ABSTRACT:
Background: Oxidative stress involving ischemia followed by reperfusion are major etiological factors in obstructive bladder dysfunction in both men and rabbits. Specific natural products such as Ganoderma lucidum with significant antioxidant activity have proven to be useful in their treatment. In two recent studies we demonstrated that pretreatment with Ganoderma was effective preventing bladder dysfunction using both in-vivo and in-vitro models of ischemia-reperfusion. The current study is a follow-up study using the in-vitro model of ischemia-reperfusion. Methods: Eight New Zealand White rabbits were divided into 2 groups. One group (Group-1) was fed Ganoderma (100 mg/Kg) daily for 3 weeks while the other group (Group-2) were given saline. At the end of a 3 week period, each rabbit was euthanized and the bladder separated into twelve full thickness strips and mounted in individual baths. After 1 hour in oxygenated Tyrodes with glucose, 4 strips from each group were removed frozen and stored at -80°C. At this time, the oxygenated Tyrodes + glucose were changed to Tyrodes equilibrated with nitrogen in the absence of glucose (ischemia) for 1 hour. After 1 hour of ischemia the buffer was then changed back to normal oxygenated Tyrode’s with glucose and allowed to recover for 2 hours (reperfusion). After this time period, the four remaining strips from each group were frozen and stored. The isolated strips were analyzed for the following biomarker enzymes: citrate synthase (mitochondria), calcium ATPase (intracellular calcium movement through the cell wall), and sarco-endoplasmic reticular ATPase (intracellular calcium uptake into the sarcoplasmic reticulum following stimulation. Results: Ischemia-reperfusion resulted in a significant decrease (~50%) in all three enzyme activities. Pretreating with Ganoderma protected all three enzyme activities which were approximately at control levels. Conclusion: Pretreatment of rabbits for three weeks with Ganoderma prior to subjecting them to in-vitro ischemia-reperfusion significantly protected the bladder
enzymes.

Key Words: Calcium ATPase, Citrate synthase, Ganoderma lucidum, Oxidative Stress, Rabbits, SERCA, Urinary bladder

BACKGROUND:
Oxidative stress (free radical damage) has been found to be a major factor in a number of major human health problems including heart disease [1-3], diabetes [4, 5], intestinal diseases [6-8], liver disease [9], renal disease [10, 11] and obstructive bladder dysfunction [12-15]. In virtually all of these oxidative stress-linked dysfunctions, antioxidants have proven to be very valuable in their treatment [13, 16-19]. In addition to specific antioxidants such as coenzyme Q10, alpha lipoic acid and vitamin E, certain natural products have been shown to be effective treatments in regard to these specific diseases [20-24]. Two advantages that natural products have over specific antioxidants is that natural products can have a variety of active components that work synergistically in the treatment of specific ischemic diseases. This was observed when comparing the beneficial effects of a whole grape preparation with pure resveratrol which is believed to be the major beneficial component in grapes [25, 26]. Our results clearly demonstrated that although resveratrol had over 100 times the antioxidant activity of the grape suspension (using the cupric ion reducing antioxidant capacity (CUPRAC) chemical test for total antioxidant capacity), 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 [25, 26]. In addition, several clinical studies have demonstrated that purified antioxidants were not as effective as originally believed in the treatment of a variety of diseases [27-30]. Two reasons for this are: 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 and bioavailability properties than that of the purified “active ingredient”.

One of the major advantages of the urinary bladder as a model to study oxidative stress, I/R, antioxidants, and natural products is that inducing ischemic injury in the heart, liver, kidney, lung and intestine has significant dysfunctional effects on a variety of other organ systems which can significantly modify the responses of the original organ system. The urinary bladder is a relatively closed system: that is, I/R can be induced in the bladder with little or no damage to other organ systems [31, 32]. Antioxidants have been proven to be very effective in protecting the rabbit bladder from oxidative damage mediated by OBD [16, 33]. OBD, induces a variety of bladder dysfunctions in addition to I/R including hypertrophy, hyperplasia, apoptosis, and inflammation. In order to eliminate these other effects, we have developed in-vivo and in-vitro models of bladder I/R [19, 34].

In addition to antioxidants, specific natural products with significant antioxidant properties have also been demonstrated to be effective treatments, and pre-treatments of OBD These products includes tadenan, which is a plant extract of Pygeum africanum. It is generally used in dispersion in peanut oil and administered orally in capsules. It is used in Europe for the treatment of BPH, and has been shown to be a strong antioxidant. A second natural product demonstrated useful as an antioxidant is Kohki tea. Extracts of the leaves of Engelharditia chrysolepis, a subtropical plant that grows wild in southern China, have been used medicinally in east Asia for hundreds of years. A standard extract named Kohki tea is sold over the counter in Japan as a sweet tea for treating fever, abdominal pains and cold symptoms. The tea contains strong antioxidants and membrane protective components, including several dihydroflavanol glycosides. In the current studies, we evaluated Ganoderma lucidum (GL) [34-36]. GL has been shown by pharmaceutical studies and clinical research that Ganoderma is useful in helping to prevent certain types of discomfort and helps prolong human life in part by acting as an antioxidant. The Chinese government classified GL as both a food and an herbal medicine and approved GL to be a safe herb which can be used as a dietary supplement with no toxicity. There are numerous scientific publications demonstrating the strong antioxidant properties of GL which make this the perfect natural product to utilize in these studies [37-42].

The preparation of GL that we used for these studies were broken spore shell extracts (GLSSSE) which have been demonstrated to have the greatest beneficial effects in the treatment of a variety of pathologies especially those relating to I/R. The commercial capsule of the BeiJing Tong Ren Tong product (used to treat humans) is 350mg in each capsule. The dose recommended for human use is 2 ~ 4 capsules, as an antioxidant, and 8~10 capsules per day for cancer patients.

In our first study on GL, the study was designed to evaluate GL’s ability to reduce the damage from in-vivo I/R. Using an in-vivo model of I/R, we have investigated the ability of GL to protect bladder function from oxidative damage mediated by I/R [34]. Our studies demonstrated that in-vivo ischemia followed by reperfusion resulted in a significant decrease in bladder compliance and decreases in the contractile responses to a variety of forms of contractile stimulation. Pretreatment of rabbits with GL prior to subjecting the rabbits to in-vivo I/R completely inhibited the negative effects of I/R on both the compliance and contractile responses. These results
demonstrate that GL provided excellent protection of bladder function following I/R (oxidative stress).

The second used an in-vitro model of rabbit urinary bladder I/R [31, 43]. This model utilizes a direct ischemia (incubation of the tissues in the absence of oxygen and glucose) followed by a period of reperfusion (incubation of the tissues in the presence of both oxygen and glucose) to induce significant oxidative stress resulting in decreased contractile responses of the bladder strips [19] as well as increased oxidative stress markers. The specific aim of this second study was to determine if GL pretreatment could protect the isolated bladder strips from direct I/R. The results demonstrated that pretreatment with GL protected the bladder strip contraction to all forms of stimulation: field stimulation (FS), carbachol, KCl, and ATP. Whereas the responses to field stimulation were only partially protected, the responses to ATP, carbachol, and KCl were fully protected.

The current study was utilized the in-vitro model of I/R to determine if GL pretreatment would protect the effect of oxidative stress on three key biomarker enzymes within the bladder smooth muscle: citrate synthase (the rate limiting step for the metabolism of glucose by mitochondria); calcium ATPase (intracellular calcium movement through the cell wall), and sarco-endoplasmic reticular ATPase (SERCA) (intracellular calcium uptake into the sarcoplasmic reticulum following stimulation).

**METHODS:**

All studies were approved by the institutional animal care and use committee (IACUC) and the research and development committee (R&D) of the Stratton VA Medical Center, Albany, NY.

Eight adult New Zealand white rabbits (approximately 3 kgs each) were divided into 2 groups of 4 each. One group was fed by gavage GL at 100 mg/Kg (as a powder suspended in distilled water) daily for 3 weeks while the other group were given distilled water and served as controls. This dose was chosen to be consistent with the dose given to humans. Humans generally take GL as capsules containing the powder. At the end of the 3 week feeding period, each rabbit was anesthetized using Ketamine/ Xylazine (25mg/10mg, im) and the rabbit was then euthanized using 1 ml Socumb Euthanasia Injection. The bladder was then rapidly removed and placed in a beaker of warmed Tyrodes buffer. Twelve full thickness strips of the bladder body were mounted in individual 15ml water baths containing Tyrode’s solution warmed to 37°C containing 1 mg/ml glucose and equilibrated with 95% oxygen, 5% carbon dioxide. After 1 hour incubation, 4 strips from each group was removed, weighed, frozen under liquid nitrogen and stored at -80°C. At this time, the baths were filled with Tyrode’s solution without glucose and equilibrated with 95% nitrogen and 5% carbon dioxide for 1 hour. After the one hour ischemia, the buffer was changed back to normal oxygenated Tyrode’s solution with glucose and the strips allowed to recover for 2 hours. After that time period, the remaining 4 strips from each group were weighed, frozen, and stored.

**BIOCHEMICAL ANALYSES:**

**Citrate Synthese Assay [26, 44, 45]**

Strips from each group were homogenized in 0.05M Tris buffer (100 mg/mL). Samples were then spun at 800g for ten (10) minutes. 0.9ml of supernatant plus 0.1ml of Triton X-100 were combined in a test tube for each sample. Samples (40 µL) were added to ten 0.5 cm 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). 10mg/mL grape powder was added to all cuvettes to give a final concentration of 1 mg/mL. The final volume in each cuvette was 1400 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.

**Ca²⁺ ATPase and SERCA Assays [45, 46]**

40 mgs of tissue (10 mg/ml) were homogenized in 50mM TRIS buffer-pH 7.4. The sample was then centrifuged at 800g for 10 minutes. The supernatant was saved and the pellet discarded. Each sample had (two) 2 tubes for each condition. The conditions were sample plus thapsigargin (10 μM), sample minus thapsigargin, control with no homogenate, and control with no ATP. All sample tubes contained: 375µls sample, 50µls CaCl₂, 50µls EDTA, (+/-) 5µls thapsigargin, 25µls ATP, and (+/-) grape suspension or (+/-) resveratrol. Sample and control tubes were incubated at 37°C for 40 minutes. At the end of the incubation, 0.5ml trichloroacetic 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 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 Ca²⁺ ATPase and SERCA. Thapsigargin is a non-competitive inhibitor of SERCA [47], thus total ATPase activity – activity in the presence of thapsigargin = SERCA activity.

**STATISTICAL ANALYSES:**

Power analyses determined that a minimum of 4 rabbits per group was required to produce standard errors of 10% which would allow a 20% difference in the means to be statistically different at p < 0.05. Statisti-
cal analyses utilized one-way analysis of variance followed by the Tukey test for individual differences. A p < 0.05 was required for statistical significance.

RESULTS:
Figure 1 displays the citrate synthase activity. I/R reduced the activity by nearly half, whereas pretreatment with GL completely protected the enzyme activity.
Figure 2 shows the Calcium ATPase activity. I/R reduced the enzyme activity by over 50%. Pretreatment with GL significantly protected the enzyme activity, although the activity Post I/R was still significantly lower than control.
Figure 3 displays the SERCA activity. I/R reduced the activity by nearly half, whereas pretreatment with GL completely protected the enzyme activity.

Figure 1 displays the citrate synthase activity. Each bar is the mean +/- SEM of 4 individual rabbits. * = significantly different from control; x = significantly different from Control – I/R; p < 0.05.

Figure 2 shows the Calcium ATPase activity. Each bar is the mean +/- SEM of 4 individual rabbits. * = significantly different from control; x = significantly different from Control – I/R; p < 0.05.

Figure 3 displays the SERCA activity. Each bar is the mean +/- SEM of 4 individual rabbits. * = significantly different from control; x = significantly different from Control – I/R; p < 0.05.

Figure 4 shows a representative schematic of a bladder smooth muscle cell.
To give some context, figure 4 shows a schematic of a bladder smooth muscle cell showing that Citrate synthase is the rate-limiting step for mitochondrial function; converting glucose to CO$_2$, H$_2$O and NADH that is then converted to ATP by the electron transport chain; which also takes place within mitochondria. Calcium ATPase provides the energy (ATP = ADP + P$_i$ + energy) for translocating intracellular calcium through the outer cell membrane. This is a relatively slow process when compared to SERCA. SERCA provides the energy for the translocation of intracellular calcium into the sarcoplasmic reticulum for storage. This is a very fast reaction and is responsible for the rapid decrease in contractile tension after a bladder contraction.
ABBREVIATIONS:

<table>
<thead>
<tr>
<th>Term</th>
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<tr>
<td>adenosine triphosphate</td>
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<td>benign prostatic hyperplasia</td>
<td>BPH</td>
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<td>cupric ion reducing antioxidant capacity</td>
<td>CUPRAC</td>
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<td>field stimulation</td>
<td>FS</td>
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<td>Ganoderma lucidum</td>
<td>GL</td>
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<td>institutional animal care and use committee</td>
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<td>ischemia followed by reperfusion</td>
<td>I/R</td>
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<tr>
<td>malondialdehyde</td>
<td>MDA</td>
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<td>obstructive bladder dysfunction</td>
<td>OBD</td>
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<tr>
<td>partial bladder outlet obstruction</td>
<td>PBOO</td>
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<tr>
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<td>KCl</td>
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AUTHORS CONTRIBUTIONS:
Robert M. Levin PhD and Alpha D-Y Lin MD were responsible for the research design and final analysis of the data obtained. They were also responsible for the writing of all reports, abstracts, and manuscripts from this study.

Li-Xia and Wu Wei were responsible for the organizing and integration of the essential scientific and historical information of the Ganoderma lucidum into the manuscript.

Catherine Schuler and Robert E. Leggett were responsible for the performance of the technical aspects of these studies. They also did the primary accumulation and analysis of the data from the study. In addition they also proofread the final manuscript for the accuracy of the methods and data analysis.

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