## Hypoxic changes to the urothelium as a bystander of end-stage bladder disease

***Introduction***

The normal urinary bladder experiences variation in perfusion and hence oxygenation throughout the filling and voiding phases of the micturition cycle. Compression of intramural vasculature during filling resulting in intermittent hypoxia is generally accepted, but there is scant information regarding physiological oxygen tensions in the functioning human bladder. In an *in vivo* guinea pig bladder model ([1](#_ENREF_1)), oxygen saturation during filling was high (92-95%), with a slight reduction in oxygen saturation seen during voiding. These reported fluctuations in oxygen saturation were negatively impacted by detrusor overactivity and outlet obstruction.

Chronic interruption of the vasculature or nervous supply can progress to end-stage bladder disease; typified by the small, fibrotic bladder of limited capacity and increased risk of upper urinary tract damage ([2](#_ENREF_2)). Various animal models have been established to examine the compensatory and decompensatory effects of partial outflow obstruction on bladder function and these have been used variously to report tissue oxygen tensions ([1](#_ENREF_1), [3](#_ENREF_3), [4](#_ENREF_4)). A porcine outflow obstruction model used to investigate the effects of hypoxia on the bladder ([5](#_ENREF_5)) found similar oxygen tensions (2.5-5.5 %) to those quoted generically for smooth muscle ([6](#_ENREF_6)). The authors suggested that chronic bladder outlet obstruction (BOO) led to the detrusor experiencing both a reduction in blood flow and increased hypoxia, potentially contributing to the functional and structural changes characteristic of obstructed bladders. Hypoxia-dependent pathways were upregulated in a murine model of chronic intermittent bladder over-distension ([3](#_ENREF_3), [7](#_ENREF_7)), with a 2.6-fold increase in HIF-1α transcript and downstream genes associated ontologically with fibrosis and inflammation ([7](#_ENREF_7)).

Hypoxia inducible factors (HIFs) are a family of transcription factors that mediate the adaptive response to hypoxia in cells and tissues. Active HIF is heterodimeric, consisting of the constitutively expressed HIF-1β subunit (also known as aryl hydrocarbon receptor nuclear translocator or ARNT) and one of three oxygen-sensitive alpha subunits 1α, 2α or 3α ([8](#_ENREF_8)). During normoxia, HIF-1α subunits are degraded in the cytoplasm by the hydroxylation-ubiquitination-proteosomal system (HUPS)([9](#_ENREF_9)). Under hypoxic conditions, nuclear translocation of HIF-1α and heterodimerisation with HIF -1β results in the induction of target genes responsible for angiogenesis, glucose metabolism, cell survival and microenvironment remodelling ([10](#_ENREF_10)).

Hypoxia has been associated with adult human bladder disease, with the detection of nuclear HIF in the bladder wall being used as a marker of hypoxia pathway activation in both benign and malignant conditions, including BOO ([11](#_ENREF_11)). In our translation of novel bladder reconstructive techniques ([12](#_ENREF_12)), we unexpectedly found that urothelial cells from children with end-stage bladder diseases displayed a compromised phenotype in vitro even though the urothelium itself was not implicated in the disease process ([13](#_ENREF_13)).

Surgery or therapy to alleviate high pressures, such as intravesical Botox or augmentation may reduce active hypoxia signalling and temporarily relieve symptoms. However, the possible phenotypic changes already undergone by the urothelium, due to the prior chronic hypoxia exposure, also remains poorly understood.

The aim of this work was to test the hypothesis that the urothelium itself encounters hypoxia, associated with high pressures, during end-stage bladder disease. Immunohistochemistry of archived tissues was used to quantitatively examine nuclear expression of HIF-1α in the urothelium and correlate this to the results of urodynamic investigations.

 Additional immunohistochemistry (non-quantified) was performed on samples to look at the expression of one of the downstream targets of HIF-1α, vascular endothelial growth factor (VEGF). VEGF is an autocrine and paracrine signalling molecule whose primary role is the activation of angiogenesis. However, it has also been implicated in tissue inflammatory responses and, specifically, the recruitment of monocytes and macrophages ([14](#_ENREF_14)) and ([15](#_ENREF_15)).

The link between hypoxia, VEGF and inflammation, coupled with the knowledge that

inflammation is a feature of the neuropathic bladder condition, suggests that VEGF may be

up-regulated in neuropathic bladders.

***Materials and Methods***

*Tissue samples*

All human urothelial tissue samples used were collected from three different hospitals between 2007 – 2015. All specimens were covered by NHS Research Ethics Committee approvals, with stipulated patient consent, as indicated below REC 12/YH/0507, REC 99/095, REC99/04/003 and REC04/Q1206/143.

Resection specimens of bladder were collected from paediatric patients with neuropathic bladders (n=15): 14 due to myelomeningocele and one due to non-neuropathic neuropathic bladder). Other paediatric specimens included bladder from children with vesicoureteric reflux (n = 3), vesicoureteric junction obstruction (n=1), and ureter from a heminephrouretectomy (n=1).

Adult specimens, without known neuropathic disease included: healthy renal transplant donor ureter (n=2), bladder in a patient with benign, non-obstructive prostatic hypertrophy (n = 1).

Biopsies from four post-enterocystoplasty native bladders acquired at the time of surveillance cystoscopy for malignancy; enabled a subjective comparison between augmented bladders and those that had not. Bladder tissue from adults with BOO secondary to prostatic disease (n=1), urothelial carcinoma (n = 1) and tonsil (n=1) were used as hypoxic tissue controls for immunohistochemistry ([11](#_ENREF_11)).

All tissue samples were fixed in 10% (v/v) formalin, dehydrated and processed into paraffin wax. Urodynamic studies and clinical notes were, where available, retrospectively reviewed in order to assess whether the bladder and upper tracts were under abnormally high pressures, according to reported criteria (16).

*Immunohistochemistry*

Immunohistochemical studies were performed to seek objective evidence for the activation of hypoxia-related pathways. Within the urothelial compartment, quantification of nuclear HIF-1α was performed and in addition, the presence of VEGF was subjectively sought. Labelling for HIF-1α and VEGF were performed on one section each from all appropriate test and control tissue samples in a single immunohistochemistry run.

De-waxed five μm tissue sections were blocked for endogenous peroxidase activity with 3% (v/v) hydrogen peroxide for 10 minutes. Antigen retrieval was performed by microwave boiling of tissue sections in a 10 mM citric acid buffer (pH 6.0) (2.4 g citric acid, 1050 mL double-distilled water purified by reverse osmosis in a Purelab Ultra Purite system (Elga Veolia) to a resistivity of 18.2 Ω and sodium hydroxide pellets to achieve pH 6.0) for 10 minutes, followed by 10 minutes cooling on ice.

Tissue sections were treated with an Avidin/Biotin blocking kit (Vector labs, Peterborough, UK), before applying 10% rabbit serum for 5 minutes to prevent non-specific binding of the secondary antibody. Mouse anti-HIF1α monoclonal antibody (Hα111A), Enzo Life Sciences, Exeter, UK; at 1:300 was applied, followed by biotinylated rabbit anti-mouse secondary antibody (Dako Cytomation Ltd, Ely, UK) at 1:200, each for 15 minutes at ambient temperature, with washing between. Antibody binding was detected with 3, 3′-diaminobenzidine (DAB), before counterstaining in haematoxylin, dehydration and mounting in DPX.

Immunohistochemistry for VEGF was performed in exactly the same fashion, using an Avidin/Biotin blocking kit (Vector labs, Peterborough, UK); except 100 μl goat serum (Dako) was applied to each slide and incubated for 5 minutes at ambient temperature followed by 100μl rabbit anti-VEGF primary antibody (Thermo Scientific, RB-9031) at 1:50. Tonsil tissue, known to express VEGF (based on high protein expression levels reported by <https://www.proteinatlas.org>), was used as the positive tissue control and negative (no primary antibody) control to ensure experimental validity (See Figure 1b).

*Image analysis*

HIF-1α labelled sections were scanned on a Zeiss AxioScan.Z1 slide scanner. Analysis of urothelial nuclear HIF-1α labelling was performed using HistoQuest (TissueGnostics) image analysis software and involved first demarcating the urothelium (red-dashed line in ***Figure 1a***), followed by supervised automated identification of haematoxylin-stained urothelial nuclei (red-filled object in ***Figure 1a***) and scoring for intensity of DAB deposition (arbitrary scale). At least 100 individual urothelial nuclei were assessed per tissue specimen and DAB intensity was expressed as the median. Intra-urothelial lymphocytes, where present, were excluded from the count based on morphology (see arrow in ***Figure 1a***).

*Statistical Analysis*

Comparison of nuclear labelling in the urothelium of end-stage bladder disease against positive and negative hypoxia control specimens was performed by one-way ANOVA with post-hoc Tukey HSD ( Honsetly Significant Difference) test (Instat™ v3, GraphPad Software Inc., La Jolla). Significance was assumed when the *p* value was <0.05 and where available the precise p value was provided.

In the datasets where urodynamic and clinical information was available, Spearman’s Rank correlation was used to test the null hypothesis that there was no correlation between either initial or maximal detrusor pressures obtained and HIF-1α nuclear labelling intensity of the specimens.

***Results***

*Immunolabelling of human urothelial tissues for evidence of hypoxia*

Microscopically nuclear labelling of HIF-1α in the urothelial compartment from neuropathic bladder samples (*n* = 15) appeared more intense than in the various control tissues, including non –obstructed, samples (*n* = 9) (***Figure 1a***). Evaluation of VEGF expression in neuropathic bladder versus bladder tissue from VUR and VUJO is shown in ***Figure 1b***. Subjectively there may be a difference in expression, which is predominantly in the cytoplasm, but no image analysis was performed.

*Image analysis*

In total, urothelial nuclei from 28 individual tissue sections were analysed for intensity of nuclear HIF-1α by immunoperoxidase labelling based on DAB deposition (see table 1). The total number was 28 not 29 as urothelial carcinoma specimen (number 10) was excluded as represented a pathology not related to intra-vesical pressure but just used as an immunohistochemistry process control. The descriptive statistics are summarised in ***Figure 2***. Although the series represented a range of conditions, the average urothelial nuclear HIF-1α labelling intensity was higher in the neuropathic disease group than in the controls. There was no apparent difference between HIF-1α labelling intensity in male and female samples in each group.

Overall, median nuclear HIF-1α labelling in all controls was 29.98 ± 3.10 s.d. (*n* = 9) and 74.29 ± 7.55 s.d. in the neuropathic samples (*n* = 15). Comparison of median scores indicated a significant difference between the control and neuropathic tissue groups (*p<0.05*) (***Figure 3***).

Of the 15 neuropathic samples, 11 had traceable clinical information regarding urodynamic studies (Table 2). According to the ICCS guidelines ([16](#_ENREF_16)); three patients had low pressure systems (opening pressures <10 cm H2O, maximum detrusor pressures <40 cm H2O) and eight had high detrusor pressures recorded (opening pressures >10 cm H2O and maximum detrusor presures >40 cmH2O). Histology samples taken from the four patients with the lowest recordable detrusor pressures were found to have the lowest intensity of labelling for HIF‑1α within the neuropathic group (***Figure 2***).All the patients in this study, with neuropathic bladders, used clean intermittent catheterization (CIC). Despite the use of CIC six patients still suffered with recurrent urinary tract infections; of which four had high-pressure and two low-pressure systems. In three patients culture evidence of infections were unavailable. None of the patients had documented bladder stones.

Initial and maximum detrusor pressures recorded at urodynamic assessment both indicated a positive relationship when plotted against HIF-1α labelling intensity (***Figures 4a and 4b***). . All 11 datasets were analysed using Spearman’s rank correlation, with no missing events. Significant correlations between both initial or maximum detrusor pressure and nuclear HIF-1α labelling intensity (median score) were found *p ≤ 0.05* for both datasets. The null hypothesis was accordingly rejected.

***Discussion***

Bladders from neuropathic patients frequently acquire the features of end-stage bladder disease including compromised capacity, fibrosis and raised pressures with a resulting increased risk to the upper renal tracts. The effect on the urothelium is unknown. Here evidence, in the form of significantly increased nuclear HIF-1α, indicates that in paediatric neuropathic bladder disease the urothelium is exposed to hypoxia-related pathway activation, which is further supported by the subjective increase of VEGF expression seen in the neuropathic samples (***Figure 1b***).

Nuclear HIF-1α was notably increased, in individuals with high-pressure, neuropathic systems, identified on previous urodynamic evaluation (*p < 0.05).* This finding is in keeping with a previous study that reported HIF-1α expression in 70 patients with BOO secondary to benign prostatic hyperplasia ([11](#_ENREF_11)), although the this study did not detail any specific effect on the urothelium. Our study shows a clear correlation between clinically proven high-pressure systems and the presence of nuclear HIF-1α in the urothelium; but with small patient numbers and four missing urodynamic studies, the possibility for skewing of results should be considered.

The final surgical option for end-stage bladder disease is augmentation, a procedure performed to reduce intravesical pressure. Results reported here appear to demonstrate a reduction in nuclear HIF-1α in the urothelium of four native bladders that have undergone previous augmentation. Such an observation, albeit in a small number of patients, may support a pressure/hypoxia relationship. Bladder augmentation is

most commonly performed using bowel, known as an enterocystoplasty, the associated side-effects include recurrent infection, mucus production ([17](#_ENREF_17)), stone formation ([18](#_ENREF_18)), metabolic disorders ([19](#_ENREF_19)) and malignancy ([20](#_ENREF_20)).

The surgical composite cystoplasty strategy has been proposed wherein the mucus-producing absorptive bowel epithelium of the augmenting bowel segment is replaced by autologous urothelium expanded *in vitro*. Success was reported in a healthy porcine surgical model([21](#_ENREF_21)), however translation to patients has been hindered by problems in expanding and generating functional urothelium from end-stage diseased paediatric bladders ([13](#_ENREF_13)).

As the urothelium is not directly implicated in the disease process, the reasons for such compromise were unknown. Of interest a mouse model for spinal cord injury has also identified a degenerative urothelial phenotype, postulated to be as a result of signals produced from afferent nerves ([22](#_ENREF_22)) ; potentially adding to the complexity of pathological pathways and processes potentially involved in the entity of end-stage bladder disease

This work suggests that the urothelium is exposed to hypoxia during bladder disease and provides a candidate pathway for explaining the patho-biological changes observed, such as fibrosis. However, as demonstrated by the fact that 50 % of our neuropathic samples were from children who also had a history of UTIs, hostile bladder systems are likely to arise from complex, multifactorial pathogenic programmes with the endpoint being the small, fibrosed, poorly compliant bladder.

As an immunohistochemical study on retrospective archived tissues, this study has inevitable limitations, although we have tried to overcome the predominantly descriptive method used by performing image analysis. Further *in vitro* studies of cells originating from human neuropathic specimens would be the obvious next-step to support this work. Although access to matching human urothelial cells were available, previous published work on these had demonstrated the difficulties in expanding and differentiating diseased urothelial cells taken from neuropathic bladder specimens (13).

Using traditional protein evaluation methods with whole tissue lysates to evaluate total HIF1-α would be inherently erroneous. As described in the introduction HIF-1α is activated by hypoxia and translocates into the nucleus to dimerise with HIF1-β and therefore a nuclear extraction technique or single cell genomic/epigenetic analysis would be required to evaluate nuclear HIF-1α expression. In addition, we were analysing only the epithelial compartment, which would be impossible using whole tissue lysates. This is why in the image analysis performed, only the epithelial nuclei were identified and analysed for HIF-1α expression.

Conclusion:

Our study indicates that the presence of urothelial nuclear HIF-1α may be a biomarker of end-stage bladder disease. In particular, nuclear expression of this transcription factor was significantly associated with high-pressure systems. HIF-1α as evidence of the pathological endpoint does not help clarify the multifactorial prequel involving infection, recurrent inflammation, fibrosis etc. The understanding of these feeding pathways would help identify modifiable mechanisms, which may prevent such hostile neuropathic bladders from occurring.

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