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# Antioxidant levels of common fruits, vegetables, and juices versus protective activity against in vitro ischemia/reperfusion

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**Abstract** It is well known that antioxidants present in various fruits, vegetables, and juices have the potential to protect the urinary bladder from free radical damage. What is not well understood, however, is how well antioxidant activities detected by chemical methods such as the CUPRAC assay for total antioxidant activity (TAA) predict the level of physiological protection available. It is hypothesized that the level of antioxidant reactivity found by the CUPRAC assay will positively correlate with increased protection in a model of in vitro ischemia/reperfusion. To test this hypothesis, the CUPRAC assay was utilized to determine the antioxidant reactivity of a series of fruits, vegetables, and juices, and the results were compared to the protective ability of selected juices in an established in vitro rabbit bladder model of ischemia/reperfusion. The results of the CUPRAC test showed that cranberry juice had the highest level of antioxidant reactivity, blueberry juice had an intermediate activity, and orange juice had the lowest. It was determined, however, that contrary to the hypothesis, the orange juice was significantly more potent in protecting the bladder against ischemia/reperfusion damage than either blueberry or cranberry juice. Thus, it is concluded that chemical tests for TAA do not necessarily correlate with their physiological activity.

**Keywords** Ischemia · Antioxidants · Ischemia/reperfusion · Juice · Fruit

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## Introduction

One major pathology of the lower urinary tract is obstructive bladder dysfunction secondary to benign prostatic hyperplasia (BPH). BPH is a slowly progressing disorder that is associated with age, which eventually results in bladder obstructive dysfunction if it is not relieved. The characteristic ailments of obstructive bladder dysfunction are decreased urinary flow, decreased compliance and post-void residual urine volume [1–5]. Ultimately, this affliction can lead to serious complications such as renal dysfunction [1–5].

The process of bladder decompensation due to outlet obstruction is divided into three stages. The initial stage shows an increase in urethral resistance to urine flow due to the enlarging prostate, which results in bladder distention. Due to the increasing pressure, the bladder subsequently shows increases in mass, vascularization, and blood flow. The hypertrophy of the bladder compensates for the increasing urethral resistance maintaining a relatively normal

bladder function. This is known as the compensated stage [6]. Although normal bladder function is maintained, cycles of ischemia/reperfusion occur during and following micturition [7, 8] and as a result hypoxic foci appear [9]. Initially, tissue hypoxia occurs in the mucosa and sub-mucosa tissue. As the tissue hypoxia spreads to the interstitial spaces and smooth muscle bundles, the bladder enters the decompensation stage [9].

The cyclical periods of ischemia/reperfusion generate both reactive nitrogen species of free radicals (RNS) and reactive oxygen species of free radicals (ROS), which proceed to cause cellular and sub-cellular damage [10, 11]. It has been suggested that these free radicals play a key role in the molecular, membrane, and sub-cellular damage that leads to the decompensated stage of bladder dysfunction [10, 11]. Injuries include protein oxidation and membrane lipid peroxidation, and the consequence of this is damage to the cellular and sub-cellular membranes of organelles such as the mitochondria, synapses, and the sarcoplasmic reticulum [6]. Nitrotyrosine, which is generated upon reaction of RNS with the protein tyrosine, is known to affect the function of enzymes, receptors, and signal transduction mechanisms [12]. This suggests that if the damage due to free radicals can be prevented, then the bladder dysfunction that occurs with outlet obstruction can be avoided and possibly reversed when damage has already occurred.

One way that free radicals can be sequestered is through the use of antioxidants found within natural food products. Antioxidants are defined as any substance that, when present at low concentrations compared with those of the oxidizable substrate, significantly delay or inhibit oxidation of that substrate. One major group of antioxidants present in natural food products are flavonoids. These molecules are able to exhibit their antioxidant activity by hydrogen atom transfer or electron donation to a reactive oxygen species (ROS) or towards lipid peroxidizing radicals ($R^{\cdot}$, $RO^{\cdot}$, and $ROO^{\cdot}$) [13]. It has been shown that oral administration of grape products, which have a significant amount of antioxidants present, reduced bladder dysfunction in a rabbit model [14, 15]. The studies suggest that the antioxidants present offered membrane protection by reducing the amount of free radical damage.

For further antioxidant investigations, the level of protection offered by antioxidants present in different food types should be determined. There are several chemical assays available that provide a method to compare the total antioxidant capacity in food products. The assay that this study utilized is the CUPRAC (Cupric Reducing Antioxidant Capacity) assay, which is an electron transferring assay that relies on the reduction of copper [13]. The CUPRAC assay was chosen because it has been found to be sensitive to lipophilic, hydrophilic, and thiol bearing antioxidants [13]. It is also able to work at a physiological pH [13], doing so by replicating more closely the reactivity of antioxidants found within the isolated baths of the in vitro model of ischemia/reperfusion.

Although the CUPRAC assay does show the relative reactivity of antioxidants, it is unknown how well this capability translates into the protection offered by antioxidants in vivo. To investigate if there is a correlation between the antioxidant capabilities in a chemical assay and their ability to offer bladder protection (or protection from free radical damage), an in vitro model of ischemia/reperfusion damage of the rabbit bladder was utilized. The in vitro analysis mimics the damage found in bladders due to obstruction by utilizing isolated rabbit bladder strips that have undergone ischemia/reperfusion damage [16–18]. Our hypothesis states that with increasing reactivity of antioxidants found within the natural food products, the level of protection offered to the bladder tissue will increase proportionally.

## Methods

### Chemical assays

#### *Juices, fresh fruits, and vegetables*

The following juices were made from fresh fruits: blueberry, grape, orange, tomato, and cranberry. The following 100% juice from bottles was used: acai, black currant, blueberry, carrot, cherry, cranberry, orange, and tomato. Antioxidant reactivity was normalized to the carbohydrate concentration.

#### *CUPRAC assay*

The CUPRAC assay was utilized to determine the antioxidant capacity of the juices and fresh fruits/

vegetables as developed by Resalt et al. [13]. This assay relies on the electron donating capabilities of antioxidants to reduce the copper ion. The CUPRAC working solution consisted of 10 mM copper (II) chloride dihydrate, 1 M ammonium acetate, and 7.5 mM neocuproine. 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 at 450 nm in a Hitachi U-2001 spectrophotometer. The standard curve utilized in this assay was ascorbic acid with the following concentrations: 1,000, 500, 250, 125, 62.5, 31.25, and 0 μM.

*Carbohydrate assay*

The carbohydrate assay was used to determine the carbohydrate concentration present in the juices and fresh fruits/vegetables. The antioxidant activity was quantitated as ascorbic acid equivalents per μg carbohydrate. The carbohydrate assay was adapted from Brooks [19]. About 20 μl of various concentrations of the samples was added to 1 ml of an anthrone–sulfuric acid solution and allowed to react for 2 min in a 90°C hot water bath. The samples were then cooled for 10 min at room temperature, after which they were read at 630 nm in a Hitachi U-2001 spectrophotometer.

In vitro ischemia/reperfusion bladder model

This project was approved by the IACUC of the Stratton VA Medical Center.

*Surgical procedure*

Four male New Zealand white rabbits were euthanized, and their bladders were harvested. Eight full thickness bladder strips were obtained from each rabbit bladder and used in the contractile studies. The in vitro ischemic/reperfusion model is described in detail previously [16–18]. The following is a summary of the protocol and the juices that were utilized in this part of the study. The bladder strips were mounted in glass water baths containing 20 ml of Tyrode's Solution with glucose (1 mg/ml) at a constant 37°C and aerated with a mixture of 95% $O_2$ and 5% $CO_2$. Two grams of tension was applied to each strip (previously shown to result in maximal active tension), and the strips were equilibrated for 30 min in the water baths. Strips from the same rabbit were then exposed to three different concentrations of the juice, and a control strip with no juice was also tested (in duplicate). The contractile responses to field stimulation (2, 8, and 32 Hz, 80 V, 1 ms), carbachol (20 μM), and KCl (120 mM) were determined using a Grass polygraph. Data were digitized using the Polyview system.

The juices that were chosen were those which featured high (cranberry), medium (blueberry), and low (orange) antioxidant capacity by the CUPRAC assay. The concentrated juices were diluted between 10- and 20-fold to obtain the desired carbohydrate concentrations. The pH of the baths was maintained at 7.4 with the addition of dilute sodium hydroxide. The juices were diluted sufficiently that we do not feel that hyperosmotic shock was a problem. If it was, we do not believe that contractility would have recovered.

The strips were then subjected to in vitro ischemia for 1 h. The in vitro ischemic model is produced by changing the aeration to 95% $N_2$ and 5% $CO_2$ and the buffer to Tyrode's solution plus juices without glucose present. During the ischemic period, the bladder strips were stimulated with 32-Hz field stimulation every 5 min for 20 s over a 1 h period. At the end of the hour, the bathing medium was changed to the oxygenated physiologic medium plus glucose with specific juice concentrations for an additional hour without stimulation. At the end of this recovery time, a second set of contractile responses was recorded. This method has been shown to be very useful in the identification of natural products having antioxidant activity [16–18].

Physiological data are presented as the percent of the control response obtained before the in vitro ischemia/reperfusion.

Statistics

Analysis of variance followed by the Bonferoni test for individual differences was used. A $P < 0.05$ was required for statistical significance.

## Results

The total antioxidant activity (TAA) of a variety of fresh juices is shown in Fig. 1. These were 100%

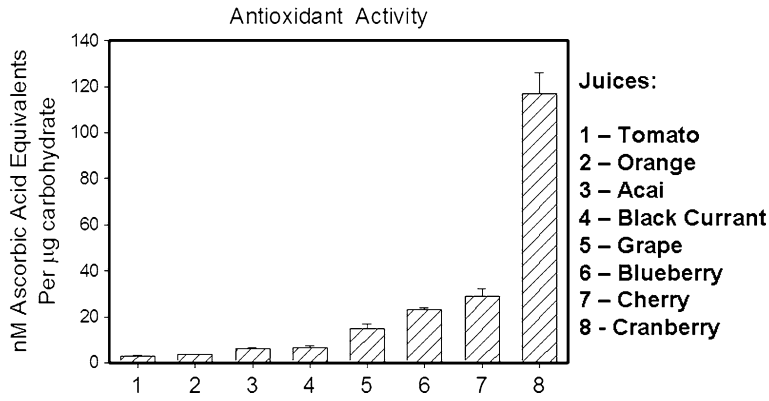


**Fig. 1** Comparison of TAA of a variety of fresh fruits and vegetable juices. Each *bar* is the mean ± SEM of four individual experiments

juice without any supplemented antioxidants. The cranberry had the highest TAA followed by the cherry, blueberry, grape, black current, acai, orange, and lastly tomato.

The TAA of selected juices compared with juice made from the fresh fruit and vegetables is displayed in Fig. 2. The pulp was removed from the fresh fruit and vegetables by low speed centrifugation (1,000×*g* for 15 min). The blueberry and tomato juices had a higher TAA compared to their fresh counterparts. This was the opposite for the grape and orange juices, which had a higher TAA in the fresh form.

The effect of in vitro ischemia/reperfusion (I/R) on the bladder strips in the presence of cranberry, blueberry, and orange juice is presented in Fig. 3. I/R resulted in a significant inhibition of contractile responses in all experiments to approximately 15% of control for 2-Hz stimulation; 20% for 8 Hz, 34% for 32 Hz, 59% for carbachol, and 44% of control for KCl. Thus, as has been shown previously, field stimulation is inhibited to the greatest extent by I/R, while carbachol stimulation is affected the least. Cranberry juice at 41 AA Eq/ml provided significant protection for all forms of stimulation; 20 AA Eq/ml provided significant protection for all forms of stimulation except at 32-Hz field stimulation; 10.25 AA Eq/ml provided protection only for KCl.

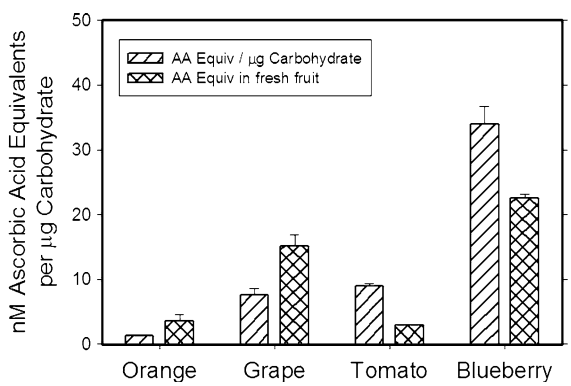


**Fig. 2** Comparison of TAA of selected fresh juices with their fresh fruit and vegetable counterparts. Each *bar* is the mean ± SEM of four individual experiments

Blueberry juice at 27.3 AA Eq/ml provided significant protection for all forms of stimulation; 13.6 AA Eq/ml provided significant protection for all forms of stimulation except at 2-Hz field stimulation, while 6.8 AA Eq/ml provided some degree of protection for all forms of stimulation except for KCl.

Orange juice at 3.0 AA Eq/ml provided significant protection for 2- and 8-Hz field stimulation and for KCl; 1.5 AA Eq/ml provided significant protection for all forms of stimulation; 0.75 AA Eq/ml provided some degree of protection only for KCl.

Figure 4 displays a comparison between TAA of the three juices with the potency of these juices in the in vitro physiological experiment. We used the AA equivilents of the juices that produce a significant 20% increase in contraction, since this was the approximate mean increase if all the significant increases in contraction for all juices were averaged. The cranberry juice had the highest TAA equivalents followed by blueberry and then orange juice, which is shown by the bars on the left.

Orange juice required less ascorbic acid equivalents in order to produce a 20% increase in contraction when compared to blueberry or cranberry. Cranberry required the highest level of ascorbic acid equivalents in order to produce the same level of increased contractile response.

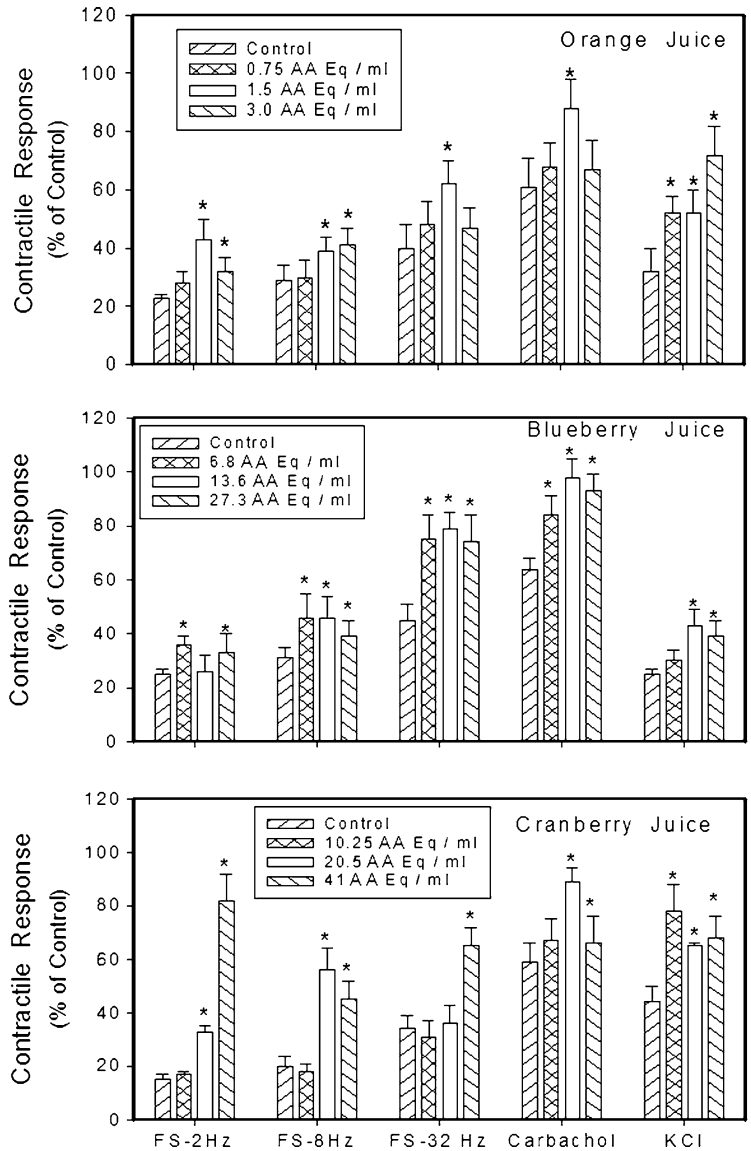


**Fig. 3** Effect of cranberry, blueberry, and orange juices on the response of isolated bladder strips to in vitro ischemia/reperfusion. Each *bar* is the mean ± SEM of four individual experiments. * Significantly greater than control, $P < 0.05$

## Discussion

Determining the reactivity of antioxidants found within juices, fresh fruits, and vegetables is only as useful as how well it accurately predicts the level of protection offered by antioxidants in the body. It was expected that the level of reactivity found with the CUPRAC assay would positively correlate with the level of protection offered in our in vitro model of ischemia/reperfusion. The CUPRAC assay as shown in Fig. 1 produced very reproducible levels of TAA. It was not surprising that the TAA of fresh fruits and vegetables did not correlate directly with their juices. Through this comparison, it can be seen that the blueberry and tomato juices had a higher level of antioxidant reactivity than their fresh fruit equivalent, when compared to the orange and grape juices that had a higher TAA in their fresh fruit form.

Of the juices tested, those with a high (cranberry), medium (blueberry), and low (orange) antioxidant capacity were chosen for the physiological study. When each of the three juices was tested in the in vitro ischemia/reperfusion model, it was found that all three offered approximately the same maximal level of protection. Field stimulation contracts the strips through the release of neurotransmitters and

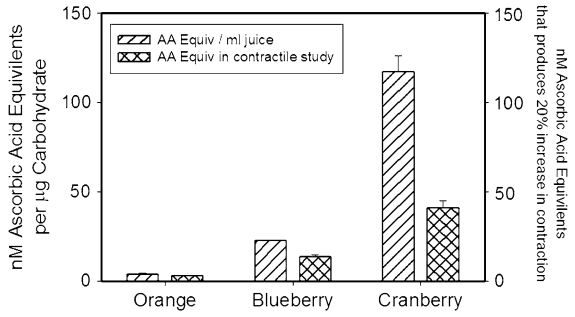


**Fig. 4** Comparison of the TAA of orange, blueberry, and cranberry juices with their physiological potency in the in vitro I/R model. Each *bar* is the mean of four individual experiments. For TAA, the higher the *bar*, the greater the activity; for the physiological studies, the lower the *bar*, the higher the activity

activation of post-synaptic receptors. The concentrations of ascorbic acid equivalents per ml that were able to preserve a significant level of contraction when compared to the control for all three frequencies of stimulation for cranberry, blueberry, and orange juice were as follows: 41 AA Eq/ml, 6.8 AA Eq/ml, and 1.5 AA Eq/ml, respectively.

Carbachol stimulates contraction through the direct stimulation of the muscarinic receptor. The concentrations of ascorbic acid equivalents per ml of juice required to maintain a significant level of contraction when compared to the control for the cranberry, blueberry, and orange juice were as follows: 20 AA Eq/ml, 6.8 AA Eq/ml, and 1.5 AA Eq/ml, respectively. The muscarinic receptors are known to be much more stable against ischemia/reperfusion damage [20], which explains why both the cranberry and the orange juices were able to protect the receptor at lower concentrations when compared to what was needed to protect the nerve.

Potassium chloride was used to determine the integrity of the cell membrane after ischemia/reperfusion. The concentrations of ascorbic acid equivalents per ml of juice needed to maintain a significant level of contraction for the cranberry, blueberry, and orange were as follows: 10.25 AA Eq/ml, 13.6 AA Eq/ml, and 0.75 AA Eq/ml, respectively. Both the cranberry and the orange juices were able to protect at the lowest concentration used in this study, suggesting that they are capable of protecting at even lower levels.

These studies clearly demonstrated that the order of potency for physiological activity of these three juices did not correlate with the TAA as demonstrated by CUPRAC. This shows that antioxidant detecting assays such as the CUPRAC assay do not necessarily accurately reflect the level of physiological protection. Possible explanations for this could be that the antioxidants present in the orange juice are more soluble and/or available, and thus, less orange juice is required in order to cause the same effect when compared to the blueberry or cranberry juices. It could also be that the orange juice has more of a certain type of antioxidant present that is capable of penetrating the muscle more efficiently and thus give better protection.

One caveat to this study is that it is clear that the components of the juices that are acting as antioxidants in this in vitro system may not be the active components when these juices are ingested because of absorption and metabolism.

In man, obstruction induced decompensation takes years to develop, and in the rabbit, obstruction induced bladder decompensation can take months. Performing these comparative studies in vivo would require 12 individual conditions (three juices, four concentrations including control) with an N of 5 rabbits for each condition, for statistical purposes would require 60 rabbits and about 1 year to complete. Although this would probably be the more physiological model to use, the use of this in vitro ischemia model can give us a comparative antioxidant potential using far fewer rabbits and in a much more time-effective basis.

The use of acute exposure to anoxia in the absence of substrate to mimic ischemia/reperfusion has been used by us and other investigators to both investigate the physiological and biochemical response to ischemia/reperfusion and test the effects of a variety of potential antioxidants and protective materials to protect against ischemia/reperfusion [16–18, 21–23].

Based on our previous published studies using this model, we feel confident that this in vitro model of ischemia/reperfusion can identify products with the potential to protect the bladder from oxidative damage; however, the order of potency as determined by the in vitro test may not be the same as in vivo potency. Thus, it would be necessary to perform in vivo studies to confirm these results [24–26].

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