyme Linked Immunosorbent Assay

ESSIC – European Society for the Study of Interstitial Cystitis

GAG – Glycosaminoglycan

GCP – Good Clinical Practice

GLP – Good Laboratory Practice

GRA – Global Response Assessment

HA – Hyaluronic Acid

HPCs – Haematopoietic Progenitor Cells

HRU – Histochemistry Research Unit

HS – Heparin Sulphate

ICAM-1 – Intercellular Adhesion Molecule-1

ICSI – Interstitial Cystitis Symptom Index

IHC – Immunohistochemistry

IL – Interleukin

IPF – Idiopathic Pulmonary Fibrosis

mAbs – Monoclonal antibodies

MC – Mast Cell

MC_T – Tryptase positive, Chymase negative Mast Cells

MC_{TC} – Tryptase positive, Chymase positive Mast Cells

NIDDK – National Institute of Diabetes and Digestive and Kidney Diseases

NGF – Nerve Growth Factor

NHS – National Health Service

NK-1R – Neurokinin-1 Receptor

NO – Nitric Oxide

PB – Painful Bladder

PBS/IC – Painful Bladder Syndrome/Interstitial Cystitis

PI – Propidium Iodide

PPS – Pentosan Polysulphate Sodium

PST – Potassium Sensitivity Test

SCF – Stem Cell Factor

SGH – Southampton General Hospital

SP – Substance P

TBS – Tris Buffered Saline

TNF-α – Tumour Necrosis Factor-α

UK – United Kingdom

UoS – University of Southampton

VAS – Visual Analogue Scale

WHO – World Health Organisation

DECLARATION OF AUTHORSHIP

I, [SHABANA TABASSUM MALIK] declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Interstitial Cystitis: Aetiology and Therapeutic Potential of Honey

....

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signed: ....

Date: 01/01/2018..

Conflicts of Interest

The author declares a conflict of interest in regards to a reference used as part of the assessment of the economic cost of PBS/IC in Section 2.8. The data was taken from a presentation produced in 2010 by Galen Limited; a pharmaceutical company that manufactures an intravesical agent for the treatment of PBS/IC.

.

Acknowledgements

They say it takes a village to raise a child…and I think the same could be said for a PhD.

Firstly, thank you to my family for their support, both financial and emotional, during this journey. Thank you to my parents, Mr. Riaz Ahmed and Mrs. Ghulam Kubra, for teaching me to work hard for what I want from life, and for believing that I can achieve anything I put my mind to. Thank you for providing me with an education, supporting my endless degrees, and for always being there for me. Without the example that you've set me, I would not be where I am today, and I would not the person that is currently writing this acknowledgment. So, thank you from the bottom of my heart, for being such amazing people, fantastic role models, and wonderful parents.

Thank you to my siblings for constantly questioning my PhD, and for pushing me to get the thesis done…FYI, it is finally finished! Mr Shakeel Malik, Mrs. Iram Malik, Mr. Aqeel Malik, and Miss. Humayra Malik, you all helped in your own ways, and I am truly grateful to have you all in my life. Amongst many other things, thank you for taking on my share of the household chores, making sure I was actually working and not online shopping, helping with the formatting of this document, and regularly reminding me that I had a PhD to finish so that I could get a real job. I am lucky to have such fantastic siblings, and I love you all so dearly.

After thanking my family, it's only fair that I now take the time to thank my friends. I feel privileged to have had the love and support from friends that I consider as my chosen family. To my inner circle of friends (Emma, Rachel, Micayla, Sarah, Harriet, Teresa, Aileen, Florence, Justine, Charlotte, Jennifer, Kate, Rob, Joanna, Natt, and Gessa), your love, generosity, care, and knowledge, have played a vital role in this PhD, and I am truly humbled by your kindness. I am lucky to have met you, and you all hold a dear place in my heart. Thank you for putting up with me, feeding me, housing me, and occasionally helping me to procrastinate.

Miss. Emma Louise Reeves, you my darling, have provided me with the longest and loveliest friendship since our chance meeting at school when we were only 4 years of age, and I am so grateful to still have you as a friend. You have always brought such joy to my life, and even on my most miserable days, you can turn my frown upside-down. You've been by my side, not just through my PhD, but throughout my life, and I cannot express just how much I appreciate you and your friendship. Thank you.

Thank you to one of my dearest friends, Mrs. Micayla MacDermot-Flynn. You are a marvellous woman, who told me to believe in myself when I needed it the most. Your kind gestures and support have helped drag me through some of the most difficult points of

this PhD, and I can't put into words how lucky I feel to have you in my life. Thank you for being you.

I would also like to thank my good friend, Dr. Florence Gannon. Darling, your kindness and support have been priceless during my academic journey. Although miles apart, we went through our PhDs together, and you always knew the right thing to say. You are inspirational, and your kind words of encouragement helped me so much. Thank you for being such a brilliant friend.

I would also like to thank my wonderful friend, Miss. Joanna Gould. I met you halfway through my time at the University of Southampton, your friendship, care, and support have been key in the success of this PhD. Thank you for the occasional lab supplies, listening to me rant, providing me with a place to live, and talking me into the most entertaining escapades. You are a remarkable human being, and I'm fortunate to have your friendship. Thank you for being a part of my life.

Mr. Robert Gray, you were by my side in real-life whilst I undertook this endeavour, and there is no doubt that you have contributed to the successful completion of this PhD. My fellow PhD-er and desk buddy, it has been a pleasure working beside you. Not only have you helped with the practical parts of this PhD, but you also gave me your time, kindness, and friendship. You are one of the nicest individuals I have ever met, and thank you so much for being part of this journey. I am going to miss our coffee breaks and lab parties!

Thank you to my star of a primary supervisor, Associate Professor Dr. Bashir Lwaleed. His supervision, guidance, and advice these past five years have been invaluable, and I am truly thankful to have been under his supervision. I would also like to thank my secondary supervisor, Dr. David Voegeli, for his on-going support and guidance. Without them, this doctorate could not have been completed.

I would also like to extend my most sincere thanks to Mr. Brian Birch for his helpful and thought provoking discussions. Thank you to consultant histopathologist, Dr. Vipul Foria, for his guidance and help with the histology work. I would also like to express my most sincere gratitude for the support provided by Professor Mandy Fader, Dean of Health Sciences. Without her continued support and belief in my work, this PhD would not have been successfully completed.

To the staff within the Faculty of Health Sciences, Biomedical Imaging Unit (NHS), the Histochemistry Research Unit (UoS), and the Histology Department (NHS); thank you for your kindness and assistance, it has been truly appreciated. Special thanks to Dr. David Johnston for his priceless knowledge and assistance with the fluorescent imaging portion of this study, and Miss. Jenny Norman for her invaluable immunohistochemical advice and for double-checking my dilutions. Without the two of you, a vast deal of this PhD wouldn't

have been possible, so thank you from the bottom of my heart. The amount of cookies that I would have to bake to repay you both for your support is infinite!

I would also like to thank you my examiners for reading this document and examining me in the defence of my doctorate. The end result is truly something that I am proud of, and this could not have been achieved without your guidance and corrections.

Lastly, I suppose I should thank myself. Thank you (soon-to-be-Doctor) Shabana Tabassum Malik. Although the journey was long, it was worth it. And just in case you forget the experience, to Future Dr. Shabana, one PhD is enough!

Chapter 1: Literature Review

This chapter will begin by discussing Painful Bladder Syndrome/Interstitial Cystitis (PBS/IC), the role of inflammation, and then critically evaluate the existing literature on the treatment options for PBS/IC. It will then conclude with a brief outline of the benefits of honey as an anti-inflammatory agent, and the possibility of using this as a novel therapeutic option in the treatment of PBS/IC.

1.1 Interstitial cystitis

Painful Bladder Syndrome/Interstitial Cystitis (PBS/IC) is a chronic inflammatory bladder condition, and symptoms include excessive urgency and frequency of urination, dyspareunia, chronic pelvic pain and pathogen-negative urine on culture (Metts 2001). Disturbances in the storage function of the bladder results in the symptoms that are associated with PBS/IC, such as urgency, and frequency.

The aetiology of PBS/IC remains poorly understood (Nordling et al. 2012) despite extensive efforts by the research community. A range of theories have been postulated including neuronal inflammation and proliferation (Christmas et al 1990), mast cell (MC) degranulation (Bjorling et al. 1999; Peeker et al. 2000; Theoharides et al. 2001), and defects in bladder permeability due to damage to the glycosaminoglycan (GAG) mucin layer (Figure 1.1; Bassi et al. 2011).

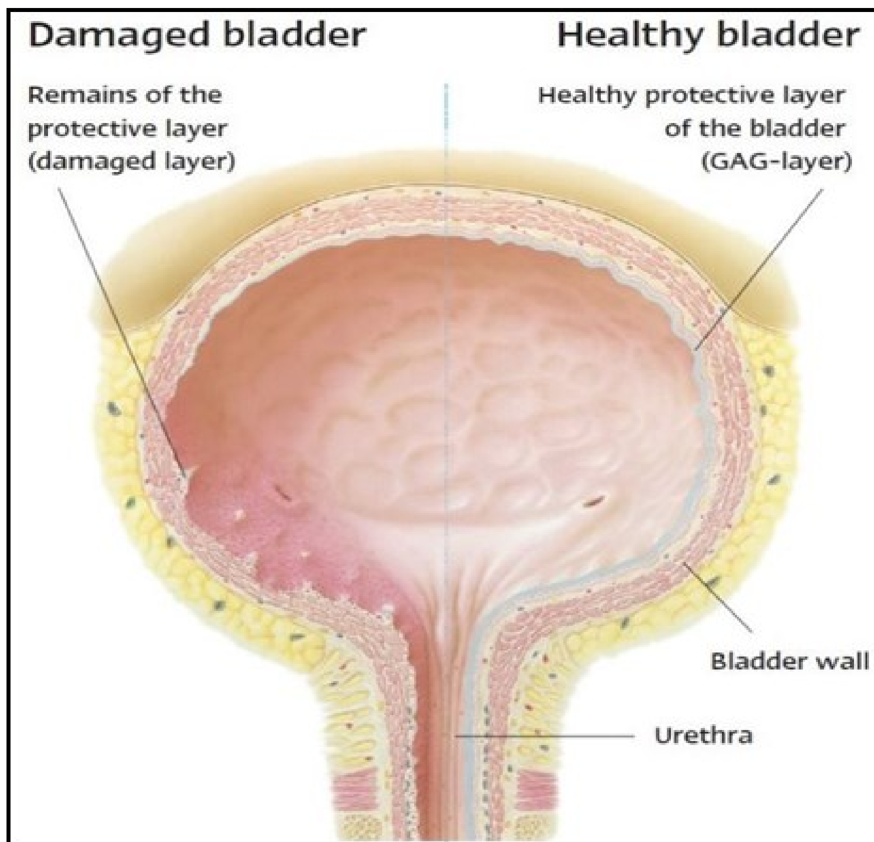


Figure 1-1: The bladder with a damaged GAG layer (left) and an undamaged GAG layer (right). (Reproduced from http://gepan-instill.co.uk/medical-conditions/; accessed on 14/05/15).

1.1.1 The glycosaminoglycan layer

The bladder's first line of defence against pathogens is a 10 – 20 μM thick GAG layer (Nickel et al. 1993; 1994). Common examples of GAGs include hyaluronic acid (HA), heparin sulphate (HS), and chondroitin sulphate (CS; Anand et al. 2013). GAGs are linear, negatively charged polysaccharide chains that can be found throughout the body, involved in a variety of cellular functions, including interacting with the cellular matrix, and activating chemokines, growth factors, and enzymes (Hiebert et al. 2014).

The GAG layer of the luminal surface of the bladder is composed mostly of negatively charged, sulphated GAGs (Hurst et al. 1987). This thin layer lines the luminal surface of the bladder, and is composed of a series of large linear polysaccharides, which in turn are composed of repeating disaccharide units of two simple sugars; an amino sugar (namely

N-acetylgalactosamine; derived from galactose) and a uronic acid (such as glucuronic acid), attached to a protein core (Favretto et al. 2014). The process of GAG formation is modulated by processing enzymes, and in the bladder, urothelial cells synthesize and secrete proteoglycans to form the GAG layer (Cervigni 2015).

The role of the GAG layer within the bladder is to act as an impenetrable and protective layer that prevents any potentially harmful substances from permeating and damaging the deeper layers of the bladder wall (Figure 1.2; Parsons et al. 1990). A functional deficiency in the structure of the GAG layer is thought to cause approximately 65% of cases of PBS/IC. (Hanno 2007). The GAG components thought to be affected in PBS/IC include CS, HS and HA (Hurst 1994), although the exact proportions of each GAG is currently unknown. This defective GAG layer leads to the leakage of harmful urinary components, including potassium which is able to depolarise nerves (Parsons 2010), through the damaged urothelium and into the interstitium where it may trigger an inflammatory response which involves the stimulation of MCs and cytokine release (Bjorling et al. 1999).

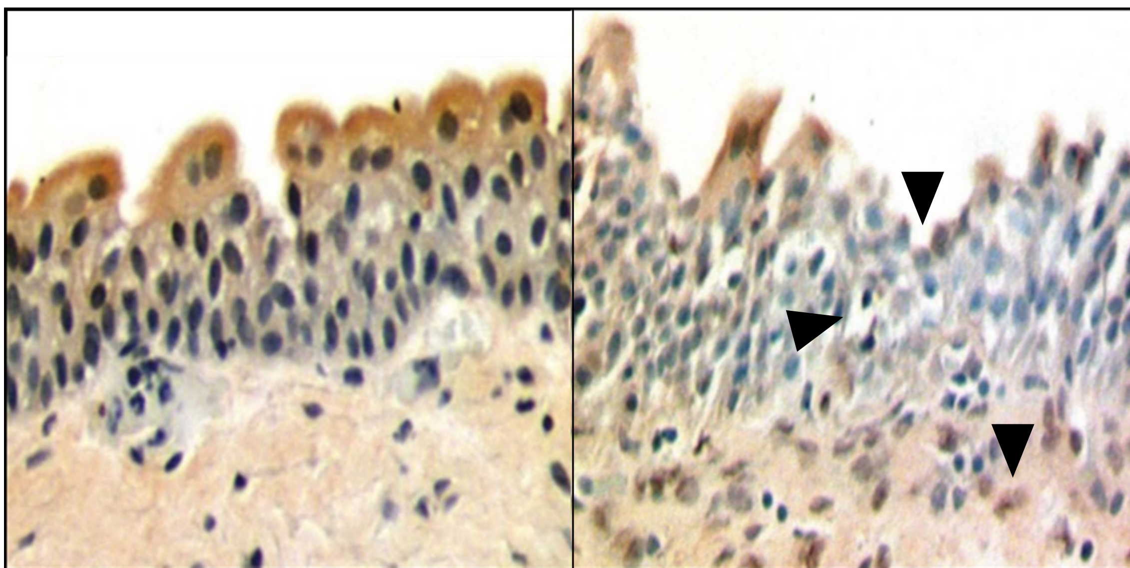


Figure 1-2: Bladder biopsies showing the presence (control; left) and absence (PBS/IC; right) of the GAG layer in human urothelium. Arrow heads indicate loss of structure. (Adapted from www.uracyst.co.uk; accessed on 12/05/2015).

1.2 Inflammation and painful bladder syndrome/interstitial cystitis

1.2.1 Inflammation

Inflammation is the protective or destructive (non-specific) response of body tissues to irritation or injury. The inflammatory pathway consists of inducers that initiate the inflammatory response and are detected by sensors (such as toll-like receptors) which lead to the production of mediators (Figure 1.3) and initiation of proteolytic cascades (such as the clotting cascade, the complement system, and the kinin system). The inflammatory mediators produced act on various target tissues to help adapt them to the noxious condition. Inflammation can be acute (i.e., of a short duration and self-limiting) or chronic (i.e., lasting from days to years).

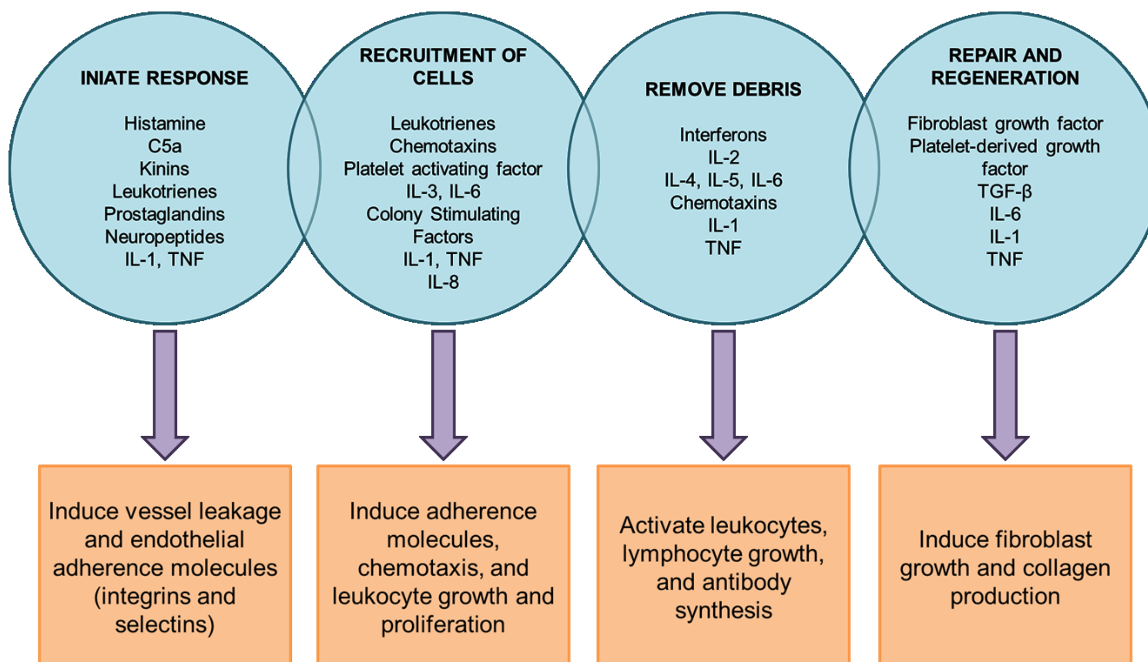


Figure 1-3: The main events and mediators in the inflammatory response. Note how each stage overlaps. (Adapted from McCance and Huether 2002).

The acute inflammatory response begins within seconds to minutes after tissue injury, and can be caused by trauma, oxygen or nutrient deficiency, or extreme temperatures. Acute inflammation is short in duration and self-limiting, and only lasts between a few minutes to several days. The main physiological processes that occur are an increased blood flow to the site of injury, an increased permeability of capillaries, the migration of neutrophils from the blood vessels into the surrounding damaged tissue, and the production of exudate as monocytes arrive at the site of inflammation to phagocytose microorganisms and foreign particles. Resolution of the acute inflammatory response occurs when the trigger is removed, the infection (if any) is cleared, and the damaged tissue has been repaired. If the inflammatory trigger is not removed by this initial inflammatory response, or if inflammation persists and resolution is not reached, a state of chronic inflammation is entered. Chronic inflammation is caused by unrepaired tissue damage, chronic infection, persistent allergens, or man-made foreign particles that cannot be digested. Under these circumstances, the chronic inflammation is usually localised to the site of the inflammatory trigger, and results in the remodelling of local tissues. Chronic inflammation lasts from days to years, and is often self-perpetuating. In addition to an increased blood flow and vascular permeability, chronic inflammation also involves a shift in the cell populations that are recruited to the site of inflammation from neutrophils to macrophages and lymphocytes, and the proliferation of fibroblasts, instead of the production of exudate seen in acute inflammation.

In addition to this, an increasing amount of chronic inflammatory disorders have been described in which the initial inflammatory trigger is not obvious, and does not seem to involve tissue damage or infection. These conditions are of particular interest as the number of individuals affected is rapidly increasing, and coincides with diseases that are more common in first-world countries (e.g. the UK and USA), such as cancer, Type 2 diabetes, obesity, and neurodegenerative diseases. These disorders are particularly self-perpetuating; for example, obesity can lead to chronic inflammation, and this in turn can induce insulin resistance and obesity-associated diabetes (Hotamisligil 2006). This positive-feedback loop can also be seen in other chronic inflammatory diseases (including neurodegenerative diseases, cancer, and atherosclerosis), and could be partially to blame for the chronic nature of these inflammatory conditions.

1.2.2 Painful bladder syndrome/interstitial cystitis

Painful Bladder Syndrome/Interstitial Cystitis is a chronic inflammatory disorder, which has periods of remission, and intense irritation. Initial bladder injury leads to a cascade of events, in which noxious substances leak through the damaged GAG layer into the bladder wall, leading to the activation of inflammatory cells and the release of inflammatory molecules which, in turn, continue to activate the inflammatory response, leading to further injury, and thus, becoming a self- perpetuating cycle (Figure 1.4; Evans 2002).

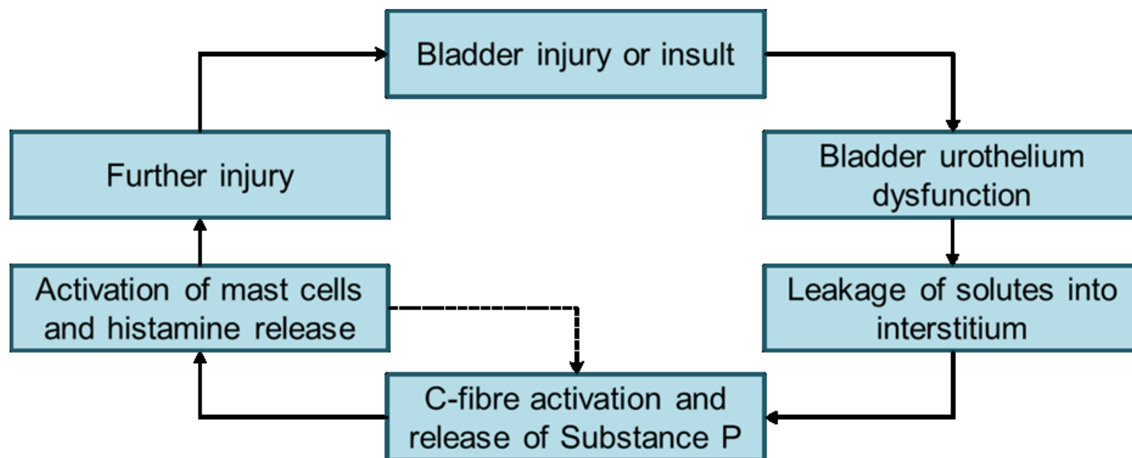


Figure 1-4: A possible model for the pathogenesis of PBS/IC. (Adapted from Evans 2002).

1.2.3 Mast cells and painful bladder syndrome/interstitial cystitis

The MC is a key player in the inflammatory process; responsible for the release of a variety of preformed mediators synthesised *de novo* and stored in cytoplasmic granules (e.g., tryptase, histamine, TNF-α, proteoglycans, lipid mediators, and cytokines such as IL-1, IL-6, IL-8) (Kobayashi et al. 2000). MC degranulation is triggered by a variety of molecules including Substance P (SP), stem cell factor (SCF), and nerve growth factor (NGF); factors that all have been implicated in the pathology of PBS/IC (Theoharides et al.

2012). It has been reported that dysfunctional or damaged urothelial cells generate cytokines such as SCF (Metcalfe 2008) that can promote activation and even proliferation of MCs, as bladder MCs are maximally activated by SCF (Sant et al. 2007).

Histological findings regularly identify a redistribution and proliferation of MCs into the subepithelium and detrusor layer (Aldenborg et al. 1986). The redistributed MCs then act on local smooth muscle and vascular epithelium, and also have a substantial effect on inflammation (Bouchelouche et al. 2006). Christmas et al. (1990) reported an increase in the density of nerve fibres within the sub-urothelial and detrusor muscle layers in patients with PBS/IC. This increase in the density of nerve fibres could possibly be due to the proliferative effects of vasoactive and inflammatory SP. In addition, a large proportion of these newly proliferated nerve fibres contain SP (Pang et al. 1995). SP is a potent stimulator of MC degranulation, and the consequent histamine release, in turn, induces the release of SP from sensory fibres (McCary et al. 2010), thus creating a positive feedback loop.

Additionally, Peeker et al. (2000) found that in classic PBS/IC there is a 6 to 10-fold increase in the redistribution of MCs into the epithelium and bladder wall, compared to only a 2-fold increase seen in non-ulcer/early PBS/IC. This characteristic proliferation of MCs in ulcerative PBS/IC may be implicated in the aetiopathogenesis of the disease, although this remains to be elucidated further.

While the aetiopathogenesis of PBS/IC is largely unknown, it has been shown that it is related to MC proliferation and activation at least in a subset of patients. Over the past decade, there have been an accumulating number of studies focussing on the role of MCs and subsequent inflammation in PBS/IC (Lv et al. 2012; Sant et al. 2007). These studies provided a substantial body of evidence suggesting that MC activation may be involved in the pathogenesis of PBS/IC, thus furthering the development of therapeutic agents aimed at inhibiting inappropriate MC activity in PBS/IC.

1.2.4 Basophils in painful bladder syndrome/interstitial cystitis

In addition to the increase in MCs in PBS/IC, it has been suggested that there is another inflammatory cell that takes part in PBS/IC; the basophil (Rebuck et al. 1963; Rivas 1997). These findings are not commonly reported in the literature, but the reasoning in support of the possibility of basophils playing a role in PBS/IC can be understood, as basophils and

MCs originate from the haematopoietic progenitor cell (Figure 1.5), and the cell into which they develop is dictated by the surrounding concentrations of cytokines and factors.

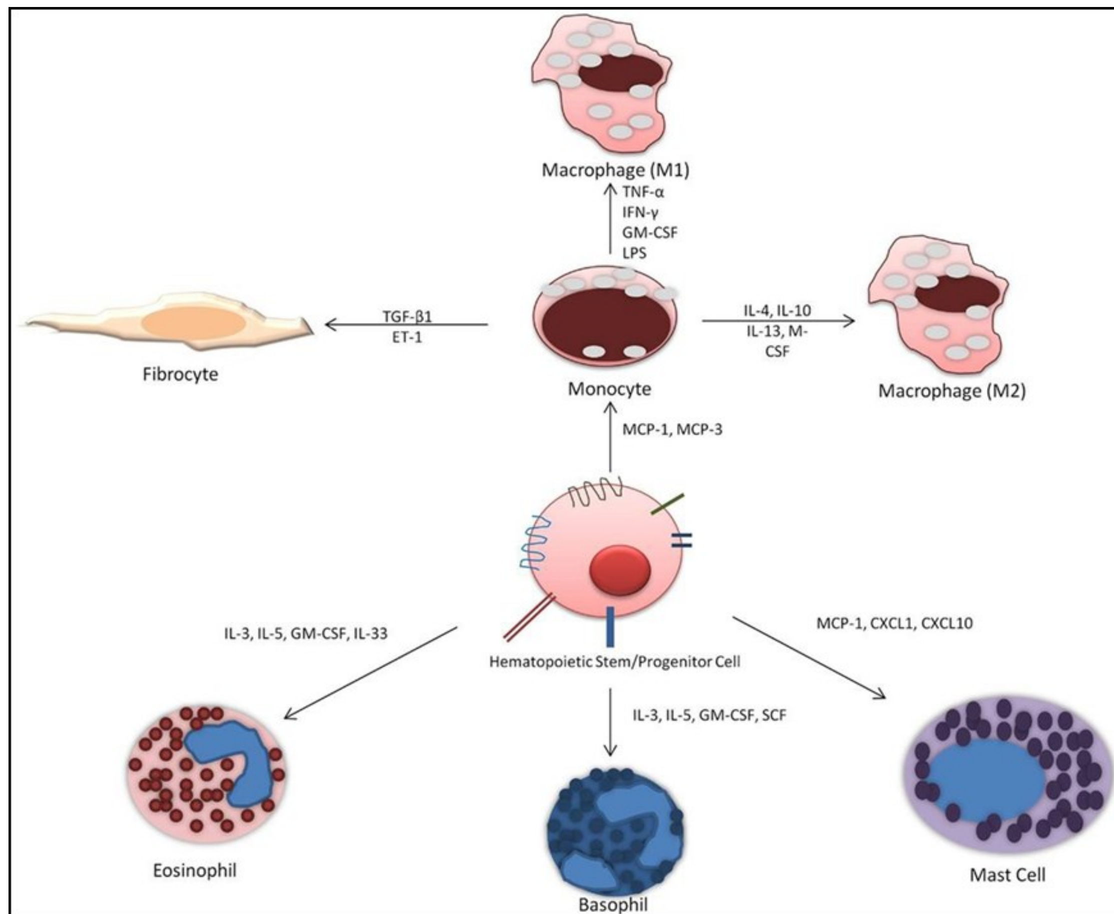


Figure 1-5: The differentiation of HPCs into immune cells, such as basophils and mast cells, dependent upon the local surrounding levels of cytokines and chemokines. (Adapted from Fischer and Agrawal 2013).

Basophils are a large granulocytic cell that make up approximately 1% of total blood count (Ito et al. 2011), mature in the bone marrow, contain histamine and leukotriene C4 granules, and degranulate upon Immunoglobulin E (IgE) cross linking. Unlike MCs, basophils have not been extensively researched, however there are certain key concepts in regards to the role of basophils that have been suggested.

Despite being the least numerous population of granulocytic cells, the accumulation of basophils has been noted in a range of diseases, including allergy, organ rejection, and

cancer (Siracusa et al. 2013). Basophils are increased during inflammation, especially in inflammatory skin disorders such as bullous pemphigoid and urticaria (Ito et al. 2011), atopic dermatitis (Mukai et al. 2009), parasitic infection (Eberle and Voehringer 2016), and asthma (Kimura et al. 1975).

Basophils and MC subtypes share many common features, including the types of mediators they release (Table 1.1). Mediators which could be of interest to PBS/IC sufferers are GAGs such as heparin in MCs (Lassila and Jouppila 2014), and chondroitin sulphate in MCs (Metcalfe 2008) and basophils (Dvorak and Morgan 1998). The commonality in regards to the mediators that MCs and basophils release mean that these inflammatory cells can have overlapping roles. Yet, the difference in their mediators and location within the human body also allows for MCs and basophils to play their own distinct roles, for example, basophils are primarily linked to in the induction of allergic diseases or in immune responses against parasites or ticks (Mukai and Galli 2013).

Table 1-1: The main mediators released from activated mast cell subtypes, MC_T and MC_{TC}, and basophils. (Adapted from http://what-when-how.com).

	Mediators Released from Activated Mast Cells and Basophils			
	Mediator	**MC_T**	**MC_{TC}**	**Basophils**
Granule Mediators; released after 1-5 minutes	Histamine	*	*	*
	Heparin	*	*	
	Chondroitin Sulphate	*	*	*
	Tryptase	*	*	
	Chymase		*	
	MC Carboxypeptidase		*	
	Cathepsin G		*	
	2D7 Antigen		*	*
Lipid Mediators; released after 5-30 minutes	LTC_4	*	*	*
	PGD_2	*	*	
Cytokines and Chemokines; released after minutes to hours	TNF-α	*	*	
	IL-4	*	*	*
	IL-8	*	*	*
	IL-13	*	*	*
	IL-3, 5, 6, 10, 16	*	*	

Much like MCs, basophils are activated upon cross-linking of IgE receptors (Figure 1.6). The specialized antibody IgE, has an anchoring site with a high affinity for corresponding receptors on the surface of MCs and basophils, and in the case of IgE, these are Fc-epsilon receptors (FcεR). When an antigen attaches to the basophil or MC-bound IgE antibodies, FcεR are cross-linked, and this leads to the release of inflammatory mediators such as histamine and interleukins.

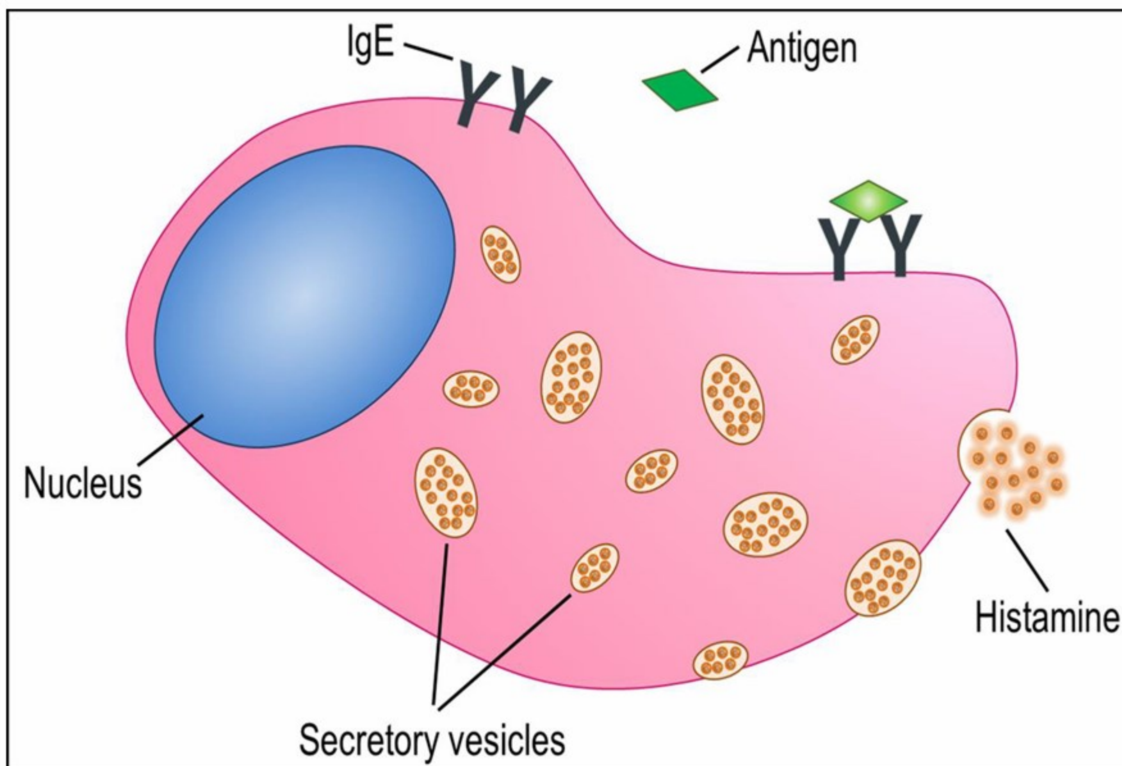


Figure 1-6: Representative image of a mast cell, showing the binding of an antigen to IgE leading to histamine release.

1.3 Current treatment options for painful bladder syndrome/interstitial cystitis

The majority of current therapies aim to repair the GAG layer that is thought to be damaged in PBS/IC, thus inhibiting the leaking of noxious substances from the urine into the surrounding tissues, and any subsequent activation of nerve endings and MCs.

Most commonly, patients are administered a short-acting intravesical instillation of a “cocktail” of heparin, lidocaine, and HA, in conjunction with exogenous oral sodium pentosan polysulphate (Parsons 2005). Intravesical pharmacotherapy provides an instant high drug concentration directly into the bladder by-passing any systemic side-effects and removing any problems related to the low level urinary elimination of orally administered treatments (Bassi et al 2011).

Although these current therapeutic options provide some relief to a subset of PBS/IC individuals, with a widely varying response between these individuals, there is still no wholly effective and well-tolerated treatment for PBS/IC. Rovner et al. (2000) found that for the 581 patients enrolled in the International Cystitis Data Base Study there were 183 different treatment modalities reported, thus indicating the variety of ineffective treatments currently offered to PBS/IC patients.

1.4 Honey

The benefits of honey have been described in a number of ancient texts (such as The Qur’an, the New Testament, the Iliad, and the Hebrew Bible), as well as being used in the repair of battle wounds during modern history e.g. World War I. The powerful healing effect of honey is well documented, as exemplified by its use in the successful treatment of wounds and infection (Cooper et al. 1999). The practice of using honey in wound healing was primarily rooted in folklore and traditional medicine, until the late 19th century, with the first reports of successful healing of infected wounds occurring mid-20th century (Oryan et al. 2016). It was then when investigators began to characterize its biologic and clinical effects, which have identified a number of its properties that make it a useful therapeutic agent. These include:

- An acidic pH (3.2 – 4.2)
- Ability to produce hydrogen peroxide by the action of the naturally occurring constituent enzyme, glucose oxidase, on glucose present in honey (Bang et al. 2003).
- Provide a suitably moist wound environment
- High sugar content which induces osmotic activity (Lusby et al. 2002).

The antimicrobial and wound healing properties of honey differ depending on its floral source (Irish et al. 2011). Manuka honey (sourced from the manuka bush, Leptospermum scoparium, which is found in New Zealand [Mullai and Menon 2007]) and Eucalyptus honey (typically sourced from Eucalyptus globules species) have been shown to have anti-inflammatory (Tonks et al. 2003; Lwaleed et al. 2010), antibacterial (Molan 2011), and pro-angiogenic properties (Rossiter et al. 2010).

These factors all contribute to a shorter healing time in cutaneous wounds (Lusby et al. 2002). Weston et al. (2000) utilised high performance liquid chromatography to highlight the active components (phenolic compounds such as methyl syringate, chrysin galangin, and phenolic acids such as vanillic acid and syringic acid) of a range of honeys (including manuka, heather, clover, and beech honeydew).

In recent years, there has been an upsurge of research investigating the anti-inflammatory property/activity of honey, which is also of importance to this researcher. In 2010, Kassim et al. found that the phenolic compounds, ellagic acid and ellagitannin, found in Malaysian honey, are able to inhibit inflammatory mediators such as TNF and Nitric Oxide (NO) *in vitro*. More recently, Kamaruzaman et al. (2014) looked at the effect of aerolised honey on airway inflammation in a rabbit model of ovalbumin-induced chronic asthma. Upon analysis, it was found that inhalation of aerolised honey resulted in structural changes of the epithelium, mucosa, and submucosal regions of the airways, as well as reducing the density of inflammatory cells present in lavage fluid.

1.4.1 Honey and mast cell degranulation

Results from an enzyme-linked immunosorbent assay (ELISA) indicated that in the presence of honey, there was an increase in cytokine production (IL-6, IL-1β, TNF- α) from monocytes (Tonks et al. 2003). These factors are known to play a crucial role in wound healing, accumulation of collagen, and angiogenesis (Molan 2011). In addition,

glucose in honey is converted to the proangiogenic molecule HA (Topham 2002). There are many cytokines involved in the inflammatory response, which act by increasing inflammation, reducing inflammation, or contributing to both (Figure 1.7). A cytokine that is known to be both pro-inflammatory and anti-inflammatory is IL-6. Tilg et al. (1994) found that following the application of honey, there was an increase in IL-6 levels that led to an increase in inflammation, but high levels of IL-6 then led to a decrease in inflammation by inhibiting the production of TNF-α, and stimulating the synthesis of an IL-1 receptor antagonist.

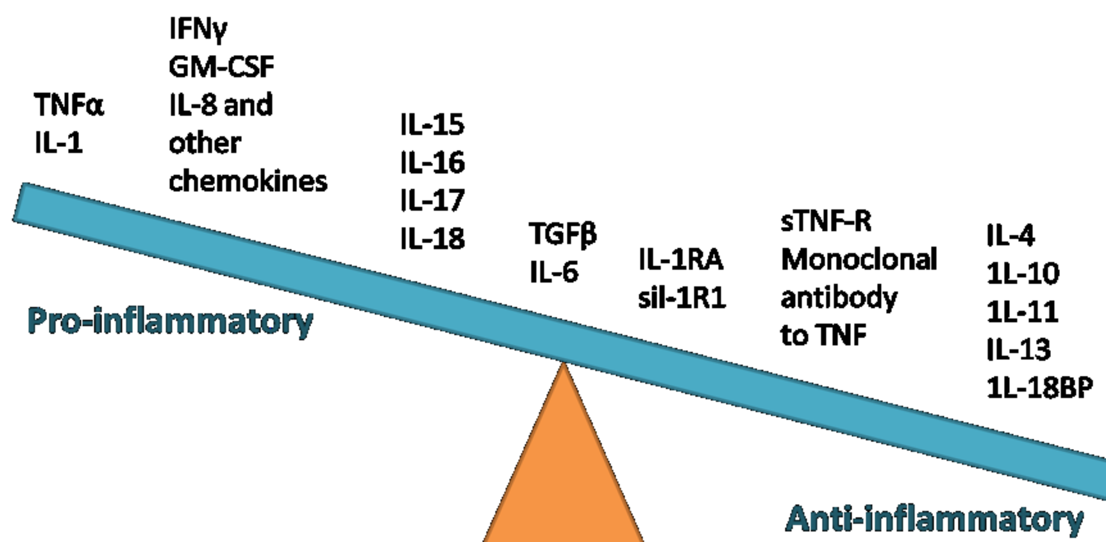


Figure 1-7: The cytokines that drive chronic inflammation. (Adapted from Arend 2001).

Honey is already known to significantly inhibit calcium-ionophore-induced MC degranulation (Nakashima et al. 2005), but there has been very little research into targeting MC in the development of PBS/IC treatments. Research from our lab (Birch et al. 2011) highlighted the protective effects of MediHoney® (a medical grade manuka honey from New Zealand) by pre-treating bladder explants with the honey before applying hydrochloric acid (HCl). The HCl was added to induce some damage to the bladder urothelium (i.e. representative of a damaged GAG layer as seen in PBS/IC). Staining with acridine orange revealed that honey does not have a damaging effect on the tissue (up to concentrations of approximately 20%), and furthermore had protected the cells in the middle of the explants from damage by HCl. In order to assess the effect of MediHoney® on histamine release, whole bladder was pre-treated with the honey before instilling SP and HCl, separately. Both instillations were found to provoke the release of histamine, yet pre-treatment reduced level of cell damage and led to a lower level of histamine release

when compared to bladders that had not been pre-treated with MediHoney®. Recently, Lwaleed et al. (2010) presented an abstract outlining the inhibitory effects of honey on MC degranulation by measuring histamine released from LAD-2 cells (a human MC line) after they had been stimulated by calcium ionophore A23187, or SP. This result in conjunction with other research implies that there is an association between honey and MC degranulation which needs further investigation.

1.5 Literature review search methods

A literature search was performed using the online databases PubMed, Embase (OvidSP), and Cochrane Database of Systematic Reviews (CDSR; The Cochrane Libraries), Web of Science (Topic Search) to find studies that had investigated current therapies used in the treatment of interstitial cystitis, and to specifically identify research into using honey as a treatment for wounds. The tables in Appendix A show the keywords and combinations used to search each of the aforementioned databases, and the total number of articles found in each database. The title and abstract of each of the identified papers were then reviewed, with exclusion of any unpublished research (with the exception of conference abstracts), and papers in a language other than English. The inclusion criteria were purposefully broad in order to collate all relevant research on the topics to be discussed, thus the searches were not limited by date or type of study.

1.6 Search results

The most studied treatments for PBS/IC were found to be the oral therapies sodium pentosan polysulphate, and amitriptyline, and the intravesical therapies HA, CS, dimethyl-sulfoxide (DMSO) “cocktail”, and lidocaine. These are briefly discussed below. In addition, a selection of the key papers assessing honey as a wound care agent will also be discussed in order to provide evidence for its anti-inflammatory activity, and thus support the rationale behind the use of honey as an intravesical therapy.

1.6.1 Oral therapies

1.6.1.1 Sodium pentosan polysulphate

Pentosan polysulphate sodium (PPS; marketed as Elmiron® by Ortho-McNeil Pharmaceutical) is an orally administered, highly sulphated, semisynthetic GAG, i.e. it has a similar chemical and structural properties to naturally occurring GAGs, and thus functions by providing a coating for the bladder. Much of the literature focuses on the modest benefits of the short-term administration of PPS in PBS/IC individuals. Although the exact mechanism of action of PPS in PBS/IC is currently unknown, it could be assumed that as PPS is binding to damaged portions of the GAG layer the drug is interacting at a molecular and perhaps enzymatic level.

One of the most comprehensive studies (Parsons et al. 1993) involved a randomized, prospective, double-blind, placebo-controlled study using 148 patients over 7 clinical centres. Findings indicated that 32% of the PPS treated group showed a statistically significant improvement in their PBS/IC symptoms (p=0.01; including a decrease in pain [p=0.04] and urgency [p=0.01], respectively), compared to only 16% in the placebo group. Although the treatment was well tolerated and side-effects (such as diarrhoea, nausea, and reversible alopecia) were mild, it should be noted that in some cases it took between three and six months for PBS/IC individuals to respond to the PPS therapy. In reality this waiting period for symptom improvement may cause some individuals to discontinue with treatment in search of a more rapid method to provide relief from particularly distressing symptoms.

More recently a study was conducted by Al-Zahrani and Gajewski (2011) in order to assess the long-term efficacy of PPS in patients with PBS/IC. They suggested that a longer period of exposure to PPS produced an improved response rate. The study failed to document any other therapies being undertaken by the participants and the primary outcome was measured by Global Response Assessment (GRA), which is not validated for long-term studies and creates a highly inaccurate prediction for studies longer than three months. Although the study provided a unique 'real-life' observation of the safety and efficacy of PPS, demonstrating that response to PPS increased with the duration of therapy, results were collected using retrospective techniques. This method of data collection has a higher likelihood of providing inaccurate or poor data (from incomplete or

falsely completed forms) as it relies on a patient's ability to recall how they felt more than three months ago. It should also be noted that many of the PBS/IC participants treated with PPS did not achieve the level of symptom relief they expected. It also seems that the researchers also either failed or chose not to provide data relating to any other therapies that may have been undertaken over the course of the study. Thus, the individuals that did persist with the PPS treatment and experienced a beneficial response may have had other forms of direct or indirect PBS/IC therapy.

1.6.1.2 Amitriptyline

One of the most used but least studied oral treatments for PBS/IC is the tricyclic antidepressant amitriptyline. To the best of our knowledge, the only prospective, placebo-controlled, double-blind study that has been conducted was by van Ophoven and colleagues (2004). Forty-four women and six men with PBS/IC were randomly allocated to either a titrated dose of amitriptyline (up to 100mg/day) or a placebo. The study was conducted over a period of four months, and although side-effects (such as tiredness, nausea, blurred vision, and/or dry mouth) were well tolerated, one patient from each treatment group dropped out from the study.

Results showed that 18 patients of the amitriptyline treatment group had a 30% or greater reduction in their O'Leary/Sant symptom and problem scores (Appendix B; French and Bhambore 2011), compared to only 13% in the placebo group.

Although the amitriptyline group showed a statistically significant improvement in symptom scores and improvement of pain and urgency intensity when compared with placebo, the improvement in frequency and functional bladder were not statistically significant from the placebo group ($p > 0.05$ in both cases). The study included relatively small numbers and so the conclusion that amitriptyline is an effective and safe treatment that leads to a reduction in pain, and nocturia in PBS/IC sufferers, needs to be accepted with caution.

Recently Hertle and van Ophoven (2010) conducted a prospective open-label study to examine the safety and efficacy of amitriptyline as a long-term treatment for PBS/IC. The results inferred that the long-term administration of amitriptyline is both safe and effective in the treatment of PBS/IC, with the patient group who had been selected using the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) criteria showing a uniform beneficial therapeutic response to the drug. However, the study was not blinded and thus is open to bias by both patient and researcher; patients were able to

self-titrate with no limitation on the maximum dose per day. This self-dosing may have contributed to the 79 patients (84%) who experienced an adverse effect and, ultimately, to the 31% drop-out rate due to either side-effect (mainly dry-mouth and/or weight-gain) or the lack of perceived improvement in PBS/IC symptoms.

1.6.2 Intravesical therapies

1.6.2.1 Hyaluronic acid

Hyaluronic acid (HA) is an anionic, non-sulphated GAG, which can be found in several types of tissue, including epithelial, connective, and neural. HA has gained popularity as a therapeutic biomaterial due to its versatility.

Applications are broad and varied, including wound healing, drug delivery, vascular repair, and gene delivery. In patients with PBS/IC, intravesical treatment with HA is able to partially replenish the damaged GAG layer. HA is one of the main constituents of the extracellular matrix, and is a major contributor to cell proliferation and migration. Within the urothelium, as part of the GAG layer, HA exhibits a variety of properties that contribute to several cellular processes, including inhibiting the adherence of immune complexes to polymorphonuclear and endothelial cells via intercellular adhesion molecule-1 (ICAM-1), augmentation of the healing process of connective tissue, and regulating proliferation of fibroblast and endothelial cells. In PBS/IC individuals, the degradation of the urothelial layer is thought to be responsible for the leaking of harmful constituents into the bladder wall, thus causing depolarisation of nerve fibres, urinary dysfunction, and the chronic pain associated with the disorder. It was previously discovered that urinary HA is higher in PBS/IC individuals compared to age-matched healthy controls, thus indicating that HA is leaking from the epithelium (and perhaps deeper layers of the bladder wall) into the urine (Erickson et al. 1998). Therefore, from a clinical perspective, GAG replacement therapy using HA intravesical installations appears to be a valid and justified treatment option for PBS/IC. However, this is yet to be reflected sufficiently in the literature, and as such, due to the considerable shortage of reliable data, the exact mechanism of action of HA for GAG replenishment in PBS/IC is still unknown. This is surprising, as amongst the PBS/IC intravesical therapeutic options, HA is one of the most researched, most likely due to its long-established use as a PBS/IC GAG replenishment therapy and popularity for use in combination therapy with CS (Cervigni et al. 2008, Porru et al. 2012, Gülpinar et al. 2014).

However, there has been some attempt at elucidating the mechanism of action of HA in PBS/IC. Leppilahti et al. (2002) conducted a small study (n=11) to evaluate the effect of intravesical HA on the intensity of ICAM-1 expression in PBS/IC patients. Before treatment, urine culture and cytology, and urodynamic studies were performed, along with deep-bladder biopsies from each patient during bladder distention, and specimens were stored at -70°C. Participants were asked to keep a voiding diary and VAS scores to assess severity of pain (Downie et al. 1978). All patients were then treated weekly with intravesical instillations of HA (Healon GV, 40mg/50ml) for 2 hours, over a period of 4 weeks. Following the final instillation, clinical examinations and VAS scores were repeated at 2 weeks, 4, 5, 8, and 12 months. Guided bladder biopsies were also taken at 2 weeks after the final instillation. By comparing the VAS scores, patients were grouped into 'long-term responders' (n=3; VAS scores improved by 50% and were sustained at the 4.5-month time-point), 'short-term responders' (n=5; those who had the same VAS scores at 2 weeks after the final installation, but requiring further treatment at 4.5 months), and 'non-responders' (n=3; those who had no improvement in symptoms or VAS scores over the entire study). Interestingly, there were no marked differences between the 'responders' and 'non-responders' before the treatment period, but after two weeks of treatment with HA, there was a 75% reduction in pain in the 'responders' group. Immunohistochemical analysis of the bladder biopsies, using anti-ICAM-1 monoclonal mouse antibody, revealed an increased intensity of ICAM-1 expression in PBS/IC individuals (in comparison to control tonsil tissue), with a marked increase in the 'responders' group. Although diagnostic bladder distention and intravesical HA instillations alleviated symptoms in most PBS/IC individuals, the exact reason for the improvements was unclear as the researchers failed to study patient groups who only underwent the bladder distention, the HA intravesical instillations, or no therapeutic intervention. Thus, the results should be interpreted with care. This is unfortunate, as there is a limited amount of research into the use of intravesical HA instillations and its effect on ICAM-1.

More recently, Rooney et al. (2015) attempted to clarify the mechanistic effect of intravesical HA on the secretion of cytokines, trans-epithelial permeability, and GAG secretion, using an *in vitro* model of IC (cultured human urothelial cells HTB4). In order to create a sufficiently representative model of PBS/IC, the cell monolayers were stripped of their GAG layer using protamine sulphate. The cells were then washed before allocation to treatment groups; basal media (control), HA (0.4 mg/ml), TNFα (10 ng/ml), or a combination of HA and TNFα (0.4 mg/ml and 10 ng/ml, respectively). The experiment was also conducted with the same groups but with intact cell monolayers, i.e., the cell monolayers had not been pre-treated with protamine sulphate. As the researchers were only interested in the acute effect of HA, all cells were treated for a total of 24 hours. At the end of this period, various assays (most notably; inflammatory protein array, sandwich

ELISA, and immunofluorescence, and scratch analysis) were conducted using the supernatant, and urothelial cells, to measure cytokine, and tight junction expression. Additionally, the effect of HA on the presence of GAG and cell infiltration was measured histochemically using bladders from female Sprague Dawley rats (n=12). Rats were randomly assigned to treatment groups; either PBS (500 µl), HA (0.4 mg/ml, 500 µl) which were delivered intravesically by catheterisation, or cyclophosphamide (CYP; 50mg/250g) via interpretational injection. The animals were then culled 24 hours later, and the bladders were collected and processed for analysis via standard histochemistry. This study found that HA can significantly (4 to 5 fold) decrease the induced secretion of pro-inflammatory cytokines IL-6 and IL-8, double the production of secreted sulphated GAGs (at both *in vitro* and *in vivo* levels), and a decrease trans-epithelial permeability without altering the expression of tight junctions. These results suggest that the mechanism of action of HA in PBS/IC bladder is of great importance and a crucial target for the development of any potential intravesical therapy. It is commendable that Rooney et al. (2015) created a controlled and randomised study that thoroughly evaluated the effect of HA in PBS/IC. This study was the first to provide molecular-level evidence of the direct effect that HA has on inflammation, epithelial permeability and the synthesis of GAG. Additionally, the decrease in IL-6 following intravesical HA was replicated *in vivo* in a chronic cystitis rat model (Lv et al. 2013). In this study, chronic cystitis in female rats (n=10) was created using long-term intermittent intravesical instillations of protamine sulphate (0.5 ml; 30 mg/ml) to damage the GAG layer. Intravesical HA (0.5 ml; 0.8 mg/ml) was also instilled in an alternating fashion. Cystometrographic analysis showed that protamine-sulphated induced rats (before undergoing instillation of HA) had significantly shorter intervals between bladder contractions, a smaller bladder capacity ($p<0.001$), and severe chronic inflammation. Additionally, analysis using immunohistochemistry, reverse transcriptase polymerase chain reaction, and ELISA revealed a significantly increased expression of IL-6 ($p<0.001$). However, after intravesical instillation of HA, these results were markedly improved; both bladder capacity and inter-contraction intervals were significantly increased ($p<0.001$), and degree of bladder inflammation and IL-6 levels were significantly decreased ($p<0.001$). In addition, statistical analysis showed a positive correlation between IL-6 levels and degree of inflammation ($r= 0.727$; $p<0.001$), and a strong negative correlation between IL-6 levels and frequency of voiding ($r= -0.761$, $p<0.001$). It was concluded that intravesical administration of HA led to a decrease in IL-6 levels, in PBS/IC induced rat bladders. Also, the researchers suggested that due to the correlation between IL-6 and degree of inflammation, and frequency of voiding, IL-6 should be regarded as a key marker and target for therapeutic intervention in PBS/IC.

As the research of inflammatory modulators in PBS/IC has grown, researchers have looked to investigate a range of lesser known cytokines that are thought to play a role in

PBS/IC, including NGF. This signalling protein is known to be produced by the urothelium and smooth muscle of the bladder, and this would make it an appropriate marker in the study of IC. A recent study conducted by Jiang et al. (2014) found that PBS/IC patients who were given intravesical HA instillations experienced an improvement in symptoms, and a reduction in pain. These beneficial results were thought to be linked to the significant decrease ($p<0.05$) in urinary NGF, however as with other popular PBS/IC treatments, this response was only seen in those who responded to intravesical HA treatment. Of the 33 PBS/IC patients, 14 individuals experienced a reduction in pain (reflected in a reduction in VAS score by ≥2), and 7 experienced a decrease in GRA of ≥2.

Additionally, after HA therapy, NGF levels in responders and non-responders' groups remained higher than controls (34.2±10.6 compared to only 2.25±0.91 in controls; $p<0.01$). In addition to the small sample size (n=33 PBS/IC patients), another main limitation of this study was that, although urine samples were taken from all subjects involved in the study for cytokine analysis using an ELISA, the researchers did not perform any cystometrographic studies in order to evaluate the bladder function of their participants.

In accordance with the NIDDK, a bladder capacity of more than 350 ml (during awake cystoscopy) is an exclusion criterion in the diagnosis of PBS/IC, yet clinically, PBS/IC sufferers possessing a bladder capacity of above 350 ml are also recognised, and thus, cystoscopy is often used as an investigative method in the study of intravesical PBS/IC therapies. A previous study of note that investigated the value of cystometry and bladder capacity, in relation to predicting the benefit of intravesical HA therapy in certain PBS/IC sufferers, was conducted by Daha et al. in 2005. A moderate number (n=48) of PBS/IC patients exhibiting clinical symptoms and a positive potassium sensitivity test (0.4M) were included in this study. First, a control value was taken for the C-max (maximum bladder capacity) of the participants, using a saline solution (0.9% NaCl). The results from this were used to separate the subjects into two groups; Group 1 with a C-max of more than 350 ml, and Group 2 with a C-max of less than or equal to 350 ml. The participants then underwent cystometry with the 0.2 M KCl solution, and results were used to further subdivide the groups according to the percentage reduction in C-max; Group 1a/2a had a reduction of less than or equal to 30%, and Group 1b/2b who had a reduction of greater than 30%. Patients then underwent 10 weeks of a weekly 40mg HA installation. Patients were also asked to use the VAS to score PBS/IC pain before, during, and after the therapy. VAS scores showed that HA instillations led to symptom relief in all groups, i.e., irrespective of C-max. However, interestingly, the improvement in VAS scores post-treatment was particularly noticeable in Group 1a/2a (i.e., those who had a reduction of ≥30% after cystometry with 0.2 M KCl), which indicates that intravesical HA instillations

may be of the most benefit to PBS/IC sufferers that have a C-max of ≥350 cc, and an increased potassium sensitivity. When this study is considered in conjunction with the previously discussed study by Jiang et al. (2014), the individuals that did not respond successfully to the HA therapy may have had a C-max of below 350 ml, or may not have possessed an increased potassium sensitivity.

In terms of randomised trials assessing the use of HA in human, Lai et al. (2013) recently designed a prospective randomised study to effectively compare the clinical effectiveness of a range of different regimes of intravesical HA instillations in PBS/IC patients (n=60, which fell to n=59 at the end of the study). The patients were split into two groups; in the 'hyaluronic acid-9 group', 30 patients received four weekly 40 mg HA intravesical instillations followed by five monthly instillations. In the 'hyaluronic acid-12 group', 30 patients received a fortnightly intravesical instillation of 40 mg HA, every 2 weeks, for a total of 12 weeks. Symptoms were monitored using the VAS to measure pain, Interstitial Cystitis Symptom Index (ICSI), Interstitial Cystitis Problem Index (ICPI), urinary frequency and nocturia (via a voiding diary), functional bladder capacity, maximum flow rate, volume of voided urine, post-void residual volume, and Quality of Life Index (QoLI; this was only at 1, 3, and 6 months). At the end of the study, there was a significant ($p \leq 0.003$) improvement in symptoms (as shown by the improvement in the ICSI, ICPI and total score, VAS score, functional bladder capacity, flow rate, and the QoLI) after 6 months, for both groups. Additionally, the 'hyaluronic acid-12' group also benefitted from a significant improvement in urinary frequency ($p<0.05$) and void volume ($p<0.01$). Results also showed that although the two groups differed in response at the 1-month mark (the 'hyaluronic acid-9' responded better than the 'hyaluronic acid-12' group), the response rate at 3 and 6 months between the two groups did not differ significantly. These results imply that the initial difference in success that is seen within the first month is due to the quick repeat in installations as experienced with the weekly instillation regime of the 'hyaluronic acid-9' group, but by the month 3, the bladder has received an adequate dose of HA, and the frequency or interval between instillations no longer causes much effect. Overall, there was no significant difference between the two regimes, but HA still had a beneficial effect in PBS/IC patients. As with most research on PBS/IC treatment, there was no control group or placebo. This is understandable, especially when the individuals who are enrolled into the study may be undertaking existing treatments for symptom relief or management. This study also failed to replicate results from a much cited pilot investigation by Morales et al. 1996. This study looked into the use of intravesical HA (40 mg) in the treatment of PBS/IC patients (n=25) and suggested that after an initial successful response rate of 56% at 1 month, there was an increase in positive response to 71% at 3 months, which was maintained until week 20. According to the authors the results were "gratifying" and may lead to the start of a large, controlled, multi-centre

clinical trial. However, they could be accused of over interpretation of their findings due to the nature of PBS/IC. Indeed, subsequently the same group conducted a trial, which was designed to provide more objective evidence for the use of the same intravesical HA that had been used in their pilot study. It should be noted, that this multicentre study cannot be found currently in the literature as a full publication.

As with many of the PBS/IC therapies, there is a need for good quality research and randomised controlled trials to further elucidate mechanisms of action, especially when the treatment is already in use in clinical practice. Randomisation avoids both selection bias and any unintentional bias, and along with sufficient numbers, controls, and placebos, are key experimental measures in elevating a therapy to a trustworthy 'gold-standard'. Until such studies are available, intravesical instillations of HA should not be unquestionably endorsed for use for the treatment of PBS/IC (Iavazzo et al. 2007), or at least, they should be considered more for use in PBS/IC individuals with a C-max of >350 ml on awake cystometry (which is an NIDDK exclusion criterion for the diagnosis of PBS/IC) and a >30% reduction in bladder capacity with 0.2 M KCl (which indicates increased potassium sensitivity), as the previous study by Daha et al. (2005) indicated that it is these individuals who are more likely to have a successful outcome with HA intravesical therapy. The popularity of HA for therapeutic intervention in PBS/IC is not unfounded, and thus, in an effort to improve symptom relief, a combination HA and CS has also been looked at. The rationale behind this is that HA and CS would act by improving the function and integrity of the GAG layer as they are both important compounds of the GAG layer, with anti-inflammatory properties. In 2012, Cervigni et al. conducted a small study (n=12; female) looking at the long-term effect (3 years) of intravesical HA (1.6%) and CS (2.0%) instillations.

Patients underwent bladder emptying via catheterisation, followed by a 1 hr HA- CS intravesical installation every week for 20 weeks, then once every 2 weeks for 4 weeks, and then once a month for 3 months. Patients were asked to keep a 3- day voiding diary, a VAS for pain, urinary urgency and frequency, and answer various questionnaires (such as ICSI and ICPI). Results showed that improvements in bladder function were sustained for 3 years, with daily voids falling from an average of 17.8 times, to 15.5 at 9 months, and 11.9 at 3 years, and voiding volume increasing from 136.8 ml, to 143.9 ml at 9 months, and 180.9 ml at the end of the 3-year period. This quantitative data was supported by the qualitative data from the quality of life assessments. Although the study was small, and lacked a control group, results seemed both interesting and promising.

Several years later, the drawbacks of the Cervigni study were addressed by another research team (Gülpinar et al. 2014) who decided to also look into HA-CS intravesical installations. In addition, Gülpinar et al. (2014) investigated the possible clinical benefits of

HA alone, versus HA-CS. They recruited patients with a history of PBS/IC who had previously responded poorly to PBS/IC treatments (such as dietary restriction, amitriptyline, and oral pentosan polysulphate). Patients were either treated with 50 ml HA (n=32) or a combination of 1.6% HA and 2% CS (n=33). In total, all patients received 8 doses spread over the duration of the study

(4 months). Following a potassium sensitivity test (PST), patients received weekly intravesical instillations for a month, then every 15 days for the 2nd month, and once a month for the 3rd and 4th months. Symptoms and pain were evaluated using a VAS, ICSI, ICPI, voiding diary (for nocturia and frequency), cystometry (for bladder capacity), and voided volume at the beginning of the study, and then at 6 months. Results showed that approximately 72% of patients had a positive PST, with no significant difference between the two groups ($p>0.05$). At 6 months, the responses for VAS, ICCS, ICPS, voiding frequency, nocturia, had improved for both groups, but there was no significant difference in the improvement of symptoms ($p>0.05$). Eight patients were also reported to have experienced mild adverse events such as urinary tract infection (UTI) (n=6), or an increase in urinary frequency, urgency, or incontinence (n=6). Although, this was the first published study comparing the two treatments in PBS/IC individuals who had previously responded poorly to other treatments, results were not as promising as expected. The properties of HA combined with CS should have produced a successful treatment, yet this was not the case in this study. The study did show that there was no significant difference between the two treatments, although the addition of controls and a placebo group would have provided more valuable comparisons. This study was not a Randomised Clinical Trial (RCT), and as stated previously, this type of study design is difficult to implement for a variety of reasons, but mainly for this study by Gülpinar et al. (2014), the main consideration was ethical.

1.6.2.2 Chondroitin sulphate

Chondroitin sulphate (CS) is a sulphated GAG which is thought to provide relief from PBS/IC symptoms by binding to the damaged portion of the PBS/IC bladder and temporarily restoring impermeability of the mucous layer, by covalent bonding of the GAG attachment sites (Iozzo and Schaefer 2015). CS differs from GAG, HA, as it is the only sulphated GAG that is located on the luminal surface on the bladder urothelium, and contributes directly to urothelial barrier function (Janssen et al. 2013). Akin to HA, CS has also been shown to play a role in the inflammation associated with PBS/IC. Engles et al.

(2012) demonstrated that treatment with CS led to a decrease in the recruitment of inflammatory cells (such as neutrophils and MCs) in a rat bladder model of acute IC. Hauser et al. (2009) assessed the efficacy of CS in binding to damaged urothelium and restoring the permeability barrier of the bladder wall, as this was previously undetermined. Binding was determined quantitatively using fluorescence microscopy and CS-bound with Texas Red® fluorochrome (a red fluorescent dye with a peak of absorption at 589 nm) in a mouse model. After damage to the bladder urothelium with HCl had been induced, the ability of CS to restore normal barrier function was measured by the movement of intravesically instilled 86Rb (a potassium ion mimetic) from within the bladder, across the damaged urothelium lining, and into the bloodstream. Although the degree of urothelial damage varied from mouse to mouse, it was uniformly shown that CS bound more tightly to impaired urothelium than it did to any undamaged lining, thus restoring the permeability of 86Rb to that of control levels. This suggests both a primary role for the GAG layer in PBS/IC, and highlights the preference of CS for binding to damaged urothelium and effectively restoring normal GAG permeability. Although this study was conducted in a mouse model, there are many other aspects of PBS/IC in humans that cannot be represented in a mouse model of urothelial acid damage. It is thought that over 95% of the mouse genome is similar to human (Mural et al. 2002), and this in addition to low maintenance and storage costs, space, lifespan, and ease of ethical approval (in comparison to acquiring approval for human studies), explains why mouse models are commonly used, and are still regarded as a cornerstone of the advancement in biomedical research.

A non-interventional study was conducted in 2008 (Nordling and van Ophoven 2008) to study the effectiveness, tolerability and safety of a popular commercially available CS instillation (Gepan®instill). Although the study was not focussed purely on PBS/IC, it included patients (n=286) with a range of chronic forms of cystitis, such as PBS/IC, radiation cystitis, overactive bladder syndrome, and chronically-recurring cystitis. Over three months, patients received 8 instillations, and symptoms were documented. Results found that all of the main symptoms of chronic cystitis disorders (urgency, nocturia, pain, and bladder capacity), decline significantly ($p<0.0001$) and consistently over the duration of the study. The CS instillation was well tolerated, and an effective therapy in the treatment of the chronic forms of cystitis that were investigated in this study. This was most likely due to a deficiency in the GAG layer of these individuals, as the components of this layer includes sulphated GAGs such as HS, dermatin sulphate, and CS. Although the results were statistically significant, there is a need for confirmation with further controlled studies.

Following promising results from preliminary work, Nickel et al. conducted a small pilot study (2012) into the safety and effectiveness of CS. The study randomised PBS/IC women into either a drug treatment group to receive 8 weekly instillations of 20ml of 2% CS, or a control group in which they received 8 weekly instillations of 20ml of an inactive control solution. Positive results gathered from the GRA at week 11 (3 weeks after the last instillation) formed the primary endpoint, with the secondary endpoint comprising a positive response to the ICSI also at week 11. Additional data for endpoint effectiveness included changes from the baseline to week 11 in the total ICSI score voiding diary, and VAS for pain/discomfort. At the endpoint visit, results showed that a slightly higher proportion (38.0 %) of patients from the group treated with CS reported either a marked or moderate improvement in their PBS/IC, compared to the control group (31.3%), however the difference was not statistically significant ($p>0.05$). This response pattern was also seen in the ICSI and VAS pain scores, with the active treatment group reporting a larger decrease in ICSI and VAS pain scores in comparison to the control group, although the difference was again not statistically significant. Thus, this study shows that although using intravesical CS therapy in the treatment of PBS/IC yields a small positive difference in some individuals, the results do not support this therapy as an adequate form of treatment for individuals with PBS/IC.

Furthermore, the non-significant difference between the groups was thought to be, at least partially, down to a poor patient selection strategy. It was suggested that results would have been better if the selection strategy had focussed on recruiting patients who had a bladder-specific clinical phenotype. This method of patient recruitment may have introduced selection bias, the pathology of a patients PBS/IC does vary subset-to-subset, and so, implementing a more focussed recruitment strategy concentrating on patients that would benefit most from GAG-replenishment instillations may be a consideration for future CS research. Although this is a valid criticism, other factors may have influenced the study findings. For example, based on previous studies reporting that CS has a low success rate in treating IC.

1.6.2.3 Dimethyl-sulfoxide

Dimethyl-sulfoxide (DMSO) was the first of two Food and Drug Administration (FDA) approved drug for bladder therapy and has since been one of the main pharmacological treatments for PBS/IC (Hung et al. 2012) due to its success in treating PBS/IC symptoms (Melchior et al. 2003). Indeed, DMSO is one of the most studied and recommended

intravesical therapies (Fall et al. 2008; Hanno et al. 2010) as a result of its anti-inflammatory, analgesic, and MC stabilisation characteristics (Hung et al. 2012). This drug is usually administered as a single- agent solution at a concentration of 50%, and DMSO treated bladders have been reported to exhibit a reduction in inflammation and expression of inflammatory markers e.g. the presence of polymorphonuclear and MCs (Soler et al. 2008), and a dose-dependent impairment of effector T-cells (Kim et al. 2011).

Although DMSO is the main clinical choice in the pharmacological intervention of PBS/IC, its use has been declining since its discovery as an intravesical agent as it is considered to be toxic and cause irreversible changes in bladder structure. For example, Melchoir and colleagues (2003) examined the effect of DMSO on the structure and contractility of the bladder wall by exposing strips of rat bladder tissue to concentrations of DMSO varying between 25% and 50%. Results showed that at a threshold DMSO concentration of 40% or above, there was a total and irreversible abolishment of contractility. In order to increase symptom relief in patients who do not respond to single-agent intravesical therapy and decrease likelihood of toxicity, a lower concentration of DMSO can be used in conjunction with other therapeutic drugs (most commonly heparin, lidocaine, and sodium hyalurinate) and is often referred to as a DMSO “cocktail” (Hung et al. 2012). This intravesical “cocktail” seems to be particularly effective due to the DMSO acting as a vehicle agent that aids the diffusion of the other constituents into the bladder wall in order to evoke improved relief of symptoms (Parkin et al. 1997). Despite the frequent prescription of intravesical DMSO “cocktail” as a PBS/IC therapy (Dasgupta and Tincello 2009), not all users will experience relief from PBS/IC symptoms. In a twelve-month study, Stav et al. (2012) reported an efficacy of 61% in PBS/IC patients treated with a weekly instillation of 50% DMSO (50ml), 0.5% bupivacaine (10ml), 10,000 U HS (10ml) and 100mg of hydrocortisone (5ml) for twelve weeks. The patients were assessed every three weeks during the treatment, and then followed-up 1,3,6,9 and 12 months after the treatment period had ended. A subset of participants was still feeling symptom relief from the intravesical therapy at the 12-month evaluation. Although generalisation is difficult as the study was only made up of 51 individuals, the results are typical of what is seen clinically.

1.6.2.4 Lidocaine

Lidocaine, also known as lignocaine and zylocaine, is used as a local anaesthetic to numb a specific area, usually before surgery. Lidocaine is used in a range of disorders, and over

the past decade or so, it seems to have also gained popularity as an intravesical therapy for PBS/IC. The interest in its use in PBS/IC, dates back to 1989, when Swedish researchers, Asklin and Cassuto (1989), published a case report on a patient with severe interstitial cystitis, who exhibited the hallmark symptoms of PBS/IC (urinary incontinence and painful urgency). The patient was administered repeat intravesical instillations of lidocaine, and the result was a long lasting relief from pain, and an effective reduction in inflammation of the bladder wall visualized using cystoscopy.

Almost a decade later, Gurpinar et al. (1996) conducted a small study involving 6 patients with PBS/IC to evaluate the possible use of electromotive administration for intravesical lidocaine. All patients were treated with an aqueous solution of lidocaine (1.5%) and 1: 100,000 epinephrine, delivered with intravesical electromotive drug-assisted (EDMA) therapy. This form of drug-delivery is achieved by passing an electric current between a urethral catheter which contains an electrode (inserted into the bladder), and an electrode applied to the suprapubic skin. The current allows ionized drugs to bypass the lining of the bladder (via the process of active transport), and penetrate into the deeper layers of the bladder wall, thus leading to a greater quantity of drug delivered further into tissue, in comparison to passive diffusion. The rate at which the medication is delivered can be controlled by altering the strength of the current. In this study, the electrical current was slowly increased from 0 to 15 mA whilst the lidocaine and epinephrine solution was held within the bladder, for 40 minutes. Immediately post-treatment, there was a decrease in voiding symptoms, and both perineal and suprapubic pain, and 4 of the 6 patients had longer lasting benefits. A similar pattern of results was seen in a study conducted by Rosamilia et al. (1997). This research team also used EDMA for drug delivery, but the solution used in this study was a combination of lidocaine and dexamethasone (a steroid used in the treatment of rheumatic arthritis, asthma, severe allergies, and inflammatory skin conditions, such as purpura). A total of 21 female PBS/IC patients underwent EDMA with lidocaine and dexamethasone, followed by cystodistention to assess the level of bladder anaesthesia. Results were promising, with an increase in the mean tolerated cystodistention volume by three-fold (200ml to 600ml), following therapy. Follow-up at 2 weeks showed that 85% of individuals had a reduction in frequency and pain by 3 points, and at 2 months, 63% were still responding, with 25% of individuals having a pain score of 0. Although these results were encouraging, there is a need for a similar study to be conducted, but with randomisation and blinding, in order to truly assess the effect of lidocaine, and the potential benefits of EDMA as a drug delivery system.

After the release of the above publication (Rosamilia et al. 1997), the literature showed a shift away from using EDMA as a method for intravesical lidocaine delivery in PBS/IC. Instead, research temporarily focussed on developing the right combination of lidocaine

and another PBS/IC drug, that could be delivered intravesically as was normally achieved using other instillations, such as HA and CS. Parsons (2005) had great success with an intravesical instillation, that was composed of 40,000 U heparin and either 1% or 2% alkalinized lidocaine. The solution was administered intravesically to newly diagnosed PBS/IC patients (n=82; Group 1% n=47, Group 2% n=35) who had significant urinary urgency, frequency, and pain. Both groups showed improvements, with the greatest success seen in Group 2%. All patients of Group 2% reported symptom relief within 20 minutes of the instillation, with 94% reporting a significant immediate relief in their symptoms. After 4 hours, this dropped to just 50% still experiencing symptom relief. However, following six repeat instillations over the course of two weeks, 80% reported a significant level of sustained symptom relief. It was concluded that intravesical treatment with a combination of heparin and alkalinized lidocaine could greatly benefit newly diagnosed PBS/IC individuals. As symptom relief was sustained beyond the duration of the anaesthetic effect of lidocaine, it was suggested that the solution may have had a suppressive effect on neurologic upregulation. This is of great interest; as neurogenic inflammation plays an important role in the pathogenesis of PBS/IC. As has been previously mentioned, activated MCs in turn will activate capsaicin-sensitive fibres, which release SP, and other neuropeptides, that also activate MCs and cause cellular damage. The success of a combination of alkalinized lidocaine and heparin was later repeated by Welk and Teichman (2008), and although the study was not as large (n=23), there was still a marked improvement in symptoms such as voiding, pain, and dyspareunia.

Following the success of Parsons (2005), he went on to conduct a single-blind study comparing a solution of heparin and alkalinized lidocaine, against alkalinized lidocaine alone, for the treatment of PBS/IC (Parsons et al. 2015). In this pilot study, 14 PBS/IC patients were instilled with either a heparin-lidocaine instillation, or alkalinized lidocaine alone. Separately from this, the serum levels of the lidocaine for heparin- alkalinized lidocaine and USP un- alkalinized lidocaine alone were measured by high performance liquid chromatography (HPLC). When compared to lidocaine alone, results showed the heparin-lidocaine combination therapy to be more effective in the treatment of the main symptoms of PBS/IC. There was a significant reduction in the percentage of bladder pain (38% vs. 13%; p=0.029), and urgency (42% vs. 8%; p=0.003). In addition, the GAR was also significantly improved in the heparin-lidocaine group, after 1 hour (77% vs.50% in the lidocaine only group, p= 0.04) and at 24 hours after treatment (57% vs. 23% in the lidocaine only group, p=0.002). Mean serum lidocaine levels revealed significantly (p= 0.019) higher values for the heparin-lidocaine combination (0.45 µg/mL), compared to the USP unalkalinized lidocaine alone (0.20 µg/mL). The higher serum levels from the heparin-lidocaine combination indicates better and greater absorption of lidocaine, thus

indicating that this may be responsible for the significant symptom improvement seen in the PBS/IC patients of the heparin- lidocaine combination group.

The potential of lidocaine as an intravesical agent for PBS/IC was clear, yet a new delivery method needed to be developed in order to ensure a continuous high dose directly to the bladder wall. Nickel et al. (2010) developed a coil shaped continuous 'lidocaine-releasing intravesical system' (LiRIS) that could be retained within the bladder and release a therapeutic dose of lidocaine into the urine over a period of two weeks. The team tested the device in healthy and PBS/IC individuals, and found it to be well-tolerated by both groups. This was thought to be due to its compact size, thus allowing it to move freely within the bladder, whilst dispersing lidocaine into the urine, and subsequently, the bladder wall in PBS/IC women (n=16) who met the NIDDK criteria for bladder haemorrhages or Hunner's lesions. Participants received either LiRIS 200mg or LiRIS 650mg for 2 weeks. Results showed that both groups tolerated the lidocaine well, and experienced a clinically meaningful decrease in urgency, voiding frequency, and pain. On day 14, the LiRIS was removed, and cystoscopic examination showed improvements in bladder wall appearance, including resolution of Hunner's lesions in 5 of the 6 individuals who had baseline lesions. The GRA is a favoured questionnaire used as the primary end-point in PBS/IC therapy clinical trials as it is balanced at 0 (i.e. the patient experienced no change), and in this trial, results from the GRA showed a responder rate of 64%, which was sustained at a follow-up at day 28. Furthermore, an extended follow-up several months after removal of the device suggested that the participants were still benefitting from a reduction in pain, possibly due to the promotion of some healing by the drug.

1.7 Honey as a therapeutic agent

All species of honey are made up of approximately 40% fructose, 35% glucose, 20% water and 5% sucrose. This basic composition provides energy and also contains many amino acids, vitamins, minerals, and enzymes such as glucose oxidase which produce the antimicrobial hydrogen peroxide (Sato and Miyata 2000). In addition to this, its effectiveness is attributed to its physical properties of hygroscopocity, hypertonicity, and complex chemical composition (Al-Waili et al. 2011). Honey also creates an acidic environment with a pH ranging between 3.2 and 4.2 (Al-Waili et al. 2011), which contrasts with the raised alkaline environment associated with chronic non-healing wounds (Gethin

et al. 2008). Thus honey will lower the wound pH by reducing protease activity, raising fibroblast action, and increasing oxygen release, all of which should aid wound healing (Al-Waili et al. 2011). Much of the existing evidence for the use of honey involves its use in wound healing as it possesses many properties, such as anti-bacterial, anti-inflammatory, and promotion of angiogenesis, that make it a useful therapeutic agent.

Tonks et al. (2003) used an ELISA assay to assess the release of inflammatory cytokines (TNF-α, IL-1β and IL-6) from MM6 cells (derived from human acute monocytic leukaemia cell line) in the presence of three different honey formulations, namely manuka, pasture and jelly bush. It was found that when incubated with each honey MM6 cells (and human monocytes) had a significantly higher release of TNF-α, IL-1β and IL-6, in comparison to untreated cells and those that had been incubated with artificial honey (a sugar syrup composed of 1.5 g sucrose, 7.5 g maltose, 40.5 g fructose and 33.5 g glucose dissolved in 17 ml of endotoxin-free distilled water). These results suggest that honey has a stimulatory effect on the release of inflammatory cytokines from monocytes, which are known to play a vital role in wound healing and tissue repair. Stimulation of inflammation is advantageous to the healing process as it aids blood flow to the site of injury, recruitment of the cells necessary for successful wound healing, production of exudate and expulsion of debris, and resolution. It could be assumed that an increase in IL-6 levels as a result of application of honey would lead to an increased inflammatory effect due to an amplified release of IL-6, but high levels (> 10ng/ml [Tilg et al. 1994]) of this cytokine act as an anti-inflammatory agent by preventing the release of IL-1 and TNF-α, and stimulating the production of an IL-1 receptor antagonist (Tilg et al.1994).The main uses for honey in wound care were found to be in the treatment of topical wounds, infected wounds, non-healing wounds, and burns. Maghsoudi et al. (2011) conducted a study in order to assess the efficacy of topical honey dressings versus the antibiotic mafenide acetate on fresh partial thickness burns. Histological evidence of healing in the form of epithelialisation and a reduction in acute inflammation was seen by day 7 in 80% (40 patients) of the honey-treated wound group, in comparison to only 52% (26 patients) in the antibiotic-treated group. By day 21, all 100% of the wounds treated with honey dressing had healed, compared to only 84% in the mafenide acetate-treated group. Furthermore, there was a higher incidence of a subjective alleviation of pain in the honey-treated group, which is of importance when considering the desired outcomes of PBS/IC sufferers. Oryan and Zaker (1998) applied 5ml of pure untreated honey topically, via a dressing, to cutaneous wounds on rabbits. By day 7 there was noticeably less oedema, fewer polymorphonuclear cells (neutrophils; typically seen in early acute inflammation) and mononuclear cell infiltrations (lymphocytes; associated with chronic inflammation), improved epithelialisation, and enhanced wound contraction. These effects ultimately led

to an improvement in both tissue organisation and improved tissue strength as was seen postoperatively on day 21.

The angiogenic properties of honey are not as well represented in the literature, however research by Rossiter et al. (2010, addressed this niche by using *in vitro* analogues of angiogenesis in addition to an endothelial proliferation assay to investigate the angiogenic potential of a variety of honeys, including medicinal honey (Activon®), European and non-European mixed-flora honey (Rowse®), and a honey based ointment (Mesitran®), versus a sugar syrup artificial honey (70% w/w sugar glucose/fructose). Results showed that all honey preparations were able to successfully stimulate the formation of pseudo-tubules from a rat aortic ring *in vitro*, with maximum growth at a honey concentration of 0.2%. Stimulation of angiogenesis, along with the stimulation of, granulation, and epithelialization, leads to an increased rate of healing (Molan 2011).

Research has highlighted honey as a satisfactory therapy that provides a moist and osmotic environment for sterile and accelerated wound healing (Benhanifia et al. 2011; Sukur et al. 2011; Maghsoudi et al. 2011; Mohd Zohdi et al. 2012), with few to no adverse effects (Al-Waili et al. 1999; Subrahmanyam 1991). It is also considered to be cost-effective (Subrahmanyam 1991) in comparison to antibiotics and other conventional treatments (Al-Waili et al. 1999; Maghsoudi et al. 2011; Subrahmanyam 1998). However, it must be noted that none of these studies were blinded and so it can be argued that there may have been researcher and/or patient bias. Moreover, the majority (approx. 87%) of randomized trials involving honey were conducted by the same researcher (Subrahmanyam 1991; 1993; 1994; 1996; 1998; 1999).

1.8 Discussion

As the pathophysiology of PBS/IC is not fully known, treatment options largely aim to improve symptoms and have mixed success. The literature on PBS/IC treatments is vast and therefore varied, with no single therapy declared as the “gold standard”. The current treatment options that have been discussed here fail to deliver satisfactory results, with, on average, each treatment option alleviating PBS/IC symptoms and pain scores in ≤61% of the PBS/IC therapeutic intervention group (Stav et al. 2012).

The majority of studies collated through this literature review support the finding that honey is superior to conventional treatments. As honey has already shown success in treating disorders that involve varying levels of inflammation (i.e. superficial wounds, ulcers, and partial-thickness burns), it may have a great potential for other inflammatory disorders that also require healing (by reepithelialisation), such as PBS/IC. An intravesical honey-based therapeutic has the potential to reduce the level of MC degranulation (Lwaleed et al. 2010), and the chronic inflammation and pain associated with the condition.

The management of PBS/IC is evolving, and due to the existence of sub-sets of PBS/IC patients, it can be argued that there is no singular fully inclusive etiological mechanism that can adequately explain the disease. As such, it is highly unlikely that there is one “gold standard” treatment that can treat all PBS/IC patients, and the search for this non-existent “cure” is therefore virtually pointless. Rather, as has been indicated by the literature, treatments that have previously deemed unsuccessful can be considered as successful in a particular sub-set of patients. Thus, it is the pathophysiology of each of the sub-sets of PBS/IC patients that needs to be researched and established. Once this has been achieved, the findings can be extrapolated to the clinical setting to develop treatments that address the underlying pathology of a particular sub-set of PBS/IC patient.

Chapter 2: Painful Bladder Syndrome/Interstitial Cystitis

2.1 The urinary system

The urinary system is a group of organs that play a key role in the removal of the waste product, urine, from the human body. This major excretory system consists of the kidneys, ureters, urinary bladder, and urethra. Figure 1 outlines the position of the organs and vessels involved in the urinary system, and the location of this system within the female body. The urinary system produces urine through the process of filtration, re-absorption, and tubular secretion. In addition to the filtration and elimination of waste, the urinary system also helps to maintain homeostasis by maintenance of osmoregulation, blood pH, levels of ions (such as Cl-, Na+, K+, and Ca2+), normal blood pressure (by secretion of the enzyme renin), red blood cell production (by secretion of the hormone erythropoietin), and re-absorption of required substances into the blood.

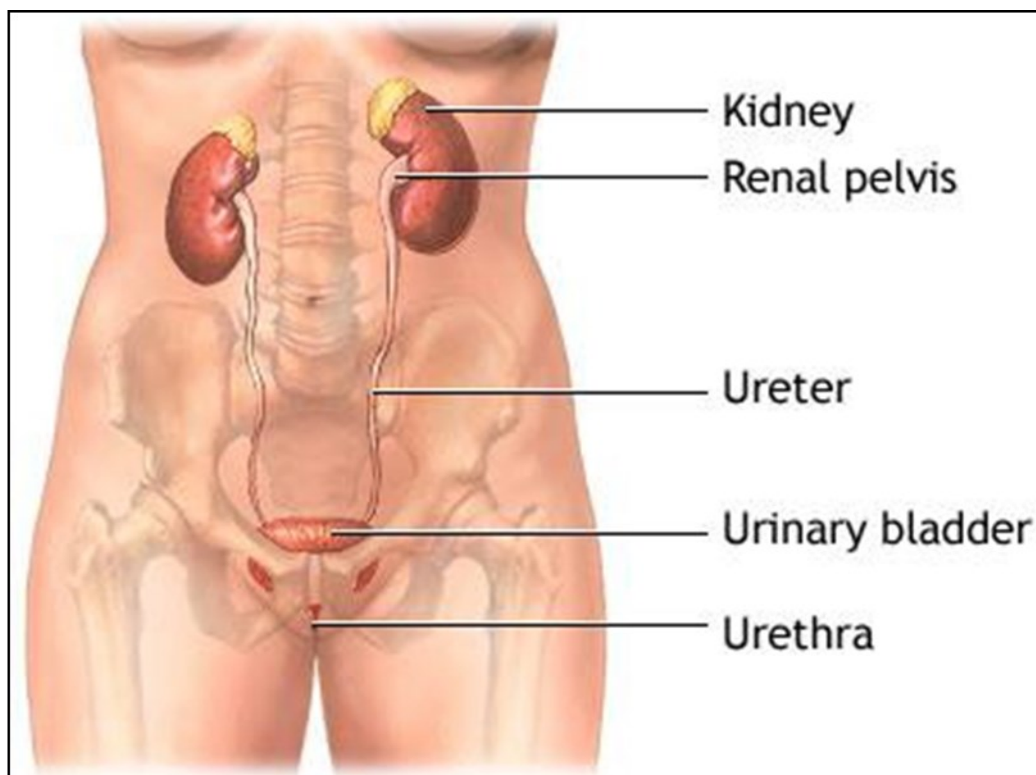


Figure 2-1: An overview of the urinary system. (Reproduced from: Keck Medicine http://keckmedicine.adam.com; accessed on 29/06/17).

The production of urine begins with the synthesis of urea in the liver, via the urea cycle, either by oxidation of amino acids (from protein), or by the donation of amino groups from ammonia and L-aspartate. Urea then dissolves into the blood, and is transported to the kidneys for filtration. It is crucial for organisms to convert ammonia to the larger and less volatile compound urea, as a high concentration of ammonia would increase the pH within cells to a toxic level.

After filtration by the kidneys, urine flows through the ureters to the bladder. Although the exact composition of urine varies according to diet, urine is primarily composed of water (at least 95%), followed by urea, Cl-, Na+, K+, and creatinine. Once the urine reaches the urinary bladder, it is stored until it can be completely expelled from the body through the tubular structure, the urethra, via the process of urination.

2.2 The urinary bladder

The urinary bladder is an elastic and hollow muscular organ that sits within the pelvic cavity, on the pelvic floor; above and behind the pubic bone (although the exact location differs between men and women due to the presence of the reproductive organs), and below the parietal peritoneum.

The urinary bladder is a temporary reservoir for urine, and thus the two main purposes of the bladder are to store a variable volume of urine that has been filtered by the kidneys and, upon complete filling, expel the urine from the body through the urethra. In adults, a healthy functional bladder is able to painlessly maintain a low intravesical pressure (<10 cm H_2O) during filling, and hold up to 500 ml of urine (Tasian et al. 2010).

The bladder fills from the apex of the trigone (a smooth triangular region of the internal urinary bladder) via the ureters, which are tubular structures that allow for the transfer of urine from the kidneys to the bladder via the process of peristalsis (waves of contraction produced by the muscular walls of the ureters). The bladder fills in a relaxed phase (adaptive relaxation) in order to create the low pressure required for filling, and empties through the simultaneous action of the distal urethral sphincter relaxing, and the

contraction of the bladder. Although the role of the bladder seems simple, micturition is the result of a complex interaction between the inner lining of the bladder, bladder smooth muscle (the detrusor), and the nervous system.

2.3 The bladder wall

The bladder wall is made up of three well-defined layers; the mucosa, the detrusor muscle, and the adventitia (although the submucosa can also be classed as its own layer). The overall structure of the bladder can be seen in Figure 2.2, with clear definition between the layers of the bladder wall.

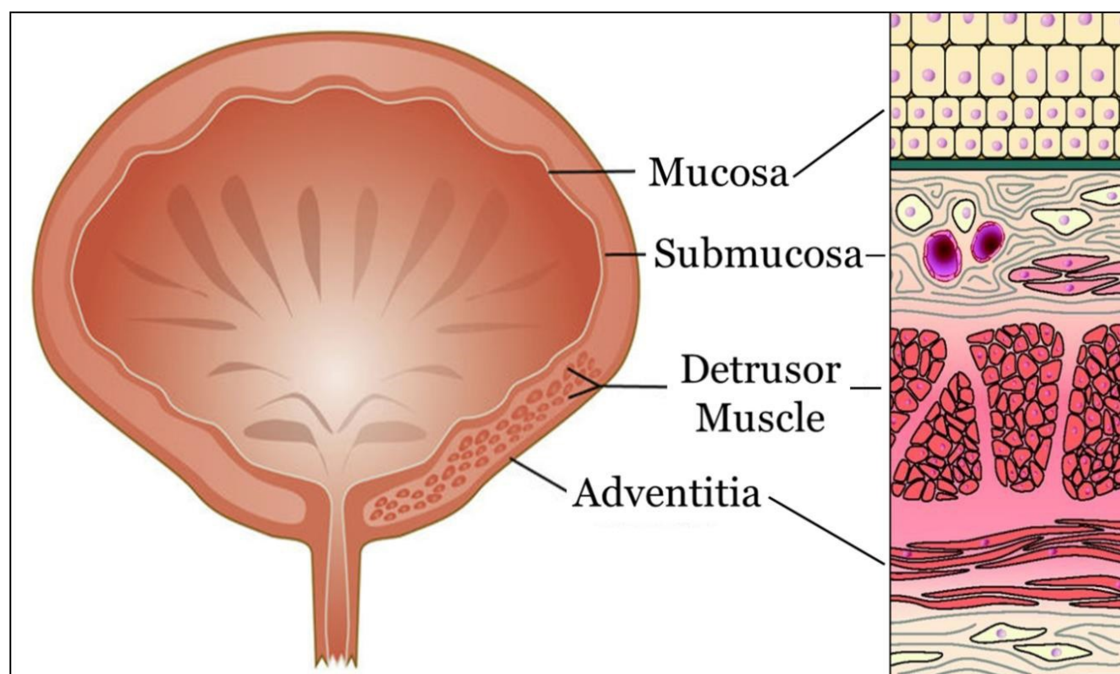


Figure 2-2: The main layers of the bladder wall from the luminal surface (mucosa) to the deepest layer (adventitia). (Adapted from Birder and Andersson 2013).

2.3.1 The mucosa

The innermost layer of the bladder, the mucosa (mucus membrane), is mainly made up of the urothelium, submucosa, and lamina propria, in addition to smooth muscle cells, and the muscularis mucosae. The urothelium, is a layer of transitional epithelium that uninterruptedly lines the majority of the urinary system, from the renal pelvis through to the ureters, bladder, and the upper urethra (and the glandular ducts of the prostate in men). Transitional epithelium itself is made up of several layers of cells, which are able to compress and flatten when the bladder fills with urine. When the bladder is empty, the transitional epithelial cells are relaxed and unstretched, and the folds (rugae) of the mucosa are visible. The epithelial layer of the mucosa contains no blood or lymphatic vessels, and has a single-celled basement membrane that lies between the epithelium and the lamina propria.

2.3.2 The lamina propria

The second layer, that supports the mucous membrane, is the submucosa. It is composed of connective tissue with elastic fibres. It has been noted that the human bladder may not have a true submucosal layer, so, the mucosa layer is most distinguishable by the lamina propria. The lamina propria, also known as the submucous coat, is an areolar connective tissue layer interlaced with a muscular coat which provides elasticity. The lamina propria is made up of several types of cells (such as fibroblasts, adipocytes, interstitial cells, and sensory nerve endings), in addition to lymphatic vessels, blood vessels, elastic fibres, nerves, smooth muscle fascicles, and in some areas, glands.

2.3.3 The detrusor muscle

Directly below the submuscosa, is the detrusor muscle (muscularis propria of the urinary bladder); a thick muscular layer that itself is made up of three layers of smooth muscle; the inner longitudinal, middle circular, and outer longitudinal. The muscle fibres are interwoven in all directions, and collectively, these layers are referred to as the detrusor muscle. When voiding is appropriate, the parasympathetic nervous system is activated,

and causes the detrusor muscle to contract. Upon contraction of this muscle, urine is expelled from the bladder through the internal urethral sphincter, into the urethra, and out of the body.

2.3.4 The adventitia

The outermost layer of the bladder is the perivesical fat layer, which contains the adventitia and serosa (made up of fibrous tissue and blood vessels). The serous coat is a reflection of the peritoneum, and only covers the superior surface and the superior part of the lateral surfaces. Any area that is not covered by the serosa, allows for the joining of the connective tissue between organs.

2.3.5 The GAG layer

At the innermost surface of the bladder, above the mucosa, exists a glycosaminoglycan layer (GAG). The GAG layer is formed from a string of repeating disaccharide molecules attached to a protein core, through the action of sulfotransferase enzymes (Kusche-Gullberg and Kjellén 2003). This layer can be visualized using histochemistry techniques with either by Alcian blue staining, or by staining for chondroitin sulfate (Engles et al. 2012). This GAG layer exists to act as a barrier from the harmful substances within urine, such as potassium ions and urea. The GAG layer is also thought to have an osmotic barrier function, and acts as an antibacterial lining that ensures a reduced adherence of potentially harmful bacteria (Gomelsky and Dmochowski 2012). In healthy individuals, the GAG layer is intact and acts as a sufficient barrier, however, a damaged GAG layer, caused by noxious pathogens (chemotherapy, exposure to radiation, autoimmune diseases, and chronic bacterial infections) may lead to loss of the GAG layer protective function.

2.4 Bladder disorders

Although the bladder may appear as a simple organ, there are still many bladder- related disorders that can occur. Common bladder conditions include urinary incontinence (loss of bladder control), overactive bladder (involuntary urination), bladder cancer, bacterial cystitis (inflammation of the bladder involving a bacterial infection), and the disease of interest in this project, interstitial cystitis.

2.5 Painful bladder syndrome/interstitial cystitis

Painful bladder syndrome/Interstitial cystitis (PBS/IC) is classified as a chronic inflammatory bladder condition, hallmarked by the presence of chronic bladder pain. At present, approximately 400,000 people in the United Kingdom (UK) have PBS/IC, and of these, over 90% are women. Although PBS/IC affects females in a 9:1 ratio (Payne et al. 2007), the incidence of PBS/IC amongst men has been increasing, possibly due to a higher number of men seeking medical aid, or being correctly diagnosed after a misdiagnosis of Chronic prostatitis/Chronic pelvic pain syndrome; a condition which presents with symptoms very similar to PBS/IC.

2.5.1 History

The first indication of a condition similar to PBS/IC was made in 1808 by the prominent surgeon, Philip Syng Physick (1768-1837). He was the first to describe an inflammatory condition of the bladder with an ulcer, which produced the same symptoms as those caused by a bladder stone (Parsons and Parsons, 2004).

Physick then continued to develop this concept by incorporating the observed symptoms of chronic frequency, urgency, and pain in the absence of an obvious aetiology, which he described as “tic douloureux of the bladder”. The term “tic douloureux” was taken from the disorder of the same name, which is also known as trigeminal neuralgia, a severe

stabbing pain to one side of the face. Although Physick never personally documented these observations, a former mentee by the name of Joseph Parrish (1779-1840), also a surgeon, published Physick's observations in 1836. He used the term "tic douloureux" in the diagnoses of three cases of severe lower urinary tract symptoms in the absence of bladder stones.

The term "interstitial cystitis" was not created until 1876 by Samuel D Gross, an American academic and surgeon. The term was used in the 3rd edition of his book, 'A Practical Treatise on the Diseases, Injuries and Malformations of the Urinary Bladder, the Prostate Gland and the Urethra' (Gross 1876). The term was then used again by the gynaecologist, Alexander JC Skene, in the 1st edition of his book, 'Diseases of the bladder and urethra in women' (Skene 1887). He described PBS/IC as a bladder disease that was characterised by inflammation, and had "destroyed the mucous membrane partly or wholly and extended to the muscular parietes."

The invention and refinement of the cystoscope in Europe, revolutionised bladder investigation, as it allowed researchers and surgeons to examine the bladder *in vivo*, rather than following post-mortem excision the bladder post-mortem. This advancement led to the development of, arguably, one of the most important anatomical diagnostic features of PBS/IC; Hunner's ulcer.

In the early 20th century, Guy LeRoy Hunner, a gynaecologist and obstetrician from Baltimore, published a series of papers describing PBS/IC (Hunner 1918), beginning with a paper in 1914 which described a study of eight women with a history (of an average of 17 years) of frequency, nocturia, urgency, and suprapubic pain. In 1918, Hunner published a paper entitled 'Elusive ulcer of the bladder', in which he described what he could see in the bladder, using a cystoscope. Unfortunately, at the time, the magnifying cytoscope had its limitations, and these led to the erroneous description of the bladder fissures as true ulcers. Although it is now known that the Hunner's ulcer has more of a fissure- like appearance, the term has remained largely unchanged, and the identification of the Hunner's ulcer remains as a gold standard for the diagnosis of PBS/IC for years to come.

Hunner also appealed for the standardisation of the nomenclature for PBS/IC, as after reviewing the existing literature, he questioned whether or not all of the researchers were describing the same bladder disorder. Unfortunately, this request was ignored, as the name of PBS/IC continues to describe a spectrum of disease to this day.

In 1949, the first comprehensive study of PBS/IC was published by John R. Hand, a contemporary of Hunner's. The paper was a report of 223 cases of PBS/IC, of which 204

were in women (Hand 1949). Hand separated these subjects into three grades, in accordance with the severity of cystoscopic findings (Table 2.1).

Table 2-1: The grading categorising that Hand developed to separate PBS/IC subjects in accordance to the main observations during cystoscopy.

	Main Observations
Grade 1	• Minimal bladder involvement • Small and discrete submucosal haemorrhages • Dotlike bleeding points • Single or parallel linear streaks • Little impact on bladder capacity
Grade 2	• More advanced stage with larger lesions • Considerably diminished bladder capacity
Grade 3	• Most advanced stage with diffuse lesions • Linear scars of old lesions • Zigzag fissuring suggesting a split of the underlying vascular network and muscle bundles • Greatly diminished bladder capacity (≤150 cc)

Hand's observations correlated with the scarred bladder that Hunner had described in his work, yet there was no appearance of a true ulcer. Although Anthony Walsh coined the term 'glomerulations' in 1978 to describe the submucosal haemorrhages that Hand had observed, it was not until Messing and Stamey (1978) published a paper that there was a change in how IC was diagnosed. Instead of basing the assessment on the presence of Hunner's ulcers, attention was turned to making a diagnosis that was instead based primarily on the exclusion of other disorders, the subject's symptoms, and glomerulations under bladder distention.

Hand also observed that there was an issue with the nomenclature of the disease, but until a better name was found, IC should continue to be used as it seemed to be the only name that covered the breadth in the variability of the grades of the disease. The following year, James Seaman wrote that the term 'interstitial cystitis' was the only name to 'epitomise the pathological picture' (Seaman 1950). In 1951, the term 'painful bladder' (PB) was introduced by JP Baroque, as an overall term to be used for all bladder pain disorders, including IC, but not as a direct replacement for IC. The introduction of the PB term led to decades of confusion in regards to the nomenclature for IC, and eventually, to

the development of the PBS/IC terminology coined by the International Continence Society (Abrams et al. 2002).

2.5.2 Classification

Typically, PBS/IC is classified into two categories; namely, ‘classic’ and ‘non-ulcer’ (Figure 2.3). Approximately 90% of those who experience PBS/IC present with the less serious non-ulcer form (Metts 2001). The classic form of PBS/IC is that which presents with Hunner’s ulcers upon cystoscopic examination. These ulcers are seen as small areas of reddened mucosa surrounded by small vessels radiating to a central pale scar (Hunner 1918) and are observed in between 5% and 20% of patients (Ho et al. 1997), with some research indicating that Hunner’s ulcers are more frequent in male PBS/IC individuals (Tamaki et al. 2004).

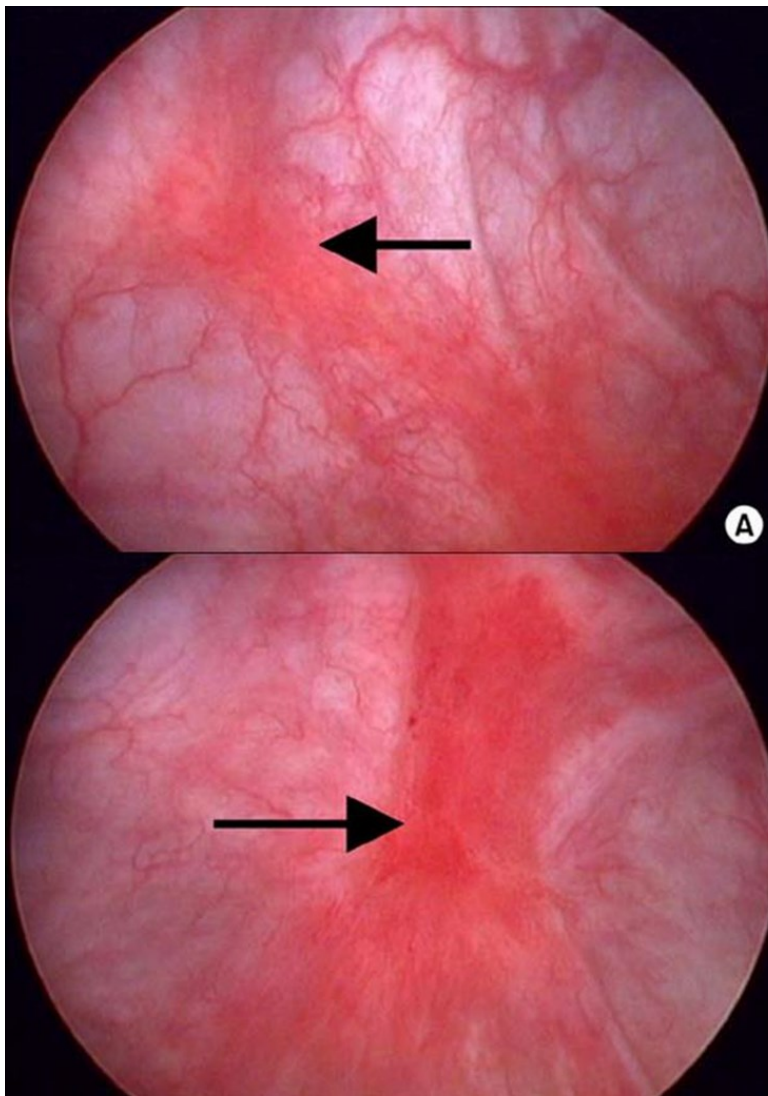


Figure 2-3: Typical cystoscopic appearance of the classic interstitial cystitis bladder. Arrows indicate Hunner's ulcers/lesions. (Adapted from Lee et al. 2013).

Although the 'classic' and 'non-ulcer' classifications have existed for some time, there is debate as to whether or not these two groups represent different stages of PBS/IC, or if they are two different disorders entirely. For example, there is a lack of research representing patients that progress from the non-ulcer type to the classic type with Hunner's lesions, but it is thought by some that the two types of PBS/IC represent two different disorders (Moutzouris and Falagas 2009). Contradicting this, was the finding that although there seems to be no correlation between pain scores and the presence of Hunner's ulcers in groups of PBS/IC individuals, it has been shown that bladder pain and other symptoms improve when all Hunner's ulcers are treated by electrocoagulation (Ryu et al. 2013), resection (Lee et al. 2013), or lasercoagulation.

Through research, clinical observation of PBS/IC patients, symptom variability, and the mixed success rates of PBS/IC therapeutic options, it is now recognised that sub-sets of PBS/IC patients exist, such as those with the Hunner's ulcer subtype. Although a classification system for these sub-sets does not yet exist within the official National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) or European Society for the Study of Interstitial Cystitis (ESSIC) guideline, the existence of the sub-sets is thought to be due to a difference in the underlying pathology of the disorder. The sub-set of interest in this project is those that experience PBS/IC caused by the proliferation and degranulation of MCs (mastocytosis) in the bladder wall (Theoharides et al. 2001; Sant et al. 2007; Rossberger et al. 2010).

2.5.3 Symptoms

Painful Bladder Syndrome/Interstitial Cystitis is a severely debilitating urological condition and is characterised by excessive urgency and frequency of urination, dyspareunia, chronic pelvic pain, and urine that does not lead to the culturing of pathogens (Metts 2001). Additionally, patients may also experience episodes involving flare-ups and remissions commonly associated with PBS/IC (Suskind et al. 2015). Those who are in the earlier stages of PBS/IC may only experience a few of the common symptoms, with or without urgency, and without pain.

The pain associated with PBS/IC can range from a feeling of discomfort to a burning sensation within the bladder, bladder spasms, a stabbing or burning vaginal pain, or a feeling of fullness or pressure despite the bladder containing very little urine (Nickel and Tripp 2015). In the majority of PBS/IC individuals, the pain is temporarily relived upon urination and emptying of the bladder, however, in others, there is an immediate intensification in pain following urination. Individuals who are in the initial stages of PBS/IC may not experience pain, but instead, a feeling of discomfort, fullness, heaviness, pressure, or a sensation of irritation within the lower abdomen.

Urinary urgency, a necessary symptom that must be present in order for the diagnosis of PBS/IC to be made, refers to the overwhelming need to void in order to relieve any bladder discomfort or pain that the individual is suffering. Retention of urine during an episode of urgency leads to an increase in distress, pain, and may also be associated with a feeling of nausea or malaise.

2.5.4 Bladder pathology

A key observation of PBS/IC bladder pathology is the lack of a continuous GAG layer (Parsons 2007). As stated previously, an inadequate GAG layer allows for the passing of noxious substances and molecules into the bladder wall (Figure 2.4), as the underlying urothelium is in direct contact with these harmful molecules, which trigger an MC led inflammatory response within the bladder wall.

The leakage of harmful substances through the defective GAG layer can cause inflammation within the sub-epithelial layer, and also delay or prevent healing of the damaged GAGs and any damaged urothelial cells (Hurst et al. 1987). The absorption of solutes through the defective GAG layer has been studied previously using PBS/IC patients. Parsons et al. (1991) conducted a study measuring the amount of instilled urea absorbed by PBS/IC patients, in comparison to controls, and the results showed a significantly higher amount of urea absorption in the PBS/IC group. The control subjects absorbed 4.3% urea in 45 minutes, compared to PBS/IC individuals, who absorbed 25% ($p<0.005$). Interestingly, PBS/IC individuals of the Hunner's ulcers subtype had a 34.5% absorption rate. The increase in absorption across the bladder wall in PBS/IC individuals may be clinically assessed using a potassium sensitivity test.

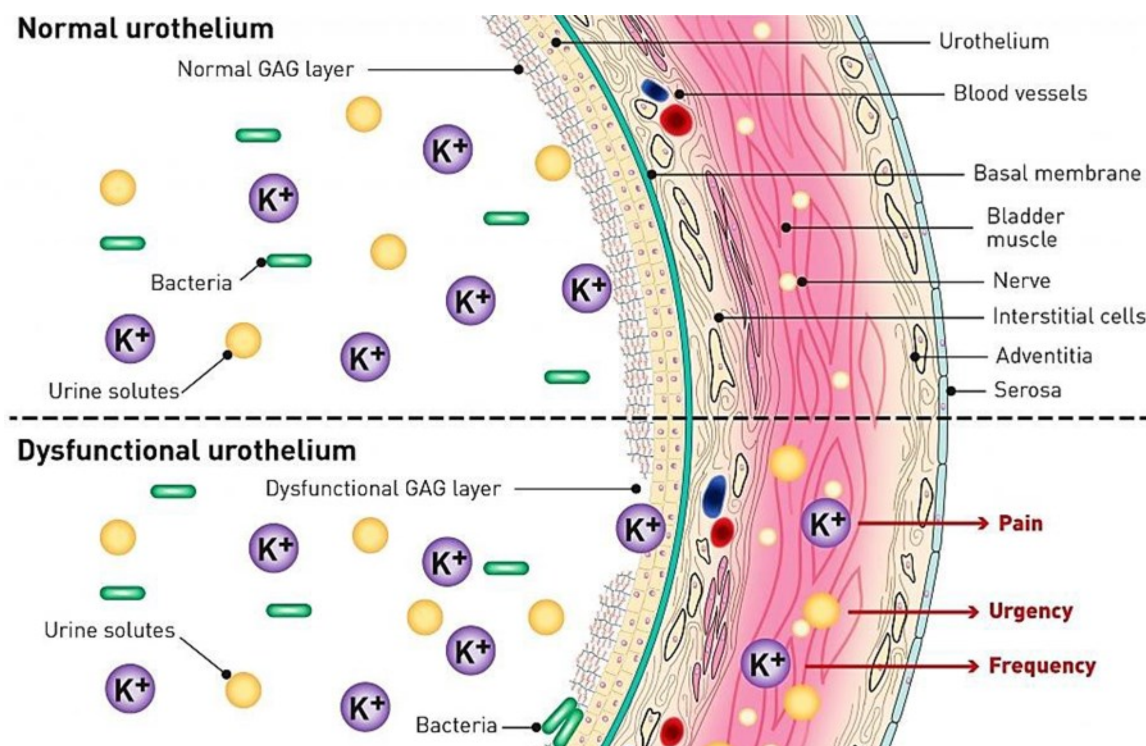


Figure 2-4: Normal urothelium compared to the dysfunctional bladder lining, as seen in PBS/IC. Note the lack of a continuous GAG layer in the dysfunction urothelium image. (Adapted from http://perthurologyclinic.com.au/).

The overall result of this move of noxious stimulatory substances into the sub- urothelium is the stimulation of capsaicin sensitive unmyelinated C-fibres (Maggi and Meli, 1988). These nerve fibres contain neuropeptides such as SP, which once released, lead to the activation and recruitment of inflammatory cells, and smooth muscle contraction. Clinically, this nerve stimulation in PBS/IC individuals results in pain sensation and activation of a visceral contraction of the bladder (Dubin and Patapoutian 2010).

When the defect in the GAG layer persists or fails to self-heal, chronic stimulation of the bladder wall may result in visceral hypersensitivity of bladder C-fibres nociceptors. Clinically, the neuronal hypersensitivity, and the amplified perception to a normal stimulus, is experienced as pain during bladder filling, an exaggerated sense of pain, i.e. a feeling of intense pain in response to mild inflammation (Doyle et al. 1997).

2.6 Diagnosis

Due to the variability in the symptoms exhibited by PBS/IC patients in a clinical setting, diagnosis following a standard PBS/IC exclusion and inclusion list (such as that provided by the NIDDK; Table 2.2) leads to many patients being left undiagnosed or misdiagnosed. Although the NIDDK diagnostic criteria for PBS/IC calls for the definite presence of Hunner's ulcers or glomerations, for the diagnosis of PBS/IC to be made, the majority of PBS/IC individuals do not present with Hunner's ulcers.

The NIDDK states that for an affirmative PBS/IC diagnosis to be made, the patient must have a waking urinary frequency of over eight times a day. However, this figure is an approximate value, as it is dependent on the volume of liquid that the patient consumes, the climate of the patient's environment, how much the patient perspires, and the diuretic effect of any medication that the patient may be taking. The frequency of urination can be severe, with some PBS/IC patient's voiding over 60 times a day (Meijlink 2014). Night-time voiding (nocturia) is also a part of the PBS/IC diagnosis as stated by the NIDDK.

However, due to the lack of appropriate clinical-based diagnosis criteria, PBS/IC diagnosis is empirical, and based on the patient's symptoms and urological assessment, involving cystoscopy, urodynamic study, potassium-sensitivity test, and exclusion of other common urinary diseases such as bacterial cystitis, urethritis, and neoplasia (Nigro et al. 1997).

PBS/IC may resemble a bacterial infection symptomatically, but individuals fail to respond to antibiotic therapy, and have negative urine cultures (French and Bhambore 2011). There are many diseases that PBS/IC is commonly misdiagnosed as, including carcinoma, overactive bladder, bacterial cystitis, and bladder stones (Table 2.3). Thus clinicians must incorporate a variety of investigative techniques in order to increase the likelihood of a correct diagnosis.

Patients may also provide additional qualitative information for diagnosis via the O'Leary-Sant Interstitial Cystitis Symptom and Problem Index (O'Leary et al. 1997), and the Visual Analogue Scale (VAS; a self-report scale widely used for the measurement of pain).

Table 2-2: The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) diagnostic criteria for PBS/IC. Note the list of exclusion criteria for a positive diagnosis for PBS/IC to be made. (Adapted from Hanno 2002).

NIDDK Diagnostic Criteria for Interstitial Cystitis
Interstitial cystitis patients should have both symptoms 1 and 2:
1) Hunner's ulcers or glomerulations
2) Bladder pain or urinary urgency
And excluding:
1) Bladder capacity greater than 350 ml on awake cystometry
2) Absence of urgency upon filling the bladder with 150 ml of liquid filling medium or 100ml of gas
3) A demonstration of phasic involuntary bladder contraction using cystometry
4) Symptoms have been present for less than 9 months
5) An absence of nocturia
6) Symptoms are relieved by antimicrobial, antiseptic, anticholinergic, or antispasmodic agents
7) A waking urination frequency of less than 8 times per day
8) Diagnosis of bacterial cystitis or prostatitis within the previous three months
9) Bladder or ureteral calculi
10) Active genital herpes
11) Cancer of the uterus, cervix, vagina, or urethra
12) Urethral diverticulum
13) Drug induced cystitis, such as cyclophosphamide
14) Tuberculosis cystitis
15) Radiation cystitis
16) Benign or malignant bladder tumours
17) Vaginitis
18) An age of less than 18 years

Table 2-3: The European Society for the Study of Interstitial Cystitis (ESSIC) list of diseases commonly confused with PBS/IC. (Adapted from van de Merwe et al. 2008).

Diseases commonly confused with PBS/IC			
Carcinoma	Infection with intestinal bacteria	Urethral diverticulum	Radiation cystitis
Carcinoma *in situ*	Tiaprofenic acid-induced cystitis	Benign prostatic obstruction	Cervical, uterine, and ovarian cancer
Infection with: *Mycobacterium tuberculosis* *Chlamydia trachomatis* *Ureaplasma urealyticum* *Mycoplasma hominis* *Mycoplasma genitalis* *Corynebacterium urealyticum* Candida species *Herpes simplex* *Human Papilloma Virus*	Overactive bladder	Chronic bacterial prostatitis	Cyclophosphamide -induced cystitis
	Bladder neck obstruction	Chronic non-bacterial prostatitis	Vaginal candidiasis
	Neurogenic outlet obstruction	Pudendal nerve entrapment	Endometriosis
	Bladder stone	Pelvic floor muscle related pain	Chemotherapy-induced cystitis
	Lower ureteric stone	Incomplete bladder emptying (retention)	Prostate cancer

2.7 Current therapies

Most commonly, patients are administered a short-acting intravesical instillation of a "cocktail" of heparin, lidocaine, and hyaluronic acid, in conjunction with an exogenous oral sodium pentosan polysulphate (Parsons 2005). Intravesical pharmacotherapy provides an instant high drug concentration directly into the bladder by-passing any systemic side-effects and removing any problems with low level urinary elimination of orally administered treatments (Bassi et al 2011).

In combination with medication, patients are also advised to alter their diet by eliminating foodstuffs that aggravate the condition, and incorporating those that may calm symptoms. Nearly 90% of patients with PBS/IC report sensitivity to a wide variety of dietary products including citrus fruits, caffeinated drinks (such as coffee and cola), and spicy foods (Friedlander et al. 2012). Patients also reported that calcium glycerophosphate and sodium bicarbonate tend to improve symptoms (Bassaly et al. 2011).

Although these current therapeutic options provide some relief to a subset of PBS/IC individuals, with a widely varying response between these individuals, there is still no wholly effective and well-tolerated treatment for PBS/IC. Rovner et al. (2000) found that for the 581 patients enrolled in the International Cystitis Data Base Study there were 183 different treatment modalities reported, thus demonstrating the variety of ineffective treatments currently presented to PBS/IC patients. This is also indicative of the difference between subsets of patients in terms of the underlying pathology of their PBS/IC.

2.8 Cost and financial burden

Quantifying the economic burden of PBS/IC is difficult due to the lack of sufficient data on this subject, however, a value can be estimated using the existing information that is available. Although more data is required to calculate the exact cost of PBS/IC, it was found that in the UK, from 2006 to 2007, there were 4,138 consultant appointments (563 inpatients and 3,575 day cases) that were associated with a diagnosis of PBS/IC. It is estimated that the average cost to the NHS per day case is £682, an elective inpatient is £3215, and a non-elective inpatient is £1436. Thus, when these figures (taken from 2011-2012) are taken into consideration with the number of PBS/IC diagnoses, it can be seen that the NHS are incurring a considerable cost nearing £4 million per year due to PBS/IC. In addition to this estimation of direct medical costs, PBS/IC also has an effect on economic costs due to PBS/IC patients taking an average of 16.5 days of absence due to ill health, per year (Galen Limited, 2010). Due to the difficulty in accurately diagnosing PBS/IC, and the number of individuals who don't seek help, these figures can be assumed to be an underestimate. PBS/IC is a huge financial burden on the patient, and the direct average cost per PBS/IC patient is greater than that of asthma, depression, diabetes mellitus, and hypertension (Clemens et al. 2009).

2.9 Summary

Not only is PBS/IC a financial, physical, and emotional burden to the patient; it also has a vast economic cost that rivals many other better understood disorders. In order to understand how to treat the disease, the role of inflammation and inflammatory cells in PBS/IC must first be clarified. Once this is understood, the efficacy of a novel anti-inflammatory treatment can then be assessed.

2.10 Hypotheses

There are three hypotheses that will be tested in this project:

- There will be a significant difference in the density and distribution of MC subtypes between PBS/IC subjects, and normal controls.
- Other inflammatory cells, such as basophils, will be present in PBS/IC subjects, and not in normal controls.
- Honey will have an effect on the release (by degranulation) of histamine from MCs.

2.11 Aims

The aims of this project are as follows:

- To quantify the number of inflammatory cells (MCs and basophils) in paraffin embedded PBS/IC patient tissue using immunohistochemistry (IHC) techniques.
- To assess the distribution and density of MC subtypes in paraffin embedded PBS/IC patient tissue using IHC techniques.

- To use commercially available enzyme linked immunosorbent assays (ELISA) to assess a range of concentrations of honey in order to evaluate their effect on histamine induced MC degranulation in rat bladder explants

Chapter 3: Mast Cells in Painful Bladder Syndrome/Interstitial Cystitis

3.1 Introduction

PBS/IC sufferers have a defective GAG layer, that normally acts as a protective lining over the bladder urothelium (Bassi et al. 2011). Research has shown that dysfunctional or damaged urothelial cells generate cytokines such as SCF (Metcalfe 2008) that can promote activation and proliferation of MCs, as bladder MCs are maximally activated by SCF (Sant et al. 2007). Following the leakage of urinary components (especially potassium due to its ability to depolarise nerves [Parsons 2010]) through the damaged urothelium, an inflammatory response involving MCs is triggered (Bjorling et al.1999) A histological feature of PBS/IC is a redistribution of MCs into the detrusor muscle layer. These redistributed MCs act on local smooth muscle and vascular epithelium and also have a significant effect on inflammation (Bouchelouche et al 2006). Christmas et al. (1990) reported a significant increase in the density of nerve fibres in PBS/IC patients. Most of these newly proliferated nerve fibres were found to contain SP (Pang et al. 1995), a potent stimulator of MC degranulation, and the consequent histamine released feeds back to induce the release of further SP (McCary et al. 2010). MC degranulation is triggered by molecules such as SP, SCF and nerve growth factor (NGF); factors that have all been implicated in the pathology of PBS/IC (Theoharides et al. 2012). Once activated, MCs release molecules (such as histamine, IL-6, and TNF-α) which play a key role in MC activation, MC recruitment, and inflammation.

MCs express a high level of two serine proteases; chymase and tryptase. To distinguish the location of human MCs, antibodies that target MC proteases can be used. Tryptase is considered to be an important marker of MC activity and so the antibody AA1 (anti-mast cell tryptase) can be used to identify tryptase containing MCs (i.e. MC_T and MC_{TC}). Chymase can be found in MC_{TC}, and can be identified using the antibody CC1 (anti-mast cell chymase). Sparse research exists utilising anti-tryptase and anti-chymase antibodies in the identification of MCs in PBS/IC

(Peeker et al. 2000, Yamada et al. 2000) and additionally, there is currently no research comparing the densities of MC subtypes in full-thickness PBS/IC bladder tissue using a co-localisation technique.

As the literature highlighted the importance of MCs in the pathogenesis of PBS/IC (Lv et al. 2012; Sant et al. 2007), a series of assays were developed to elucidate the role of MCs in this disorder. This involved acquiring tissue from PBS/IC and control patients, and using immunohistochemistry (IHC) techniques to identify the presence of MCs.

3.2 Immunohistochemistry

3.2.1 Background

The IHC technique is commonly used for the detection of a range of cell or tissue antigens (including amino acids, proteins, infectious agents, and cells of interest) in biological tissue. The approach typically involves the use of a labelled antibody to identify the corresponding antigens within the tissue of interest. Once the antibody is bound to its complementary antigen, it is visualised via the label which is attached to the antibody, which can be detected using light microscopy (LM) or electron microscopy (EM).

The immunostaining method dates back to 1934, when Marrack used a red stain conjugated to benzidin tetraedo to develop a reagent against typhus and cholera microorganisms (Marrack 1934). The resulting success of this labelling method also led Marrack to conclude that maximum precipitation occurred at optimal concentrations of antigen and antibody. However, it was Professor Coons from the Harvard School of Medicine (Boston, USA) who refined the method. He believed that when under optical microscopy, the level of antigen detected by the red staining was very low. With the help of organic chemists, Coons then demonstrated that it was possible to use fluorescent dyes to aid visualisation. He developed a fluorescein-labelled anti-pneumococcal antibody to successfully detect bacteria that had been ingested by macrophages, and was also able to establish reversibility and specificity of the reaction. This work laid out the guidelines for the development of basic immunolabelling procedures (Matos et al. 2010).

Following further investigation into antibodies and more specifically, the bivalent IgG molecule, Coons developed the direct immunolabelling approach, whereby the primary antibody is attached to the label in a one-step protocol. Although this method requires an abundancy of primary antibodies, it is still used today by researchers who favour a

protocol that subjects the target cell to as few steps as possible. As Coons and his team unravelled the potential of using antibodies as antigens, they developed a new indirect two-step protocol that used a secondary antibody developed against the species in which the primary antibody had been made. The secondary antibodies are raised against the IgG of the animal species that made the first antibody, in another species that has an abundancy of serum.

The secondary anti-species IgG can then be used in any reaction that involves IgG from the same species. This approach, ‘indirect immunolabelling’, is commonly used today, and was the selected method for this study.

3.2.2 The indirect immunohistochemistry technique

The IHC technique is a multistep process, but these can be grouped into two phases: 1) Slide preparation; including fixation of the specimen, tissue processing, and the stages required for the reaction to take place (including: antigen retrieval, blocking of non-specific sites, endogenous peroxidase blocking, incubation with the primary antibody, application of the secondary antibody, detection method, counterstaining, and mounting); 2) Quantification and study of the molecule or cells of interest. An advantage to the IHC technique is that this basic multistep protocol can be easily adapted for the researcher’s purpose. Below is an overview of the IHC technique that was used in this project (See Appendix C for further details).

3.3 Aim

- PBS/IC may be associated with the proliferation and activation of MCs in the bladder wall, thus the aim of this study was to determine the subpopulations and location of MCs in bladder tissue using antibody-based techniques. Any clarification of the role of MC subtypes would aid understanding of and help to address the clinical shortfalls of PBS/IC diagnosis and treatment.

3.4 Materials and methods

3.4.1 Participants

Bladder sections were obtained from the tissue bank at the Department of Histopathology, University Hospital Southampton NHS Foundation Trust. The tissue was originally collected during cystoscopy, with patient consent. Inclusion into this study was based on the amount of tissue present within the block (full- thickness samples were selected whereas biopsy samples were excluded due to a lack of adequate tissue). These selection criteria resulted in the inclusion of fourteen patients at the start of the study. The medical history of the subject was also considered, i.e., subjects with a histological diagnosis of PBS/IC were selected (all other patient information was blinded to the researcher). The study included control tissue from subjects (n=4) that appeared histologically normal and did not show cellular abnormalities associated with inflammation. These controls were individuals who had lower urinary tract symptoms, but had no inflammatory cell infiltrate or other markers of inflammation, upon histological examination.

3.4.2 Bladder tissue preparations

Bladder sections were obtained from the tissue bank. Tissue had been fixed in 10% buffered formalin before a conventional wax embedding process.

3.4.3 Ethical considerations

Due to the nature of this study, ethical approval was required, as is normal practice. The study fell under the umbrella ethical approval granted to the research team, and so it was not necessary to apply for ethics specific to this study. Additionally, this study complied fully with Good Laboratory Practice (GLP) and registrations such as the Human Tissue Act (2004). The researcher successfully completed Good Clinical Practice (GCP) training, and utilised this during the study. The researcher carefully considered the use of archival

tissue in this study and as the end-point of the study was to develop a treatment for PBS/IC to be used in humans, archival bladder tissue had to be used. The researcher was careful to minimize wastage with efficient laboratory practice and accurate protocol development.

3.4.4 Immunohistochemistry

3.4.4.1 Antibodies

Tryptase is present in abundance in all MCs (immune system and non-immune system associated MCs) and is often used as a marker for MC activation. The AA1 antibody is highly sensitive and specific for tryptase, with minimal background staining of other tissue structures and thus is routinely used for the identification of MCs. Chymase is often studied when looking at mastocytosis-associated diseases. This proteinase is less prominent than tryptase, as it is only found in non-immune system associated MCs and is thought to play a key-role in fibrosis and smooth muscle degradation. The CC1 antibody is able to detect chymase successfully, with minimal background staining of other tissue structures.

A conventional IHC technique was employed. Blocks were cut at 4µm and sequential sections applied to Apex-coated slides. The slides were dewaxed and rehydrated to 70% alcohol. A combination of hydrogen peroxidase and methanol was applied to all slides for 10 minutes to inhibit exogenous peroxidase and minimise background staining. Slides were then washed with Tris- buffered saline (TBS) before the antigen retrieval step. As MCs are the cell-type of interest, a pronase solution was applied for 10 minutes in order to unmask antibody binding sites for chymase and tryptase. To further increase the specificity of antigen binding and reduce background staining, endogenous biotin was also blocked by incubating with avidin for 20 minutes, washing in TBS and then incubating for 20 minutes with biotin. The slides were then rinsed before the application of culture medium and left to incubate at room temperature for 20 minutes. The slides were drained and either the monoclonal antibody AA1 or CC1 was applied at a dilution of either 1:50000 or 1:100 respectively (as determined by previous titrations using tonsil and nasal polyp tissue) and left overnight (≥18 hours) at 4ºC. Subsequently, the slides were brought to room temperature for 30 minutes. The slides were washed with TBS and rabbit anti-mouse 2Y antibody at a dilution of 1:400 was applied for 30 minutes. They were then

washed with TBS before the application of a streptavadinbiotin biotin-peroxidase complex (1:1+75µl TBS) for 30 minutes. The slides were then washed with TBS and the chromagen diaminobenzidine (prepared in accordance with the manufacturer's instructions) was applied for 5 minutes, before counterstaining with Haematoxylin and placed under running tap water for 5 minutes. The slides were then dehydrated, cleared and mounted.

3.5 Outcome measurements

The slides were analysed using the dotSlide virtual slide system (Olympus Corporation; Tokyo, Japan) and a blue 83-A Wratten filter (Møller et al 1984) (Eastman Kodak Company; New York, USA). The filter was used to enhance the contrast for easier detection of stained cells of interest. Each layer of the bladder (mucosa, lamina propria, detrusor muscle and adventitia) was photographed in 5 separate areas within the layer, and the images were analysed using a cell counting programme routinely used to count the number of particles of interest (Yeo et al 2013). This programme, ImageJ™, was used in conjunction with a macro that was developed by the researcher in order to identify positively stained MCs using an unbiased yet accurate method. An attempt at counting by eye was made, but the method was both time consuming and prone to bias (Appendix D).

3.5.1 Macro development

A skeleton macro for use with ImageJ™ was acquired from the Biomedical Imaging Unit (BIU) at University Hospital Southampton NHS Foundation Trust. The macro was adapted using basic coding language compatible with the ImageJ™ program, and underwent development before a final version was used for analysis of all images. The macro was designed to be robust enough to detect positive red staining and ignore blue background structural staining, but also contained parameters that could be adjusted in cases of weak staining or particularly large or small cells of interest.

3.5.2 Cell counting

The following guidelines were used to develop the automated cell counting method described above, in order to ensure unbiased, reproducible and reliable results:

- Only MCs that positively stained brown were counted.
- MCs that were on the edge of a photograph were only included if more than or equal to 50% was present in the image.
- If inadequate tissue for analysis was present, cross-pattern objective fields were selected at random.

3.6 Statistical analysis

Data were analysed using GraphPad Prism 6 (Version 6.07; La Jolla California, USA). Data were normally distributed; thus descriptive statistics are expressed as mean ± standard errors of the mean. In order to compare the density of each subtype of MC in the four layers of bladder wall that were analysed (adventitia, detrusor muscle, lamina propria and mucosa), individual paired t-tests comparing the average value of each of the layers against the average of a different layer, for each subtype, were conducted. Results were determined to be significant at 95% confidence levels.

3.7 Results

Immunolocalisation of mast cells within normal and PBS/IC bladder tissues

Immunostaining of tryptase containing MCs was moderate to intense, and MCs were found distributed throughout the entire bladder wall (Figure 3.1). The staining was exclusive to the cytoplasm and granules of MCs (Figure 3.2). MCs which were actively degranulating during the time of tissue sampling had tryptase staining of granules beyond the outer membrane. AA1 positively identified MC_T and MC_{TC} as these both contain

tryptase (Figure 3.3), and CC1 positively identified MC chymase, i.e., MC_{TC} only. Thus, some MCs stained positively with both antibodies, and this is depicted in Figure 3.4.

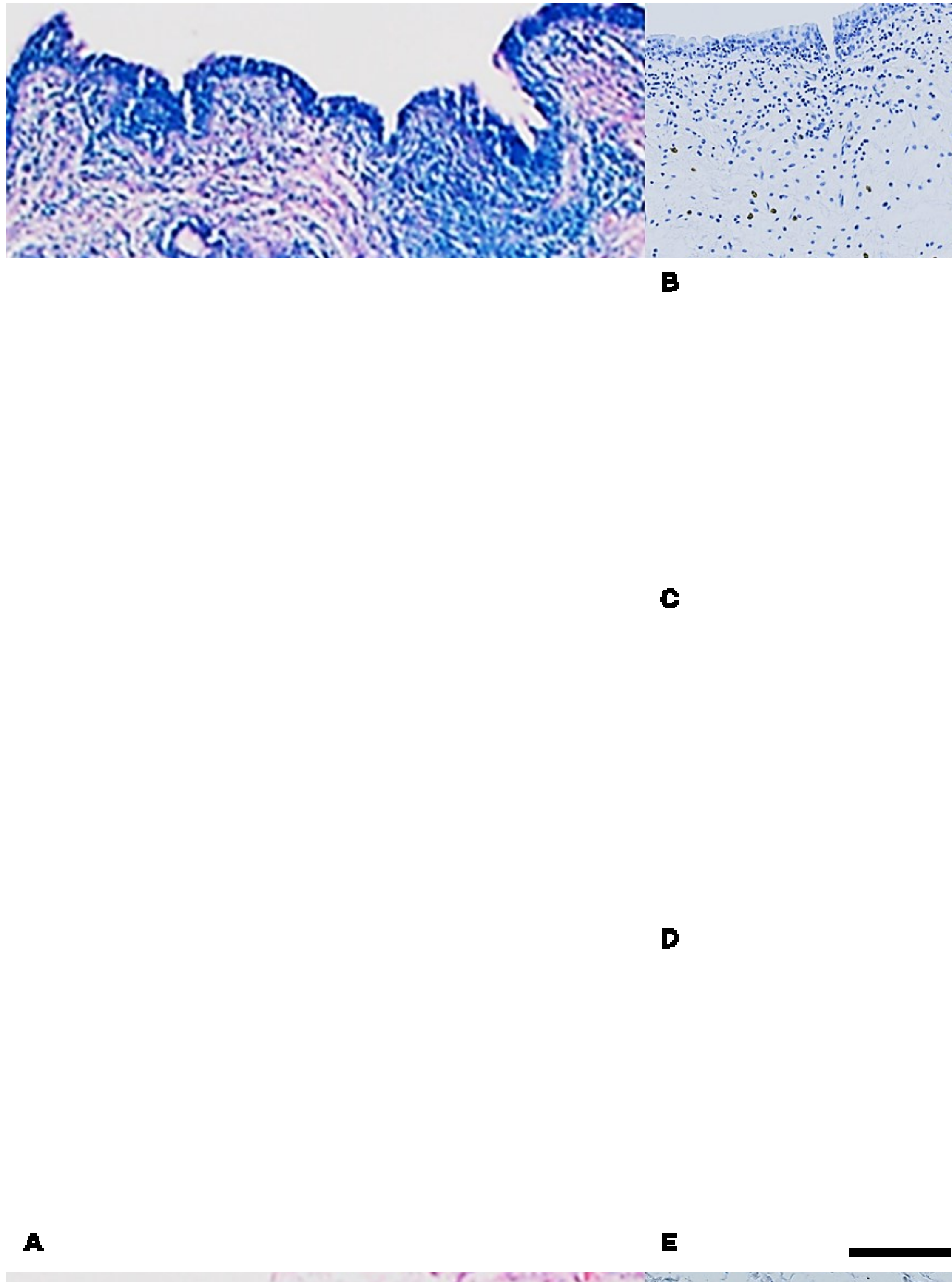


Figure 3-1: Representative images of AA1 positively stained human full thickness bladder wall, taken using light microscopy, and either standard histochemical or immunohistochemical procedures. Scale bar= 50µm. A) GIEMSA stained full thickness human bladder tissue at x4 magnification. The GIEMSA stain is a contrasting stain that will stain erythrocytes pink, platelets a light pale pink, lymphocyte cytoplasm sky blue, monocyte cytoplasm stains pale blue, and leukocyte nuclear chromatin stains magenta. In this image it can be seen that the structural bladder wall has been stained as pink/red, and the granulocytic cells of interest as blue. B) Positively stained bladder mucosa using the mAb AA1 and toluidine blue, with positively stained mast cells stained brown. Magnification is x60. C) Positively stained bladder lamina propria using the mAb AA1 and toluidine blue, with positively stained mast cells stained brown. Magnification is x60. D) Positively stained bladder detrusor muscle using the mAb AA1 and toluidine blue, with positively stained mast cells stained brown. Magnification is x60. E) Positively stained bladder adventitia using the mAb AA1 and toluidine blue, with positively stained mast cells stained brown. Magnification is x60.

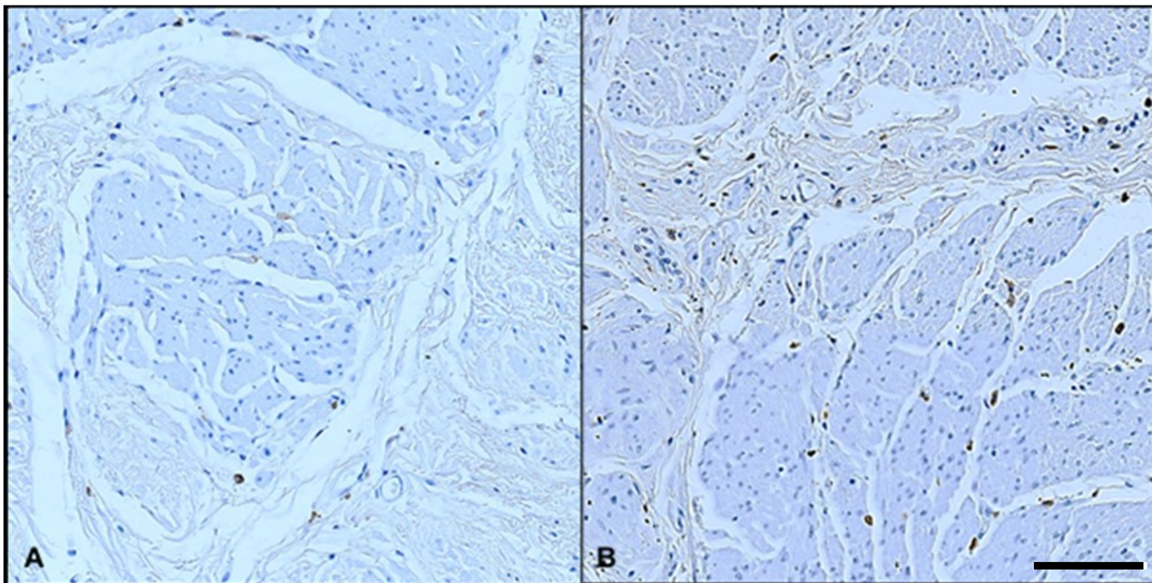


Figure 3-2: Representative images of human PBS/IC bladder detrusor tissue at x60 magnification using light microscopy. Human bladder tissue samples have been immunohistochemically stained using toluidine blue and mAb AA1. A) Control bladder tissue immunohistochemically stained using toluidine blue and mAb AA1. Positively stained cells are identified by brown colouring. B) PBS/IC bladder tissue immunohistochemically stained using toluidine blue and mAb AA1. Positively stained mast cells are identified by brown colouring. Scale bar= 50μm.

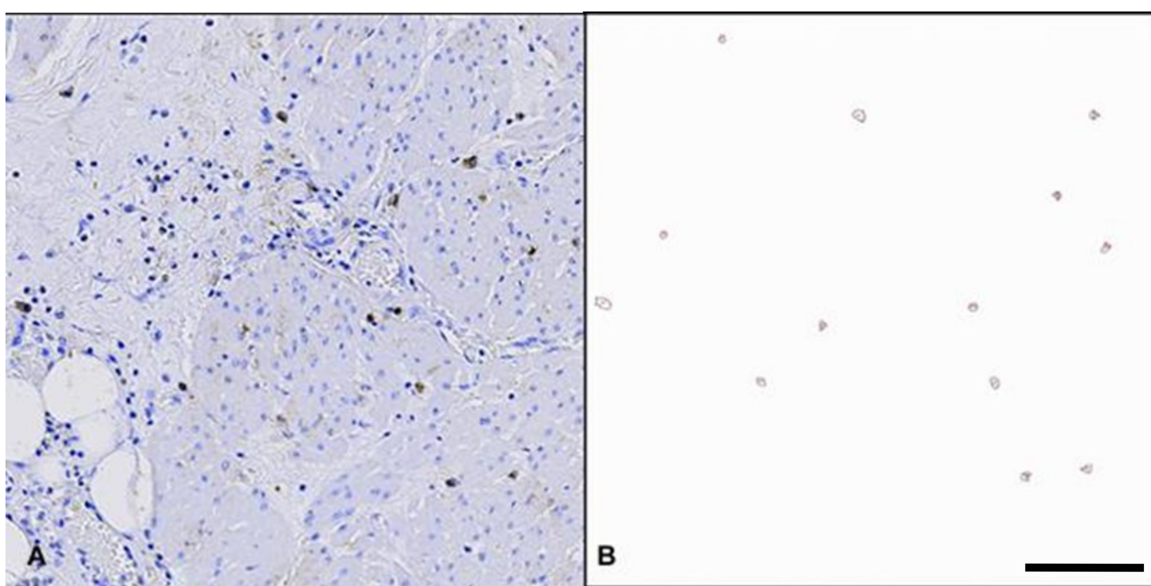


Figure 3-3: Representative images of human PBS/IC bladder detrusor tissue at x60 magnification using light microscopy. Human bladder tissue samples have been immunohistochemically stained using toluidine blue and mAb AA1. A: Detrusor muscle layer positively stained for mast cell tryptase with the AA1 antibody (brown). B: 'Skeleton' left after the image has been successfully processed by ImageJ^{TM}. Scale bar= 50μm.

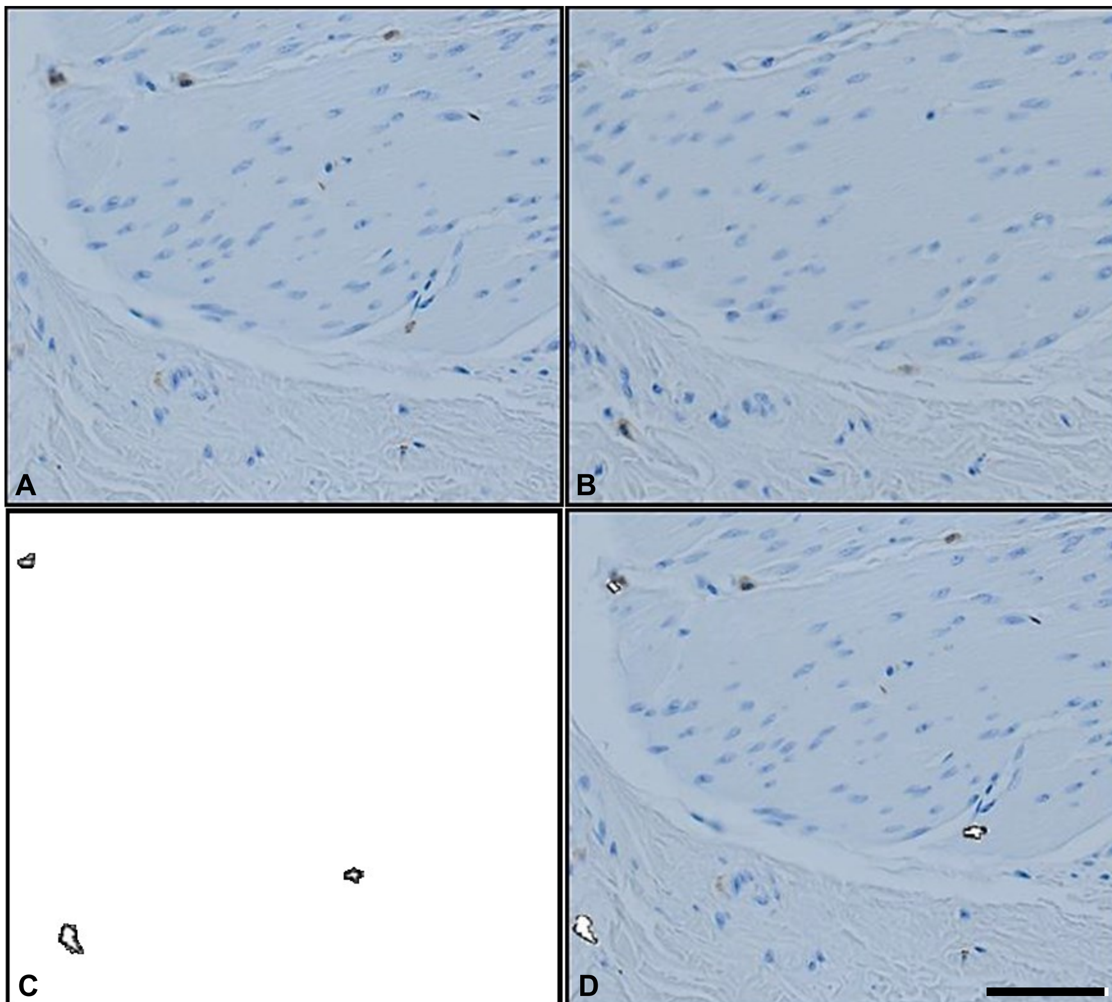


Figure 3-4: Schematic representation of CC1 positively stained mast cells amongst AA1 positively stained mast cells in PBS/IC bladder detrusor tissue. Human PBS/IC bladder detrusor tissue were stained using mAbs and toluidine blue, and imaged at x80 magnification using light microscopy Scale bar= 50µm. A) AA1 positively stained mast cells in the detrusor layer of PBS/IC bladder. B) CC1 positively stained mast cells in the detrusor layer of PBS/IC bladder. C) CC1 positively stained mast cells as detected by ImageJ™ processing of image (B). D) Image (A) superimposed with Image (C) to show the distribution of CC1 positive mast cells as constituents of AA1 positive mast cells.

Distribution of mast cell subtypes in PBS/IC bladder tissues

AA1 and CC1 positive MCs show a similar pattern of distribution (Figure 3.5). In CC1 positive MCs in PBS/IC individuals, there was a statistically significant difference in the density of MC_{TC} between the detrusor ($p=0.01$) and the lamina propria ($p<0.01$), when compared to controls (Figure 3.6). AA1 positive MC proliferation was increased in PBS/IC tissue, with a difference in density related to location within the bladder wall (Figure 3.7; mean values ±SEM). Within the detrusor, the mean number of AA1 positive MCs present in PBS/IC was 25.6 cells/0.5mm x 0.5mm (n=14) compared to only 2.7 cells/0.5mm x 0.5mm in the control group (n=4) ($p<0.001$). In the lamina propria the mean number of AA1 positive MCs found in PBS/IC was 21.5 cells/0.5mm x 0.5mm (n=14), compared to 1.7 cells/0.5mm x 0.5mm (n=4) in controls ($p<0.001$). The difference in the density of AA1 positive MCs between PBS/IC and control was highly statistically significant within all layers of the bladder wall ($p<0.001$; Figure 3.8).

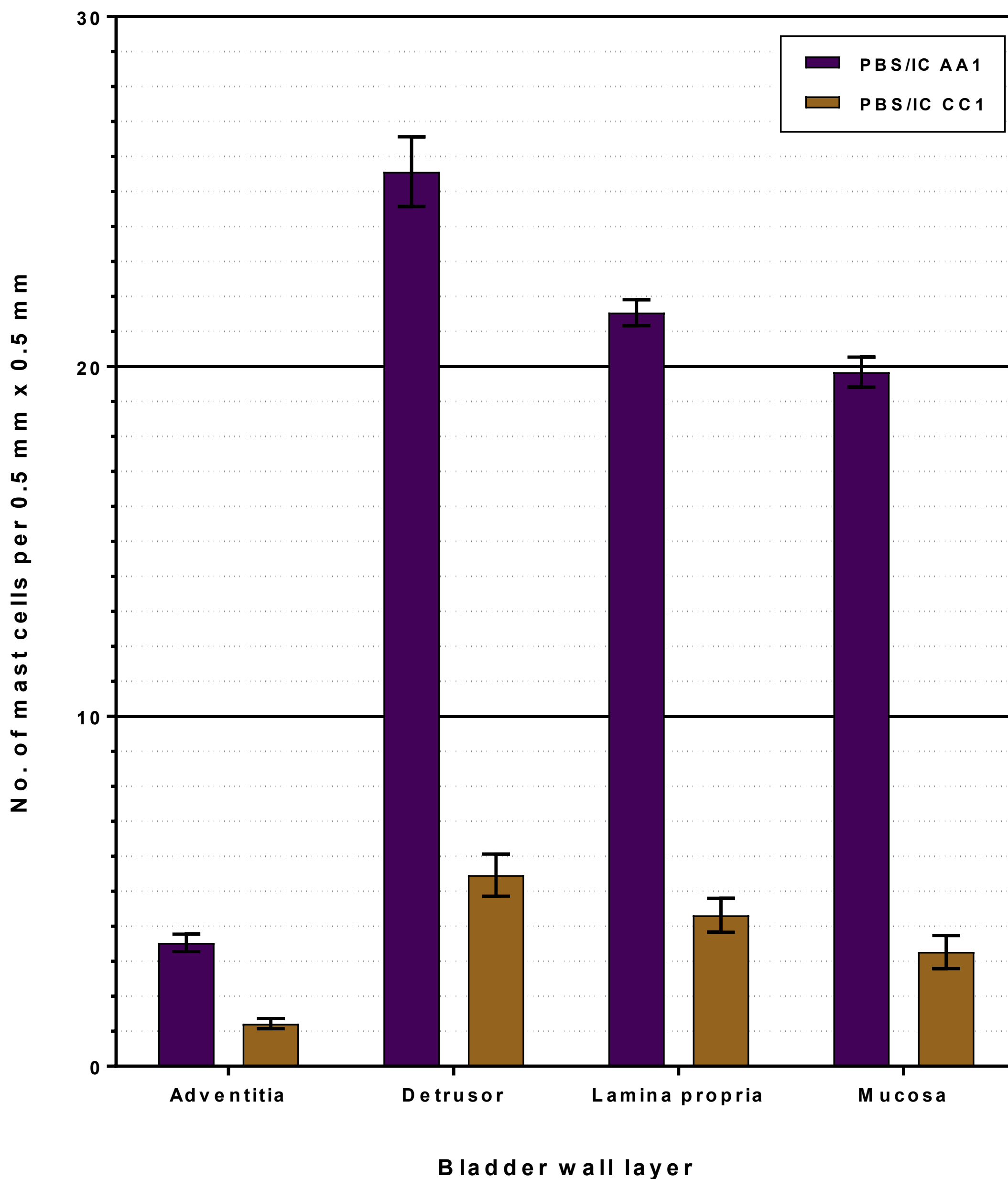


Figure 3-5: Distribution of AA1 positive and CC1 positive mast cells in PBS/IC bladder tissue (±SEM bars). Both AA1 and CC1 positive mast cells show a similar pattern of distribution, although the presence of AA1 positive mast cells is the greatest overall. Within each layer of the bladder wall, the difference in density of AA1 and CC1 mast cells is statistically significant at $p<0.001$. Mast cells were identified using the mAbs AA1 and CC1, and a standard immunohistochemical procedure.

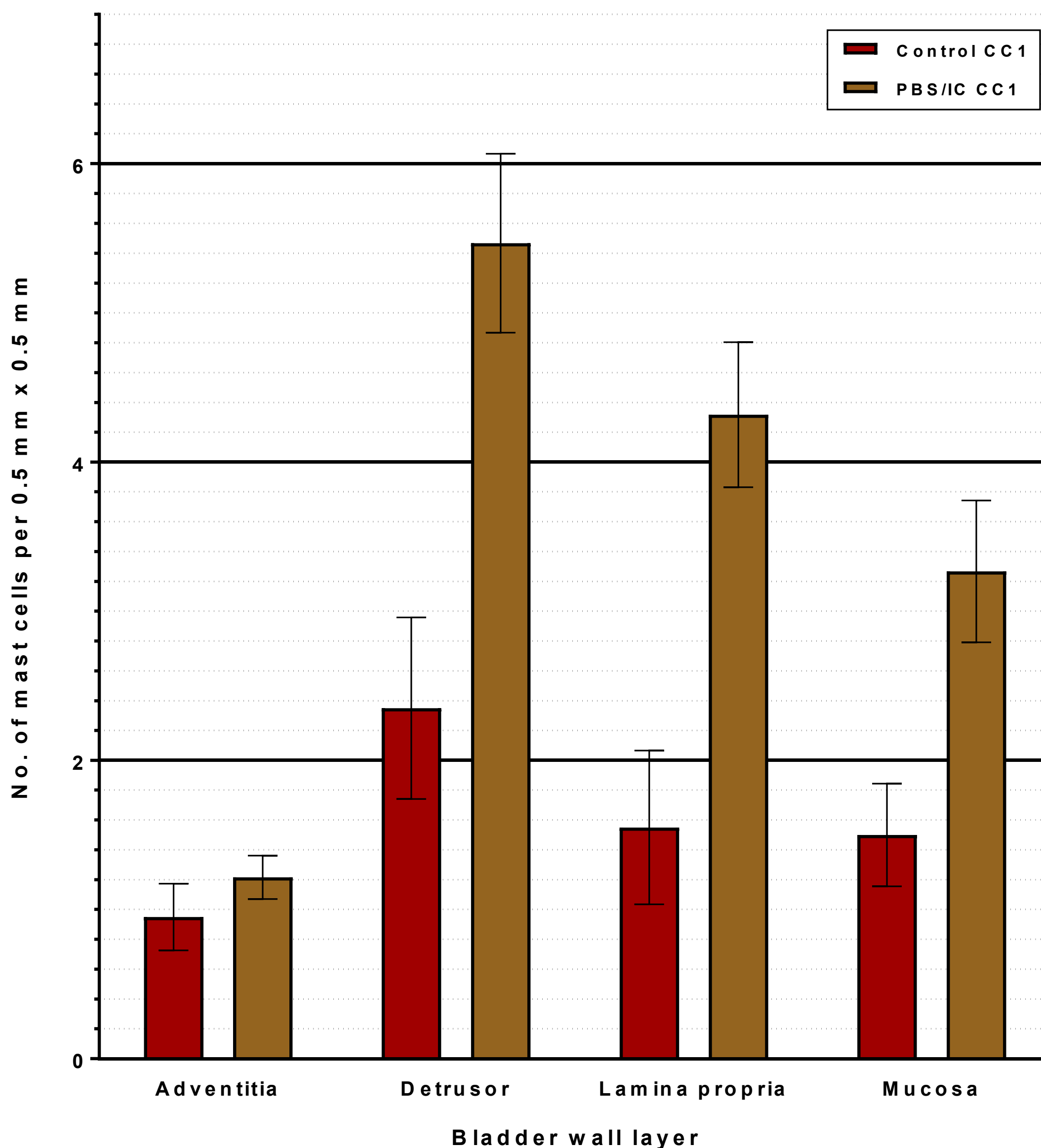


Figure 3-6: Distribution of CC1 positive mast cells in PBS/IC bladder tissue and controls (±SEM bars). The difference in the density of CC1 positive mast cells between the PBS/IC and control group was statistically significant within the detrusor (p=0.01), and within the lamina layer (p<0.01).

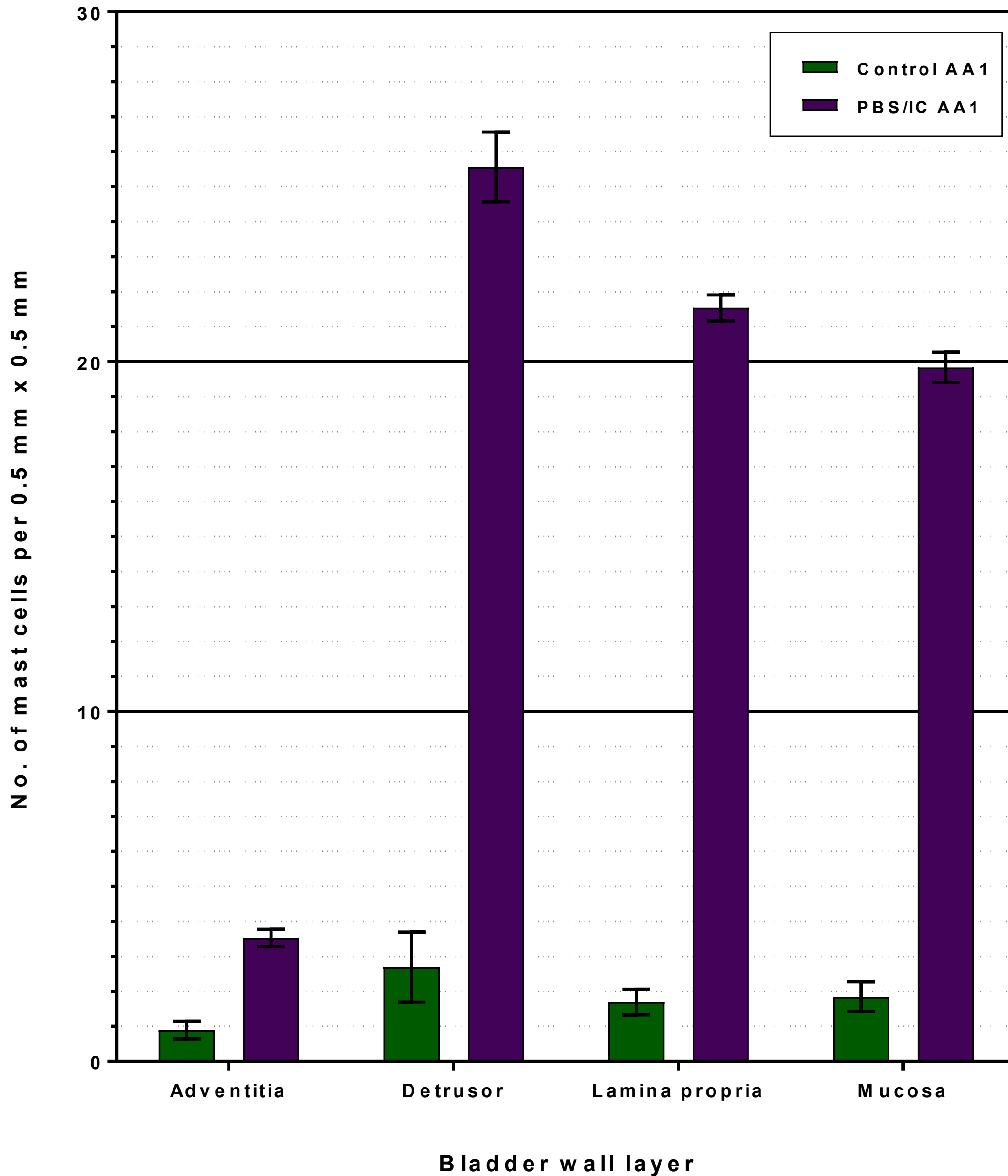


Figure 3-7: Distribution of AA1 positive mast cells in PBS/IC bladder tissue and controls (±SEM bars). The difference in the density of AA1 positive mast cells between PBS/IC and control was statistically significant within all layers of the bladder wall ($p<0.001$).

Distribution of AA1 positive and CC1 positive mast cells in PBS/IC and control bladder tissue (±SEM bars)

The average number of AA1 and CC1 positive MCs within the bladder wall of PBS/IC and control individuals is shown in Figure 3.8. There is a similar pattern of distribution between the MC subtypes in PBS/IC tissue. Note the three-fold increase in the number of AA1 positive MCs in the detrusor of PBS/IC subjects, in comparison with controls.

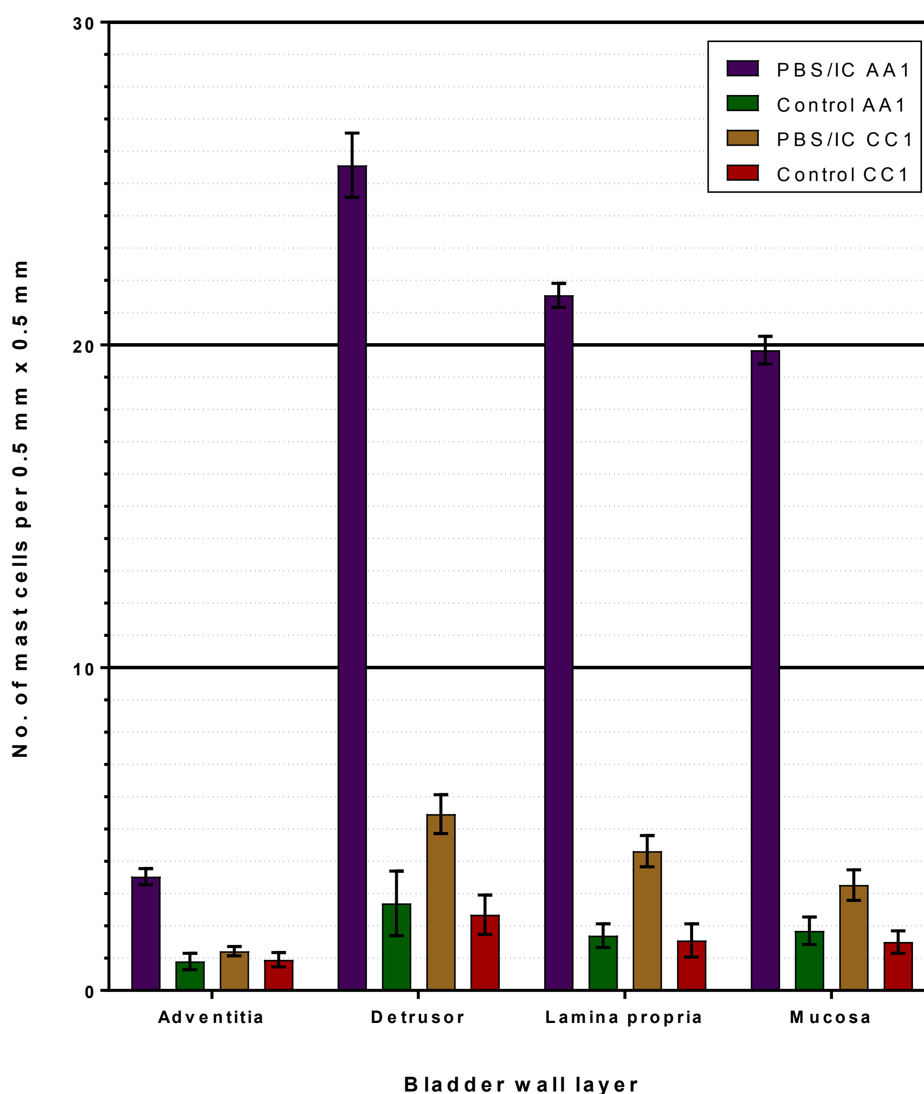


Figure 3-8: Distribution of AA1 positive and CC1 positive mast cells in PBS/IC and control bladder tissue (±SEM bars). Both mast cell subtypes show a similar pattern of distribution in both groups, although the presence of AA1 positive mast cells is the greatest overall. Mast cells were identified using the mAbs AA1 and CC1, and a standard immunohistochemical procedure.

Males are more likely to be diagnosed at a later age than females, and have a similar MC distribution

The mean age of diagnosis for men was older (66.75 years; n=4) than that for women (44.6 years; n=10) (Figure 3.9). In addition to this, both sexes show a similar profile of distribution of MCs, but males have a higher number of AA1 and CC1 positive MCs (Figure 3.10).

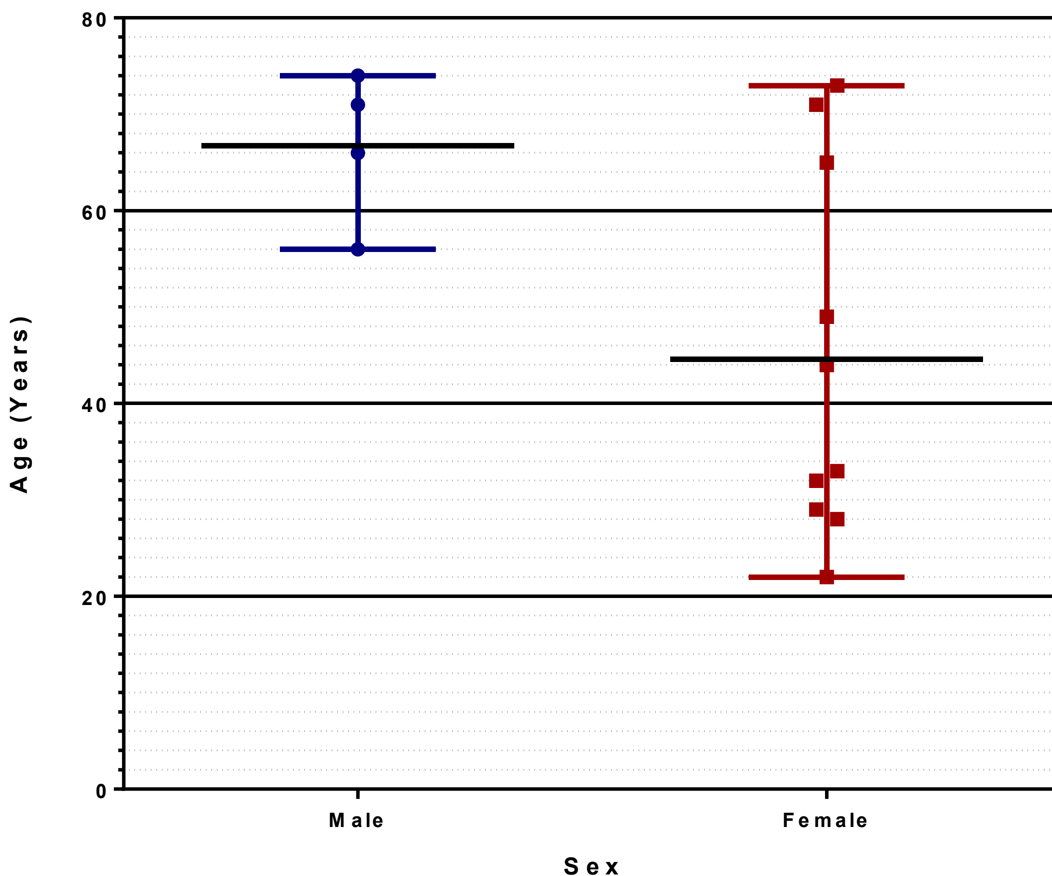


Figure 3-9: Distribution of patient diagnosis of PBS/IC in years, grouped by sex. The age at which patients are positively diagnosed with PBS/IC was recorded during standard histochemical analysis of the bladder wall, and combined with routine patient information, this data was correlated in order to identify any relationship between the age of diagnosis and the sex of the individual. PBS/IC males are statistically significantly more like to be successfully diagnosed at a later age when compared to females ($p<0.05$).

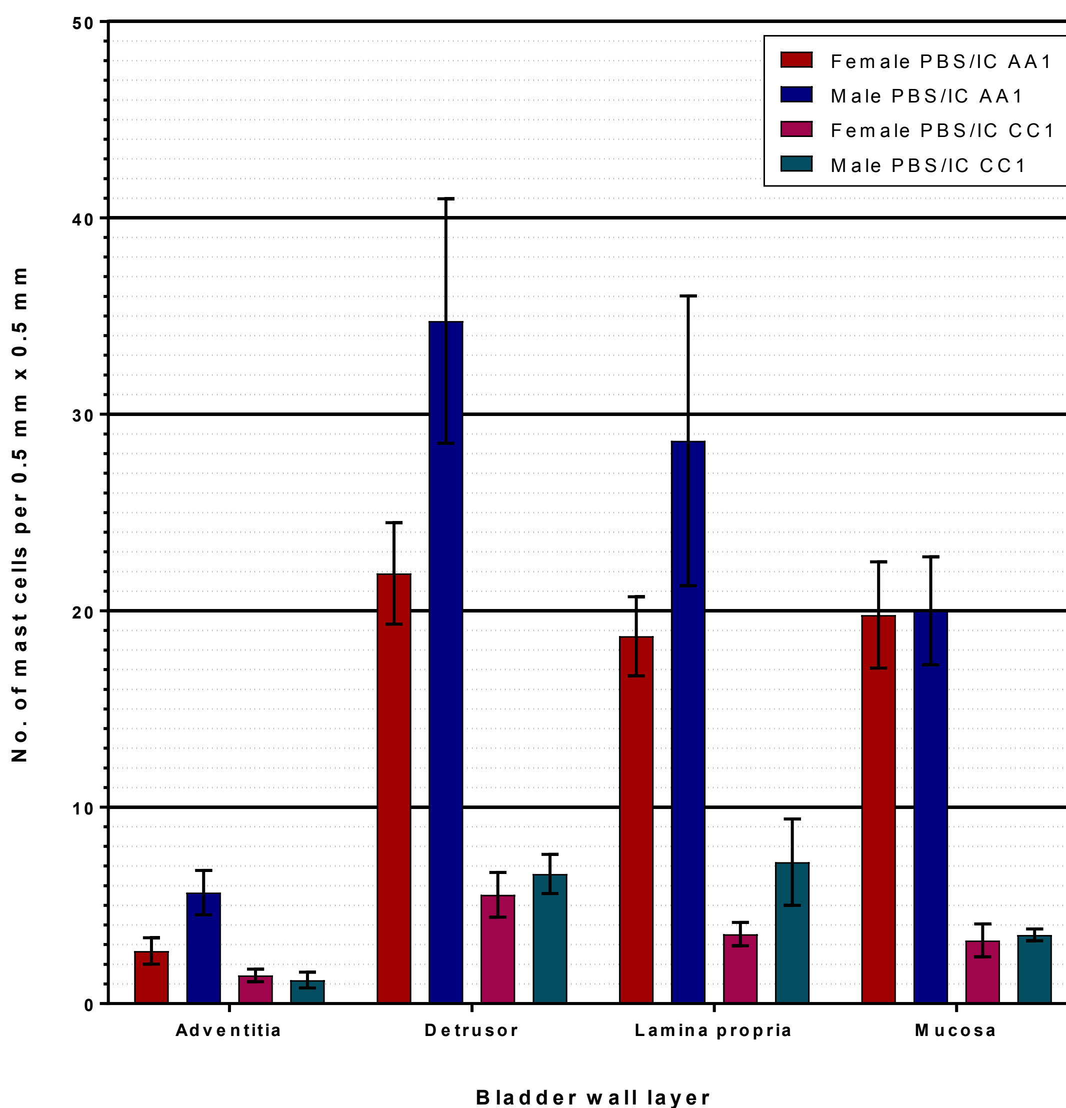


Figure 3-10: The mean no. of AA1 and CC1 mast cells within the bladder wall of PBS/IC individuals, when subjects have been separated according to sex. Note, there is a difference in the distribution of mast cell subtypes between females and males, the most obvious being that males have a higher count of CC1 mast cells in comparison to females.

3.8 Discussion

MCs have long been considered as a key cell type in a range of inflammatory conditions and are currently thought to play a significant role in the development, chromicity and pain

associated with PBS/IC (Peeker et al. 2000, Theoharides et al. 2001, McCary et al. 2010). Results from this study add further support to this hypothesis. Previous studies indicated that MC accumulation (mastocytosis) was only present in a subset of patients (Theoharides et al. 2001, Roth 2007, Rössberger et al. 2010). In this study mastocytosis was present in all PBS/IC individuals. However, it must be noted that the samples were taken from a tissue bank and so this sub-group represented PBS/IC patients with more severe disease.

Interestingly, the distribution of MCs according to sex highlighted that males had been diagnosed at a later stage in life (as seen in Figure 3.9). This may be for one of three reasons; either males develop PBS/IC at an older age, males are more likely to be misdiagnosed and so the correct diagnosis takes longer to reach, or, most likely, men are more likely to wait to see a clinician about their symptoms. The last reason may also explain the higher number of AA1 positive MCs in the bladder wall, when compared to females.

In the present study, we observed differences in the distribution and density of MCs within the bladder wall between normal and PBS/IC bladders, with the difference in MC density within the detrusor being statistically significant ($p<0.001$). This is in addition to the differences in MC distribution seen in the layers of PBS/IC bladder. Generally, both MC subtypes are located close to blood vessels within the lamina and close to nerve fibres (Figure 3.11) within the detrusor. This difference in the location of MC accumulations supports the suggested pathology of PBS/IC; that MC recruitment and degranulation is triggered by inflammatory factors (Theoharides et al. 2012). The statistically significant increase in MC density within the detrusor of PBS/IC may be attributed to the increase in inflammatory cytokines such as SCF. Activated MCs also release various factors such as SP. Thus, the detrusor muscle mastocytosis seen in this study can be explained by the self-perpetuating cycle of MC activation and recruitment. Additionally, this variance in the distribution of MCs within a particular layer of the bladder wall would suggest that the current practice of using a biopsy sample of bladder wall in the diagnosis of PBS/IC should be reconsidered. Although easier to acquire, a biopsy bladder wall sample will only capture tissue from the mucosa to the outer layer of the detrusor muscle, and the results of this study suggest that it is the deeper layers of the bladder wall, i.e. the entire detrusor and the lamina propria, that hold the key to a more detailed and accurate PBS/IC diagnosis (in regards to MC subtype presence and distribution).

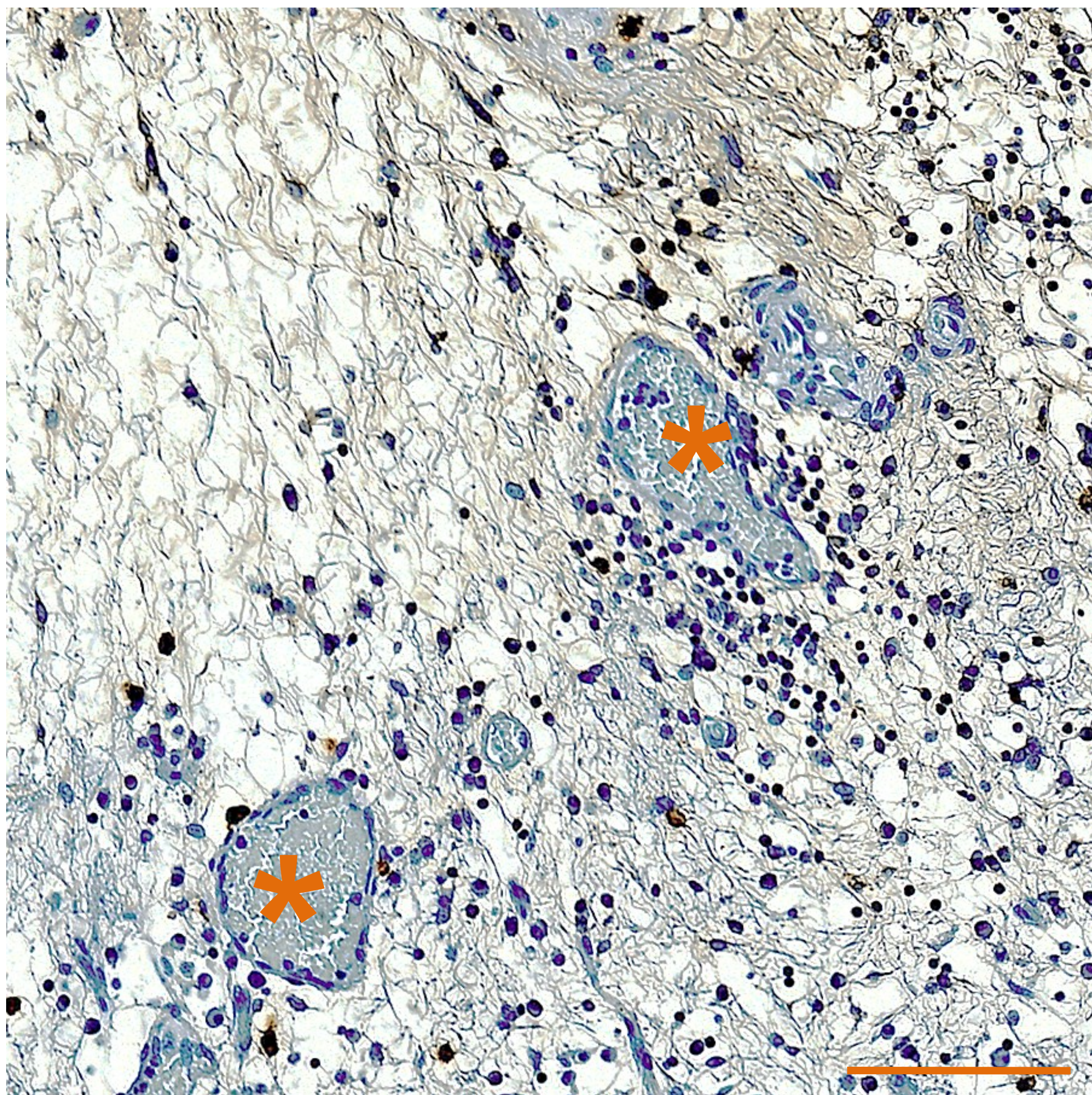


*Figure 3-11: Distribution of AA1 positive mast cells in human PBS/IC bladder tissue, imaged at a magnification of x80 using light microscopy. Note that positively stained brown MCs are predominantly localised around transversely cut nerve fibres (as denoted by * Mast cells were identified using the mAbs AA1 and CC1, the toluidine blue stain, and a standard immunohistochemical procedure. Scale bar= 100µm.*

The results also indicate that although there is no significant difference in the density of MC subtypes in controls (Figure 3.8), the increased density of both subtypes within the detrusor supports the currently accepted pathogenesis of PBS/IC as discussed above. This may explain why the majority of current treatment options have a success rate of

61% (Stav et al. 2012) or less; these therapies are failing to target the stimulation of nerve fibres, which occurs when urinary components leak through a damaged GAG layer.

The significant increase of the MC_{TC} subtype in the detrusor ($p<0.01$) and lamina ($p<0.01$) of PBS/IC individuals is an interesting finding, as chymase has a greater destructive potential than tryptase and may explain the variability in severity of symptoms between PBS/IC sufferers. The number of MC_{TC} within the adventitia is almost identical between normal and PBS/IC bladder. This suggests that MC_{TC} is a constitutive component of the bladder, and that the larger influx of MC_T is the more important event in PBS/IC. MC subtypes have different roles within the body; MC_T are associated with the immune system and are found predominantly at mucosal surfaces, and MC_{TC} are non-immune system associated and are found predominantly in connective tissue.

As MC_{TC} are increased in fibrotic disease, it could be suggested that the statistically significant influx of MC_{TC} in the lamina propria and detrusor of PBS/IC bladder may be linked to a higher risk of fibrosis. Clinically this would be reflected as a low bladder capacity due to thickening and stiffening of the bladder wall. To confirm this, further histological staining of bladder wall tissue samples would be required. If these findings can be confirmed, then PBS/IC patients who have a significant influx of MC_{TC} may benefit from therapies that are specific for the treatment of fibrotic bladder. In addition, upon unblinding to the patient data and diagnostic notes, a link between a substantial presence of MC_{TC} in the mucosa, and the presence of ulcers was observed. This novel finding may have significant clinical ramifications i.e.; it would allow the identification of patients who suffer from the less common Hunner's ulcer subtype of PBS/IC (Yamada et al. 2000).

The main limitation of the study is that the number of patients in both test groups was small. Only full-thickness bladder sections were selected, and this type of tissue sample is less likely to be taken during cystoscopy. Although the paraffin- embedded tissue block may have been classed as full-thickness in the tissue bank database, once the tissue had undergone processing, paraffinisation, storage, and preparation for this study, shrinkage and tissue loss occurred thus resulting in incomplete representation of the bladder wall. Consequently, a number of patients were excluded from the study.

In conclusion, PBS/IC is an under-researched disorder, thus it is poorly understood, diagnosed and managed. In addition, the diagnostic criteria and treatment options for PBS/IC need modernising. The study highlights the clinical importance of MCs in the pathogenesis of PBS/IC. A diagnosis of PBS/IC is based on the patient's symptoms and urological assessment, including cystoscopy, urodynamic study and exclusion of other common urinary diseases such as bacterial cystitis, urethritis and neoplasia (Nigro et al. 1997). Despite this, only the ESSIC criteria incorporate the presence of detrusor

mastocytosis or inflammatory infiltrates for PBS/IC diagnosis. This study and other similar work suggest that the MC count should be incorporated into all official diagnostic criteria. Although not all patients exhibit mastocytosis, inclusion of a minimum MC count would aid the identification of individuals who are otherwise misdiagnosed. Following further confirmation, these findings could be used to develop much-needed comprehensive diagnostic criteria for the successful identification of PBS/IC sufferers, and for the development of novel tailored therapeutic options, that would benefit patients.

Chapter 4: Basophils in Painful Bladder Syndrome/Interstitial Cystitis

4.1 Introduction

Basophils are part of the same lineage as MCs, and so it is unsurprising that this inflammatory cell is often studied in a variety of inflammatory disorders. However, the research literature exploring the role of basophils in PBS/IC is scarce (Aldenborg et al. 1986). This may be because PBS/IC is such an understudied area of urological disease, in addition to basophils being a rare blood-based cells that would not be involved in chronic tissue inflammation (Irani et al. 1998) as is characteristic of PBS/IC. However, the increasing quantity of research based on the role of the basophil in disease has shown that basophils can be detected in skin tissue of those with atopic dermatitis and bullous pemphigoid (Ito et al. 2011), and so seem to have the ability to migrate to the site of inflammation in tissue. In addition to this, basophils are granulocytic cells that release histamine as part of their role in inflammation (Siracusa et al. 2013), and PBS/IC individuals have elevated of histamine in urine (Lv et al. 2012). Histamine also recruits other inflammatory cells, such as MCs to the site of inflammation, which leads to activation of MCs, and further release of histamine and inflammatory molecules, thus creating a positive- feedback loop (Mazzoni et al. 2001).

An immunohistochemistry protocol to test and refine the 2D7 and BB1 antibodies specific for basophils was designed and developed (following the standard IHC procedure as outlined in Appendix C). Initially, the protocol was developed using tissues and dilutions suggested in the literature (Irani et al. 1998, Ito et al 2011, McEuen et al 1999, Agis et al. 2006). Once successfully stained basophils were able to be positively identified by several histochemistry specialists, the protocol was considered to be developed enough for use with human bladder tissue.

4.2 Basophils

The basophil was first described by Paul Ehrlich during his work on MCs and other blood cell types at the end of the 18th century. They are granulocytic inflammatory cells, which make up approximately 1% of total blood count (Ito et al. 2011), and yet, despite this, basophils are evolutionary preserved in numerous animal species. This indicates that the basophil plays a crucial role in the maintenance and proper functioning of the body.

Basophils have a similar call lineage to MCs, mature in the bone marrow, contain histamine and leukotriene C4 granules, and degranulate upon IgE cross linking. Basophils have not been extensively researched as they make up less than 1% of blood leukocytes and have a short life span. Up until recent advancements in detection methods, it was thought that basophils may play a role in IgE mediated Type 1 Hypersensitivity reactions, and in chronic allergic reactions. In recent years, the development of a basophil-deficient mouse model, in conjunction with the discovery of more reliable detection methods, has led to the progression in the study of basophils. It is also generally thought that basophils do not migrate to sites of inflammation, like MCs, and can only be found in circulation, but research has shown that a small percentage of basophils can be observed in the tissues of asthmatic patients, and those of atopic dermatitis (Mukai et al. 2009). Previous work by our group demonstrated the possible presence of basophils in bladder tissue obtained from patient with interstitial cystitis, however, the results are ambiguous as the cells thought to be positively-stained basophils seem to have more of a MC appearance. Therefore, these results are unclear and require further investigation.

When stimulated, basophils release a range of mediators, including cytokines (such as IL-4 and IL-13), histamine, and leukotriene C4. However, these are non- specific to basophils, and are also released by other cells types such as MCs and eosinophils. To address this, much immunological research has been conducted in order to identify basophil-specific markers that can be identified using antibodies. By raising antibodies to whole basophils, several basophil-specific monoclonal antibodies have been identified, including 2D7 (Kepley et al. 1995) and BB1 (McEuen et al. 1999), which are able to react with the granular constituent of basophils.

In addition to using the IHC technique for the detection of MCs in PBS/IC bladder tissue samples, it was also used to identify the presence or absence of basophils in the same tissues. Unlike the antibody AA1 for the detection of tryptase in MCs, there is no ‘gold

standard' antibody for the detection of basophils. Two monoclonal antibodies currently exist for the successful identification of basophils. The only commercially available antibody is '2D7', and it is claimed to be specific for 2D7 secretory granules in basophils, without any cross-reaction with MCs (Arock et al. 2002). However, it has been suggested that the 2D7 antigen is present in both mature MCs and basophils (Idoate et al. 2013), thus making it non-specific for basophils. Although 2D7 has been used successfully for the detection of basophils using the IHC technique, the problem with cross-reactivity with MCs has led to the development of an antibody that targets basogranulin, a secondary granule thought to be specific to activated basophils (Mochizuki et al. 2003).

Due to the variability in the reliability of 2D7 and BB1 (Siracusa et al. 2013, Crome 2012), it was decided that both antibodies would be tested, in order to increase the chance of the successful detection of basophils in PBS/IC tissue. As is routinely done, before the antibodies can be used to procure results with the tissue of interest, the optimal dilution and control tissue need to be established. These both need to be optimised in order to minimise false positives and false negatives, increase cost effectiveness, and test that the antibody is the correct match for the antigen of interest. A positive tissue control must be used in order to validate IHC staining, and in cases where it is unknown as to whether or not the cell of interest is present in the tissue sample, a tissue known to express that particular cell of interest must be used. A positive result from the positive tissue control will validate that the cell of interest can be detected, and if it is not present in the tissue sample of interest, then the cell of interest is not present in that tissue.

With most commercially available antibodies, a data sheet providing the optimal experimental protocol is provided. This includes information regarding the correct dilution, species reactivity, antigen specificity, antigen retrieval method, suitable applications, and the positive control tissue. In the case of AA1, this data sheet is thorough, well-tested, and can be closely followed to produce trustworthy results. However, in regards to 2D7, this is not the case. Unfortunately, the existing data on 2D7 is scarce, and has led to the laborious and time consuming task of creating and developing these experimental standards during this project, in order to then implement the procedure with PBS/IC tissue to produce trust-worthy and reproducible results. It should be noted that during the time of this project, a partial data sheet for 2D7 was made available; however, research utilising this protocol remains uncommon.

Generally, an antibody will have a recommended dilution and/or control tissue, however, these data are still in their preliminary format for 2D7 and BB1. The standardisations of

the control tissue and antibody dilution to be used are not mutually exclusive tasks, and so had to be considered almost simultaneously. To begin this process, a thorough search of the literature for existing research that had successfully used 2D7 and/or BB1 to detect basophils was conducted.

Results were scarce, with little correlation in relation to control tissues, success of antibody detection, dilutions, disease states that have an elevated basophil count, or even the technique that would be best employed to detect basophil infiltration. There was also a discrepancy with regards to the recommended dilution for 2D7, with results in the literature ranging from 1:100 (Idoate et al. 2013), to 1:300 (Irani et al. 1998), and the data sheet recommending a 1:50 dilution (Abcam, UK). This controversy in regards to the best dilution for 2D7 led to the decision of establishing a new protocol as part of this project. Although time- consuming, it was a necessity in order to ensure the consistency of results, and reproducibility of this study.

4.3 Validity of control tissues

Of the research that utilised the 2D7 and BB1 antibodies, the healthy tissues that were reported as containing a detectable quantity of basophils, were tonsil (Walls & Amalinei 2014) and bone marrow (Agis et al. 2006), and the disease states that were cited as having basophilia (an abnormally elevated number of basophils), were chronic myeloid leukaemia (CML; Apperley 2015), asthmatic lung (AL; Nabe et al. 2013), sarcoidosis (Gaik et al. 1992), and a variety of human skin diseases (skin lesions of atopic dermatitis, prurigo, urticaria, bullous pemphigoid, drug eruptions, eosinophilic pustular folliculitis, insect bites, scabies, Henoch-Schonlein purpura, and dermatomyositis) (Ito et al. 2011).

In conjunction with the results from the literature, several histopathologists and researchers within SGH were consulted in order to help finalise a list of tissue controls to use for the validation of the 2D7 and BB1 antibodies. A list of tissues of interest was put together using the process depicted in Figure 4.1, and an attempt to collect adequate amounts of each of the tissues was made. This process was both time-consuming, and

led to the exclusion of tissues that could not be acquired due to issues with supply or ethics that were beyond the scope of this project.

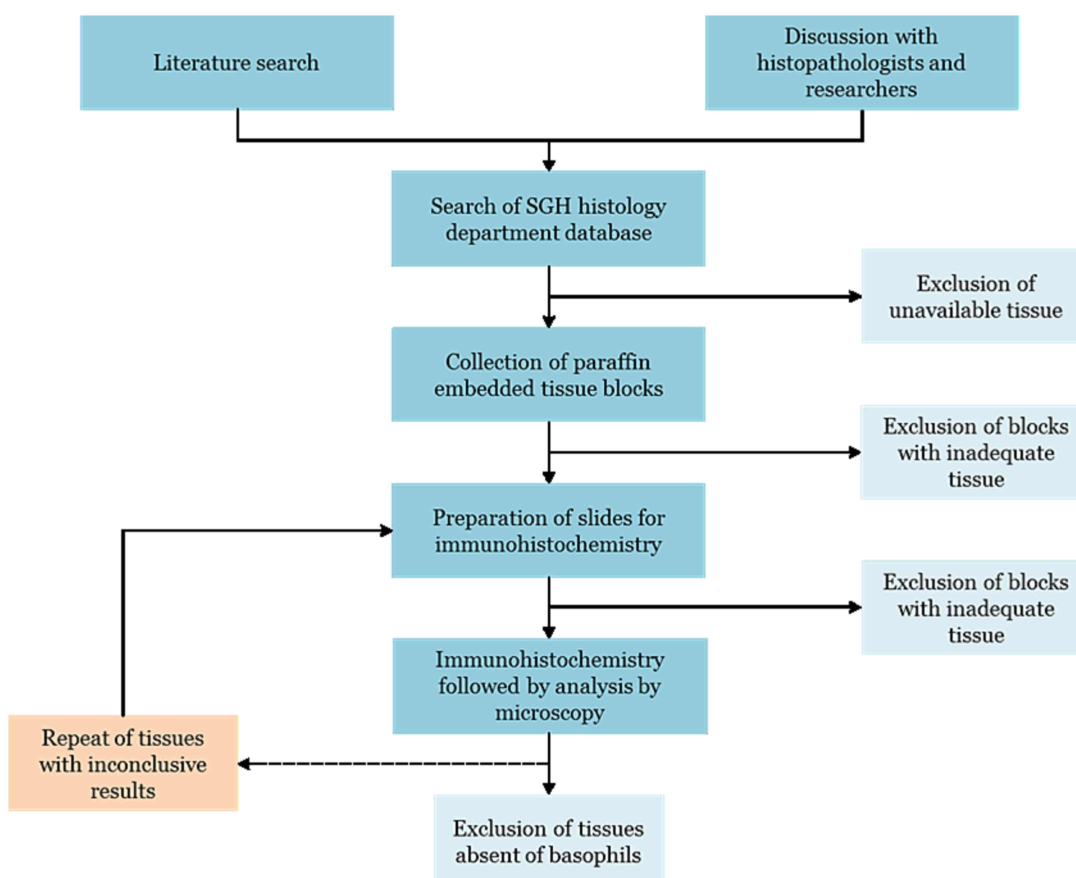


Figure 4-1: A flowchart depicting the decision process for the selection and inclusion of tissue as a control for the validation of the basophils antibodies.

4.3.1 Ethical considerations

Due to the nature of this study, ethical approval was required, as is normal practice. The study fell under the umbrella ethical approval granted to the research team, and so it was not necessary to apply for ethics specific to this study. Additionally, this study complied

fully with Good Laboratory Practice (GLP) and registrations such as the Human Tissue Act (2004). The researcher successfully completed Good Clinical Practice (GCP) training, and utilised this during the study. The researcher carefully considered the use of archival tissue in this study and an aim of the study was to advance the understanding of inflammatory cells in PBS/IC, and to develop an anti-inflammatory treatment for PBS/IC to be used in humans, archival tissue had to be used. The researcher was sure to minimize wastage with efficient lab practice and accurate protocol development.

4.3.2 Tonsil

Tonsil tissue is routinely used as a control tissue for the detection of MCs in IHC (Walls et al. 1990), and has also been suggested as positive control tissue for the detection of basophils (Arock et al. 2002). Tonsil was tested many times throughout this study, yet yielded no positive results for basophils.

4.3.3 Bone marrow

Basophils originate from haemopoietic stem cells, and typically, mature within the bone marrow before entering blood circulation (Arinobu et al. 2009). For this reason, bone marrow was thought to be a good candidate for the positive control tissue for the basophil arm of this study. Several paraffin blocks of bone marrow were tested, and although most appeared to be void of basophils, the bone marrow tubular biopsy produced doubtful results. There appeared to be some stained basophil-like cells, yet due to the deformation that occurs when a delicate tissue, such as bone marrow, has undergone IHC with a microwave pre-treatment, the resulting staining was sparse and was difficult to analyse confidently. This block was then retested several times, and when compared to TBS control slides, results for basophils were negative.

As an extra validation step, it was decided that all sections would be cut onto X- tra® Slides (Leica Biosystems, Germany) and then tested. These slides have a positively charged surface which helps to adhere more fragile paraffin embedded tissue sections for

use during arduous histochemical procedures. Thus, bone marrow tissue sections from the same block were applied to the X-tra® Slides and the immunohistochemistry process was repeated as before. Unfortunately, this still resulted in negative results when compared to TBS controls.

4.3.4 Inflamed appendix

It was suggested by the histopathology team within the Histochemistry Research Unit (HRU) that inflamed appendix should be tested for the presence of basophils as during the process of inflammation there is the recruitment of many inflammatory cells, including basophils and MCs. Inflamed appendix was regularly used during this study, but did not show positively stained basophils with either of the monoclonal antibodies (mAbs).

4.3.5 Sarcoidosis

Although the basophil infiltration associated with this disorder is not of the same density as seen in asthma (Gaik et al. 1992), sarcoidosis is another disorder that is associated with basophilia (Codeluppi et al. 2014). Sarcoidosis is a multi-systemic granulomatous disorder that results in the development of multiple non-necrotising granulomas (collections of inflammatory cells that appear as small, red, swollen patches) on various locations throughout the body, but most often affecting the lung parenchyma and lymphatic system (Mondoni et al. 2015). Multiple blocks of cutaneous sarcoidosis tissue were acquired and underwent routine IHC with the 2D7 antibody. Unfortunately, these tissues did not yield a positive result for basophils during this study.

4.3.6 Asthmatic lung

An infiltration of basophils has been noted in the lung tissue of asthma sufferers (Nabe et al. 2013), and the 2D7 antibody has been used previously to detect basophils in post-mortem lung sections from individuals who had died from fatal asthma (Kepley, 2001). These findings in conjunction with advice from the histopathology department at Southampton General Hospital (SGH), gave strong evidence supporting the use of AL tissue as a positive control for basophils; however, histological specimens are usually not taken as the disorder can be treated without surgery. It was then suggested that an attempt to locate AL from post mortem patients should be made. This required contacting clinical lung pathologists at SGH, in order to acquire AL samples, yet unfortunately, it was found that AL samples are extremely rare, and it was unlikely that the tissue could be supplied for this study. Over the course of this study, no AL tissue was made available, and thus it was concluded that acquiring the tissue was beyond the scope of this study, and so, AL was excluded from basophil investigation.

4.3.7 Skin disorders

As mentioned briefly above, Ito et al. (2011) successfully detected basophils in a variety of inflammatory skin disorders using the Immunohistochemistry method and the antibody BB1. Amongst others, basophils were found in skin lesions of dermatomyositis, purpura, bullous pemphigoid, urticarial, and prurigo. Of these, urticara and bullous pemphigoid were found to be particularly basophil rich, and other disorders such as atopic dermatitis contained much fewer numbers. Further research of the literature (Ito et al. 2011, Siracusa et al 2013) seemed to confirm these results, and so, these tissue blocks were requested from the SGH histology department.

Due to the delicate composition of skin, it was decided that all sections would be cut onto X-tra® Slides (Leica Biosystems, Germany). These slides have a positively charged surface which helps to adhere more fragile paraffin embedded tissue sections for use during arduous histochemical procedures. Each block was tested several times with the 2D7 antibody, yet yielded no positive results.

4.3.8 Chronic myeloid leukaemia

As with the previously listed control tissues, a similar issue with the availability of adequate tissue was found when attempting to procure histological samples of Chronic Myeloid Leukaemia (CML). This disorder is characterised by a proliferation of the myeloid cell line, with up to a 50% increase in myeloblastic subtypes, and more specifically, up to a 20% increase in circulating basophils (Apperley 2015). Due to this large increase of basophils in the blood, it was decided that CML tissue would be preferential as the control tissue for basophils. However, acquiring the tissue was troublesome; of the 8,600 leukaemia diagnoses made in the U.K. in 2011, only 600 cases were CML (NHS statistic, 2011), thus making CML a rare cancer type. Several attempts were made to acquire CML bone marrow tissue, but none could be acquired from within SGH, and due to ethical issues, CML samples could not be acquired from other hospitals. It was possible, however, to acquire CML skin punctures, embedded in paraffin. Of those diagnosed with leukaemia, 3% will also develop leukaemia cutis; which is an infiltration of neoplastic leukocytes into the dermis, resulting in cutaneous lesions (Rao & Danturty 2012).

The CML skin biopsies were tested for basophils using a range of dilutions, based on recommended dilutions from the literature and data sheet. Positive staining was identified in the CML tissue, however only in samples that contained blood. This is unsurprising as basophils are a blood-based cell type, and although some evidence suggests that basophils can be detected in dermal disorders (Ito et al. 2011), the majority of literature states that basophils do not migrate into tissue sites of inflammation. The CML skin biopsies were then tested for MCs using the AA1 antibody, so as to clarify that the 2D7 had identified basophils and not MCs. The results are shown below.

In order to confirm results, a total of five repeats were conducted, and matching positive results were yielded each time. When analysing the 2D7 tissue, it can be seen (Figure 4.2) that the cells that have been detected have a typical basophil appearance; i.e. they are smaller in diameter than MCs, contain larger but fewer granules, and have a bi-lobed nucleus. The cells that were identified by the AA1 antibody have the distinct appearance of MCs; a large diameter (10 µm; twice the size of a basophil), small granules (approx. 0.2 µm in size), a cytoplasm densely packed with granules (~1000, compared to ~80 in basophils), and a round nucleus. When comparing the AA1 positive and 2D7 positive images side-by-side, it could be argued that the AA1 antibody has also detected the

inflammatory cells that have been picked up by the 2D7 antibody. However, close inspection revels that these cells are not identical, and basophils have been correctly identified only by the 2D7 antibody.

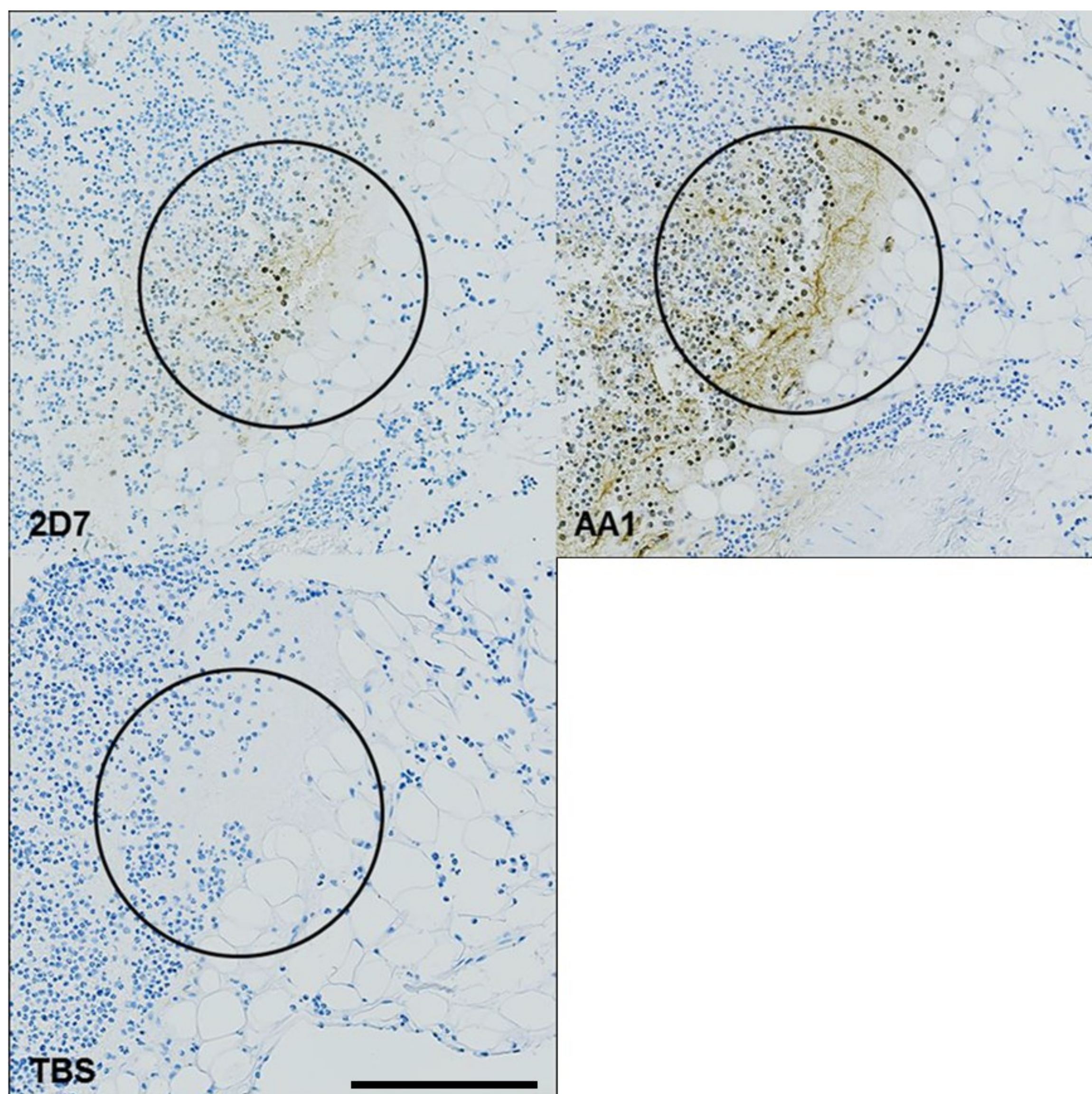


Figure 4-2: A series of immunohistochemically stained chronic myeloid leukaemia skin biopsies, with positive staining for mast cells and basophils. A series of immunohistochemically stained chronic myeloid leukaemia skin biopsies, with positive staining for mast cells and basophils. Tissues have been stained using toluidine blue, and imaged at a magnification of x4 using light microscopy. 2D7 positive identification of basophils in CML tissue; AA1 positive identification of mast cells in CML tissue; TBS image with no positive staining of cells of interest in CML. Scale bar= 500µm.

4.4 Dilution optimisation

During testing of the possible positive control tissues, a variety of dilutions of both the antibodies were also tested. 2D7 was tested at the dilution suggested in the data sheet (1:50) as well as 1:40, 1:30, 1:60, and neat. BB1 was tested at 1:4, 1:5, and neat, on most of the tissues discussed above, using both the microwave citrate, and pronase antigen retrieval methods. As BB1 was acquired from another research group within the UoS, it was not practical to repeatedly test BB1, as multiple batches from different production dates were tested, and all appeared to be ineffective (Figure 4.3). In an effort to reduce the wastage of BB1 antibody, archival diseased tissue, especially tissue that was not available in large quantities (such as the CML), it was decided that BB1 would no longer be included in the detection of basophils, and only 2D7 would be utilised, 2D7 is a commercially available antibody, and so has gone through vigorous testing and quality checks before being made available to the public. In addition to this, the discrepancy between batches would be negligible, although as part of the study, each new batch was titrated to find the optimal dilution specific to it, before continuing with the protocol.

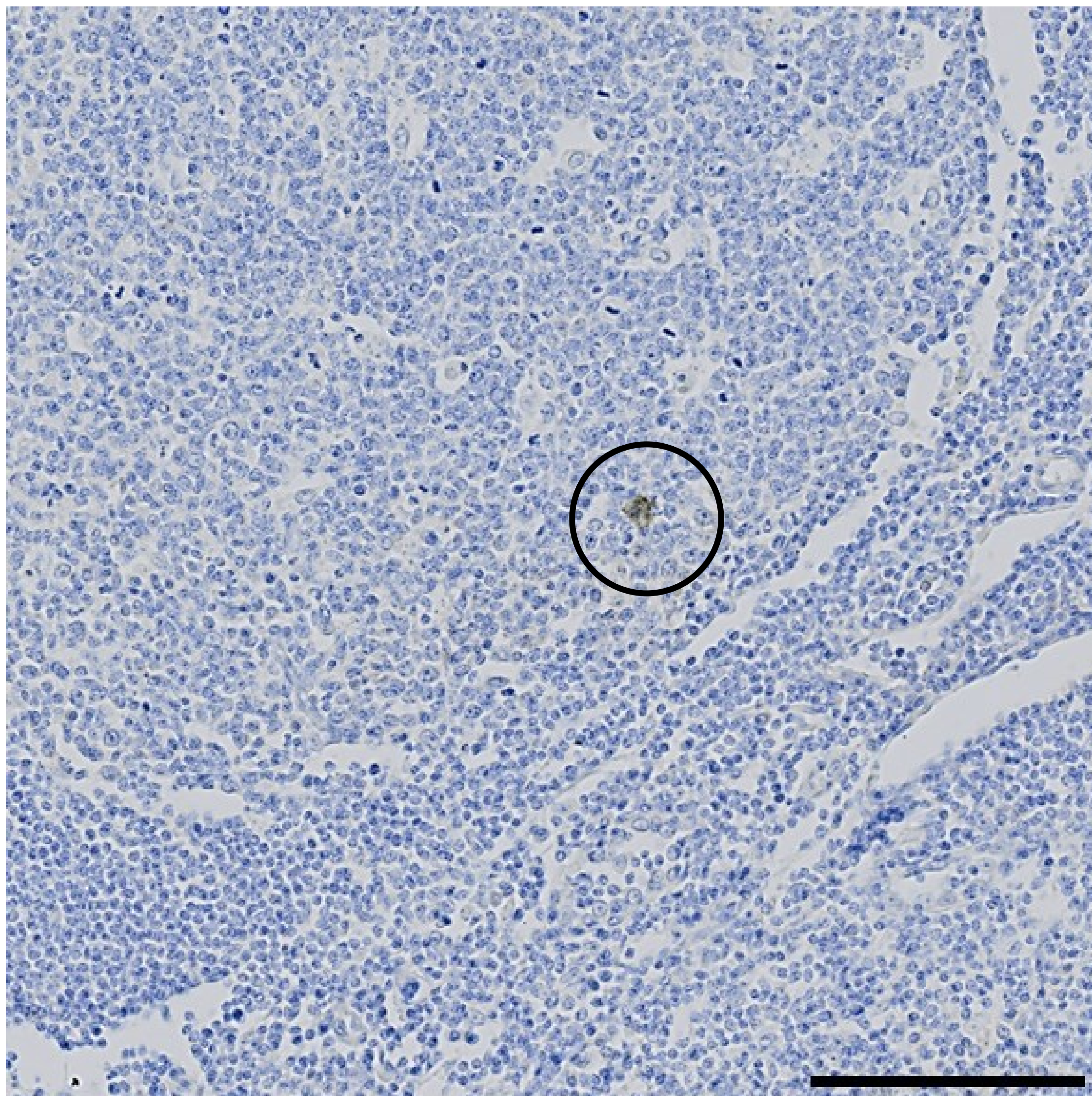


Figure 4-3: An image of nasal polyp, taken at x20 magnification using light microscopy. The tissue has been immunohistochemically stained using the mAb BB1, and toluidine blue. The image shows no positive staining for basophils or any other inflammatory cells, and an area of brown staining that is irregular and considered as an artefact. Scale bar= 100µm.

4.5 Painful bladder syndrome/interstitial cystitis

After the success of the 2D7 antibody with chronic myeloid leukaemia skin punch samples, it was decided that 2D7 was effective in the detection of basophils, and chronic myeloid leukaemia was a suitable positive control tissue. Additionally, an attempt to acquire chronic myeloid leukaemia bladder tissue was made, but there was no such tissue available at the time of this study.

The successful protocol that was developed involved a microwave pre-treatment with citrate, and a 2D7 dilution of 1:40. Using this protocol, all interstitial cystitis blocks were tested several times, however no basophils were detected. A summary of all results can be seen in Table 4.1 below.

Table 4-1: A summary of the tissues used in the development of the 2D7 mAb immunohistochemistry protocol for the detection of basophils.

Tissue type	Sourced	Stained	Basophils
PBS/IC bladder	Yes	Yes	No
Chronic myeloid leukaemia skin punch	Yes	Yes	Yes
Chronic myeloid leukaemia bladder	No	No	No
Asthmatic lung	No	No	No
Bone marrow	Yes	Yes	No
Inflamed appendix	Yes	Yes	No
Tonsil	Yes	Yes	No
Sarcoidosis (cutaneous)	Yes	Yes	No
Bullous pemphigoid (cutaneous)	Yes	Yes	No
Dermatomyositis (cutaneous)	Yes	Yes	No
Purpura (cutaneous)	Yes	Yes	No
Prurigo (cutaneous)	Yes	Yes	No
Urticaria (cutaneous)	Yes	Yes	No

4.6 Limitations

The lack of positive staining of basophils in PBS/IC was not unexpected. The basophil is highly elusive, and with circulating levels of less than 1% (Ehrlich, 1878) and a lifespan of 60-70 hours for mature basophils (Siracusa et al. 2011), the likelihood of positive identification of a basophil is slim. In regards to the positive control tissue that was found, CML, the chance of the positive identification of a basophil was much higher due to the 20% increase in circulating basophils in this disorder (Apperley 2015). The positive identification of basophils was also helped by the fact that the tissue block contained a sample of blood, as following the IHC procedure, the positively stained basophils were all found to be located within this area (Table 4.1 and Figure 4.2). This is unsurprising as basophils are a blood-based cell type, and although some research shows that they successfully infiltrate skin lesions during inflammatory skin disorders (Ito et al. 2011, Otsaka and Kabashima 2014), these results were not reflected in this study.

Another limitation of this portion of the study has been discussed in a previous chapter which looked at the distribution of MC subtypes in PBS/IC using the IHC method. The IHC technique has its limitations, and these must be kept in mind for the basophil aspect of this work.

Secondly, the suitability of the 2D7 and BB1 antibodies for IHC work, and for the detection of basophils must be questioned. As stated previously, these antibodies are largely untested, and with such a range of titrations and variability in results, the comparably smaller scale of this work may have been the reason for the lack of success. Also, 2D7 and BB1 may be particularly susceptible to degrading if stored incorrectly, or if permitted to go through repeated freeze-thaw cycles, which can occur during transport of the antibodies from freezer to bench, or if the freezer experiences a lack of power. The limitation of degradation and storage was a chief reason for excluding BB1 from further testing during this study; the source of the BB1 antibody used here was non-commercial, and so the guarantees that are usually in place for such products could not be in place for this study, and thus issues with purity, degradation, and storage were encountered.

4.7 Discussion

Basophils are thought to play a non-redundant role in the inflammatory process via the release of several cytokines and mediators, as well as antigen presentation. Although basophils are mainly present in peripheral blood, they are able to migrate into tissues such as lymph nodes, skin and the lungs, during inflammation and pathogenic disease states (Mukai and Galli 2013).

Basophils are activated by IgE and antigens, and so play a role in acute allergic diseases such as anaphylaxis, and chronic allergic disease states such as atopic dermatitis and hay fever. Although some of this role may overlap with MCs, in PBS/IC, it does not.

Despite the limitations of this study as discussed previously, the finding that basophils are not present in PBS/IC bladder tissue remains a crucial finding and contribution to the scientific literature. This key finding suggests that PBS/IC is a chronic inflammatory disorder that is driven by the presence of MCs, and supports the need for tailored treatment options that target mast cells and its derivatives/subtypes specifically.

Chapter 5: Honey and Rat Bladder Explants

5.1 Introduction

The initial concept of this study was to elucidate the potential of honey as an anti-inflammatory agent for the treatment of PBS/IC patients. Analysis of the existing honey-related research has provided statistically significant data indicating the anti-inflammatory capabilities of honey. A thorough literature search has also established the poor treatment options for PBS/IC, and the presence of inflammatory cells in the bladder of these individuals. This, in combination with the assays conducted earlier in this study are the basis for hypothesising that honey could act as a potential anti-inflammatory agent for the treatment of PBS/IC. Thus a series of experiments were designed, refined, and conducted in order to help gain an insight into this potential.

Initially, the tissue culture assay was developed to discover what would transpire over a broad timeframe. Thus capturing the initial response of the tissue, the response over the "treatment window" (i.e. the timeframe over which the treatment would be instilled and held within the bladder), and the response after prolonged exposure to the honey solution (to help understand the tolerance and potential toxic effect of honey on bladder tissue). Whilst undertaking this 8-day assay, a number of findings led to the development of further assays that will be discussed in stage II of this chapter.

5.2 Hypothesis

Honey stabilises mast cells degranulation through its anti-inflammatory effect.

5.3 Aims

The aims of this project are as follows:

- To use commercially available ELISA to assess a range of concentrations of honey in order to evaluate their effect on stabilising mast cell degranulation reducing spontaneous histamine release in rat bladder explants.
- To establish a realistic working concentration of honey to be utilised in future experiments in vivo in PBS/IC bladders.

5.4 Stage I

The response of bladder tissue explants in exposure to varying concentrations of honey was assessed, by measuring the level of histamine in the medium, in which the tissue had been incubated. Histamine is a short-acting endogenous biogenic amine found widely throughout the body. It is also a key mediator of immediate allergic reactions (such as anaphylaxis), and so typically is circulating in insignificant amounts. As histamine is released in response to an attack on the immune system, it has a short half-life, and is rapidly converted to N-methyl histamine. The histamine ELISA is able to react equally to N-methyl histamine and histamine, and so is an ideal choice for this study.

The ELISA was selected as the most appropriate assay to measure histamine in samples. The histamine ELISA is a competition based assay, in which the substrate is in direct competition with a competitive inhibitor for the active site of the enzyme. Thus, this means that increasing the concentration of honey substrate will decrease the likelihood of the inhibitor binding to the enzyme. Measuring the level of histamine provides a direct representation of the possible anti-inflammatory effect that honey may be having on the MCs within the bladder explant tissue.

5.4.1 Materials and methods

5.4.1.1 Tissue culture of rat bladder

Recent work within our research group has shown the anti-inflammatory activity of honey against the degranulation of LAD-2 cell line and release of histamine and IL-6 (Lwaleed et al. 2014), indicating a similar result could be achieved in bladder tissue explant.

The full protocol for the tissue culture assay is shown in Appendix E. Bladders were harvested from normal Albino Wister rats (<300g) freshly sacrificed under a Home Office Schedule-1 method, and then cut into 2mm by 2mm fragments. For each run of the assay, 2 bladders are required to produce 20 2mm2 tissue explants. Following the randomisation list produced via Sealed Envelope (Appendix F), the tissue fragments were distributed evenly between the 5 treatment groups (0%, 5%, 10%, 20% and 40% honey). The tissue explants were placed into a well plate with a medium made up of Dulbecco's Modified Eagle's Medium (DMEM), 10% foetal bovine serum, and L-glutamine-penicillin-streptomycin solution (Sigma Gx114, UK). After a "settling" period of 5 days, the rat bladder explants were exposed to varying concentrations (5%, 10%, and 20% v/v) of eucalyptus honey for a total of 8 days. Sampling of 500 µl of medium from each treatment well occurred every second day and these samples were then stored at -80 ºC until required for histamine analysis by ELISA (Figure 5.1).

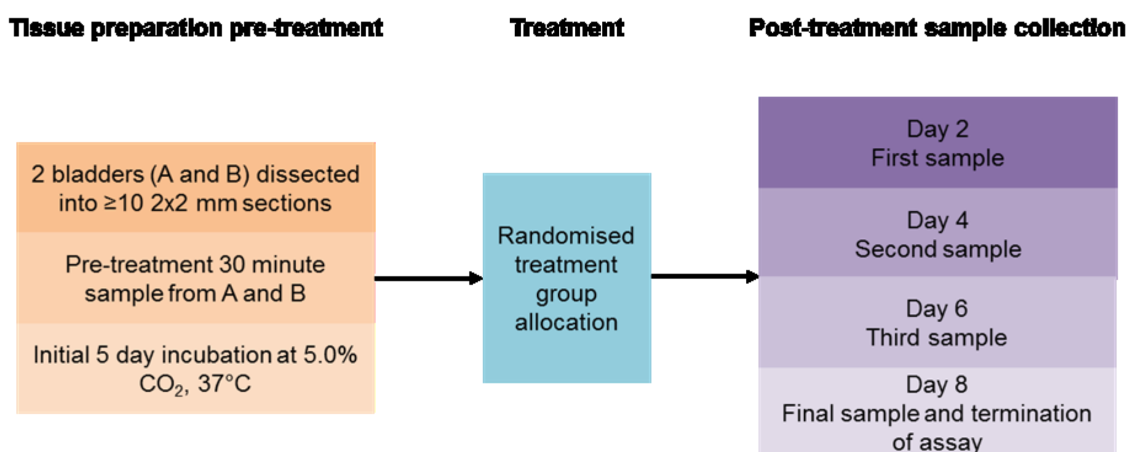


Figure 5-1: Sampling timeline for the tissue culture portion of the study.

5.4.1.2 Honey selection and preparation

As previously stated, eucalyptus honey was used for this part of the study. Previous research conducted by the group found that eucalyptus honey provided a high level of anti-inflammatory activity (Lwaleed et al. 2014), at a cost effective price (£0.86/100g) (Tesco PLC, Hertfordshire, UK). As the overall aim of this research is to produce a feasible treatment option for PBS/IC, the cost of the materials also plays a role in the larger-scale testing and production of the treatment in the future.

In order to ensure zero contamination of the honey, it was handled using aseptic technique and the standard lab procedures used when dealing with reagents. When utilizing the honey for preparation of the testing medium, the honey was first left to warm for 5 minutes at 37.5°C in order to make it less viscous and easier to measure accurately. The honey jar was then sprayed with disinfectant (90% ethanol 10% filtered water) before being taken under the hood to be opened and utilized.

5.4.1.3 Honey concentration and histamine assay

Whilst undertaking the bladder explant assay and conducting ELISAs, a key issue was raised; the effect of honey alone on the release of histamine remained unknown. Using the previous honey and LAD-2 cell work conducted by Lwaleed et al. 2014, an assay was developed to assess the spontaneous release of histamine from MCs. It was hypothesised that honey would stabilise MCs and there would be a reduction in the release of histamine.

A simple ELISA-based protocol was designed to assess if there was any effect of honey concentrations on the level of a known amount of 100% histamine. A volume of 10µl 100% histamine was added to each well of a small section of a 96- well plate, followed by a dilution of honey (0%, 1.25%, 2.5%, 5%, 10%, and 20%), in duplicate. Additionally, standard histamine calibrators were used to provide a standard histamine curve, and to serve as a control. The ELISA was then conducted following a standard procedure, and data was collected using a plate reader.

5.4.1.4 Histamine assay

The concentration of histamine within the medium was assessed using an ELISA in accordance with the manufacturer's instructions (Beckman Coulter UK Ltd, High Wycombe, UK) (Appendix H). According to the manufacturer, this test has an analytical sensitivity of 0.5nM (with a measurement range of 0.5- 100nm), and is 100% specific for histamine.

Results were obtained from a standard curve by interpolation (Figure 5.2) and this will be calculated following the manufacturer's guidelines; i.e. a semi-logarithmic curve fit (a logistic model) would be used with histamine concentration of the calibrators on the horizontal axis (nM) and absorbance values on a vertical axis. All results were entered into a database for analysis using GraphPad Prism 6.

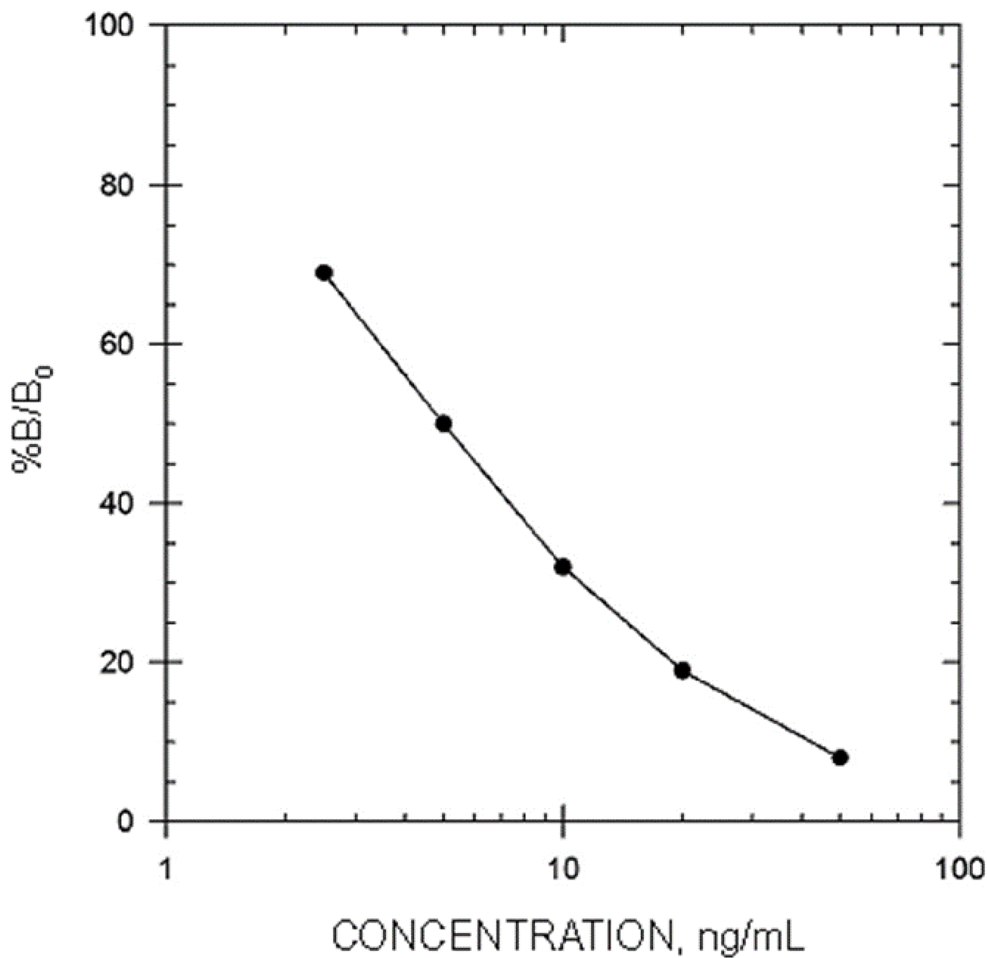


Figure 5-2: A typical histamine standard curve. (Reproduced from: Oxford Biomedical Research, http://www.oxfordbiomed.com/histamine-eia-kit; accessed on 28/05/15).

5.4.2 Ethical considerations

Due to the nature of this laboratory based study, ethical approval is not required, as is normal practice. This study complied fully with Good Laboratory Practice (GLP) and registrations such as the Human Tissue Act (2004). The researcher successfully completed Good Clinical Practice (GCP) training, and utilised this during the study. The researcher carefully considered the use of animal tissue in this study and as the end-point

of the study was to develop a treatment to be used in humans, animal tissue had to be used. The researcher understood the need for replacement, refinement, and reduction of the use of animals in research (Russell & Burch, 1959) and adhered to the strict regulations governing the use of animals in scientific research (Animals [Scientific Procedures] Act 1986).

5.4.3 Optimisation and standardisation

The protocol that was produced by the researcher for the tissue culture work has undergone several optimising and standardisation procedures during its use. One great improvement in the protocol was the introduction of randomisation. The implementation of randomisation strengthened the validity of results, by minimising selection bias and providing a satisfactory basis for generalisation. For this study, it was decided that the most suitable randomisation method would be block randomisation (Appendix G). This method allowed for each bladder to be represented equally in each treatment group, and the tissue explant that was taken from each bladder was randomly picked using a pre-generated block randomisation list (Sealed Envelope, 2001).

An additional standardisation step was introduced into the protocol in order to ensure that each explant was of the same size, and thus each well would contain approximately the same area of bladder tissue. Previous work by the researcher’s group standardised the amount of tissue in each well by weighing each tissue explant before it was added to the test medium, and then weighed again before destruction in order to calculate tissue loss or gain, and correct for this in conjunction with the histamine ELISA results. For this study, we decided that it was more important to keep the tissue as fresh as possible, reduce the likelihood of contamination, and to streamline the protocol. Thus, it was decided that this tissue-weighing standardisation step would be replaced with something simpler, quicker, and more efficient. This led to the development of a laminated cutting mat (Appendix F), which could be kept within the fume hood, wiped clean with 70% ethanol, and used under any transparent object (such as a petri-dish, or 6 well plate). The laminated cutting mat has gridlines marking out 1mm squares, and ensured that the researcher was cutting each explant to 2mm by 2mm, whilst the tissue was immersed in growth medium to maintain its viability and to reduce the likelihood of cellular damage from dehydration.

5.4.4 Statistical analysis

Data was compiled and analysed by GraphPad Prism 6 (Version 6.07; La Jolla California USA). Data normality was tested by the Shapiro-Wilk method. Data was normally distributed, and so statistical analysis using multiple independent t-tests were conducted. A p value of <0.05 was considered to be statistically significant.

5.4.5 Results

Increasing concentrations of honey reduces the spontaneous histamine release by rat bladder explants when compared to a 0% control

Over a period of 8 days, honey had a stabilising effect on the release of histamine, when compared to a 0% honey control solution (Figure 5.3). Results were only found to be significant when comparing 0% and 5% ($p<0.001$), and when comparing 0% and 10% ($p= 0.001$).

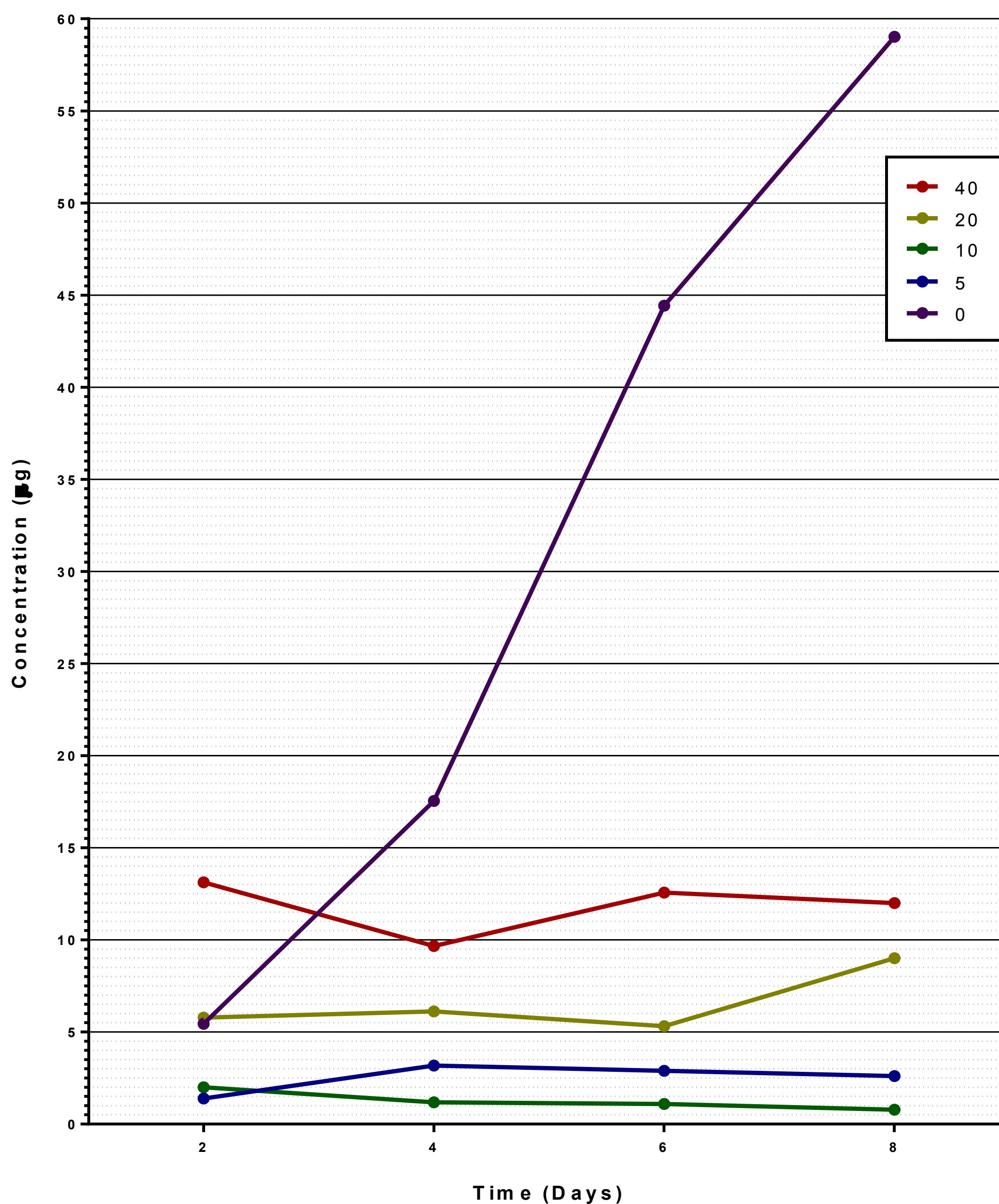


Figure 5-3: The effect of an increasing concentration of honey on the concentration of histamine release (µg) by rat bladder explant, over the duration of 8 days. Note that in comparison to the 0% control, lower concentrations of honey (5% and 10%) have the greatest effect on the reduction in the release of histamine from explants (p<0.001 and p<0.01 respectively), in comparison to higher honey concentrations (20% and 40%).

A honey concentration between 10% and 40% leads to a greater range in the concentration of histamine released over time

The difference in the range of results was highest in 0% honey, and honey concentrations above 10%, as reflected by the SD bars in Figure 5.4 below. The greatest range between repeated assays is seen in 0% honey. In addition, as the assay reached the Day 6 and Day 8 mark, the range in histamine concentration increased greatly.

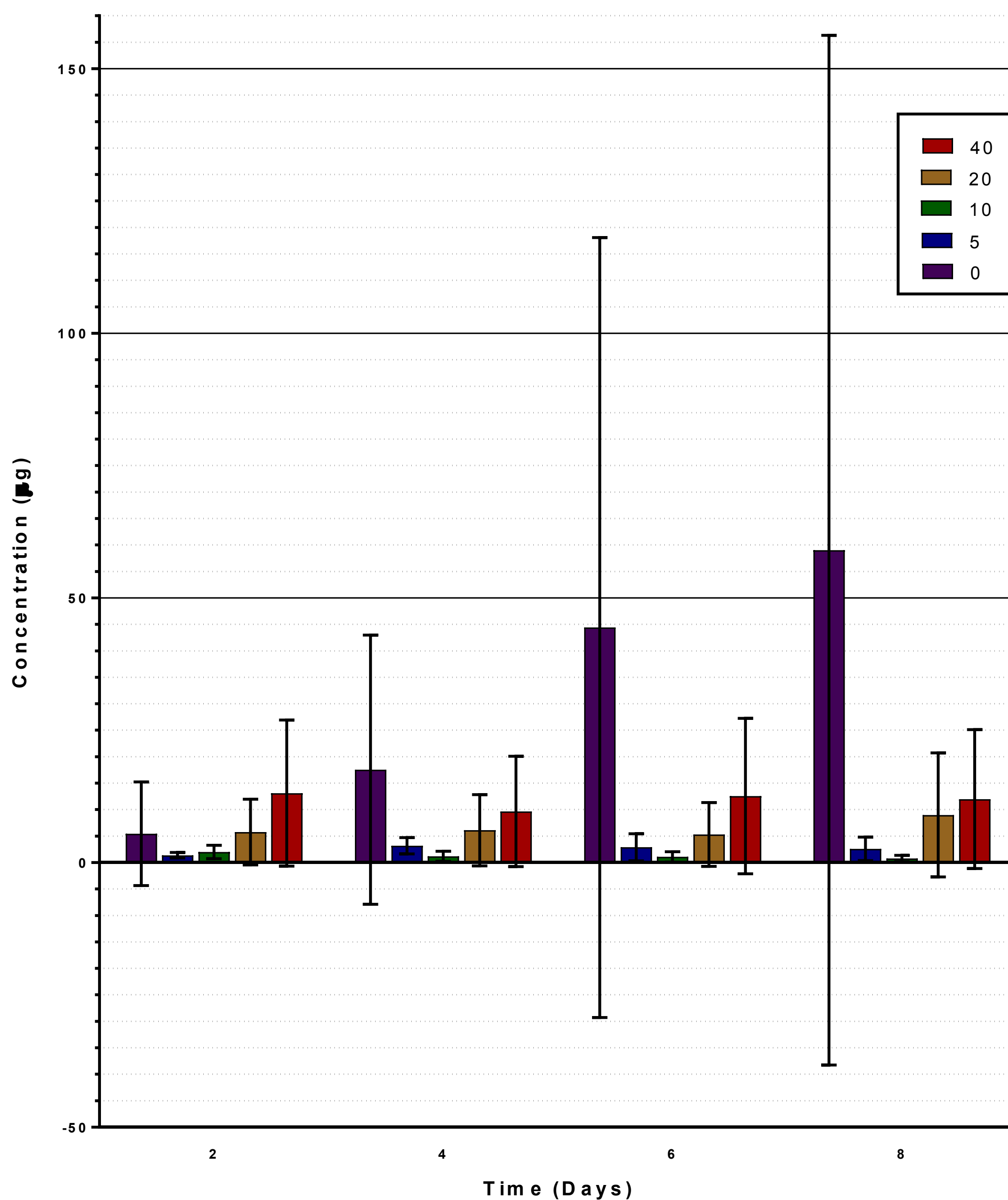


Figure 5-4: The effect of an increasing concentration of honey on the concentration of histamine release (µg) by rat bladder explant (n=5), over the duration of 8 days (±SD bars). Note that the greatest difference within a group is seen with 0% honey, and the least variance within the 10% honey group.

When assessing % inhibition of histamine release, 5% has the greatest inhibition on the release of histamine by rat bladder explants

Using the formula ‘Inhibition = [(normal activity - inhibited activity) / (normal activity)] x 100’, the inhibitory effect of honey was calculated and graphed (Figure 5.5). Results showed that 5% has the greatest inhibition over the longest period of time (approx. to Day 7), and 40% had the weakest level of inhibition over the total length of the assay. Interestingly, honey concentrations 10%-40% appear to increase the amount of histamine inhibition over time.

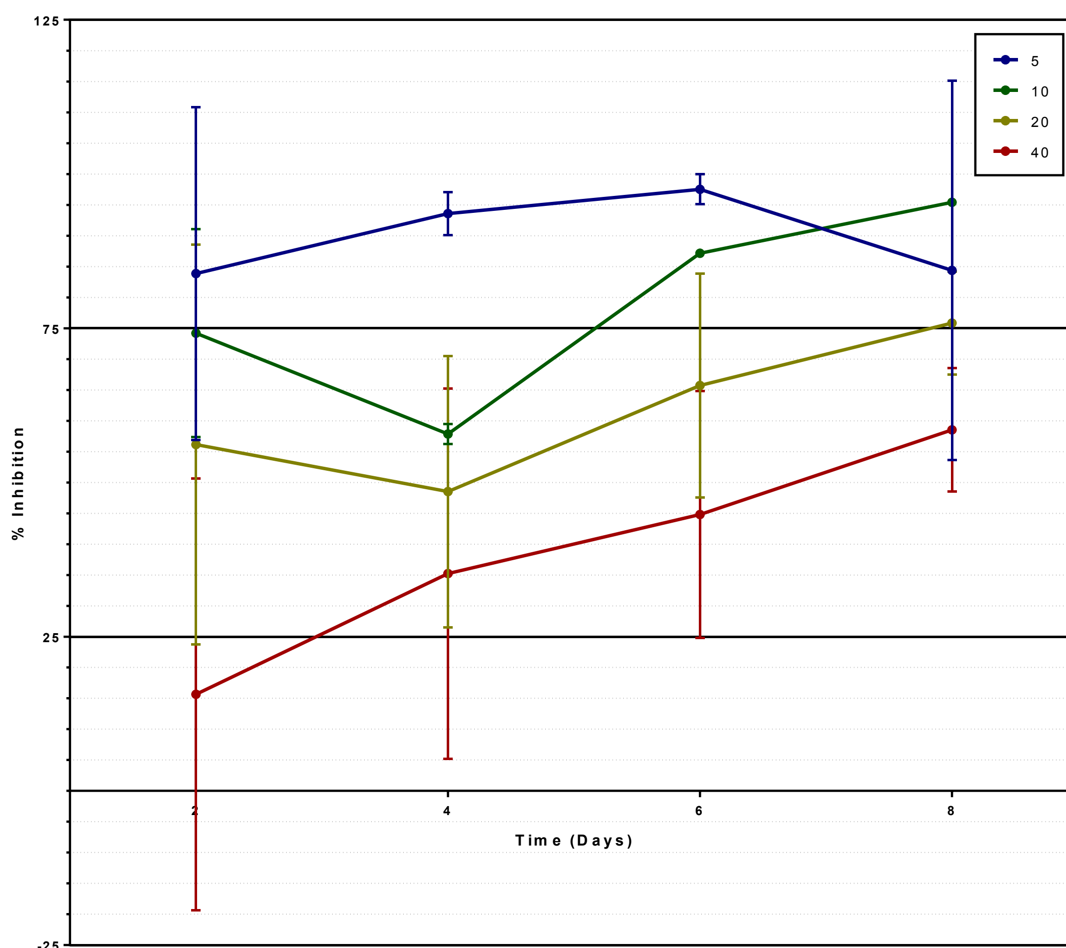


Figure 5-5: The effect of honey concentration on the % inhibition of histamine release from rat bladder explants over a period of 8 days (±SD bars). Note that 5% honey has the greatest inhibitory effect on the release of histamine from mast cells, and 40% honey has the weakest inhibitory effect. Additionally, 5% also exhibits the least inter-group variability as shown by the short standard deviation bars.

Honey does not bind histamine

Increasing concentrations of honey (1.25%, 2.5%, 5%, 10%, and 20%) does not greatly alter the concentration of histamine when compared to a control of 0% honey with histamine (n=2), thus suggesting that honey only minimally binds histamine (Figure 5.6).

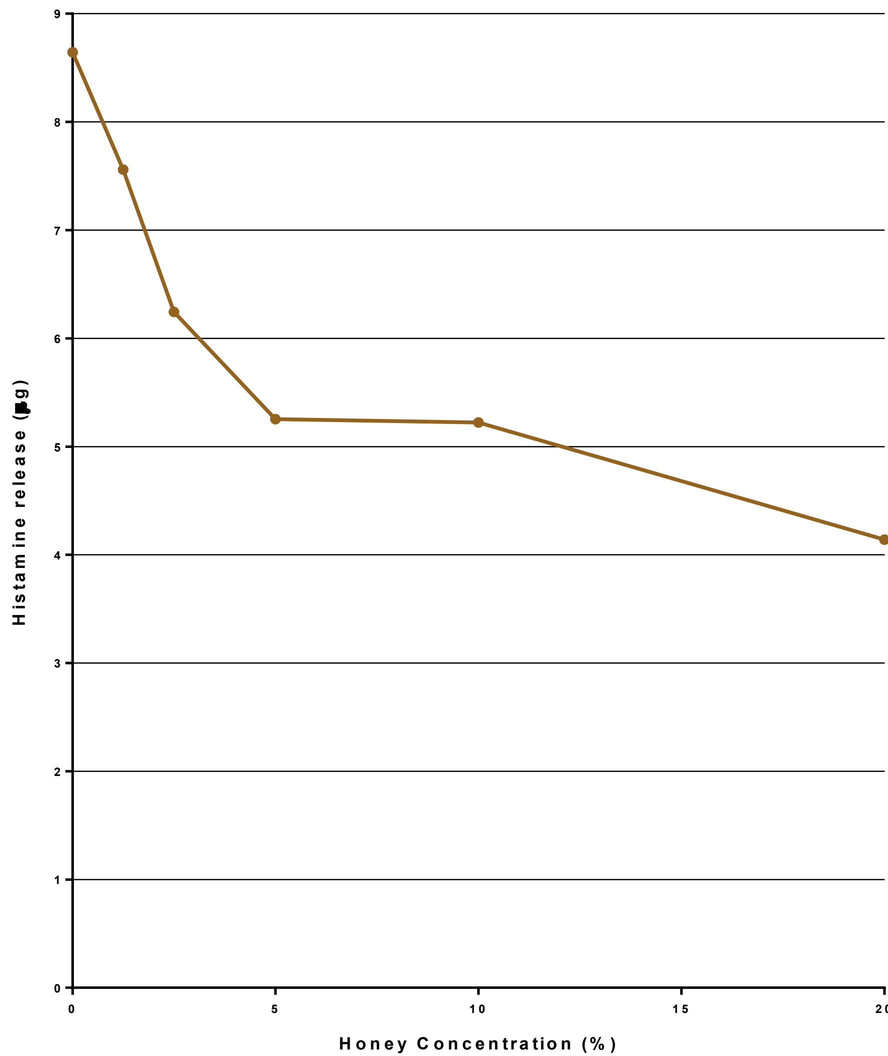


Figure 5-6: The effect of an increasing concentration of honey on the concentration of histamine released (µg) by rat bladder explant. It can be seen that as the percentage concentration of honey is increased, there is a reduction in the quantity of histamine release from explants. The greatest reduction in the release of histamine is seen as honey is increased from 0 to 5%.

5.4.6 Discussion

This part of the study was designed to assess a range of honey concentrations in regards to their effect on spontaneous histamine release as seen in LAD-2 cells (Lwaleed et al. 2014), and to establish a realistic working concentration of honey which could be used for

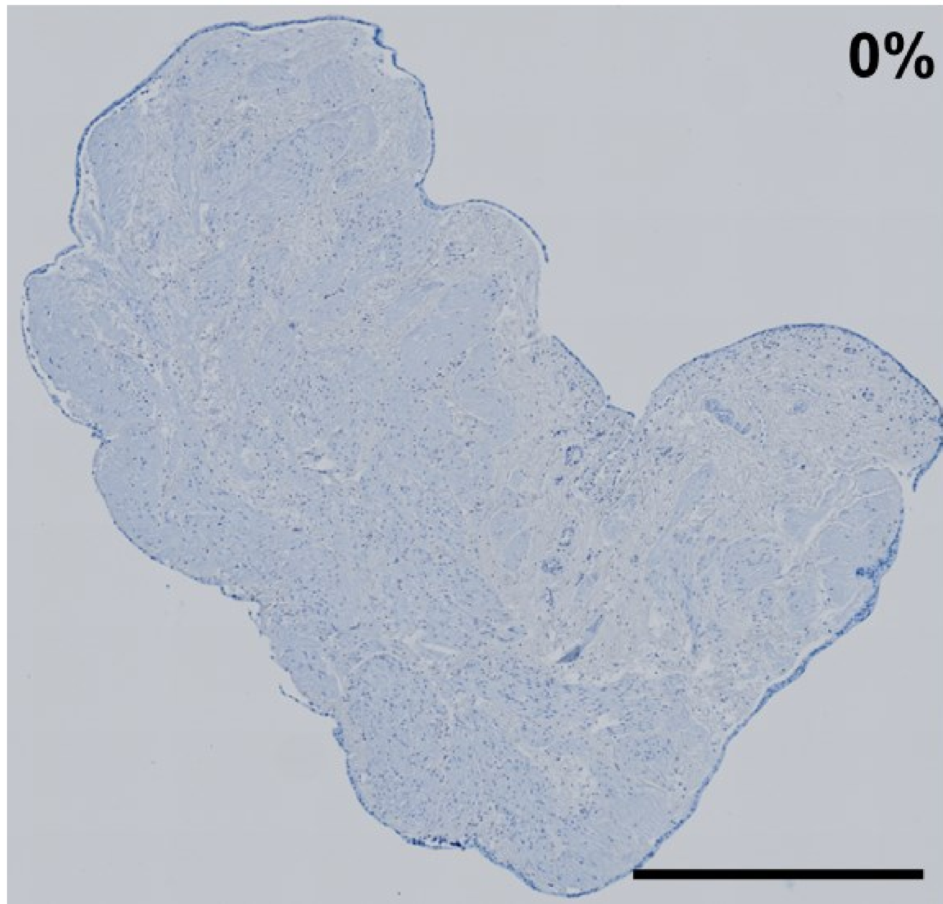
the further development of an intravesical agent for the treatment of PBS/IC. The results from the tissue culture assay have helped to meet these aims.

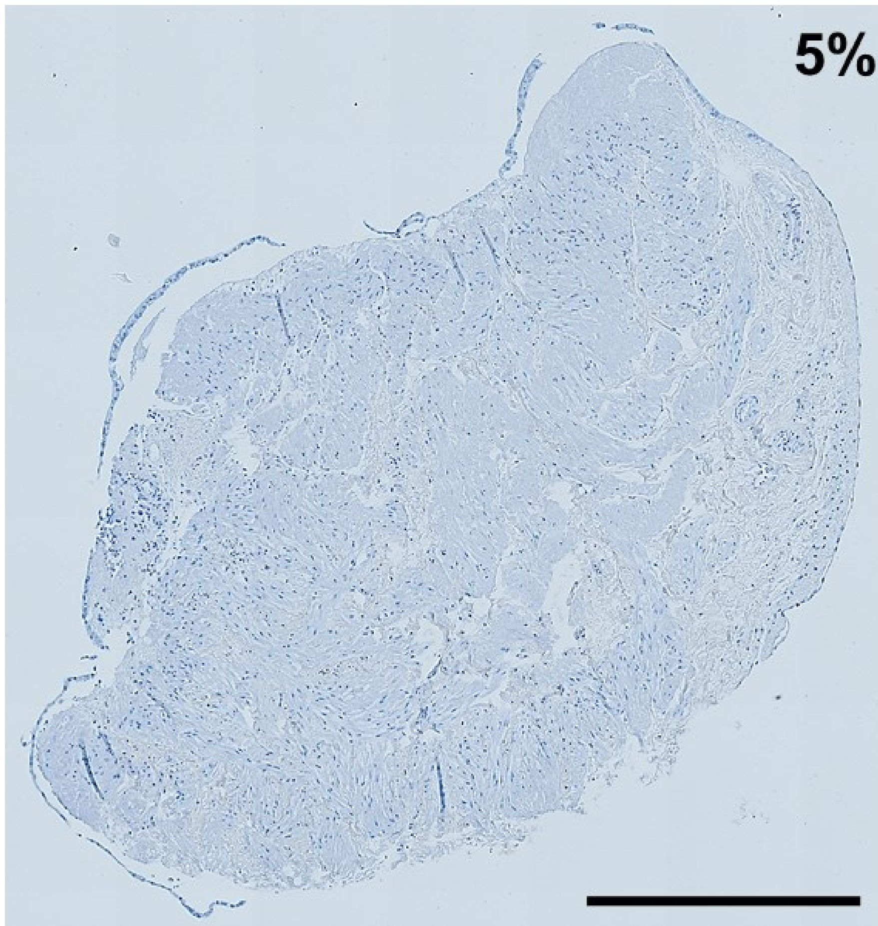
The tissue culture assay was repeated 5 times and the overall results concurringly suggest that the ideal concentration to be used in developing a successful treatment for PBS/IC would lie between 5% and 10%. These concentrations of honey exhibited the highest % of histamine inhibition, and reduction in histamine release.

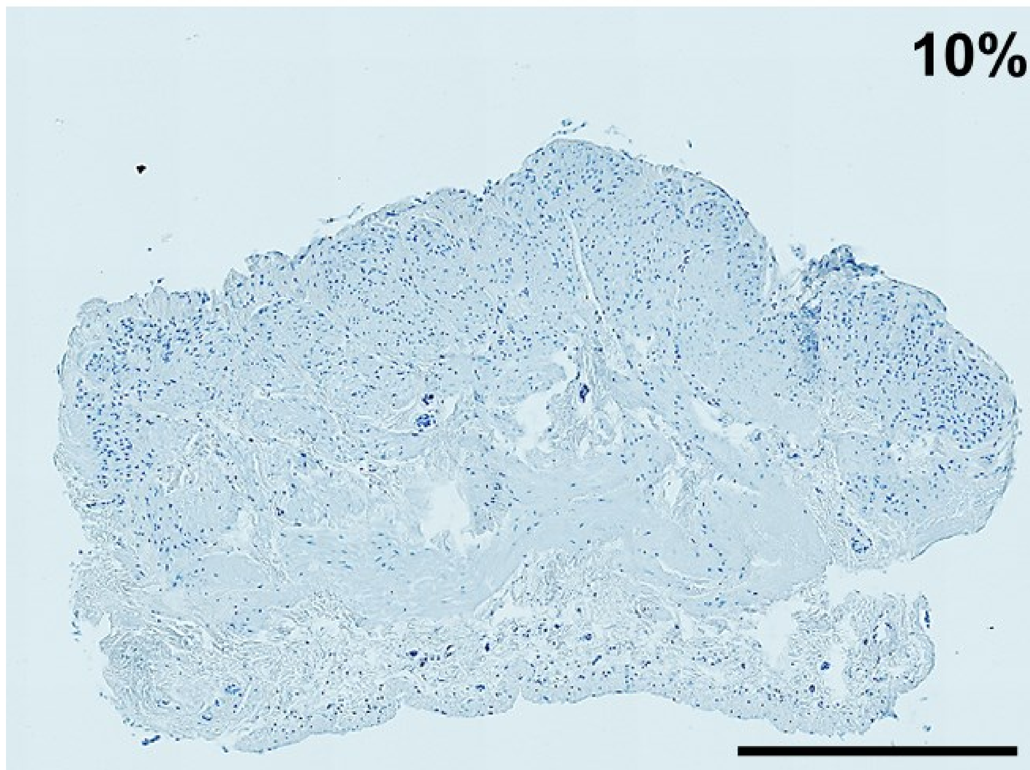
It can be seen that honey leads to a decrease in the release of histamine at 48 hours after treatment allocation. This may be due to the honey stabilising the MCs, and thus interrupting the degranulation mechanism of MCs by interacting with the receptor that activates histamine release (McNeil et al. 2015). The 5% honey maintained an almost negligible concentration of histamine over the 8-day length of the assay. When compared to the other honey concentrations, especially the 0%, it seems that the 5% honey is better at suppressing the release of histamine. Upon noticing this effect, an additional assay (n=2) was conducted to help glean a clearer idea of what was occurring between the honey and histamine. It was found that as the % of honey concentration was increased steadily, the levels of histamine concentration remained relatively stable. Results from this additional assay show that there is minimal and insignificant binding, and it could therefore be suggested that honey in fact interacting with histamine degranulation at the MC level. However, this assay would need to be repeated in order to confirm these findings.

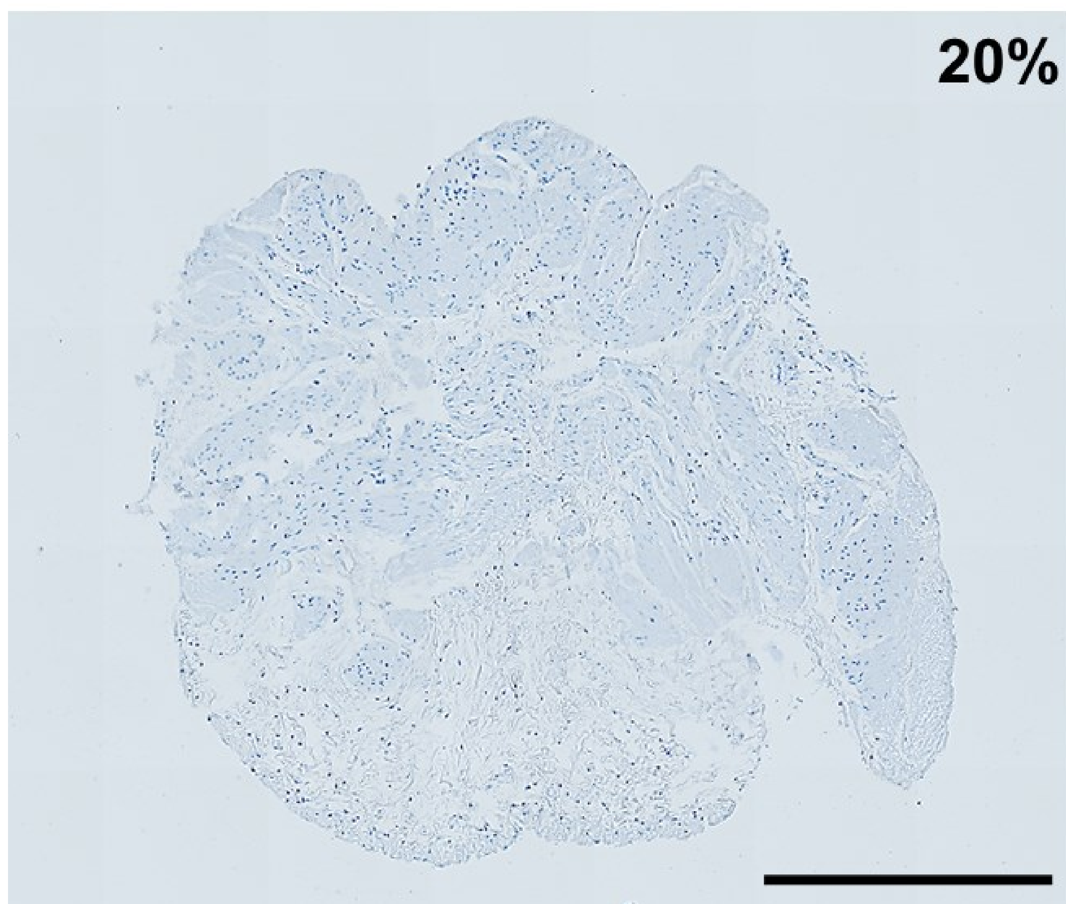
Although little research exists to explain the exact process of histamine release from MCs, a ground-breaking study was recently published in the journal Nature, that provides a hypothetical framework as to what is occurring at the molecular level (McNeil et al. 2015). The group discovered that histamine degranulation occurs via the MrgprB2 receptor in mice (an orthologue of the MrgprX2 receptor in humans), which is a target receptor of inflammatory molecules such as SP. This is of great importance in PBS/IC, as these individuals have a higher density of MCs, and so an increase in MCs, leads to an increase in the release of autocrine compounds such as SP, which results in a higher concentration of histamine in the bladder wall. The higher concentration of histamine is released from resident MCs within the bladder wall. Interestingly, Azimi et al. (2016) demonstrated that standard neurokinin-1 receptor (NK-1R) antagonists also have an off-target effect on the MrgprB2 receptor. Although this is yet to be seen clinically with the human MrgprX2 receptor, the research holds potential for the development of small molecular MrgprX2-specific antagonists for the treatment of MC-mediated inflammatory disorders, such as PBS/IC.

The increase in the range of values for histamine concentration over time is an intriguing finding in its own right. Results suggest that 5% honey consistently and successfully reduced histamine levels over a longer period of time, whereas honey concentrations above 10% showed the greatest variation in values for histamine concentration. Although there was variation in the results, the addition of honey still had some stabilising effect, even when at 40%. The variability could be explained by the notion that a honey concentration above 10% leads to toxic effect on the bladder explants. This is not surprising, as visually, the explants appear less viable (i.e. darkened, rigid, and shrunken) from Day 4 (Figure 5.7). The medium in which the explants are incubated also darkens in colour, and become more viscous after Day 6. This toxicity and apoptosis potential of honey has been noted in the literature and warrants further investigation in this project. The discrepancy in the results may also be due to human and machine error. The ELISA is a time-sensitive assay that requires speed, precision, and a high level of aseptic practice. Any discrepancy in the volume of solution, the time difference between the first well and last, and purity of the sample can negatively influence the results. This was seen to an extent during plate reading when wells that had been completed in duplicates, and so should have similar values, were undesirably different.


0%


5%


10%


20%

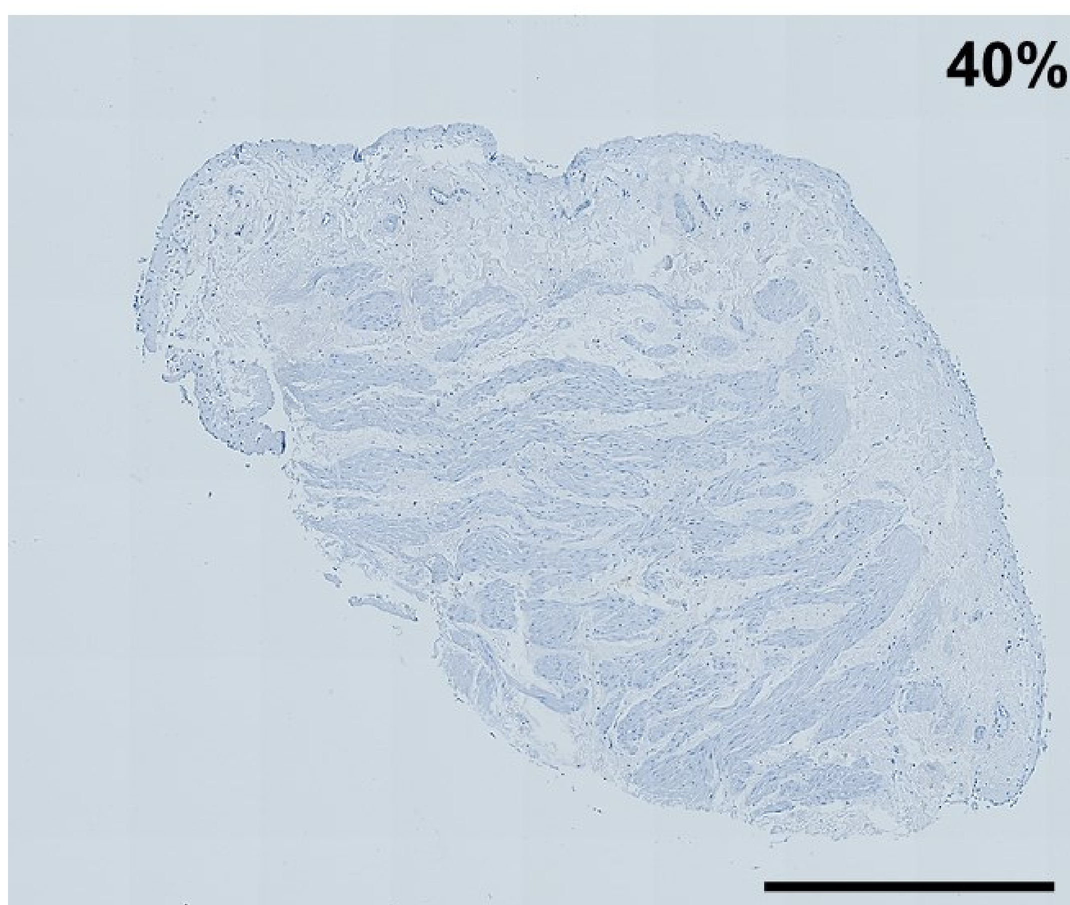

40%

Figure 5-7: Toluidine blue stained rat bladder explants that were incubated for 4 days in either 0%, 5%, 10%, 20%, or 40% honey. Tissues have been stained using a standard histochemistry procedure, and imaged at a magnification of x2 using light microscopy. Notice the gradual loss of structure throughout the entire bladder explant, and the disintegration of the outer epithelial layer, as the honey concentration increases from 10% to 40%. Scale bar= 1mm.

Another limitation of the study was the size and preparation of the bladder explants. Previous research guided the size to which the bladder explants must be cut, but using such small rat explants has many downfalls, namely the lack of generalisability to whole human bladder. As stated in the methodology, two rat bladders were used per assay. These rat bladders were cut into 2mm by 2mm explants, and a minimum of 12 were created per bladder. Using small sections of a whole bladder does not provide an accurate idea of what occurs within a complete organ, as cutting the bladder severs the unity in which an organ works. A suggestion for future work beyond this project would involve the use of whole rat bladders, before the advancement to whole human bladders, to ensure adequate testing is carried out to gain a detailed idea of the effect of honey in a more plausible and realistic setting.

The set of assays that were conducted during this part of the study focused on unravelling the potential of honey as an anti-inflammatory agent. Results suggest that honey concentrations between 5% and 10% hold the most promising potential in the development of a novel anti-inflammatory agent for the treatment PBS/IC. Additionally, the inclusion of scientific standardisation steps and protocol refinement help to increase the reproducibility and reliability of the results. Although results varied, the overall picture is one which not only supports existing research conducted by the team, but also leads the way in the selection of a suitable concentration of honey for the development of a therapeutic agent for the treatment of PBS/IC.

Initially, existing work conducted within the research team aided the development of the hypothesis and the protocols, however, once underway, it became apparent that the initial work had some technical issues in terms of methods', reproducibility, etc., and thus the hypothesis and aim of this arm of the study required revisions.

The discrepancy in repeat results may have been in part due to the toxicity that was occurring post-Day 4 of the assay, and when bladder explant was left to incubated in

honey concentrations above 10%. Although n=5 was deemed as an adequate number of repeats, a minimal number of repeats increases the effect of variance, which may otherwise have been levelled out when a large number of repeats had been conducted. One main reason for having 5 repeats was due to the lack of adequate materials, more specifically, a limited supply of adult Wister rats and ELISA kits. Both of these are crucial to the bladder explant work, but the availability and cost of such materials must be considered. As the explant honey work was conducted to establish a working dilution of honey for further research into the development of a honey-based intravesical agent for the treatment of PBS/IC, it could be argued that an adequate number of repeats were conducted. Additionally, as mentioned previously, whilst undertaking the honey-explant assays, a number of other areas for further study were raised, which utilised the same resources. The importance of discovering if honey was binding histamine, and assessing the potential of honey for toxicity, were crucial to understand in order to develop a safe and effective honey-based PBS/IC therapy for human use.

In conclusion, initially the tissue culture assay was developed to discover what would transpire over a broad timeframe, to capture the response of the tissue over the “treatment window” (i.e. the timeframe over which the treatment could be instilled and held within the bladder), and the response after prolonged exposure to the honey solution. This was so that the results would be more clinically translational, and more comparable to the PBS/IC installations and drug therapies that are utilised in the clinical setting. However, the toxicological effects of honey were an unexpected, but not unsurprising development. In order to better understand the tolerance and potential toxic effect of honey on bladder tissue, a series of fluorescent imaging assays were developed and conducted.

5.5 Stage II: Fluorescent imaging of bladder explants

5.5.1 Introduction

Whilst conducting stage 1 of this chapter, it became apparent that a reliable visualisation of what was occurring at the cellular surface level of the explant was required. A search of

the literature in regards to the possible toxicity of the topical application of honey to tissue failed to reveal applicable or scientifically dependable information. Although the results from stage 1 of this chapter were statistically significant, the discrepancy in results from repeat assays, and the incidence of outliers, led to the concern that honey toxicity and tissue apoptosis may be occurring. Another indication of apoptosis of the explants was the change in appearance of the honey solution in which the tissue was incubated. At the beginning of this assay, each dilution of honey solution was transparent and aqueous, yet by day 6, all honey dilutions appeared less transparent and slightly more viscous.

5.5.2 Materials and methods

In order to assess tissue viability, a live/dead assay was designed to simultaneously stain for live and dead cells, using two fluorescent dyes. Stained and fixed tissues were then analysed using confocal microscopy, and visualised using specialised software (Leica Application Suite X; LAS X).

5.5.2.1 Confocal microscopy

In conventional fluorescence microscopy, a wide-field of visualisation is used, so that the entire specimen is illuminated by a light source. Thus, all parts of the sample are excited at approximately the same time, and detected by a camera or photodetector. The resulting image includes fluorescent staining of interest, and a substantial amount of unfocused background. Confocal microscopy, or confocal laser scanning microscopy, is a relatively recent advancement in the field of scientific imaging, designed to address this key limitation of fluorescence microscopy.

Confocal microscopy uses the point illumination method, in conjugation with a pinhole in an optically conjugate plane (Figure 5.8). This results in only the points of interest of the specimen being detected, i.e. whatever structure or cell the excitation light is focused on. An image is compiled by logging the intensity of fluorescent light at each point of interest sequentially (Sanderson et al. 2014) Confocal can be used to create 2D and 3D images, that in turn, provide a non- invasive but direct visualisation of a section of tissue, without the need for much preparation. Specimens are often prepared using fluorescent dyes in

minimal concentrations, in order to reduce the likelihood of disruption to the tissue, excess noise in the end image, or any false positive results. The selection of the appropriate fluorescent dye at the correct concentration for the specimen type is an important part of the imaging process.

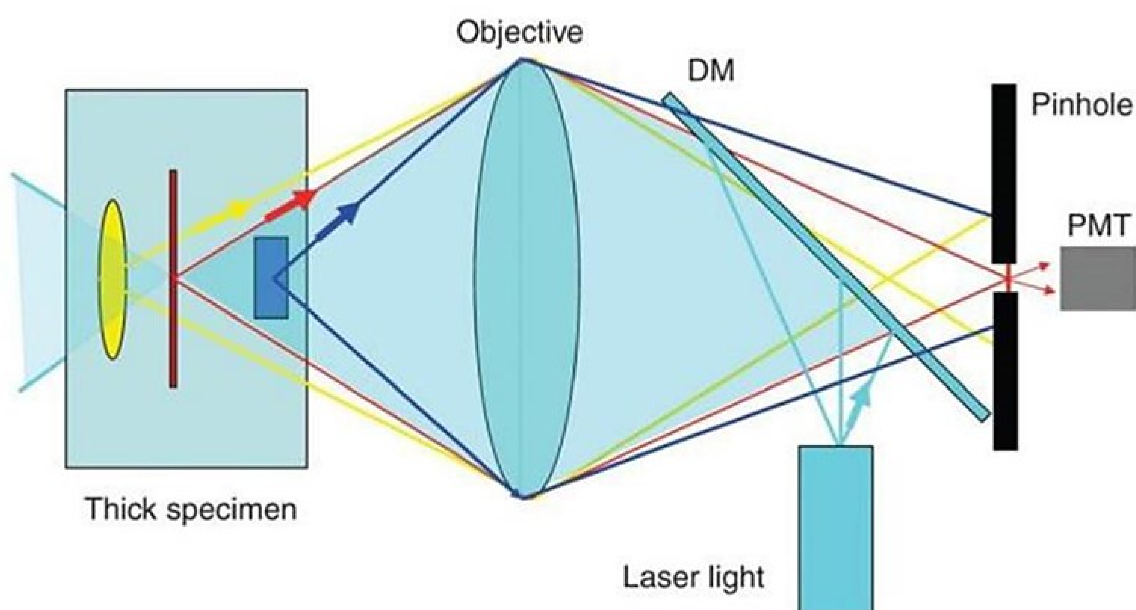


Figure 5-8: A representative schematic of confocal fluorescence microscopy. Laser light is focused onto a thick specimen by reflection from a dichroic mirror (DM) and the objective lens. The laser excites fluorescence throughout the specimen that passes through the DM and is focused onto the image plane. A pinhole only allows light from the confocal plane of the specimen to reach the photomultiplier tube (PMT). The image is then visualised using appropriate software. (Adapted from Sanderson et al. 2014).

5.5.2.2 Tissue preparation

A key aspect of confocal microscopy, is preparation of the tissue specimen. An advantage of confocal microscopy is the ability to scan fairly large tissue samples, but the specimen must be prepared in the correct way and made transparent. Following staining with fluorescent dyes, the tissue is fixed by being submerged in 10% neutral buffered formalin (NBF) for a period of 24 hours. This fixation step ensures that the tissue does not putrefy, or undergo autolysis, and thus is kept in a preserved state for analysis. NBF disables essential biological processes and enzymes which may otherwise digest or destroy the tissue specimen. Using a fixative also allows protection from external opportunistic microorganisms, such as bacteria, by creating a toxic or indigestible barrier around the tissue sample. Fixatives also stabilise the tissue specimen by increasing the stability and mechanical strength on a molecular level. This rigidity helps to reduce the chance of tears during handling, and maintain the morphology for further processing before analysis. However, as vital as the fixation step is, there is a chance of the development of artefacts that can alter or interfere with the visualisation and interpretation of the specimen. These artefacts can lead to the occurrence of false positive or false negative results. A fixation period of 24 hours is ideal, especially for small tissue samples, as it allows for adequate penetration of the entire specimen, yet reduces the likelihood of over-fixation and the formation of artefacts.

After a fixation period of 24 hours, the tissue is then transferred into 90% Glycerol to replace the NBF and preserve the tissue until ready for microscopy. After approximately 24-48 hours, the tissue is ready to view. The confocal microscope is able to scan to a total depth of 100-200 um, but this is in the most ideal conditions, and includes the tissue within its mounting structure. The mount that was used for the bladder explants is displayed below (Figure 5.9). It was built using coverslips held together with a sticky tape frame, in which the tissue is encapsulated within mounting medium (50%PBS 50% Glycerol). The coverslips must not touch the tissue, in order to avoid significant and possibly detrimental distortion of the end image, and so tape and mounting medium is used to keep the tissue apart from the coverslips. The use of a ‘tape chamber’ and mounting medium within the coverslips does allow for some flattening of the tissue which is desirable in this case, as the bladder explants tended to have a curl to their cut edges. This indirect gentle flattening of the tissue specimens allowed for clearer viewing of the fluorescent cells. The use of mounting media increases the refractive index, and so, should be used with care.

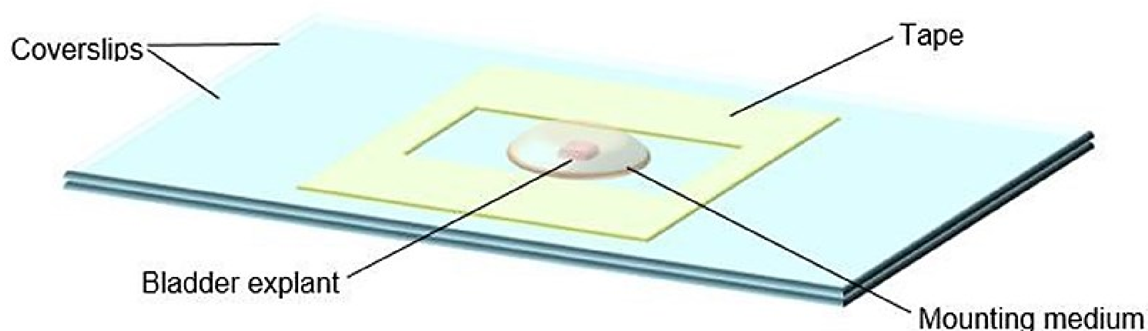


Figure 5-9: Mounting structure used for confocal microscopy. The structure consists of two coverslips secured together with a sticky tape frame, enclosing the tissue specimen of interest in mounting medium.

5.5.3 Fluorescent dyes

It was decided that Acridine Orange (AO) and Propidium Iodide (PI) would be used in this study. These dyes are widely used for fluorescent confocal imaging, and were deemed the favourable dyes for the assay as the standard protocols for dilution and usage were readily available. The double staining method was first described by Borel et al. in 1998. A combination of a cationic dye (acridine orange) and anionic dye (propidium iodide) was used to distinguish between live and dead *Toxoplasma gondii* (an obligate intracellular parasite that causes toxoplasmosis (Montoya and Liesenfeld 2004). AO has long been used in fluorescent microscopy to identify viable cells (Beers 1964). AO is able to permeate cells and provide clear staining of nuclear DNA, and cytoplasmic RNA in endothelial cells (Rouse and Gmitro 2000). AO also possesses the characteristic of emitting red fluorescence when bound to RNA or single-stranded DNA, and green when bound to double-stranded DNA. Thus making it a versatile and useful fluorescent dye. PI is a fluorescent red dye that stains the nucleic acid of dead cells. PI is often used to identify dead cells, and as a counter stain in live/dead fluorescent imaging, due to its ability to preferentially penetrate cells that have a damaged membrane, and thus exclude live cells that have an intact membrane (Pasini et al. 2013). During research of the literature, SYTO-9 was also identified as a potential fluorescent dye for non-viable cells, however, in contrast to PI, SYTO-9 is able to enter both live and dead cells (Stiefel et al. 2015).

5.5.3.1 Dilution optimisation

As AO and PI are typically used only to stain cells, a protocol had to be adapted in order to accommodate for the use of a tissue explant specimen. A range of dilutions to use in the preparation of tissue specimens were created using manufacturer guidelines (Thermo Fisher Scientific, USA), and advice from specialist imaging staff within the Biomedical Imaging Unit (BIU) at the University of Southampton.

In the early stages of protocol development, it was decided that untreated bladder explants would be used to assess the optimal dilution for each fluorescent dye in separate procedures. Following manufacture guideline, PI was diluted to 1/10, 1/100, 1/1000, 1/10000, 1/30, 1/300, and 1/3000 using PBS. Additionally, the incubation period for staining also needed refining, and thus initially each tissue specimen was incubated at room temperature within the dye for either 5, 10, or 30 minutes. Control tissue specimens were incubated in PBS. Analysis of the tissue using confocal microscopy revealed several interesting points. Firstly, the tissue specimens that had been incubated for longer than 5 minutes had excessive staining that created hazy images with structures that could not be concisely identified. Additionally, it was also noted that the 1/10 PI dilution was not as bright as expected, and this may have been for one of two reasons, either the PI (which had been acquired from another research team) had lost some intensity due to storage, or the bladder tissue was still highly viable. The assay was repeated with only a 5-minute staining incubation period. Results revealed that a concentration of 1/300 provided the best staining in regards to clarity and conciseness.

The 5-minute staining assay was repeated with AO. The literature suggested dilutions between 1/100 and 1/2000. Thus the dilutions that were selected for testing were 1/5, 1/10, 1/25, 1/50, 1/75, 1/100, 1/150, 1/200, 1/250, 1/400, 1/500, 1/750, 1/1000, 1/2000, and 1/3000. After the concern that the quality of PI may have degraded after storage, it was decided that tissue would also be stained using 100% AO, to ensure that the dye was active before being diluted. Results revealed that the most appropriate AO dilution was 1/1000.

5.5.4 Protocol development

Using the dilutions selected above, a final protocol was developed (Figure 5.10). Bladders were gathered from standard Albino Wister rats (<300g) sacrificed under a Home Office Schedule-1 method. The bladders were then cut into 2mm by 2mm fragments, using the cutting mat developed earlier in this chapter. The tissue explants were placed in a Petri dish of growth medium; made up with a medium made up of Dulbecco's Modified Eagle's Medium (DMEM), 10% foetal bovine serum, and L-glutamine-penicillin-streptomycin solution (Sigma Gx114, UK).

After undergoing Stage I (8-day honey treatment assay), the explants were transferred (one explant per well) to a 48-well plate containing 100 µl of 1/150 PI and 100 µl of 1/500 AO in each well. The 48-well plate was placed on a plate shaker, and left to oscillate for 5 minutes. After this staining period, the fluorescent dye was quickly pipetted from each well, and 200 µl PBS was added to each well. The plate was then returned to the shaker, and left to oscillate for 5 minutes. After this washing period, the explants were placed in plastic centrifuge tubes containing 1ml of 10% NBF. After 24 hours, the tissue explants were then transferred into 5ml tubes containing 90% Glycerol (with 10% PBS), and stored in the dark at 4 °C for a minimum of 24 hours. Tissue explants are then analysed individually using the coverslip mount described earlier.

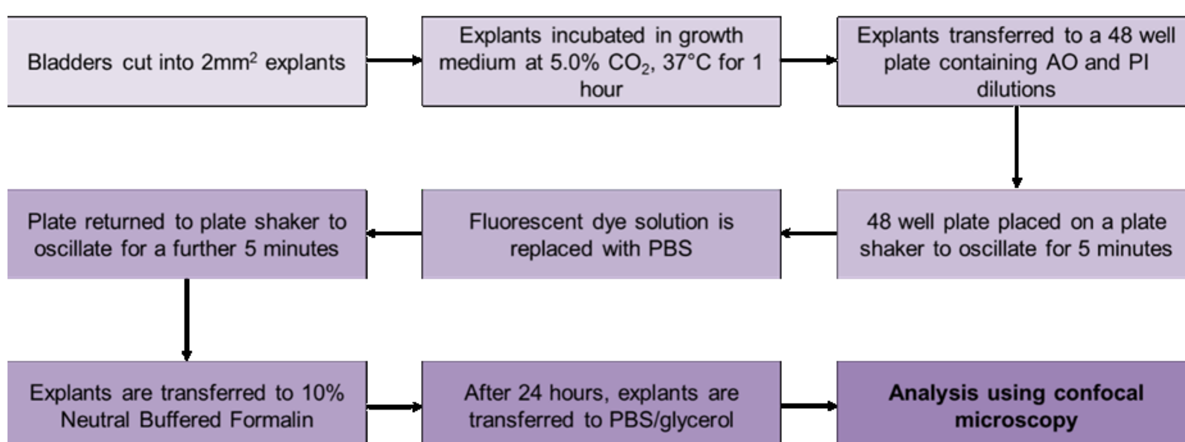


Figure 5-10: Fluorescent staining procedure.

5.5.5 Analysis

Analysis was performed under the guidance and supervision of the BIU at the University of Southampton. Explants were analysed using confocal microscopy a x20 or x63 water/glycerine multi lens, dependent upon the thickness of the specimen. The software used was the Leica Application Suite X. The excitation wavelength used during analysis was selected using the data sheets for each of the fluorescent dyes. The correct wavelength is one that aids to avoid crosstalk between the different dyes utilised, PI and AO, and between the dyes and any pigments that may already exist within the specimen. Selecting an incorrect excitation wavelength can lead to false-positive signals.

Although some sources differ, the standard peak excitation and emission wavelengths of AO and PI are 490nm (within the blue part of the light spectrum) and 530nm (within the green part of the light spectrum), respectively (Table 5.1). During analysis, the excitation wavelength was adjusted slightly dependent upon the image under investigation. Emission filters were also selected to be specific for the fluorescent dyes under investigation.

Table 5-1: Fluorochrome data table showing the excitation and emission wavelengths of Propidium Iodide and Acridine Orange. (Adapted from Microscopy Resource Centre, 2012).

Fluorochrome	Excitation Wavelength	Emission Wavelength	Target
Propidium Iodide	530	615	DNA•RNA
Acridine Orange	490	530, 640	Single/Double-stranded Nucleic Acid

As this assay was conducted to provide descriptive data, no requirement for statistical analysis was made. If in the event quantitative data could be collected, a % of live vs. dead cells would have been calculated.

The following criteria was created and used to assess the images:

- Only epithelial cells would be assessed as they were in direct contact with the honey mixture.
- If possible, the whole tissue would be scanned in order to see if any structural changes had occurred deeper within the tissue.
- If deemed necessary, the protocol would be altered and the assay would be repeated.

5.5.6 Results

The effect of time on the viability of bladder explants

Analysis of explants revealed that by day 2, very little epithelium remained, and tissues were considered to be non-viable, regardless of the concentration of honey (Figures 5.11, 5.12, and 5.13). Many of the explants appeared to have no epithelium present, and if present, the cells appeared non-viable and appeared to be moving from the remainder of the explant (Figure 5.11).

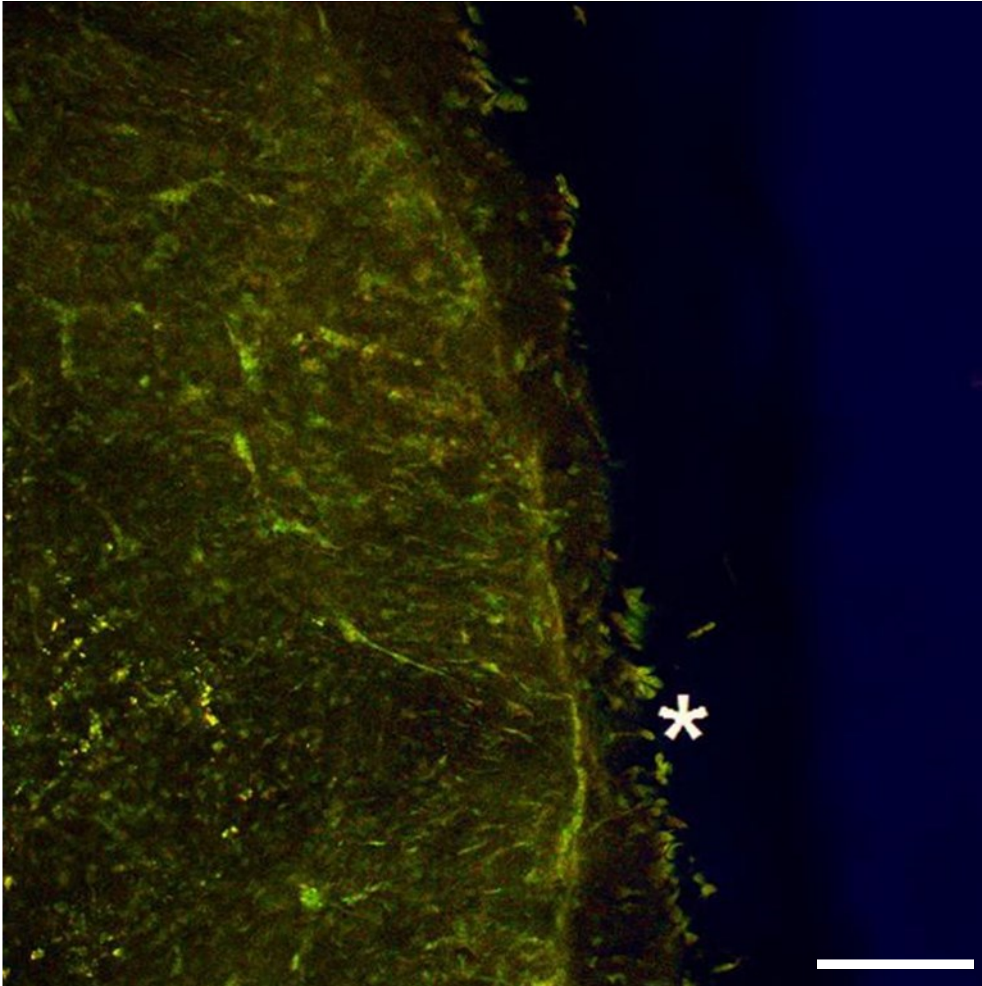


*Figure 5-11: Rat bladder tissue explant at day 4 in 40% honey. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide, and appear red. Image taken at x20 magnification using confocal microscopy. Note the appearance of bright green spots, which are indicative of clumped chromatin from non-viable cells. The * denotes possible epithelial cells disassociating from the remainder of the explant. Scale bar= 100μm.*

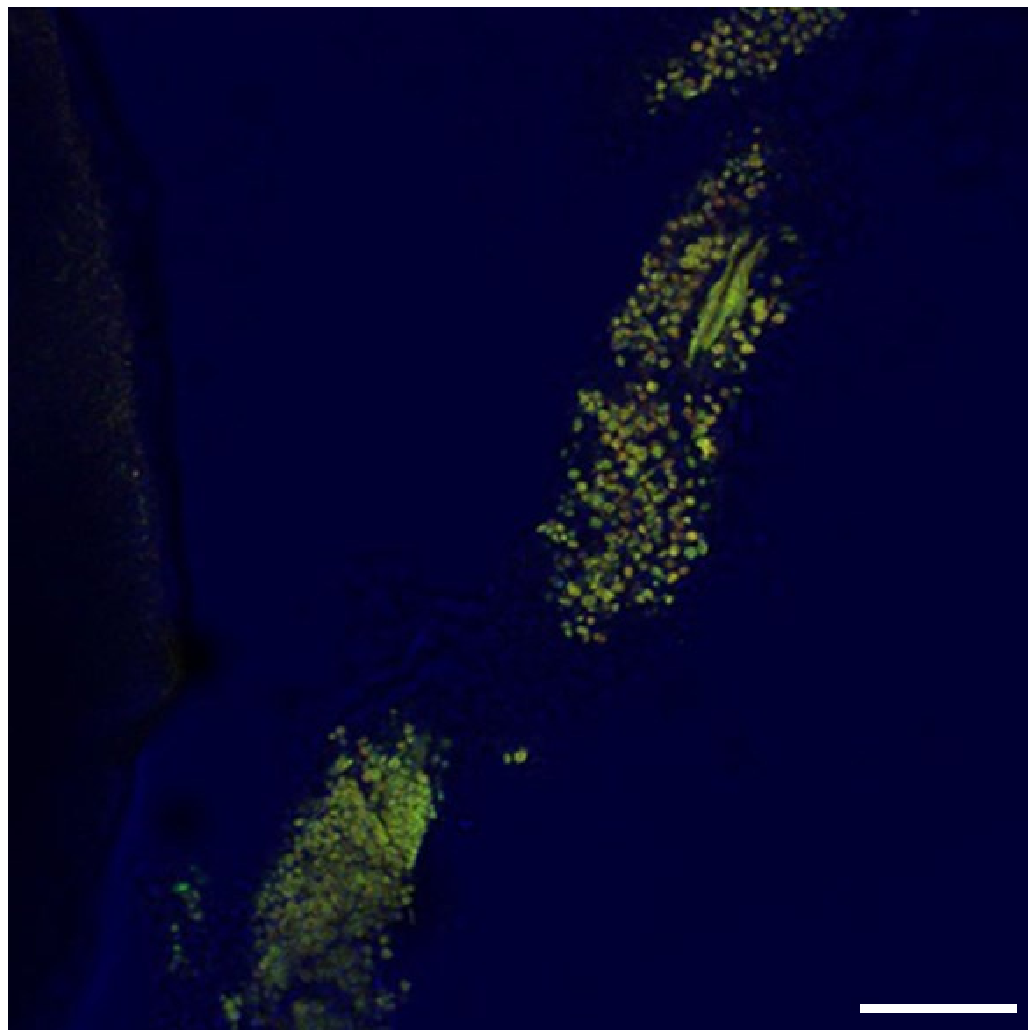


Figure 5-12: Rat bladder tissue explant at Day 4 in 0% honey. Rat bladder tissue explant at Day 4 in 0% honey. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide, and appear red. Image taken at x20 magnification using confocal microscopy. Note the absence of any obvious epithelial structure. Scale bar= 100µm.

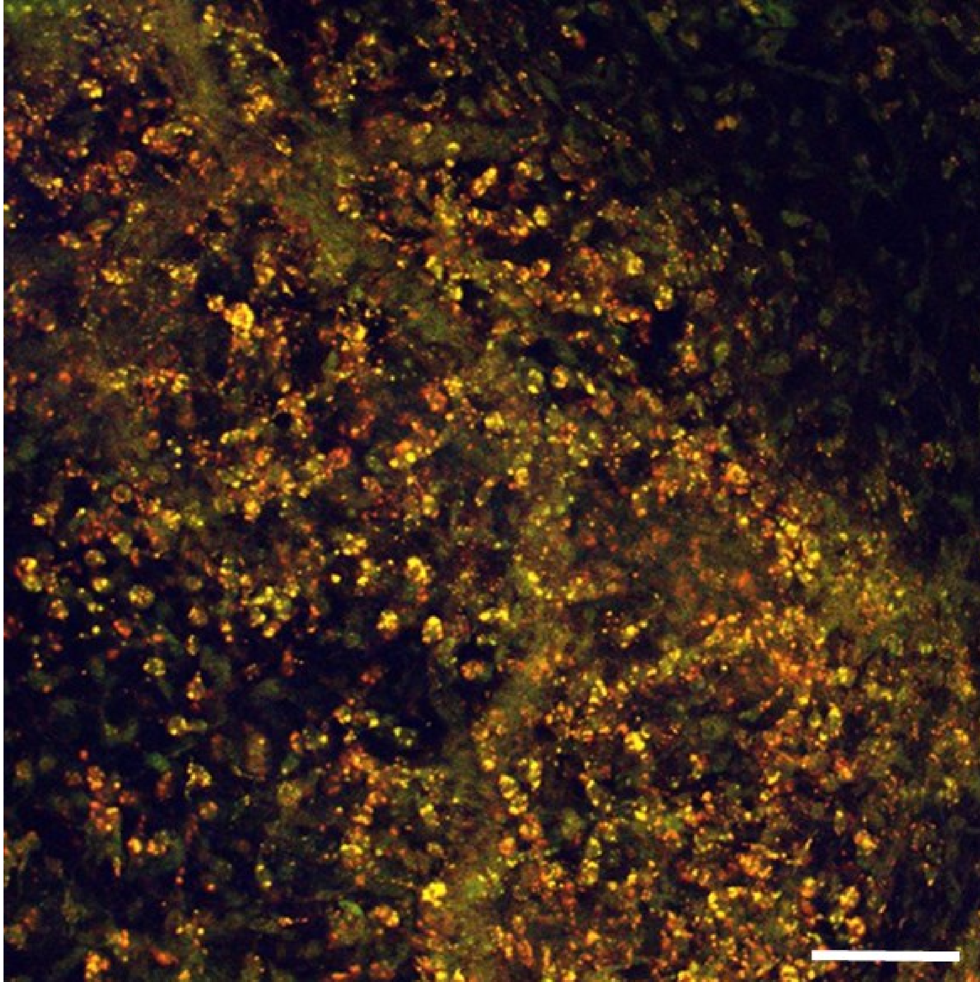


Figure 5-13: Rat bladder tissue explant at Day 4 in 5% honey. Rat bladder tissue explant at Day 4 in 5% honey. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide and appear red. Image taken at x20 magnification using confocal microscopy. Note the absence of any epithelial structure. Scale bar= 100µm.

The effect of concentration of honey on viability of bladder explants

An increasing concentration from 1.25% to 40% shows a gradual decrease in viability of epithelial cells (Figures 5.14). Irrespective of the length of time in which the explant was incubated with medium, the pattern of the increase in cell death was directly related to the increase on concentration of honey. This is best seen with explants that have been treated for a total of 1 hour (Figure 5.14). As the concentration of honey increases, the amount of red fluorescent cells also increases, and the amount of viable and identifiable epithelium decreases. However, explant that has been incubated in 0% honey (i.e. 100% growth medium) has a similar appearance to explant that has been treated with 20% honey. Apoptosis was also noted in bladder explant treated with 20% honey (Figure 5.15).

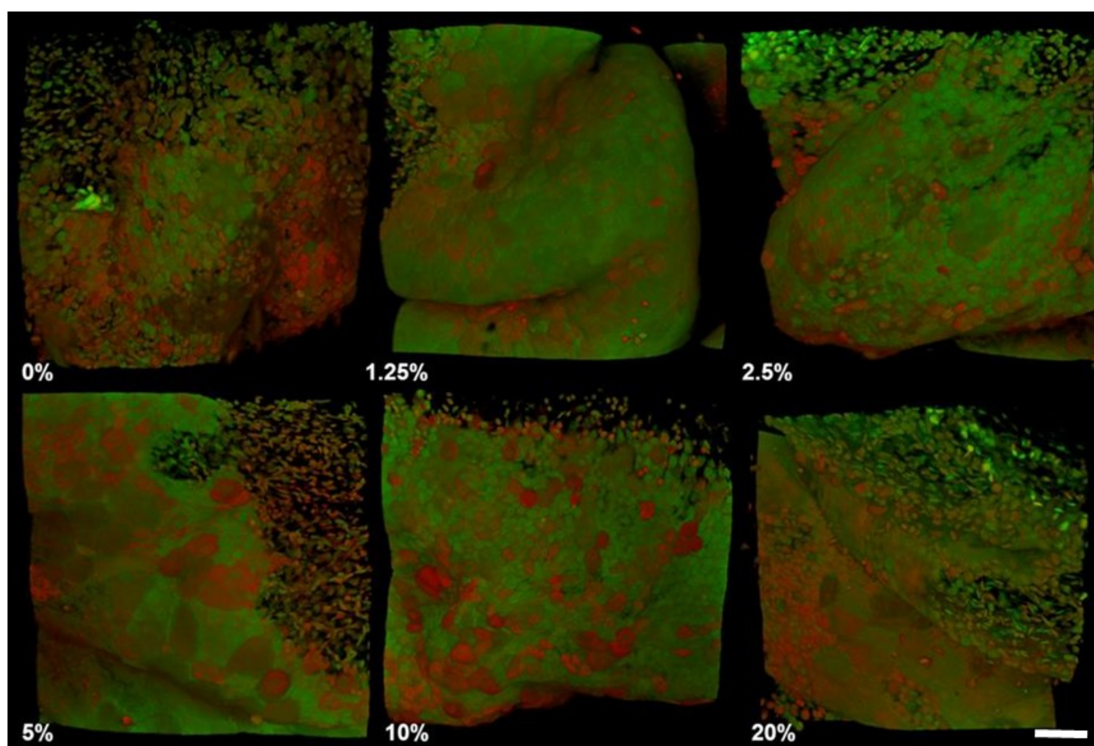


Figure 5-14: Rat bladder tissue explants in increasing concentrations of honey. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide, and appear red. Images taken at x20 magnification using confocal microscopy. The red staining is indicative of non-viable cells that appear to be undergoing apoptosis. Scale bar= 100μm.

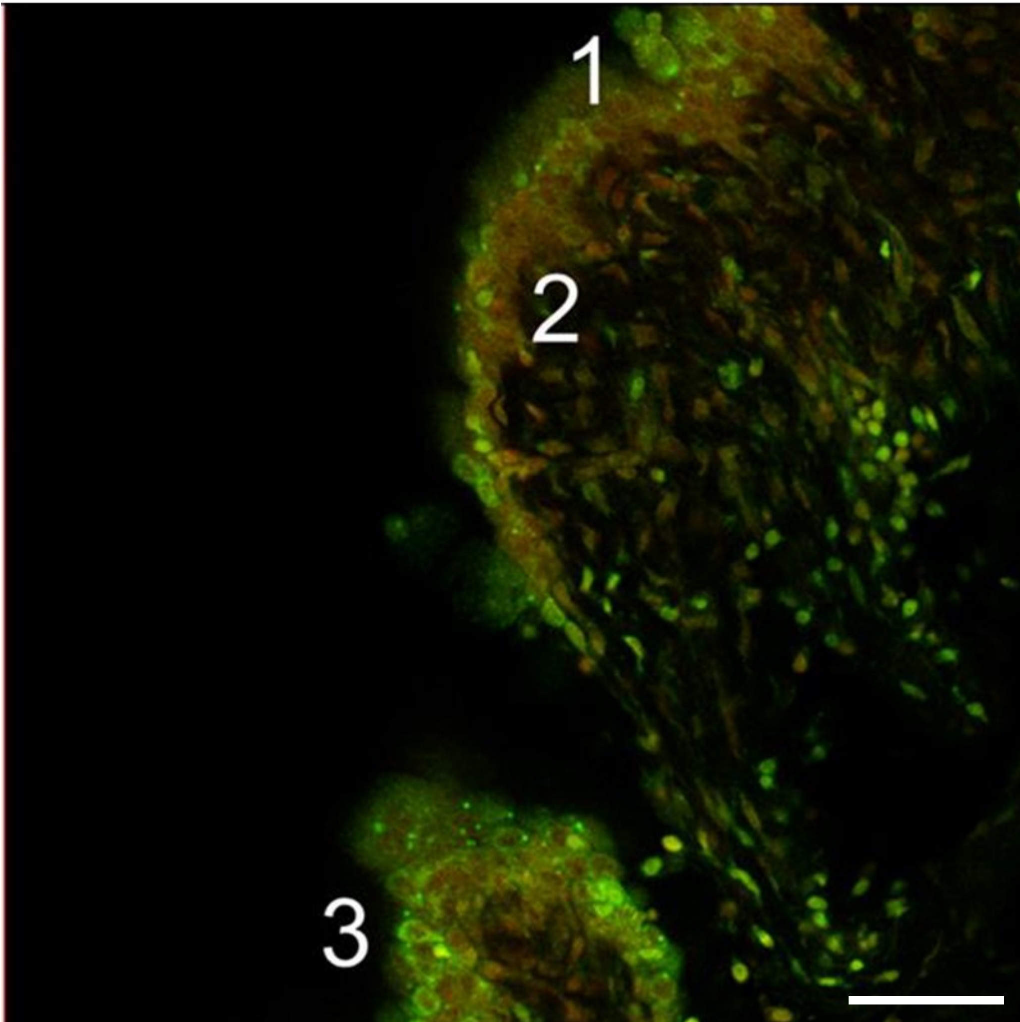


Figure 5-15: Signs of toxicity and apoptosis at the epithelial surface of rat bladder tissue. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide (PI), and appear red. Image taken at x20 magnification using confocal microscopy. 1) Blebbing of epithelial cells, 2) Excessive PI staining and loss of clear structure of tissue and cells, and 3) Nuclei are clumped, and these fragments appear brightly stained but are not indicative of viable cells. Scale bar= 100µm.

The effect of tissue shape and size on the viability of the epithelium of bladder explants

Tissue shape and size also plays a role in the viability of the bladder explant epithelium (Figure 5.16). Cutting the bladder into rings increases the likelihood of cell viability, when compared to 4mm x 4mm, and 2mm x 2mm squares (after a 1- hour incubation in 1.25% honey).

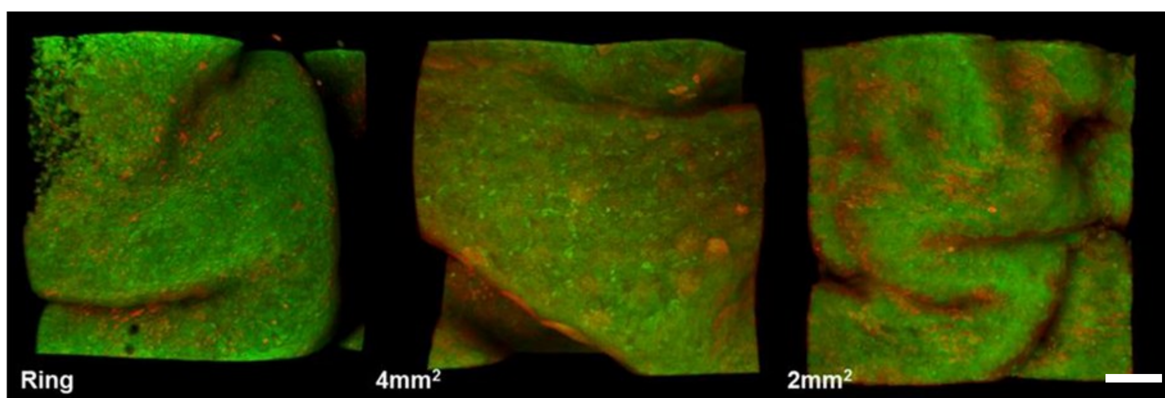


Figure 5-16: Rat bladder tissue explants of different sizes and shapes after incubation in 1.25% honey for 1 hour. Rat bladder tissue explants of different sizes and shapes after incubation in 1.25% honey for 1 hour. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide, and appear red. Images taken at x20 magnification using confocal microscopy Scale bar= 100µm.

5.5.7 Discussion

Stage II was conducted in order to identify the effect of honey on bladder tissue explants, at a structural level. As the assays were designed and conducted, it became apparent that further dilutions were required (1.25% and 2.5%), as well as the assessment of shape on the viability of tissue. Initially, the assays were conducted using 2mm by 2mm squares, but upon fluorescent analysis, the lack of epithelium raised the question of whether or not

the bladder was being damaged by excessive handling and cutting was raised. Thus, 4mm by 4mm explants were cut, in addition to cutting the bladder into 2 mm thick rings, in order to reduce the amount of handling and exposure of the bladder tissue to a sharp blade. Results showed that the bladder explants that fared the best were those that had been cut into rings. This is unsurprising as the bladder rings required the least amount of handling and exposure to the scalpel, and thus the epithelium had a lesser chance of mechanical damage.

The assays also identified that in order to ensure epithelium viability, a honey dilution of 1.25% to 5% should be considered. The assay was repeated several times (n=3) and revealed consistent results, and so this dilution range is a suitable continuation point for the development of a honey-based therapeutic agent for PBS/IC. In addition, it should be acknowledged that this assay has utilised bladder explants rather than whole bladder, thus the results are merely an indication of what may be suitable within the whole bladder. It is suggested that the assays are adapted for whole bladder in order to assess the effect of honey concentrations on the viability of bladder tissue.

The results also clarified the effect of time of exposure to honey, on the bladder explants. Initially, the assay was designed to look at the toxicity of honey, and results highlighted the lack of viability, and the increase of cell death, as time increased from Day 0 to Day 8. This then lead to the development of the 1-hour incubation assay, which is also more clinically relevant.

The toxicity of honey is of key importance, as although honey may be a possible therapeutic option for chronic inflammatory disorders such as PBS/IC, the potential of honey to further harm a PBS/IC sufferer needed to be identified. A particularly key disease subgroup that honey toxicity may effect is PBS/IC individuals who are also diabetic. Much of the existing honey and wound care research fails to truly assess the effect of honey on glucose levels of healthy or diabetic individuals, but it has been considered safe to use in diabetic patients, for the treatment of ulcers (Kateel et al. 2016). In the context of PBS/IC, a deficient GAG layer leads to the leakage of harmful molecules into the underlying urothelium, and thus, in a diabetic PBS/IC patient being treated with a honey-based intravesical therapy, the risk of honey toxicity is higher, and needs to be addressed to ensure the safety of a honey-based intravesical wash in PBS/IC diabetics.

Stage II of this honey work has identified that honey does indeed have a toxic effect when used in concentrations of 10% and beyond, on bladder explants. Honey also has a toxic effect when bladder explants are exposed for a period longer than 24 hours. This can be seen by the lack of epithelium in the explants that were exposed for too long, and/or to a

too high concentration of honey. The toxic effect is visualised by the increase in red PI staining, the decrease in the presence of epithelium and lack of tissue structure, and the increase in apoptotic cells.

The main limitation of Stage II of this honey and explant work is the lack of statistical analysis. Although, gathering quantitative data from the fluorescent images was highly desirable, it was made impossible due to several factors. The staining of the explants appears “noisy”, i.e. the fluorescent dyes, especially PI, have not stained the nuclei as concisely as desired. Additionally, the contrast of the staining isn’t bright enough to count nuclei for quantitative analysis, and so discrepancy between the cytoplasm and nuclei would be highly subjective, and thus reliability and reproducibility of results would be poor. As epithelium cells were of key importance for analysis, the lack of epithelium tissue in the majority of the images mean that no relevant quantitative analysis could be made. A recent advancement in image analysis software has led to the development of powerful rendering software that can clarify and sharpen confocal images, and produce 3D renders. The 3D rendered image is easier to analyse using quantitative techniques, and provide a clearer and more accurate visualisation (Figure 5.17).

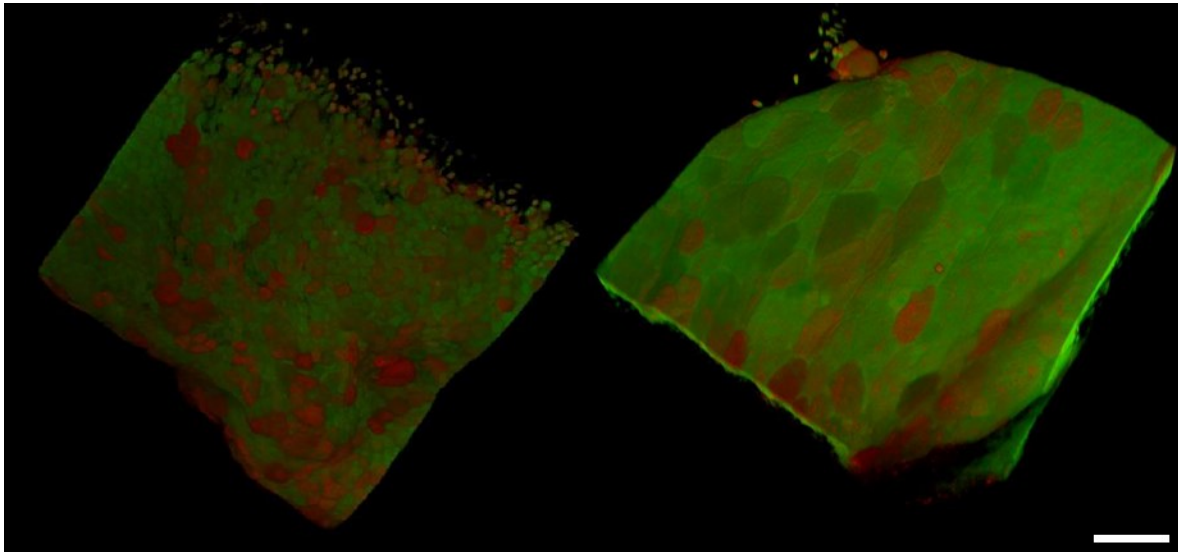


Figure 5-17: An example of how 3D rendering can be used to enhance a poor confocal image. On the left is a bladder explant ring which has been incubated for 1 hour in 10% honey and on the right is the same image after 3D rendering. Note that the 3D rendered image gives a clearer visualisation of the contours of the tissue, and the epithelial layer. Live cells are stained using Acridine Orange, and appear green. Dead cells are stained using Propidium Iodide, and appear red. Images taken at x20 magnification using confocal microscopy. Scale bar= 100µm.

It is apparent that the quality of images produced is highly dependent upon tissue preparation, handling, staining, storage, software, and knowledge of confocal imaging, however, the pilot nature of this work has provided useful novel data, and warrants further repeats. These findings are highly pivotal in the development of assays that are more relevant to the development of a honey-based therapeutic agent in the treatment of PBS/IC, and live/dead staining in conjunction with confocal microscopy should be utilised during the refinement process to help ensure that the best concentration of honey is selected and used.

Chapter 6: General Discussion

6.1 Introduction

The main purpose of this study was to address the discrepancies concerning the role of inflammatory cells in the pathogenies of PBS/IC. Previous literature has identified the presence of MCs within the PBS/IC bladder wall, with a particular emphasis on a large presence of MCs within the detrusor layer. Yet very little exists in terms of analysing the subtypes of MC in PBS/IC, and the potential role of basophils in this PBS/IC.

Once the role of inflammation in PBS/IC has been fully elucidated, the efficacy of a novel anti-inflammatory treatment can then be assessed. Thus, our working hypothesis was that there would be a significant difference in the density and distribution of inflammatory cells (such as MCs, as well as basophils) between PBS/IC subjects, and histologically normal controls. In order to achieve this, several aims were identified and successfully fulfilled. These were as follows:

- To quantify the number of inflammatory cells (such as MCs and basophils) in paraffin embedded PBS/IC patient tissue using IHC techniques.
- To assess the distribution and density of MC subtypes in paraffin embedded PBS/IC patient tissue using IHC techniques.
- To use commercially available ELISA to assess a range of concentrations of honey in order to evaluate their effect on histamine induced MC degranulation in rat bladder explants.

6.2 Summary of key findings

A) There is an influx of MCs in PBS/IC bladder, compared to controls

- Mastocytosis is seen in PBS/IC individuals, and is predominately made up by the MC_T subtype.

- All PBS/IC full thickness bladder samples exhibited an influx of both types of MCs.
- As already established in the literature, a high number of MCs were found in the detrusor layer of PBS/IC individuals.
- An influx of MC_{TC} into the mucosa may be linked to Hunner's ulcers, and an influx of MC_{TC} in the lamina propria may be linked to fibrotic bladder seen in some patients.
- The discrepancy in the distribution of MCs between PBS/IC sufferers may help explain the variability of symptoms seen clinically during the diagnosis period.

B) There are no basophils present in all PBS/IC bladder tissue studied

- In this study, basophils were not found in most of the inflammatory conditions suggested by the literature.
- BB1 was not found to be useful in the identification of basophils.
- Basophils were found using the 2D7 mAb, but only in chronic myeloid leukaemia skin punch samples that contained traces of blood.

C) Honey decreased spontaneous histamine release by MCs in bladder explant tissues

- A low % concentration of honey leads to a respectable decline in spontaneous histamine release, when assessed using an ELISA.
- A high % concentration of honey does not lead to a corresponding decrease in the amount of spontaneous histamine released by MCs.
- An appropriate dilution is within the same range as previous reported by our team i.e., approximately 5%.

D) Excessive honey leads to toxic effect and loss of surface cells

- Confocal microscopy revealed that honey concentrations of 20% and above lead to death of the endothelial cells.

- Fluorescent imaging showed that high concentrations of honey also lead to sloughing off of dead cells, and loss of the endothelial layer.

6.3 Importance of key findings

6.3.1 Mast cells

The project was conducted to clarify some of the pathology of PBS/IC, and to assess the possibility of utilising honey successfully as an intravesical instillation for the treatment of PBS/IC. This debilitating and poorly understood inflammatory condition is clearly representing an under-researched area of urology, yet the number of sufferers is higher than often reported. PBS/IC disorder is more prevalent than previously thought; 3 to 8 million women in the US are thought to be affected by PBS/IC, and 1 to 4 million men; although this figure is most likely lower than the true figure due to PBS/IC often being misdiagnosed as chronic prostatitis in male patients (Interstitial Cystitis Association 2015; Berry et al. 2011). One of the most important set of findings from this project was the clarification in regards to the role of MCs. Existing literature is in agreement with the finding that MCs are present in significantly high numbers in the detrusor of PBS/IC sufferers. In addition, a significant influx of MC_{TC} was seen in the lamina propria, which is not as reported previously (Aldenborg et al 1986, Yamada et al. 2000). These findings are of importance because they identify the possibility of sub-groups of sufferers who have a large influx of the more destructive MC subtype, and would benefit from therapy targeted to MC tryptase and/or MC chymase.

The significant increase of the MC_{TC} subtype in the detrusor ($p<0.01$) and lamina ($p<0.01$) of PBS/IC individuals, when compared to controls, is an interesting finding, as chymase has a greater destructive potential than tryptase and may explain the variability in severity of symptoms between PBS/IC sufferers. The statistically significant influx of MC_{TC} in the lamina propria and detrusor of PBS/IC bladder may be linked to a higher risk of fibrosis. Analysis of the diagnosis data, after unblinding, showed a positive correlation between the presence of MC_{TC} within the lamina propria and the occurrence of bladder fibrosis.

Although this has not been previously reported in PBS/IC, a similar link has been identified in other renal disorders. Chymase containing MCs were found to be increased in IgA nephropathy, and increased levels correlate with an increase in the severity of renal fibrosis (Wasse et al. 2012). This correlation between fibrosis and MC_{TC} has also been identified in children with crescentic glomerulonephritis; where the increase in MC_{TC} is associated with an incidence of tubolointestinal fibrosis and a loss in renal function (Togawa et al. 2009). Additionally, Takai et al. (1997) noted that chymase can be inhibited by certain broad spectrum serine protease inhibitors (such as chymostatin) and thus, this could be of benefit to PBS/IC sufferers who have a particularly large influx of the MC_{TC} subtype. Conversely, a study into MC subtypes in PBS/IC, conducted by Yamada et al. (2000), suggested that it was the increase in tryptase from the influx of MC_{TC}, that was responsible for detrusor stiffening, as MC_{TC} contain significantly more tryptase (approx. three times more) when compared to the MC_T subtype. The findings from the present work do not concur with Yamada's theory, as when compared to controls, the overall influx of the MC_{TC} subtype in PBS/IC is not statistically different. Thus this suggests that MC_{TC} is a constitutive component of the bladder, and that the larger influx of MC_T is the more important event in PBS/IC.

Unblinding of the diagnostic data also identified a possible correlation between the presence of ulcers (known as Hunner's ulcers, or Hunner's lesions) and an increase in the presence of MCs in the mucosal layer. Hunner's ulcers affects only 5-10% of PBS/IC sufferers, and is classed as the "classic" subtype of PBS/IC. Clearly, Hunner's ulcers appear as distinct areas of inflammation on the bladder wall, but do not have a typical ulcer-like appearance. As mentioned previously, chymase-positive MCs are particularly destructive, in comparison to MC_T, and so the presence of ulcers may be due to the influx of MC_{TC}. Thus, if a PBS/IC individual shows a high count of MC_{TC} and the presence of ulcers, it would be wise to tailor their therapeutic treatment to target chymase.

The findings from this project support the clinical importance of the influx of MCs in the detrusor of PBS/IC being of key importance in regards to pathogenesis and a positive diagnosis. Currently, a positive diagnosis of PBS/IC is based on the observed and self-reported symptoms of the patient, urological assessment (including cystoscopy and urodynamic assessment), and the exclusion of other common urinary disorders. The findings from this project, in conjunction with other similar work, strongly support the incorporation of a MC count into all official diagnostic criteria, as currently it is only the ESSIC diagnostic criteria that incorporates the presence of detrusor mastocytosis or inflammatory infiltrates for a positive PBS/IC diagnosis. Hence all patients exhibit detrusor mastocytosis, is diagnosed when the MC count is >28 MCs/mm^2 (Nordling et al. 2004).

This variability was seen to an extent in this project, as some PBS/IC individuals had a markedly higher detrusor MC count (52.0 MC/mm^2) or lower count (9.4 MC/mm^2) than what is suggested for a positive detrusor mastocytosis diagnosis. Interestingly, a positive PBS/IC diagnosis was made in all cases. This may be for one of several reasons; the areas that were randomly selected during this project happened to contain fewer MCs and did not represent the mastocytosis that was seen during the official diagnosis made by specialist histopathological clinicians, initial diagnosis of PBS/IC was made without the pre-requisite of detrusor mastocytosis, or the PBS/IC individuals involved in this study truly represent the variability in the pathology of the disorder. Regardless, the inclusion of a minimum MC count in all diagnostic criteria as a suggestion, would aid the identification of individuals who are otherwise misdiagnosed, and provide a baseline to mark the progression of the patient over time, and any success of treatments undertaken.

This project sought to assess the entire PBS/IC bladder; from epithelium to adventitia. There is currently no existing research in the literature that assesses MC subtypes (MC_T and MC_{TC}) in the adventitia of human PBS/IC tissue. The adventitia is the outermost layer of the bladder wall, and consists of loose connective tissue, autonomic nerve fibres (that continue to extend into the bladder wall), and blood vessels. Our findings suggest that the influx of MCs into the adventitia of PBS/IC layer is significantly higher compared to controls ($p<0.001$). Additionally, the number of MC_{TC} within the adventitia is almost identical between normal and PBS/IC bladder. This suggests that MC_{TC} is a constitutive component of the bladder adventitia, and that the larger influx of MC_T is the more important event in this disorder. By providing data for the whole bladder, a more representative indication of what is happening at the cellular level can be created. PBS/IC is already an under-researched area of urology, and part of the purpose of this project was to raise the quality and quantity of research focussing on this disorder.

This project highlights that there is a difference in the pathologies between PBS/IC subgroups. Interestingly, although the hypothesis was supported in regards to discovering MCs in the detrusor layer of all subjects, additional findings, such as an influx of MCs in the lamina propria, were less consistent. This supports the idea of the existence of subtypes of patients in accordance to their disease pathogenesis. However, the results from this study support the theory that a damaged GAG layer is much to blame for the consequent MC influx and damage of the bladder wall layers. Thus, the GAG layer and protecting the inner lining of the bladder of PBS/IC patients should be a main therapeutic target. Providing an intravesical agent that not only reduces inflammation, inhibits bacterial infection, and promotes post-inflammatory healing, but also targets MCs could be achieved by using a honey-based therapeutic agent.

6.3.2 Honey

Honey is not often studied in a scientific capacity; much of the research lacks scientific standards, or are randomised clinical trials using animal models (Subrahmanyam 1991; 1993; 1994; 1996; 1998; 1999). This study utilised key aspects of scientific research such as randomisation and standardisation during protocol development. Through the continued development and refinement of protocols, the project became a standardised and refined set of experiments, which aimed to contribute to the reputation of honey as a potential therapeutic agent for clinical use. The series of honey and bladder tissue experiments that were conducted used standard scientific assurances such as randomisation techniques, repeats, optimisation, and researcher blinding.

The finding that honey did not bind histamine is crucial to the development of honey as a therapeutic agent for PBS/IC and other inflammatory disorders that involve MCs. This finding suggests that honey is stabilising MCs at both cellular and possibly molecular levels, i.e. it is interfering with the degranulation and subsequent release of histamine. MC degranulation is mediated by the binding of an antigen to the IgE surface receptor complex. After exposure to the antigen, the IgE molecules cross-link, leading to the activation of adenylate cyclase, the activation of protein kinase, the phosphorylation of specific proteins, and the eventual release of histamine via exocytosis. There are several stages or cellular components with which honey can interact in order to halt the degranulation and histamine release. Findings and existing research have already shown that honey is capable of activating apoptosis at a molecular level by inhibiting Bcl-2, and stimulating Caspase 9 and Caspase 3 (Fernandez-Cabezudo et al., 2013), and so, if honey is able to interact with the apoptotic pathway, there is also the likelihood of interactions with other cellular pathways, such as degranulation.

Results showed that a concentration of between 5-10% of eucalyptus honey displayed the most effective inhibition of histamine release, and minimal apoptosis of rat bladder explants. In regards to the bladder explant tissue work that was conducted, a honey concentration of below 5% may not exhibit much effect in the expanse of a whole human bladder filled with urine, i.e. the concentration may not be realistic when being used in a living patient whose bladder would be filling with urine. Honey concentrations above 10% led to apoptosis of the endothelial layer of bladder tissue explants. Although some apoptosis may have also occurred during preparation of the bladder explants, the apoptosis was slowed down in explants that had been incubated in 5 and 10% honey in both the 1-hour assay, and the 8-day assay.

6.3.3 Basophils

Basophils are rare cell type that is involved in many inflammatory processes. However, our results indicate that basophils do not play a role in PBS/IC. This is unsurprising, as basophils make up only 1% of circulating blood, and are not known to migrate into tissue.

The findings also helped established a control tissue and protocol for the 2D7 antibody, for the successful detection of basophils. The basophil portion of this study required much protocol development, and following thorough research of the literature, the control tissue was established as chronic myeloid leukaemia skin punch, however, the success of this tissue as the control lay in the presence of blood within the tissue, as the basophils were detected within this area. Repeats exhibited the same pattern of staining. Thus, results suggest that in order to detect basophils in tissue samples using a standard IHC technique, a sample of blood must also be present. This is unsurprising, as it is already known that basophils are only found within the blood, and MCs are predominantly found within tissue.

6.4 Limitations

There are some limitations that occurred during the study that may have restricted generalisability of some findings. These are briefly discussed below.

The IHC portion of this study faced a number of limitations, which are a common occurrence within this practice. Although regarded as a fairly simple scientific procedure, the success of IHC relies on many factors. The reliability of the results is highly dependent upon the quality of the reagent, preparation of tissue samples, the researcher performing the assay, and the individual who interprets the outcomes. Thus, a seemingly simple analytical method becomes a more complex procedure that requires specialist technique and rigor.

A key part of any scientific assay is the protocol. An inadequate protocol will undoubtedly lead to flawed and untrustworthy results. The idea that part of this study involved much protocol development, could be seen as a limitation in this study. Protocol development

begins with defining a question that needs to be answered, and determining which approaches and assays to use in order to answer the question effectively. Protocol development is often required for novel work, and when successful, is seen as a positive contribution to the scientific research world. However, the nature of protocol development is complex and prone to faults. The development of a protocol was a key aspect of the immunohistochemistry work with the mAb 2D7 for the detection of basophils. Although the protocol was developed effectively, and results can be successfully reproduced, it could be argued that the results should be regarded with caution as they have been gathered using a new protocol that has not been tested beyond the host lab.

As with all self-designed and implemented research, bias is an almost inevitable consequence. Bias must be identified and avoided both pre-emptively, as well as during the later stages of data collection and analysis. Bias occurs when ‘systematic error introduced into sampling or testing by selecting or encouraging one outcome or answer over others (Merriam Webster 2017). Bias exists in many forms and can occur at different stages throughout the study. Chance and confounding bias can be eliminated by adequate study design and analysis.

During the design phase of this study, there were several possible sources where bias could have been inadvertently introduced. As part of the study utilised tissue of a disease state, the expectation of finding inflammatory cell infiltrate was already in place before any assays were conducted. In addition to this, much of the study used existing literature as the basis for the assay design, and so there was some indication of what would be seen. These pre-conceived notions can infiltrate the data collection process, by the observation of positive results and trends which may not be there, and lead to false positive results which become incorporated into statistical analysis and conclusions. Several efforts were made to minimise bias throughout this study. For example, as immunohistochemistry involves much human visual analysis, the identification of positively stained cells or structures of interest is open to interpretation. During this project, in a bid to remove this interpretation bias, a macro was created to independently identify and quantify positively stained MCs when the images of the stained slides were evaluated using the image analysis software programme, ImageJ^{TM} (Schneider et al. 2012). Additionally, during the basophil arm of the is study, an independent histopathologist was consulted in order to interpret results without bias, as to the untrained eye, basophils and MCs have a similar appearance, and as previous work had already misidentified MCs as basophils in PBS/IC tissue (Crome 2012), the correct identification of basophils was of key importance.

An inherent problem with using humans to critically assess results is that they are open to bias. Thus, several scientific means were used in order to minimise bias in this study. One such method was randomisation during the rat bladder tissue and honey portion of the study. As the name suggests, randomisation is the process of creating a random sequence of variables, which does not follow a deterministic pattern. Findings from randomised control trials are considered the most reliable.

An unexpected limitation of the study occurred during unblinding of the patient data. Although every effort was made to only incorporate PBS/IC individuals into this study, access to the diagnostic criteria revealed that not all patients had a positive PBS/IC diagnosis. Participants were found to have a range of cystitis-type disorders, including PBS/IC, chronic and eosinophilic cystitis, and inflammatory and reactive cystitis. Upon analysis of the SNOMED search terms used, results suggest that the inclusion of non-PBS/IC patients into this study occurred due to the key words used producing a positive match, regardless of context. Unblinding to the diagnostic data occurred after MC counts had been analysed, and so knowing the data beforehand would have introduced bias during data analysis. Once the correct diagnostic data was known, analysis revealed that all those that had signs of fibrosis also had a positive PBS/IC diagnosis. Thus, this suggests that fibrosis is a key histopathological feature in more serious cases of this disorder, and should be regarded as such during diagnosis.

6.5 Future work

6.5.1 Painful bladder syndrome/interstitial cystitis

First and foremost, future work should aim to establish the findings of this project. This can be achieved by repeating the IHC protocol using a larger cohort of PBS/IC and control tissue, and further statistical analysis to confirm the significance seen in this project. In addition to this, the presence of mastocytosis could be confirmed by a serum tryptase analysis of the participant's blood. The World Health Organisation (WHO) diagnostic criteria for mastocytosis includes possessing a serum baseline value for tryptase of 20

ng/mL, and a greater than 25% of MCs having a morphologic abnormality (such as a spindle-like shape, cytoplasmic projections, and hypogranulation). Identification of a baseline serum tryptase level and continued regular tryptase assessment of the PBS/IC sufferer could provide information of disease progression, and success of anti-inflammatory PBS/IC treatments. A stable level over a period of time is indicative of successful treatment and disease management, yet rising levels would indicate a poor prognosis, inadequate and unsuitable treatment options, and poor management. Continued IHC assessment in conjunction with serum tryptase tests, may also be of benefit. During this project, an effort was made to acquire tryptase values of the participants involved, but this was deemed as an unnecessary request (for the project), by the histopathology team, and not possible for this project. Additionally, due to the finding that chymase positive MCs may play a key role in a subpopulation of PBS/IC sufferers, it may be suggested that measuring chymase serum levels could provide some useful information in regards to the severity and progression of an individual's PBS/IC.

Another key area for future work would be further elucidating the role of chymase- positive MCs in PBS/IC. In this project, a possible link between ulceration and an increased presence of the MC_{TC} subtype was identified. A literature search revealed that a link has previously been identified between chymase and disorders involving the presence of ulcers, mainly bullous pemphigoid (Briggaman et al. 1984), and chronic leg ulcers (Huttunen et al 2000). In regards to mode of action, research suggests that chymase degrades cell-to-cell and cell-to-membrane junctions, specifically, by altering tight junction proteins such as occludin (Scudamore et al. 1998). Ebihara et al. (2005), found that human corneal epithelial cells incubated with a level of chymase (representative of that found in the tears of vernal keratoconjunctivitis patients) were found to have a decreased barrier function, and when human corneal epithelial layers are damaged, incubation with the same level of chymase led to an inhibition in the migration of cells. This wound assay suggests that when tissue is damaged, mid-high levels of chymase inhibit epithelial healing due to chymase cleaving occludin. In the bladder, tight junctions exist between the transitional epithelium cells of the endothelium, below the GAG layer. In PBS/IC, the GAG layer is damaged, and so noxious components leak into the bladder wall and stimulate inflammation. A damaged GAG layer in addition to a loosening of the tight junctions would increase the amount of inflammation- inducing agents reaching the inner layers of the bladder wall i.e. the lamina propria and detrusor muscle thus perpetuating the inflammatory process, further provoking PBS/IC symptoms, and increasing the recruitment of MCs to the site of inflammation. It could therefore be proposed that the PBS/IC individuals with a higher proportion of chymase positive MCs within the

endothelial layer and a particularly damaged GAG layer may also have worse symptoms in comparison to those that predominantly have the MC_T subtype within the endothelial layer.

Another key area for the future of PBS/IC research should be to focus on the diagnostic criteria, and possible prevention measures. Interestingly, adult PBS/IC sufferers state that their symptoms seem to have begun during childhood (Interstitial Cystitis Association 2016). In 1978, the idea of ‘early IC’ was popularised. This type of PBS/IC had milder symptomology and occurred in younger individuals, but as the diagnostic criteria has been modernised and narrowed, these ‘early IC’ patients are passing undiagnosed, as the diagnostic criteria focusses on the secondary effects of the PBS/IC, rather than the origins of the disorder (Messing and Stamey 1978). It seems that the diagnostic criteria for PBS/IC needs further improvement to include the diagnosis of younger patients. Once this is achieved, preventative measures could be adopted by the sufferer to reduce the deterioration of bladder function, or even stop the development of PBS/IC. This could be achieved through a combination of dietary modifications, drug therapy, and bladder training.

An earlier diagnosis could aid in treating and slowing down the progression of the disorder. The diagnostic criteria are mainly based on the exclusion of more common or easily detectable bladder-related disorders, and this often leads to misdiagnosis, and PBS/IC remaining undiagnosed longer than need be. The variability in symptomology reflects the variability in the pathogenesis of the disease, but it can be agreed that all PBS/IC patients will have a damaged GAG layer and an influx of MCs. The results from this project raise several improvements that could be made to the inclusion criteria for PBS/IC. One improvement would be to incorporate an MC count of the entire bladder wall, and although this may be impractical, it could be adopted for particularly difficult cases. Another important improvement would be to incorporate a histopathology examination of the bladder wall for signs of fibrosis. Unblinding to the diagnostic data reinforced the variability in MC subtype distribution and density seen during this study; PBS/IC symptomology varies from individual to individual and in conjunction with unsatisfactory diagnostic criteria, it is unsurprising that PBS/IC is so often misdiagnosed. It is important for clinicians to exhibit some flexibility in the interpretation of the criteria during diagnosis, in addition to conducting IHC of a full-thickness sample of the bladder wall.

6.5.2 Honey and painful bladder syndrome/interstitial cystitis

The key future direction of this project is to continue to refine a honey-based therapeutic agent for the treatment of PBS/IC. The project established the need for a new anti-inflammatory therapeutic agent in the treatment of PBS/IC, the presence of MCs in this disorder, and the potential of honey to dampen down the degranulation of MCs. Results suggest that further work in the development of a honey-based intravesical treatment is potential feasible and arguably necessary due to it anti-inflammatory, antibacterial and proangiogenic/healing properties. It would also be of interest to discover if a honey-based intravesical agent should have an adverse effect on diabetic patients. Much of the existing honey and wound care research focuses on the treatment of diabetic ulcers (Surahio et al. 2014, Imran et al. 2015, Kamaratos et al. 2015) and honey is considered safe to use in diabetic patients, for the treatment of ulcers (Kateel et al. 2016). However, it has been proposed that methylglyoxal (MG), the compound responsible for the high antibacterial capability of Manuka honey, is a powerful protein-glycating agent, and a key precursor molecule of advanced glycation end products (AGEs). These AGEs play a crucial role in the pathogenesis of impaired diabetic wound healing (Majtan 2011), and so further study into this subgroup of PBS/IC sufferers is crucial to ensure the safety of a honey-based intravesical wash in PBS/IC diabetics.

Results from this project suggest that the optimal honey concentration for use in an intravesical therapy is between 5 and 10%. The first area to be addressed should be the refinement of the exact concentration of honey required to successfully treat PBS/IC without also inducing apoptosis using ex vivo assays in whole rat bladder, and following success, human bladder tissue. Following this, the next key step would be to develop an intravesical agent for in vivo testing, in whole rat bladders (prepared with cyclophosphamide cystitis to represent the PBS/IC bladder), and human bladders. As translation into live testing in human bladders is a key step, it must be completed as safely as possible. This would be achieved through utilising an incrementally progressive series of experiments, that would allow gradual development of the safest and most effective clinically-successful honey concentration. The gradual move into live human bladder creates a thorough and robust study that allows time to identify and improve on any possible adverse effects of the intravesical agent. Each step would need the scientific measures that were implemented during this project, that is, controls, randomisation, repeats, and protocol refinement. Additionally, the testing of a control solution of glucose-fructose should be used in order to provide a direct comparison to honey, to ensure that it

is not just the sugar content and viscosity of honey that is preventing MC degranulation of histamine. As in this project, histamine release will be assessed by measuring the histamine within the supernatant using a standard ELISA, bladder morphology will be assessed using H&E staining, MC distribution will be identified with IHC, and tissue viability using live/dead staining and confocal microscopy.

This project also highlighted the potential role of honey in the degranulation of histamine from MCs. Results suggested that honey does not bind histamine, and so is interacting with the degranulation process of the MCs. Although the molecular analysis of this interaction was beyond the scope of this project, it would be highly beneficial to elucidate the possible mechanism of action, as has been achieved with the effect of honey on apoptosis. Recently, the exact receptor involved in histamine release from MCs in mice was identified by a ground- breaking study by McNeil et al. (2015). Using MrgprB2-null mutant mice, they reported that inflammation, secretagogue-induced histamine release, and airway narrowing were eliminated. They found that basic stimulatory compounds activate MCs in mice, both in vivo and in vitro through the receptor Mrgprb-2 (Figure 6.1), which is representative of Mrgpr-2, the corresponding human G-protein couple receptor. Thus, it was concluded that it is the activation of this receptor that leads to the release of histamine. When these findings by McNeil are taken into account, along with the results from this project, several potential areas of inhibition are evident. The honey may be blocking the binding of the secretagogue to the Mrgprb-2 receptor, or honey may be inhibiting the influx of calcium required for degranulation. Interestingly, results from the McNeil study also suggested that as well as basic stimulatory molecules such as SP, peptidergic drugs are also activate the mouse MC MrgprB2 receptor. Peptidergic drugs induce an allergic-type reaction at the site of injection, thus implicating the involvement of histamine. Further studies should be conducted in order to pinpoint the exact role of the MrgprB2 receptor, and it’s potential as a therapeutic target in the treatment of PBS/IC. Elucidating the role of this receptor in PBS/IC could aid in the development of honey-based therapies that target the receptor and modulate its activity, and thus, reduce the inflammation and symptoms associated with PBS/IC.

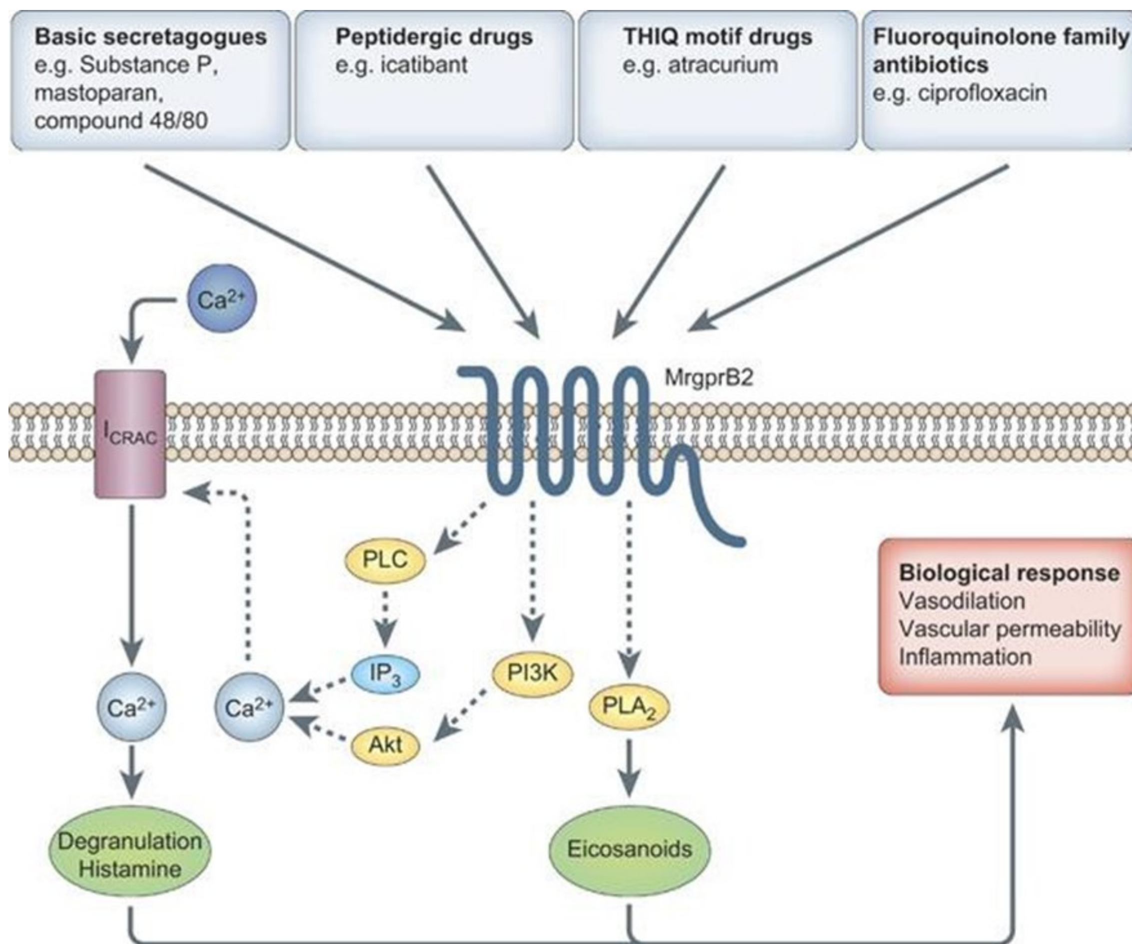


Figure 6-1: MrgprB2 (the mouse mast cell basic secretagogue receptor) and mediates side- effects of peptidergic and small molecule therapeutic drugs. (Adapted from Grimbaldeston 2015).

Although this project utilised honey in a liquid form (combined with growth medium), a development to this would be to truly assess the best form in which to deliver honey to the bladder. In-line with most other PBS/IC treatments, a honey- based intravesical instillation could be developed to be administered via catheterisation as and when is needed. The drawback to this, is the same as with all instillations, the patient must be catheterised, the instillation must be held within the bladder for a minimum of 30 minutes, and several catheterisations per week may be required. Thus an alternative delivery method may be beneficial. Over the past few years, there has been a development in the technology of intra-bladder devices that can deliver a therapeutic dose of PBS/IC treatment directly into the bladder that treats symptoms for a long period of time. Nickel et al. (2012) are proving successful with the development of a continuous lidocaine-releasing intravesical system

(LiRIS) (Figure 6.2). This “pretzel-shaped” device was designed to fold in order to be inserted during cystoscopy, and once free in the bladder, unfold into a “pretzel shape” that sits within the bladder. The system can be retained for two weeks in the bladder to locally deliver therapeutic amounts of the treatment drug into urine, and thus, the bladder wall. This system is currently undergoing clinical trials, and has promising results thus far. Another option for longer-term drug delivery is to hydrolyse the honey into a gel-form to ensure slow drug dissipation. Momin et al. 2016 have developed a biodegradable super-porous hydrogel sponge, impregnated with honey and curcumin, for the treatment of ulcers. Although the model was developed to absorb liquid from a wound whilst adhering and delivering treatment to the area, the concept that honey can be manipulated into other forms is shown clearly. The sponge has only been tested using in vitro methods, but results are promising. The sponge contributed to a faster and more effective wound healing, whilst also delivering treatment to the site of injury for a total of 20 days. With the incidence of PBS/IC increasing, the risk of infection due to catheterisation, and the need for patients to have a convenient yet effective treatment option, the need to develop an adequate delivery method for a honey based treatment is vital.

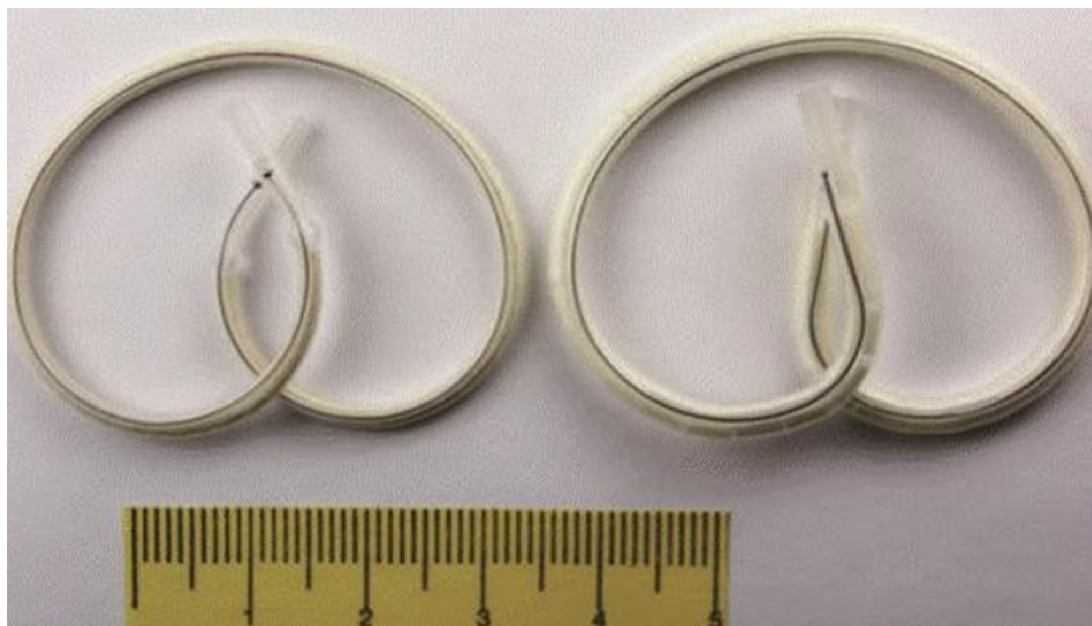


Figure 6-2: Lidocaine-releasing intravesical system; LiRIS (TARIS Biomedical, Lexington, MA, USA). Ruler= 5 cm. (Adapted from Nickel et al. 2012).

6.5.3 Honey and apoptosis

A particularly interesting finding during this project was in relation to the honey toxicity noted during the ELISA work, and the live-dead staining assay. A honey concentration of 20% and above, led to noticeable Propidium Iodide staining of dead cells within bladder explant specimens. Although the toxicity was not further looked into in this project, the finding is worth further investigating. Questions that should be addressed include the concentration at which honey is regarded as toxic to cells and tissues, the length of incubation that leads to apoptosis due to honey, and if the floral source of honey has an impact on the level of toxicity and apoptosis. Additionally, as apoptosis can be assessed by measuring the associated markers, such as cleaved cytokeratin-18, cleaved caspase-3, and translocation of apoptosis-inducing factor (Holubec et al. 2005), immunohistochemical techniques can be used to identify the possible presence of apoptosis in tissues incubated in honey.

The anti-inflammatory properties of honey would be of great benefit to a variety of difficult to treat inflammatory disorders, including cancer. Chronic inflammation is linked to the formation and progression of cancer, and so sufferers could benefit from an anti-inflammatory and apoptosis inducing therapeutic agent such as honey. In the UK, cancer causes 1 in 4 deaths, with approximately 163,000 deaths being caused by cancer in 2014 (Cancer Research UK, 2017). Over time, a range of cancer treatment and prevention therapies have been developed (such as surgery, chemotherapy, and radiotherapy), but perhaps in a bid to reduce visible side-effects and multi-drug resistant cancers, there has been an increasing interest in alternative or more natural therapies. Existing literature in regards to utilising the toxicity of honey has identified the possibility of using honey to develop potential chemo-preventive therapies (Ahmed and Othman 2013).

6.6 Conclusion

In conclusion, PBS/IC is an under-researched urological disorder, and thus, is poorly understood, diagnosed, and managed. Advancements in medicine have led to the development of targeted tailored therapeutic options for many disorders, and the

development of thorough and highly successful diagnostic criteria, yet this modernisation has eluded PBS/IC. The diagnostic criteria and treatment options for PBS/IC patients need updating and improving.

This project was designed to; 1) Further elucidate the role of mast cells (MC) in PBS/IC, 2) Identify any difference in the distribution of MC subtypes (MC_T and MC_{TC}), in order to shed light on the pathogenesis of the disorder, and 3) Evaluate the potential of honey as a novel anti-inflammatory intravesical agent for the treatment of PBS/IC. The project was successful in that it identified a difference in the distribution of MCs within the bladder wall, and a difference in distribution of MC subtypes, in comparison to controls. It was also successful in establishing a narrow range from which to develop a honey-based intravesical therapy for the treatment of inflammation associated with PBS/IC.

Novel findings from this study included the discovery that a particularly high presence of MC_{TC} in the mucosa may be linked to the presence of ulcers, and thus, identified the patients that may have had the Hunner's Ulcer PBS/IC subtype. It was also found that some patients had a higher amount of MC_{TC} in the lamina propria, and this may indicate those patients that were also suffering from fibrotic bladder. These discoveries could explain the variability seen in patient symptomology, and could reflect disease severity. In regards to honey, results reaffirmed that honey can be used to treat inflammation by targeting MC degranulation. It was also discovered that bladder tissue will undergo apoptosis when exposed to a honey concentration of above 10%.

The suggestions for future work are important for establishing honey as an option for the treatment of inflammatory disorders, and for advancing our knowledge of PBS/IC and how to target such a difficult disorder. After further validation, these findings should be used to develop much-needed comprehensive diagnostic criteria for the successful identification of PBS/IC sufferers, and for the development of honey-based intravesical agent, that has the potential to treat the histamine-related inflammation seen in PBS/IC. Due to the variety of healing properties that honey possesses (antibacterial, anti-inflammatory, angiogenic, and reepithelialisation), the potential for the development of honey-based therapies for other difficult to treat chronic and acute inflammatory conditions, such as methicillin-resistant *Staphylococcus aureus* (Hammond et al. 2016), cancer, and arthritis (Owoyele et al. 2011), is extraordinary yet achievable.

Appendices

Appendix A Search Terms

Appendix B O'Leary/Sant Voiding and Pain Indices

Appendix C Protocol: Immunohistochemistry

Appendix D Mast Cell Counting

Appendix E Protocol: Bladder Explant

Appendix F Cutting Mat

Appendix G Randomisation Lists

Appendix H Standard Histamine ELISA Procedure

Appendix A Search Terms

Search Terms for PBS/IC

Database	Search terms	Filter	Results
PubMed	Interstitial cystitis OR painful bladder syndrome AND Treatment OR intravesical OR therapy	English Language	1827
CDSR (The Cochrane library)	Interstitial cystitis OR painful bladder syndrome AND Treatment OR intravesical OR therapy	None	202
Medline (OvidSP)	Interstitial cystitis OR painful bladder syndrome AND Treatment OR intravesical OR therapy	None	1279

Process for literature selection:

- Removal of duplicates
- Omission of articles that are not in English, or do not have interstitial cystitis/PBS as the main focus
- Cross-referencing of each article/paper, and then grouping into main drug therapies
- Exclusion of any unpublished research (with the exception of conference abstracts)
- Selection of key papers from main drug therapies for PBS/IC

Search Terms for Honey

Database	Search terms	Filter	Results
PubMed	Honey AND Interstitial cystitis OR painful bladder syndrome OR treatment OR therapy OR anti-inflammatory OR inflammation OR wound	English Language	1363
CDSR (The Cochrane library)	Honey AND Interstitial cystitis OR painful bladder syndrome OR treatment OR therapy OR anti-inflammatory OR inflammation OR wound	None	222
Medline (OvidSP)	Honey AND Interstitial cystitis OR painful bladder syndrome OR treatment OR therapy OR anti-inflammatory OR inflammation OR wound	None	1440

Process for literature selection:

- Removal of duplicates
- Omission of articles that are not in English, or do not have honey as the main focus
- Cross-referencing of each article/paper, and then grouping into main themes of research
  - Exclusion of any unpublished research (with the exception of conference abstracts)
  - Selection of key papers

Appendix B O'Leary/Sant Voiding and Pain Indices

O'Leary/Sant
VOIDING AND PAIN INDICES

INTERSTITIAL CYSTITIS SYMPTOM INDEX

1.
During the past month, how often have you felt the strong need to urinate wiht little or no warning?

0. _______ not at all
1. _______ less than 1 time in 5
2. _______ less than half the time
3. _______ about half the time
4. _______ more than half the time
5. _______ almonst always

2.
During the past month, have you had to urinate less than 2 hours after you finised urinating?

0. _______ not at all
1. _______ less than 1 time in 5
2. _______ less than half the time
3. _______ about half the time
4. _______ more than half the time
5. _______ almost always

3.
During the past month, how often did you most tupically get up at noght to urinate?

0. _______ never
1. _______ once
2. _______ 2 times
3. _______ 3 times
4. _______ 4 times
5. _______ 5 times
6. _______ 5 or more times

4 .
During the past month, have you experienced pain or burning in your bladder?

0. _______ nat at all
1. _______ once
2. _______ a few times
3. _______ fairly often
4. _______ almost always
5. _______ usually

Add the numerical values of the checked entries;
Total score __________.

INTERSTITIAL CYSTITIS PROBLEM INDEX

During the past month, how much has each of the following been a problem for you?

1. Frequent urination during the day?

0. _______ no problem
1. _______ very small problem
2. _______ small problem
3. _______ medium problem
4. _______ big problem

2. Getting up at nigth to urinate?

0. _______ no problem
1. _______ very small problem
2. _______ small problem
3. _______ medium problem
4. _______ big problem

3. Need to urinate with little warning?

0. _______ no problem
1. _______ very small problem
2. _______ small problem
3. _______ medium problem
4. _______ big problem

4. Burning, pain, discomfort, or pressure in your bladder?

0. _______ no problem
1. _______ very small problem
2. _______ small problem
3. _______ medium problem
4. _______ big problem

Add the numerical values of the check entries:
Total score __________.

Appendix C Protocol: Immunohistochemistry

Histochemistry Research Unit, University of Southampton

AVIDIN BIOTIN-PEROXIDASE TECHNIQUE FOR PARAFFIN SECTIONS
Including avidin-biotin blocking steps

The full risk assessment (see safety folder) must be read before undertaking this procedure.

SUMMARY OF RISKS:
ANTIBODIES – YELLOW
CLEARENE – RED 1
AEC, DAB, HAEMATOXYLIN, HYDROGEN PEROXIDE – RED 2
ALCOHOLS, PERTEX – RED 4

METHOD:

Includes avidin biotin and culture medium blocking stages. All stages are at room temperature unless otherwise stated.

1. Deparaffinise sections in clearene (2 x 5 mins) and rehydrate through graded alcohols (5 mins in each) to 70%.
2. Inhibit endogenous peroxidase with 0.5% hydrogen peroxide in methanol (make fresh), 10 mins.
3. Wash TBS 3 x 2 mins.

IF SECTIONS DO NOT REQUIRE ANTIGEN RETRIEVAL TREATMENT OMIT STEP 4.

4. Perform appropriate antigen retrieval procedure for primary antibody (see separate protocols).
5. Drain slides and apply avidin solution, 20 mins.
6. Rinse TBS, 3 x 2 mins.
7. Drain slides and apply biotin solution, 20 mins.
8. Rinse TBS, 3 x 2 mins,
9. Drain slide, apply culture medium, 20 mins.
10. Drain slides and apply primary antibodies at appropriate dilutions, incubate overnight at 4°C.
11. Wash TBS, 3 x 5 mins.
12. Drain slides and apply biotinylated second stage antibodies at appropriate dilutions, 30 mins.
13. Wash TBS, 3 x 5 mins.
14. Drain slides and apply avidin biotin-peroxidase complexes at appropriate dilution, 30 mins.
15. Wash TBS, 3 x 5 mins.
16. Drain slides and apply substrate.
    a: DAB, 5 mins
    b: AEC, 10 mins
17. Rinse in TBS.
18. Wash in running tap water, 5 mins.
19. Counterstain sections with Mayer's haematoxylin.
20. Blue sections in running tap water.
21. Mount sections either
    [a] DAB sections – dehydrate through graded alcohols, clear in clearene and mount in pertex,
    or
    [b] AEC sections – apply aqueous mounting medium, bake at 80°C for 20 mins, allow to cool and mount in pertex.

Version 4, 24th January 2013 Page 1 of 1

Histochemistry Research Unit, University of Southampton

PRONASE PRETREATMENT

The full risk assessment (see safety folder) must be read before undertaking this procedure.

SUMMARY OF RISKS:

No known risks

REAGENTS

a. Pronase:

1% pronase stock. Solution is made by dissolving the lyophilised contents of one vial (100mg) of pronase (Dako) in 10mls of TBS. It is then stored in 0.1ml aliquots at -20°C. This stock solution is stable for at least one year. Avoid repeated freezing and thawing. The activity of each new batch of pronase should not vary considerably, but each batch must be tested before use.

b. TBS pH7.6

See separate protocol for preparation

METHOD

1. Paraffin sections should be mounted on APES coated slides and dried for at least 24 hours.
2. Sections should be dewaxed and endogenous peroxidase blocked as described in the IHC protocol.
3. Prepare the pronase solution by thawing one vial of 0.1mil of pronase stock (1%) and adding 1.9mls of TBS. Mix well. The working solution is stable for 5 days at -28°C.
4. Drain the slides and cover with working pronase solution. Incubate at room temperature for approximately 10 mins. The optimum incubation time depends on the length and type of formalin fixation and the batch of pronase.
5. Wash in TBS 2 x 5 mins, before continuing with the IHC protocol.

Version 2, 30th June 2010

Page 1 of 1

Histochemistry Research Unit, University of Southampton

MICROWAVE PRETREATMENT

The full risk assessment (see safety folder) must be read before undertaking this procedure
This procedure must not be undertaken outside normal working hours.

SUMMARY OF RISKS:
Chemical
CITRIC ACID – YELLOW
Physical
The containers of buffer will become hot during microwaving so a face shield and insulated gloves must be worn when removing the containers from the microwave.

BUFFER FORMULAS

0.01M Citrate buffer pH6.0:

Citric acid crystals 2.1g

Distilled water 1000ml

Mix and adjust pH to 6.0 with 1M sodium hydroxide (approximately 25ml)

Or

1mM EDTA buffer pH8.0:

EDTA 0.37g

Distilled water 1000ml

Mix and adjust pH to 8.0 with 0.1M sodium hydroxide (approximately 8ml)

Consult antibody register for recommended buffer to be used for each antibody. These instructions are for a SHARP microwave, R-27STM-A 800W. The settings and times for other models may vary.

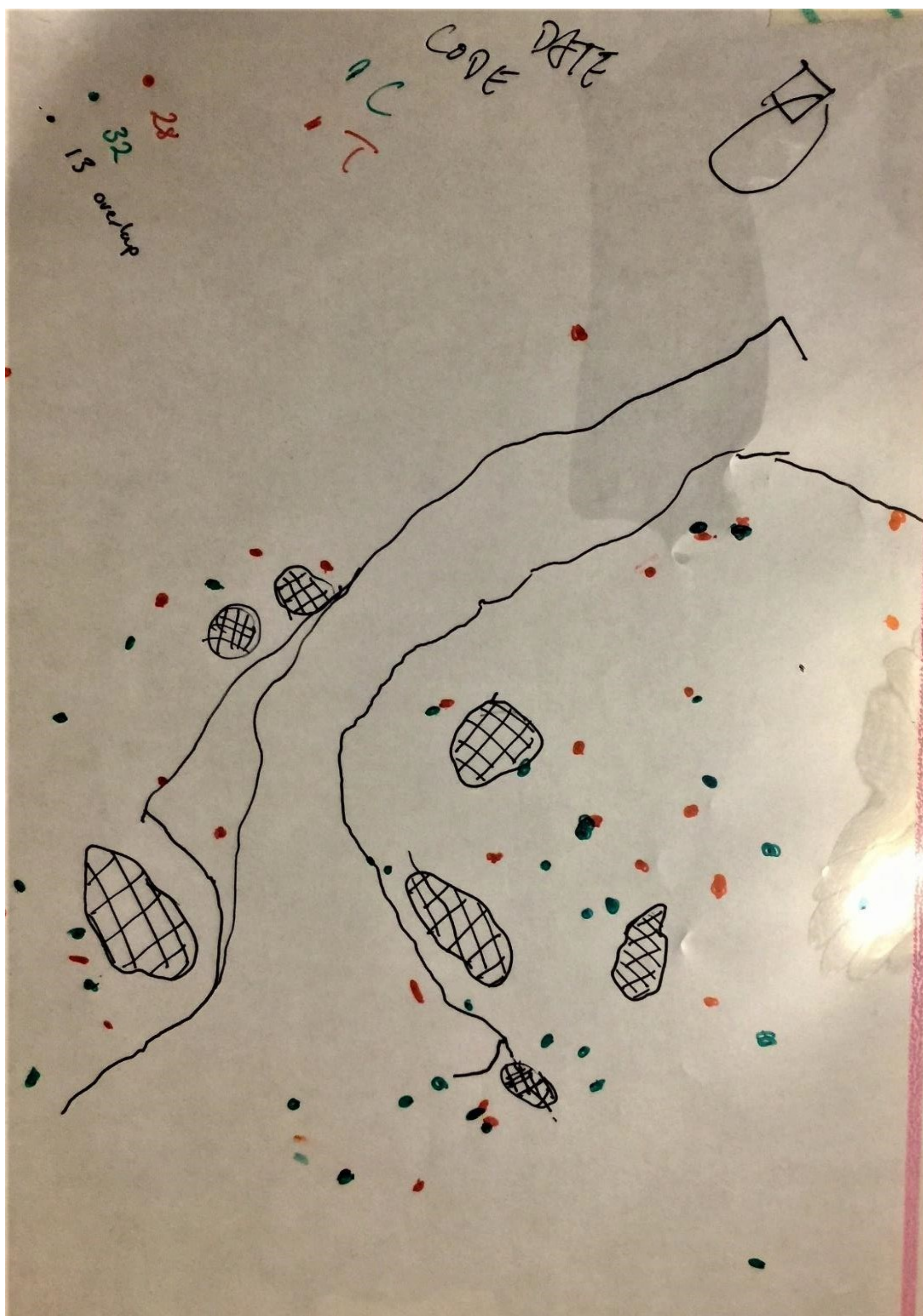
METHOD

1. Paraffin sections should be mounted on APES coated slides and dried for at least 24 hours.
2. Sections should be dewaxed and endogenous peroxide blocked as described in the IHC protocol
3. Fill the plastic staining racks with 24 slides and place in the polythene box. To maintain a constant load 3 polythene boxes are always used, together with 72 slides. Use blank slides if necessary.
4. Fill each box with 330ml of prepared buffer. Place the perforated lid firmly on the box.
5. Place the 3 boxes in the microwave, placing each one at the edge of the plate, evenly spaced with a small gap between each box.
6. Set the microwave to 25 minutes and 50% power. Start and allow to run.
7. When the time has elapsed, remove one box at a time CARE AS IT WILL BE HOT – WEAR INSULATED GLOVES AND FACE SHIELD. Remove the lid and fill quickly with cold running water. Leave all 3 racks in running water for 2 – 3 mins.
8. Place the slides back in the staining trays, wash in TBS 2 x 5 mins, before continuing with the IHC protocol.

Version 3, 12th October 2012

Appendix D Mast Cell Counting

Manual cell counting method for co-localisation staining of mast cell subtypes. Chymase-positive mast cells are identified in green and tryptase-positive mast cells are identified in red. Any mast cell identification marks that overlap are considered as mast cells that contain both chymase and tryptase (MC_{TC}).



Appendix E Protocol: Bladder Explant

STAGE I

- Set up lab by turning on the hood and checking that the incubator is set to 37°C + 0.5% CO_2.

- Clean work area and any apparatus to be used with alcohol spray. Place all of the required apparatus in the fume cupboard, including glass pipettes, Pasteur pipettes, medium, PBS solution, tweezers, scalpel, and a beaker that has been sprayed thoroughly with alcohol.

- Prepare tissue growth medium;

Growth Medium- Dulbecco's modified Eagle's medium (DMEM) 500 ml

Nutrient- Calf Serum 5%/50 ml

Antibiotics- L-glutamine penicillin 5 ml

All individual components of this growth medium have been sterile-filtered through a 0.22µm sterile filter during production.

- Under the hood, add 30ml PBS to a sterile Universal tube. Add freshly acquired rat bladder to the tube.

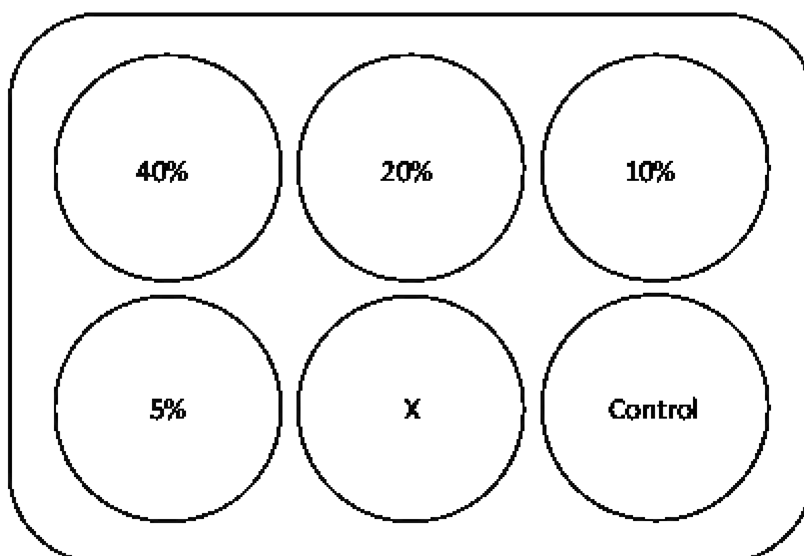
- Place the laminated cutting guidelines (Appendix F) underneath a Petri dish. Add 10 ml of growth medium to the Petri dish. Place the bladders into this medium. There should be enough medium to keep the bladder tissue from dehydrating, and thus keep the tissue viable (and minimise damage).

- Remove the fat from each bladder using tweezers and a scalpel, and then dissect the bladder into 2mm by 2mm sections. The researcher acknowledges that the scalpel will damage the tissue and cause an inflammatory response, so the bladder should be cut carefully and swiftly.

- Place the lid onto the petridish and incubate at 37°C and 0.5% CO_2 for 5 days (120 hours). This incubation period is required to help the tissue stabilise after being cut, and become acquired

- The tissue and medium must be checked daily, to ensure that there is no contamination.

- After the 5-day incubation, stage II of the protocol will commence.



STAGE II

- Place honey into the incubator to warm through for 5 minutes
- Clean work area and any apparatus to be used with alcohol spray. Place all of the required apparatus in the fume cupboard, including glass pipettes, Pasteur pipettes, honey, growth medium, PBS solution, tweezers, filter unit, and a beaker that has been sprayed thoroughly with alcohol
- Line up 4 sterile tubes, and label each one as 40%, 20%, 10%, or 5%.
- Label a large universal tube with ‘40% unfiltered’

- Alcohol-spray all tubes and place under the hood.
- Using the glass pipette, add 5ml of growth medium to the 20%, 5ml to the 10%, and 6ml to the 5%
- Prepare 20 ml of 40% tissue growth medium and honey mix. Add12ml growth medium and 8ml honey to the universal tube labelled as 40% unfiltered. Whirlimix for 30 seconds, and then invert shake for 30 seconds. Withdraw all of the mixture into a syringe, and fix a single use 0.22 μm filter unit to the tip.
- Push 10 ml of the mix through the filter into the tube labelled 40%
- Whirlimix for 30 seconds and invert shake for 30 seconds
- Using a sterile syringe, withdraw 5ml of the filtered 40% mix, and add to the 20% tube.
- Whirlimix for 30 seconds and invert shake for 30 seconds
- Using a sterile syringe, withdraw 5ml of the filtered 20% mix, and add to the 10% tube.
- Whirlimix for 30 seconds and invert shake for 30 seconds
- Using a sterile syringe, withdraw 4ml of the filtered 10% mix, and add to the 5% tube
- Whirlimix for 30 seconds and invert shake for 30 seconds
- To a 6-well plate, label as 5%, 10%, 20%, 40%, and control (medium only, no honey)
- To each well, add 5ml of the corresponding medium-honey mixture. Leave one well empty
- Distribute the bladder tissue explants equally between the 5 wells.
- Record the appearance of the tissue and medium
- Place the plate in the incubator (37°C and 0.5% CO_2)

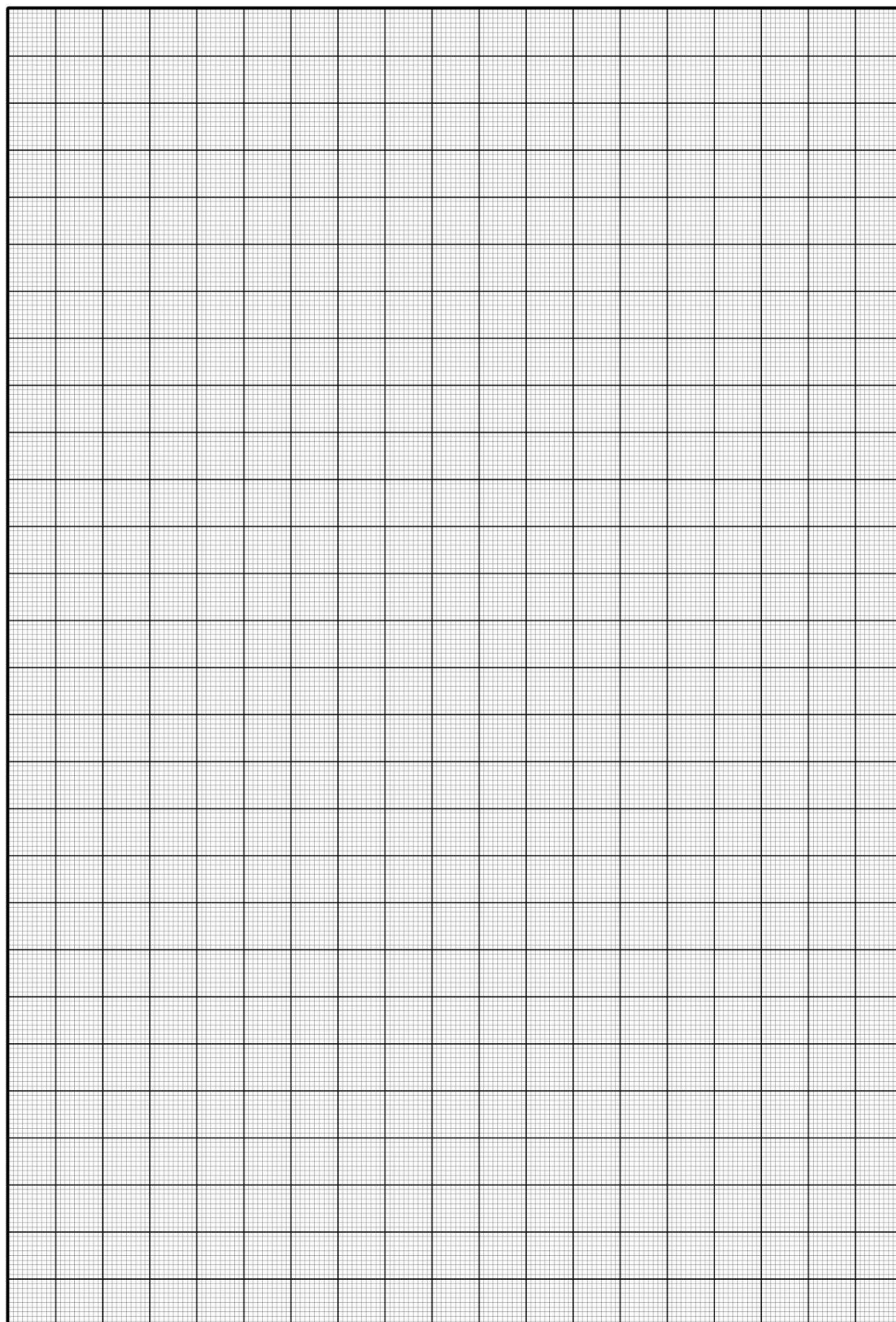
STAGE III

- Sampling will take place every 2 days/48 hours from Day 0 - Day 18. Once samples have been taken, they will be placed in a -80 ºC freezer, and the appearance of the tissue and medium in each well will be recorded

- Day 2 – First sample
- Turn on the fume hood
- Clean work area and any apparatus to be used with alcohol spray. Place all of the required apparatus in the fume cupboard, including the 250 μl pipette, sterile pipette tip box, labelled Eppendorf tubes, and a beaker that has been sprayed thoroughly with alcohol.
    - Proceed to withdraw 500 μl from each well and add to the corresponding Eppendorf tube. Make sure each tube is closed securely, and a new tip is attached to the pipette for each well.
    - Once all samples have been taken, place the lid back on the 6-well plate, and return the plate to the incubator (37°C and 0.5% CO_2)
    - Put the Eppendorf's containing the medium samples in the -80 ºC freezer
    - Tidy work area and remove of waste appropriately
    - Clean work area using alcohol spray, and turn off the fume hood
    - Repeat Stage III of the protocol until the last samples have been taken on Day 18.
    - On Day 18, remove the tissue from each well, blot on tissue, and weigh.
    - Record weights
    - Dispose of tissue in the appropriate method

Appendix F Cutting Mat

A representation (not to scale) of the cutting guidelines used in the bladder explant assay protocol (Appendix E)



Appendix G Randomisation Lists

https://www.sealedenvelope.com/simple-randomiser/v1/lists

CREATE A RANDOMISATION LIST

Use this tool to create a blocked randomisation list for your trial. The generated lists are suitable for use with our simple randomisation service

Create a list

Seed: 71859971163267

Treatment groups A, B, C, D, E

Block sizes 10, 10

List length 11

Strata (optional) e.g. Age group: under 30, 30 or over +

name: category 1, category 2, ...

Generate unique randomisation code?

Create list | Download as CSV

Your list

Seed: 71859971163267

Block sizes: 10, 10

Actual list length: 20

block identifier, block size, sequence within block, treatment

```
1, 10, 1, B
1, 10, 2, D
1, 10, 3, B
1, 10, 4, A
1, 10, 5, A
1, 10, 6, E
1, 10, 7, C
1, 10, 8, C
1, 10, 9, E
1, 10, 10, D
2, 10, 1, B
2, 10, 2, A
2, 10, 3, A
2, 10, 4, C
2, 10, 5, E
2, 10, 6, E
2, 10, 7, C
2, 10, 8, D
2, 10, 9, B
2, 10, 10, D
```

CREATE A RANDOMISATION LIST

Use this tool to create a blocked randomisation list for your trial. The generated lists are suitable for use with our simple randomisation service

Create a list

Seed: 102078634825123

Treatment groups A, B, C, D, E

Block sizes 10, 10

List length 11

Strata (optional) e.g. Age group: under 30, 30 or over +

name: category 1, category 2, ...

Generate unique randomisation code?

Create list | Download as CSV

Your list

Seed: 102078634825123

Block sizes: 10, 10

Actual list length: 20

block identifier, block size, sequence within block, treatment

```
1, 10, 1, B
1, 10, 2, C
1, 10, 3, A
1, 10, 4, D
1, 10, 5, A
1, 10, 6, E
1, 10, 7, D
1, 10, 8, E
1, 10, 9, B
1, 10, 10, C
2, 10, 1, B
2, 10, 2, C
2, 10, 3, D
2, 10, 4, B
2, 10, 5, D
2, 10, 6, A
2, 10, 7, C
2, 10, 8, A
2, 10, 9, E
2, 10, 10, E
```

CREATE A RANDOMISATION LIST

Use this tool to create a blocked randomisation list for your trial. The generated lists are suitable for use with our simple randomisation service

Create a list

Seed:
158161586231925

Treatment groups
A, B, C, D, E

Block sizes
10, 10

List length
11

Strata (optional)
e.g. Age group: under 30, 30 or over +

name: category 1, category 2, ...

☐ Generate unique randomisation code?

Create list | Download as CSV

Your list

Seed: 158161586231925

Block sizes: 10, 10

Actual list length: 20

block identifier, block size, sequence within block, treatment

```
1, 10, 1, B
1, 10, 2, B
1, 10, 3, D
1, 10, 4, A
1, 10, 5, D
1, 10, 6, C
1, 10, 7, E
1, 10, 8, A
1, 10, 9, E
1, 10, 10, C
2, 10, 1, A
2, 10, 2, D
2, 10, 3, B
2, 10, 4, B
2, 10, 5, D
2, 10, 6, C
2, 10, 7, E
2, 10, 8, A
2, 10, 9, E
2, 10, 10, C
```

CREATE A RANDOMISATION LIST

Use this tool to create a blocked randomisation list for your trial. The generated lists are suitable for use with our simple randomisation service.

Create a list

Seed:
28567379906369

Treatment groups
A, B, C, D, E

Block sizes
10, 10

List length
11

Strata (optional)
e.g. Age group: under 30, 30 or over +

name: category 1, category 2, ...

☐ Generate unique randomisation code?

Create list | Download as CSV

How to cite this tool

Sealed Envelope Ltd. 2015. Create a blocked randomisation list. [Online] Available from:

Your list

Seed: 28567379906369

Block sizes: 10, 10

Actual list length: 20

block identifier, block size, sequence within block, treatment

```
1, 10, 1, C
1, 10, 2, A
1, 10, 3, C
1, 10, 4, B
1, 10, 5, E
1, 10, 6, D
1, 10, 7, B
1, 10, 8, A
1, 10, 9, E
1, 10, 10, D
2, 10, 1, D
2, 10, 2, C
2, 10, 3, C
2, 10, 4, A
2, 10, 5, E
2, 10, 6, B
2, 10, 7, E
2, 10, 8, D
2, 10, 9, B
2, 10, 10, A
```

CREATE A RANDOMISATION LIST

Use this tool to create a blocked randomisation list for your trial. The generated lists are suitable for use with our simple randomisation serv

Create a list

Seed:
153117420413950

Treatment groups
A, B, C, D, E

Block sizes
10, 10

List length
11

Strata (optional)
e.g. Age group: under 30, 30 or over +

name: category 1, category 2, ...

☐ Generate unique randomisation code?

Create list | Download as CSV

How to cite this tool

Sealed Envelope Ltd. 2015. Create a blocked randomisation list. [Online] Available from: https://www.sealedenvelope.com/simple-randomiser/v1/lists [Accessed 21 Jul 2015].

Your list

Seed: 153117420413950

Block sizes: 10, 10

Actual list length: 20

block identifier, block size, sequence within block, treatment

```
1, 10, 1, B
1, 10, 2, A
1, 10, 3, B
1, 10, 4, E
1, 10, 5, A
1, 10, 6, E
1, 10, 7, C
1, 10, 8, D
1, 10, 9, C
1, 10, 10, D
2, 10, 1, A
2, 10, 2, D
2, 10, 3, B
2, 10, 4, E
2, 10, 5, C
2, 10, 6, E
2, 10, 7, A
2, 10, 8, C
2, 10, 9, B
2, 10, 10, D
```

Appendix H Standard Histamine ELISA Procedure

Histamine EIA Kit
Product Number: EA31
Store at 4°C
FOR RESEARCH USE ONLY
Document Control Number: EA31.081006
Page 1 of 6

Enzyme Immunoassay for
Histamine (Life Science Format)
For Research Use Only

INTRODUCTION

Histamine is a heterocyclic primary amine derived from the decarboxylation of the amino acid histidine. It is a mediator of inflammation closely associated with the initial phase of immediate hypersensitivity response (anaphylaxis). Histamine is synthesized by the enzyme histidine decarboxylase and is present in most cells, but typically stored in metachromatic granules of basophils and mast cells (granulocytes) (1). Histamine in the intracellular granules is bound to proteins and inactive until it is released from the cells.

During anaphylactic response, an antigen-IgG antibody complex formed in vivo activates the complement cascade and cleaves bioactive complement associated peptides called anaphylatoxins. Among anaphylatoxins, C3a, which is derived from the complement component C3, and C5a derived from C5 releases histamine from mast cells (2). In IgE-mediated immediate hypersensitivity response, an IgE antibody is produced by B lymphocytes upon stimulation by an allergen and under the control of IL-13 and IL-4. Such IgE antibodies are secreted from B lymphocytes and bound to a high affinity receptor (FceR I High binding IgE receptor) on mast cells in the tissue or on basophilic leukocytes in the peripheral blood leukocytes (3). When IgE bearing mast cells or basophils encounter an allergen to which the IgE antibody was directed, the allergen (antigen) binds to the cell-bound IgE and agglutinates on the surface of these cells. This event triggers the release of granules into the blood stream. Degranulation of the mast cell involves the release of mediators such as leukotriene C_4, D_4, B_4, thromboxane A_2, PGD_2, Platelet Activating Factor, histamine, heparin, tryptase, kallikrein, ECF-A, IL-8 and other cytokines. Histamine released from mast cell acts on smooth muscle and blood vessels, causing bronchoconstriction, vasodilation and increased vascular permeability (erythema)(4).

Histamine exerts its biological effects through three distinct receptors on various tissues and cells: H1, H2 and H3. Among these histamine receptors, H2 receptor is best recognized as associated with secretion of acid in the stomach leading to peptic ulcer. Thus, an H2 receptor antagonist is used for treatment of peptic ulcers (5).

Tissue bound mast cells (such as in the skin, nasal mucosa) respond to incoming allergen and manifest as erythema (e.g. in skin test) and wheezing response. In various research areas, it is important to study in vitro histamine release from peripheral blood basophils. When whole blood from a sensitized animal is exposed to a given allergen, basophils respond to the allergen by releasing histamine into the incubation mixture. Using a whole blood sample, one can assess ex vivo response to a sensitizing antigen as a function of histamine released from the basophils (6).

Other than histamine being an important mediator of immediate hypersensitivity, histamine is found in decaying fish meat, especially of scombroid fish such as tuna. For this reason histamine is called "Scombrotoxin"(7). Histamine is also found in wine (8) and cheese (9).

PRINCIPLES OF PROCEDURE

This Histamine kit is a competitive direct ELISA (Enzyme-Linked Immunosorbent Assay) in a microwell format that allows users to obtain exact concentrations of histamine in nanograms per milliliter.

The microwells in this assay kit are pre-coated with a monoclonal antibody to histamine. The sample or standard solution is first added to the antibody-coated microplate. Next, the enzyme conjugate is added and the mixture is shaken and incubated at room temperature for 45 minutes. During the incubation, unbound (free) histamine in the samples or standards is allowed to compete with enzyme (horseradish peroxidase: HRP)-labeled histamine (conjugate) for antibody binding sites. The plate is then washed, removing all the unbound material. The bound enzyme conjugate is detected by the addition of a one-component substrate that generates color by horseradish peroxidase. An optimal color is generated after 30 minutes. A microplate reader is then used to take an absorbance reading at 650 nm.

Quantitative test results may be obtained by measuring and comparing the absorbance reading of the sample wells against the standard curve with the use of a log-logit curve-fitting model. The extent of color development is inversely proportional to the amount of histamine in the sample or standard. For example, the absence of histamine in the sample will result in a bright blue color, whereas the presence of histamine will result in decreased or no color development.

INTENDED USE

This kit is designed for in vitro quantification of histamine in various biological fluids by competitive direct enzyme-linked immunosorbent assay (CD-ELISA). This kit is intended for use in investigative research only and not for human clinical diagnostic use.

This Histamine kit (Life Science Format) should not be used for determining histamine levels in scombroid fish.

Intended User: Researchers in biomedical fields.

MATERIALS PROVIDED

Component	Description	Volume	Storage
PBS Buffer	Buffer used to dilute extracted and non-extracted samples.	1 pouch	4°C
25x Wash Buffer	Buffer used to wash the plate prior to color development.	30 mL	4°C
TMB Substrate	TMB substrate used for color development.	20 mL	4°C
Histamine Conjugate	Histamine horseradish peroxidase conjugate (ready-to-use).	6 mL	4°C
Histamine Standards	6 vials of Histamine standard solutions at 0, 2.5, 5, 10, 20, 50 ng/mL.	500 μL	4°C
Coated Plate	96-well microplate coated with monoclonal anti-Histamine antibody.	1 plate	4°C

MATERIALS NEEDED BUT NOT PROVIDED

1. Microplate reader with a 450 nm or 650 nm filter
2. Adjustable micropipettes (10 – 1000 μL) and tips
3. Deionized water
4. Plate cover or plastic film
5. 1 N HCl (optional)

STORAGE

1. Store the components of this kit at the temperatures specified on the labels.
2. Unopened reagents are stable until the indicated kit expiration date.
3. Desiccant bag must remain in foil pouch with unused strips. Keep pouch sealed when not in use to maintain a dry environment. Remove excess air before sealing.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.

PROCEDURAL NOTES

1. Glassware should not be used for extraction purposes. As Histamine may adhere to glass, using glassware may affect test results.
2. The kit should be brought to room temperature prior to use.
3. To minimize errors in absorbance measurements due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel prior to inserting into the plate reader.

SAMPLE PREPARATION

The amount of Histamine in your samples may differ. It is recommended that you conduct a preliminary test to determine the optimum dilution for your samples. Typically, tissue culture media, tissue extracts, cell and cell-free extracts can be used.

The samples to be analyzed with this kit must have a pH of 6-8. Excessively acidic or alkaline samples should be adjusted using 0.1 N NaOH or HCl.

REAGENT PREPARATION

1. **PBS Buffer:** Add the contents of the pouch to 1.0 L of deionized water prior to use.
2. **25x Wash Buffer:** Add 30 mL to 720 mL of deionized water prior to use.

STANDARD CURVE PREPARATION

The Histamine Standards are provided ready-to-use in the following concentrations: 0, 2.5, 5, 10, 20, and 50 ng/mL.

ASSAY PROCEDURE

1. Add 50 μL of Standards or Samples (may require diluting) to the corresponding wells on the microplate in duplicate. See **Scheme I** for a sample plate layout.
2. Add 50 μL of Histamine-HRP Conjugate to each well. Incubate at room temperature for 45 minutes.
3. Wash the plate three times with 300 μL of diluted Wash Buffer per well. Wash 5 times if using an automated plate washer.
4. Add 150 μL of TMB Substrate to each well. Incubate at room temperature for 30 minutes.
5. Read the plate at 650 nm.

   Alternately, the color reaction can be stopped after 15-20 minutes by adding 50 μL of 1 N HCl and read at 450 nm.

   NOTE: If accounting for substrate background, use 2 wells as blanks (BLK) with only 150 μL TMB Substrate in the wells. Subtract the average of these absorbance values from the absorbance values of the wells being assayed.

Scheme I: Sample Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S_0	S_0	U_3	U_3	U_{11}	U_{11}	U_{19}	U_{19}	U_{27}	U_{27}	U_{35}	U_{35}
B	S_1	S_1	U_4	U_4	U_{12}	U_{12}	U_{20}	U_{20}	U_{28}	U_{28}	U_{36}	U_{36}
C	S_2	S_2	U_5	U_5	U_{13}	U_{13}	U_{21}	U_{21}	U_{29}	U_{29}	U_{37}	U_{37}
D	S_3	S_3	U_6	U_6	U_{14}	U_{14}	U_{22}	U_{22}	U_{30}	U_{30}	U_{38}	U_{38}
E	S_4	S_4	U_7	U_7	U_{15}	U_{15}	U_{23}	U_{23}	U_{31}	U_{31}	U_{39}	U_{39}
F	S_5	S_5	U_8	U_8	U_{16}	U_{16}	U_{24}	U_{24}	U_{32}	U_{32}	U_{40}	U_{40}
G	U_1	U_1	U_9	U_9	U_{17}	U_{17}	U_{25}	U_{25}	U_{33}	U_{33}	U_{41}	U_{41}
H	U_2	U_2	U_{10}	U_{10}	U_{18}	U_{18}	U_{26}	U_{26}	U_{34}	U_{34}	BLK	BLK

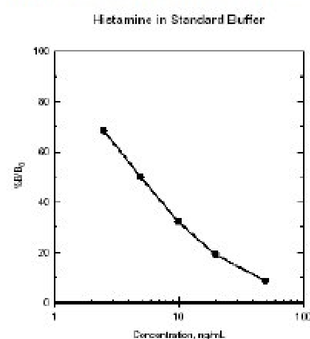
CALCULATIONS

1. After the substrate background has been subtracted from all absorbance values, average all of your duplicate well absorbance values.
2. The average of your two S_0 values is now your B_0 value. (S_1 now becomes B_1, etc.)
3. Next, find the percent of maximal binding (%B/B_0 value). To do this, divide the averages of each standard absorbance value (now known as B_1 through B_7) by the B_0 absorbance value and multiply by 100 to achieve percentages. Transform the ratio into a logit function using the following equation:

$$Logit = \ln\left(\frac{(\%B / \%B_0)}{100 - (\%B / \%B_0)}\right)$$

4. Graph your standard curve by plotting the logit for each standard concentration on the y-axis against the log of the standard concentrations on the **x**-axis. Draw a curve by using a curve-fitting routine (i.e. 4-parameter or linear regression). A log-logit curve is recommended for this assay.
5. Find the percent of maximal binding and the logit function of each sample.
6. Using the standard curve, the concentration of each sample can be determined by comparing the %B/B_0 of each sample to the corresponding concentration of histamine standard.
7. If the samples were diluted, the concentration determined from the standard curve must be multiplied by the dilution factor.
8. To convert mass based concentration of Histamine into molarity the following equation can be used: ng/mL **x** 9.005 = nmole/L (nM). E.g. 1.0 ng/mL = 9 nM.

Figure 1: Typical Standard Curve



P.O. Box 522 | Oxford, MI 48371 | tel:248.852.8815 | fax:248.852.4466 | www.oxfordbiomed.com | info@oxfordbiomed.com

Cadaverine	0.003%	Trimethylamine	<0.01%
Tyramine	<0.01%		

PERFORMANCE CHARACTERISTICS

Limit of quantification: 2.5 ng/mL.
Range of quantification: 2.5 - 50.0 ng/mL.
Intra-assay Precision: < = 10%
Inter-assay Precision: < = 10%

DISCUSSION

Histamine release reactions in vivo and in vitro are investigated by various researchers. Some investigators use HPLC with fluorimetric detector, radioimmunoassay, and enzyme immunoassay to determine histamine contents of biological fluids. One attractive feature of studying histamine release using ELISA is that one can use whole blood to activate cells with stimulants and measure histamine in the same reaction mixture. The normal plasma level of histamine is less than 1 ng/mL, and 3-7 ng/mL is found in animals or patients with allergic response. Histamine contents of whole blood from humans are between 20 to 200 ng/mL. In clinical situations, arterial hypotension is observed in patients whose plasma histamine reached 6-8 ng/mL, bronchospasm at 7-12 ng/mL. If plasma histamine exceeds 100 ng/mL, it is lethal. Animal and fish tissues contain 1-100 μg/g tissue.

Ferrer, *et. al.* (10) showed that histamine can be released from whole blood of patients in response to antigenic response. Histamine can also be released from mouse mast cell line. Histamine release is modulated by the addition of tetracosahexaenoic acid in the culture media (11). Eugenol (a major component of clove) reduced Compound 48/80-induced systemic anaphylaxis in rat. Eugenol also inhibited cutaneous anaphylaxis in response to anti-DNP-IgE and reduced serum histamine levels (12). Demoly, *et. al.* used histamine release to predict allergic response to therapeutic drugs (13). In this paper, drug specific histamine release from whole blood was compared with the total histamine released by freeze-thawing the cells. The total histamine release by freeze-thawing was 61 ng/mL (median value).

REFERENCES

1. Stites, D.P., *et. al.*; (1987) "Basic and Clinical Immunology, a Lange Medical Book" (Sixth Edition), Appleton & Lange, Chapter 15, pp 208-209
2. Roitt, I.; (1988) "Essential Immunology", Blackwell Scientific Publications, pp 8-11
3. Wiley, J., *et. al.*; (1999) Biochemical Pathways: An atlas of biochemistry and molecular biology, p 246
4. Roitt, I., *et. al.*; (1998) "Immunology" (Fifth Edition), Mosby, pp 68-69
5. Stites, D.P., *et. al.*; (1987) "Basic and Clinical Immunology, a Lange Medical Book" (Sixth Edition), Appleton & Lange, Chapter 24, p 441
6. Roitt, I., *et. al.*; (1998) "Immunology" (Fifth Edition), Mosby, Chapter 23, pp 301-317
7. Scoging, A.; (1998) *Commun Dis Public Health* **1**:204-205
8. Buteau, C. and Duitschaever, C.L.; (1984) *J Chromatography* **284**:201-210
9. Vale, S.R., *et. al.*; (1997) *J AOAC International* **80(5)**:1006-1012
10. Ferrer, M., *et. al.*; (1998) *Clin Exp Allergy* **28**(6):709-714
11. Ishihara, K., *et. al.*; (1998) *Lipids* **33(11)**:1107-1114
12. Kim, H.M., *et. al.*; (1997) *Pharmacol Res* **36**(6):475-480
13. Demoly, P., *et. al.*; (1999) *Allergy* **54**:500-506

P.O. Box 522 | Oxford, MI 48371 | tel:248.852.8815 | fax:248.852.4466 | www.oxfordbiomed.com | info@oxfordbiomed.com

DISCLAIMER

This information is believed to be correct but does not purport to be all-inclusive and shall be used only as a guide. Oxford Biomedical Research, Inc. shall not be held liable for any damage resulting from handling or from contact with the above product. See catalog for additional terms and conditions of sale.

ORDERING INFORMATION

For additional kits or a complete catalog please call 800-692-4633.

TECHNICAL SUPPORT

If you need technical information or assistance with assay procedures, call our Technical Support Department at 800-692-4633 or 248-852-8815. Our staff will be happy to answer your questions about this or any other product in the Oxford Biomedical line.

GUARANTEE AND LIMITATION OF REMEDY

Oxford Biomedical Research, Inc. makes no guarantee of any kind, expressed or implied, which extends beyond the description of the materials in this kit, except that these materials and this kit will meet our specifications at the time of delivery. Buyer's remedy and Oxford Biomedical Research, Inc.'s sole liability hereunder is limited to, at Oxford Biomedical Research, Inc.'s option, refund of the purchase price of, or the replacement of, material that does not meet our specification. By acceptance of our products, Buyer indemnifies and holds Oxford Biomedical Research, Inc. harmless against, assumes all liability for the consequence of its use or misuse by the Buyer, its employees, or others. Said refund or replacement is conditioned on Buyer notifying Oxford Biomedical Research, Inc. within thirty (30) days of the receipt of product. Failure of Buyer to give said notice within thirty (30) days of receipt of product shall constitute a waiver by the Buyer of all claims hereunder with respect to said material(s).

Oxford Biomedical Research, Inc.
P.O. Box 522
Oxford, MI 48371 U.S.A.

Orders: 800-692-4633
Technical Service: 248-852-8815
Fax: 248-852-4466
E-mail: info@oxfordbiomed.com

Made in the U.S.A.

List of References

Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U, van Kerrebroeck P, Victor A, Wein A; Standardisation Sub-committee of the International Continence Society (2002) The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-committee of the International Continence Society. *American Journal of Obstetrics & Gynecology* 187(1): 116-126

Agis H, Krauth MT, Böhm A, Mosberger I, Müllauer L, Simonitsch-Klupp I, Walls AF, Horny HP and Valent P (2006) Identification of basogranulin (BB1) as a novel immunohistochemical marker of basophils in normal bone marrow and patients with myeloproliferative disorders. *American Journal of Clinical Pathology* 125(2): 273-281

Ahmed S and Othman NH (2013) Honey as a potential natural anticancer agent: a review of its mechanisms. *Evidence-based Complementary and Alternative Medicine: eCAM* 829070. doi: 10.1155/2013/829070

Aldenborg F, Fall M and Enerbäck L (1986) Proliferation and transepithelial migration of mucosal mast cells in interstitial cystitis. *Immunology* 58(3): 411-416

Al-Waili NS and Saloom KY (1999) Effects of topical honey on post-operative wound infections due to gram positive and gram negative bacteria following caesarean sections and hysterectomies. *European Journal of Medical Research* 4(3): 126-130

Al-Waili NS, Salom K and Al-Ghamdi AA (2011) Honey for wound healing, ulcers, and burns; data supporting its use in clinical practice. *Scientific World Journal* 5(11): 766-787

Al-Zahrani AA and Gajewski JB (2011) Long-term efficacy and tolerability of pentosan polysulfate sodium in the treatment of bladder pain syndrome. *Canadian Urological Association Journal* 5(2): 113-118

Anand P, Singh B, Jaggi AS and Singh N (2012) Mast cells: an expanding pathophysiological role from allergy to other disorders. *Naunyn-Schmiedeberg's Archives of Pharmacology* 385(7): 657-670

Apperley JF (2015) Chronic myeloid leukaemia. *Lancet* 385(9976): 1447-1459
Arend WP (2001) Physiology of cytokine pathways in rheumatoid arthritis. *Arthritis & Rheumatology* 45(1): 101-106

Arinobu Y, Iwasaki H and Akashi K (2009) Origin of basophils and mast cells. *Allergology International* 58(1): 21-28

Arock M, Schneider E, Boissan M, Tricottet V and Dy M (2002) Differentiation of human basophils: an overview of recent advances and pending questions. *Journal of Leukocyte Biology* 71(4): 557-564

Asklin B and Cassuto J (1989) Intravesical lidocaine in severe interstitial cystitis. Case report. *Scandinavian Journal of Urology and Nephrology* 23(4), 311-312

Azimi E, Reddy VB, Shade KTC, Anthony RM, Talbot S, Pereira PJS and Lerner EA (2016) Dual action of neurokinin-1 antagonists on Mas-related GPCRs. *JCI insight; American Society for Clinical Investigation* 1(16), e89362. doi: 10.1172/jci.insight.89362

Bang LM, Buntting C and Molan P (2003) The Effect of Dilution on the Rate of Hydrogen Peroxide Production in Honey and Its Implications for Wound Healing. *The Journal of Alternative and Complementary Medicine* 9(2): 267-273

Bassaly R, Downes K and Hart S (2011) Dietary Consumption Triggers in Interstitial Cystitis/Bladder Pain Syndrome Patients. *Female Pelvic Medicine & Reconstructive Surgery* 17(1): 36-39

Bassi P, Costantini E, Foley S and Palea S (2011) Glycosaminoglycan Therapy for Bladder Diseases: Emerging New Treatments. *European Urology Supplements* 10(6): 451-459

Beers RF (1964) Acridine Orange binding by Micrococcus lysodeikticus. *Journal of Bacteriology* 88(5): 1249-1256

Benhanifia MB, Boukraâ L, Hammoudi SM, Sulaiman SA and Manivannan L (2011) Recent patents on topical application of honey in wound and burn management. *Recent Patents on Inflammation & Allergy Drug Discovery* 5(1): 81- 86

Berry SH, Elliott MN, Suttorp M, Bogart LM, Stoto MA, Eggers P, Nyberg L and Clemens JQ (2011) Prevalence of Symptoms of Bladder Pain Syndrome/Interstitial Cystitis Among Adult Females in the United States. *Journal of Urology* 186(2): 540–544

Birch B, Murray T, Cooper A and Lwaleed BA (2011) The effect of honey on mast- cell degranulation: a possible role in painful bladder syndrome/interstitial cystitis (PBS/IC). *Journal of Urology* 185: 381-382

Birder L and Andersson KE (2013) Urothelial Signaling. *Physiological Reviews* 93(2): 653-680

Bjorling DE, Jerde TJ, Zine MJ, Busser BW, Saban MR and Saban R (1999) Mast cells mediate the severity of experimental cystitis in mice. *Journal of Urology* 162(1): 231-236

Borel E, Mayenon M., Kaiser K, Picot S and Peyron F (1998) Fluorogenic detection of viable Toxoplasma gondii. *Parasite* 5(4): 371-373

Bouchelouche K, Alvarez S, Horn T, Nordling J and Bouchelouche P (2006) Human detrusor smooth muscle cells release interleukin-6, interleukin-8, and RANTES in response to proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha. *Urology* 67(1): 214-219

Bourque JP (1951) Surgical management of the painful bladder. *Journal of Urology* 65(1): 25-35

Briggaman RA, Schechter NM, Fraki J and Lazarus GS (1984) Degradation of the epidermal-dermal junction by proteolytic enzymes from human skin and human polymorphonuclear leukocytes. *The Journal of Experimental Medicine* 160(4): 1027-1042

Cancer Research UK, http://www.cancerresearchuk.org/health- professional/cancer-statistics#heading-Zero, Accessed June 2017

Caughey GH (2007) Mast cell tryptases and chymases in inflammation and host defence. *Immunological Reviews* 217: 141-154

Cervigni M (2015) ‘Interstitial cystitis/bladder pain syndrome and glycosaminoglycans replacement therapy.’, Translational andrology and urology. AME Publications, 4(6): 638–642

Cervigni M, Natale F, Nasta L and Mako A (2012) Intravesical hyaluronic acid and chondroitin sulphate for bladder pain syndrome/interstitial cystitis: long-term treatment results. *International Urogynecology Journal* 23(9): 1187-1192

References

Cervigni M, Natale F, Nasta L, Padoa A, Voi RL and Porru, D. (2008) 'A combined intravesical therapy with hyaluronic acid and chondroitin for refractory painful bladder syndrome/interstitial cystitis. *International Urogynecology Journal* 19(7): 943–947

Christmas TJ, Rode J, Chapple CR, Milroy EJ and Turner-Warwick RT (1990) Nerve fibre proliferation in interstitial cystitis. *Virchows Archive A: Pathological Anatomy and Histology* 416(5): 447-451

Cima K, Vogelsinger H and Kähler CM (2010) Sensory neuropeptides are potent chemoattractants for human basophils in vitro. *Regulatory Peptides* 160(1-3): 42- 48

Clemens JQ, Markossian T and Calhoun EA (2009) Comparison of Economic Impact of Chronic Prostatitis/Chronic Pelvic Pain Syndrome and Interstitial Cystitis/Painful Bladder Syndrome. *Urology* 73(4): 743-746

Codeluppi L, Spagnolo P, Tondelli M, Malaguti MC and Mandrioli J (2014) Recurrent cerebrospinal fluid basophilia in neurosarcoidosis. *Acta Neurologica Belgica*: 1-3

Cooper RA, Molan PC, and Harding KG (1999) Antibacterial activity of honey against strains of Staphylococcus aureus from infected wounds. *Journal of the Royal Society of* Medicine 92(6): 283-285

Crome G (2012) 'Basophils and Mast Cell Subtypes in Interstitial Cystitis'. Undergraduate. University of Southampton

Daha LK, Riedl CR, Lazar D, Hohlbrugger G and Pflüger H (2005) Do Cystometric Findings Predict the Results of Intravesical Hyaluronic Acid in Women with Interstitial Cystitis? *European Urology* 47(3): pp. 393-397

Dasgupta, J. and Tincello, D. G. (2009) Interstitial cystitis/bladder pain syndrome: An update. *Maturitas* 64(4): 212-217

Diaconu NC, Kaminska R, Naukkarinen A, Harvima RJ and Harvima IT (2007) The increase in tryptase- and chymase-positive mast cells is associated with partial inactivation of chymase and increase in protease inhibitors in basal cell carcinoma. *Journal of the European Academy of Dermatology* 21(7): 908-915

Doyle C, Palmer J, Munglani R and Hunt S (1997) Molecular consequences of noxious stimulation. In: Borsook D., editor. (ed.), *Molecular Neurobiology of Pain, Progress in Pain Research and management*, Vol. 9 Seattle, WA: IASP Press, pp. 145–170

Downie WW, Leatham PA, Rhind VM, Wright V, Branco JA and Anderson JA (1978) Studies with pain rating scales. *Annals of the rheumatic diseases* 37(4): 378-381

Dubin AE and Patapoutian A (2010) Nociceptors: the sensors of the pain pathway. *The Journal of Clinical Investigation* 120(11):3760–3772

Dvorak AM and Morgan ES (1998) Ribonuclease-gold labels chondroitin sulphate in guinea pig basophil granules. *The Histochemical Journal* 30(8): 603-608

Eberle JU and Voehringer D (2016) Role of basophils in protective immunity to parasitic infections. *Seminars in Immunopathology* 38(5): 605–613

Ebihara N, Funaki T, Murakami A, Takai S and Miyazaki M (2005) Mast Cell Chymase Decreases the Barrier Function and Inhibits the Migration of Corneal Epithelial Cells. *Current Eye Research* 30(12): 1061–1069

Ehrlich P (1878) Beitrage zur Theorie und Praxis der histologischen Farbung [Thesis] Leipzig University

Engles CD, Hauser PJ, Abdullah SN, Culkin DJ and Hurst RE (2012) Intravesical chondroitin sulfate inhibits recruitment of inflammatory cells in an acute acid damage “leaky bladder” model of cystitis. *Urology* 79(2): 483.e13-17

Erickson DR, Sheykhnazari M, Ordille S and Bhavanandan VP (1998) Increased urinary hyaluronic acid and interstitial cystitis. *Journal of Urology* 160(4): 1282-1284

Evans RJ (2002) Treatment approaches for interstitial cystitis: multimodality therapy. *Reviews in Urology* 4(1): S16-20

Fall M, Oberpenning F and Peeker R (2008) Treatment of bladder pain syndrome/interstitial cystitis 2008: can we make evidence-based decisions? *European Urology* 54(1): 65-75

Favretto ME, Wallbrecher R, Schmidt S, van de Putte R and Brock R (2014) Glycosaminoglycans in the cellular uptake of drug delivery vectors - bystanders or active players? *Journal of Controlled Release* 180: 81-90

References

Fernandez-Cabezudo MJ, El-Kharrag R, Torab F, Bashir G, George JA, El-Taji H and Al-Ramadi BK (2013) Intravenous Administration of Manuka Honey Inhibits Tumor Growth and Improves Host Survival When Used in Combination with Chemotherapy in a Melanoma Mouse Model. *PLoS ONE Edited by A. Facchiano* 8(2): p. e55993. doi: 10.1371/journal.pone.0055993

Fineman SM (2014) Optimal treatment of anaphylaxis: antihistamines versus epinephrine. *Postgraduate Medicine* 126(4): 73-81

Fischer KD and Agrawal DK (2013) Hematopoietic Stem and Progenitor Cells in Inflammation and Allergy. *Frontiers in Immunology* 4: 428

French LM and Bhambore N (2011) Interstitial Cystitis/Painful Bladder Syndrome. *American Family Physician* 83(10): 1175-1181

Friedlander JI, Shorter B and Moldwin RM (2012) Diet and its role in interstitial cystitis/bladder pain syndrome (IC/BPS) and comorbid conditions. *British Journal of Urology International* 109(11): 1584-1591

Gaik A, Brzezińska-Błaszczyk E, Jarzebska M, Prochowski M, and Karczewska M (1992) Reactivity of pulmonary mast cells and peripheral blood basophils in patients with sarcoidosis and bronchial asthma. *Polish Pneumonology and Allergology* 60(1-2): 23-27

Galen Limited (2010) Management of PBS/IC (Painful Bladder Syndrome/Interstitial Cystitis) [PowerPoint presentation]. Retrieved from www.uracyst.co.uk [Accessed on 11th May 2015]

Gepan Instill (n.d.) Medical Conditions [Image]. Retrieved from http://gepan-instill.co.uk/medical-conditions/ [Accessed on 27th May 2015]

Gethin GT, Cowman S and Conroy RM (2008) The impact of Manuka honey dressings on the surface pH of chronic wounds. *International Wound Journal* 5(2): 185-194

Giberti C, Gallo, F, Cortese P and Schenone M (2013) Combined intravesical sodium hyaluronate/chondroitin sulfate therapy for interstitial cystitis/bladder pain syndrome: a prospective study. *Therapeutic Advances in Urology* 5(4): 175-179

Gomelsky A and Dmochowski RR (2012) GAG Layer Replenishment Therapy for Recurrent Infectious Bladder Dysfunction. Current Bladder Dysfunction Reports. *Current Science Inc*. 7(2): 113-119

Grimbaldeston MA (2015) Mast cell-MrgprB2: sensing secretagogues or a means to overreact? *Immunology and Cell Biology* 93(3): 221-223

Gross SD. *A Practical Treatise on the Diseases, Injuries, and Malformations of the Urinary Bladder, the Prostate Gland and the Urethra*, 3rd edition. (Revised and edited by Samuel W. Gross). Henry C. Lea, Philadelphia, PA, 1876

Gülpinar Ö, Kayış A, Süer E, Gökçe MI, Güçlü AG and Arikan N (2014) Clinical comparision of intravesical hyaluronic acid and hyaluronic acid-chondroitin sulphate therapy for patients with bladder pain syndrome/interstitial cystitis. *Canadian Urological Association Journal* 8(9-10): 610

Gürpinar T, Wong HY and Griffith DP (1996) Electromotive administration of intravesical lidocaine in patients with interstitial cystitis. *Journal of endourology / Endourological Society* 10(5): 443 - 447

Hall C and Moldwin R (1995) Interstitial cystitis: An overview and discussion of treatment options. *Primary Care Update for OB/GYNS* 2(5): 181-187

Hammond EN, Duster M, Musuuza JS and Safdar N (2016) Effect of United States buckwheat honey on antibiotic-resistant hospital acquired pathogens. *Pan African Medical Journal* 25: 212

Hand JR (1949) Interstitial cystitis: Report of 223 cases (204 women and 19 men). *Journal of Urology* 61: 291

Hanno P, Lin A, Nordling J, Nyberg L, van Ophoven A, Ueda T and Wein A (2010) Bladder Pain Syndrome Committee of the International Consultation on Incontinence. *Neurourology and Urodynamics* 29(1): 191-198

Hanno PM (2002) Interstitial cystitis-epidemiology, diagnostic criteria, clinical markers. *Reviews in Urology* 4(1): S3-8

Hanno PM (2007) Painful Bladder Syndrome/Inerstitial Cystitis and Related Disorders. Retrieved from: http://med-stud.narod.ru/med/urology/pbs.html [Accessed on 11th May 2015]

Harvima IT, Naukkarinen A, Harvima RJ and Horsmanheimo M (1989) Enzyme- and immunohistochemical localization of mast cell tryptase in psoriatic skin. *Archives of Dermatological Research* 281(6): 387-391

Hauser PJ, Buethe DA, Califano J, Sofinowski TM, Culkin DJ and Hurst RE (2009) Restoring barrier function to acid damaged bladder by intravesical chondroitin sulfate. *Journal of Urology* 182(5): 2477-2482

Hertle L and van Ophoven A (2010) Long-term results of amitriptyline treatment for interstitial cystitis. *Aktuelle Urologie* 41(Supplement 1): 61-65

Hiebert LM, Han J and Mandal AK (2014) Glycosaminoglycans, hyperglycemia, and disease. *Antioxidants & Redox Signaling* 21(7): 1032-1043
Ho N, Koziol J and Parsons CL (1997) Epidemiology of interstitial cystitis, in: G Sant (ed) *Interstitial cystitis*. Philadelphia: Lippincott-Raven 9-16

Holubec H, Payne CM, Bernstein H, Dvorakova K, Bernstein C, Waltmire CN, Warneke JA and Garewal H (2005) Assessment of Apoptosis by Immunohistochemical Markers Compared to Cellular Morphology in Ex Vivo-stressed Colonic Mucosa. *Journal of Histochemistry & Cytochemistry* 53(2): 229-235

Hotamisligil GS (2006) Inflammation and metabolic disorders. *Nature* 444(7121): 860–867

Hung MJ, Chen YT, Shen PS, Hsu ST, Chen GD and Ho ES (2012) Risk factors that affect the treatment of interstitial cystitis using intravesical therapy with a dimethyl sulfoxide cocktail. *International Urogynecology Journal* 23(11): 1533- 1539

Hunner GL (1918) A rare type of bladder ulcer: Further notes, with a report of eighteen cases. *Journal of the American Medical Association* 70(4): 203-212

Hurst RE (1994) Structure, function, and pathology of proteoglycans and glycosaminoglycans in the urinary tract. *World Journal of Urology* 12(1): 3-10

Hurst RE, Rhodes SW, Adamson PB, Parsons CL and Roy JB (1987) Functional and structural characteristics of the glycosaminoglycans of the bladder luminal surface. *Journal of Urology* 138(2): 433-437

Huttunen M and Harvima IT (2005) Mast cell tryptase and chymase in chronic leg ulcers: chymase is potentially destructive to epithelium and is controlled by proteinase inhibitors. *British Journal of Dermatology* 152(6): 1149-1160

Huttunen M, Aalto ML, Harvima RJ, Horsmanheimo M and Harvima IT (2000) Alterations in mast cells showing tryptase and chymase activity in epithelializating and chronic wounds. *Experimental Dermatology* 9(4): 258-265

Iavazzo C, Athanasiou S, Pitsouni E and Falagas ME (2007) Hyaluronic Acid: An Effective Alternative Treatment of Interstitial Cystitis, Recurrent Urinary Tract Infections, and Hemorrhagic Cystitis? *European Urology* 51(6): 1534-1541

Idoate MA, Echeveste J, Gil P, Sanz ML and Ferrer M (2013) Expression of the basophil-specific antibodies 2D7 and BB1 in patients with cutaneous Mastocytosis. *Journal of Investigational Allergology and Clinical Immunology* 23(6): 392-397

Ikeda Y, Zabbarova IV, Birder LA, de Groat WC, McCarthy CJ, Hanna-Mitchell AT and Kanai AJ (2012) Botulinum neurotoxin serotype a suppresses neurotransmitter release from afferent as well as efferent nerves in the urinary bladder. European Urology 62(6): 1157-1164

Imran M, Hussain MB and Baig M (2015) A Randomized, Controlled Clinical Trial of Honey-Impregnated Dressing for Treating Diabetic Foot Ulcer. *Journal of the College of Physicians and Surgeons-Pakistan: JCPSP* 25(10): 721-725

Interstitial Cystitis Association. 2015. *4 to 12 Million May Have IC* [ONLINE] Available at: https://www.ichelp.org. [Accessed 29 June 2017]

Interstitial Cystitis Association. 2016. *Interstitial Cystitis Association* [ONLINE] Available at: https://www.ichelp.org/. [Accessed 29 June 2017]

Iozzo RV and Schaefer L (2015) Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. *Matrix Biology* 42: 11–55

Irani AM, Huang C, Xia HZ, Kepley C, Nafie A, Fouda ED, Craig S, Zweiman B and Schwartz LB (1998) Immunohistochemical detection of human basophils in late-phase skin reactions. *Journal of Allergy and Clinical Immunology* 101(3): 354-362

Irish J, Blair S and Carter DA (2011) The antibacterial activity of honey derived from Australian flora. *PLoS ONE* 6(3): e18229

Ito Y, Satoh T, Takayama K, Miyagishi C, Walls AFand Yokozeki H. (2011) Basophil recruitment and activation in inflammatory skin diseases. *Allergy* 66(8): 1107-1113

Janssen DAW, van Wijk XMR, Jansen KCFJ, van Kuppevelt TH, Heesakkers JPFA and Schalken JA (2013) The Distribution and Function of Chondroitin Sulfate and Other Sulfated Glycosaminoglycans in the Human Bladder and Their Contribution to the Protective Bladder Barrier. *Journal of Urology* 189(1): 336-342

Jiang YH, Liu HT and Kuo HC (2014) Decrease of Urinary Nerve Growth Factor but Not Brain-Derived Neurotrophic Factor in Patients with Interstitial Cystitis/Bladder Pain Syndrome Treated with Hyaluronic Acid. *PLoS ONE. Edited by J. J. Barchi* 9(3): p. e91609. doi: 10.1371/journal.pone.0091609

Kamaratos AV, Tzirogiannis KN, Iraklianou SA, Panoutsopoulos GI, Kanellos IE and Melidonis AI (2014) Manuka honey-impregnated dressings in the treatment of neuropathic diabetic foot ulcers. *International Wound Journal. Blackwell Publishing Ltd* 11(3): 259-263

Kamaruzaman NA, Sulaiman SA, Kaur G and Yahaya B (2014) Inhalation of honey reduces airway inflammation and histopathological changes in a rabbit model of ovalbumin-induced chronic asthma. *BMC Complementary and Alternative Medicine* 14: 176

Kassim M, Achoui M, Mustafa MR, Mohd MA and Yusoff KM (2010) Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. *Nutrition Research* 30(9): 650-659

Kateel R, Adhikari P, Augustine AJ and Ullal S (2016) Topical honey for the treatment of diabetic foot ulcer: A systematic review. *Complementary Therapies in Clinical Practice* 24: 130-133

Keck Medicine of USC. 2016. Kidney Stones. [ONLINE] Available at: http://keckmedicine.adam.com/content.aspx?productId=10&pid=10&gid=000081. [Accessed 29 June 2017]

Kepley CL, McFeeley PJ, Oliver JM and Lipscomb MF (2001) Immunohistochemical detection of human basophils in postmortem cases of fatal asthma. *American Journal of Respiratory and Critical Care Medicine* 164(6): 1053- 1058

Kepley CL, Craig SS and Schwartz LB (1995) Identification and partial characterization of a unique marker for human basophils. *Journal of Immunology* 154(12): 6548-6555

Kikuchi Y and Kaplan AP (2001) Mechanisms of autoimmune activation of basophils in chronic urticaria. *Journal of Allergy and Clinical Immunology* 107(6): 1056-1062

Kikuchi HA, Tsujimura A, Takao T, Yamamoto K, Nakayama J, Miyagawa Y, Nonomura N, Takeyama M and Okuyama A (2009) Increased vascular endothelial growth factor expression in patients with bladder pain syndrome/interstitial cystitis: its association with pain severity and glomerulations. *BJU International* 104(6): 826-831

Kim R, Liu W, Chen X, Kreder K and Luo Y (2011) Intravesical Dimethyl Sulfoxide Inhibits Acute and Chronic Bladder Inflammation in Transgenic Experimental Autoimmune Cystitis Models. *Journal of Biomedicine and Biotechnology* 2011: 937061

Kimura I, Tanizaki Y, Saito K, Takahashi K, Ueda N and Sato S (1975) Appearance of basophils in the sputum of patients with bronchial asthma. *Clinical Allergy* 5(1): 95-98

Kobayashi H, Ishizuka T and Okayama Y (2000) Human mast cells and basophils as sources of cytokines. *Clinical & Experimental Allergy* 30(9): 1205-1212

Kosanovic D, Dahal BK, Wygrecka M, Reiss I, Günther A, Ghofrani HA, Weissmann N, Grimminger F, Seeger W, Schermuly RT and Banat GA (2013) Mast cell chymase: an indispensable instrument in the pathological symphony of idiopathic pulmonary fibrosis? Histology and Histopathology 28(6): 691-699

Lai MC, Kuo YC and Kuo HC (2013) Intravesical hyaluronic acid for interstitial cystitis/painful bladder syndrome: A comparative randomized assessment of different regimens. *International Journal of Urology* 20(2): 203-207

Lassila R. and Jouppila A. (2014) Mast Cell-Derived Heparin Proteoglycans as a Model for a Local Antithrombotic. *Seminars in Thrombosis and Hemostasis* 40(8): 837-844

Lee ES, Lee SW, Lee KW, Kim JM, Kim YH and Kim ME (2013) Effect of transurethral resection with hydrodistention for the treatment of ulcerative interstitial cystitis. *Korean Journal of Urology* 54(10): 682-688

Leppilahti M, Hellström P and Tammela TLJ (2002) Effect of diagnostic hydrodistension and four intravesical hyaluronic acid instillations on bladder ICAM-1 intensity and association of ICAM-1 intensity with clinical response in patients with interstitial cystitis. *Urology* 60(1): 46-51

Lichtenstein LM and Bochner BS (1991) The role of basophils in asthma. *Annals of the New York Academy of Sciences* 629: 48-61

Lucioni A, Bales GT, Lotan TL, McGehee DS, Cook SP and Rapp DE (2008) Botulinum toxin type A inhibits sensory neuropeptide release in rat bladder models of acute injury and chronic inflammation. *British Journal of Urology International* 101(3): 366-370

Lusby PE, Coombes A and Wilkinson JM (2002) Honey: A potent agent for wound healing? *Journal of Wound Ostomy & Continence Nursing* 29(6): 295-300

Lv J, Huang Y, Zhu S, Yang G, Zhang Y, Leng J, Bo J and Liu D (2012) MCP-1- Induced Histamine Release from Mast Cells Is Associated with Development of Interstitial Cystitis/Bladder Pain Syndrome in Rat Models. *Mediators of Inflammation* 358184 (10): 19

Lwaleed BA, Kambara T, Elmutairi A, Murray T, Voegeli D, Fader M, Cooper A and Birch B (2010) Honey inhibits mast cell degranulation: implication for management of cystitis. *ICS/IUGA August* (568)

Lwaleed BA, Kambara T, Birch BR, Lau LC, Voegeli D, Fader M and Cooper AJ (2014) Inhibition of mast cell degranulation by honey in-vitro: potential as a treatment option in inflammatory conditions. *British Journal of Haematology* 165: 25

Maggi CA and Meli A (1988) The sensory-efferent function of capsaicin- sensitive sensory neurons. *General pharmacology* 19(1): 1-43

Maghsoudi E, Salehi F, Khosrowshahi MK, Baghaei M, Nasirzadeh M and Shams R (2011) Comparison between topical honey and mafenide acetate in treatment of burn wounds. *Annals of Burns and Fire Disasters* 30(24): 132-137

Majtan J (2011) Methylglyoxal-a potential risk factor of manuka honey in healing of diabetic ulcers. *Evidence-based complementary and alternative medicine: eCAM* 295494. doi: 10.1093/ecam/neq013

Marrack, J (1934). Nature of Antibodies. *Nature* 133(3356):.292-293

Matos LL, de Trufelli DC, de Matos MGL. and da Silva Pinhal MA (2010) Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomarker insights Libertas Academica* 5: 9-20

Mazzoni A, Young HA, Spitzer JH, Visintin A and Segal DM (2001) Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization. *Journal of Clinical Investigation* 108(12): 1865-1873

McCance KL and Huether SE (2002) *Pathophysiology: The Biologic Basis for Disease in Adults & Children, 4th ed*. Mosby, St. Louis. pp: 1,616

McCary C, Tancowny BP, Catalli A, Grammer LC, Harris KE, Schleimer RP and Kulka M (2010) Substance P downregulates expression of the high affinity IgE receptor (FcεRI) by human mast cells. *Journal of Neuroimmunology* 220(1-2): 17-24

McEuen AR, Buckley MG, Compton SJ and Walls AF (1999) Development and characterization of a monoclonal antibody specific for human basophils and the identification of a unique secretory product of basophil activation. *Laboratory Investigation; A journal of technical methods and pathology* 79(1): 27-38

McNeil BD, Pundir P, Meeker S, Han L, Undem BJ, Kulka M and Dong X (2015) Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature* 519(7542): 237-241

Meijlink JM (2014) Interstitial cystitis and the painful bladder: A brief history of nomenclature, definitions and criteria. *International Journal of Urology* 21: 4-12

Melchior D, Packer CS, Johnson TC and Kaefer M (2003) Dimethyl Sulfoxide: Does It Change the Functional Properties of the Bladder Wall? *Journal of Urology* 170(1): 253-258

Merriam Webster. 2017. Merriam Webster Dictionary. [ONLINE] Available at: https://www.merriam-webster.com/dictionary/bias. [Accessed 29 June 2017]

Messing EM and Stamey TA (1978) Interstitial cystitis: early diagnosis, pathology, and treatment. *Urology* 12(4): 381–392

Metcalfe DD (2008) Mast cells and mastocytosis. *Blood* 112(4): 46-56
Metts JF (2001) Interstitial Cystitis: Urgency and Frequency Syndrome. *American Family Physician* 64(7): 1199-1206

Microscopy Resource Centre. 2012. *Fluorochrome Data Tables* [ONLINE] Available at: http://olympusmicro.com/primer/techniques/fluorescence/ fluorotable1.html. [Accessed 30 June 2017]

Mochizuki A, McEuen AR, Buckley MG and Walls AF (2003) The release of basogranulin in response to IgE-dependent and IgE-independent stimuli: validity of basogranulin measurement as an indicator of basophil activation. *Journal of Allergy and Clinical Immunology* 112(1): 102-108

Mohd Zohdi R, Abu Bakar Zakaria Z, Yusof N, Mohamed Mustapha N and Abdullah MNH (2012) Gelam *(Melaleuca spp.)* Honey-Based Hydrogel as Burn Wound Dressing. *Evidence-Based Complementary and Alternative Medicine* 1-7

Molan PC (2011) The evidence and the rationale for the use of honey as wound dressing. *Wound Practice and Research* 19(4): 204-220

Møller M, Glistrup OV and Olsen W (1984) Contrast enhancement of the brownish horseradish peroxidase-activated 3,3'-diaminobenzidine tetrahydrochloride reaction product in black and white photomicrography by the use of interference filters. *Journal of Histochem & Cytochemistry* 32(1): 37-42

Momin M, Kurhade S, Khanekar P and Mhatre S (2016) Novel biodegradable hydrogel sponge containing curcumin and honey for wound healing. *Journal of Wound Care. MA Healthcare London* 25(6): 364-372

Mondoni M, Radovanovic D, Valenti V, Patella V and, Santus P (2015) Bronchoscopy in sarcoidosis: union is strength. *Minerva Medica* 22: 22

Montoya J and Liesenfeld O (2004) Toxoplasmosis. *The Lancet* 363(9425): 1965-1976

Morales A, Emerson L, Nickel JC and Lundie M (1996) Intravesical hyaluronic acid in the treatment of refractory interstitial cystitis. *Journal of Urology*, 156(1): 45-48

Moutzouris DA and Falagas ME (2009) Interstitial cystitis: an unsolved enigma. *Clinical Journal of the American Society of Nephrology* 4(11): 1844-1857

Mukai K, Obata K, Tsujimura Y and Karasuyama H (2009) New insights into the roles for basophils in acute and chronic allergy. *Allergology International* 58(1):11-19

Mukai K and Galli S (2013) Basophils. In eLS. Chichester, UK: John Wiley & Sons, Ltd. Available at: http://doi.wiley.com/10.1002/9780470015902.a0001120.pub3 [Accessed May 15, 2017]

Mullai V and Menon T (2007) Bactericidal activity of different types of honey against clinical and environmental isolates of Pseudomonas aeruginosa. *The Journal of Alternative and Complimentary Medicine* 13(4): 439-441

Mural, R. J., Adams, M. D., Myers, E. W., Smith, H. O., Miklos, G. L. G (2002) A comparison of whole-genome shotgun-derived mouse chromosome 16 and the human genome. *Science* 296(5573): 1661-1671

Nabe T, Matsuya K, Akamizu K, Fujita M, Nakagawa T, Shioe M, Kida H, Takiguchi A, Wakamori H, Fujii M, Ishihara K, Akiba S, Mizutani N, Yoshino S and Chaplin DD (2013) Roles of basophils and mast cells infiltrating the lung by multiple antigen challenges in asthmatic responses of mice. *British Journal of Pharmacology* 169(2): 462-476

Nakashima K, Takeuchi T and Shirakawa T (2005) Differentiation, distribution, and chemical state of intracellular trace elements in LAD2 mast cell line. *Biological Trace Element Research* 108(1-3): 105-114

National Health Service. 2011. Chronic myeloid leukaemia. [ONLINE] Available at: https://www.nhsinform.scot/illnesses-and-conditions/cancer/cancer-types-in-adults/chronic-myeloid-leukaemia. [Accessed 29 June 2017]

Nickel JC, Emerson L and Cornish J (1993) The bladder mucus (glycosaminoglycan) layer in interstitial cystitis. *Journal of Urology* 149(4): 716-718

Nickel JC and Cornish J (1994) Ultrastructural study of an antibody-stabilized bladder surface: a new perspective on the elusive glycosaminoglycan layer. *World Journal of Urology* 12(1): 11-14

Nickel JC (2008) Treatment of chronic prostatitis/chronic pelvic pain syndrome. *International Journal of Antimicrobial Agents* 31 Suppl 1: 112-116

Nickel JC, Egerdie B, Downey J, Singh R, Skehan A, Carr L and Irvine-Bird K (2009) A real-life multicentre clinical practice study to evaluate the efficacy and safety of intravesical chondroitin sulfate for the treatment of interstitial cystitis. *British Journal Urology International* 103(1): 56-60

Nickel JC, Egerdie RB, Steinhoff G, Palmer B and Hanno P (2010) A multicentre, randomized, double-blind, parallel group pilot evaluation of the efficacy and safety of intravesical sodium chondroitin sulfate versus vehicle control in patients with interstitial cystitis/painful bladder syndrome. *Urology* 76(4): 804-809

Nickel JC, Moldwin R, Lee S, Davis EL, Henry RA and Wyllie MG (2009) Intravesical alkalinized lidocaine (PSD597) offers sustained relief from symptoms of interstitial cystitis and painful bladder syndrome. *British Journal of Urology International* 103(7): 910-918

Nickel JC, Tripp DA, Pontari M, Moldwin R, Mayer R, Carr LK, Doggweiler R Yang CC, Mishra N and Nordling J (2011) Childhood sexual trauma in women with interstitial cystitis/bladder pain syndrome: a case control study. *Canadian Urological Association Journal* 5(6), pp. 410-415

Nickel JC, Hanno P, Kumar K and Thomas H (2012) Second multicentre, randomized, double-blind, parallel-group evaluation of effectiveness and safety of intravesical sodium chondroitin sulfate compared with inactive vehicle control in subjects with interstitial cystitis/bladder pain syndrome. *Urology* 79(6): 1220-1224

Nickel JC, Jain P, Shore N, Anderson J, Giesing D, Lee H, Kim G, Daniel K, White S, Larrivee-Elkins C, Lekstrom-Himes J and Cima M (2012) Continuous intravesical lidocaine treatment for interstitial cystitis/bladder pain syndrome: safety and efficacy of a new drug delivery device. *Science Translational Medicine* 4(143): 100

Nickel JC and Tripp JD (2015) Clinical and Psychological Parameters Associated with Pain Pattern Phenotypes in Women with Interstitial Cystitis/Bladder Pain Syndrome. *Journal of Urology* 193(1): 138-144

Nigro DA, Wein AJ, Foy M, Parsons CL, Williams M, Nyberg LM Jr, Cook YL and Simon LJ (1997) Associations among cystoscopic and urodynamic findings for women enrolled in the Interstitial Cystitis Data Base (ICDB) Study. *Urology* 49: 86-92

Nordling J, Fall, M and Hanno P (2012) Global concepts of bladder pain syndrome (interstitial cystitis). *World Journal of Urology* 30(4): 457-464

O'Leary MP, Sant GR, Fowler FJ, Whitmore KE and Spolarich-Kroll J (1997) The interstitial cystitis symptom index and problem index. *Urology* 49 (Supplement 5A): 58-63

Oryan A and Zaker SR (1998) Effects of topical application of honey on cutaneous wound healing in rabbits. *Zentralbl Veterinarmed A* 45(3): 181-188

Oryan A, Alemzadeh E and Moshiri A. (2016) Biological properties and therapeutic activities of honey in wound healing: A narrative review and meta-analysis. *Journal of Tissue Viability* 25(2): 98–118

Otsuka A. and Kabashima K (2015) Mast cells and basophils in cutaneous immune responses. *Allergy* 70(2): 131–140

Owoyele BV, Adenekan OT and Soladoye AO (2011) Effects of honey on inflammation and nitric oxide production in Wistar rats. *Zhong xi yi jie he xue bao; Journal of Chinese Integrative* Medicine 9(4): 447-452

Pang X, Marchand J, Sant GR, Kream RM and Theoharides TC (1995) Increased number of substance P positive nerve fibres in interstitial cystitis. *British Journal of Urology* 75(6): 744-750

Parkin J, Shea C and Sant GR (1997) Intravesical dimethyl sulfoxide (DMSO) for interstitial cystitis- a practical approach. *Urology* 49 (Supplement 5A): 105-107

Parsons CL, Benson G, Childs SJ, Hanno P, Sant GR and Webster G (1993) A quantitatively controlled method to study prospectively interstitial cystitis and demonstrate the efficacy of pentosan polysulfate. *Journal of Urology* 150(3): 845-848

Parsons CL (1997) Epithelial coating techniques in the treatment of interstitial cystitis. *Urology* 49(Supplement5A): 100-104

Parsons CL (2005) Successful down regulation of bladder sensory nerves with combination of heparin and alkalinized lidocaine in patients with interstitial cystitis. *Urology* 65(1): 45-48

Parsons CL (2010) The role of a leaky epithelium and potassium in the generation of bladder symptoms in interstitial cystitis/overactive bladder, urethral syndrome, prostatitis and gynaecological chronic pelvic pain. *British Journal of Urology International* 107(3): 370-375

Parsons CL, Boychuk D, Jones S, Hurst R and Callahan H (1990) Bladder surface glycosaminoglycans: an epithelial permeability barrier. *Journal of Urology* 143(1): 139-142
Parsons JK and Parsons CL (2004) The Historical Origins of Interstitial Cystitis. *Journal of Urology* 171(1): 20-22

Parsons CL, Lilly JD and Stein P (1991) Epithelial dysfunction in nonbacterial cystitis (interstitial cystitis). *Journal of Urology* 145(4): 732–735

Pasini EM, van den Ierssel D, Vial HJ and Kocken CHM (2013) A novel live-dead staining methodology to study malaria parasite viability. *Malaria Journal; BioMed Central* 12:190

Payne CK, Joyce GF, Wise M and Clemens JQ (2007) Interstitial cystitis and painful bladder syndrome. *Journal of Urology* 177(6): 2042-2049

Peeker R, Enerback L, Fall M and Aldenborg F (2000) Recruitment, distribution and phenotypes of mast cells in interstitial cystitis. *Journal of Urology* 163(3): 1009-1015

Perez-Marrero R, Emerson LE and Feltis JT (1988) A controlled study of dimethyl sulfoxide in interstitial cystitis. *Journal of Urology* 140(1): 36-39

Porru D, Leva F, Parmigiani A, Barletta D and Choussos D (2012) Impact of intravesical hyaluronic acid and chondroitin sulfate on bladder pain syndrome/interstitial cystitis. *International Urogynecology Journal* 23(9): 1193-1199

Rao AG and Danturty I (2012) Leukemia Cutis. *Indian Journal of Dermatology* 57(6): 504-504

Rebuck JW, Hodson JM, Priest RJ and Barth CL (1963) Basophilic granulocytes in inflammatory tissues of man. *Annals of the New York Academy of Sciences* 103: 409-426

Rivas DA, Chancellor MB, Shupp-Byrne S, Shenot PJ, McHugh K and McCue P (1997) A Molecular Marker for the Development of Interstitial Cystitis in a Rat Model: Isoactin Gene Expression. *Journal of Urology* 157(5): 1937-1940

Rooney P, Srivastava A, Watson L, Quinlan LR and Pandit A (2015) Hyaluronic acid decreases IL-6 and IL-8 secretion and permeability in an inflammatory model of interstitial cystitis. *Acta Biomaterialia* 19: 66-75

Rosamilia A, Dwyer PL and Gibson J (1997) ‘Electromotive drug administration of lidocaine and dexamethasone followed by cystodistension in women with interstitial cystitis. *International Urogynecology Journal and Pelvic Floor Dysfunction* 8(3): 142-145

Rössberger J, Fall M, Gustafsson CK AND Peeker R (2010) Does mast cell density predict the outcome after transurethral resection of Hunner's lesions in patients with type 3C bladder pain syndrome/interstitial cystitis? Scandinavian *Journal of Urology and Nephrology* 44(6): 433-437

Rossiter K, Cooper A, Voegeli D and Lwaleed BA (2010) Honey promotes angiogeneic activity in the rat aortic ring assay. *Journal of Wound Care* 19(10): 440-446

Roth TM (2007) Interstitial cystitis in a woman with systemic mastocytosis. *International Urogynecology Journal and Pelvic Floor Dysfunction* 18(8): 963-965

Rouse AR and Gmitro AF (2000) Multispectral imaging with a confocal microendoscope. *Optics Letters* 25(23): 1708-1710

Rovner E, Propert KJ, Brensinger C, Wein AJ, Foy M, Kirkemo A, Landis JR, Kusek JW and Nyberg LM (2000) Treatments used in women with interstitial cystitis: the interstitial cystitis data base (ICDB) study experience. The Interstitial Cystitis Data Base Study Group. *Urology* 56(6): 940-945

Russell WMS and Burch RL (1959) *The Principles of Humane Experimental Technique* London: Methuen and Co. Ltd. P238

Ryu J, Pak S, Song M, Chun JY, Hong S and Choo MS (2013) Elimination of Hunner's Ulcers by Fulguration in Patients with Interstitial Cystitis: Is It Effective and Long Lasting? *Korean Journal of Urology* 54(11): 767-771

Sanderson MJ, Smith I, Parker I and Bootman MD (2014) ‘Fluorescence Microscopy. *Cold Spring Harbor Protocols* (10): 071795

Sant GR, Kempuraj D, Marchand JE and Theoharides TC (2007) The mast cell in interstitial cystitis: role in pathophysiology and pathogenesis. *Urology* 69(Supplement 4): 34-40

Sato T and Miyata G (2000) The nutraceutical benefit, part iii: honey. Nutrition 16(6): 468-469

Schneider CA, Rasband WS and Eliceiri KW (2012) NIH Image to ImageJ™: 25 years of image analysis. *Nature Methods* 9(7): 671-675

Scudamore CL, Jepson MA, Hirst, B. H. and Miller, H. R. (1998) 'The rat mucosal mast cell chymase, RMCP-II, alters epithelial cell monolayer permeability in association with altered distribution of the tight junction proteins ZO-1 and occludin. *European Journal of Cell Biology* 75(4): 321-330

Sealed Envelope (2001) *Create a blocked randomisation list* [ONLINE] Available at: https://www.sealedenvelope.com/. [Accessed 30 June 2015]

Seaman JA (1950) Interstitial cystitis. *Journal of Urology* 63(1): 105-15

Siracusa MC, Comeau MR and Artis D (2011) New insights into basophil biology: initiators, regulators and effectors of type 2 inflammation. *Annals of the New York Academy of Sciences* 1217: 166-177

Siracusa MC, Kim BS, Spergel JM and Artis D (2013) Basophils and allergic inflammation. *The Journal of Allergy and Clinical Immunology; NIH Public Access* 132(4): 789-801

Siracusa MC, Saenz SA, Wojno EDT, Kim BS, Osborne LC, Ziegler CG, Benitez AJ, Ruymann KR, Farber DL, Sleiman PM, Hakonarson H, Cianferoni A, Wang ML, Spergel JM, Comeau MR and Artis D (2013) Thymic stromal lymphopoietin-mediated extramedullary hematopoiesis promotes allergic inflammation. *Immunity; NIH Public Access* 39(6): 1158-1170. doi: 10.1016/j.immuni.2013.09.016

Skene AJC (1887) Diseases of Bladder and Urethra in Women. *New York: Wm Wood*, p167

Smith KJ, Chess-Williams R and McDermott C (2014) Luminal DMSO: effects on detrusor and urothelial/lamina propria function. *BioMed Research International 347616* (10): 18

Soler R, Bruschini H, Truzzi JC, Martins JR, Camara NO, Alves MT, Leite KR, Nader HB, Srougi M and Ortiz V (2008) Urinary glycosaminoglycans excretion and the effect of dimethyl sulfoxide in an experimental model of non-bacterial cystitis. *International Braz J Urol* 34(4): 503-511

Stav K, Beberashvili I, Lindner A and Leibovici D (2012) Predictors of response to intravesical dimethyl-sulfoxide cocktail in patients with interstitial cystitis. *Urology* 80(1): 61-65

Stiefel P, Schmidt-Emrich S, Maniura-Weber K and Ren Q (2015) Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. *BMC Microbiology. BioMed Central* 15: 36

Subrahmanyam M (1991) Topical application of honey in treatment of burns. *British Journal of Surgery* 78(4): 497-498

Subrahmanyam M (1993) Honey impregnated gauze versus polyurethane film (OpSite) in the treatment of burns- a prospective randomized study. *British Journal of Plastic Surgery* 46(4): 322-323

Subrahmanyam M (1994) Honey-impregnated gauze versus amniotic membrane in the treatment of burns. *Burns* 20(4): 331-333

Subrahmanyam M (1996) Honey dressing versus potato peel in the treatment of burns: a prospective randomised study. *Burns* 22(6): 491-493

Subrahmanyam M (1998) A prospective randomised clinical and histological study of superficial burn wound healing with honey and silver sulfadiazine. *Burns* 24(2): 157-161

Subrahmanyam M (1999) Early tangential excision and skin grafting of moderate burns is superior to honey dressing: a prospective randomised trial. *Burns* 25(8): 729-731

Sukur SM, Halim AS, Singh KK (2011) Evaluations of bacterial contaminated full thickness burn wound healing in Sprague-Dawley rats treated with tualang honey. *Indian Journal of Plastic Surgery* 44(1):112-7

Surahio AR, Khan AA, Farooq M and Fatima I (2014) Role of honey in wound dressing in diabetic foot ulcer. *Journal of Ayub Medical College, Abbottabad* 26(3): 304-306

Suskind AM, Berry SH, Suttorp MJ, Elliott MN and Clemens JQ (2014) Symptom persistence in a community cohort of women with interstitial cystitis/bladder pain syndrome (IC/BPS): 3-, 6-, 9-, and 12-month follow-up from the RICE cohort. *International Urogynecology Journal. NIH Public Access* 25(12): 1639-1643

Takai S, Shiota N, Sakaguchi M, Muraguchi H, Matsumura E and Miyazaki M (1997) Characterization of chymase from human vascular tissues. *Clinica Chimica Acta* 265(1): 13-20

Tamaki M, Saito R, Ogawa O, Yoshimura N and Ueda T (2004) Possible mechanisms inducing glomerulations in interstitial cystitis: relationship between endoscopic findings and expression of angiogenic growth factors. *Journal of Urology* 172(3): 945-948

Tasian G, Cunha G and Baskin L (2010) Smooth Muscle Differentiation and Patterning in the Urinary Bladder. *Differentiation; Research in biological diversity* 80(0): 106-117

Tasian GE, Bagga HS, Fisher PB, McCulloch CE, Cinman NM, McAninch JW and Breyer BN (2013) Pediatric Genitourinary Injuries in the United States from 2002 to 2010. *Journal of Urology* 189(1): 288-294

Theoharides TC, Alysandratos KD, Angelidou A, Delivanis DA, Sismanopoulos N, Zhang B, Asadi S, Vasiadi M, Weng Z, Miniati A and Kalogeromitros D (2012) Mast cells and inflammation. *Biochimica et Biophysica Acta (BBA); Molecular Basis of Disease* 1822(1): 21-33

Theoharides TC, Kempuraj D and Sant GR (2001) Mast cell involvement in interstitial cystitis: A review of human and experimental evidence. *Urology* 57(Supplement 6A): 47-55

Tilg H, Trehu E, Atkins M, Dinarello C and Mier J (1994) Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumour necrosis factor receptor p55. *Blood* 83(1): 113-118

Togawa H, Nakanishi K, Shima Y, Obana M, Sako M, Nozu K, Tanaka R, Iijima K and Yoshikawa N (2009) Increased chymase-positive mast cells in children with crescentic glomerulonephritis. *Pediatric nephrology (Berlin, Germany)* 24(5): 1071-1075

Tonks AJ, Cooper RA, Jones KP, Blair S, Parton J and Tonks A (2003) Honey stimulates inflammatory cytokine production from monocytes. *Cytokine* 21(5): 242-247

Topham J (2002) Why do some cavity wounds treated with honey or sugar paste heal without scarring? *Journal of Wound Care* 11(2): 53-55

Tzan CJ, Berg J and Lewis SA (1993) Effect of protamine sulfate on the permeability properties of the mammalian urinary bladder. *Journal of Membrane Biology* 133(3): 227-242

van de Merwe JP, Nordling J, Bouchelouche P, Bouchelouche K, Cervigni M, Daha LK, Elneil S, Fall M, Hohlbrugger G, Irwin P, Mortensen S, van Ophoven A, Osborne JL, Peeker R, Richter B, Riedl C, Sairanen J, Tinzl M and Wyndaele JJ (2008) Diagnostic Criteria, Classification, and Nomenclature for Painful Bladder Syndrome/Interstitial Cystitis: An ESSIC Proposal. *European Urology* 53(1): 60-67

van Ophoven A, Pokupic S, Heinecke A and Hertle L (2004) A prospective, randomized, placebo controlled, double-blind study of amitriptyline for the treatment of interstitial cystitis. *Journal of Urology* 172(2): 533-536

Walls AF, Jones DB, Williams JH, Church MK and Holgate ST (1990) Immunohistochemical identification of mast cells in formaldehyde-fixed tissue using monoclonal antibodies specific for tryptase. *The Journal of Pathology* 162(2): 119-126

Walls AF and Amalinei C (2014) Detection of Mast Cells and Basophils by Immunohistochemistry. In BF Gibbs & FH Falcone, eds. *Basophils and Mast Cells: Methods and Protocols.* New York, NY: Springer New York. p117-134. Available at: http://dx.doi.org/10.1007/978-1-4939-1173-8_9

Wasse H, Naqvi N and Husain A (2012) Impact of Mast Cell Chymase on Renal Disease Progression. *Current Hypertension Reviews; NIH Public Access* 8(1): 15-23. doi: 10.2174/157340212800505007

Welk BK and Teichman JM (2008) Dyspareunia response in patients with interstitial cystitis treated with intravesical lidocaine, bicarbonate, and heparin. *Urology* 71(1): 67-70

Weston RJ, Brocklebank LK and Lu Y (2000) Identification and quantitative levels of antibacterial components of some New Zealand honeys. *Food Chemistry* 70(4): 427-435

Yamada T, Murayama T, Mita, H and Akiyama K (2000) Subtypes of bladder mast cells in interstitial cystitis. *International Journal of Urology* 7(8): 292-297

Yang CC, Burks DA, Propert KJ, Mayer RD, Peters KM, Nickel JC, Payne CK, FitzGerald MP, Hanno PM, Chai TC, Kreder KJ, Lukacz S, Foster HE, Cen L, Landis JR, Kusek JW and Nyberg LM (2011) Early termination of a trial of mycophenolate mofetil for treatment of interstitial cystitis/painful bladder syndrome: lessons learned. *Journal of Urology* 185(3): 901-906

Yeo JC, Wall AA, Stow JL and Hamilton NA. (2013) High-throughput quantification of early stages of phagocytosis. *Biotechniques* 55(3):115-124