The effect of α–and δ–tocopherol–lipoic acid ester co–drugs on the response of the rabbit bladder to *in vitro* ischemia/reperfusion

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ABSTRACT

Objective: Obstructive bladder dysfunction (OBD) caused by benign prostatic hyperplasia is a common medical problem in ageing men. As the prostate enlarges and compresses the urethra, the bladder wall thickness and the bladder is termed "compensated" because its function is still relatively normal. Subsequently, bladder function begins to fail and this change is termed "decompensation." The extent of decompensation progresses from mild through severe. Bladder decompensation is mediated by cyclical ischemia followed by reperfusion (I/R) resulting in an increased generation of free radicals and oxidative stress. Previous studies demonstrated that both vitamin E (tocopherol) and alpha-lipoic acid (LA) showed significant antioxidant activity in experimental urinary bladder oxidative stress models. We hypothesized that co-drugs derived from these antioxidants would result in enhanced antioxidant activity relative to either individual compound for the treatment of oxidative stress in the lower urinary tract.

Material and methods: Two ester co-drugs of TOC and LA, tocopherol ester (α-TOCE) and δ-TOCE were synthesized. Six adult male New Zealand White (NZW) rabbits were divided into two groups of three rabbits each. Eight full thickness strips from each rabbit bladder were taken for in vitro I/R experiments. The strips from the first set were control rabbits (24 strips). Six strips were not incubated, while the remaining strips were incubated in α-TOCE dissolved in 1% (n=6) or 2.5% ethanol (n=6) solutions. These strips were not subjected to in vitro I/R. The strips from the second set were processed as follows: 6 strips were not incubated, while the remaining strips were incubated in α-TOCE dissolved in 1% (n=6) or in δ-TOCE dissolved in 2.5% ethanol. These strips were subjected to 1 hour in vitro ischemia followed by two hours reperfusion.

Results: Preliminary studies demonstrated that neither antioxidant had any effect on the contractile responses to 1% or 2.5% ethanol. Neither antioxidant had any effect on the control contractile responses. Both antioxidants protected the tissue from the initial effects of ischemia. Both antioxidants had significant protective effects on the contractile responses to all forms of stimulation after the reperfusion period.

Conclusion: Incubation with both antioxidants had similar protective effects on responses both to ischemia and to reperfusion.

Keywords: Alpha-lipoic acid; antioxidants; bladder; ischemia/reperfusion; oxidative stress; vitamin E.

Introduction

The urinary bladder is a smooth muscle (SM) organ whose function is to collect and store urine at low intravesical pressures and then to periodically expel the urine via highly coordinated, sustained contractions.[1,2] Bladder function depends upon several factors including state of innervation, structure of the organ as a whole, contractile response of the SM elements to autonomic stimulation and availability of metabolic energy sources (cytosolic ATP and mitochondrial oxidative metabolites).[1,2] These factors are intimately associated with an alteration in bladder function in one and can induce substantial adaptive changes in the others. Partial outlet obstruction of urinary bladder is a common medical problem. More than 80% of males older than 50 years of age have varying degrees of bladder outlet obstruction secondary to benign prostatic hyperplasia (BPH).[3,4] Recent

evidence has demonstrated that ischemia is a major etiological factor in bladder dysfunction secondary to BPH and bladder outflow obstruction. Experimentally, bladder ischemia has been shown to produce significant changes in bladder function and structure leading to noncompliance and hyperreflexia.[5-7] Ischemia-induced cellular and molecular changes in the bladder have been shown to be similar to those induced by over-distension and partial outlet obstruction.[8,9] Ischemia-induced bladder fibrosis and decrease in the volume fraction of smooth muscle contribute to the development of bladder dysfunction.[8-10]

In order to understand the effects of outlet obstruction on bladder morphology, physiology, biochemistry and pharmacology, several animal models of obstruction have been developed using different species.[11,12] One problem is that partial outlet obstruction (PBOO) has a variety of effects on bladder structure and function other than ischemia/reperfusion (I/R) including SM hypertrophy, mucosal hyperplasia, changes in blood flow and blood vessel density.[13,14] Several I/R models have been developed which eliminates these non-ischemic responses to PBOO, including in vivo bilateral I/R studies[15,16], and in vitro I/R studies.[16,17] The advantage of the in vitro models are that the experiments can be completed within several hours whereas the completion of in vivo models can take several weeks. Additionally the in vivo models require substantially greater number of animals relative to in vitro models. Several antioxidants and natural products with antioxidant activity have been shown to protect the bladder from dysfunctions mediated by PBOO[18,19], in vivo I/R[19,20], and in vitro I/R.[17,21] Two of the major antioxidants shown to be effective in protecting the bladder from oxidative stress are tocopherol (vitamin E)[22,23] and α-lipoicacid.[21,24] Tocopherol exists in eight different forms, four tocopherols and four tocotrienols. All forms feature a chromane ring, with a hydroxyl group that can donate a hydrogen atom to reduce free radicals, and a hydrophobic side chain which allows for penetration into biological membranes. Compared to the other forms, α-tocopherol is preferentially absorbed and accumulated in humans, and it is the most widely utilized form.[25] α-Tocopherolis an orally bioavailable form of the naturally-occurring fat-soluble vitamin E with antioxidant activity that contains a phenolic hydrogen, but has two fewer methyl groups associated with the chromane ring. Although the exact mechanism of action of α-tocopherol has yet to be fully identified, its phenolic hydrogen has the same ability to scavenge free radicals as α-tocopherol, and is thus able to protect cells against oxidative damage, presumably through the same mechanism. We chose these two forms of vitamin E because of their different structural, absorption and bio-availability characteristics.

Tocopherol (TOC) and lipoic acid (LA), either alone or in combination, have been previously shown to exhibit antioxidant activity. Formulations containing TOC, or prodrugs of TOC, have been developed to maintain or restore concentrations of this protective antioxidant. Ester prodrugs of TOC analogs have also been developed to improve formulation stability and enhance bioavailability of TOC. Co-drugs are single chemical entities derived from synergistic drugs that are chemically linked together in order to improve drug delivery properties and efficacy of one or both drugs. We have prepared two ester co-drugs (Figure 1), derived from TOC and LA that are designed to simultaneously deliver these antioxidants where they undergo hydrolytic activation to the parent compounds which then act synergistically to inhibit ischemia-induced ROS in the bladder.The ester co-drugs protect the susceptible phenolic group of TOC against thermal and photo-degradation, and mask the carboxylic acid of LA to optimize bioavailability. In vivo hydrolytic metabolism of the co-drugs triggers release of TOC and LA and allows for synergistic antioxidant activity.

As stated above, TOC and LA have been shown to be active antioxidants for the treatment of oxidative stress of the lower urinary tract. We hypothesized that co-drugs derived from these antioxidants would result in enhanced antioxidant activity relative to either individual compound for the treatment of oxidative stress in the lower urinary tract. Both α-and δ-TOC, lipoic acid, and the ester co-drugs are lipophilic with low water solubility, but all are soluble in a water-ethanol mixtures. Figure 1 shows the structure of the two novel co-drugs derived from TOC and LA.

Material and methods

All studies were approved by the Institutional Animal Care and Use Committee and Research and Development Committee of the Stratton VA Medical Center, Albany, NY, USA (Approval, 545016-1]. In vitro Studies on the Ability of Natural Products and Antioxidants to Protect Rabbit Bladders from İn vitro Models of Ischemia/Reperfusion).

Synthesis of Ester Co-Drugs (α-and δ-TOCE)
A solution of dicyclohexylcarbodiimide (DCC, 1.1 mmol) in dichloromethane (DCM, 10 mL) was added in 5 min to a solution of TOC (1 mmol), LA (1.25 mmol) and DMAP (0.8mmol) in DCM (10 mL) at 0^0 C under N_2. After the addition was completed the cooling bath was removed and the reaction was stirred overnight. The solvent was evaporated and the crude reaction mixture was purified by flash column chromatography on silica gel using hexane:ethyl acetate 100:0-98:2-96:4 to yield the pure product as a yellow viscous oil.
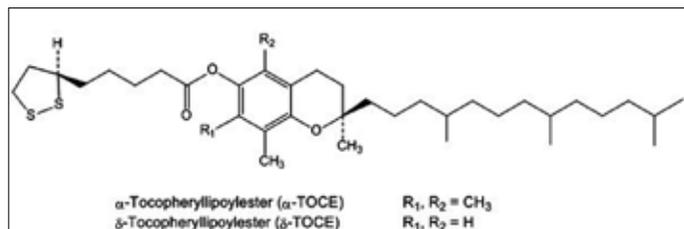
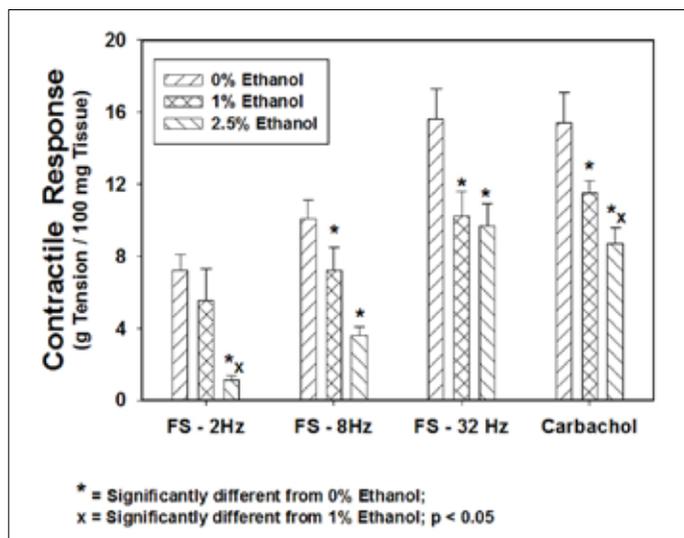Figure 1. Structures of ester co-drugs derived from tocopherols and lipoic acid

Figure 2. Effect of 1 and 2.5% ethanol on the contractile responses to FS and carbachol. Each bar is the mean +/-SEM of 6 individual full thickness strips of bladder body; *significantly different from 0% ethanol, x = significantly different from 1% ethanol, p<0.05
FS: field stimulation

α-TOCE: Yield: 90%APCI-MS (m/z):619.41 (M⁺+1).Anal calcd for $C_{37}H_{62}O_3S_2$ (618.41): C, 71.79; H, 10.10; O, 7.75; S, 10.36. IR (neat): 2926cm⁻¹; 2865 cm⁻¹, 1758cm⁻¹.¹H NMR (CDCl₃): δ 3.65-3.55 (1H, q), 3.2 (2H, t), 2.65-2.54 (4H, m), 2.51-2.4 (2H, m), 2.1 (3H, s), 1.95 (3H, s), 1.94-1.89 (2H, m), 1.85-1.71 (5H, m), 1.65-1.45 (6H, m), 1.45-1.32 (10H, m), 1.15-1.31 (4H, m), 1.24 (3H, s), 1.14-1.0 (5H,m). 0.89 (3H,s), 0.85 (3H, s), 0.81 (3H, s); ¹³C NMR (CDCl₃): δ 172.13 (CO, ester), 149.98, 145.02, 126.73, 124.92, 123.14, 117.48, 74.98, 56.44, 40.34, 39.49, 38.61, 37.51, 36.49, 34.76, 33.99, 32.89, 32.33, 29.05, 28.09, 28.01, 24.99, 24.93, 24.91, 24.55, 24.31, 22.83, 22.74, 20.72, 19.86, 19.80, 13.11, 12.27, 11.94.

δ-TOCE: Yield: 98% APCI-MS (m/z): 591.38 (M⁺+1).Anal calcd for $C_{35}H_{58}O_3S_2$ (590.38): C, 71.13; H, 9.89; O, 8.12; S, 10.85. IR (neat): 2925cm⁻¹, 2865 cm⁻¹, 1755cm⁻¹.¹H NMR (CDCl₃): δ 6.65 (1H, s), 6.60 (1H, s), 3., s), 1.96-1.88 (1H, m), 1.83-1.70 (6H, m), 1.58-1.50 (5H, m), 1.38-1.34 (4H, m), 1.27-1.24 (9H, m), 1.16-1.04 (7H, m), 0.87 (3H, s), 0.85 (3H, s), 0.84
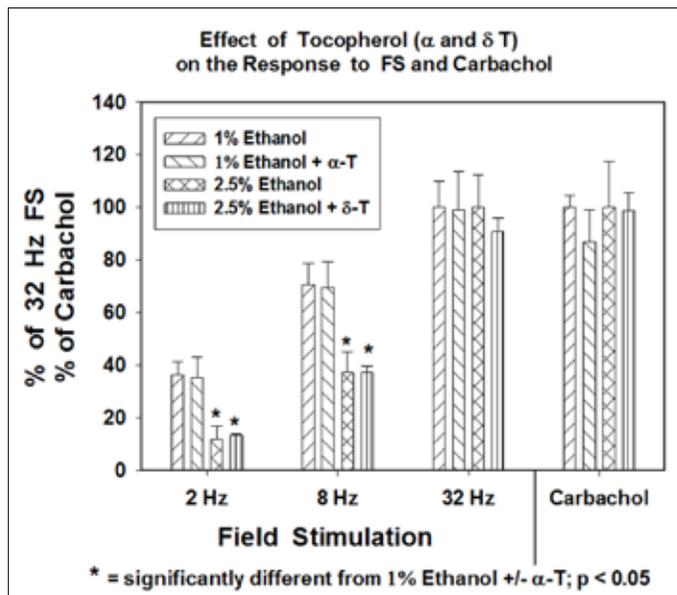

Figure 3. Effect of α-TLA and δ-TLA on the contractile responses to FS and carbachol. Each bar is the mean +/-SEM of 6 individual full thickness strips of bladder body; *significantly different from1% ethanol +/-α-TLA, p<0.05
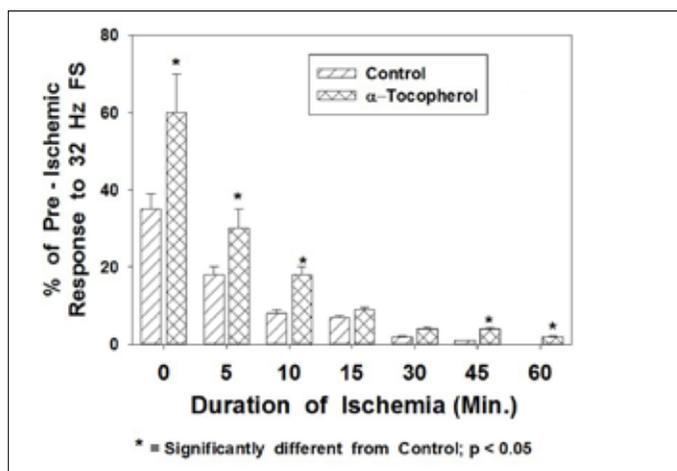

Figure 4. Effect of α-TLA on the response to 60 minutes ischemia. Each bar is the mean +/-SEM of 6 individual full thickness strips of bladder body; *significantly different from 1% Ethanol, p<0.05

(3H, s), 0.83 (3H, s). ¹³C NMR (CDCl₃): δ 172.75 (CO, ester), 149.86, 142.64, 127.51, 121.03, 119.05, 56.44, 40.35, 39.52, 38.61, 37.56, 37.53, 37.40, 34.73, 34.24, 32.90, 32.80, 31.09, 28.84, 28.09, 24.91, 24.87, 24.56, 24.34, 22.84, 22.75, 22.57, 21.07, 19.87, 19.76, 16.24.

Six adult male New Zealand white rabbits (approximately 3.5 kg each) were divided into two groups of three rabbits each. Each rabbit was anesthetized with 3% isoflurane. The bladder of each

animal was rapidly removed and weighed and the rabbit was euthanized with 2 mL Fatal Plus euthanasia fluid IV. Each bladder was opened longitudinally and eight full thickness strips (1.5 cm long and 2 mm wide) of bladder body (separated from the bladder base at the ureteral orifices) were placed in individual 15 mL organ baths containing Tyrode's solution with glucose (1 mg/mL) at 37°C and equilibrated with 95% oxygen and 5% carbon dioxide.

The strips from the first set were harvested from control rabbits (24 strips). Six strips were not incubated, while 6 strips were incubated in α-TOCE (10 mg/mL dissolved in Tyrodes containing 1% ethanol); 6 strips in Tyrode's solution containing δ-TOCE (10 mg/mL dissolved in Tyrode's solution containing 2.5% ethanol). These strips were not subjected to in vitro I/R. The strips from the second set were separated as follows: 6 strips were not incubated, while the remaining strips were incubated in Tyrode's solution containing α-TOCE (n=6) dissolved in 1% ethanol or in Tyrode's solution containing δ-TOCE dissolved in 2.5% ethanol (n=6).

Both antioxidants were not soluble in Tyrode's solution without ethanol. The concentration of ethanol required to solubilize the antioxidants were determined in preliminary experiments. The concentrations of ethanol given were the final concentrations in the baths.

Each strip was incubated for two hours prior to the start of the experiment. Each strip was set at 2 gm passive tension and stimulated with field stimulation (FS: 2 Hz, 8 Hz, 32 Hz, 1 ms duration for 20 seconds with 3 minutes in between stimulations). Carbachol (20 μM) was then applied to each strip for 3 minutes. After the control stimulations, each bath was then filled with Tyrode's solution +/-antioxidant without glucose and equilibrated in the presence of 95% nitrogen and 5% carbon dioxide for 1 hour with stimulation at 32 Hz every 5 minutes (ischemic period). The buffer was then switched back to standard oxygenated Tyrode's with glucose +/-antioxidant, and the strips were allowed to recover for two hours (reperfusion period). The strips were again stimulated as originally described. Each strip was then removed from the bath, weighed and frozen in liquid nitrogen and stored at -80°C for biochemical analyses.

Field stimulation mimics neurotransmitter stimulation of muscle contraction. Carbachol is a muscarinic agonist stimulating the receptor directly without resorting to neuro-transmission.[26]

Statistical analysis

One way analysis of variance followed by the Tukey test for individual differences were used for all studies. A p<0.05 was required for statistical significance.

Results

The contractile responses of the bladder body +/-ethanol is presented in Figure 2. At 2 and 8 Hz FS, 2.5% ethanol inhibited the contraction to a significantly greater degree than 1% ethanol. At 32 Hz and in the presence of carbachol, 2.5% ethanol inhibited contraction to a similar degree than 1% ethanol, although 2.5% ethanol inhibited carbachol-induced contraction to a statistically greater degree than 1% ethanol. Although not shown in the figure both α-and δ-TOCE had no effects on the contractile responses to ethanol.

Figure 3 shows the effects of α-and δ-TOCE on the contractile responses to FS and carbachol. Both forms of tocopherol had no effect on the contractile responses to FS or carbachol. Please note that the control strips from the α-TOCE were incubated in 1% ethanol and the control strips from the δ-TOCE were incubated in 2.5% ethanol.

Figure 4 shows the effects of α-TOCE on the response to 60 minutes of ischemia. The "0" time represents the initial response after change to the ischemic medium. Ischemia at all time points significantly and progressively inhibited the contractile response to both control and α-TOCE groups. α-TOCE significantly protected the contractile response to 32 Hz FS at virtually all time points, including at 60 minutes.

Figure 5 shows the effects of δ-TOCE on the response to 60 minutes of ischemia. Ischemia at all time points significantly and progressively inhibited the contractile response to both control and δ-Tocopherol groups. δ-TOCE significantly protected the contractile response to 32 Hz FS at only 0, 10 and 15 minutes. Thus α-TOCE was more protective against ischemia than δ-TOCE.

Figure 6 shows the effect of 1 and 2.5% ethanol on the contractile responses following the 2 hours of reperfusion period. Ethanol (2.5%) was significantly more inhibitive for FS at all frequencies of stimulation and for carbachol.

Figure 7 shows the post-ischemic contractile responses in the presence of α-TOCE. α-TOC was protective at all frequencies of FS and for carbachol.

Figure 8 shows the post-ischemic contractile responses in the presence of δ-TOCE. δ-TOCE was protective at all frequencies of FS and for carbachol. It should be noted that the post-ischemic responses to all forms of stimulation were significantly lower for δ-TOC than for α-TOCE. This would be due to the use of 2.5% ethanol for δ-TOCE which is much more inhibitory on contraction than α-TOCE dissolved in 1.0% ethanol.
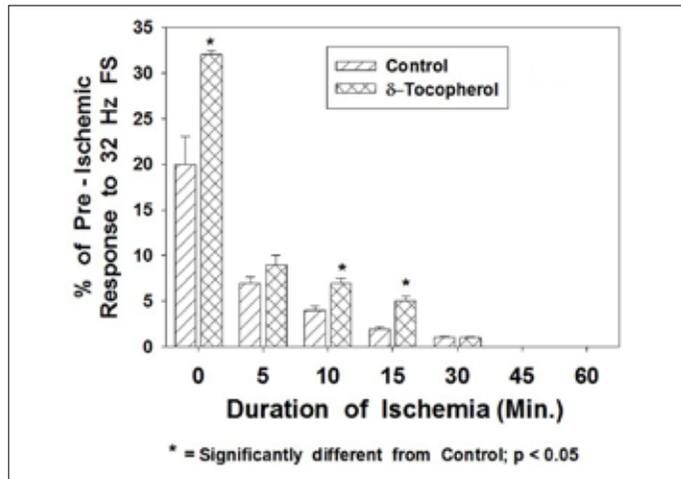
Figure 5. Effect of δ-TLA on the response to 60 minutes ischemia. Each bar is the mean +/-SEM of 6 individual full thickness strips of bladder body; *significantly different from 1% Ethanol, p<0.05

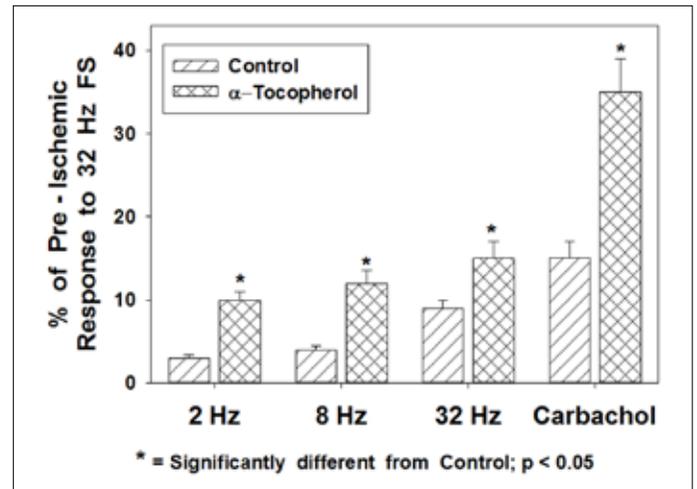Figure 7. Effect of α-TLA on the contractile responses to FS and carbachol at the end of the reperfusion period; *significantly different from 1% ethanol, p<0.05
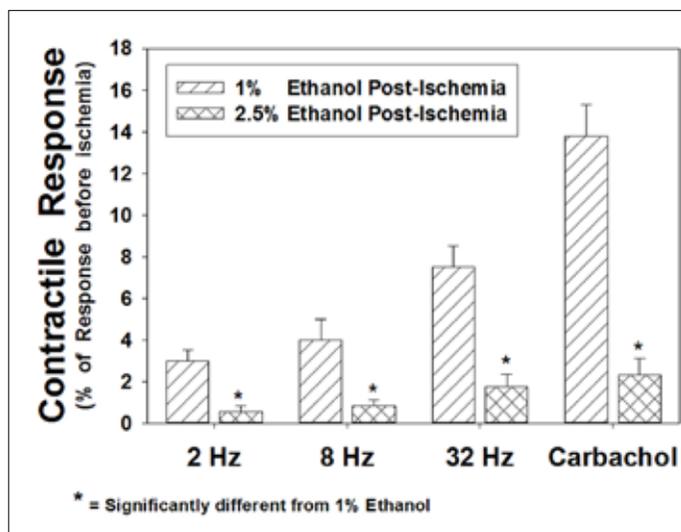

Figure 6. Effect of 1 and 2.5% ethanol on the contractile responses to FS and carbachol at the end of the reperfusion period. *significantly different from 1% ethanol, p<0.05
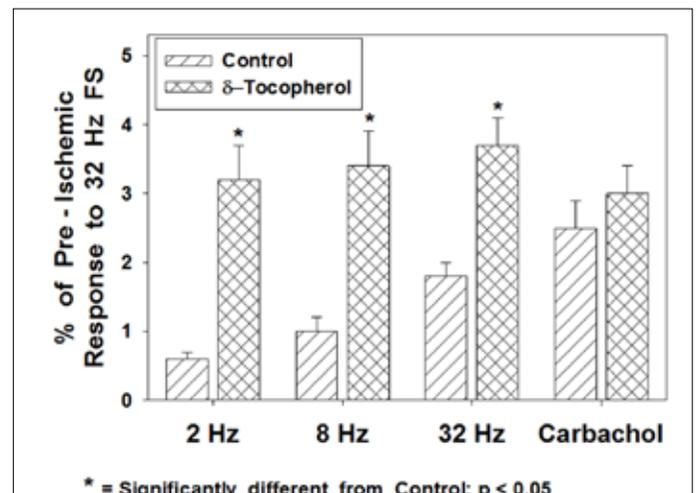

Figure 8. Effect of δ-TLA on the contractile responses to FS and carbachol at the end of the reperfusion period; * = significantly different from 1% ethanol, p<0.05

Discussion

Oxidative stress plays important roles in a variety of specific pathophysiologies and in the ageing process. Urinary bladder outlet obstruction is a common medical problem in men. More than 80% of males older than 50 years of age have varying degrees of bladder outlet obstruction secondary to BPH.[27,28] Although it is a common knowledge that in man progressive bladder dysfunction occurs in association with ageing, there is also excellent evidence that both bladder physiological and biochemical dysfunctions of bladder occur in rabbits.[29,30] We have strong evidence that progression of obstructive bladder dysfunction (OBD) in men is very similar to the progression of OBD in rabbits subjected to PBOO.[14,31,32]Results of our recent studies provided direct evidence that one of the major etiologies for OBD in both men and rabbits is ischemia followed by reperfusion (I/R). Ischemia resulting from decreased blood flow during contraction followed by reperfusion results in the generation of free radicals and oxidative damage to muscle and mucosal cellular and subcellular membranes.[33,34] Indirect evidence for an I/R etiology of OBD comes from a series of published studies using a variety of in vivo and in vitro models, and demonstrating that several specific natural antioxidant products can protect the bladder from oxidative stress.[17,20]

As mentioned in the introduction, in addition to hypoxia, ischemia, and reperfusion, OBD also induces SM hypertrophy, mucosal hyperplasia, and collagen infiltration into the smooth muscle compartment.[11,13] In order to discriminate the I/R effects of OBD from the structural effects, we have utilized a recognized in vitro model of I/R.[35,36]

We have clearly identified that the primary etiology of the pathological effects of I/R is due to the generation of free radicals and oxidative stress[16,37-39]; thus the protective effects of α and δ-TOCE would be due to their strong antioxidant effects.

Although specific antioxidants and natural products have been shown to protect bladder contractile responses to I/R and partial bladder outlet obstruction (PBOO)[18,20,21,40], not all antioxidants have this effect. This was best observed when we compared the effects of a freeze dried preparation of whole grapes with the effects of pure resveratrol. These studies demonstrated that although resveratrol (the proposed major antioxidant of grapes) had an extremely potent in vitro antioxidant activity, it was not nearly as effective as the whole grape preparation in protecting the bladder from oxidative stress.[41,42] As mentioned previously, several clinical studies on the effectiveness of specific antioxidants on specific diseases linked to oxidative stress have been disappointing.[43-46]

The bladder SM and mucosal cells have both lipid components (cell wall, intracellular organelle wall) and aqueous components (intracellular fluid). For this reason we thought a combination antioxidant with a lipid-soluble component (lipoic acid) and aqueous component (tocopherol) would have better access into the bladder cells as a whole. The major difference between the two antioxidants was that α-TOCE was extremely effective of protecting the contractile response of carbachol from I/R, while δ-TOCE had no significant effect.

This preliminary study indicates that this composite antioxidant has extremely potent activity in protecting the isolated bladder strips from significant oxidative stress. Additional dose-response studies are planned to directly compare the potency of these two composite antioxidants with their two components namely TOC and LA.

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