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Research Article

Effect of Hydrogen Peroxide on Citrate Synthase and SERCA activities of the Rabbit Urinary Bladder in the presence and absence of Jack Bean -

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ABSTRACT

Background: Ischemia / reperfusion are major etiologies related to obstructive bladder dysfunction. Jack bean is a natural product used in the treatment of a variety of human pathologies in traditional Chinese medicine. One mechanism of action is as an antioxidant to relieve oxidative stress.

Methods: Six New Zealand White rabbits were used. The urinary bladders were excised and separated into muscle and mucosa, frozen and stored at -80°C for biochemical analysis. Dose response studies to hydrogen peroxide for citrate synthase, total calcium ATPase, and sarco-endoplasmic reticular Calcium ATPase activities were performed in the presence and absence of Jack Bean. In addition, the CUPRAC test for total antioxidant activity was performed using ascorbic acid as a standard and jack bean as the experimental agent.

Results: 1) Calcium ATPase and SERCA were significantly less sensitive than citrate synthase to H₂O₂. 2) For all enzymes, hydrogen peroxide showed dose-response inhibitions of activity. 3) For all enzymes, the tissue homogenates incubated with jack bean showed significantly higher activity in the presence of hydrogen peroxide than the homogenates incubated with placebo. 4) JB showed a linear antioxidant activity with concentration.

Conclusions: These data demonstrate that jack bean showed protective effects against oxidative stress mediated by hydrogen peroxide.

Keywords: Rabbit; Bladder; Cystitis; Oxidative Stress; Ischemia; Reperfusion; Hydrogen peroxide

INTRODUCTION

Benign Prostatic Hyperplasia (BPH) is a major dysfunction in aging men [1]. The major consequence of BPH is Obstructive Bladder Dysfunction (OBD) which is mediated by the compression of the urethra [2]. OBD affects 80% of men over the age of 50 [2,3]. Ischemia followed by reperfusion (I/R) is a primary etiology in OBD [4,5]. Ischemia results when oxygen delivery to tissues is reduced which in turn also reduces the delivery of glucose. Reperfusion occurs when the blood flow is rapidly restored. Reperfusion results in a marked increased production of free radicals which results in oxidative stress resulting in significant cellular damage. Tissue hypoxia with the resulting ischemia is induced by the decreased blood flow which is mediated by OBD. This response to OBD results in oxidative stress which is accompanied by the generation of free radicals and oxidative damage to cellular and subcellular structures [5-7]. Hydrogen peroxide (H₂O₂) causes tissue damage even at the low concentrations [8]. Two enzymes responsible for antioxidant defence are Superoxide Dismutase (SOD) and catalase. SOD catalyses the formation of oxygen and H₂O₂. The enzyme catalase is then responsible for converting H₂O₂ to water and oxygen [9,10]. Natural products having strong antioxidant and membrane protective properties have been demonstrated to reduce and reverse bladder damage initiated by oxidative stress [11]. Mitochondria are one of the most sensitive subcellular organelles to respond to oxidative stress damage from Partial Bladder Outlet Obstruction (PBOO) and ischemia / reperfusion (I/R) [4,12]. Citrate Synthase (CS) is an enzyme that is used as a quantitative bio-marker for mitochondrial activity [13,14]. CS plays a major role in the citric acid cycle as it is the rate limiting step [14]. A second major subcellular organelle affected by oxidative stress is the sarcoplasmic reticulum (SR) [13]. The SR functions to store calcium (Ca²⁺) and release it during contractile stimulation (calcium-induced calcium release - CICR). Immediately following contraction, the Ca²⁺ is actively pumped into the SR via the enzyme sarco-endoplasmic reticular ATPase (SERCA). SERCA damage causes a significant increase in intracellular free calcium and results in the activation of specific calcium-dependent proteases such as calpain [15,16], and lipases such as phospholipase A₂ [16,17]. One natural product that has been used extensively in China to treat a variety of dysfunctions in humans is Jack Bean (JB). One of the proposed mechanisms of action for JB is as an antioxidant [18,19].

In our previous study, we determined the effect of feeding rabbits a suspension of JB daily for 2 weeks prior to inducing chemical cystitis using intravesical administration of protamine sulphate + uric acid [20]. Control rabbits received saline instead of the protamine sulphate + uric acid. Rabbits were fed JB for two weeks following chemical cystitis. *In-vivo* I/R mediated significant decreases in bladder compliance and contractile responses to field stimulation, carbachol, ATP, and KCl. The results clearly demonstrated that the rabbits fed JB showed no decreases in compliance and no contractile dysfunctions to any form of stimulation. In addition, JB had significant antioxidant activity [21].

JACK BEAN PREPARATION

Canavalia ensiformis

Common name Jack bean is a **legume** which is used for human nutrition. Two of the major components of JB are urease and concalectin A. Concalectin A (ConA) is a **lectin (carbohydrate-binding protein)** and member of the **legume lectin** family. It binds specifically to certain structures found in various **sugars, glycoproteins, and glycolipids**. ConA is a plant **mitogen** which is known for its ability to stimulate mouse T-cells. It has also been found to be effective in the treatment of liver cancer and other tumors. Ureases belong to the **super family of amidohydrolases** and phosphotriesterases. It is an **enzyme that catalyzes the hydrolysis of urea into carbon dioxide and ammonia. These toxic properties have been completely eliminated by proprietary methodologies**. Thus the preparation of JB used for these studies had no toxic properties. In the current study, the ability of JB to protect the enzyme activities of CS, total calcium ATPase, and SERCA from the oxidative effect of H₂O₂ was determined.

MATERIALS AND METHODS

Six male New Zealand white rabbits were anesthetized with ketamine/xylazine (25mg/10mg, im) and the bladder exposed through a midline incision. Each bladder was then removed and sectioned between body and base at the level of the urethral orifices. The bladder body was opened longitudinally and the bladder body was separated by blunt dissection into muscle and mucosal compartments. The bladder body smooth muscle was separated into 12 individual strips, frozen in liquid nitrogen and stored at -80°C for biochemical evaluation.

Citrate Synthase activity [13,22]

Samples of muscle were homogenized in 0.05M Tris buffer (100 mg/mL). Samples were then spun at 250 x g 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). 100mg/mL JB was added to ½ of the cuvettes to give a final concentration of 10 mg/ml. An equal volume of water was given to the other ½ of the cuvettes. The final volume in each cuvette was 1400 ml excluding the 50 µL oxaloacetate (10 mM) used to start the reaction. Before the oxaloacetate was added, 1.4 µL of the mixtures in cuvettes 1 and 2 were removed and 1.4 µL of Tris buffer added to serve as controls. Similarly, for the remainder of the cuvettes, 1.4 µL of the mixtures were removed and 1.4 µL of H₂O₂ added to give final concentrations of 0, 0.031, 0.063, and 0.125 % H₂O₂. A pair of cuvettes contained no sample served as additional controls. A magnetic stir bar was placed in each cuvette to mix the chemicals and after 1 minute 50 µL of 10ml oxaloacetate was added to each cuvette. The activity was read every 2 minutes for 30 minutes in a Hitachi U-2001 spectrophotometer. The free coenzyme-A generated by CS activity reacted with DTNB to form a coloured compound that was quantified at 412 nm.

Plasma Membrane Calcium ATPase and SERCA Methodologies [13]

40 mgs of smooth muscle (10 mg/ml) was homogenized in 50mM TRIS buffer-pH 7.4. The sample was then centrifuged at 250 xg 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, (+/-) JB (10 mg/ml) and one of the following concentrations of H₂O₂ as percentages: 0, 0.125, 0.250, 0.5, and 1.0. The reaction was started with 25µl of a 20µM solution of ATP. 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 sulphate 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 membrane Ca²⁺ATPase and SERCA. Thapsigargin is a non-competitive inhibitor of SERCA [23], thus total ATPase activity – activity in the presence of thapsigargin = SERCA activity.

CUPRAC Assay for total Antioxidant Activity

The CUPRAC assay was utilized to determine the total antioxidant capacity of the JB preparation. This assay relies on the electron donating capabilities of antioxidants to reduce the copper ion. The CUPRAC working solution consisted of 10mM copper (II) chloride dihydrate, 1M ammonium acetate, and 7.5 mM neocuproine. 0.15 mL of the above three solutions were added to 0.15 mL of each sample and allowed to react for 30 minutes at room temperature after which the absorbance was read at 450 nm in a Hitachi U-2001 spectrophotometer. The concentrations of ascorbic acid (standard) and JB were 10 – 80 mg/ml. The data was quantitated as Optical Density (mOD) / mg/ml.

Statistical analysis

Each set of data was analysed individually. One way analysis of variance was used followed by the TUKEY test for individual differences among the groups. *P* < 0.05 was required for statistical significance.

RESULTS

Figure 1 displays the effect of H₂O₂ on CS activity. H₂O₂ resulted in a dose-dependent decrease in the CS activity. However, at 0.075% and 0.150% H₂O₂ the activities in the presence of JB were significantly higher than in the absence of JB (at a significance value of *p* < 0.05).

Figure 2 presents the effect of H₂O₂ on the plasma membrane calcium ATPase activity. H₂O₂ resulted in a dose-dependent decrease in the plasma membrane calcium ATPase activity. However, at 0.5% and 1.0% H₂O₂ the activities in the presence of JB were significantly higher than in the absence of JB (at a significance value of *p* < 0.05).

Figure 3 presents the effect of H₂O₂ on SERCA activity in the presence and absence of JB. H₂O₂ resulted in a dose-dependent decrease in the SERCA activity. However, at 0.25%, 0.5%, and 1.0% H₂O₂ the activities in the presence of JB were significantly higher than in the absence of JB (at a significance value of *p* < 0.05).

For all three enzymes, JB had no effect at the lowest concentrations

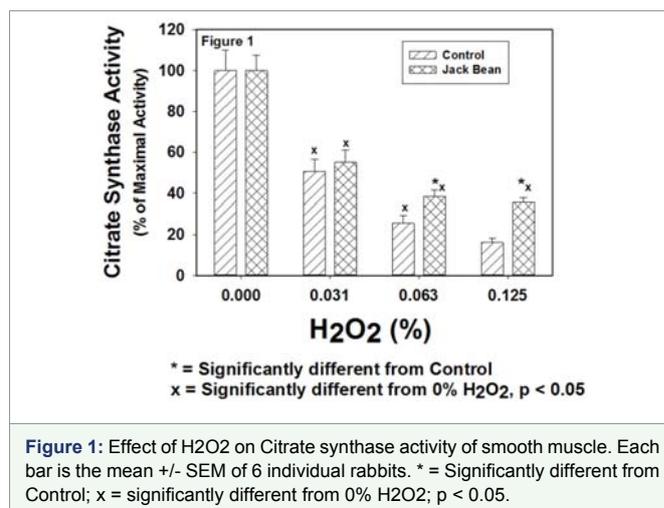


Figure 1: Effect of H₂O₂ on Citrate synthase activity of smooth muscle. Each bar is the mean +/- SEM of 6 individual rabbits. * = Significantly different from Control; x = significantly different from 0% H₂O₂; *p* < 0.05.

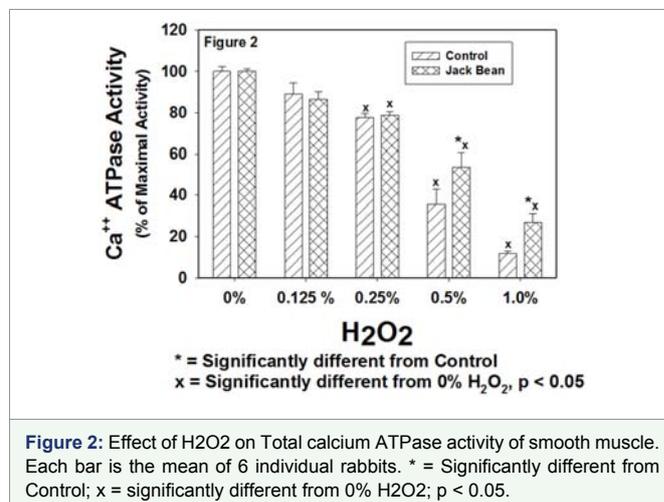


Figure 2: Effect of H₂O₂ on Total calcium ATPase activity of smooth muscle. Each bar is the mean of 6 individual rabbits. * = Significantly different from Control; x = significantly different from 0% H₂O₂; *p* < 0.05.

of H₂O₂. At the higher, more effective, concentrations of H₂O₂, JB significantly protected the enzymes from the destructive effects of H₂O₂.

Table 1 displays the ED₅₀ for the effect of H₂O₂ on the enzyme activities in the presence and absence of JB: * = significantly different from CS; X = significantly different from -JB at *p* < 0.05.

At the ED₅₀ concentration, CS was significantly more sensitive to H₂O₂ than either plasma membrane Ca ATPase or SERCA; although there were no differences in the ED₅₀ between + or - JB. Plasma membrane Ca ATPase + JB was significantly less sensitive to H₂O₂ than plasma membrane Ca ATPase - JB. Similarly, SERCA + JB was significantly less sensitive to H₂O₂ than SERCA - JB.

Figure 4 shows the total antioxidant capacity of JB and ascorbic acid (standard). JB and ascorbic acid showed linear antioxidant activity. Quantitative analysis is presented in figure 5.

DISCUSSION AND CONCLUSIONS

Urinary bladder dysfunctions in both men and women increase with age. The most common age-related urinary dysfunctions in women are incontinence and cystitis [24]. In men, the most common age-related dysfunctions include Obstructive Bladder Dysfunction (OBD) and metabolic syndrome, both of which are secondary to BPH [2,3]. Oxidative stress plays an important part in the etiologies of most forms of lower urinary tract dysfunctions in both men and women [4,5,24]. In rabbits, PBOO in males and experimental cystitis in females result in an increased frequency of urination and a decreased volume per urination. Using in-vivo cystometry, there is also a decreased compliance (increased bladder stiffness) and a decreased volume at micturition. In addition, there is a decreased contractile response of the bladder smooth muscle to various forms of stimulation. Metabolically, there is decreased CS activity, decreased

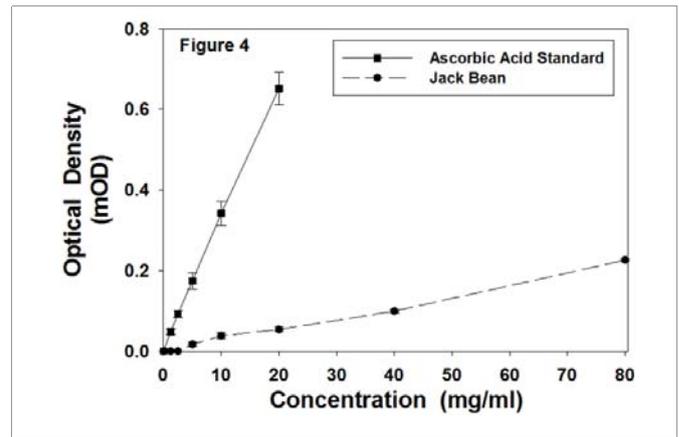


Figure 4: Total antioxidant capacity of JB and ascorbic acid (standard) using the CUPRAC test. Each point is the mean +/- SEM of 6 individual assays.

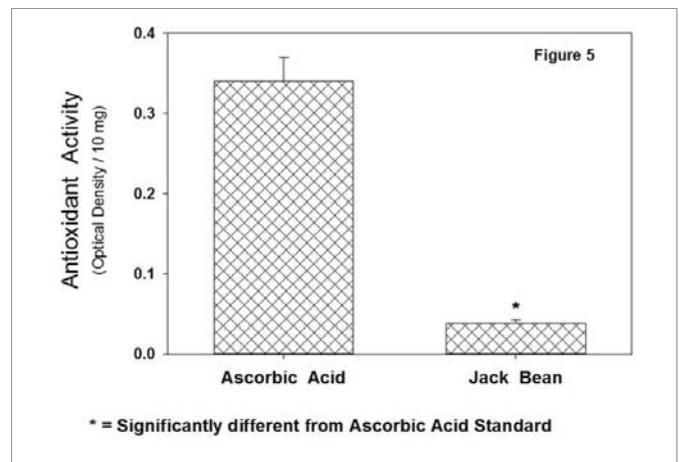


Figure 5: Quantitative antioxidant capacity of JB and ascorbic acid (standard) using the CUPRAC test. Each bar is the mean +/- SEM of 6 individual assays. * = Significantly different from ascorbic acid standard, *p* < 0.05.

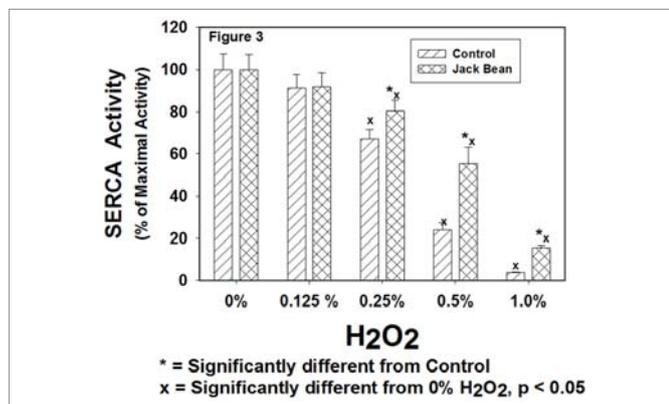


Figure 3: Effect of H₂O₂ on SERCA activity of smooth muscle. Each bar is the mean +/- SEM of 6 individual rabbits. * = Significantly different from Control; x = significantly different from 0% H₂O₂; *p* < 0.05.

Table 1: Table displays the ED₅₀ for the effect of H₂O₂ on the enzyme activities in the presence and absence of JB: * = significantly different from CS; X = significantly different from -JB at *p* < 0.05.

CS - JB:	0.04 +/- 0.003 %	CS + JB:	0.03 +/- 0.005%
Ca ATPase-JB:	0.65 +/- 0.05% *	Ca ATPase + JB:*	0.4 +/- 0.05%*x
SERCA - JB:	0.6 +/- 0.04% *	SERCA + JB:*	0.32 +/- 0.04%*x

* = significantly different from CS, *p* < 0.05
X = significantly different from - JB, *p* < 0.05

calcium ATPase and SERCA activities, and a significant increase in oxidative damage [24]. In regards to the current study, it was observed that CS was significantly more sensitive to H₂O₂ than was calcium ATPase or SERCA. At the higher concentrations of H₂O₂, the tissues treated with JB were significantly more resistant to the oxidative stress induced by H₂O₂ than the tissues without JB. This was similar for both all three enzymes, that is, at the higher concentrations of H₂O₂ the tissues treated with JB were significantly more resistant to the oxidative stress induced by H₂O₂ than the tissues not treated with JB. Interestingly, SERCA showed a significantly greater effect of JB than did plasma membrane calcium ATPase. This is extremely important since the SR is the organelle that releases intracellular free calcium during a contraction and pumps the calcium out of the cell and into the SR for storage after the contraction. Decreased SERCA activity results in increased intracellular free calcium and results in decreased bladder compliance and decreased contractile responses; as observed in the previous studies. The CUPRAC assay demonstrated that JB had significant antioxidant activity [25]. One question that should be discussed: Is the beneficial effect of JB in these studies due to a single active ingredient or is it due to the synergistic action of a variety of active components? Although JB has a number of active ingredients such as berginine, p-hydroxybenzoylberginin, 11-O-galloylberginin and methyl gallate [26], in our experience,

natural products have greater physiological or biochemical activity than individual components of the natural product due to the better pharmacokinetic properties of the natural product, and the synergistic activity among the components. For example, although resveratrol is supposed to be the major active antioxidant in grapes, we compared the ability of a standardized whole grape preparation (supplied by the California Table Grape Commission) with pure resveratrol in their ability to protect in-vitro bladder strips from oxidative stress [27,28]. Although the resveratrol had over 100 times the antioxidant activity of the grape preparation, the grape preparation had significantly more potent biological activity. Two possibilities are 1) that the active ingredients in the grape preparation have a greater bioavailability than resveratrol, or that the beneficial effects of grapes depend on the synergistic activity of a variety of bio-active components. We tend to go with the second possibility. Similarly, as mentioned above, we believe the beneficial effects of JB in these studies depend on the synergistic activity of a variety of bio-active components and not on the activity of a single active component. These data support the conclusions from the previous study on JB [21] that it is the antioxidant properties of JB that contribute to its physiologic and biochemical protective effects.

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ETHICAL APPROVAL

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

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