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Investigating Detrusor Muscle Concentrations of Oxybutynin after Intravesical Delivery in an *Ex Vivo* Porcine Model

Nicholas A. Williams ^a, Kay M. Lee ^a, Chris J. Allender ^a, Jenna L. Bowen ^a, Mark
Gumbleton ^a, Tim Harrah ^b, Aditya Raja ^c, Hrishu B. Joshi ^c

^a School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Redwood Building, King Edward VII Ave., Cardiff, UK.

^b Department of Research and Development in Urology and Gynaecology, Boston Scientific, Marlborough, MA 01752, USA.

^c Department of Urology, University Hospital of Wales, Heath Park, Cardiff, UK.

INTRODUCTION

Overactive bladder (OAB) is a syndrome described by a collection of lower urinary tract symptoms (LUTS). These typically include urgency, with or without urge incontinence, along with frequency and nocturia, in the absence of proven infection or other obvious pathology¹. Considering these are storage, rather than voiding symptoms, OAB is most accurately described as a storage syndrome. In combination with behavioural therapies, first - line pharmacotherapy is treatment with anti - muscarinic drugs². Unfortunately for some patients oral antimuscarinics are unsuitable; either due to insufficient suppression of detrusor overactivity³ or the experience of significant adverse effects⁴. For such patients, conservative management with intravesically delivered oxybutynin is an option⁵. Intravesical oxybutynin is highly effective in increasing mean bladder capacity and decreasing mean maximum filling pressure^{6,7}. It is also well tolerated and associated with fewer adverse effects than its oral counterpart⁸. The mechanism of action (MOA) of intravesical oxybutynin was believed to be the same as that of oral delivery, that is cholinergic blockade of the M₃ muscarinic receptors in the bladder detrusor muscle⁹. However, the last decade has seen significant changes in opinion as to the MOA of antimuscarinics as a whole¹⁰. Accepted theory was that storage LUTS arose as a result of abnormal, involuntary detrusor contractions during bladder filling¹¹. Since antimuscarinics had been shown beneficial in treating OAB¹², their MOA was therefore believed to be antagonism of the detrusor M₃ muscarinic receptors and subsequent inhibition of these involuntary contractions^{13,14}. However, evidence now suggests that this may not be the case and alternative MOAs should be considered¹⁵. Therefore, although it

is well established that antimuscarinics reduce the symptoms of OAB¹⁶, whether this is brought about by an inhibition in parasympathetic mediated detrusor contraction is in doubt¹⁵. Finney *et al* conducted a review of all articles containing cystometric data for both storage and voiding phases in OAB patients before and after antimuscarinic therapy¹⁷. They found that although at clinically relevant doses antimuscarinics significantly improve variables associated with storage, there was little evidence to support the notion that antimuscarinics significantly alter voiding variables such as maximum detrusor pressure during voiding or maximum urinary flow. During bladder filling the parasympathetic switch is off and post - junctional muscarinic receptors in the detrusor are presumed inactive¹⁸. Therefore the ability of antimuscarinics to improve storage symptoms is unlikely to be explained exclusively by antagonism of the parasympathetic - controlled, acetylcholine - activated M₃ receptors of the detrusor¹⁹. Rather, a growing evidence base suggests antimuscarinics elicit their effects by modifying afferent pathways in the storage phase of the micturition cycle^{20,21}.

To date the majority of studies investigating intravesical oxybutynin have focused on post - intravesical pharmacokinetics and systemic levels of oxybutynin and N - desethyl - oxybutynin (the major metabolite responsible for its adverse effects)^{22,23}. Furthermore, despite the significant recent interest in the MOA of antimuscarinics in OAB, the fate of intravesically delivered oxybutynin in the bladder wall has received little attention. To our knowledge only one study has investigated the transurothelial permeation and resulting bladder wall concentrations achieved following topical application of oxybutynin to the urothelium²⁴. Results from this study report average bladder wall

oxybutynin concentrations rather than drug concentration as a function of each tissue layer. We report a more detailed analysis of drug concentrations achieved in the different layers of the bladder wall (urothelium, lamina propria and detrusor muscle), following the intravesical instillation of a clinically relevant oxybutynin solution in an *ex vivo* setting.

MATERIALS AND METHODS

Transurothelial permeation profile of oxybutynin

En bloc porcine urinary tracts, from pigs weighing 70 - 90 kg, were obtained fresh from a local abattoir. Within 5 min of excision, they were immersed in cold, oxygenated Krebs buffer (composition: NaCl 118.3 mM, NaHCO₃ 25 mM, CaCl₂ 2.5 mM, MgSO₄ 1.2 mM, KCl 4.7 mM, KH₂PO₄ 1.2 mM and D - glucose 11 mM, pH 7.4). Excess perivesical fat was trimmed and the bladder dissected out. Bladders were filled and drained with 37 °C Krebs to remove any residual urine. Bladder tissue sections (~ 2 cm²) were excised from the lateral sides and dome area and loaded into custom - built Franz - type diffusion cells (average surface area of tissue exposure 1.32 cm²) with the urothelium facing upwards. The receiver compartments of the diffusion cells were filled with oxygenated Krebs buffer and equilibrated at 37 °C for 30 min with continuous stirring. A 0.75 ml aliquot of oxybutynin chloride (0.167 mg ml⁻¹ in deionised water, oxybutynin chloride, ≥ 98 %, Sigma - Aldrich Company Ltd, Dorset, UK) was pipetted into the donor chamber, which was then covered to prevent evaporative loss. The sampling arm was capped and the Franz - type cells placed in a thermostatically controlled water bath (37 °C). At fixed time points (20, 40 and 60 min), the contents of the receiver and donor chambers were collected and the tissue sample removed. To

ensure removal of surface - bound drug, the urothelium was subjected to three saline rinses. Full thickness bladder wall from the area of drug contact was excised with a scalpel and immediately snap frozen between two metal plates using liquid nitrogen before being fixed to cork mounts with optimal cutting temperature medium (OCT) (Tissue-Tek™, CRYO - OCT Compound, Fisher Scientific UK Ltd, Leicestershire, UK). The time between experiment end and freezing was less than 2 min. Samples were serially sectioned (50 µm) parallel to the urothelial surface using a cryostat (Leica CM3050 S, Leica Microsystems, Buckinghamshire, UK) and sections collected in pre - weighed 1.5 ml eppendorf tubes. Tissue sections between 0 and 250 µm (urothelium) were collected individually for analysis. Groups of four 50 µm tissue sections between 250 and 1,050 (upper lamina propria) and ten 50 µm sections between 1,050 and 1,550 µm (lower lamina propria) were collected and pooled for analysis. Similarly, groups of ten 50 µm tissue sections between 1,550 and 3,550 µm (detrusor muscle) were collected and pooled for analysis. For pooled samples, tissue depths were expressed as the midpoint depth of the sections. Tissue sections were weighed, homogenised and oxybutynin extracted in 0.75 ml of HPLC mobile phase for 36 h with 10 min sonication per sample. Samples were then centrifuged for 5 min (7000 RPM, 2680 g) and the supernatant isolated for analysis. The amount of oxybutynin in the bladder wall at each time point was quantified using HPLC - MS and average tissue concentrations achieved in the urothelium, lamina propria and detrusor muscle calculated by dividing the total amount of drug recovered by the total weight of tissue in that layer.

Intravesical instillation of oxybutynin

Ex vivo porcine bladders were prepared as described. Working in a shallow bed of oxygenated Krebs buffer, both ureters were ligated by suturing 0.5 cm away from the bladder and 30 ml of oxybutynin chloride solution (0.167 mg ml⁻¹ in deionised water) instilled via the urethra using an open - ended ureteral catheter (5 Fr, 70 cm, Cook medical Inc, Bloomington, IN, USA). Care was taken to avoid contact with the urothelial surface and post - instillation the urethra was sutured. Bladders were submerged in oxygenated Krebs buffer and maintained at 37 °C. To half of the bladders the catheter remained *in situ* and synthetic urine (composition: NaCl 105.5 mM, NaH₂PO₄ 3.2 mM, Na₃C₆H₅O₇·2H₂O 3.2 mM, MgSO₄ 3.9 mM, CaCl₂ 4.0 mM, Na₂SO₄ 17 mM, KCl 64 mM, Na₂C₂O₄ 0.3 mM and NaNO₃ 1.0 mM, pH 5.8, pre - equilibrated at 37 °C²⁵) was introduced at a rate of 1 ml min⁻¹ for the duration of the experiment (urine - diluted group). For the remaining bladders the catheter was retracted after drug instillation (undiluted group). After 60 min bladders were removed, emptied and opened with a single vertical incision along the length of the organ. To remove surface - bound drug, the urothelium was subjected to three saline rinses. Samples of bladder wall from areas of drug contact were excised using a scalpel. Samples were frozen in liquid nitrogen, sectioned and drug extracted and quantified as described.

Quantification of oxybutynin in the bladder wall

Oxybutynin was analysed by HPLC - MS (Quattro Ultima liquid chromatography - tandem mass spectrometer, Waters Micromass, Elstree, Herts, UK, coupled to a Thermo Finnigan Spec - traSYSTEM). The column was a Telos C18, 5 µm, 150 x 4.6 mm i.d column, Supelco Inc and the mobile phase was 50 % aqueous formic

acid (0.1 %) and 50 % acetonitrile. The injection volume was 10 μl and the flow rate 0.4 ml min^{-1} . Positive ion mass spectrometry was used for the detection, with single ion monitoring for the parent ion between 357.7 and 358.15 m / z . The limits of detection and quantification were 1 and 3 ng ml^{-1} respectively.

Histology

Samples of tissue were taken from bladders after 60 min intravesical instillation of oxybutynin chloride (undiluted and urine - diluted groups). Samples were fixed in 4 % buffered formaldehyde (Fisher Scientific UK Ltd, Leicestershire, UK) for 48 h at room temperature. Tissues were embedded in paraffin, sectioned at 10 μm thickness and stained with Mason's trichrome prior to examination under light microscopy.

Statistical analysis

All statistical analysis was performed using GraphPad Prism version 6.0c (GraphPad Software, Inc, San Diego, California, USA). For all comparisons, multiple t - tests with multiple comparisons corrected using the Holm - Šidák method were used. For all tests, the significance level was set at 5 % ($p = 0.05$).

Results

Transurothelial permeation profile of oxybutynin

The rate of permeation of oxybutynin into the bladder wall over 60 min was constant (Figure 1A) with concentrations achieved in the urothelium, lamina propria and detrusor muscle generally increasing with time. The exception to this was the slight decrease between the 40 and 60 min timepoints in the lamina propria layer (Figure 1C). An apparent transurothelial permeability coefficient

(K_p) of $1.36 \times 10^{-05} \text{ cm s}^{-1}$ was determined. Importantly we have made the assumption that sink conditions are maintained in the lower tissue layers throughout these experiments. This assumption was based upon the low concentrations of oxybutynin achieved in these layers (maximum concentration of $\sim 62 \mu\text{g g}^{-1}$ achieved in the lamina propria, Figure 1C) compared to the drug's aqueous solubility ($\sim 50 \text{ mg ml}^{-1}$ ²⁶). Given these tissue concentrations represent $\sim 0.12 \%$ of saturated solubility, a value far lower than the $\sim 10 - 20 \%$ sufficient to maintain sink conditions²⁷, this assumption is reasonable. However it is appreciated that aqueous solubility is only an estimation of tissue solubility and therefore permeation parameters are referred to as 'apparent'.

Oxybutynin concentrations in the detrusor muscle (more clearly visible in Figure 1D) increased with time with concentrations of 0.27, 0.82 and $1.77 \mu\text{g g}^{-1}$ achieved at 20, 40 and 60 min respectively. Mass balance studies showed that on average 94 % of the applied dose was recovered per Franz - type cell setup (Figure 1B).

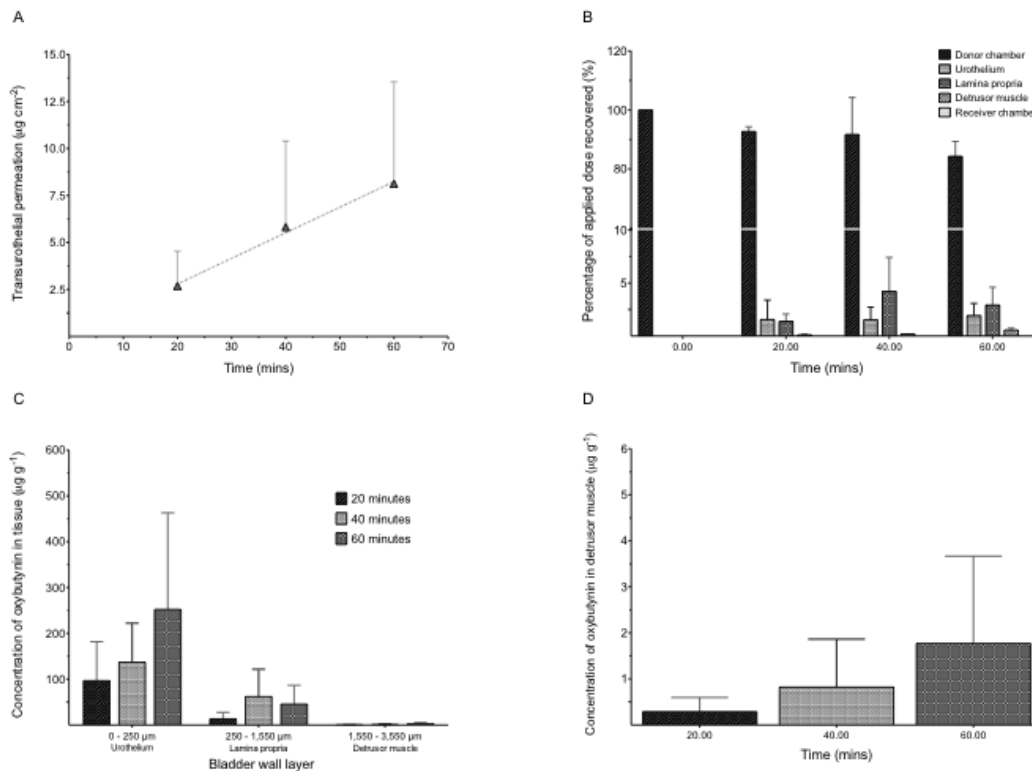


Figure 1. A) Transurothelial permeation of oxybutynin into the bladder wall after 20, 40 and 60 min. An apparent transurothelial K_p of $1.36 \times 10^{-5} \text{ cm s}^{-1}$ was calculated by normalising the apparent flux (J , calculated from the slope of the permeation profile) to the concentration of oxybutynin applied to the urothelium ($167 \mu\text{g ml}^{-1}$). B) Percentage recovery of oxybutynin from the Franz - type apparatus during permeability studies. Average recovery across the three sampling timepoints was 94 % of the applied dose. Drug concentrations in the receiver chamber were below detection limits at all timepoints. C) Average oxybutynin concentrations achieved in the urothelium, lamina propria and detrusor muscle after 20, 40 and 60 min. D) Average oxybutynin concentrations achieved in the detrusor muscle after 20, 40 and 60 min. For all figures, $n = 4$ tissue sections from 4 bladders \pm SD.

Intravesical instillation of oxybutynin

Following 60 min intravesical instillation with 30 ml of 0.167 mg ml⁻¹ oxybutynin solution (undiluted dose), average tissue concentrations of 298.69, 43.65, 0.93 and 25.81 µg g⁻¹ were achieved in the urothelium, lamina propria, detrusor muscle and whole bladder wall respectively (Figures 2 – 3, blue symbols and bars). Urothelial concentrations were significantly greater than those in the lamina propria, which in turn were significantly greater than those in the detrusor muscle ($p < 0.05$). When urine - dilution of the intravesical dose was simulated, tissue oxybutynin concentrations were significantly reduced (102.73, 23.81, 0.56 and 10.14 µg g⁻¹ for the urothelium, lamina propria, detrusor muscle and whole bladder wall respectively) ($p < 0.05$, Figures 2 - 3, yellow symbols and bars). As was the case with the undiluted instillation, urothelial concentrations were significantly greater than those in the lamina propria, which in turn were significantly greater than those in the detrusor muscle. For both instillations, the concentration of drug achieved in the urothelium and lamina propria was greater than reported IC₅₀ values for oxybutynin in isolated detrusor muscle (Figure 2B)²⁸. However for both instillations, the drug concentrations achieved in the detrusor muscle fell below this value.

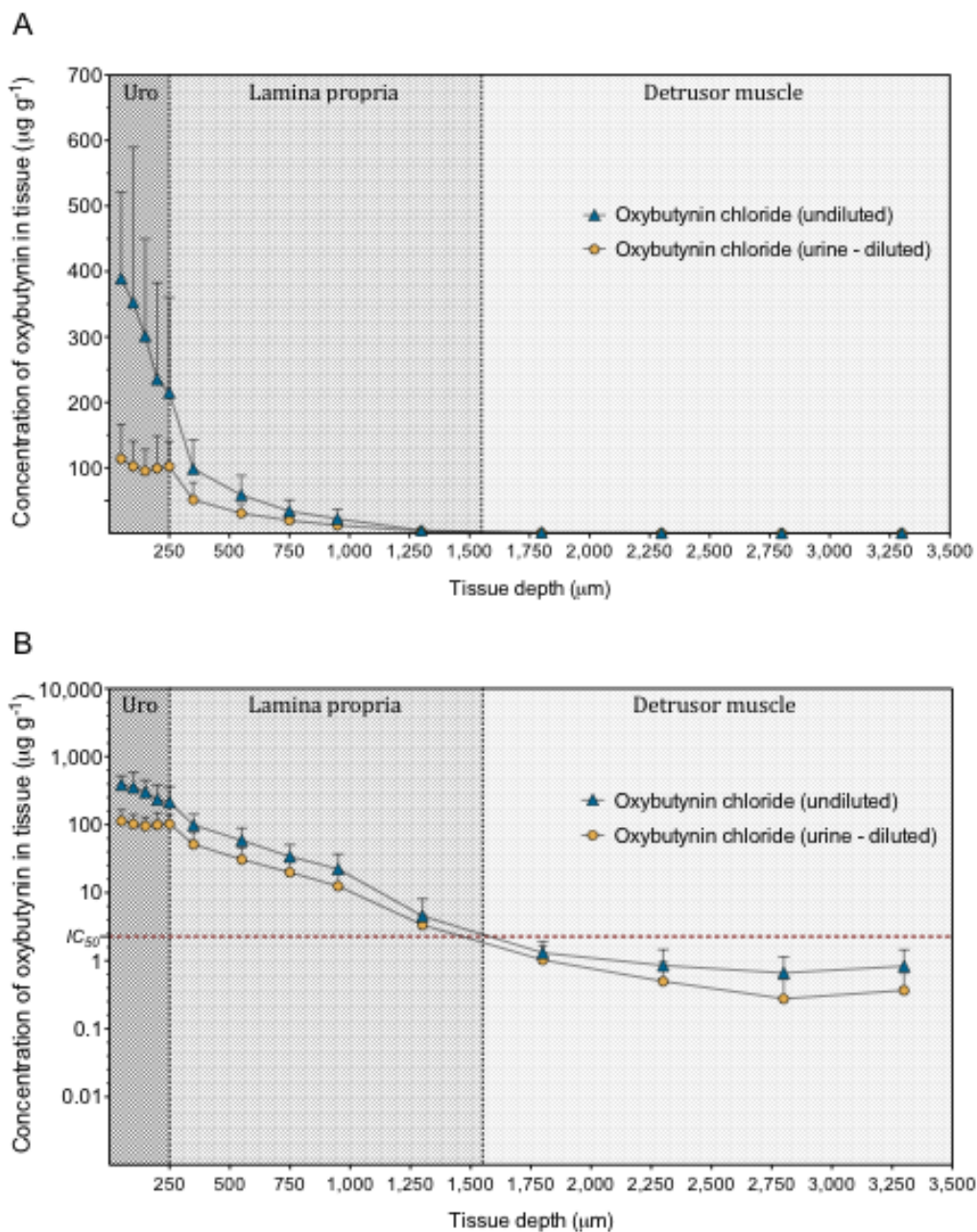


Figure 2. Concentration - depth profile (A) and Log - concentration - depth profile (B, same data as A) showing the concentrations of oxybutynin achieved at different depths of the bladder wall following 60 min intravesical instillation with undiluted (blue triangle) and urine - diluted (yellow circle) oxybutynin solution (0.167 mg ml^{-1}). From a reported pIC_{50} value of 5.24 mol L^{-1} ²⁶, an IC_{50} of 2.27 µg g^{-1} (red dotted horizontal line in figure 2B) was calculated for oxybutynin²⁷. For both figures, bladder wall layers are represented by the following depths; Urothelium ($0 - 250 \text{ µm}$), lamina propria ($250 - 1,550 \text{ µm}$),

detrusor muscle (1,550 – 3,500 μm). For pooled samples, tissue depths were expressed as the midpoint depth of the sections. For both figures, $n = 10$ tissue samples from 2 bladders \pm SD.

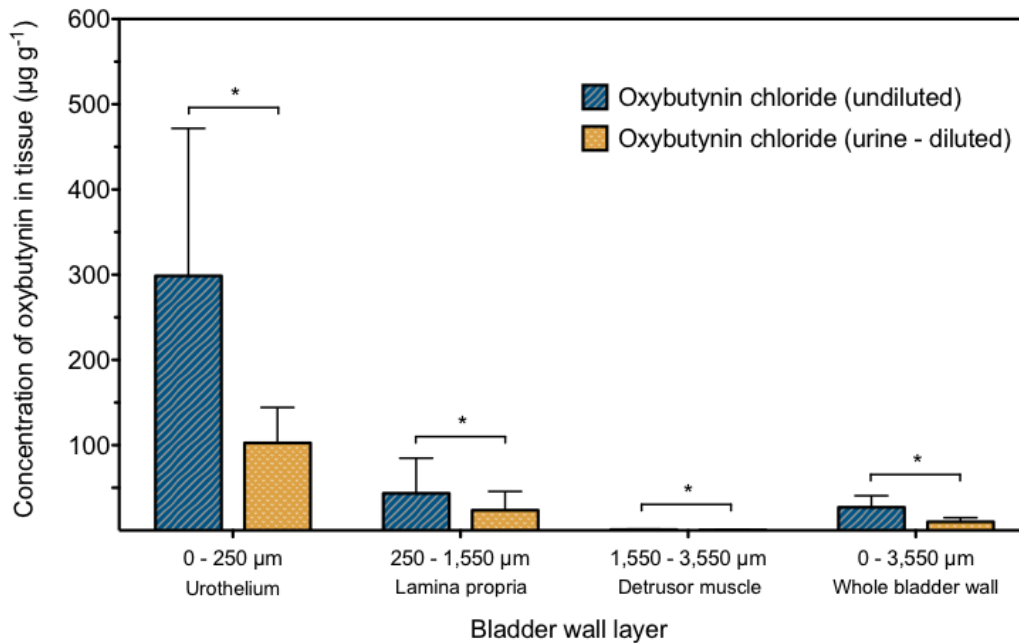


Figure 3. Average drug concentrations achieved in the different layers of the bladder wall following 60 min intravesical instillation with undiluted (blue) and urine - diluted (yellow) oxybutynin solution (0.167 mg ml^{-1}). In all bladder wall layers, concentrations achieved following instillation with the undiluted oxybutynin solution were significantly greater than those achieved with the urine - diluted dose (multiple unpaired t - tests, $*p < 0.05$). $n = 10$ tissue samples from 2 bladders \pm SD.

Histology

Analysis of histological sections taken from *ex vivo* bladder tissue after 60 min intravesical instillation with oxybutynin solution (Figure 4A and B) shows normal bladder morphology with the presence of an intact, multi - cell urothelium (pink) and normal lamina propria (blue).

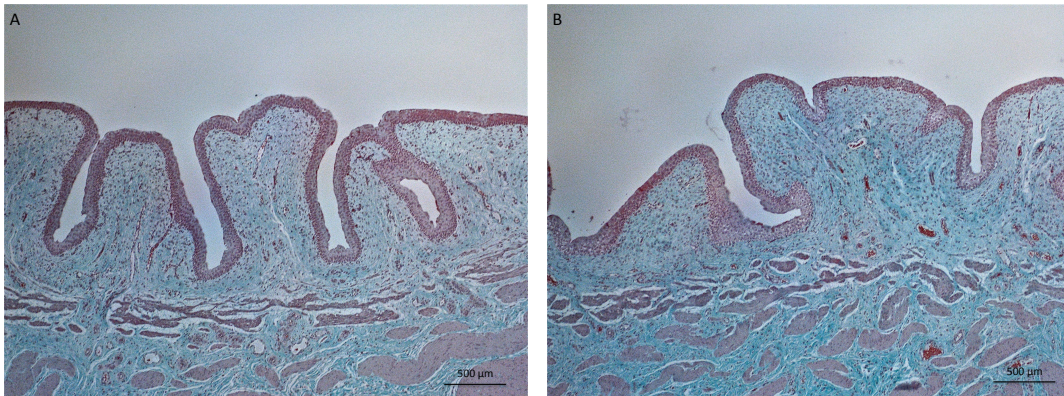


Figure 4. Photomicrographs of Masson's trichrome stained porcine bladder tissue taken from bladders A) following 60 min instillation with undiluted oxybutynin solution (0.167 mg ml^{-1}) and B) following 60 min instillation with urine - diluted oxybutynin solution (0.167 mg ml^{-1}). All images 50x magnification (scale bar represents $500 \mu\text{m}$).

DISCUSSION

Despite the recent introduction of Mirabegron (β_3 agonist), antimuscarinics remain first - line pharmacotherapy for the treatment of OAB² with oxybutynin the most widely prescribed drug³⁰. It is well established that intravesical oxybutynin is beneficial for the treatment of OAB in patients refractory to oral treatment⁶, although the MOA remains unclear. Originally believed to act by inhibiting M_3 muscarinic receptors in the detrusor muscle, there is now considerable evidence that antimuscarinics activate muscarinic receptors at the

urothelial and / or suburothelial level to modulate the afferent arc of the micturition cycle³¹⁻³⁶. When instilled intravesically at the same concentration used in this study, oxybutynin significantly increased bladder capacity, intercontraction interval and pressure threshold (indicators of bladder storage function) without decreasing detrusor contractility in the rat³². Instillations were retained in the bladder for short periods of time and cystometric effects observed immediately after emptying suggesting a local action on muscarinic receptors in the urothelium or suburothelial layer rather than the underlying detrusor muscle. Oxybutynin is a non - selective muscarinic acetylcholine receptor antagonist and therefore the expression of muscarinic receptors in the bladder wall is significant. In addition to the detrusor muscle, all five subtypes of the muscarinic receptor (M₁ - M₅) are expressed by the human urothelium³⁷, whilst the interstitial cells of Cajal (ICCs) found in the lamina propria have been shown to express both M₂ and M₃ receptors³⁸. Interestingly there is evidence that in OAB, the M₂ and M₃ receptors on the ICCs exhibit increased immunoreactivity³⁸. The hypothesis that oxybutynin acts on muscarinic receptors at the mucosal level may explain the increased effectiveness of intravesical oxybutynin given the relatively high concentrations of drug presented to the urothelium in comparison to the low bladder bioavailability following oral dosing. Considering this, bladder wall concentrations achieved following intravesical delivery of oxybutynin are highly valuable in ascertaining target concentrations necessary to modulate sensory pathways. Additionally, quantifying drug concentrations in the different layers of the bladder wall may provide more information regarding the site of action of antimuscarinics. A recurring limitation to understanding the pharmacological site of action of

antimuscarinics in modulating bladder afferent activity has been the lack of understanding of the extent of permeation (and hence bladder layer and cell type likely to be exposed) of drug after direct application to the urothelium²¹. Quantifying concentrations of drug in the different layers of the bladder wall (concentration - depth profiling) is becoming an established technique and one which has been used to investigate the local delivery of chemotherapeutics^{39,40} and NSAIDs⁴¹. However such studies have not been carried out for anticholinergics. In this study we aimed to quantify the concentrations of oxybutynin achieved in the different layers of the bladder wall after intravesical instillation of a clinically relevant dose. Intravesical oxybutynin is usually administered in doses ranging between 0.3 and 0.6 mg kg⁻¹ per day divided over two or three instillations⁴². Although a range of concentrations have been used, most clinics crush and dissolve a single 5 mg oxybutynin chloride tablet in 30 ml of distilled water, making a 0.167 mg ml⁻¹ solution⁴³. It was for this reason that 0.167 mg ml⁻¹ oxybutynin in deionised water was used in this study. Like many intravesical therapies, oxybutynin is commonly instilled for 1 h⁴⁴ and therefore an exposure time of 60 min was chosen.

In agreement with others^{39,40} we recently showed that *ex vivo* porcine tissue is suitable for use in permeability studies with urothelial integrity maintained for several hours post excision⁴¹. To assess the effect of oxybutynin on the intactness of the urothelium and general gross morphology of the *ex vivo* porcine bladder tissue, sections from bladders instilled with drug solution were treated to a Masson's trichrome stain. Histological results suggested no significant changes in the overall morphology of the tissue with intact, normal urothelium (ranging 4 - 8 cells) present in both the undiluted and urine - diluted oxybutynin instillations

(Figure 4A and B). This concurs with other studies which have reported no evidence of mucosal or bladder wall abnormality after exposure to oxybutynin^{24,45}.

Results of the transurothelial permeation study (Figure 1) show oxybutynin is capable of crossing the urothelium and penetrating into the underlying bladder wall (apparent transurothelial $K_p = 1.36 \times 10^{-05} \text{ cm s}^{-1}$). Mass balance studies showed good recovery of drug from the diffusion apparatus (94 % on average), especially considering the complex process involved in quantifying drug in each of the bladder wall layers. As one may expect, given the urothelial barrier function and the short experimental time frame, the vast majority of the drug was recovered from the donor compartment. To date only one group has investigated the penetration of oxybutynin into the bladder wall²⁴. Di Stasi *et al* investigated the differences in bladder wall concentration achieved after the passive or electromotive administration of oxybutynin into *ex vivo* human bladder tissue²⁴. From a reported apparent flux value of $0.16 \mu\text{g cm}^{-2} \text{ min}^{-1}$ after passive delivery, an apparent transurothelial K_p of $5.93 \times 10^{-05} \text{ cm s}^{-1}$ can be calculated for the passive diffusion of oxybutynin. Although higher than the apparent K_p of $1.36 \times 10^{-05} \text{ cm s}^{-1}$ calculated in this study, values are comparable in that they are notably higher than those reported previously for other drugs^{41,46,47}. This, in conjunction with the circumvention of first - pass metabolism, may explain recent work showing that intravesical oxybutynin results in significantly higher systemic bioavailability in comparison to oral oxybutynin²². Interestingly in guinea pigs, oxybutynin has been shown to increase bladder permeability to technetium compared to phosphate buffered saline (~ 5 times increase in permeability)⁴⁸. It is suggested that oxybutynin, a

tertiary amine, increases permeability by inactivating the glycosaminoglycan (GAG) layer in a similar fashion to the mechanism of the quaternary amine protamine. Disruption of the GAG layer could explain the high apparent K_p value although it should be noted that the concentration of oxybutynin instilled (5 mg ml^{-1}) was ~ 30 times greater than that used in this study.

Using a different experimental setup, the intravesical delivery of oxybutynin was investigated in a whole bladder model. Whilst applying drug solution to the urothelial surface of bladder tissue sections is a valid way of investigating urothelial permeability, it is not truly representative of intravesical drug delivery. The major limitation being the absence of urine dilution of the instilled oxybutynin dose. By using a whole bladder we were able to incorporate this variable into our model. 1 ml min^{-1} is an accepted rate of urine production in humans hence its choice⁴⁹. Although urine drains into the bladder from the ureters, this study introduced synthetic urine via the urethra. Several factors influenced this decision: Firstly, oxybutynin solution was introduced via the urethra making it necessary to ligate both ureters to prevent any leakage of the instilled dose. Furthermore, urine was instilled to represent the dilution effect on the instilled dose that occurs *in vivo*. The same dilution effect will be achieved regardless of the site of urine introduction. Resulting concentration - depth profiles (Figure 2) were typical of those obtained for other drugs⁴¹ with the majority of drug sequestered in the superficial urothelium. For both instillations (diluted and undiluted), urothelial concentrations were significantly higher than those achieved in the underlying lamina propria which in turn were significantly higher than those in the detrusor muscle (Figure 3). The introduction of synthetic urine at a physiological rate had a marked effect on the permeation of

oxybutynin into the bladder wall with significantly lower concentrations of drug achieved in each layer compared to the undiluted drug solution (Figure 3). We and others have previously shown using pharmacokinetic modelling that the production of urine would have significant effects on drug concentrations achieved in the bladder wall^{41,50}. Urine production is therefore an integral part of the intravesical delivery process.

Average oxybutynin concentrations achieved in the detrusor muscle after 60 min instillation with the undiluted and urine - diluted drug solutions were 0.93 and 0.56 $\mu\text{g g}^{-1}$ respectively. These concentrations were 325 - fold and 183 - fold lower than the urothelium and 47 - fold and 43 - fold lower than that found in the lamina propria for the undiluted and urine - diluted oxybutynin doses respectively. Based on reported IC_{50} values for oxybutynin in isolated detrusor muscle²⁸ (red dotted line in Figure 2B) and that systemic clearance would further decrease these concentrations *in vivo*, it is unlikely that concentrations achieved after instillation under either condition would significantly inhibit detrusor contraction. This was the case at all depths of detrusor muscle investigated including the most superficial layers located immediately below the lamina propria.

This study sought to investigate the distribution and tissue concentrations achieved following the intravesical delivery of oxybutynin. Although the effect of oxybutynin concentration on detrusor contractility was not investigated, drug distribution data provides further supportive evidence that an antimuscarinic MOA based exclusively on direct detrusor inhibition is unlikely.

CONCLUSIONS

A detailed analysis of oxybutynin concentrations achieved in the urothelium, lamina propria and detrusor muscle after intravesical delivery is provided. Oxybutynin permeated the bladder wall at a higher rate than other drugs previously investigated. For the first time the effect of intravesical dose dilution, as a result of urine production, has been incorporated into *ex vivo* investigations and the significant effect this has on drug delivery reported. Concentration - depth profiles suggest that following intravesical therapy, oxybutynin concentrations achieved in the detrusor muscle would be insufficient to directly inhibit bladder wall contraction. This adds weight to the argument that intravesical antimuscarinics do not exclusively exert their effect through direct inhibition of the detrusor muscle.

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None

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