BAOJ Urology and Nephrology

Robert M Levin, et al., BAOJ Urol Nephrol 2017, 3: 2
3: 011

Review

The Rabbit Urinary Bladder as a Model System to Study the Antioxidant Activity of Ganoderma Lucidum: A Review

***Robert M Levin[1,2],Catherine Schuler[1], Robert E Leggett[1] and Alpha D-Y Lin[3, 4, 5]**

[1]Stratton VA Medical Center, Albany, NY

[2] Albany College of Pharmacy and Health Science, Albany, NY

[3]Beijing Tong Ren Tang Chinese medicine Co., LTD. Beijing, China

[4]The Central-Clinic Hospital, Taipei, Taiwan

[5]Urology Department, National Yang-Ming University, Taiwan

Abstract

Micturition is a complex neuromuscular process. Although control mechanisms have been identified at several levels of the central nervous system and spinal cord, the final pathway in the control of micturition is the autonomic innervation of the urinary bladder and related structures. Micturition is ultimately dependent on the ability of the urinary bladder to contract, generate intravesical pressure, and modify its shape in such a way as to efficiently expel its contents without leaving a high residual volume [1,2].

In order to understand the various elements involved in regulation of bladder function, a wide variety of animal models have been published. In many cases, animal models have been utilized to describe the effect of specific experimental pathologies on the lower urinary tract. One major advantage that the urinary bladder has in the study of pathological processes is that the urinary bladder is a relatively closed system: that is, specific pathologies can be induced in the bladder with little or no damage to other organ systems.

Oxidative stress has been linked to a wide variety of pathologies of almost all organs, neural systems, and brain. Specifically, specific experimental oxidative damage to one organ system such as the heart can secondarily damage other organ systems such as the kidney, liver, and lung; which in turn can further damage the heart. However, specific experimental oxidative damage to the urinary bladder is limited only to urinary bladder function without these secondary effects on other organ systems.

Key Words: Ganoderma Lucidum; Oxidative Stress; Anti-Ageing ; Rabbits Urinary Bladder

Introduction

Oxidative stress plays an important role in the etiology of specific pathophysiologies and in the ageing process[3,4]. In the history of medicine many herbs were (and still are) utilized for disease prevention and treatment[5,6]. One important question is: What is the most useful model to study the effects of oxidative stress and the use of medicinal herbs to treat and / or prevent specific diseases linked to oxidative stress?

Although there are many species of animals used for medical research; and each has advantages and disadvantages, we have found that the rabbit urinary bladder has many advantages in the study of oxidative stress. Many of the functional changes associated with pathology of human urinary bladder can be induced in the rabbit (see reviews [7-9]). Rabbit urinary bladders capacity is between 50 to 100 mL. Compliance can be evaluated cystometrically using a Foley catheter to catheterize the bladder through the urethra (not a surgical procedure). The cystometric curve of the rabbit is similar in shape to that of humans: the bladder fills at low intravesical pressure until capacity is reached at which time the pressure rises sharply. Similar to humans, bladder emptying occurs during the tonic phase of contraction; whereas the rat and mouse urinate during the phasic contraction[10,11]. The bladder's ability to sustain increased

***Corresponding Author:** Robert M Levin, Senior Research Career Scientist, Stratton VA Medical Center, Albany, NY 12208518-369-0173 E-mail: Robert.levin2@va.gov

Sub Date: March 17th 2018, **Acc Date**: March 31st 2018, **Pub Date**: April 4th,2018.

Citation: Robert M Levin, Catherine Schuler, Robert E Leggett and Alpha D-Y Lin (2018) The Rabbit Urinary Bladder as a Model System to Study the Antioxidant Activity of Ganoderma Lucidum: A Review. BAOJ Urol Nephrol 3: 011

pressure in response to stimulation is significantly reduced by partial bladder outlet obstruction (PBOO – model for obstructive bladder dysfunction secondary to benign prostatic hyperplasia (BPH)) before any change in maximal pressure generation occurs. This decreased ability to sustain increased pressure during stimulation is the reason that, in rabbits and humans, the bladder's ability to empty is reduced at times when the organ is capable of maximal pressure generation. Urethral pressure profiles can also be performed without surgical intervention, and is representative of urethral pressure profiles in humans.

Major characteristics of the rabbit's response to PBOO are an increase in bladder mass to a stable level, reduced compliance during bladder filling, and development of overactive bladder syndrome (unstable bladder contractions during filling) [7,8,12], and are similar to changes in bladder function secondary to BPH in men. Ultrasound studies have confirmed that not only do men with obstructive uropathies exhibit an increase in bladder mass, but bladder wall thickness has been shown to be the most accurate non-invasive way to identify men with obstructive bladder dysfunction (OBD)[13-15]. Another common feature of obstruction in both rabbits and man is denervation which has been demonstrated immuno histochemically and biochemically in both rabbits [16-19] and in men [16,19,20]. In addition, both obstructed rabbits and men show an increase in the density and distribution of connective tissue (CT) within the bladder wall resulting, in decreased compliance and higher pressure during filling [8,21]. We believe that these CT alterations contribute significantly to the contractile failure of the obstructed bladder [22].

In two major studies we demonstrated that the level of contractile dysfunction in rabbits subjected to PBOO correlated with the degree of ultra structural damage to nerves, synapses, mitochondria, and sarcoplasmic reticulum (SR) [16,17]. Similarly, men with significant obstructive symptoms had structural damage to the same cellular and subcellular membranes as did the obstructed rabbits [16,17].

A second part of these studies directly correlated the effect of PBOO in rabbits with obstructive dysfunction in men secondary to BPH. In these studies, we compared the enzyme activities of citrate synthase (CS), a marker for mitochondrial function; sarco-endoplasmic calcium ATPase (SERCA), a marker for SR function; and choline acetyltransferase (ChAT), a marker for acetylcholine synthesis and cholinergic innervations, in smooth muscle (SM) biopsies from men with significant obstructed bladder dysfunction with biopsies and tissue samples from age-matched men with no bladder dysfunction (organ donors). These studies demonstrated that although men and rabbits had very different basal activities of these three enzymes, obstructive dysfunction in both species significantly reduced these marker enzyme activities to approximately the same extent [16,19].

An additional extremely important relevant aspect of the obstructive bladder model to study the effectiveness of the nanopreparations of resveratrol is the relatively closed system of the rabbit urinary bladder. This means that the oxidative stress aspects of OBD are limited almost exclusively to the urinary bladder, and did not directly involve other organ systems. This may not true with oxidative stress dysfunction of other organ systems, for example, congestive heart failure (CHF) or chronic kidney disease (CKD); where both CHF and CKD significantly affect other organ systems leading to widespread dysfunctions, which can in turn significantly increase the severity of CHF and CKD. It is primarily for this reason we propose that the rabbit urinary bladder is an excellent model to study oxidative stress, and the effects of potential treatments.

As mentioned above, oxidative stress has been found to be a major factor in a number of human health problems including heart disease [23-25], diabetes [26,27], intestinal diseases [28-30], liver disease [31], renal disease [32,33], and obstructive bladder dysfunction[34-37]. In virtually all of these oxidative stress-linked dysfunctions, antioxidants have proven to be very valuable in their treatment [35,38-41]. In addition to specific antioxidants such as coenzyme Q10, alpha lipoic acid, vitamins C, E, and D; certain natural products have been shown to be effective for treatment of these specific diseases [42-46].

Several clinical studies have demonstrated that purified antioxidants were not as effective as originally believed in the treatment of a variety of diseases [47-50]. We compared the beneficial effects of a whole grape preparation with pure resveratrol, which is believed to be the major beneficial component in grapes [51,52], our results clearly demonstrated that although resveratrol had over 100 times antioxidant activity of that of grape suspension, the grape suspension (freeze dried preparation made from all varieties of California grapes) was significantly more effective in preventing oxidative stress induced in the rabbit bladder using a variety of oxidative stress models [51,52].Two reasons for this may be: 1) natural products have many components that may work synergistically in the treatment of the pathology; and 2)many of the individual active components of natural products have significantly better solubility, bioavailability, and pharmacokinetic properties than that of the purified "active ingredient".

In many ways, congestive heart failure (CHF) is very similar to obstructive bladder function (OBF)[53,54]. In CHF models, arterial blood flow is restricted causing an increase in the muscle strength needed to maintain blood flow, resulting in cardiac muscle hypertrophy and ischemic dysfunctionsthat can lead to death[55,56]. Similarly,OBFresults in increased resistance to urine flow (by placing a ring or suture loosely around the urethra to increase resistance), resulting in compensated increase in smooth muscle thickness, ischemia and decreased bladder contractile function[57,58]. One major difference is that CHFresults in altered blood flow throughout the entire body resulting in the failure of other organs[59,60], leading to death; whereas OBF can result in urinary retention (which can be relieved easily by catheterization through the

urethra). Other organ systems are not affected, and thus the ischemic disease is limited to the bladder and can be more easily and specifically studied than other organ-models of ischemia / reperfusion (I/R).

Urinary bladder dysfunction secondary to benign prostatic hyperplasia (BPH) is a major affliction of ageing men. As the prostate gradually enlarges, the response to increased urethral resistance results in increase in bladder mass via mucosal hyperplasia, hypertrophy of the smooth muscle layer and a net increase in bladder-wall thickness[16,61,62]. In turn, the increased wall thickness results in ischemia/reperfusion (I/R) injury, characterized by damage to nerves, synapses and smooth muscle cells within the bladder wall which leads to generation of reactive oxygen species (ROS) and reactive nitrogen species (RNOS), that oxidize cellular and subcellular membrane proteins and lipids[34,63,64], resulting in lipid peroxidation. The damage to the cellular and subcellular membraneslead to dysregulation of Ca^{+2} homeostasis, which results in a net increase of intracellular Ca^{+2}due to increased release from the sarcoplasmic reticulum (SR) and mitochondria[65-68]. The high intracellular Ca^{+2}concentrationsactivate Ca^{+2}-dependent hydrolytic enzymes such as calpain and phospholipase A_2[67,68]. The hydrolytic activity of these enzymes, is responsible fordamagesof neurons and synapses, resulting in the observed denervation and damaged membranes of the SR and mitochondria[34,63,64,69].

Two major advantages of using the rabbit bladder model of I/R are: 1) I/R leads to a direct oxidative damage to the bladder smooth muscle and mucosa whereas the model of PBOO in addition to I/R also induces progressive hypertrophy, hyperplasia, angiogenesis, connective tissue synthesis and inflammation; making linking treatments to relieving oxidative stress complicated. 2)As mentioned previously the rabbit bladder is a relatively closed system. That is inducing specific pathologies of the bladder such as I/R and PBOO have little or no effects on other organ systems; unlike pathological models of cardiac, liver, lung, kidney and other organ systems that have significant secondary effects on other organ systems, which can feed back and induce tertiary damage to the organ system under investigation; thus significantly complicating analysis.

As mentioned above, the cellular dysfunctions and disease etiologies are very similar to those of other organ systems, especially to congestive heart failure. Thus we believe that the bladder is an excellent organ system to study the etiology of cellular dysfunctions induced by ischemia, obstruction, and reperfusion without the complications of other organ failures, which can result in additional damage to the primary organ system.

Antioxidants have been proven to be very effective in protecting the rabbit bladder from oxidative damage mediated by I/R induced by partial bladder outlet obstruction (PBOO)[35,38, 41,70,71].PBOO, as demonstrated above, induces a variety of bladder dysfunctions in addition to ischemia / reperfusion. In order to eliminate these other effects, we have developed an *in-vivo* model of bladder I/R in the rabbit by exposing the bladder and placing vascular clamps on the vesical arteries for 2 hours (ischemia) and then releasing the clamps to initiate reperfusion for a variable period of time [72-75]. Using this method, we have demonstrated that the reperfusion period induces the formation of both ROS and RNOS resulting in oxidative stress and contractile and biochemical dysfunctions[74,76].

One of our most interesting studies using this model of I/R evaluated the effects of Ganoderma Lucidum (GL) on oxidative stress. GL has been shown by Chinese pharmaceutical studies and clinical research to be useful in preventing certain types of discomfort and helps in prolonging human life in part by acting as an antioxidant. The popular ancient Chinese medical dictionary written in the Ming Dynasty provides detailed descriptions of the effects of GL, showing that it has been used effectively for several thousand years[77].

Chinese scientific studies have confirmed that the polysaccharides and triterpenes in GL are key ingredients needed to maintain a physiological balance within the human body and also help prolonging life[78-80]. There are numerous scientific publications (both basic science and clinical studies) demonstrating the strong antioxidant properties of GL which make this the perfect natural product to utilize in these studies [77, 81-86].

The preparation of GL that we used for these studies were broken spore shell extracts which have been demonstrated to have the greatest beneficial effects in the treatment of a variety of pathologies, especially those relating to I/R [87-89], which was carried out in a rabbit urinary bladder model for I/R.

Methods

All studies were approved by the Institutional Animal Use and Care Committeeand the Research and Development Committee of the Stratton VA Medical Center, Albany, NY.

Twenty four adult male New Zealand White rabbits were divided into 4groups (6 rabbits in each group). Group 1:control rabbits (control rabbits received sham operations),Group 2:control rabbits receivingGL (100 mg/kg body weight/dayby gavage for 2 weeks prior to sham surgery and for 4 weeks following sham surgery, Group 3:rabbits who received bilateral ischemia by clamping the vesicular arteries (arteries entering the bladder) for 2 hours and then having the clamps removed and allowing the rabbits to recover for 4 weeks(reperfusion period) and Group 4:rabbits were given a suspension of GL (100 mg/kg body weight/day)by gavage daily for 2 weeks prior to surgery and for 4 weeks following surgery. Previous studies demonstrated that two weeks pre-treatment showed a maximal effect of antioxidants[71,90].

Cystometry

Before surgery and at the end of the reperfusion period, each rabbit was sedated using ketamine/xylazine(10mg / 4 mg / kg). The bladder was catheterized with an 8French Foley catheter and the bladder emptied. A

filling cystometrogram was performed using warmed saline at a filling rate of 1 mL per minute until a micturition contraction occurred. The French scale or French gauge system is commonly used to measure the size of a catheter. It is most often abbreviated as **Fr.** The French size is three times the diameter in millimeters. A round catheter of 1 French has an external diameter of $\frac{1}{3}$ mm, and therefore the diameter of a round catheter in millimeters can be determined by dividing the French size by 3:

Surgery

Each rabbit was sedated with ketamine/xylazine (10mg/4mg / kg,im) while surgical anesthesia was maintained with isoflurane (1-3%). Under sterile conditions, the urinary bladder was catheterized with an 8 French. Foley catheter, emptied, and exposed through a midline incision. Ischemia was initiated by placing vascular clamps on both vesicular arteries at the point of entranceto the bladder. Reperfusion was mediated by removing the clamps after 2 h of ischemia and allowing the rabbit to recover for 4 weeks. Sham surgeries were performed as controls: each sham-operated rabbit underwent the same procedures (catheterization, anesthesia, and surgery) except no vascular clamps were applied.

Contractile Studies

After the 4 week reperfusion period, the rabbits were anesthetized, and their bladders were excised intact. Three full thickness longitudinal strips were then cut from the mid-bladder and placed in individual isolated baths containing 15 ml of an oxygenated Tyrode's physiological solution (137 mMNaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$,0.2 mM Na_2HPO_4, and 12 mM $NaHCO_3$) containing 1 mg/mL glucose. During a 30 minute period of equilibrium at 37° C, 2 gof tension were placed on each strip and they were then stimulated as follows: field stimulation at 2, 8, and 32 Hz; carbachol; ATP; and KCl. After each drug stimulation, the bladder strips were rinsed with fresh warmed, oxygenatedTyrode's three timesat 15 minute intervals. Tension was recorded continually using a Grass Polygraph and the signal digitized using a Polyview system.Maximal contractile responses were recorded from the digital recording.

The balance of the bladder was separated into smooth muscle and mucosal tissues by blunt dissection, frozen by directly dipping the tissues under liquid nitrogen and stored at -80°C for biochemical studies.

Citrate Synthase Assay [52, 91, 92]

Frozen tissue samples were homogenized in 0.05M Tris buffer (100 mg/mL). Samples were then centrifuged at 800g for10 min and supernatant was separated. 0.9mL of supernatant was added to 0.1mL of Triton X-100 in a 10 mL test tube for each sample. Samples (40 µL) were added to 3 mL cuvettes, along with 1.1mL 0.05M Tris buffer (pH 7.6), 30µL 24.6mM acetyl-coenzyme A, and 100 µL 1mM 5.5'- dithiobis-2-nitrobenzoic acid (DTNB). The final volume in each cuvette was 1.4 mL excluding the 50 µL oxaloacetate (10 mM- substrate) used to start the reaction. The activity was read every 2 minutes for 30 minutes in a Hitachi U-2001 spectrophotometer at 412 nm.

Sarco / endoplasmic reticular calcium ATPase (SERCA)Assay [92, 93]

40 mg samples of frozen tissue were homogenized at 10 mg/mL in 50mM TRIS buffer (pH 7.4). The sample was then centrifuged at 800g for 10 min. The supernatant (sample) was saved and the pellet discarded. The conditions were sample (1 mL) plus thapsigargin (10 µM), sample minus thapsigargin, control with no homogenate, and control with no ATP. Sample and control tubes were incubated at 37°C for 40 min. At the end of the incubation, 0.5mLtrichloroacetic acid (TCA) was added to stop the reaction after which the tubes were vortexed. 0.5mL ferrous sulfate molybdate was then added to all tubes and the phosphate levels were measured spectrophotometrically at 650nm.

The values for SERCA were determined by subtracting the values of sample with thapsigargin from the values of sample without thapsigargin. This was done to differentiate between the enzyme activity of plasma Ca^{2+}ATPase and SERCA. Thapsigargin is a non-competitive inhibitor of SERCA [94], thus total ATPase activity – activity in the presence of thapsigargin = SERCA activity.

Antioxidant Activities:

The antioxidant activity of the GL preparation was performed using the CUPRAC test for total antioxidant activity with ascorbic acid as the standard. The CUPRAC working solution consisted of 10 mM copper (II)chloride dihydrate, 1 M ammonium acetate, and7.5 mMneocuproine. Volumes of 0.15 ml of the

above three solutions were added to 0.15 ml of each sample and allowed to react for 30 min at room temperature, after which the absorbance was read at450 nm in a Hitachi U-2001 spectrophotometer. The standard curve utilized in this assay was ascorbic acid with the following concentrations (mg/ml): 20, 10, 5, 2.5, 1.25, 0. The antioxidant activity of the GL was calculated as the mg ascorbic acid equivalents..

Protein Concentration

The protein concentration was performed using the bicinchoninic acid (BCA) method (Eliza kit from Thermo Fisher Scientific). Specifically, the BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate.

In the second step of the color development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/

copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The BCA reagent is approximately 100 times more sensitive (lower limit of detection) than the pale blue color of the first reaction..[95].

Statistics

We utilized one way Analysis of Variance followed by the Tukey test for comparison among individual groups (Sigmastat). A $P< 0.05$ was considered statistically significant.

Results

Bladder Weight

Theischemia / reperfusion (I/R)bladders showed a small but significant increase in bladder weight compared to the control group.

Cystometry

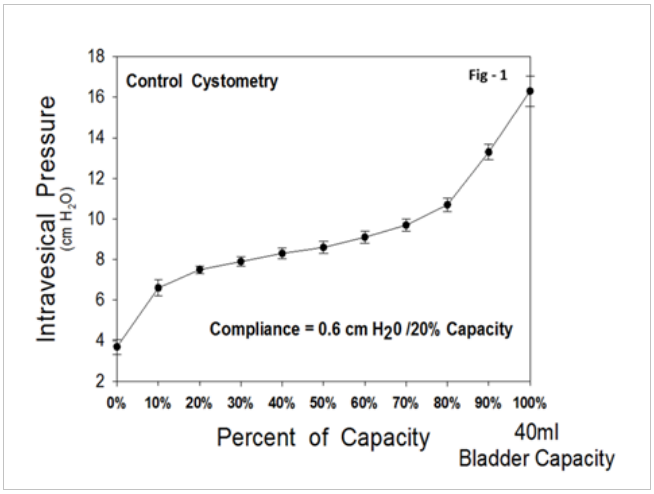


Figure 1 displays a control cystometry from the study. It has the same shape and characteristics of a cystometrogram from humans. The compliance is given as cm H_2O / 20% of capacity taken from the plateau portion of the curve. In the case of the curve shown it was between 20% and 40%.

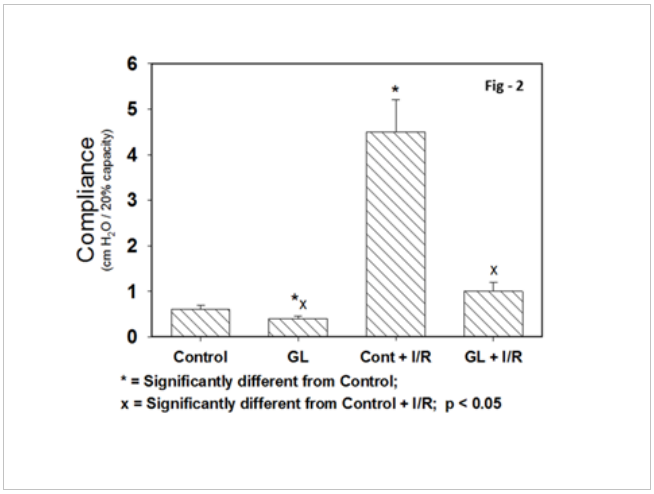
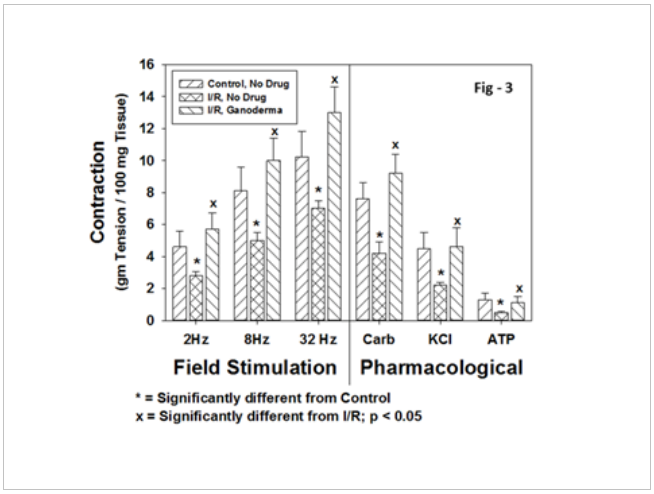


Figure 2 shows the quantitative comparison of the compliance of the 4 groups. The GL group showed significantly increased compliance compared to the Control + I/R group. GL + I/Rreturned the cystometric curve to control levels; thus showing a significant beneficial effect for the GL + I/Rgroup.There were no significant differences in the functional bladder volume (bladder volume at micturition)[96].



The contractile responses to all forms of stimulation were statistically lower for the I/R group compared to the control–no GL group **(Figure 3).** Pretreatment with GL completely inhibited the dysfunctional effects of I/R on the contractile responses to all forms of stimulation[96]. There were no significant differences between the control – no GL group and the control GL group, thus the Control GL group is not shown on the figure to avoid crowding the figure.

Field stimulated contraction represents neurotransmission responses and is a consequence of neurotransmitter release, postsynaptic receptor activation, calcium induced calcium release (CICR) and smooth muscle contraction. Carbachol contracts smooth muscle by direct muscarinic receptor activation resulting in CICR. ATP contracts smooth muscle by direct purinergic receptor activation. KCl depolarizes the smooth muscle membrane resulting in calcium entry into the cytoplasm of the smooth muscle and smooth muscle contraction; no CICR or receptor stimulation is involved. The fact that all forms of stimulation were reduced to approximately the same degree indicates that the major contractile dysfunction was in the smooth muscle rather than in the synapses, receptors or smooth muscle membranes.

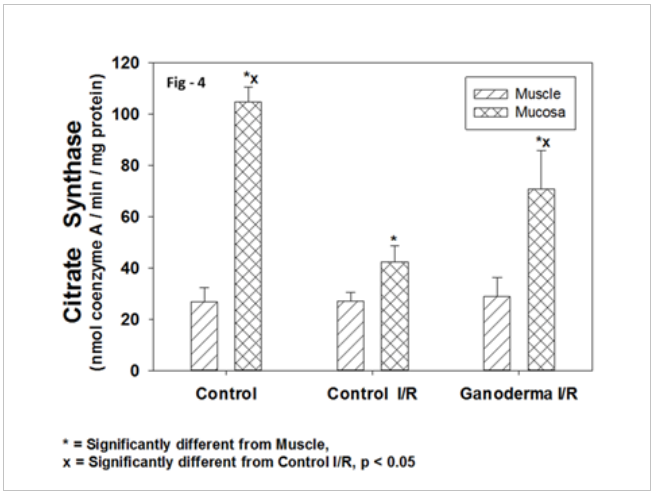


Figure 4 displays the citrate synthase activity (CS) for both muscle and mucosa. CS is involved in the rate limiting step in the mitochondrial citric acid cycle; and is a biomarker for mitochondrial health and activity.

As has been previously demonstrated the mucosa has a 5 fold higher activity than the muscle (in the control tissues [97, 98]. After I/R, the CS activity of the mucosa was reduced significantly by approximately 60% whereas the CS activity of the muscle remained the same as control. Pre-feeding rabbits with GL significantly prevented the the decrease in mucosal CS activity.

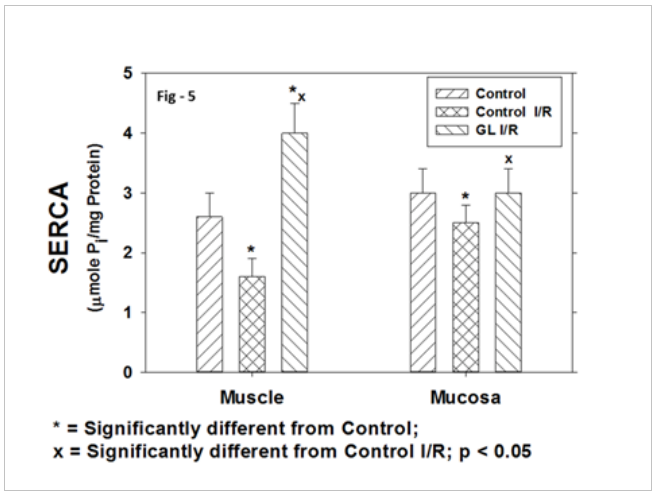
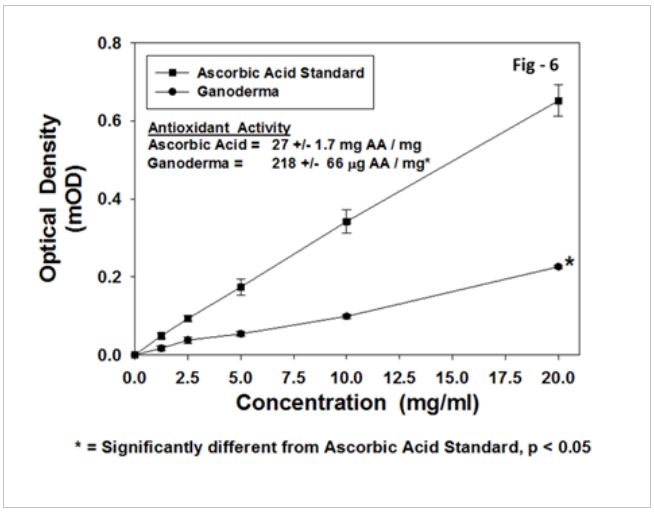


Figure 5 displays the SERCA activity. SERCA is the enzyme localized on the membrane of the sarcoplasmic reticulum responsible for the uptake and subsequent storage of intracellular calcium following a contraction. It is essential in the maintenance of intracellular calcium balance. An increase in intracellular free calcium would result in decreased compliance and decreased contraction. I/R resulted in significant decreases in the activities of SERCA in both muscle and mucosa; being reduced in a significantly greater extent in the muscle than in the mucosa. Pre-treatment with GL prevented reduction in SERCA activity and actually increased the SERCA activity of the muscle above control. These results would contribute significantly to the protective effect of GL on the contractile responses presented above.



The Cuprac test for total antioxidant activity for the ascorbic acid (standard) and for the GL is presented in Figure 6. The antioxidant activity for both ascorbic acid (AA) and GL are calculated as AA in mg per optical density reading (mg AA / mOD). The ascorbic acid standard curvewas linear with an antioxidant value of 27 +/- 1.7 mg AA per mOD.The GL has an antioxidant value of 4.6 +/- 0.3 mg AA equivalents per mg GL. At 100 mg / day GL,eachrabbit received 460 mg ascorbic acid equivalents of antioxidant activity per day.

The protein concentration of the was 213 µg protein per mg GL. Thus the rabbits received 10.8 mg ascorbic acid equivalents of antioxidant activity per mg protein.

Discussion

Oxidative stress plays important roles in a variety of specific pathophysiologies and in theageing 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 benign prostatic hyperplasia (BPH) [99,100]. Although it is common knowledge that in man progressive bladder dysfunction occurs in association with ageing, there is also excellent evidence that in rabbits both bladder physiological dysfunctions and biochemical dysfunctions occur [73,101-103]. 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 partial bladder outlet obstruction (PBOO) [16,17,19]. 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 significantly decreased blood flow during contraction followed by reperfusion resulting in the generation of free radicals and the resulting oxidative damage to muscle and mucosal cellular and subcellular membranes [7,8,34,64,104,105]. Indirect evidence for an I/R etiology of OBD includes a series of published studies demonstrating that several specific natural antioxidant products can protect the bladder from oxidative stress [38,41,106-108].

PBOO induces a variety of bladder responses in addition to I/R including hypertrophy of the smooth muscle [37,109], hyperplasia of the mucosa [110,111], angiogenesis [112,113], and connective tissue replacement of smooth muscle which reduce the compliance of the bladder [22], and inflammation[114,115]. In order to eliminate these other effects, we have developed an *in vivo* model of bladder I/R in the rabbit by exposing the bladder and placing vascular clamps on the vesical arteries entering the bladder for 2 hours (ischemia) and then releasing the clamps to initiate reperfusion for a variable period of time [72-75]. Bladder mass was increased in the Control + I/R group. Although the magnitude of this increase was considerably lower than that seen following PBOO, it could be due to increased inflammation of the bladder [114,115] and / or hypertrophy of the smooth muscle[37,109] to compensate for the decreased contractile responses.

Although we did not quantitate either oxidative stress bio-markers such as DNP or nitrotyrosine; or the cellular antioxidant enzymes such as superoxide dismutase (SOD) and Catalase (CAT) in the present study; other published studies utilizing the *in vivo* bilateral ischemia / reperfusion (I/R) model have demonstrated that the I/R resulted in similar decrease in contraction and compliancewhich correlated with increased DNP and nitrotyrosine concentrations, and decreased activities of SOD and CAT. In addition, in a study in which AntrodiaCamphorata (AC) pre-treatment was used in similar studies, it was shown that AC pre-treatment was effective in protecting against I/R induced contractile and compliance dysfunction, decreased citrate synthase activity, decreased nerve density, and increased apoptosis[45,71,72,74, 76,90].

Pre-treatment of rabbits for two weeks with GLprior to subjecting them to ischemia followed by reperfusion completely inhibited the dysfunctional effects of I/R on both the compliance and contractile responses. We have performed similar studies with both specific antioxidant supplements (vitamin E, coenzyme Q10, and alpha lipoic acid) [35,38,71,116,117] and natural products (whole grape preparations, Antrodia camphorate and pygeumafricanum)[45,72,118, 119]. Based on these studies, GL provided equal or better protection. We have clearly identified that the primary etiology of the pathological effects of I/R is due to the oxidative stress due to higher generation of free radicals[72-75]; thus the beneficial effects of GL would be due to strong antioxidant effects as demonstrated by the CUPRAC assays [120,121].

Decreased compliance (increased stiffness) is a function of two intracellular processes within the bladder. The first is an increase in the connective tissue within and between the smooth muscle cells which restricts the ability of the bladder to fill and thus increases the intravesical pressure during bladder filling[110,122-125].The second is an increase in intracellular free calcium during bladder filling which results in an increase in baseline contraction during filling, andalso leadsto decreased compliance [67,68,126,127].Antioxidants have been shown to have antifibrotic activity in several biological systems [128-130].This action explainsin part for positive effect of GL on bladder compliance.

Although specific antioxidants and natural products have been shown to protect bladder contractile responses to I/R and PBOO [35,38,70,71], not all antioxidants have this effect[51, 52, 131]. 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 although it was not nearly as effective as the whole grape preparation was in protecting the bladder from deleterious effects of oxidative stress [51,52]. As mentioned previously, several clinical studies on the effectiveness of specific antioxidants on specific diseases linked to oxidative stress have been disappointing[47-50].

GL has been demonstrated to have a variety of properties that may be useful in the treatment of a number of diseases such as cancer, diabetes, cardiac, inflammatory and oxidative stress related diseases[132-137]. In a related study, GL has been shown to be effective in the treatment of ischemic heart disease in an isolated rat heart model[138,139], presumably via its antioxidant effects. This model has many similarities to the functional isolated whole bladder model[21,140, 141].

A second physiological property of Ganoderma is the protection of cardiac capillary permeability against penetration of small particles that can damage underlying cardiac muscle, and similar protection of the gut lining against increased permeability[142,143]. Urothelial permeability can be easily evaluated in our *in vivo* and *in vitro* bladder models using a variety of methodologies[144-146]. Thus, the rabbit urinary bladder is an excellent model to study the beneficial effects of GL on the response of organ system to oxidative stress. Oxidative stress has significant negative effects on mucosal linings and mucosal permeability, vascular permeability, muscle contractile activity, and smooth muscle responses to a variety of forms of stimulation. It has the advantage of being a closed system; that is, there are no dysfunctions of other organ systems which can significantly complicate the evaluation of bladder studies.

As can be observed in the above references, GL has a variety of bioactive components, and the bio-activity of one specific component would not give the same beneficial effects as that of a preparation of the whole GL preparation. We have completed one additional study on GL using an *in vitro* model of I/R[147-149]

In this study, 8New Zealand White rabbits were divided into 2 groups. One group was fed Ganoderma Lucidum (GL) (100 mg/Kg) daily for 3 weeks while the second group received water. The GL preparation was a powder suspension in water. At the end of the 3 week period, each rabbit was euthanized and the bladder separated into six strips and mounted in individual baths. Each strip was stimulated with field stimulation (FS) (Please see page 12, last paragraph for description), Carbachol, potassium chloride (KCl), and adenosine triphosphate (ATP). After the control stimulations, the oxygenated baths were subjected to nitrogen gas equilibration (replacing the oxygen in the bath and was at 37° C)in the absence of glucose (ischemia) for 1 h while being stimulated at 32Hz at 5 min intervals. After 1 h of ischemia the buffer was changed back to normal oxygenated Tyrode's with glucose and allowed to recover for 2 h. After this time period, the strips were stimulated as described earlier. The results are summarized as follows: 1)There was a rapid and near complete inhibition of the contractile responses during the ischemic period for both control and GL groups, 2) For the control group, following I/R there was a 86% decrease in the response to FS, 11% decrease in the response to ATP, 17% decrease in the response to carbachol, and 20% decrease in the response to KCl. For the GL group, there was a significantly less decrease in the response to FS, and no decrease in the responses to ATP, carbachol, or KCl. Thus, pretreatment of rabbits for three weeks with GL prior to subjecting them to *in vitro* ischemia/reperfusion significantly protected the bladder strips from all forms of contractile dysfunctions.

One additional interesting observation is that GL has been shown in several studies to have significant anti-ageing properties. Proposed mechanisms for these anti-ageing effects are neuroprotection against age-related neural-degeneration [150,151], age-related oxidative stress (antioxidant) [152], novel ergo sterols shown to have anti-ageing properties [153,154], and ageing effects on mitochondrial function [155,156].These studies are extremely relevant to continued studies of GL in ageing studies in the bladder.

We know that the progressive bladder dysfunction in during ageing in both man and rabbit involve the progressive decrease in antioxidant defense mechanisms [157,158], progressive mitochondrial damage[102,159], progressive synaptic degeneration[160,161], and increased content of collagen and decreased content of smooth muscle in response to progressive smooth muscle apoptosis and degeneration[101,160]. Thus, the anti-ageing properties of GL would make this an excellent candidate to determine its anti-ageing effects in a closed organ system such as the rabbit urinary bladder.

Acknowledgement

These studies were based upon work supported in part by Well strong Biotech Company, Beijing Tong Ren Tang Chinese medicine Co., LTD, the Office of Research and Development Department of the Veterans Affairs, and by the Capital Region Medical Research Foundation.

References

1. Levin RM, Monson FC, Longhurst PA Wein AJ (1994) Rabbit as a model of urinary bladder function. Neurourol Urodyn 13(2): 119-135.

2. Andersson KE , Arner A (2004) Urinary bladder contraction and relaxation: physiology and pathophysiology. Physiol Rev 84(3): 935-986. doi: 10.1152/physrev.00038.2003

3. Manoharan S, Guillemin GJ, Abiramasundari RS, Essa MM, Akbar M, et al. (2016) The Role of Reactive Oxygen Species in the Pathogenesis of Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease: A Mini Review. Oxid Med Cell Longev 2016:8590578. doi: 10.1155/2016/8590578

4. Akhtar MJ, Ahamed M, Alhadlaq HA, Alshamsan A (2017) Mechanism of ROS scavenging and antioxidant signalling by redox metallic and fullerene nanomaterials: Potential implications in ROS associated degenerative disorders. Biochim Biophys Acta 1861(4): 802-813. doi: 10.1016/j.bbagen.2017.01.018

5. Hugel HM (2015) Brain Food for Alzheimer-Free Ageing: Focus on Herbal Medicines. Adv Exp Med Biol 863: 95-116. doi: 10.1007/978-3-319-18365-7_5

6. Li CS, Deng HB, Li DD, Li ZH (2013) Advances and challenges in screening traditional Chinese anti-aging materia medica. Chin J Integr Med 19(4): 243-252. doi: 10.1007/s11655-013-1432-8

7. Levin RM, Brading AF, Mills IW, Longhust PA (2000) Experimental Models of Bladder Obstruction. In: Lepor H (ed) Prostatic Diseases, W.B. Saunders Co., Philadelphia pp. 169-196.

8. Mannikarottu A, Kogan, B, and Levin, RM (2005) Ischemic etiology of obstructive bladder dysfunction: A review. Recent Res. Devel. Mol Cell Biochem 2: 15-34.

9. Levin RM, Longhurst PA, Monson, FC, Haugaard N, Wein A J (1993) Experimental studies on bladder outlet obstruction. In: H. Lepor RKL (ed) Prostate Diseases, W.B. Saunders, Philadelphia pp. 119-130.

10. Yoon JY, Zderic SA, Duckett JW, Levin RM (1994) Effect of partial outlet obstruction on the biphasic response to field stimulation at different concentrations of calcium. Pharmacology 49(3): 167-172. doi: 10.1159/000139231

11. Saito M, Wein AJ and Levin RM (1994) Biphasic response of the rabbit detrusor muscle to changing the extracellular concentrations of potassium and calcium. Gen Pharmacol 25:1101-7.

12. Levin RM, Chichester P, Hass MA, Gosling JA, Buttyan R (2002) Obstructive bladder dysfunction: Morphological, Biochemical, and Molecular Changes. European Urology Supplements 1(9): 14-20.

13. Manieri C, Carter SS, Romano G, Trucchi A, Valenti M, et al. (1998) The diagnosis of bladder outlet obstruction in men by ultrasound measurement of bladder wall thickness. J Urol 159(3): 761-765.

14. Tubaro A, Mariani S, De Nunzio C and Miano R (2010) Bladder weight and detrusor thickness as parameters of progression of benign prostatic hyperplasia. Curr Opin Urol 20(1): 37-42. doi: 10.1097/MOU.0b013e32833307e0

15. Chalana V, Dudycha S, Yuk JT and McMorrow G (2005) Automatic Measurement of Ultrasound-Estimated Bladder Weight (UEBW) from Three-Dimensional Ultrasound. Rev Urol 7(6): 22-28.

16. Levin RM, Haugaard N, O'Connor L, Buttyan R, Das A, et al. (2000) Obstructive response of human bladder to BPH vs. rabbit bladder response to partial outlet obstruction: a direct comparison. Neurourol Urodyn 19(5): 609-629.

17. Gosling JA, Kung LS, Dixon JS, Horan P, Whitbeck C, et al. (2000) Correlation between the structure and function of the rabbit urinary bladder following partial outlet obstruction. J Urol 163(4): 1349-1356.

18. Levin RM, Saito M, Wein AJ, Packard D, Cohen A, et al. (1993) Effect of partial outlet obstruction on choline acetyltransferase activity in the rat and rabbit. Neurourol Urodyn 12(3): 255-261.

19. Levin RM, Haugaard N, Mogavero L, Leggett RE and Das AK (1999) Biochemical evaluation of obstructive bladder dysfunction in men secondary to BPH: a preliminary report. Urology 53(2): 446-450.

20. Harrison SC, Hunnam GR, Farman P, Ferguson DR, Doyle PT (1987) Bladder instability and denervation in patients with bladder outflow obstruction. Br J Urol 60(6): 519-522.

21. Levin RM, Yu HJ, Kim KB, Longhurst PA, Wein AJ and Damaser MS (1997) Etiology of bladder dysfunction secondary to partial outlet obstruction. Calcium disregulation in bladder power generation and the ability to perform work. Scand J Urol Nephrol Suppl 184: 43-50.

22. Levin RM, Reed TP, Whitbeck C, Chichester P and Damaser M (2005) Effect of strip length on the contractile dysfunction of bladder smooth muscle after partial outlet obstruction. Urology 66(3): 659-664. doi: 10.1016/j.urology.2005.03.057

23. Sovari AA (2016) Cellular and Molecular Mechanisms of Arrhythmia by Oxidative Stress. Cardiol Res Pract 2016:9656078. doi: 10.1155/2016/9656078

24. Dhalla NS, Golfman L, Takeda S, Takeda N and Nagano M (1999) Evidence for the role of oxidative stress in acute ischemic heart disease: a brief review. Can J Cardiol 15(5): 587-593.

25. Reddy S and Bernstein D (2015) Molecular Mechanisms of Right Ventricular Failure. Circulation 132(18): 1734-1742. doi: 10.1161/CIRCULATIONAHA.114.012975

26. Nikooyeh B and Neyestani TR (2016) Oxidative stress, type 2 diabetes and vitamin D: past, present and future. Diabetes Metab Res Rev 32(2): 260-267. doi: 10.1002/dmrr.2718

27. Rani V, Deep G, Singh RK, Palle K and Yadav UC (2016) Oxidative stress and metabolic disorders: Pathogenesis and therapeutic strategies. Life Sci 148: 183-193. doi: 10.1016/j.lfs.2016.02.002

28. Stone JR, Wilkins LR (2015) Acute mesenteric ischemia. Tech Vasc Interv Radiol 18: 24-30. doi: 10.1053/j.tvir.2014.12.004

29. Stoney RJ and Cunningham CG (1993) Acute mesenteric ischemia. Surgery 114(3): 489-490.

30. Bhattacharyya A, Chattopadhyay R, Mitra S and Crowe SE (2014) Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. Physiol Rev 94(2): 329-354. doi: 10.1152/physrev.00040.2012

31. Nastos C, Kalimeris K, Papoutsidakis N, Tasoulis MK, Lykoudis PM, et al. (2014) Global consequences of liver ischemia/reperfusion injury. Oxid Med Cell Longev 2014:906965. doi: 10.1155/2014/906965

32. Tucker PS, Scanlan AT, Dalbo VJ (2015) Chronic kidney disease influences multiple systems: describing the relationship between oxidative stress, inflammation, kidney damage, and concomitant disease. Oxid Med Cell Longev 2015:806358. doi: 10.1155/2015/806358

33. Tamma G, Valenti G (2016) Evaluating the Oxidative Stress in Renal Diseases: What Is the Role for S-Glutathionylation?25(3): 147-164. Antioxid Redox Signal. doi: 10.1089/ars.2016.6656

34. Juan YS, Lin WY, Kalorin C, Kogan BA, Levin RM , et al. (2007) The effect of partial bladder outlet obstruction on carbonyl and nitrotyrosine distribution in rabbit bladder. Urology 70:1249-53. doi: 10.1016/j.urology.2007.09.047

35. Li HT, Schuler C, Leggett RE and Levin RM (2011) Differential effects of coenzyme Q10 and alpha-lipoic acid on two models of in vitro oxidative damage to the rabbit urinary bladder. Int Urol Nephrol 43(1): 91-97. doi: 10.1007/s11255-010-9771-2

36. Nomiya M, Andersson KE and Yamaguchi O (2015) Chronic bladder ischemia and oxidative stress: new pharmacotherapeutic targets for lower urinary tract symptoms. Int J Urol 22(1): 40-46. doi: 10.1111/iju.12652

37. Levin RM, Schuler C, Leggett RE, Callaghan C and Maknuru S (2013) Partial outlet obstruction in rabbits: duration versus severity. Int J Urol 20(1): 107-114. doi: 10.1111/j.1442-2042.2012.03184.x

38. Juan YS, Levin RM, Chuang SM, Hydery T, Li S, et al. (2008) The beneficial effect of coenzyme Q10 and lipoic acid on obstructive bladder dysfunction in the rabbit. J Urol 180(5): 2234-2240. doi: 10.1016/j.juro.2008.07.022

39. Lindblom R, Higgins G, Coughlan M and de Haan JB (2015) Targeting Mitochondria and Reactive Oxygen Species-Driven Pathogenesis in Diabetic Nephropathy. Rev Diabet Stud 12(1-2): 134-156. doi: 10.1900/RDS.2015.12.134

40. Saha SP and Whayne TF, Jr (2016) Coenzyme Q-10 in Human Health: Supporting Evidence? South Med J 109(1): 17-21. doi: 10.14423/SMJ.0000000000000393

41. Radu F, Leggett RE, Schuler C and Levin RM (2011) The effect of antioxidants on the response of the rabbit urinary bladder to in vitro ischemia/reperfusion. Mol Cell Biochem 355(1-2): 65-73. doi: 10.1007/s11010-011-0839-9

42. Kim J, Fann DY, Seet RC, Jo DG, Mattson MP, et al. (2016) Phytochemicals in Ischemic Stroke.18(3): 283-305. Neuromolecular Med. doi: 10.1007/s12017-016-8403-0

43. Xie C, Xie Z, Xu X and Yang D (2015) Persimmon (Diospyros kaki L.) leaves: a review on traditional uses, phytochemistry and pharmacological properties. J Ethnopharmacol 163: 229-240. doi: 10.1016/j.jep.2015.01.007

44. Yu L, Qin Y, Wang Q, Zhang L, Liu Y, et al. (2014) The efficacy and safety of Chinese herbal medicine, Rhodiola formulation in treating ischemic heart disease: a systematic review and meta-analysis of randomized controlled trials. Complement Ther Med 22(4): 814-825. doi: 10.1016/j.ctim.2014.05.001

45. Juan YS, Mannikarottu A, Chuang SM, Li S, Lin AD, et al. (2010) Protective effect of Antrodia camphorata on bladder ischemia/reperfusion injury. Int Urol Nephrol 42(3): 637-645. doi: 10.1007/s11255-009-9642-x

46. Levin RM, Leggett RE, Whitbeck C, Matsumoto S, Ohto N, et al. (2008) Kohki tea protects the rabbit bladder from ischemia/reperfusion-induced contractile dysfunction. Urol Int 80(4): 425-430. doi: 10.1159/000132702

47. Suksomboon N, Poolsup N, Juanak N (2015) Effects of coenzyme Q10 supplementation on metabolic profile in diabetes: a systematic review and meta-analysis. J Clin Pharm Ther 40(4): 413-418. doi: 10.1111/jcpt.12280

48. Ozkanlar S and Akcay F (2012) Antioxidant vitamins in atherosclerosis--animal experiments and clinical studies. Adv Clin Exp Med 21(1): 115-123.

49. Lonn E (2001) Do antioxidant vitamins protect against atherosclerosis? The proof is still lacking*. J Am Coll Cardiol 38(7): 1795-1798.

50. McQuillan BM, Hung J, Beilby JP, Nidorf M and Thompson PL (2001) Antioxidant vitamins and the risk of carotid atherosclerosis. The Perth Carotid Ultrasound Disease Assessment study (CUDAS). J Am Coll Cardiol 38(7): 1788-1794.

51. Francis JA, Leggett RE, Schuler C and Levin RM (2015) Comparative biochemical responses and antioxidant activities of the rabbit urinary bladder to whole grapes versus resveratrol. Mol Cell Biochem 410(1-2): 121-129. doi: 10.1007/s11010-015-2544-6

52. Francis JA, Leggett RE, Schuler C and Levin RM (2014) Effect of hydrogen peroxide on contractility and citrate synthase activity of the rabbit urinary bladder in the presence and absence of resveratrol and a whole-grape suspension. Mol Cell Biochem 391(1-2): 233-239. doi: 10.1007/s11010-014-2007-5

53. Koeck I, Burkhard FC and Monastyrskaya K (2016) Activation of common signaling pathways during remodeling of the heart and the bladder. Biochem Pharmacol 102: 7-19. doi: 10.1016/j.bcp.2015.09.012

54. Evans JM, Owens TP, Jr., Zerbe DM, Rohren CH (1995) Venous obstruction due to a distended urinary bladder. Mayo Clin Proc 70:1077-10779. doi: 10.1016/S0025-6196(11)64443-1

55. Yarbrough WM and Spinale FG (2003) Large animal models of congestive heart failure: a critical step in translating basic observations into clinical applications. J Nucl Cardiol 10(1): 77-86. doi: 10.1067/mnc.2003.16

56. O'Brien PJ, O'Grady M, McCutcheon LJ, Shen H, Nowack L, et al. (1992) Myocardial myoglobin deficiency in various animal models of congestive heart failure. J Mol Cell Cardiol 24(7): 721-730.

57. Levin R, Chichester P, Levin S and Buttyan R (2004) Role of angiogenesis in bladder response to partial outlet obstruction. Scand J Urol Nephrol Suppl:37-47. doi: 10.1080/03008880410015156

58. Kibar Y, Irkilata HC, Yaman H, Onguru O, Coguplugil AE, et al. (2011) The effect of intravesical acetylsalicylic acid instillation on tissue prostaglandin levels after partial bladder outlet obstruction in rabbits. Neurourol Urodyn 30(8): 1646-1651. doi: 10.1002/nau.21153

59. Jahng JW, Song E, Sweeney G (2016) Crosstalk between the heart and peripheral organs in heart failure. Exp Mol Med 48:e217. doi: 10.1038/emm.2016.20

60. Valentova M, von Haehling S, Doehner W, Murin J, Anker SD, et al. (2013) Liver dysfunction and its nutritional implications in heart failure. Nutrition 29(2): 370-378. doi: 10.1016/j.nut.2012.06.002

61. Bauer SB (2000) The effects and challenges of bladder outlet obstruction. J Urol 163(1): 3.

62. Levin RM, Levin SS, Zhao Y and Buttyan R (1997) Cellular and molecular aspects of bladder hypertrophy. Eur Urol 32 Suppl 1: 15-21.

63. Mannikarottu A, Lin AD, Whitebeck C, Leggett R, Kogan B and Levin R (2006) Effect of partial bladder outlet obstruction on nitrotyrosine levels and their correlation with contractile function. Neurourol Urodyn 25(4): 397-401. doi: 10.1002/nau.20219

64. Levin RM, Agartan CA, Leggett RE, Whitbeck C, Chichester P, et al. (2005) Effect of partial outlet obstruction on nitrotyrosine content and distribution within the rabbit bladder. Mol Cell Biochem 276(1-2): 143-148. doi: 10.1007/s11010-005-4061-5

65. Arner A, Sjuve Scott R, Haase H, Morano I, Uvelius B (2007) Intracellular calcium in hypertrophic smooth muscle from rat urinary bladder. Scand J Urol Nephrol 41(4): 270-277. doi: 10.1080/00365590701225970

66. Scott RS, Uvelius B, Arner A (2004) Changes in intracellular calcium concentration and P2X1 receptor expression in hypertrophic rat urinary bladder smooth muscle. Neurourol Urodyn 23(4): 361-366. doi: 10.1002/nau.20047

67. Callaghan CM, Johnson A, Neumann P, Leggett RE, Schuler C, et al. (2013) The effect of partial outlet obstruction on calpain and phospholipase-2 activities: analyzed by severity and duration. Mol Cell Biochem 381(1-2): 217-220. doi: 10.1007/s11010-013-1705-8

68. Zhao Y, Levin SS, Wein AJ, Levin RM (1997) Correlation of ischemia/reperfusion or partial outlet obstruction-induced spectrin proteolysis by calpain with contractile dysfunction in rabbit bladder. Urology 49(2): 293-300. doi: 10.1016/S0090-4295(96)00452-9

69. Lin WY, Chen CS, Wu SB, Lin YP, Levin RM, et al. (2011) Oxidative stress biomarkers in urine and plasma of rabbits with partial bladder outlet obstruction. BJU Int 107(11): 1839-1843. doi: 10.1111/j.1464-410X.2010.09597.x

70. Juan YS, Chuang SM, Mannikarottu A, Huang CH, Li S, et al. (2009) Coenzyme Q10 diminishes ischemia-reperfusion induced apoptosis and nerve injury in rabbit urinary bladder. Neurourol Urodyn 28(4): 339-342. doi: 10.1002/nau.20662

71. Juan YS, Hydery T, Mannikarottu A, Kogan B, Schuler C, et al. (2008) Coenzyme Q10 protect against ischemia/reperfusion induced biochemical and functional changes in rabbit urinary bladder. Mol Cell Biochem 311(1-2): 73-80. doi: 10.1007/s11010-007-9696-y

72. Lin AD, Mannikarottu A, Kogan BA, Whitbeck C, Leggett RE, et al. (2007) Effect of bilateral in vivo ischemia/reperfusion on the activities of superoxide dismutase and catalase: response to a standardized grape suspension. Mol Cell Biochem 296(1-2): 11-16. doi: 10.1007/s11010-005-9068-4

73. Erdem E, Whitbeck C, Kogan BA, Levin RM (2006) Effect of maturation and aging on response of rabbit bladder to bilateral in vivo ischemia/reperfusion. Urology 67(1): 220-224. doi: 10.1016/j.urology.2005.07.055

74. Bratslavsky G, Whitbeck C, Horan P , Levin RM (1999) Effects of in vivo ischemia on contractile responses of rabbit bladder to field stimulation, carbachol, ATP and KCl. Pharmacology 59(4): 221-226. doi: 28323

75. Gill HS, Monson FC, Wein AJ, Ruggieri MR, Levin RM (1988) The effects of short-term in-vivo ischemia on the contractile function of the rabbit urinary bladder. J Urol 139(6): 1350-1354.

76. Bratslavsky G, Kogan BA, Matsumoto S, Aslan AR, Levin RM (2003) Reperfusion injury of the rat bladder is worse than ischemia. J Urol 170(5): 2086-90. doi: 10.1097/01.ju.0000092144.48045.13

77. Bishop KS, Kao CH, Xu Y, Glucina MP, Paterson RR, Ferguson LR (2015) From 2000years of Ganoderma lucidum to recent developments in nutraceuticals. Phytochemistry 114: 56-65. doi: 10.1016/j.phytochem.2015.02.015

78. Galvez P, Martin MJ, Calpena AC, Tamayo JA, Ruiz MA, et al. (2014) Enhancing effect of glucose microspheres in the viability of human mesenchymal stem cell suspensions for clinical administration. Pharm Res 31(12): 3515-3528. doi: 10.1007/s11095-014-1438-8

79. Mero A, Campisi M, Favero M, Barbera C, Secchieri C,et al. (2014) A hyaluronic acid-salmon calcitonin conjugate for the local treatment of osteoarthritis: chondro-protective effect in a rabbit model of early OA. J Control Release 187: 30-38. doi: 10.1016/j.jconrel.2014.05.008

80. Zehavi U, Polacheck I (1996) Saponins as antimycotic agents: glycosides of medicagenic acid. Adv Exp Med Biol 404: 535-546.

81. Mohsin M, Negi P, Ahmed Z (2011) Determination of the antioxidant activity and polyphenol contents of wild Lingzhi or Reishi medicinal mushroom, Ganoderma lucidum (W.Curt. Fr.) P. Karst. (higher Basidiomycetes) from central Himalayan hills of India. Int J Med Mushrooms 13(6): 535-544.

82. Zhao W, Jiang X, Deng W, Lai Y, Wu M, et al. (2012) Antioxidant

activities of Ganoderma lucidum polysaccharides and their role on DNA damage in mice induced by cobalt-60 gamma-irradiation. Food Chem Toxicol 50(2): 303-309. doi: 10.1016/j.fct.2011.10.071

83. Wong KL, Chao HH, Chan P, Chang LP, Liu CF (2004) Antioxidant activity of Ganoderma lucidum in acute ethanol-induced heart toxicity. Phytother Res 18(12): 1024-1026. doi: 10.1002/ptr.1557

84. Sun J, He H, Xie BJ (2004) Novel antioxidant peptides from fermented mushroom Ganoderma lucidum. J Agric Food Chem 52(21): 6646-6652. doi: 10.1021/jf0495136

85. You YH, Lin ZB (2003) [Antioxidant effect of Ganoderma polysaccharide peptide]. Yao Xue Xue Bao 38(2): 85-88.

86. Chiu HF, Fu HY, Lu YY, Han YC, Shen YC, et al. (2017) Triterpenoids and polysaccharide peptides-enriched Ganoderma lucidum: a randomized, double-blind placebo-controlled crossover study of its antioxidation and hepatoprotective efficacy in healthy volunteers. Pharm Biol 55(1):1041-1046. doi: 10.1080/13880209.2017.1288750

87. Liu T, Ma Q, Zhao L, Jia R, Zhang J, et al. (2016) Protective Effects of Sporoderm-Broken Spores of Ganderma lucidum on Growth Performance, Antioxidant Capacity and Immune Function of Broiler Chickens Exposed to Low Level of Aflatoxin B(1). Toxins (Basel) 8(10): 278 doi: 10.3390/toxins8100278

88. Li D, Zhong Q, Liu T , Wang J (2016) Cell growth stimulating effect of Ganoderma lucidum spores and their potential application for Chinese hamster ovary K1 cell cultivation. Bioprocess Biosyst Eng 39(6): 925-935. doi: 10.1007/s00449-016-1572-2

89. Wang F, Zhou Z, Ren X, Wang Y, Yang R, et al. (2015) Effect of Ganoderma lucidum spores intervention on glucose and lipid metabolism gene expression profiles in type 2 diabetic rats. Lipids Health Dis 14:49. doi: 10.1186/s12944-015-0045-y

90. Erdem E, Leggett R, Dicks B, Kogan BA, Levin RM (2005) Effect of bladder ischaemia/reperfusion on superoxide dismutase activity and contraction. BJU Int 96(1): 169-174. doi: 10.1111/j.1464-410X.2005.05589.x

91. Haugaard N, Potter L, Wein AJ, Levin RM (1992) Effect of partial obstruction of the rabbit urinary bladder on malate dehydrogenase and citrate synthase activity. J Urol 147(5): 1391-1393.

92. Spettel S, De E, Elias T, Schuler C, Leggett RE, et al. (2012) Citrate synthase, sarcoplasmic reticular calcium ATPase, and choline acetyltransferase activities of specific pelvic floor muscles of the rabbit. Mol Cell Biochem 370(1-2): 1-5. doi: 10.1007/s11010-012-1347-2

93. Levin RM, Nicholas TJ, Snitkoff GG, Mandell J, Russell D,et al. (1997) Subcellular distribution of SERCA and calcium-activated ATPase in rabbit and human urinary bladder smooth muscle. Pharmacology 55(3): 136-143.

94. Rogers TB, Inesi G, Wade R, Lederer WJ (1995) Use of thapsigargin to study Ca2+ homeostasis in cardiac cells. Biosci Rep 15(5): 341-349.

95. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, et al. (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150(1): 76-85.

96. Levin RM, Xia L, Wei W, Schuler C, Leggett RE, et al. (2017) Effects of Ganoderma Lucidum shell-broken spore on oxidative stress of the rabbit urinary bladder using an in vivo model of ischemia/reperfusion. Mol Cell Biochem 435(1-2): 25-35. doi: 10.1007/s11010-017-3053-6

97. Hypolite JA, Longhurst PA, Gong C, Briscoe J, Wein AJ ,et al. (1993) Metabolic studies on rabbit bladder smooth muscle and mucosa. Mol Cell Biochem 125(1): 35-42.

98. Hsu TH, Levin RM, Wein AJ, Haugaard N (1994) Alterations of mitochondrial oxidative metabolism in rabbit urinary bladder after partial outlet obstruction. Mol Cell Biochem 141(1): 21-26.

99. Barry M, Meigs, JB (2000) The natural history of benign prostatic hyperplasia. In: Lepor H (ed) Prostatic Diseases, WB Saunders and Co., Philadelphia pp. 106-115

100. Bushman W (2009) Etiology, epidemiology, and natural history of benign prostatic hyperplasia. Urol Clin North Am 36(4): 403-415, v. doi: 10.1016/j.ucl.2009.07.003

101. Guven A, Mannikarottu A, Whitbeck C, Chichester P, Leggett RE, et al. (2007) Effect of age on the response to short-term partial bladder outlet obstruction in the rabbit. BJU Int 100(4): 930-934. doi: 10.1111/j.1464-410X.2007.07135.x

102. Guven A, Lin WY, Leggett RE, Kogan BA, Levin RM, Mannikarottu A, et al. (2007) Effect of aging on the response of biochemical markers in the rabbit subjected to short-term partial bladder obstruction. Mol Cell Biochem 306(1-2): 213-219. doi: 10.1007/s11010-007-9571-x

103. Yu HJ, Levin RM, Longhurst PA, Damaser MS (1997) Effect of age and outlet resistance on rabbit urinary bladder emptying. J Urol 158(3): 924-930.

104. Levin RM, Monson FC, Haugaard N, Buttyan R, Hudson A, et al. (1995) Genetic and cellular characteristics of bladder outlet obstruction. Urol Clin North Am 22(2): 263-283.

105. Levin RM, O'Connor LJ, Leggett RE, Whitbeck C , Chichester P (2003) Focal hypoxia of the obstructed rabbit bladder wall correlates

with intermediate decompensation. Neurourol Urodyn 22(2): 156-163. doi: 10.1002/nau.10076

106. Agartan CA, Whitbeck C, Sokol R, Chichester P, Levin RM,et al. (2004) Protection of urinary bladder function by grape suspension. Phytother Res 18(12): 1013-1018. doi: 10.1002/ptr.1620.

107. Levin RM, Das AK, Haugaard N, Novitsky Y, Horan P, et al. (1997) Beneficial effects of Tadenan therapy after two weeks of partial obstruction in the rabbit. Neurourol Urodyn 16(6): 583-599.

108. Levin RM, Hass MA, Bellamy F, Horan P, Whitbeck K, Chow PH, et al. (2002) Effect of oral Tadenan treatment on rabbit bladder structure and function after partial outlet obstruction. J Urol 167(5): 2253-2259.

109. Levin RM, Longhurst PA, Monson FC, Kato K ,Wein AJ, et al. (1990) Effect of bladder outlet obstruction on the morphology, physiology, and pharmacology of the bladder. Prostate Suppl 17(3):9-26.

110. Monson FC, Sun L, Wein AJ, Levin RM (1995) Hyperplasia in the rabbit bladder urothelium following partial outlet obstruction. Autoradiographic evidence. Mol Cell Biochem 152(2): 167-173.

111. Roelofs M, Wein AJ, Monson FC, Passerini-Glazel G, Koteliansky VE, et al. (1995) Contractility and phenotype transitions in serosal thickening of obstructed rabbit bladder. J Appl Physiol (1985) 78(4): 1432-1441.

112. Walker A, Tanner MJ, Husson P, Schuler C, Kogan BA, et al. (2009) Differential expression of vascular endothelial growth factor, and angiopoietin 1 and 2 in functionally divergent experimental rabbit models of bladder hypertrophy. J Urol 181(6): 2790-6. doi: 10.1016/j.juro.2009.01.100

113. Chichester P, Schroder A, Horan P, Levin RM (2001) Vascular response of the rabbit bladder to chronic partial outlet obstruction. Mol Cell Biochem 226(1-2): 1-8.

114. Lin WY, Lin YP, Levin RM, Chen ML (2016) The relevance of immune responses to partial bladder outlet obstruction and reversal. Neurourol Urodyn.36(5): 1306-1312.doi: 10.1002/nau.23136

115. Kanno Y, Mitsui T, Kitta T, Moriya K, Tsukiyama T, et al. (2016) The inflammatory cytokine IL-1beta is involved in bladder remodeling after bladder outlet obstruction in mice. Neurourol Urodyn 35(3): 377-381. doi: 10.1002/nau.22721

116. Levin RM, Wein AJ (1980) Adrenergic alpha receptors outnumber beta receptors in human penile corpus cavernosum. Invest Urol 18(3): 225-226.

117. Lin WY, Rehfuss A, Schuler C, Levin RM (2008) Effect of co-enzyme Q10 and alpha-lipoic acid on response of rabbit urinary bladder to repetitive stimulation and in vitro ischemia. Urology 72(1): 214-229. doi: 10.1016/j.urology.2007.11.074

118. Lin AD, Mannikarottu A, Chaudhry A, Whitbeck C, Kogan BA et al. (2005) Protective effects of grape suspension on in vivo ischaemia/reperfusion of the rabbit bladder. BJU Int 96(9): 1397-1402. doi: 10.1111/j.1464-410X.2005.05832.x

119. Levin RM, Whitbeck C, Horan P, Bellamy F (2005) Low-dose tadenan protects the rabbit bladder from bilateral ischemia/reperfusion-induced contractile dysfunction. Phytomedicine 12(1-2): 17-24. doi: 10.1016/j.phymed.2003.10.002

120. Callaghan CM, Leggett RE, Levin RM (2013) A Comparison of Total Antioxidant Capacities of Concord, Purple, Red, and Green Grapes Using the CUPRAC Assay. Antioxidants (Basel) 2(4): 257-64. doi: 10.3390/antiox2040257

121. Bean H, Radu F, De E, Schuler C, Leggett RE et al. (2009) Comparative evaluation of antioxidant reactivity within obstructed and control rabbit urinary bladder tissue using FRAP and CUPRAC assays. Mol Cell Biochem 323(1-2): 139-142. doi: 10.1007/s11010-008-9972-5

122. Levin RM, Haugaard N, Levin SS, Buttyan R, Chen MW, et al. (1995) Bladder function in experimental outlet obstruction: pharmacologic responses to alterations in innervation, energetics, calcium mobilization, and genetics. Adv Exp Med Biol 385:7-19; discussion 75-9.

123. Stevenson K, Kucich U, Whitbeck C, Levin RM Howard PS (2006) Functional changes in bladder tissue from type III collagen-deficient mice. Mol Cell Biochem 283(1-2): 107-14. doi: 10.1007/s11010-006-2388-1

124. Eika B, Levin RM, Longhurst PA (1992) Collagen and bladder function in streptozotocin-diabetic rats: effects of insulin and aminoguanidine. J Urol 148(1): 167-172.

125. Santarosa R, Colombel MC, Kaplan S, Monson F, Levin RM,et al. (1994) Hyperplasia and apoptosis. Opposing cellular processes that regulate the response of the rabbit bladder to transient outlet obstruction. Lab Invest 70(4): 503-510.

126. Yamada A, Torimoto K, Obata K, Hirayama A, Fujimoto K et al. (2014) Persistent overexpression of SERCA2a affects bladder functions under physiological conditions, but not in bladder outlet obstruction-induced sub-acute pathological conditions. J Physiol Sci 64(1):21-30. doi: 10.1007/s12576-013-0286-3

127. Wang Y, Fang Q, Lu Y, Song B, Li W et al. (2010) Effects of mechanical stretch on interstitial cells of Cajal in guinea pig bladder. J Surg Res 164(1):e213-229. doi: 10.1016/j.jss.2010.04.040

128. Salomone F, Godos J, Zelber-Sagi S (2016) Natural antioxidants for non-alcoholic fatty liver disease: molecular targets and clinical perspectives. Liver Int 36(1): 5-20. doi: 10.1111/liv.12975

129. Kuno A, Tanno M, Horio Y (2015) The effects of resveratrol and SIRT1 activation on dystrophic cardiomyopathy. Ann N Y Acad Sci 1348(1): 46-54. doi: 10.1111/nyas.12812

130. Conte E, Fagone E, Fruciano M, Gili E, Iemmolo M et al. (2015) Anti-inflammatory and antifibrotic effects of resveratrol in the lung. Histol Histopathol 30(5): 523-529.

131. Matsumoto S, Leggett RE, Levin RM (2003) The effect of vitamin E on the response of rabbit bladder smooth muscle to hydrogen peroxide. Mol Cell Biochem 254: 347-351.

132. Jin X, Ruiz Beguerie J, Sze DM ,Chan GC (2016) Ganoderma lucidum (Reishi mushroom) for cancer treatment. Cochrane Database Syst Rev 4:CD007731. doi: 10.1002/14651858.CD007731.pub3

133. Jin X, Ruiz Beguerie J, Sze DM, Chan GC (2012) Ganoderma lucidum (Reishi mushroom) for cancer treatment. Cochrane Database Syst Rev 6:CD007731. doi: 10.1002/14651858.CD007731.pub2

134. Baby S, Johnson AJ , Govindan B (2015) Secondary metabolites from Ganoderma. Phytochemistry 114: 66-101. doi: 10.1016/j.phytochem.2015.03.010

135. Campos Ziegenbein F, Hanssen HP , Konig WA (2006) Secondary metabolites from Ganoderma lucidum and Spongiporus leucomallellus. Phytochemistry 67(2): 202-211.

136. Ma HT, Hsieh JFChen ST (2015) Anti-diabetic effects of Ganoderma lucidum. Phytochemistry 114: 109-13. doi: 10.1016/j.phytochem.2015.02.017

137. Klupp NL, Chang D, Hawke F, Kiat H, Cao H et al. (2015) Ganoderma lucidum mushroom for the treatment of cardiovascular risk factors. Cochrane Database Syst Rev 17(2):CD007259. doi: 10.1002/14651858.CD007259.pub2

138. Lasukova TV, Maslov LN, Arbuzov AG, Burkova VN ,Inisheva LI et al. (2015) Cardioprotective Activity of Ganoderma lucidum Extract during Total Ischemia and Reperfusion of Isolated Heart. Bull Exp Biol Med 158(6): 739-741. doi: 10.1007/s10517-015-2851-7

139. Kan Y, Chen T, Wu Y, Wu J, Wu J et al. (2015) Antioxidant activity of polysaccharide extracted from Ganoderma lucidum using response surface methodology. Int J Biol Macromol 72: 151-157. doi: 10.1016/j.ijbiomac.2014.07.056

140. Liu SP, Volfson I, Horan P,Levin RM (1998) Effects of hypoxia, calcium, carbachol, atropine and tetrodotoxin on the filling of the in-vitro rabbit whole bladder. J Urol 160: 913-919.

141. Zhao Y, Wein AJ, Bilgen A Levin RM (1991) Effect of anoxia on in vitro bladder function. Pharmacology 43: 337-44.

142. Tseng CY, Chung MC, Wang JS, Chang YJ, Chang JF, et al. (2016) Potent In Vitro Protection Against PM[Formula: see text]-Caused ROS Generation and Vascular Permeability by Long-Term Pretreatment with Ganoderma tsugae. Am J Chin Med 44(2): 355-376.doi: 10.1142/S0192415X16500208

143. Chang CJ, Lin CS, Lu CC, Martel J, Ko YF, et al. (2015) Ganoderma lucidum reduces obesity in mice by modulating the composition of the gut microbiota. Nat Commun 6:7489. doi: 10.1038/ncomms8489

144. Tammela T, Wein AJ, Monson FC ,Levin RM (1993) Urothelial permeability of the isolated whole bladder. Neurourol Urodyn 12: 39-47.

145. Hass MA, Nichol P, Lee L ,Levin RM (2009) Estrogen modulates permeability and prostaglandin levels in the rabbit urinary bladder. Prostaglandins Leukot Essent Fatty Acids 80(2-3): 125-129. doi: 10.1016/j.plefa.2008.11.010

146. Levin RM, Whitmore K, Monson F C, Witkowski, RuggieriM et al.(1990) Trypan blue as an indicator of urothelial integrity. Neurourol Urodyn 9(3): 269-279.

147. Levin RM , XL, Wei W, Schuler C, Leggett RE, Lin AD (2017) Effects of Ganoderma Lucidum on Contractile Dysfunction of the Rabbit Urinary Bladder using an In-Vitro Model of Ischemia / Reperfusion. Journal of Natural Products (in Press).

148. Agartan CA, Leggett RE, Kogan BA , Levin RM (2007) Effect of age on the response to in vitro ischemia and reperfusion of the rabbit bladder. Urol Int 78(2): 155-159.

149. Radu F, Leggett RE, Schuler C, Levin RM (2011) The effect of in vitro ischemia/reperfusion on contraction, free fatty acid content, phospholipid content, and malondialdehyde levels of the rabbit urinary bladder. Mol Cell Biochem 346(1-2): 179-186.

150. Shen B, Truong J, Helliwell R, Govindaraghavan S ,Sucher NJ,et al. (2013) An in vitro study of neuroprotective properties of traditional Chinese herbal medicines thought to promote healthy ageing and longevity. BMC Complement Altern Med 13: 373.

151. Lai CS, Yu MS, Yuen WH, So KF, Zee SY,et al. (2008) Antagonizing beta-amyloid peptide neurotoxicity of the anti-aging fungus Ganoderma lucidum. Brain Res 1190: 215-224.

152. Cilerdzic J, Stajic M, Vukojevic J, uletic-Lausevic S (2013) Oxidative stress and species of genus Ganoderma (higher Basidiomycetes). Int J Med Mushrooms 15(1): 21-28.

153. Weng Y, Lu J, Xiang L, Matsuura A, Zhang Y, et al. (2011) Ganodermasides C and D, two new anti-aging ergosterols from spores of the medicinal mushroom Ganoderma lucidum. Biosci Biotechnol Biochem 75(4): 800-3. doi: 10.1271/bbb.100918

154. Weng Y, Xiang L, Matsuura A, Zhang Y, Huang Q,et al. (2010) Ganodermasides A and B, two novel anti-aging ergosterols from spores of a medicinal mushroom Ganoderma lucidum on yeast via UTH1 gene. Bioorg Med Chem 18(3): 999-1002. doi: 10.1016/j.bmc.2009.12.070

155. Sudheesh NP, Ajith TA, Ramnath V ,Janardhanan KK (2010) Therapeutic potential of Ganoderma lucidum (Fr.) P. Karst. against the declined antioxidant status in the mitochondria of post-mitotic tissues of aged mice. Clin Nutr 29(3): 406-12. doi: 10.1016/j.clnu.2009.12.003

156. Ajith TA, Sudheesh NP, Roshny D, Abishek G ,Janardhanan KK (2009) Effect of Ganoderma lucidum on the activities of mitochondrial dehydrogenases and complex I and II of electron transport chain in the brain of aged rats. Exp Gerontol 44(3): 219-223. doi: 10.1016/j.exger.2008.11.002

157. Perse M, Injac R, Erman A (2013) Oxidative status and lipofuscin accumulation in urothelial cells of bladder in aging mice. PLoS One 8:e59638. doi: 10.1371/journal.pone.0059638

158. Andersson KE, Boedtkjer DB ,Forman A (2017) The link between vascular dysfunction, bladder ischemia, and aging bladder dysfunction. Ther Adv Urol 9(1): 11-27.doi: 10.1177/1756287216675778

159. Lin AT, Hsu TH, Yang C, Chang LS (2000) Effects of aging on mitochondrial enzyme activity of rat urinary bladder. Urol Int 65(3): 144-7. doi: 64860

160. Zhao W, Aboushwareb T, Turner C, Mathis C, Bennett C,et al. (2010) Impaired bladder function in aging male rats. J Urol 184(1): 378-385. doi: 10.1016/j.juro.2010.03.004

161. Yoshida M, Miyamae K, Iwashita H, Otani M, Inadome A, et al. (2004) Management of detrusor dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging. Urology 63(3):17-23. doi: 10.1016/j.urology.2003.11.003