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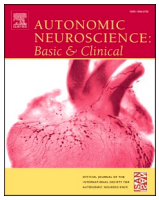

Monotherapy with tolterodine or mirabegron is insufficient for ameliorating cyclophosphamide-induced bladder overactivity in rats

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ABSTRACT

Monotherapy continues to be the most common pharmacological treatment option for patients with overactive bladder (OAB), despite evidence indicating that it may have inferior efficacy compared to combination therapy. This seems to be especially true for patients with concomitant cystitis. The current study examined the effects of monotherapy with either the antimuscarinic tolterodine or the β3 agonist mirabegron on bladder overactivity induced by bladder inflammation. Further, the possible involvement of nitric oxide (NO) was studied. For this purpose, rats were pretreated with either drug for 10 days. Bladder inflammation was induced by intraperitoneal injection with cyclophosphamide, with saline serving as control. Micturition parameters were assessed in a metabolic cage. Meanwhile, urine samples were collected and further analysed for NO content. After 16 h, the animals were euthanized, and their bladders were excised and examined immunohistochemically for signs of inflammation. Cyclophosphamide treatment led to bladder overactivity and obvious signs of inflammation. Neither treatment with tolterodine nor mirabegron could significantly alleviate the induced overactivity or the observed inflammation. Further, while induction of inflammation led to a significant increase in NO production, neither drug seemed to act by further enhancing its production. On the contrary, treatment with either tolterodine or mirabegron significantly decreased NO production in cyclophosphamide treated rats. Considering previous findings showing significant improvement by combination therapy, the current study indirectly implies this as the superior treatment option. Further studies are needed to verify the involvement, or lack thereof, of NO in the mechanism of action of drugs used to treat OAB.

1. Introduction

Regardless of its aetiology, inflammatory bladder disease displays similar symptoms including pain in the pelvic area, urgency and nocturia. These symptoms have significant impact on the patient's quality of life, but very few effective pharmacological treatment options exist. Therefore, the need for better treatment is imperative (Smaldone et al., 2009). Currently, antimuscarinics and β3 adrenoceptor agonists are utilized to alleviate symptoms of bladder overactivity and reduce urgency (Benner et al., 2010). Tolterodine is an antagonist with a proposed selectivity for the bladder over the parotid gland by acting primarily on muscarinic receptors of the M3 subtype, thereby increasing its efficacy while limiting the risk of potential side effects (Garely and Burrows, 2004). However, studies have suggested that muscarinic antagonists, including those with M3 selectivity, have the potential to decrease production of nitric oxide (NO) during bladder inflammation (Andersson et al., 2012; Andersson et al., 2011). Decreased production of NO leads to decreased formation of cyclic GMP, which may directly impact detrusor contraction but also lead to altered purinergic signaling (Chakrabarty et al., 2019). This could explain why they often lack effect in the inflamed bladder.

While a number of antimuscarinics are currently in use, only two β3 adrenoceptor agonists have been approved by the FDA. The most commonly used β3 adrenoceptor agonist worldwide is mirabegron, which has a proposed mechanism of action *via* activation of β3 adrenoceptors located in the detrusor, leading to bladder relaxation (Imran et al., 2013). The mechanism of action is also partly believed to be *via* induction of an increased release of urothelially derived inhibiting factors, mainly NO (Murakami et al., 2007). It has been demonstrated that activation of urothelial β3 adrenoceptors increases intracellular calcium

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levels through cAMP accumulation, in turn increasing NO production (Birder et al., 2002). However, the actual effect of mirabegron on the production of NO in the bladder still remains to be shown in more conclusive studies.

A recent study in rats on combination therapy with tolterodine and mirabegron showed that treatment with this combination normalized altered micturition patterns that arose upon induction of inflammation (Patel et al., 2020). The main effect was a normalization of urinary frequency, which was significantly enhanced in animals with cystitis. The results also showed an increase of NO in urine samples collected from the animals with bladder inflammation. A similar increase of NO production was seen upon combination therapy in healthy animals. However, it remained to be determined if this increase of NO was imperative for the successful treatment of symptoms of bladder overactivity during cystitis. The findings from the aforementioned article on the use of combination therapy against bladder overactivity have led to several possible hypotheses. The first is that the level of NO is not crucial for the treatment efficacy since NO production was not further enhanced by combination therapy. A second possibility is that a maximum production of NO is reached during inflammation, which makes it impossible to increase the NO level further. The third possibility is that mirabegron and tolterodine balance each other out in regards to NO, with tolterodine potentially decreasing and mirabegron potentially increasing its production (Patel et al., 2020). Lastly, it is also possible that either drug attenuates the inflammatory response seen in rat bladders treated with CYP.

To investigate this, it is necessary to study animals treated with either mirabegron or tolterodine as monotherapy. For this purpose, rats were pretreated for ten days with either saline, mirabegron or tolterodine, resulting in steady state concentrations of the drugs. On the tenth day, in order to allow for assessment of voiding behaviour, the rats were placed in a metabolic cage for 16 h with free access to water. However, 48 h prior to being placed in the metabolic cage, the rats were injected with an intraperitoneal bolus dose with either cyclophosphamide (CYP), in order to induce bladder inflammation, or saline, serving as control. During the 16 h in the metabolic cage, micturition frequency was measured, and the collected urine was subsequently analysed for NO content. After the assessment period, the rats were euthanized, and their bladders were excised and used for immunohistochemical analyses.

2. Methods and materials

2.1. Animals

The experimental procedures performed on animals in this study were approved by the local ethics committee at the University of Gothenburg (permit #1794/2018) and followed the ARRIVE guidelines. Currently, 48 male Sprague-Dawley rats (300–600 g), purchased from Charles River Laboratories (Calco, Italy) and Janvier Labs (Le Genest-Saint-Isle, France), were included in the experiments. The requirements stated in the ethical permit were followed in all the experimental procedures, and they had full adherence to the ARRIVE guidelines. Furthermore, the chosen species was used because it was evaluated to give translational data to human, and most of the existing lower urinary tract models are developed in rats. Therefore, valid comparisons with previous data are possible.

2.2. Study design

The animals were randomized to pretreatment two times a day for 10 days with either saline (0,9 % NaCl in 6% DMSO, s.c; 1 mL/kg per injection), mirabegron (0,3 mg/mL in saline with 6% DMSO, s.c; 1 mL/kg per injection; Adooq Bioscience, Irvine, USA) or tolterodine (0,025 mg/mL in saline with 6% DMSO, s.c.; 1 mL/kg per injection; Selleck Chemicals, Houston, USA). The doses were chosen to match the recommended dosing of the given drugs to OAB patients, and treatment over ten days was done to reach a steady state of the drugs before moving the animals to a metabolic cage. Forty-eight hours before moving the animals to a metabolic cage, half of the rats were randomized to be treated with a bolus dose of cyclophosphamide (in saline; 100 mg), in order to induce experimental cystitis. The corresponding control rats were treated with a bolus injection of saline (1 mL/kg, i.p.). This generated six groups (controls pretreated with saline, controls pretreated with mirabegron, controls pretreated with tolterodine, animals with cystitis pretreated with saline, animals with cystitis pretreated with mirabegron, animals with cystitis pretreated with tolterodine) with 6–8 animals in each group (Fig. 1).

2.3. Metabolic cage

Forty-eight hours after treatment with CYP or saline, the animals were placed for 16 h in a metabolic cage with free access to water, but not food due to risk of contamination of urine samples. The 16 h time-period commenced in the afternoon and ended the next morning, thus encompassing a light - dark – light cycle. The amount of water consumed and the total volume of urine was measured. Urine samples from each rat were collected and subsequently stored at −60 °C for further analysis. A WFL30-40B416 laser Doppler (SICK, St Albans, UK) near the bottom of the cage was used to detect each drop of urine. The data were recorded by a MP150WSW data acquisition system and the Acknowledge 3.8.1 software (BioPac Systems, Goleta, USA). The number of micturitions were measured by analysis of registered data and volume per micturition was measured by dividing the total volume of urine by the number of micturitions. All urine samples were sent to Dr. Bhavik Patel's lab at the University of Brighton for further analysis.

2.4. Immunohistochemistry

After spending 16 h in the metabolic cage, the animals were given an overdose of pentobarbitone (100–150 mg/kg i.p; pentobarbital; APL, Gothenburg, Sweden). Subsequently, the urinary bladders were harvested from the rats and fixed in 4% PFA solution. Thereafter, the rats were euthanized by puncture of the heart. After maximum 48 h in PFA solution, the bladders were transferred to and stored in a sucrose solution until they were sent to Histocenter (Mölndal, Sweden) for preparation of the tissues and subsequent staining. In brief, the fixed bladders were cut into 5 μm sections and mounted onto glass slides. To evaluate if the bladders were inflamed, they were stained with haematoxylin and eosin. The tissue slices were examined under a Nikon 90i brightfield and fluorescence microscope and pictures were taken with a DS-Fi camera and analysed with the NIS Element 4.3 software (Nikon Corporation, Tokyo, Japan).

2.4.1. Evaluation of inflammation

Pictures were taken at 4× magnification and given a random number so evaluation (grading) of inflammation would be blinded. In total, four persons were involved in the grading procedure and each gave an individual grade for each tissue. Similar to previous studies in which the grade of inflammation in the prostate was evaluated (Inamura et al., 2018; Aydogdu et al., 2021), each bladder was graded from 1 to 4 where “1” indicates no signs of inflammation, “2” indicates few signs of interstitial oedema and a few or no signs of inflammatory cells, “3” indicates some signs of interstitial oedema, some signs of inflammatory cells, thinner transitional epithelium and ulceration and “4” indicates serious interstitial oedema, signs of inflammatory cells, thinner transitional epithelium and ulceration. Damage to the urothelium and vasculature increase was accounted for. An aggregate grade of inflammation for each tissue was reached by determining the average of each blinded evaluator's grade.

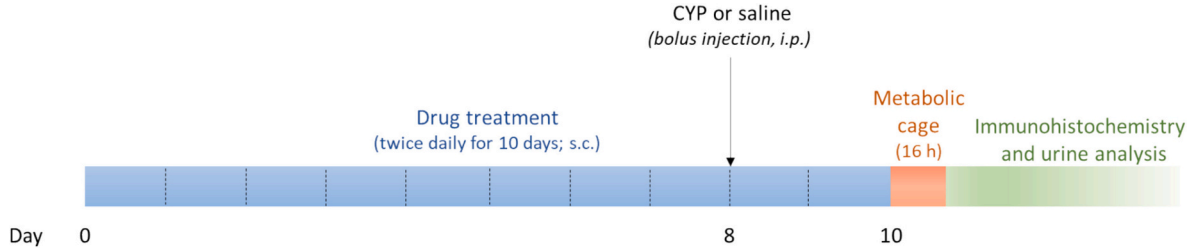

Fig. 1. Study timeline. Treatment with either the muscarinic M3 antagonist tolterodine, the β3-adrenoceptor agonist mirabegron or saline, serving as control, was initiated 10 days prior to metabolic cage experiments. On the eighth day, 48 h prior to the metabolic cage experiments, an intraperitoneal bolus injection with either cyclophosphamide (CYP), to induce experimental cystitis, or saline, serving as control, was given. Urine was collected during the metabolic cage experiments (16 h) and the level of nitrite was measured. After the metabolic cage experiments, the animals were euthanized and their urinary bladders were excised, followed by immunihistochemical analysis of grade of bladder inflammation.

2.5. Electrochemical analysis of urinary nitrite

The amount of nitrite in the urine samples was determined by electrochemical measurement. In an oxygenated environment, NO is quickly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-). In biological samples, the levels of measured nitrite are considered to correspond to levels of produced NO.

Urine samples from each animal were gathered during the metabolic cage experiments and stored at −60 °C until they were shipped on dry ice to the University of Brighton. The individual samples were continuously kept on ice and vortexed prior to analysis, which was conducted by a three-electrode setup. This setup consisted of a platinum wire counter (CH instruments, Bee Cave, USA), an AglAgCl reference and a 2 mm platinum working electrode. Next, differential pulse voltammetry (DPV) was run with 0–1.0 V as a potential window using a 760 potentiostat (CH instruments). In the differential pulse voltammetry, an amplitude of 0.05 V, pulse width of 0.06 s and a scan rate of 0.004 V was used. The calibration was performed with 0.1–5 mM sodium nitrite in 0.1 M phosphate-buffered saline (PBS). The resultant nitrite oxidation peak amplitude was measured, converted to concentration using a calibration response and then normalized to the urine volume to determine nitrite content.

2.6. Statistical analysis

Data from the metabolic cage experiments and electrochemical urine analysis were compared statistically by two-way analysis of variance (ANOVA) followed by Tukey's correction for multiple comparisons. A non-parametric Kruskal-Wallis test, followed by Dunnett's multiple comparisons test, was used to statistically compare immunohistochemical data, *i.e.* grade of inflammation. Statistical significance was considered for p-values under 0.05. All graphs were generated and data calculated by using GraphPad Prism 9.1.2 (GraphPad Software Inc., San Diego, USA). All data are presented as mean±SEM.

3. Results

3.1. Metabolic cage parameters

No significant differences could be detected between the groups when comparing water consumption or the total urine volume (Table 1). Induction of bladder inflammation with CYP led to bladder overactivity, demonstrated by a significant increase in the number of micturitions per hour (*i.e.* frequency; from 0.83 ± 0.06 to 1.51 ± 0.17 in healthy and inflamed saline treated rats, respectively; $p = 0.012$; Table 1) and a corresponding decrease in the volume per micturition (from 0.88 ± 0.09 to 0.48 ± 0.05 mL in healthy and inflamed saline treated rats, respectively; $p = 0.006$; Table 1).

Monotherapy with mirabegron or tolterodine did not alter frequency or the volume of micturition in neither healthy nor inflamed rats (Table 1). However, while the frequency remained significantly increased in all inflamed groups regardless of treatment, no significant differences could be detected between healthy and inflamed rats regarding the volume per micturition when treating with mirabegron or tolterodine (Table 1).

3.2. Nitrite levels in urine

Induction of bladder inflammation with CYP led to a significant increase in the urine levels of nitrite (*i.e.* the amount of produced NO; from 21.46 ± 11.31 to 50.95 ± 22.72 μM in healthy and inflamed saline treated rats, respectively; $p = 0.011$; Fig. 2). Monotherapy with mirabegron or tolterodine did not alter the nitrite urine levels in healthy rats (Fig. 2). However, monotherapy with either mirabegron or tolterodine led to significantly lower urine levels of nitrite in rats with CYP-induced bladder inflammation (from 50.95 ± 8.03 μM in saline-treated rats to 26.78 ± 14.42 and 25.62 ± 14.25 μM in mirabegron and tolterodine treated animals, respectively; Fig. 2).

3.3. Grading of inflammation

Intraperitoneal injection with CYP led to significant signs of bladder inflammation, as indicated by immunohistochemical staining with haematoxylin and eosin (HE) for analysis of inflammatory grade (p =

Table 1
Metabolic cage parameters. Healthy animals (Healthy) and animals with induced bladder inflammation (Cystitis) were treated for 10 days with either saline, mirabegron or tolterodine. The metabolic cage data were gathered during the 10th day of treatment. n = 6–8 in each group. Data are presented as mean ± SEM. Statistical comparisons were made by two-way analysis of variance (ANOVA) followed by Tukey's correction for multiple comparisons. *$p < 0.05$ and **$p > 0.01$, as compared to control (Healthy-saline).

	Healthy - saline	Healthy - mirabegron	Healthy - tolterodine	Cystitis - saline	Cystitis - mirabegron	Cystitis - tolterodine
Micturitions/h	0.829±0.060	0.911±0.067	0.858±0.130	1.508±0.166*	1.778±0.308**	1.491±0.203*
Vol/micturition (mL)	0.878±0.094	0.730±0.066	0.745±0.111	0.483±0.051**	0.564±0.109**	0.605±0.087*
Water consumption (mL)	13.2±6.6	10.1±5.2	13.4±4.1	12.8±4.9	17.4±5.3	13.3±5.8
Total urine volume (mL)	11.5±4.1	10.0±1.5	11.4±6.8	9.9±1.4	13.4±4.3	12.3±5.4

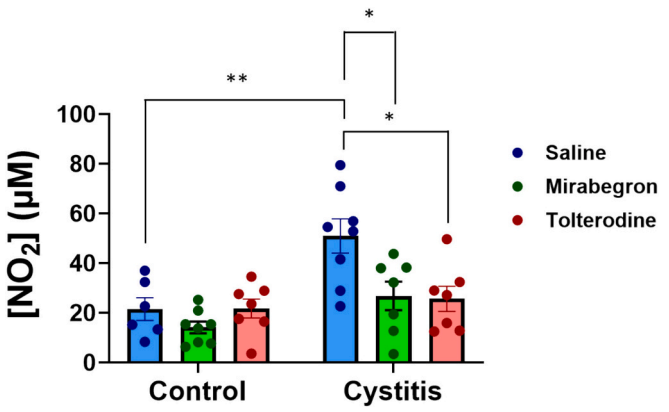

Fig. 2. Urine nitrite levels. Healthy animals (control) and animals with induced bladder inflammation (cystitis) were treated for 10 days with either saline, mirabegron or tolterodine. Urine samples were gathered on the 10th day, while the animals were in a metabolic cage. Urine nitrite levels, measured by differential pulse voltammetry, increased significantly during cystitis. This increase was significantly attenuated by treatment with either mirabegron or tolterodine. n = 6–8 in each group. Data are presented as mean ± SEM with each individual value depicted. Statistical comparisons were made by two-way analysis of variance (ANOVA) followed by Tukey's correction for multiple comparisons. *p < 0.05 and **p > 0.01, as compared to control-saline.

0.0063; Fig. 3a–b). Neither treatment with mirabegron (p = 0.0311 between healthy and inflamed bladders from mirabegron treated rats; Fig. 3a) nor tolterodine (p = 0.0157 between healthy and inflamed bladders from tolterodine treated rats; Fig. 3a) counteracted the significant increase of the inflammatory grade after CYP injection.

4. Discussion

In the current study, the effects of monotherapy with either tolterodine or mirabegron on the micturition pattern and release of NO in rats with CYP-induced cystitis were investigated. It was demonstrated that induction of bladder inflammation led to signs of bladder overactivity and that this was accompanied by an elevation of urine levels of nitrite, indicating an increase in the production of NO. Meanwhile, no significant differences were observed between the groups regarding water intake or total urine production. These findings are in line with previous studies (Patel et al., 2020; Souza-Fiho et al., 1997; Lundberg et al., 1996; Andersson et al., 2008). However, while previous studies could demonstrate how combination therapy with tolterodine and mirabegron could normalize the micturition pattern in rats with CYP-induced cystitis (Patel et al., 2020), tentatively by affecting production of NO, no significant normalization of micturition parameters could be seen in the current study. Further, the urine levels of nitrite were decreased in the cystitis treatment groups (mirabegron and tolterodine, respectively), as compared to the cystitis group that only received saline.

Clinical evidence has accrued showing that combination therapy with an antimuscarinic and mirabegron has better efficacy than respective monotherapy when treating overactive bladder (OAB) (Allison and Gibson, 2018; Andersson et al., 2018; Gratzke et al., 2018; Herschorn et al., 2017). Similar evidence has been presented in animal studies (Furuta et al., 2016). Despite this, the use of combination therapy to treat OAB is still not the clinical standard. Further, several studies have failed to show adequate efficacy of monotherapy with either drug when aiming to alleviate symptoms of inflammatory bladder disease (Yamada et al., 2018; Chapple et al., 2014). The current study strengthens the notion of monotherapy not having sufficient efficacy to treat bladder overactivity that arises due to cystitis. However, it should be pointed out that several clinical randomized trials have succeeded in showing acceptable efficacy for monotherapy with mirabegron or tolterodine against OAB (Yamaguchi et al., 2014; Chapple et al., 2013; Nitti et al., 2013; Kreder et al., 2002; Appell et al., 2001). One should therefore distinctly separate clinically diagnosed OAB from what is currently studied, namely bladder overactivity due to cystitis.

The results from the measurements of nitrite (*i.e.* NO) levels showed a significant increase in the urine from bladders with chemically induced cystitis, as compared to healthy controls. This increase was counteracted by treatment with tolterodine or mirabegron, respectively. Meanwhile, monotherapy with either drug had no effect on NO production in healthy animals. A previous study showed that combination therapy increased the urine levels of nitrite in healthy animals (Patel et al., 2020). In the same study, a similar increase in NO production was seen in urine from rats with CYP-induced cystitis, analogous to the current study. However, combination therapy did not further affect urine levels of nitrite in rats with CYP-induced cystitis. The previous study drew the conclusion that one or both of the drugs are capable of increasing NO production, also in healthy bladders, but that in a state of inflammation, during which NO production is already augmented, no additional increased production occurs. One of the main aims of the current study was to examine these interesting findings, possibly seeing if either drug was the one responsible for the augmented production of NO. However, the current data give no clear answer to that aim. Instead, it seems as if alone, none of the drugs affect NO production in the healthy bladder, while attenuating NO production in the inflamed bladder. This raises several new questions. One possible explanation for the ambiguities could be due to one or both of the drugs having anti- or pro-inflammatory effects, and thereby directly or indirectly affecting the involvement of NO in the initial inflammatory process. However, the current HE analysis showed no signs of this. On the contrary, the grade of inflammation was found to be identical in all CYP groups, regardless of treatment. Even though not indicated by macroscopic evaluation in a previous study (Patel et al., 2020), future studies should reexamine the possibility of combination therapy possibly affecting the development of bladder inflammation.

Earlier studies have indicated that after induction of bladder inflammation with CYP, activation of urothelial M3 and/or M5 muscarinic receptors can lead to production of NO (Andersson et al., 2012; Andersson et al., 2008). It was therefore not surprising that treatment with an anticholinergic drug, currently tolterodine, attenuated the production of NO. However, other studies have shown that activation of β3 adrenoceptors can increase production of NO, both in full-thickness bladder strips as well as in isolated urothelial cells from the rat bladder (Birder et al., 2002). It has therefore been assumed that mirabegron acts, at least partly, by augmenting urothelial production of NO. Considering that the combination of mirabegron and tolterodine increased the NO concentration in a previous study (Patel et al., 2020), it was expected that at least one of these drugs would do so when given as monotherapy, with mirabegron being the prime suspect. It was therefore surprising that the urine nitrite levels were significantly decreased also in the mirabegron treated CYP group. However, the importance of NO in the mechanism of action of β3 adrenoceptor agonists has previously been challenged. Animal models of acute urinary retention have failed to show significant effects of mirabegron on NO production, despite having a positive effect on bladder blood flow (Noh et al., 2021). Further, studies on rat and human corpus cavernosum have not indicated that mirabegron has any involvement of NO in its mechanism of action (Gur et al., 2016; Calmasini et al., 2015). The current study aimed to determine the importance of NO for the mechanism of action of drugs used to treat bladder overactivity, but the question remains unanswered. For this reason, it is disadvantageous that the current study did not include groups given combination therapy.

In future studies, it would be of interest to examine the effects of the drugs at additional doses and simultaneously monitor drug serum concentrations. However, the current study specifically aimed to examine the individual input of each drug as compared to a previous study in which the drugs were given as combination treatment (Patel et al., 2020). That study was preceded by a substantial number of pilot studies,

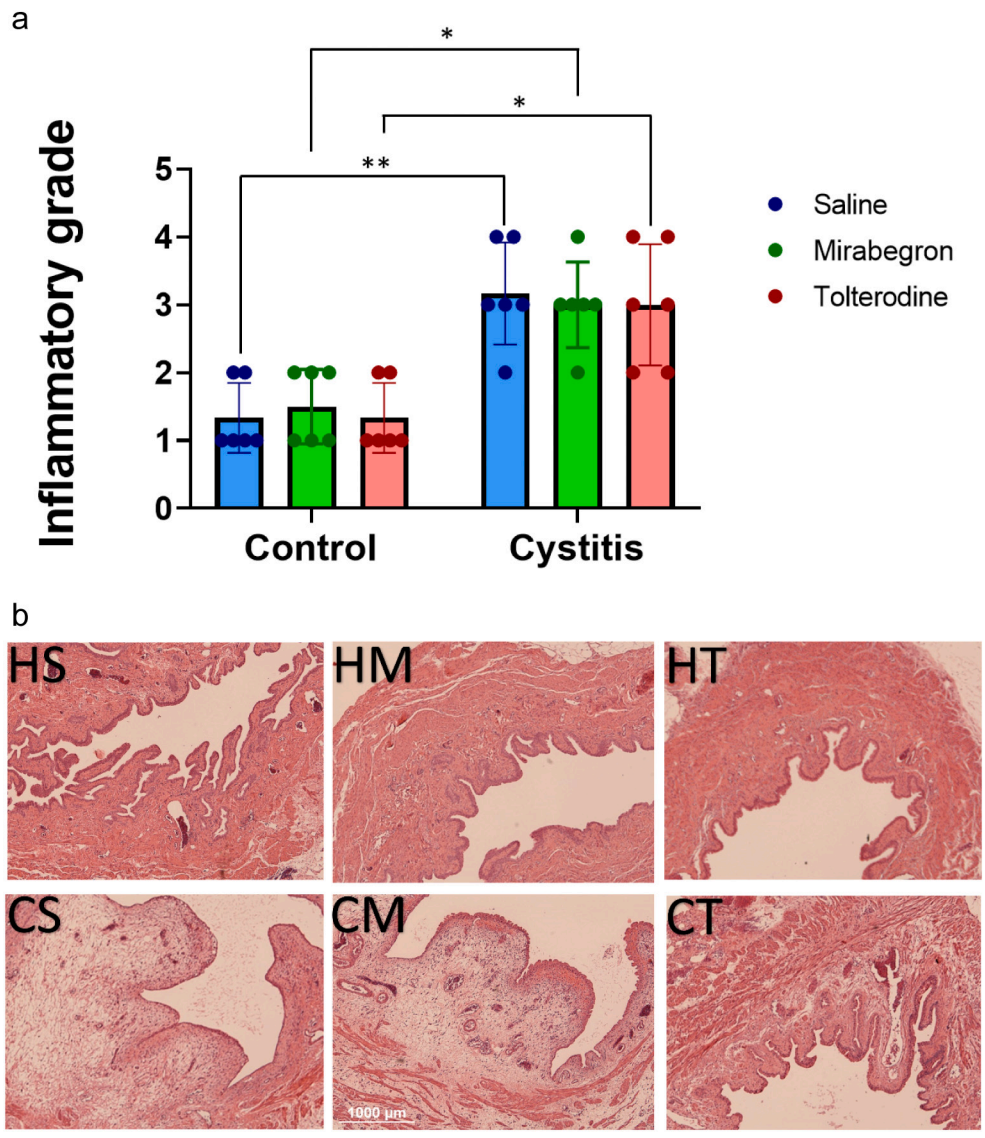

Fig. 3. a. Grading of inflammation. Healthy animals (control) and animals with induced bladder inflammation (cystitis) were treated for 10 days with either saline, mirabegron or tolterodine. Following metabolic cage experiments, urinary bladders were excised. Each bladder was given an inflammatory grade of 1–4. 1: no signs of inflammation 2: few signs of interstitial oedema and a few or no signs of inflammatory cells. 3: some signs of interstitial oedema, some signs of inflammatory cells, thinner transitional epithelium and ulceration. 4: serious interstitial oedema, signs of inflammatory cells, thinner transitional epithelium and ulceration. n = 6. Data are presented as mean ± SEM, with each individual grade depicted. Statistical comparisons were made by non-parametric Kruskal-Wallis test, followed by Dunnett's multiple comparisons test. *$p < 0.05$, **$p > 0.01$.
b. Representative images of HE stained bladders from each group. HS – saline-treated healthy (control). HM – mirabegron-treated healthy. HT – tolterodine-treated healthy. CS – saline-treated inflamed (cystitis). CM – mirabegron-treated inflamed. CT – tolterodine-treated inflamed.

to determine the correct dosing regimen. Further, the current doses are comparable to those given to treat humans. Therefore, the dosing regimen is believed to be correctly chosen. Future studies should also aim to determine the actual importance of NO for inflammatory disease in the lower urinary tract. The possibility remains that NO has detrimental effects in certain instances, while having protective effects in other. Further, other drugs, that have a more unambiguous effect on the NO/sGC/cGMP pathway, should be examined in a similar manner as was currently investigated for tolterodine and mirabegron to pinpoint the importance of altered nitrergic signaling in the treatment of lower urinary tract disease. In such a study, the effects of the drugs on the development of inflammation should be carefully examined.

5. Conclusions

The current animal study shows that CYP-induced bladder overactivity could not be counteracted by monotherapy with neither tolterodine nor mirabegron. Likewise, there were no indications that monotherapy with either drug attenuated bladder inflammation caused by CYP injection. Considering that previous similar studies have demonstrated that combination therapy with an antimuscarinic and a β3 agonist can counteract CYP-induced bladder overactivity, the current data strengthen the notion that monotherapy in many cases in lower urinary tract disease may be insufficient. Instead, combination therapy should likely be advertised as the preferred option. While previous studies implicated an involvement of NO in the efficacy of combination therapy, the current study could not demonstrate any such link when either drug is given alone.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the

principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research. This study was approved by the local ethics committee at the University of Gothenburg (permit #1794/2018).

CRediT authorship contribution statement

Håvard Fjelltveit: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Thomas Carlsson:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation. **Fernando Perez:** Writing – review & editing, Methodology, Investigation, Data curation. **Ozgu Aydogdu:** Writing – review & editing, Methodology, Investigation, Data curation. **Bhavik Patel:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Michael Winder:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

None of the authors have any conflicts to declare.

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Data availability

Data will be made available on request.

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