Original article

Effect of solifenacin plus and minus antioxidant supplements on the response to experimental outlet obstruction and overactive bladder dysfunction in rabbits—Part 2

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ABSTRACT

Objective: One of the most common forms of bladder dysfunction is related to the development of unstable bladder contractions (bladder overactivity). Solifenacin is a relatively new, selective, antimuscarinic agent that has been shown to be particularly useful in the treatment of overactive bladder (OAB) dysfunctions in both men and women. Experimentally, we have demonstrated that OAB and obstructive bladder dysfunction are associated with the generation of free radicals and oxidative damage to the bladder. The hypothesis tested was that solifenacin + coenzyme Q10 + α-lipoic acid (CoQ + LA) would be more effective in the treatment of OAB than either would be individually.

Materials and methods: Forty-eight male White New Zealand rabbits were separated into 8 groups of 6 rabbits each. The following oral treatments were given to each group: Groups 1 and 5, vehicle (saline); Groups 2 and 6, solifenacin; Groups 3 and 7, CoQ + LA; Groups 4 and 8, solifenacin + CoQ + LA. After three weeks of treatment (by oral gavage), the rabbits in Groups 1–4 received partial outlet obstruction as detailed above. The rabbits continued their treatments for four weeks following surgery. At the end of this four-week period, immediately following urodynamic and physiological studies, the bladder was excised and the muscle and mucosa were separated and frozen for biochemical research.

Results: The following marker enzymes were quantitated: choline acetyltransferase, citrate synthase, catalase, superoxide dismutase, and malondialdehyde (MDA, marker for lipid peroxidation). Solifenacin had no effect on citrate synthase activity of the bladder smooth muscle. However, pretreatment with both the antioxidants or the combination of solifenacin + antioxidants protected the citrate synthase activity such that it remained at control values. Partial outlet obstruction (PBOO) resulted in a 60% decrease in choline acetyltransferase activity (ChAT) activity, while solifenacin had no effect on reduced ChAT activity. Both the antioxidant and combination therapy resulted in maintaining ChAT activity at control values. PBOO resulted in a significant increase in MDA of both the muscle and mucosa. For both tissues, all treatments resulted in a significantly lower MDA content. In general, the results demonstrate the combination of solifenacin + antioxidants was more effective than either solifenacin or antioxidants alone.

Conclusion: The addition of the antioxidants CoQ + LA works synergistically with solifenacin in the treatment of obstructive bladder dysfunction and OAB.

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1. Introduction

The urinary bladder is a smooth muscle organ whose function is to collect and store urine at low intravesical pressures and then to periodically expel urine via a highly coordinated, sustained contraction.1–3 One of the most common forms of dysfunction is related to the development of overactive bladder dysfunction (OAB).4,5 In
mals, this condition often accompanies benign prostatic hyperplasia (BPH) and results in the symptoms of urgency and frequency. In females, unstable bladder contractions can result in urgency and frequency and can also induce urge incontinence. The incidence of incontinence increases significantly with age and is especially prevalent after menopause.5,6

Most forms of overactivity/unstable bladder contractions are mediated through the the release of acetylcholine and stimulation of muscarinic receptors localized on the detrusor smooth muscle membranes.7,8 Treatment for overactivity has traditionally used anti-muscarinic agents.

Solifenacin is a relatively new, selective, anti-muscarinic agent (M-3) that has been shown to be particularly useful in the treatment of OAB dysfunctions in both men and women.9-11 However, it is not clear whether there is a selectivity for the muscarinic mechanisms involved in OAB activity compared with the muscarinic mechanisms involved in micturition. Additionally, it is clear that BPH-induced bladder hypertrophy can alter the muscarinic receptor subtype distribution in the bladder and thus modify solifenacin’s activity.12-15 The etiology of bladder dysfunction secondary to BPH (OAB and poor bladder contraction) is, in part, caused by the generation of free radicals and oxidative damage to the bladder smooth muscle and mucosa.16,17

The combination of coenzyme Q10 (CoQ10) and alpha lipoic acid (LA) have proven to be very potent at preventing oxidative damage to the bladder caused by both direct ischemia/reperfusion and partial outlet obstruction.18-21 This combination of supplements significantly protected against the contractile, biochemical, and structural dysfunctions associated with increased free radical production.

The current study was designed to evaluate the hypothesis that solifenacin can work in synergy with CoQ10 and LA. Part one of this two-part study demonstrated that the combination of solifenacin + CoQ10 and LA were significantly more effective than either treatment alone in improving urodynamics, compliance, and in situ and in vitro contractile responses to a variety of contractile agonists. This current study presents the results of our biochemical tests.

2. Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Stratton V Medical Center, Albany, NY, USA.

2.1. Partial outlet obstruction22-24

Each rabbit was anesthetized, the bladder catheterized per urethra and then exposed through a midline incision. A 00 silk ligature was snugly tied around the catheterized proximal urethra and the catheter removed. This has been shown to be an excellent model of BPH-induced obstructive bladder dysfunction in men.25,26 Approximately 50% of these rabbits developed OAB, as observed during cystometry. Those rabbits that showed OAB progressed to severe bladder dysfunction at a significantly faster rate than rabbits that did not show OAB; this is similar to what happens with men with BPH and OAB. The etiology for obstructive bladder dysfunction has been shown to be via ischemia and free radical generation and damage.

2.2. In situ OAB27-29

Each rabbit was anesthetized and the right external carotid artery cannulated for blood-pressure monitoring. A polyethylene catheter was inserted through the rabbit’s right femoral artery until it reached the lower abdominal aorta. A heparinized, saline-filled polyethylene catheter was used for intra-arterial administration of acetylcholine (Ach). The bladder was exposed through a midline incision of the abdominal wall, and catheterized through the bladder dome for both monitoring bladder pressure and infusion of saline. At this point, a ligature was placed around the external proximal penis and the bladder filled to 30 ml. Ach was given intra-arterially and the maximal pressure generated was recorded. Within 30 minutes, the majority of rabbits developed overactivity, and the amplitude and frequency of the overactivity were recorded.

Forty-eight male White New Zealand rabbits were separated into eight groups of six rabbits each. The following oral treatments were given to each group:

- Groups 1 and 5: vehicle (saline)
- Groups 2 and 6: solifenacin (0.3 mg/kg/day)
- Groups 3 and 7: alpha lipoic acid (100 mM/kg/day) + coenzyme Q10 (5 mg/kg/day)
- Groups 4 and 8: solifenacin (0.3 mg/kg/day) + alpha lipoic acid (100 mM/kg/day) + coenzyme Q10 (5 mg/kg/day).

After three weeks of treatment (by oral gavage), the rabbits in groups 1–4 were anesthetized with isoflurane (1–3%) and received partial outlet obstruction, as detailed above. The rabbits continued their treatments for four weeks post-surgery. At the end of this four-week period, cystometry was performed on each rabbit and they then underwent the in situ study for OAB, as described above.

Immediately following the in situ experiment, the bladder was excised, weighed, and three full-thickness bladder strips were taken for in vitro contractile studies. The balance of the bladder was separated by blunt dissection into muscle and mucosal compartments, frozen under liquid nitrogen, and stored at -80°C for biochemical experiments. Part 1 reports on the urodynamic and physiological results of this study, while part 2 reports on the biochemical aspects.

2.3. Biochemical analyses

2.3.1. Citrate synthase, biomarker for mitochondrial function

Frozen tissue samples of muscle and mucosa were homogenized at 55.6 mg/ml in ice-cold Tris buffer (50 mM, pH 7.6) and centrifuged at 800 x g for 10 minutes to remove the cell membranes and nuclei. A sample aliquot (40 µl) of supernatant was added to a 0.5 cm cuvette along with 1.1 ml 0.05 M Tris buffer (pH 7.6), 30 µl 12.3 mM acetyl-coenzyme A (acetyl-CoA), 100 µl 1 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and 40 µl 10% Triton X-100. The reaction was started by adding 50 µl 10 mM oxaloacetate (substrate). The free coenzyme A generated by citrate synthase activity reacts with DTNB to form a colored compound that is quantified at 412 nm. Absorbance was recorded every 2 minutes for 16 minutes (reaching steady state) using a Hitachi spectrophotometer (Hitachi Co. Chicago, Ill.). Citrate synthase activity is given as nanomoles of coenzyme A generated per minute per mg protein.

2.3.2. Choline acetyltransferase—biomarker for cholinergic synapses

Frozen muscle tissue samples were homogenized at 50 mg/ml in a 20 mM EDTA buffer pH 7.6 and centrifuged at 20,000 x g for 30 minutes to remove the cell membranes, mitochondria, and nuclei. One hundred milliliter aliquots were incubated at 37°C for 30 minutes with 100 µl of reaction mixture consisting of 0.04 mM acetyl-CoA (substrate), 0.50 µCi 3H-acetyl-CoA (200 mCi/mmol), 8 mM choline, 50 mM sodium phosphate, 300 mM sodium chloride and 96 mM physostigmine. After incubation, each solution was diluted with 1 m1 of 0.01 M sodium phosphate and the reaction
stopped with 0.4 ml of acetonitrile containing 5 mg/ml tetraper-
nylboron. Then, the contents of each reaction tube were transferred to a 7-ml scintillation vial and 3 ml of Insta-Fluor Plus scintillation fluid were slowly added to each vial and the vials shaken lightly. Samples were stood for 1 hour while the phases separated, extracting $^3$H-Ach into the Insta-Fluor phase, while $^3$H-acetyl-CoA stayed in the aqueous phase. The aqueous phase was removed and $^3$H-Ach measured using scintillation spectroscopy. Choline acetyltransferase activity (ChAT) activity was reported as fmol acetyl-CoA generated per minute per mg protein.

2.3.3. Malondialdehyde—marker for lipid peroxidation

Frozen samples of bladder muscle and mucosa were weighed and thawed in 1.15% KCl-0.05 M Tris buffer, pH 7.4 on ice and homogenized at 20 mg/ml with a Polytron homogenizer (Brinkman Co, Westbury, NY). Homogenates were spun at 800 × g for 10 minutes and supernatants saved. The reaction system was as follows: 1.15% KCl-0.05 M Tris, 1 mM and 500 μl tissue extract. Samples were incubated at 37°C for 30, 60, and 120 minutes. At the end of incubation, the reaction was stopped by adding 100 μl of sample to microwaves containing 30 μl of 40% trichloroacetic acid. Tubes were centrifuged at 1000 × g for 2 minutes. After centrifugation, 500 μl of supernatant was incubated with 750 μl of perchloric acid (PCA)/thiobarbituric acid (TBA) solution (2.3% PCA, 0.53% TBA, pH 7.4) at 90°C for 30 minutes in 10-75 mm test tubes. After this time, the samples were placed on ice for 10 minutes to cool. To each tube, 2 ml of 1-butanol was added and they were then vortexed for 5–10 seconds. Tubes were spun at 1000 × g for 5 minutes and the basal malondialdehyde (MDA) content of the upper layer was measured fluorometrically at 532 nm.

Quantitation of total protein was performed using Micro BCA Protein Assay (Pierce, Rockford, IL, USA) and results are expressed as nmol MDA/mg protein.

2.3.4. Catalase and superoxide dismutase—endothelial antioxidant enzymes

Superoxide simutase (SOD) (total) activity was determined by the standard biochemical method30–32 using a cytochrome C reduction test. In this method, oxygen free radicals are generated by xantine oxidase reactions with ferricytochrome C. SOD activity is calculated via the degree of inhibition of this reaction and recorded as the change in optical density (mOD) at 550 nm (using a Hitachi spectrophotometer) per milligram of protein.

Specifically, bladder tissue was homogenized in a 50 mM phosphate buffer, pH 7.8, at a concentration of 200 mg/ml. The homogenate was centrifuged at 2500 rpm for 10 minutes. The pellet was eliminated, and the supernatant was used for the following assay: 1 ml of solution A (0.76 mg xantine in 10 ml 0.001 NaOH added to 50 mg cytochrome C + 3.7 mg EDTA in 100 ml 50 mM phosphate buffer at room temperature) was incubated with 25 μl of the tissue sample or Cu-Zn SOD standards in a 3-ml cuvette. One hundred microliters of solution B (5.63 μl xantine oxidase in 1 ml 0.1 mM EDTA) was used to start the reaction. After mixing, the absorbance change indicating cytochrome C reduction was measured in a spectrophotometer at 550 nm for 2 minutes. The change in absorbance with time over the first two minutes for all preparations was linear.

Quantitative comparisons were made by the concentration of enzyme (protein) that inhibited the reaction by 25% (IC50). Although the IC50 is used more widely, several of the preparations did not reach 50% inhibition.

Puriﬁed Cu-Zn SOD (purchased from Sigma Chemical Co., St Louis, MO, USA) was prepared at 25 ng/ml and diluted 1:1 down to 0.39 ng/ml for a standard curve (speciﬁcity was demonstrated by heating both preparations for 10 minutes at 90°C and demonstrating that this eliminated all activity).

2.3.5. Catalase analysis

Following the method of Abei,32 the reaction was initiated by adding 3 ml of of a buffer/H2O2 solution (60 μl of 30% in 50 mM phosphate buffer, pH 7.8) in the presence of 50 μl of tissue extract. The degradation of H2O2 was monitored at 240 nm for 6 minutes, and an extinction coefﬁcient of 43.6 M−1 cm−1 was used to calculate units of activity. The enzyme activity was expressed in units per milligram protein (1 UNIT = 1 mol of H2O2 degraded for 1 minute). For comparative purposes, the slope of enzyme reaction was utilized to demonstrate ChAT activity in units of mOD/minute/mg protein.

3. Results

Table 1 presents the values for the control groups for citrate synthase, ChAT and MDA. For these parameters, the treatments had no signiﬁcant effect on the control values and these groups were combined. Citrate synthase activity and MDA content of the mucosa were signiﬁcantly higher than the values of the muscle.

Table 2 presents the numerical means ± SEM and signiﬁcance details for all data shown in ﬁgures.

Fig. 1 presents the effect of the three treatments on citrate synthase activity as percent of control values. Partial outlet obstruction (PBOO) resulted in a 60% decrease in citrate synthase activity. Solifenacin had no effect on citrate synthase activity of the bladder smooth muscle. However, pretreatment with both the antioxidants, or the combination of solifenacin + antioxidants protected the citrate synthase activity such that it remained at control values. No treatment had any signiﬁcant effect on the reduced citrate synthase activity of the mucosa (Fig. 1).

ChAT of the bladder smooth muscle is presented in Fig. 2 as percentage of control. Similar to citrate synthase, PBOO resulted in a 60% decrease in ChAT activity, while solifenacin had no effect on the reduced ChAT activity. Both the antioxidant and combination therapy resulted in protecting ChAT activity such that it remained at control values. As ChAT is involved in cholinergic neurotransmission to the bladder smooth muscle, only the muscle was analyzed. The MDA content of the muscle and mucosa is presented in Fig. 3. Obstruction resulted in a signiﬁcant increase in lipid peroxidation (MDA content) of both the muscle and mucosa. For both tissues, all treatments resulted in signiﬁcantly lower MDA content when compared with no treatment.

The SOD of the muscle and mucosa is presented in Fig. 4. For both tissues, obstruction resulted in decreased SOD activity (increased IC50). Only the antioxidant pretreatment increased the activity of the obstructed tissue to control values for both muscle and mucosa. Catalase activity is presented in Fig. 5. In the mucosa, obstruction caused reduced catalase activity in the no treatment, antioxidant, and combined solifenacin + antioxidant groups. The control and obstructed antioxidant and combination groups had signiﬁcantly higher catalase activity compared with the no treatment control and obstructed groups. Interestingly, in the bladder

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Muscle</th>
<th>Mucosa</th>
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<tbody>
<tr>
<td>Citrate synthase (nM coenzyme A/min/mg protein)</td>
<td>12.0 ± 2.1</td>
<td>66.6 ± 6.7*</td>
</tr>
<tr>
<td>Choline acetyltransferase (fmol Ach/mg protein)</td>
<td>3.55 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>MDA (μmol/mg protein)</td>
<td>0.96 ± 0.11</td>
<td>1.06 ± 0.2*</td>
</tr>
</tbody>
</table>

* = significantly different from muscle; p < 0.05.

Ach = acetylcholine.
smooth muscle, there were slight increases in catalase activity in the obstructed bladders (compared with control bladders) in the no treatment, solifenacin, and antioxidant groups. The catalase activity of the antioxidant control and obstructed groups were significantly lower than the activity of the respective no treatment groups.

3.1. Statistics

Analysis of variance was used to analyze the data, followed by the Tukey test for individual differences among the groups. A p < 0.05 was required for statistical significance.

4. Discussion

Anti-muscarinic agents have proven to be clinically effective in the treatment of overactive bladders in both men and women.33–35 These drugs relax the bladder by muscarinic cholinergic inhibition which makes them useful for the treatment of the unstable bladder. As both bladder overactivity and micturition are primarily based on the neuronal release of Ach and muscarinic receptor stimulation, it is important to directly compare the effect of antimuscarinic agents on these two cholinergic systems in a controlled, quantitative manner, in which a true sensitivity can be determined for each system. Moreover, it would be beneficial to find treatment modalities that address overactivity without affecting bladder contractile force. This might be accomplished through the use of antioxidants, as mentioned in the Introduction. For the current studies, we have chosen to use the combination of coenzyme Q10 and alpha lipoic acid because, as mentioned earlier, this combination of antioxidants has been shown to be effective in the treatment of partial outlet obstruction and in vivo ischemia/reperfusion.19,20,36

In part one of these studies, we evaluated these treatments on bladder compliance, OAB amplitude and frequency, and in vitro contractile responses to field stimulation, carbachol, and KCl. In general, all treatments improved compliance, reduced OAB, and improved the contractile responses. However, the combination therapy of solifenacin + antioxidants was more effective than either therapy alone.

Previous studies demonstrated that partial outlet obstruction and OAB both result in the release of free radicals and oxidative damage, especially to mitochondria, and to synaptic membranes and nerve terminals.37–40 In the current studies, pretreatment with both the antioxidants alone and the combination of solifenacin + antioxidants protected mitochondrial activity (citrate synthase activity) and cholinergic synaptic function (ChAT activity) in the bladder smooth muscle. Recent studies have clearly demonstrated that there is non-neuronal Ach localized in the urothelium.41,42 The release of Ach and other bioactive substances from the urothelium may play a role in the etiology of OAB,42 and may be the reason why antimuscarinic agents are not effective against OAB in all

| Table 2 | Numerical values of data shown in figures. |
|---|---|---|---|---|
| | Muscle | | Mucosa | |
| | No treat | Sol | CoQ + LA | Sol + CoQ + LA | No treat | Sol | CoQ + LA | Sol + CoQ + LA |
| Citrate synthase | 37.2 ± 6.8| 26.6 ± 5.0 | 114 ± 8.0 x | 116 ± 13.0 x | 55.7 ± 7.6 | 41.6 ± 3.7 | 66.0 ± 9.0 | 58.0 ± 2.0 |
| ChAT | 44.0 ± 10 | 42.0 ± 18 x | 93.0 ± 11 x | 94.0 ± 8.0 x | 227 ± 59 | 112 ± 50 x | 140 ± 15.0 | 105 ± 25 x |
| MDA | 318 ± 90 x | 203 ± 60 | 152 ± 24.6 x | 138 ± 53.1 x | 1.2 ± 0.05 | 1.0 ± 0.04 | 1.0 ± 0.06 | 1.2 ± 0.03 |
| SOD−control | 1.8 ± 0.7 | 2.0 ± 0.16 | 2.0 ± 0.7 | 2.4 ± 0.04 | 2.4 ± 0.06 | 1.5 ± 0.1 | 1.2 ± 0.06 | 1.6 ± 0.3 |
| SOD−obstructed | 0.2 ± 0.14 x | 3.0 ± 0.1 x | 2.4 ± 0.08 | 3.0 ± 0.1 x | 0.55 ± 0.01 x | 0.59 ± 0.01 | 0.78 ± 0.03 x | 0.98 ± 0.03 x |
| Catalase−control | 0.63 ± 0.03 | 0.56 ± 0.03 | 0.42 ± 0.01 x | 0.61 ± 0.01 | 0.32 ± 0.01 | 0.55 ± 0.05 x | 0.6 ± 0.01 | 0.8 ± 0.15 x |
| Catalase−obstructed | 0.77 ± 0.06 x | 0.67 ± 0.02 x | 0.53 ± 0.02 x | 0.67 ± 0.01 | 0.32 ± 0.01 | 0.55 ± 0.05 x | 0.6 ± 0.01 | 0.8 ± 0.15 x |

*Significantly different from control; x = significantly different from no treatment; p < 0.05.

ChAT = choline acetyltransferase; MDA = malondialdehyde; No Treat = no treatment; Sol = solifenacin; SOD = superoxide dismutase.

a Data presented as percentage of control values.
b Data presented as the IC - 25 (concentration that inhibits 25% of the activity of SOD).
c Data presented as mOD/minute/mg protein.

d Data presented as nanomoles of Rose Bengal reduced per mg of protein.

e Data from Table 1.

Fig. 1. Citrate synthase activity of the smooth muscle and mucosal compartments of the rabbit bladder presented as percent of control activity. “Control activity” is the enzyme activity of rabbits that were not subjected to either partial outlet obstruction (PBOO) or in situ overactive bladder (OAB). The control values can be found in Table 1. The “no treatment” group is the group of rabbits that did not receive any solifenacin or antioxidants, but were subjected to PBOO and in situ OAB. Each bar is the mean ± SEM of six individual rabbits. “*” = significantly different from control; x = significantly different from no treatment; p < 0.05.

Fig. 2. Choline acetyltransferase activity of the smooth muscle compartment of the rabbit bladder presented as percent of control activity. “Control activity” is the enzyme activity of rabbits that were not subjected to either partial outlet obstruction (PBOO) or in situ overactive bladder (OAB). The control values can be found in Table 1. The “no treatment” group is the group of rabbits that did not receive any solifenacin or antioxidants, but were subjected to PBOO and in situ OAB. Each bar is the mean ± SEM of six individual rabbits. “*” = significantly different from control; x = significantly different from no treatment; p < 0.05.
Because of the rather extensive non-neuronal cholinergic system in the urothelium, we only measured ChAT activity in the muscle. There was no protection observed for the mucosa in regard to citrate synthase activity. This may be owing to the observed mucosal structural damage caused by partial outlet obstruction.\textsuperscript{45,46} The MDA studies show that all three treatments protect lipid membrane components of both the muscle and mucosa from peroxidation. In general, the increased citrate synthase activity and increased ChAT activity in association with reduced MDA levels in the bladder smooth muscle are entirely consistent with the functional improvements in bladder contractile responses and compliance observed for the antioxidant and combination therapy groups.

Similar to the conclusions from part 1, it is clear that all three forms of treatment were effective in improving the obstructed bladder’s biochemical function. Taking all the studies into account, the combination of solifenacin and antioxidants was more effective in improving more parameters than either of the other two therapies alone, thus supporting our original hypothesis.

Acknowledgments

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References


