2012-06-04

Effect of Antioxidants and Oxidative Stress on Different Cancer Cell Types

Gaytri Devi Gupta Elera
Brigham Young University - Provo

Effect of Antioxidants and Oxidative Stress on

Different Cancer Cell Types

Gaytri Gupta Elera

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

Kim L. O'Neill, Chair
Richard A. Robison
Craig Coleman

Department of Microbiology and Molecular Biology

Brigham Young University

June 2012

ABSTRACT

Effect of Antioxidants and Oxidative Stress on Different
Cancer Cell Types

Gaytri Gupta Elera
Department of Microbiology and Molecular Biology, BYU
Master of Science

Vaccinium cyanococcus, most commonly known as blueberry, is a fruit native to North America that is known for its unique taste and high antioxidant content. The skin, seed and juice of both organically and conventionally grown blueberry extract were analyzed for antioxidant content using both the Hydrophilic and Lipophilic Oxygen Radical Absorbance Capacity (ORAC) assays. Results from the Hydrophilic ORAC test showed that conventionally grown blueberries had a higher antioxidant capacity across all samples, while the Lipophilic ORAC assay showed that the antioxidant concentration of organically cultivated blueberry juice was highest, but conventionally grown blueberry seed and skin extract showed higher antioxidant content. The vitamin C content of both conventional and organic blueberries were analyzed using High Performance Liquid Chromatography (HPLC), where the organic blueberries showed a higher vitamin C concentration. In general, both organic and conventional blueberries are rich in antioxidants, and therefore, possess potential health benefits that require further study.

Abbreviations: AAPH, 2,2'-azobis(2-aminidopropane) dihydrochloride; ORAC, oxygen radical absorbance capacity; ROS, Reactive Oxygen Species; TE, Trolox Equivalents; AUC, area under the curve.

ACKNOWLEDGEMENTS

I express my gratitude to all those who contributed to my thesis. To my advisor and mentor, Kim O'Neill, for his encouragement and inspiration when I needed it the most. To my graduate committee members, Richard A. Robison and Craig Coleman, for their expertise and evaluation of my research. To Evita and Andres for helping me in any way they could. Finally, I would like to thank my family for supporting me throughout my Masters education.

TABLE OF CONTENTS

Appendix

Appendix I: The antioxidant properties of the cherimoya (*Annona cherimola*) fruit

Appendix II: The Role of Oxidative Stress in Prostate Cancer

LIST OF FIGURES

SUMMARY OF WORK

Cancer, although one of the leading causes of death in the United States, is a lifestyle disease, where correlations have been observed between lifestyle choices and cancer incidences. Due to this, interest has risen in dietary antioxidants as a means of lowering cancer risk and as potential therapeutic biomarkers for different cancer cell types. This thesis revolves around the potential health benefits of antioxidants and the role of oxidative stress on different cancer cell lines. Each study presented has been published.

The study that has been accepted for publication in the Journal of Food Research explores potential health benefits of organically and conventionally grown blueberries by comparing differences in antioxidant concentration using different antioxidant measuring techniques.

The appendix section contains two papers that have already been published. The first paper, titled "The antioxidant properties of cherimoya (*Annona cherimola*) fruit", examines the health benefits of cherimoya consumption by measuring its antioxidant content, and also explores the effect of this fruit on Raji (Burkitt's Lymphoma) and HT-29 (colon cancer) cells. An alternate cellular model was also established to investigate the effects of oxidative stress on antioxidant absorbance of these two cell lines.

The second paper, titled "The Role of Oxidative Stress on Prostate Cancer", is a review that summarizes the effects of oxidative stress on prostate cancer initiation and development, while also exploring the therapeutic potential of antioxidants and regulators of reactive oxygen species. Overall, this thesis is centered on the role antioxidants and oxidative stress might play in the prevention of different cancer cell types.

1. Introduction

In recent years, there has been a rising interest in organically cultivated foods as an alternative to conventional agricultural techniques, which has led to an increase in the acreage assigned to organic cultivations (Carbonaro, Mattera, Nicoili, Bergamo, & Cappeloni, 2002). Differences between these farming techniques are attributed to fertilizer type and pesticide use. Conventional farming techniques involve the use of mineral fertilizers and synthetic pesticides, while organic farming techniques use organic fertilizers and no synthetic pesticides.

Whether it is more beneficial for the consumer to purchase organic instead of conventional foods remains a hot topic of discussion. Although most people are under the impression that organically cultivated foods are more beneficial (Worthington, 2004), the experimental evidence to support this claim remains unclear and assessment of the health benefits of these products still requires further investigation.

Interest in the consumption of foods with the greatest health benefit potential has become more prevalent because epidemiological studies suggest that the consumption of both an antioxidant and phytochemical-rich diet contribute to the reduction of heart disease and several types of cancer (Chinery et al., 1998).

As a result, there has been substantial amount of interest in *Vaccinium corymbosum*, commonly known as blueberries, due to this fruit's anti-tumor properties in different cancer types such as colon cancer (Yi, Fisher, Krewer, & Akoh, 2005), breast cancer (Adams et al., 2010), and prostate cancer (Schmidt, Erdman, & Lila, 2006). Blueberries are native to North America, and are known to contain high contents of both antioxidants, such as phenolic acid and resveratrol, and phytochemicals, such as flavonoids and tannins (Seeram, 2008).

Antioxidants provide protection against oxidative stress, a condition that has been

1

suggested to contribute to a wide variety of diseases, including artherosclerosis, Parkinson's disease, and cancer (Amira, 2010). Oxidative stress occurs when the levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) increase to a level that propagates oxidative injury in DNA, lipids and proteins. Damage to DNA is an early event in carcinogenesis, and oxidation is known to induce 2'-4'-oxidative DNA damage that may lead to the formation of DNA-protein cross-links, alterations in the carbohydrate-phosphate backbone, or direct modification of purine or pyrimidine bases. If these alterations lead to the activation of oncogenes or the inactivation of tumor suppressor genes, cancer may develop (Gupta-Elera, Garrett, Martinez, Robison, & O'Neill, 2010). Therefore, antioxidants such as phenolic acid protect cells against oxidative stress by scavenging oxygen free radicals, and functioning as metal chelators (Russo et al., 2000). Resveratrol, another antioxidant found in blueberries, additionally reduces levels of ROS by increasing the levels of MnSOD, an enzyme that reduces superoxide to hydrogen peroxide (Robb, Page, Wiens, & Stuart, 2008), and also interferes with carcinogenesis by inducing Fas/Fas ligand-mediated apoptosis (Cao, Fu, Wang, Liu, & Han, 2005).

Phytochemicals have both antioxidant and hormone mimicking actions, and they exert their anticancer effects through different mechanisms, such as the induction of metabolizing enzymes, alteration of gene expression, modulation of cell signaling pathways and DNA repair (Seeram, 2008). Phytochemicals in blueberries have been shown to decrease the secretion of urokinase-type plasminogen activator (uPA) and increase the secretion of plasminogen activator inhibitor (PAI), both of which are cell-signaling proteins that inhibit tumor cell proliferation and metastatic potential in breast cancer cells (Adams et al., 2010). Anthocyanin, the flavonoid responsible for the pigmentation in blueberries, has both antioxidant and anti-cancer properties

(Seeram, Yanjun, & Muraleedharan, 2003). A study that explored the potential anticancer activities of polyphenols in rabbiteye blueberries on HepG2 liver cancer cells found that the anthocyanin fraction had the greatest inhibitory effect with 50% inhibition of cancer cell growth, thus suggesting that blueberries may help reduce the risk of liver cancer (Yi, Akoh, Fischer, & Krewer, 2006).

The main objective of this research was to provide preliminary results on the differences in antioxidant concentrations between conventionally and organically grown blueberries, specifically by examining extracts of their juice, skin and seed; and, therefore, provide insight into the health benefits of organic vs. conventional blueberries.

2. Materials and Methods

2.1 Chemicals

The 2,2'-azobis(2-aminidopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Fluorescein-sodium salt, Metaphosphoric Acid, methylated β-cyclodextrin, and DL-Dithiothreitol (threo-1,4-dimercapto-2,3-butanediol) were obtained from Sigma-Aldrich, Inc. (Milwaukee, WI). Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) was purchased from Enzo Life Sciences, Inc. (Plymouth, PA). Acetonitrile, methanol, and acetone were purchased from Baxter (Muskegon, MI).

2.2 Materials

Costar 96-well plate and 24-well clear culture plates were purchased from Corning Inc. (Lowell, MA).

2.3 Equipment

All fluorescence readings were taken using the BMG FLUOstar Optima (BMG

Laboratories) microplate reader (serial #413-0225). HPLC readings were made using the Agilent 1100 Zorbax SB-Aq (serial # USVD001136). Data were analyzed using Microsoft Excel and Apple Numbers.

2.4 Fruit Sample

Ten different lots of both conventional and organic highbush blueberry fruits were purchased at a local grocery store in Provo, UT on the day of importation. Organic blueberries carried the USDA organic label. Both organic and conventional blueberries were grown in Michigan.

2.5 Sample preparation

Blueberry skin, seed and juice were isolated and were then homogenized by weighing 0.1 g of sample and diluting it in 10 ml of double distilled water. Samples were centrifuged at 1800 rpm for 15 minutes, placed in aliquots of 2.5 ml and kept in a freezer at -20° C until analyzed.

2.6 Hydrophilic and Lipophilic ORAC

All samples were analyzed using a modified ORAC assay based on published methods (Garrett, Murray, Robison, & O'Neill, 2010; Huang, 2002). The ORAC assay measures oxidative degradation of a fluorescent compound (fluorescein sodium salt) after being exposed to an oxygen radical initiator, AAPH (2,2'-azobis-(2-amidinopropane) dihydrochloride). All readings were taken in Cole-Parmer black bottom 96-well plates, with 200µl Fluorescein, 25µl AAPH, and 20µl of antioxidant sample or PBS in each well.

The Lipophilic ORAC was performed in duplicate as described for the Hydrophilic ORAC, except that the solvent used to dilute the sample was 7% randomly methylated-cyclodextrin (RMCD) in acetone/water (1:1 v/v) solution.

Readings were taken every 2 minutes for 90 minutes. Measurements were taken by the BMG FLUOstar Microplate reader at 37°C; 485 nm excitation and 590 nm emission filters were used for fluorescence readings. All fluorescent measurements were expressed relative to the initial reading. The area under the curve (AUC) was calculated by using the difference of area under the fluorescein decay curves between the sample curve and the fluorescein + AAPH curve. The AUC measures the total antioxidant activity in the sample minus the fluorescein + AAPH antioxidant curve. Lipophilic and Hydrophilic ORAC values were measured separately and the Total Antioxidant Capacity (TAC) was calculated by summing the L-ORAC and H-ORAC values.

2.7 Standard Curve Data

Trolox, a water-soluble analogue of vitamin E, was used as a control standard. Trolox standards were run at concentrations of 50μM, 25μM, 12.5μM, and 6.25μM to obtain standard curves for each trial. Data from each standard curve was used to convert raw net area under the sample curve (net AUC) values to Trolox Equivalents per liter (TE/L). Subsequent calculations converted TE/L to TE/100g (see Figure A.1)

2.8 High-Performance Liquid Chromatography

All samples were measured using an Agilent 1100 (US) HPLC system coupled with an Agilent 1100 diode array detector and equipped with Agilent 1100 binary pumps based on published methods (Nielson, 2006). Samples consisting of 5 μL were injected at ambient temperature (20 °C) into a Reverse phase Zorbax SB-Aq (4.6x150 mm 3.5 μm) with C18 guard column. An Isocratic mobile phase consisting of MeOH/Sodium Acetate (3/97) with a pH of 4.0 was used with a flow rate of 1mL/min. Vitamin C content was identified by its UV spectra,

recorded with a diode array detector and by chromatographic comparison with authentic

markers. Scanning between 190 and 400 nm was performed, and data were collected by the

Agilent Chemstation data system.   Data collection time was 30 minutes.

3. Results

3.1 Hydrophilic ORAC Values

As shown in Figure 1, hydrophilic antioxidant analysis of conventional blueberry skin,

seed and juice extract resulted in higher antioxidant values when compared to the organic

blueberries, across all samples. Juice exhibited the highest measured antioxidant activity, with

the conventional yielding a value of 9,806 TE/100g (stdev=3851, N=30), and the organic

yielding a value of 5,258 TE/100g (stdev=2649, N=30). The next highest activity was found in

the skin at 7,891 TE/100g (stdev=2311, N=30) for conventional, and at 4,623 TE/100g

(stdev=2337, N=30) for organic. Finally, the lowest activity was measured in the seed, with the

conventional at 1,015 TE/100g (stdev=524, N=30), and the organic at 636 TE/100g (stdev=283,

N=30).

Figure 1. Differences in antioxidant content between organic and conventional blueberry juice, skin, and seed extract using H-ORAC. All values are reported as TE/100g (Mean of N=30).

3.2 Lipophilic ORAC Values

As shown in Figure 2, lipophilic antioxidant analysis of the conventional blueberry skin
and seed extract resulted in a higher antioxidant content in comparison to the organic blueberry
samples, while the organic juice extract resulted in a higher antioxidant content than that of the
conventional. The highest measured antioxidant activity was found in the juice, with the organic
yielding a value of 35,156 TE/L (stdev=2,183, N=30), and the conventional yielding a value of
31,535 TE/L (stdev=2,281, N=30). The next highest activity was observed in the skin at 13,755
TE/100g (stdev=2,593, N=30) for conventional, and at 11,833 TE/100g (stdev=1,687, N=30) for
organic. Finally, the lowest activity was found in the seed, with the conventional at 10,727
TE/100g (stdev=2,198, N=30), and the organic at 8,709 TE/L (stdev=1,830, N=30).
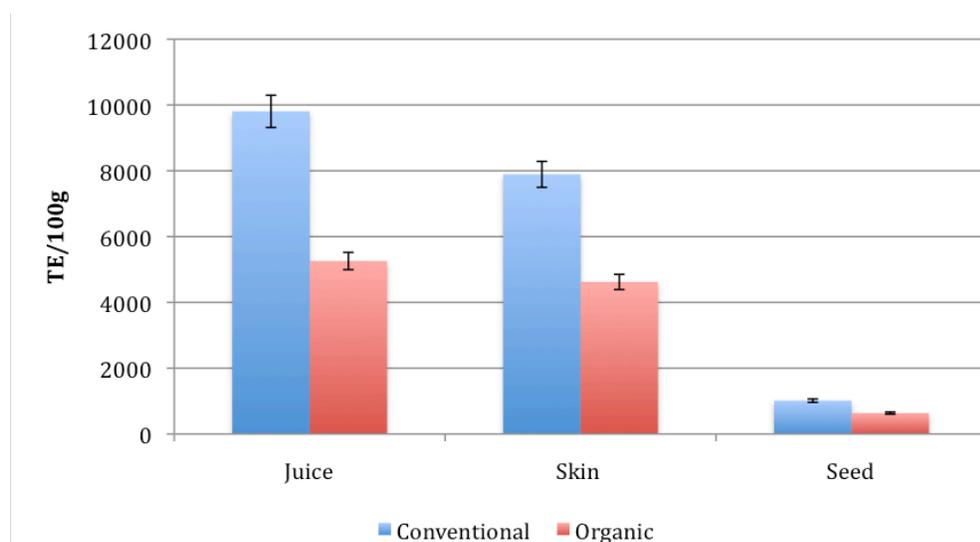

Figure 2. Differences in antioxidant content between organic and conventional blueberry juice, skin, and seed extract using L-ORAC. All values are reported as TE/100g (Mean of N=30).

<u>3.3 Vitamin C content using HPLC</u>

High-Performance Liquid Chromatography analysis of whole organic blueberries resulted in a higher vitamin C content than whole conventional blueberries, as shown in Figure 3. Organic blueberries yielded a value of 592.879 μg/g (stdev=42.406, N=5), while conventional blueberries had 386.947 μg/g (stdev=40.172, N=5).
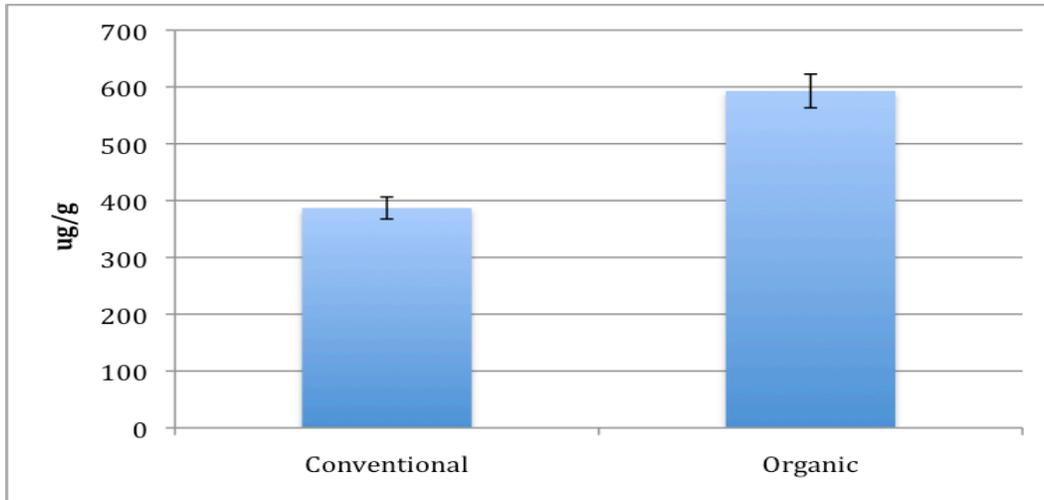
Figure 3. Difference in vitamin C content between organic and conventional blueberry using HPLC. All values are reported as µg/g.

4. Discussion

4.1 Analysis of H-ORAC and L-ORAC values

When the extracts of blueberry seed, skin and juice were analyzed for antioxidant activity using the Hydrophilic ORAC, conventionally grown blueberries exhibited higher antioxidant content across all samples. Statistical significant changes in antioxidant content were observed in blueberry skin ($p<0.01$), seed ($p<0.05$), and juice ($p<0.01$) components, with conventional samples possessing more antioxidant activity. This might be explained in part by the difference in nutrient composition between organic and mineral fertilizers. Organic fertilizers have a lower nutrient content, solubility and nutrient release rates, thus making the nutrients, such as nitrogen, less readily available to plants. Therefore, the blueberry plant grown in an organic environment has to allocate more of its resources towards the synthesis of nitrogen-containing compounds and towards its growth and development, whereas conventionally grown blueberries can allocate their resources elsewhere (Seghers et al., 2003).

The Lipophilic ORAC provided different results. The extract of organic blueberry juice showed a statistically significant increase ($p<0.001$) in antioxidant concentration when compared to conventional blueberry juice. On the other hand, conventionally grown blueberries showed a statistically significant increase of antioxidants in their skin ($p<0.001$) and seed ($p<0.001$), when compared to its organic blueberry components. Winter & Davis (2006) have suggested that a potential reason that could account for the elevated antioxidant content in organic blueberry juice is that since organically grown blueberries do not have synthetic pesticides, the plant has to build its own chemical defenses against pathogens, thus increasing their antioxidant concentrations. However, they also argued that conventionally-grown blueberry plants are also subjected to stress due to their exposure to sub-lethal doses of chemicals found in both herbicides and

9

pesticides, and in response, they increase their antioxidants and phytochemicals so that they can act as chemical barriers against pathogens (Winter & Davis, 2006).
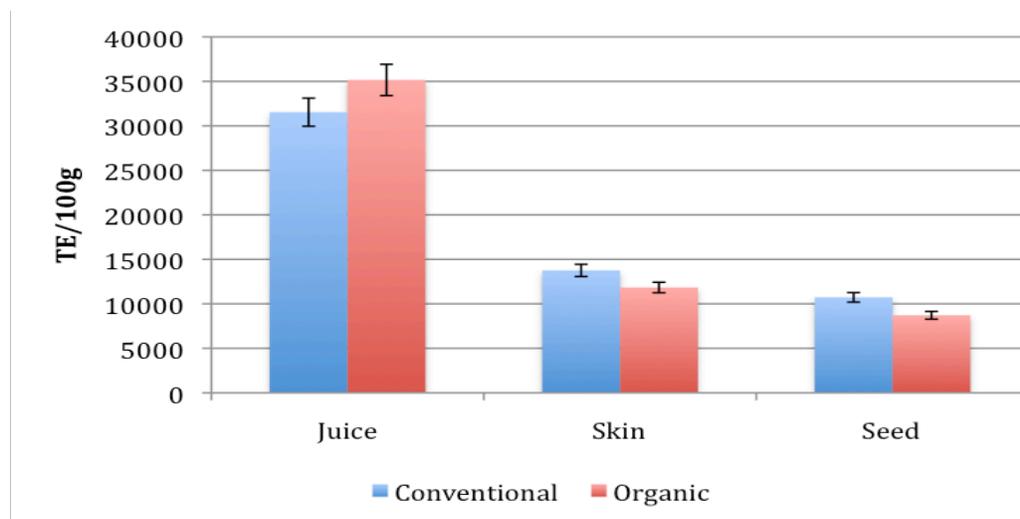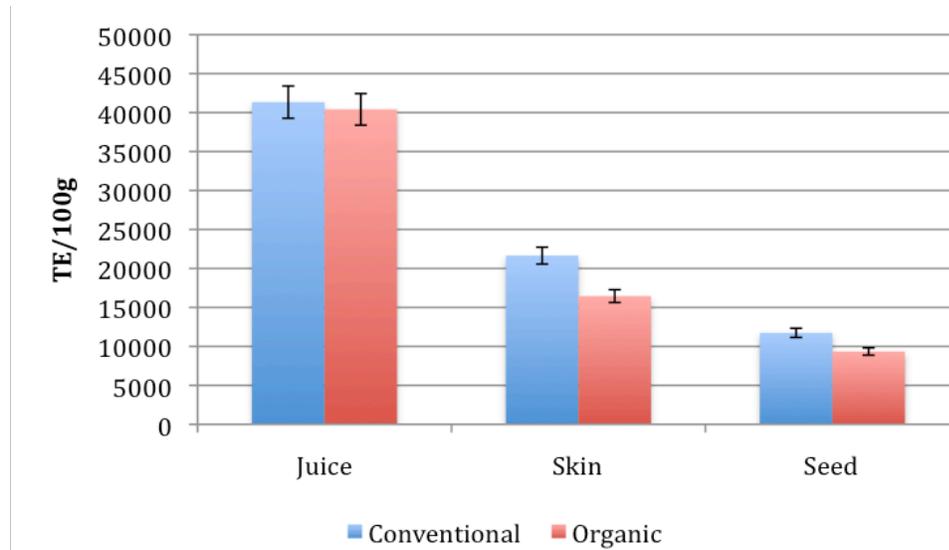

Figure 4. Differences in total antioxidant content between organic and conventional blueberry juice, skin, and seed extract. All values are reported as TE/100g (Mean of N=5).

4.2 Analysis of Vitamin C content using HPLC

Results showed that organically grown blueberries had a 53.4% antioxidant increase when compared to conventionally grown blueberries. Ascorbic acid reduces reactive oxygen species to prevent oxidative damage, and also reduces vitamin E to activate it and restore the antioxidant protection of the cell membrane (O'Neill, Standage, Hughes, & Murray, 2001). The decreased amount of vitamin C in conventionally grown blueberries could be due to the synthetic fertilizer. The amount of nitrogen in the fertilizer might affect the amount of vitamin C and nitrates, and the amount of protein produced by the plant. Since plants that are conventionally grown are presented with a higher concentration of nitrogen, its protein production is increased, while its carbohydrate production is reduced. Consequently, the amount of vitamin C produced is reduced, since it is made from carbohydrates (Seghers et al., 2003).

This is consistent with results from a review that surveyed 41 studies in the existing

literature comparing the nutritional content of organic and conventional crops in order to identify significant differences; and trends in the data found that, on average, the vitamin C content of an organic fruit or vegetable was 27% higher than that of a conventional fruit or vegetable (Worthington, 2004).

Student's one tail t-test ($6.69e^{-30}$) and two tail t-test ($1.34e^{-29}$) show that there is a statistically significant difference in vitamin C capacity between organic and conventional blueberries.

4.3 Analysis of total ORAC values

As shown in Figure 4, when the total antioxidant capacity for the H-ORAC and L-ORAC are analyzed, conventional blueberries have a higher total antioxidant content, with 33,388 TE/100g and 607,700 TE/L, respectively, in comparison to 25,800 TE/100g and 415,608 TE/L for organic blueberries. Student t-test analyses performed on data revealed that the differences in antioxidant content of juice, skin and seed between organically- and conventionally- grown blueberries were statistically statistically significant: $p<0.01$, $p<0.01$, and $p<0.01$, respectively.

Overall, the blueberry fruit is very rich in antioxidants, with ORAC values higher than blackberries, strawberries and grapes (Haytowitz & Bhagwat, 2010). A study that analyzed the flavonoid and phenolic acid profiles of 19 different berries, including cranberry, raspberry and strawberry, suggested that blueberries had the highest total phenolic content (Häkkinena et al., 1999). This suggests that the potential health benefits of blueberries is high, and may, thereby, prevent the initiation and progression of diseases that have been linked to oxidative stress, such as cancer, cardiovascular diseases and neurodegenerative diseases.

5. Conclusion

Results showed although organic and conventional blueberries are both very rich in antioxidants, conventional blueberries have higher antioxidant content, and, therefore, may have a higher overall health benefit potential in reducing cancer risk. However, organic blueberries have higher vitamin C content when analyzed with HPLC. This study also suggests that it is important to consume both the juice and skin of the fruit to maximize antioxidant intake.

Further chemical analyses are needed to more fully understand the chemical composition in these blueberry components and the specific antioxidant compounds they contain. Further research may also include establishing a cellular model, where different cancer cell lines can be exposed to both organic and conventional blueberry juice, skin and seed extract where the concentration of antioxidants that is absorbed by the cell can be measured. This may provide insight into how effectively the cells take in the antioxidants, and ultimately, add insight into the potential role of blueberries in the prevention of different cancer types.

References

Adams, L. S., Phung, S., Yee, N., Seeram,N., Li, L., & Chen, S. (2010). Blueberry Phytochemicals Inhibit Growth and Metastatic Potential of MDA-MB-231 Breast Cancer Cells through Modulation of the Phosphatidylinositol 3-Kinase Pathway. *Cancer Research, 70*(9), 3594–3605. http://dx.doi.org/10.1158/0008-5472.CAN-09-3565

Amira, A. (2010). Oxidative Stress and Disease: An Updated Review. *Research Journal of Immunology, 3*(2), 129-145. http://dx.doi.org/10.3923/rji.2010.129.145

Cao, Y., Fu, Z., Wang, F., Liu, H., & Han, R. (2005). Anti-angiogenic activity of resveratrol, a natural compound from medicinal plants. *Journal of Asian natural products research, 7*(3), 205-213. http://dx.doi.org/10.1080/10286020410001690190

Carbonaro, M., Mattera, M., Nicoili, S., Bergamo, P., & Cappeloni, M. (2002). Modulation of Antioxidant Compounds in Organic vs Conventional Fruit (Peach, Prunus persica L., and Pear, Pyrus communis L.). *Journal of Agricultural and Food Chemistry, 50*(19), 5458-5462.

Chinery, R. B., R.D., Shyr, Y., Kirkland, R., Coffey, R.J., & Morrow, J.D. (1998). Antioxidants Reduce Cyclooxygenase-2 Expression, Prostaglandin Production, and Proliferation in Colorectal Cancer Cells. *Cancer Research, 58*(11), 2323-2327.

Garrett, A., Murray, B.K., Robison, R.A., & O'Neill, K.L. (2010). Measuring antioxidant capacity using the orac and tosc assays. *Methods in Molecular Biology* (Vol. 594, pp. 251-262): Springer. http://dx.doi.org/10.1007/978-1-60761-411-1_17

Gupta-Elera, G., Garrett, A., Martinez, A., Robison, R., & O'Neill, K. L. (2010). The antioxidant properties of the cherimoya (Annona cherimola) fruit. *Food Research International, 44*(7), 2205–2209. http://dx.doi.org/10.1016/j.foodres.2010.10.038

Häkkinena, S., Heinonenc, M., Kärenlampid, S., Mykkänena, H., Ruuskanene, J., & Törrönen, R. (1999). Screening of selected flavonoids and phenolic acids in 19 berries. *Food Research International, 32*(5), 345-353. http://dx.doi.org/10.1016/S0963-9969(99)00095-2

Haytowitz, D. & Bhagwat, S. (2010). USDA Database for the Oxygen Radical Capacity (Orac) of Selected Foods. from USDA National Nutrient Database for Standard Reference.

Huang, D. (2002). Development and Validation of Oxygen Radical Absorbance Capacity Assay for Lipophilic Antioxidants Using Randomly Methylated-Cyclodextrin as the Solubility Enhancer. *Journal of Agricultural and Food Chemistry, 50*(7), 1815-1821.

Nielson, A. P., Ogden, L.V., & Pike, O.A. (2006). Sensory and Nutritional Quality of Dehydrated Potato Flakes in Long-Term Storage. *Journal of Food Science, 71*(6), 461-466. http://dx.doi.org/10.1111/j.1750-3841.2006.00084.x

O'Neill, K. L., Standage, S., Hughes, B., & Murray, B. (2001). Fruits and Vegetables and the Prevention of Oxidative DNA Damage. In R. Watson (Ed.), *Vegetables, Fruits, and Herbs in Health Promotion.* (pp. 135-143). Boca Raton: CRC Press. http://dx.doi.org/10.1201/9781420042542.ch10

Robb, E., Page, M.M., Wiens, B.E., & Stuart, J. (2008). Molecular mechanisms of oxidative stress resistance induced by resveratrol: Specific and progressive induction of MnSOD. *Biochemical and Biophysical Research Communications, 367*(2), 406-412. http://dx.doi.org/10.1016/j.bbrc.2007.12.138

Russo, A., Acquaviva, R., Campisi, A., Sorrenti, V., Di Giacomo, C., Virgata, G., Barcellona, M., & Vanella, A. (2000). Bioflavonoids as antiradicals, antioxidants and DNA cleavage protectors. *Cell Biology and Toxicology, 16*(8), 91-98. http://dx.doi.org/10.1023/A:1007685909018

Schmidt, B. M., Erdman, J.W., & Lila, M.A. (2006). Differential effects of blueberry proanthocyanidins on androgen sensitive and insensitive human prostate cancer cell lines. *Cancer Letters, 231*(2), 240-246. http://dx.doi.org/10.1016/j.canlet.2005.02.003

Seeram, N. (2008). Berry Fruits: Compositional Elements, Biochemical Activities, and the Impact of Their Intake on Human Health, Performance, and Disease. *Journal of Agricultural and Food Chemistry, 56*, 627–629. http://dx.doi.org/10.1021/jf071988k

Seeram, N., Yanjun, Z., & Muraleedharan, N.G. (2003). Inhibition of proliferation of human cancer cell lines and cyclooxygenase enzymes by anthocyanidins and catechins. *Nutrition and Cancer, 46*(1), 101-106. http://dx.doi.org/10.1207/S15327914NC4601_13

Seghers, D., Top, E.M., Reheul, D., Bulcke, R., Boeckx, P., Verstraete, W., & Siciliano, S.D. (2003). Long-term effects of mineral versus organic fertilizers on activity and structure of the methanotrophic community in agricultural soils. *Environmental Microbiology, 5*(10), 867-877.

Winter, C. K. & Davis, S.F. (2006). Organic Foods. *Journal of Food Science, 71*(9), 117-124. http://dx.doi.org/10.1111/j.1750-3841.2006.00196.x

Worthington, V. (2004). Nutritional quality of organic versus conventional fruits, vegetables, and grains. *Journal of Alternative and Complementary Medicine, 7*(2), 161-173. http://dx.doi.org/10.1089/107555301750164244

Yi, W., Akoh, C., Fischer, J., & Krewer, G. (2006). Effects of phenolic compounds in blueberries and muscadine grapes on HepG2 cell viability and apoptosis. *Food Research International, 39*(5), 628-638. http://dx.doi.org/10.1016/j.foodres.2006.01.001

Yi, W., Fisher, J., Krewer, G., & Akoh,C. (2005). Phenolic Compounds from Blueberries Can Inhibit Colon Cancer Cell Proliferation and Induce Apoptosis. *Journal of Agricultural and Food Chemistry, 53*(18), 7320-7329.

Appendix I

<u>The antioxidant properties of the cherimoya (*Annona cherimola*) fruit.</u>

The antioxidant properties of the cherimoya (*Annona cherimola*) fruit

Gaytri Gupta-Elera, Andrew R. Garrett, Andres Martinez, Richard A. Robison, Kim L. O'Neill *

Department of Microbiology and Molecular Biology, Brigham Young University, Provo, UT, USA, 84602

A B S T R A C T

Annona cherimola is an exotic fruit from the genus *Annona*, native to the Andean highlands in western South America. The cherimoya skin, flesh and juice were isolated and analyzed for antioxidant content using the oxygen radical absorbance capacity (ORAC) assay. The juice showed the highest antioxidant activity, while the flesh exhibited the lowest. The cherimoya skin, flesh and juice extracts were also incubated with Raji (Burkitt's Lymphoma) and HT-29 (colon cancer) cell lines, and the antioxidant uptake of cells was measured. Both cell lines, when subjected to cherimoya juice, showed the highest antioxidant uptake. The cells were then exposed to AAPH, a radical initiator, to simulate the conditions of cells under oxidative stress, and then subjected to cherimoya skin, flesh and juice extracts. Both cell lines absorbed more antioxidants after being pre-exposed to AAPH, indicating that cells under stress have the ability to import antioxidants.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Annona cherimola, commonly known as cherimoya, is a species of fruit found in different subtropical areas around the world, mainly in northern Peru and southern Ecuador. Cherimoya is known to have been cultivated during the times of the Incan Empire, dating back to 1200 BC. Anthropological evidence suggests that the cherimoya fruit was an active ingredient in the Incan diet (Bonavia, Ochoa, Tovar, & Palomino, 2004). The cherimoya fruit is known for its exceptional taste, its use in traditional medicine as an antimicrobial and insecticide, and as an effective treatment for digestive disorders such as stomachache and pancreatic ulcers (Amoo, Emenike, & Akpambang, 2008).

Considerable evidence suggests that oxidative stress leads to the development of conditions such as cancer and heart disease the two leading causes of death in the United States (Tribble & Frank, 1994; Xu, Kochanek, Murphy, & Tejada-Vera, 2010). Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds antioxidant capacity, which leads to oxidative damage. Partially reduced forms of oxygen, such as superoxide and hydrogen peroxide, that are produced during aerobic metabolism have the potential of causing oxidative injury to macromolecules such as nucleic acids, proteins and lipids. Oxygen radicals are capable of initiating radical chain-reactions that propagate oxidative injury (O'Neill, Standage, Hughes, & Murray, 2001). DNA damage is an early event in carcinogenesis, and oxidation is known to induce 2′-4′-oxidative DNA damage which may lead to the formation of DNA–protein cross-links, alterations in the carbohydrate-phosphate backbone, or direct modification of purine and pyrimidine bases. Tumors may develop if alterations in DNA lead to the activation of oncogenes or the inactivation of tumor suppressor genes.

Dietary antioxidants found in bioactive food components exert regulatory effects on cell proliferation and cytotoxicity. Antioxidants such as vitamin C, vitamin E, soy sterols, and polyphenols help protect the cell against oxidant-induced proliferation, lipid peroxidation, glutathione peroxidase activation (Garcia-Alonso, Ros, & Periago, 2006; Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002), and also contribute to thyroid regulation and glucose homeostasis (Panda, Kar, & Patil, 2009). Evidence indicates that *A. cherimola* is rich in antioxidants such as vitamins A and C, making it a beneficial health supplement (Garcia, Zepeda-Vallejo, Gallegos, Gonzales, & Madrigal, 2008).

Interest in this fruit has also risen due to its high concentration of acetogenins, which are polyketides that act as secondary metabolites in plants. Research has accredited beneficial effects to acetogenins, including the induction of cytotoxic, anti-inflammatory and anti-tumorous activities, particularly in mammary, pancreatic, prostatic, and colon cancer cells (Chen, Chang, Chiu, Wu, & Wu, 1999). Analysis of twelve acetogenins in *Annona cornifolia*, a fruit related to *A. cherimola*, revealed through DPPH radical-scavenging activity that acetogenins contain antioxidant capacity, comparable to that of ascorbic acid (Santos Lima, Pimenta, & Boaventura, 2010). Acetogenins are thought to exert their effects through the inhibition of the ubiquinone oxido-reductase complex in the mitochondrial electron transport chain and the inhibition of NADH oxidase in the plasma membrane. Acetogenins contained in the stems of *A. cherimola* (Chen et al., 1999) and the seeds of *Annona reticulata* (Chen, Chang, Chiu, Wu, & Wu, 1998) have been isolated and identified, however, antioxidant properties of these

acetogenins have yet to be assessed in order to evaluate their potential for use in dietary supplements.

This project explores the potential health benefits of cherimoya consumption by examining the antioxidant activity of its individual components, including skin, flesh and juice using the oxygen radical absorbance capacity (ORAC) assay. The cherimoya extracts were also incubated with Raji (Burkitt's Lymphoma) cells and HT-29 (colon cancer) cells to measure antioxidant uptake. Incubations with cherimoya juice extract exhibited the highest antioxidant uptake in both cell lines. Cells were stressed by exposure to 2,2′-azobis(2-aminopropane) dihydrochloride (AAPH) and then exposed to cherimoya extracts to measure their antioxidant uptake. Results showed that cells under oxidative stress import more antioxidants when incubated with cherimoya skin, flesh, and juice extract, thus suggesting the potential benefits of consuming antioxidants following exercise.

2. Materials and methods

2.1. Chemicals

2,2′-azobis(2-aminidopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). RPMI 1640 medium was purchased from Hyclone (Logan, UT). Cosmic Calf Serum was purchased from Hyclone (Logan, UT). L-glutamine was obtained from Fisher Scientific (Pittsburgh. PA). Fluorescein-sodium salt was obtained from Sigma-Aldrich, Inc. (Milwaukee, WI). Hank's Balanced Solution was purchased from Hyclone (Logan, UT). Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) was purchased from Enzo Life Sciences, Inc. (Plymouth, PA). Phosphate buffered saline (PBS) was purchased from Sigma-Aldrich, Inc. (Milwaukee, WI).

2.2. Materials

Costar 96- and 24-well clear culture plates were purchased from Corning Inc. (Lowell, MA). Raji cells (Burkitt's Lymphoma cell line) and colon cancer cells (HT-29) were obtained from the American Type Culture Collection (ATCC).

2.3. Equipment

All fluorescence readings were taken using the BMG FLUOstar Optima (BMG Laboratories) microplate reader (serial #413-0225). All data were analyzed using Microsoft Excel and Apple Numbers.

2.4. Burkitt's Lymphoma cell culture

2.4.1. Growth medium
RPMI 1640 Medium was supplemented with 10% Fetal Bovine Serum and 2 mM of L-glutamine. Cells were kept in an incubator with 5% carbon dioxide at 37 °C.

2.4.2. Changing media
Cells were placed in a conical vial and centrifuged at 450 g for 15 min. Spent media was discarded and new RPMI media was introduced. The conical vial containing new media was vortexed and placed in incubation plates. Cell count and viability were determined via trypan blue staining using a hemocytometer. Cell viability was maintained above 90% and cell counts were maintained between 900,000 and 1,100,000 cells/mL.

2.5. HT-29 (colon) cancer cell culture

2.5.1. Growth medium
McCoy Medium was supplemented with 10% Fetal Bovine Serum and 1.5 mM L-glutamine. Cells were kept in an incubator with 5% carbon dioxide at 37 °C.

2.5.2. Changing media
Used media was discarded and cells were washed with 5 mL of Hank's solution. One mL of 0.05% trypsin-EDTA at 37 °C was added to the flask and evenly dispersed over the surface. The flask was placed in the 37 °C incubator and observed until the cells were detached from the walls of the flask. After centrifugation at 450 g for 15 min, the trypsin was discarded, the pellet of cells was resuspended in 5 mL of McCoy media, re-seeded in a flask. The new media introduced was used to rinse the surface of the flask and was pipetted up and down to dissociate cell clumps. A cell count and viability test was performed via trypan blue using a hemocytometer. Cell viability was maintained above 90% and cell counts were maintained between 900,000 and 1,100,000 cells/mL.

2.6. Fruit sample

All the cherimoya fruits were purchased at a local grocery store in Orem, UT, and were stored in a refrigerator at 3 °C.

2.7. Sample preparation

Cherimoya skin and flesh were excised from the fruit and homogenized by weighing 0.4 g of sample and diluting it in 10 mL of distilled water. Juice was extracted through the compression of the cherimoya flesh. Juice was then homogenized to reduce possible interference from fruit constituents, according to recent methodological recommendations (Perez-Jimenez et al., 2008). Samples were centrifuged at 450 g for 15 min, filter-sterilized and kept in a freezer at −20 °C until used.

2.8. Oxygen radical absorbance capacity (ORAC) assay

All samples were analyzed using a modified ORAC assay based on published methods (Garrett, Murray, Robison, & O'Neill, 2010). The ORAC assay measures oxidative degradation of a fluorescent compound (fluorescein sodium salt) after being exposed to an oxygen radical initiator, AAPH (2,2′-azobis(2-aminidopropane) dihydrochloride). All readings were taken in Cole-Parmer black bottom 96-well plates, with 133 μL Fluorescein (0.1 μM), 50 μL AAPH (4 μM), and 13 μL of antioxidant sample or PBS in each well. Readings were taken every 2 min for 1 h, followed by readings every 15 min for 3 h. Measurements were taken by the BMG FLUOstar Microplate reader at 37 °C; 485 nm excitation and 590 nm emission filters were used for fluorescence readings. All fluorescent measurements were expressed relative to the initial reading. The area under the curve (AUC) was calculated by using the difference of area under the fluorescein decay curves between the sample curve and the fluorescein + AAPH curve. The AUC measures the total antioxidant activity in the sample minus the fluorescein + AAPH antioxidant curve.

2.9. Standard curve data

Trolox, a water-soluble analogue of vitamin E, was used as a control standard. Trolox standards were run at concentrations of 50 μM, 25 μM, 12.5 μM, and 6.25 μM to obtain standard curves for each trial. Data from each standard curve were used to convert raw net area under the sample curve (net AUC) values to Trolox Equivalents per liter (TE/L). Subsequent calculations converted TE/L to TE/100 g.

2.10. Antioxidant concentration of samples only

Fifty μL of PBS was inserted in the first and last columns of the 96-well plate and 133 μL of fluorescein was inserted into rows 2–11. The cherimoya skin, flesh and juice extract were placed in a 37 °C water bath for 10 min. 13 μL of skin sample was added to rows 2–4, 13 μL of flesh sample was added to rows 5–7 and 13 μL of juice sample was

added to rows 8–9. Mixtures were pipetted up and down to ensure homogeneity of the mixture. After the fluorescein was measured twice in a two minute interval using the FLUOstar Optima fluorescence microplate reader, 50 µL of AAPH was placed in rows 2–10. Fluorescein levels were recorded in 2 minute intervals for 1 h, followed by 15 minute intervals for 3 h.

2.11. Antioxidant uptake cellular model

One mL of cells was placed in each well of the 24-well plate, and each column of the plate was mixed with 100 µL of cherimoya skin, flesh or juice extract. One mL of cells with 100 µL of PBS served as the control. The incubation plate was placed in the incubator for 20 min. The samples from each well were then placed in labeled eppendorf vials that were centrifuged for 15 min at 450 g. The supernatant was discarded. To perform the wash, 1 mL of sterilized PBS was added to each eppendorf vial and vortexed until the cells were suspended in solution. The eppendorf vials were subjected to a 37 °C water bath for 3 min and then to a liquid nitrogen bath for 3 min. The freeze/thaw process was repeated three times. The eppendorf vials were centrifuged for 30 min at 3000 g. The supernatant (cell lysate) was then placed in a 96-well plate to perform the ORAC assay to determine antioxidant uptake in the cells.

2.12. Simulation of oxidative stress: an alternate cellular model

One mL of cells with 100 µL of a 1:16 dilution of AAPH:sterile PBS was placed in each well of the 24-well plate. The incubation plate was placed in the incubator for 10 min. The cells were then placed in eppendorf vials and centrifuged for 15 min at 450 g. The supernatant was discarded and 1 mL of PBS was added to each eppendorf vial and vortexed so the cells were re-suspended. Vials were again centrifuged for 15 min at 450 g. The supernatant was discarded and 1 mL of cell media was added to the eppendorf vials and vortexed until cells were re-suspended. The cells with the media were placed in each well of the 24-well incubation plate, and an additional 100 µL of cherimoya skin, flesh or juice extract was added. The plate was placed in the incubator for 20 min and same procedure outlined earlier was followed to measure the antioxidant uptake of the cells.

2.13. Cytotoxicity

To eliminate the possibility that the cells were exhibiting higher antioxidant uptake because the cell membrane was being damaged by AAPH exposure, Raji cells, with a cell count between 900,000 and 1,100,000 cells/mL and a viability above 90%, were treated with 1:1, 1:2, 1:4, 1:8 and 1:16 dilutions of AAPH in sterile PBS in a 24-well plate. After being exposed to the different concentrations of AAPH, cells were placed in the incubator for 24 h. The cell count and viability for each well containing the different dilutions of AAPH were determined via trypan blue staining using a hemocytometer. The 1:16 dilution of AAPH sterile PBS maintained cell viability and the cell count had increased, showing that the AAPH treatment had not substantially damaged the cell membrane, as to affect the cell integrity.

3. Results

3.1. Raw cherimoya extract

Antioxidant analyses of several cherimoya skin, flesh and juice extracts yielded the following data: The highest measured antioxidant activity was found in the cherimoya juice at 98,085 TE/100 g (stdev = 12,017, N = 48). The next highest activity was in the cherimoya skin at 74,714 TE/100 g (stdev = 6614, N = 48), and the lowest activity was found in the cherimoya flesh at 6004 TE/100 g

(stdev = 1257, N = 48). Measurements on each extract were repeated four times, with 12 replicates for each sample, giving a total of 48 measurements per sample. Data represent mean values for each.

3.2. Raji incubations

Initial assessment of antioxidant activity in a Raji cell lysate following incubation with PBS revealed a final Trolox Equivalent value of 2147 TE/100 mg sample per 1,100,000 cells (N = 36). All of the following data are reported in TE/100 g sample per 1,100,000 cells. Incubations with cherimoya skin, flesh, and juice extracts revealed higher TE values for each sample when compared to PBS controls (4136 for cherimoya skin, N = 36; 2580 for cherimoya flesh, N = 36; 5830 for cherimoya juice, N = 36). Assessments taken from each sample following pre-exposure to AAPH revealed increased antioxidant activity in each sample, including the PBS control (4097, N = 36). Sample data revealed a mean value of 5895 (N = 36) for the cherimoya skin extract, 6939 (N = 36) for the cherimoya flesh extract, and 7527 (N = 36) for the cherimoya juice extract (see Fig. 1).

3.3. HT-29 incubations

Assessment of HT-29 cell lysate antioxidant activity revealed trends similar to those found in the Raji lysates (Fig. 2). Initial measurement of PBS control-incubated HT-29 cell lysate revealed a mean TE value of 1521 (N = 24). Data for cherimoya-incubated HT-29 samples revealed a mean value of 2904 (N = 24) for the cherimoya skin extract, 1760 (N = 24) for the cherimoya flesh extract, and 5644 (N = 24) for the cherimoya juice extract. Pre-exposure to AAPH resulted in higher antioxidant assessments for each sample, including PBS controls: 7769 (N = 24) for PBS control, 10,343 (N = 24) for cherimoya skin extract, 10,433 (N = 24) for cherimoya flesh extract, and 11,929 (N = 24) for cherimoya juice extract. The same trend observed in the Raji cell model was observed in the HT-29 cells, in that the highest overall values were seen in the juice-incubated cell lysate. In contrast to data for Raji cells, however, the flesh and skin-incubated cell lysates were almost equal, where the values for the skin-incubated cell lysates were larger than the flesh-incubated cell lysate in the Raji model.

4. Discussion

4.1. Analysis of raw cherimoya extracts

The current study's antioxidant analyses of raw cherimoya extracts, to our knowledge, represent the first attempt at such quantification using the ORAC assay, although 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) assay analyses have been previously preformed (Vasco, Ruales, & Kamal-Eldin, 2008). When each individual cherimoya component was analyzed for antioxidant activity, the juice exhibited the highest antioxidant activity while the skin contained the lowest. This may be due to the higher concentrations of vitamins A and C found in the fruit flesh (16.63 µg/100 g of vitamin A and 42.38 mg/100 g of vitamin C) (Amoo et al., 2008). Further analysis and testing of the skin and fruit composition are needed to provide additional insight into the compounds present in each part of the fruit that contribute to their overall antioxidant capacities.

4.2. Incubation analysis

Raji cells and HT-29 cells were exposed to cherimoya skin, flesh and juice extracts and the antioxidant capacity of the cell lysates were measured. Antioxidant values in the cellular models were significantly different from the raw antioxidant extract levels themselves. This is likely due to the dynamic cellular processes that allow antioxidant
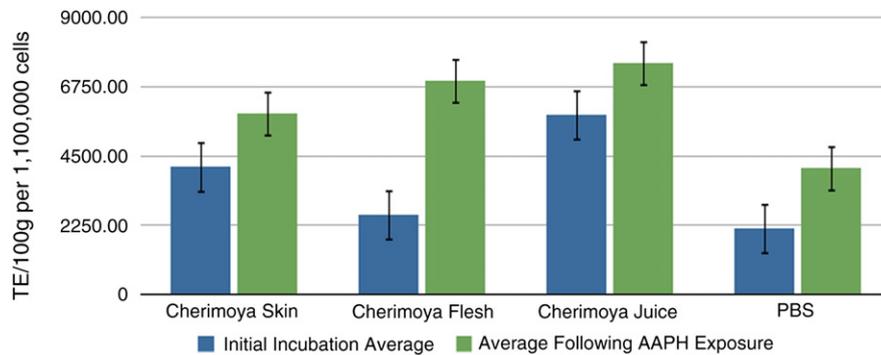
Fig. 1. Changes in antioxidant activity in Raji cell lysates before and after incubation with AAPH. All values were reported as TE/100 g sample per 1,100,000 cells.

compounds from the medium to cross the cellular membrane into the cellular interior. Individual cells are also equipped with their own processes to cope with oxidative stress, resulting in increased antioxidant protection. Both Raji and HT-29 cells exposed to cherimoya juice exhibited higher antioxidant activity in their cell lysates (when compared to those exposed to skin or flesh extract), while cells exposed to cherimoya skin extract had the lowest antioxidant activity of the group. This suggests that the antioxidant compounds in the cherimoya juice are both readily accessible and beneficial to the cells for uptake. By contrast, the cherimoya flesh, for example, either does not contain usable antioxidant compounds or is lacking in antioxidant content.

Student's t-tests performed on data also revealed statistically significant increases in antioxidant capacity in both Raji (one-tailed p = 0.016) and HT-29 (one-tailed p = 0.0006) models. This suggests that both cell lines are equipped to respond to the oxidative stresses with which they are presented, and encourages further study of more broad and diverse cell lines.

Overall, it was observed that HT-29 cells produced a much greater percent increase in their antioxidant activities following exposure to oxidative stress. The average percent increase for all Raji runs was 82.9% (max = 169%, min = 29.1%, stdev = 62.2), while the average percent increase for all HT-29 runs was 318% (max = 493%, min = 111%, stdev = 169). Student's t-test analysis revealed that the difference in HT-29 cellular response compared to that of the Raji cells was statistically significant (p = 0.013). These results suggest that the HT-29 cell line may be more robust in its ability to respond to oxidative stress and react to protect itself against these stresses. Further testing of this and other cell lines may be beneficial in determining common cellular responses in various cell types. Such analyses may also help to elucidate the mechanisms responsible for responding to oxidative stress in varying cell types. It appears from the data mentioned earlier that the cell lines tested have the ability to sense oxidative damage and correspondingly increase their uptake of available antioxidants. Further

research regarding different levels of oxidative stress exposure, as well as experiments with different classes of antioxidants, may prove beneficial in understanding the mechanisms involved in cellular responses to oxidative stress.

From the PBS results (Figs. 1 and 2), it can be seen that cells exposed to oxidants internally increase their antioxidant capacity. However, cells exposed to fruit extracts substantially increase their intracellular antioxidant levels and these increases are higher than the basal antioxidant levels in the cells, indicating that cells have the ability to import available antioxidants when experiencing oxidative stress. It is interesting to note that while the cherimoya juice is the most potent antioxidant under conditions of oxidative stress, there is a much higher percentage increase in antioxidant activity when exposed to skin and flesh. This is significant because it suggests benefits from the consumption of all components of Cherimoya fruit, including skin, flesh and juice.

5. Conclusion

The first objective of this research was to analyze the antioxidant capacities of the *A. cherimola* fruit, specifically examining its skin, flesh, and juice. Initial analyses suggested that *A. cherimola* contains high antioxidative properties, and that the highest antioxidative potential is found first in the plant's juice, and next in its skin. Further chemical analyses of *A. cherimola* are needed to more fully understand its chemical composition and the antioxidant compounds it contains. Future research may also include studying the plant's stem, leaves, and flowers for antioxidant activity and chemical composition. Regular consumption of *A. cherimola* may contribute to increased antioxidant capacity of human serum, and may thereby help to prevent the development and progression of cardiovascular disease, neurodegenerative diseases, cancer, and other diseases thought to be linked to oxidative stress. Further studies are needed to add insight into the potential role of *A. cherimola* in the prevention of these
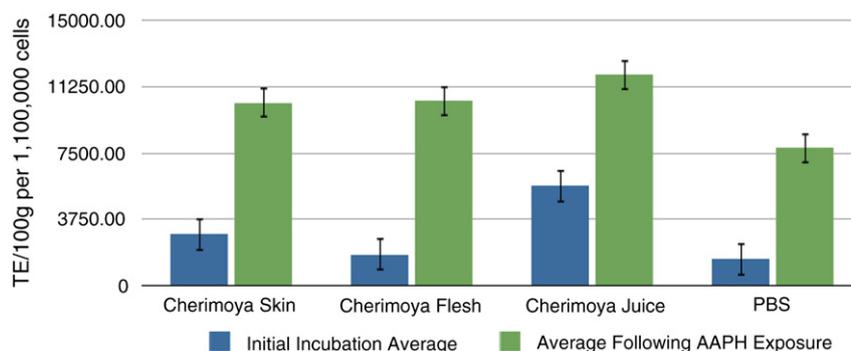

Fig. 2. Changes in antioxidant activity in HT-29 cell lysates before and after incubation with AAPH. All values were reported as TE/100 g sample per 1,100,000 cells.

conditions. Additional studies are needed to further elucidate the intracellular mechanisms that may be contributing to the increased antioxidant capacity in these and other cell lines. Further study may also provide insights on the effects of changes of baseline oxidative stress in humans and how these changes affect tolerance to oxidative stress in future stressful environments. Additional insights may be gained into the effects of exercise on oxidative stress and the body's ability to protect its tissues during future stressful events. These insights may also aid in the future study of the development and progression of oxidative stress-induced degenerative conditions, and how oxidative-antioxidant equilibria may increase prevention against such conditions.

As a second focus of this paper, the effects of pre-exposure to oxidative stress on cellular antioxidant uptake were examined, using *A. cherimola* extracts as the source of antioxidants. The data indicate that pre-exposure to oxidative stress may contribute to increased antioxidant uptake in both Raji and HT-29 cell lines. In both cell lines, cell lysate antioxidant capacity was significantly higher when cells were exposed to oxidative stress, suggesting that the presence of oxidative stress contributed to the uptake of antioxidants as part of a cellular response mechanism. These data correlate with similar studies on the effect of pre-exposure to inflammation-inducing environments on tissues (Schmid-Schonbein, 2006), and suggest that the mechanisms that influence these responses should be studied further on the cellular level.

Results show that while cherimoya juice is a potent antioxidant, flesh and skin consumption, along with the juice, will allow a higher antioxidant uptake when cells are placed under oxidative stress. Therefore, when consuming cherimoya, it is beneficial to include all parts of the fruit. Further research may shed light on the use of *A. cherimola* as a useful dietary supplement.

References

Amoo, I. A., Emenike, A. E., & Akpambang, V. O. E. (2008). Compositional evaluation of *Annona cherimoya* (custard apple) fruit. *Trends in Applied Sciences Research*, *2*, 216–220.

Bonavia, D., Ochoa, C., Tovar, O., & Palomino, R. (2004). Archaeological evidence of cherimoya (*Annona cherimolia* mill.) and guanabana (*Annona muricata* L.) in ancient Peru. *Economic Botany*, *58*, 509–522.
Chen, C., Chang, F., Chiu, H., Wu, M., & Wu, Y. (1998). Acetogenins from seeds of *Annona reticulata*. *Phytochemistry*, *47*, 1057–1061.
Chen, C., Chang, F., Chiu, H., Wu, M., & Wu, Y. (1999). Aromin-a, an Annonaceous acetogenin from *Annona cherimola*. *Phytochemistry*, *51*, 177–182.
Garcia, K., Zepeda-Vallejo, L., Gallegos, E., Gonzales, I., & Madrigal, L. (2008). Genotoxic and cytotoxic effects produced by acetogenins obtained from *Annona cherimola*. *Biological and Pharmaceutical Bulletin*, *31*, 2346–2349.
Garcia-Alonso, J., Ros, G., & Periago, M. (2006). Antiproliferative and cytoprotective activities of a phenolic-rich juice in HepG2 cells. *Food Research International*, *39*, 982–991.
Garrett, A., Murray, B., Robison, R., & O'Neill, K. (2010). Measuring antioxidant capacity using the ORAC and TOSC assays. In D. Armstrong (Ed.), *Advanced protocols in oxidative stress II* (pp. 251–262). New York: Humana Press.
O'Neill, K., Standage, S., Hughes, B., & Murray, B. (2001). Fruits and vegetables and the prevention of oxidative DNA damage. *Vegetables, Fruits, and Herbs in Health Promotion* (pp. 135–146).
Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J., & Deemer, E. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *Journal of Agricultural and Food Chemistry*, *50*, 3122–3128.
Panda, S., Kar, A., & Patil, S. (2009). Soy sterols in the regulation of thyroid functions, glucose homeostasis and hepatic lipid peroxidation in mice. *Food Research International*, *42*, 1087–1092.
Perez-Jimenez, J., Arranz, S., Tabernero, M., Diaz-Rubio, M., Serrano, J., Goñi, I., & Saura-Calixto, F. (2008). Updated methodology to determine antioxidant capacity in plant foods, oils and beverages: Extraction, measurement and expression of results. *Food Research International*, *41*, 274–285.
Santos Lima, L., Pimenta, L., & Boaventura, M. (2010). Acetogenins from *Annona cornifolia* and their antioxidant capacity. *Food Chemistry*, *122*, 1129–1138.
Schmid-Schonbein, G. (2006). Analysis of inflammation. *Annual Review of Biomedical Engineering*, *8*, 93–151.
Tribble, D., & Frank, E. (1994). Dietary antioxidants, cancer, and atherosclerotic heart disease. *The Western Journal of Medicine*, *161*, 605–612.
Vasco, C., Ruales, J., & Kamal-Eldin, A. (2008). Total phenolic compounds and antioxidant capacities of major fruits from Ecuador. *Food Chemistry*, *111*, 816–823.
Xu, J., Kochanek, K., Murphy, S., & Tejada-Vera, B. (2010). *Deaths: Final data for 2007*. National Vital Statistics Reports, 19, 1–136. Hyattsville, MD: National Center for Health Statistics.

Appendix II

<u>Role of Oxidative Stress in Prostate Cancer.</u>

The role of oxidative stress in prostate cancer
Gaytri Gupta-Elera, Andrew R. Garrett, Richard A. Robison and Kim L. O'Neill

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are not only byproducts of normal cellular metabolism, but also play important roles in cell signaling. However, when the levels of ROS and RNS increase, cells are exposed to oxidative stresses, which activate a variety of mechanisms to allow them to cope with these changes. Studies have shown that oxidative stress conditions play an important role in both the initiation and the progression of prostate cancer by regulating molecules such as DNA, enhancers, transcription factors, and cell cycle regulators. Other studies have shown that antioxidants, molecules that protect cells against oxidative stress, play a role in prevention of prostate cancer. This review summarizes the effects of oxidative stress on the development of prostate cancer and explores the potential of ROS regulators as

Department of Microbiology and Molecular Biology, Brigham Young University, Provo, Utah, USA

Correspondence to Kim L. O'Neill, PhD, 855 WIDB, Brigham Young University, Provo, UT 84602, USA
Tel: +801 422 2449; fax: +801 422 0519;
e-mail: kim_oneill@byu.edu

Introduction

Prostate cancer is the most common type of cancer in men and the second leading cause of cancer death in men after lung cancer (Jemal *et al.*, 2010). The prostate is a male gland located below the bladder that begins to develop before birth due to the presence of androgens. The function of the prostate is to store and secrete an alkaline fluid, which makes up approximately one-third of the volume of semen. This fluid contributes significantly to both the viability and motility of the sperm during the fertilization process. On average, one man in six is diagnosed with prostate cancer and one in 36 dies from it. The American Cancer Society estimates approximately 217 730 new cases of prostate cancer and 32 050 resulting deaths in 2010 (Society, 2010). Statistical data suggest that prostate cancer can be very aggressive. This, coupled with its high incidence, makes further study of prostate cancer necessary.

Although prostate cancer is relatively common, there are genetic and environmental risk factors that increase the probability of developing prostate cancer. Prostate cancer is usually an age-correlated disease, meaning the chances of developing prostate cancer increase significantly with age. Approximately two of three cases of prostate cancer are found in men over 65 years of age (Society, 2010). Race also contributes to prostate cancer mortality: there is a higher incidence and death rate from prostate cancer among African-American men. In contrast, prostate cancer is less common in the Asian-American and Hispanic populations. Although the reasons for these trends are unknown (Fedewa *et al.*, 2010), diet may play a significant role. Besides ethnicity and aging, prostate cancer can also be influenced by smoking, hormone

profiles, a diet high in saturated fats, and family predisposition (Rohrmann *et al.*, 2007; Cheung *et al.*, 2008; Heymach *et al.*, 2010), thus suggesting the importance of lifestyle in reducing the risk of prostate cancer.

Prostate disorders

Although prostate cancer is the most destructive type of prostate disease, there are other diseases of the prostate, including benign prostatic hyperplasia (BPH) and prostatitis.

Benign prostatic hyperplasia

BPH, also known as adenofibromyomatous hyperplasia, refers to the enlargement of the prostate that often occurs in middle-aged men. It is one of the most common types of prostate disease, and is present in more than 60% of men aged between 50 and 60 years (Miner *et al.*, 2006). BPH is characterized by the hyperplasia of prostatic stromal and epithelial cells, which leads to the formation of nodules that compress the urethral canal. This compression leads to an obstructed urethra, which interferes with urine flow. Studies have shown that when BPH cells are subjected to mild oxidative stress by being treated with low concentrations of hydrogen peroxide (H_2O_2), their proliferation rate increases. The treatment of these cells with vitamin E and selenium reverses the proliferation, thus suggesting a possible role of antioxidants in BPH prevention (Lonn *et al.*, 2005).

Prostatitis

Although BPH is the most common prostate disease in men over 50 years, prostatitis is the most common disease in men aged less than 50 years of age (Potts and

Pasqualotto, 2003). Prostatitis refers to the inflammation of the tissue in the prostate and is divided into four categories: (a) acute bacterial prostatitis, a bacterial infection of the prostate; (b) chronic bacterial prostatitis, a rare condition characterized by frequent urinary tract infections; (c) chronic prostatitis/chronic pelvic pain syndrome, which accounts for 90–95% of prostatitis diagnoses; and (d) asymptomatic inflammatory prostatitis, characterized by leukocytosis and infection-fighting cells in the semen. These cells in the semen are a rich source of reactive oxygen species (ROS) that may lead to oxidative stress conditions, contributing to sperm damage and infertility.

Prostate cancer malignancy

The most aggressive type of prostate disease is prostate cancer. It is classified as an adenocarcinoma and is most common in the peripheral zone. More than 40% of men diagnosed with prostate cancer experience tumor metastasis, which is the spread of the cancer to sites such as the bones, lymph nodes, rectum, and bladder (Society, 2010). A condition known as prostatic intraepithelial neoplasia (PIN) is believed to be a precursor of prostate cancer. PIN is characterized by prostatic epithelial cells that are similar, both phenotypically and genotypically, to those of cancer, as opposed to normal prostatic epithelial cells (Bostwick, 2000). The ideal time to detect and treat prostate cancer is before it has invaded other parts of the body.

Prostate cancer detection

Although late stage prostate cancer incidence can be reduced with early detection, prostate cancer diagnostic methods are limited in both specificity and sensitivity. Early diagnosis methods include prostate-specific antigen (PSA) blood testing, a digital rectal examination, and tissue biopsy. PSA is a protein made in the prostate, and most healthy men have a PSA level less than 4 ng/ml in the blood. If PSA levels are between 4 and 10 ng/ml, the chance of prostate cancer is approximately 25%, and if the PSA levels exceed 10 ng/ml, the chance of prostate cancer is more than 50% (Borgermann *et al.*, 2010). PSA, however, is not specific to prostate cancer, as PSA levels often rise due to age and prostatitis. Factors that can cause PSA levels to decrease, even when the patient has prostate cancer, include obesity and some medicines used to treat BPH and urinary symptoms. The digital rectal examination is a physical rectal examination, in which the physician feels for irregularities. This form of diagnosis is less effective than PSA blood testing and centrally located tumors are easily missed. Tissue biopsy is the most accurate; however, its limitations include its invasiveness and sampling errors that can lead to false diagnoses. Biopsy is the only detection method for PIN, as serum PSA is not usually elevated and PIN cannot be detected by ultrasound (Bostwick, 2000). Novel detection methods with an increased sensitivity and specificity

are needed, which will require a better understanding of the cellular and molecular mechanisms involved in the development of prostate cancer.

Oxidative stress and its effects on early carcinogenesis

The effect of oxidative stress on prostate cancer has been extensively studied in order to better understand the development of prostate cancer malignancy. Oxidative stress occurs when the production of ROS exceeds antioxidant capacity, leading to oxidative damage. ROS can be produced from both endogenous and exogenous sources. The body naturally produces ROS through different mechanisms, such as oxygen reacting with metal ions in the body and through reactions in the mitochondrial electron transport chain that reduce O_2 to H_2O. The body can also be exposed to ROS exogenously through cigarette smoking and chemical exposure. UV light and other radiation can also lead to increases in ROS in the body. These partially reduced forms of oxygen, such as superoxide and H_2O_2, are produced during aerobic metabolism and have the potential to cause oxidative injury to macromolecules such as nucleic acids, proteins, and lipids through radical chain reactions.

Oxidative damage to nucleic acids such as DNA is an early event in carcinogenesis. $2'$-$4'$-oxidative DNA damage leads to the formation of DNA–protein crosslinks, alterations in the carbohydrate–phosphate backbone, or direct modification of purine or pyrimidine bases. If these alterations in the DNA lead to the activation of oncogenesis or the inactivation of tumor suppressor genes, cancer may develop.

There are other factors that exist within the cell that can lead to the generation of oxidative stress and, therefore, prostate cancer. Androgens, such as testosterone, are hormones involved in the development of male organ such as the prostate. Androgens, although necessary for prostate development, can also lead to BPH and prostate cancer if BPH overgrowth occurs. Recent research has examined the role that androgens play in androgen-induced oxidative stress in androgen-sensitive human prostate adenocarcinoma (LNCaP) cells. A study showed that androgen receptor-induced ROS production is related to increased levels of JunD, a protein belonging to the activator protein 1 family of transcription factors that has been shown to inhibit cell proliferation and promote cell differentiation (Church *et al.*, 2005). A subsequent study used cell constructs that overexpressed and underexpressed JunD to examine the effects on LNCaP cells. Results showed that JunD overexpression led to an increase in ROS production in a low-androgen environment and that JunD was a necessary mediator of androgen-induced oxidative stress (Mehraein-Ghomi *et al.*, 2008). Another study showed that androgens induce the production of spermidine N1-acetyltransferase in a

polyamine catabolic pathway, in which JunD directly binds to a specific spermidine N1-acetyltransferase sequence, and leads to an increase in ROS levels, thus showing that androgen receptors need JunD as a coactivator to activate an oxidative stress generation pathway in LNCaP cells (Mehraein-Ghomi *et al.*, 2010). This illustrates how ROS, although obligate byproducts of cell metabolism, can be harmful when unregulated.

Oxidative stress and its effects on senescence and prostate cancer

Although ROS play a role in the initiation of prostate cancer either by directly affecting nuclear DNA or affecting transcription factors, they also create mutations in mitochondrial DNA (mtDNA), which in turn are believed to promote aging. Aging, also known as senescence, is characterized by a lessened ability to respond to stress stimuli and a progressive decline in body functions at both cellular and tissue level. Many theories have been devised to explain biological senescence, one of them being Harman's 'free radical theory of aging', which suggests that cells subjected to oxidative stress have damage to biomolecules that leads to aging (Harman, 2006). With age, an accumulation of somatic mutations in mtDNA causes deficiencies in oxidative phosphorylation and the electron transport chain, which in turn cause both increased production of ROS and their leakage into the cytoplasm. The accumulation of mtDNA somatic mutations seems to be an indicator of human age-related disorders, including prostate cancer, for which the highest incidence occurs between 50 and 75 years of age (Jemal *et al.*, 2010).

Nitrosative stress and its effect on prostate cancer

Increasing evidence suggests that reactive nitrogen species (RNS) also play a major role in aging and, therefore, age-related diseases such as prostate cancer (Drew, 2002). When the levels of RNS such as nitric oxide (NO) and its byproducts such as nitrate (NO_3^-), nitrite (NO_2^-), and peroxynitrite ($ONOO^-$) are increased, the cell is subjected to what is known as nitrosative stress. Studies have shown that levels of RNS are increased in prostate cancer tissues, thus suggesting a potential role in the development of prostate cancer. A clinical trial performed in Turkey and Macedonia concluded that the plasma of patients with prostate cancer contained higher levels of nitrate, nitrite, and cyclic guanosine monophosphate, an indicator of NO metabolism, than that of controls and patients with BPH (Arsova-Sarafinovska *et al.*, 2009).

The family of enzymes responsible for NO biosynthesis is known as NO synthases (NOS). These enzymes are involved in the production of neuronal NOS (NOS1), calcium-independent inducible NOS (NOS2 or iNOS), and calcium-dependent endothelial NOS (NOS3). Stu-

dies have shown that NOS2 is increased in prostate cancer cells (Uotila *et al.*, 2001). In addition, NOS3 has been suggested to protect prostate cancer cells from apoptosis by reducing their sensitivity to tumor necrosis factor-related apoptosis-inducing ligand, an anticancer agent that induces apoptosis in most tumor cells without affecting normal cells (Tong and Li, 2004).

Interestingly, iNOS has been related to both stimulatory and suppressive effects on the growth of cancer cells. One study examined its role as a potential therapeutic target for both the early and late stages of prostate cancer (Coulter *et al.*, 2010). Results showed that iNOS-induced cytotoxicity in both androgen-dependent and androgen-independent prostate cancer cell lines, and NO production, had a positive correlation with an increased cytotoxicity. Tumor growth delay was also observed in iNOS-expressing cells, thus suggesting a potential role in prostate cancer therapy.

Cell cycle and prostate cancer progression

Other targets that are being examined for their therapeutic potential besides NO species are molecules involved in cell cycle checkpoints, as an uncontrolled cell growth due to defects in the cell cycle helps in propagating the development of prostate cancer. One such molecule, cdk2ap1, is an S-phase growth suppressor known to induce apoptosis and to decrease cell proliferation in squamous cell carcinomas (Zolochevska and Figueiredo, 2009). A study showed cdk2ap1 expression correlated with a decrease in cell growth due to cell cycle arrest, apoptosis, and a decrease in the aggressive phenotype of prostate cancer (Ishikawa *et al.*, 2008).

Molecules with the ability to induce cell cycle arrest have become important in the development of therapeutic drugs that target both the early and late stages of cancer. Many chemokines and cytokines are suspected to play a role in the terminal stage of prostate cancer known as androgen-independent prostate cancer, where the tumor no longer depends on hormones for growth and is resistant to therapy. One such chemokine is interleukin-8 (IL-8). A recent study investigated the role of IL-8 in the malignant progression of androgen-independent prostate cancer by transfecting cells with IL-8 small interfering RNA and comparing them with controls. They concluded that IL-8 reduction led to cell cycle arrest at the G_1/S boundary and a decrease in cell cycle-regulated proteins cyclin D1 and cyclin B1 (Singh and Lokeshwar, 2009). A 43% increase in apoptosis was also observed in IL-8-depleted cells due to an increase in the activation of caspase 9, a protein involved in the apoptotic pathway, and a decrease in antiapoptotic proteins such as BCL-2. The biological changes these prostate cancer cells experienced made them more susceptible to the cytotoxic activity of chemotherapeutic drugs such as docetaxel, staurosporine, and rapamycin.

Two other chemopreventive agents that have therapeutic potential for prostate cancer are selenite and genistein. Although selenite induces apoptosis only, genistein induces both apoptosis and G_2/M cell cycle arrest. When these two compounds are combined, their effects are enhanced and p53, a cell cycle regulator and tumor suppressor protein, is increased in LNCaP cells (Zhao et al., 2009). These two agents may serve as potential ingredients in anticancer drugs and help slow down the aggressive growth of prostate cancer during its latter stages.

Cell cycle regulators have promising potential as therapeutic targets, as they can slow down the aggressive phenotype of cells by making cancer cells more susceptible to cytotoxic agents and chemotherapeutic treatment, and can also arrest the cell cycle and, therefore, prevent the uncontrolled development of cancer.

Oxidative stress and its effect on apoptosis and prostate cancer progression

An alternative way to prevent the progression of cancer is through cell death. The two major mechanisms for cell death are apoptosis, an organized, regulated process in which the cell programs its own death, and necrosis, a disorganized process in which the cell swells, disintegrates, and exudes cell contents that lead to inflammation. Decreased apoptosis plays a major role in tumor progression, as failure of apoptosis leads to continual cell growth and proliferation.

Studies have shown that the concentration of ROS in the cell helps to determine which mechanism of death the cell will undergo. Exposure of cancer cells to ROS depletes the cells of their antioxidant capacity, and when ROS levels exceed a certain threshold, the apoptotic pathway is triggered (Kong et al., 2000). Depletion of antioxidant defenses leads to the activation of stress-responsive transcription factors responsible for the activation of genes involved in apoptosis. A recent study showed how the increase in ROS leads to the activation of the Jun N-terminal kinases/stress-activated protein kinase signaling pathway, which is involved in both the activation of genes and the posttranslational modifications of proteins involved in apoptosis (Haddad, 2004). Other studies have demonstrated that H_2O_2 activates caspase, a protein involved in the apoptotic pathway, and that this is dependent on the release of cytochrome c from the mitochondria, thus suggesting the importance of H_2O_2 in mitochondrial permeability (Burkitt et al., 2005).

Although certain concentrations of ROS trigger apoptosis, higher levels of ROS have also been shown to cause necrosis in lymphoid cells (Villena et al., 2008) and liver cancer cells (Lu et al., 2010). The effect of ROS in necrosis of prostate cancer cells has yet to be examined. Two possible mechanisms have been proposed to explain how cell death through apoptosis can be switched to necrosis. The first mechanism involves the inactivation of caspase proteins due to the oxidation of their active thiol group or S-nitrosylation, which can lead to cell death by necrosis (Melino et al., 1997). The second mechanism that could lead to a switch from apoptosis to necrosis is the depletion of cellular ATP levels due to the failure of energy production in the mitochondria caused by high levels of oxidants (Miyoshi et al., 2006).

Although many studies have shown free radical species as mediators of apoptosis, it has become more apparent that different levels of ROS can have different effects on cellular mechanisms. One study explored the effects of varying concentrations of H_2O_2 on the proliferation of PC-3 prostate carcinoma cells, which lack functional p53, a tumor suppressor protein (Bataller and Portugal, 2005). Results showed that in the presence of $50\,\mu mol/l$ of H_2O_2, cell cycle arrest was observed in the G_2 phase, along with p53-independent apoptosis. When the H_2O_2 concentration was increased to $500\,\mu mol/l$, an increased percentage of apoptosis was observed, suggesting that DNA damage by ROS can have adverse effects on the cell and is far more complex than was initially thought.

Hypoxia and its effect on prostate cancer progression

Another condition that aids in prostate cancer progression and metastasis is hypoxia, which is characterized by a low oxygen environment. Tumor cell growth is dependent on a constant supply of oxygen and nutrients; however, when the tumor mass reaches a critical size, the vasculature becomes inadequate to sustain additional growth. When cells are exposed to hypoxic conditions, tumor cells release angiogenic growth factors that aid in the development of necessary vasculature to maintain the progression of the tumor (Cheema et al., 2010). Hypoxic conditions cause the ROS levels to increase within the tumor, thus contributing to the malignant phenotype of prostate cancer and its resistance to radiation therapy (Anastasiadis et al., 2003).

Hypoxia-inducible factor-1a (HIF-1a) is used as a marker of hypoxia, and several studies have explored the role of hypoxia in tumor growth and progression. It has been shown that HIF-1a is overexpressed in primary prostate cancers (Zhong et al., 1999) and in LNCaP cells in vitro (Ghafar et al., 2003). Other studies have suggested that HIF-1a upregulation occurs in the early stages of carcinogenesis (Zhong et al., 2004), and it is believed to help induce angiogenesis through the release of signals that bind to rapamycin (mammalian target of rapamycin) and thus activate a series of pathways (Vaupel, 2004). In contrast, results of another study showed that systemic hypoxia aided in tumor growth in athymic mice that were xenografted with LNCaP cells regardless of HIF-1a expression levels, thus suggesting the existence of

HIF-1a-independent hypoxia-inducing pathways (Terraneo *et al.*, 2010). Hypoxic conditions contribute to the aggressive phenotype in prostate cancer cell lines by turning on cell survival pathways that make tumor cells unresponsive to many therapeutic treatments.

Endogenous antioxidants and their effect on oxidative stress and prostate cancer

Although many commercial companies are investigating therapeutics that may reduce concentrations of ROS in the cell, the body has its own defense mechanisms to prevent and combat oxidative stress. Antioxidants, molecules that inhibit oxidation, are known to protect the cell against oxidative stress conditions by preventing oxidant-induced damage. The antioxidant defense system involves enzymes such as those of the glutathione redox system and enhancer elements such as antioxidant response elements (ARE) that are activated in response to high levels of ROS.

Glutathione redox system

The body maintains the antioxidant/oxidant balance using the glutathione redox system, which involves enzymes such as glutathione-s-transferases (GST) and glutathione peroxidase (GPx). GST are a group of enzymes that detoxify activated metabolites of procarcinogens produced by phase I reactions, and also determine the cell sensitivity to different toxic chemicals (Hayes and Pulford, 2003). One study found that genetic polymorphisms that altered the detoxifying activity of GSTs affected the levels of procarcinogenic metabolites (Nock *et al.*, 2009). It specifically found that certain types of GST, GSTTI null, and GSTP1, increased the risk of prostate cancer biochemical recurrence in African-Americans but not in Caucasians. Although explanations for these results remain undetermined, the data still suggest the potential of GSTs as therapeutic targets for prostate cancer.

GPx is another enzyme that is being investigated for its therapeutic potential, as it acts as an electron donor to peroxyl radicals, thus reducing oxidative stress within the cell and thereby potentially helping in the prevention of the development of prostate cancer. It is located in the cytoplasm of prostate epithelial cells, and its depletion has been associated with prostate cancer metastasis (Aydin *et al.*, 2009). One study revealed that mice with mutations that suppressed GPx activity in the prostate gland developed prostate cancer (Ouyang *et al.*, 2005). In another study, it was observed that increasing the expression of GPx in prostate cancer cells suppressed tumor growth both *in vitro* and *in vivo* by downregulating the expression of c-met, a tyrosine receptor tumor-transforming gene and proto-oncogene that encodes for a protein called hepatocyte growth factor receptor (Bottaro *et al.*, 1991).

Another study found similar results, where the expression of GPx was inactivated in prostate cancers, and deletions of the GPx gene were observed in 39% of the prostate cancer samples studied (Yu *et al.*, 2007). It also showed that GPx suppressed colony formation, and when cells expressing GPx were xenografted into mouse models, they showed a reduced tumor volume and inability to metastasize. All these studies suggest that GPx is a tumor suppressor gene with therapeutic potential.

In addition to measuring levels of GPx, one study measured the levels of additional antioxidant enzymes such as catalase and copper zinc superoxide dismutase (Arsova-Sarafinovska *et al.*, 2009). This clinical trial, which was performed to explore the prooxidant/antioxidant balance in patients with prostate cancer in Turkey and Macedonia, showed a decrease in GPx and copper zinc superoxide dismutase in patients with prostate cancer when compared with controls and patients with BPH, thus suggesting the importance of these antioxidant enzymes in limiting the progression of prostate growth. Another study confirmed these results by reporting that GST activity was significantly decreased in patients with prostate cancer (Surapaneni and Venkata, 2006), whereas others have observed an increase in ROS production in prostate cancer cells compared with normal prostate cells (Kumar *et al.*, 2008).

Antioxidant response elements (AREs)

Another defense mechanism that the body uses to combat ROS is the activation of transcription factors that bind to the AREs. These sequences are found in promoter regions of antioxidant genes, and when activated, lead to the transcription of these genes (Jain *et al.*, 2010). AREs are regulated by a transcription factor called erythroid-derived 2-related factor 2 that plays a role in regulating ROS levels by regulating antioxidants such as NADPH, quinone oxidoreductase, and heme oxygenase. A study using transgenic adenocarcinoma of the prostate mice showed that the loss of erythroid-derived 2-related factor 2 led to elevated ROS levels and DNA damage associated with tumorigenesis in the prostate (Frohlich *et al.*, 2008). This suggests the potential of these regulatory components as therapeutic targets, as they are responsible for maintaining the antioxidant/oxidant balance that plays a major role in regulating the development of prostate cancer malignancy.

Dietary antioxidants and their effect on oxidative stress and prostate cancer

One of the predispositions that leads to the development of prostate cancer is diet, which is why many studies have emphasized the importance of certain vitamins in decreasing prostate cancer risk. Dietary antioxidants such as vitamin D, carotenoids, and lycopene aid in the body's defense against oxidation events.

Role of vitamin D in prostate cancer risk

Vitamin D is involved in the synthesis of NO synthase and in the increased levels of glutathione. In a case study of 19 000 men with prostate cancer, those with levels of

25(OH)D, a vitamin D metabolite, below 16 ng/ml had a 70% higher incidence rate of prostate cancer than those with higher levels of this metabolite. In addition, the incidence of prostate cancer for younger men was 3.5 times higher if their levels were below 16 ng/ml (Ahonen *et al.*, 2000). This shows that vitamin D administration may have significant effects on the initiation of colon and prostate cancer. A study that examined the levels of vitamin D receptors (VDR) of 841 patients with prostate cancer showed that men with tumors that had a VDR upregulation had a lower risk of lethal prostate cancer (Hendrickson *et al.*, 2010). There was also a positive correlation observed between VDR expression and androgen/estrogen receptors, suggesting that VDR acts in an androgen-dependent or estrogen-dependent manner, thus emphasizing the potential role of vitamin D in preventing the progression of prostate cancer.

Role of carotenoids in prostate cancer risk
Another important class of antioxidant compounds in dietary supplements are carotenoids, which are naturally occurring pigments found in plants that are known to be powerful antioxidants. In one case–control study, carotenoid plasma levels of 118 non-Hispanic Caucasian men suffering from nonmetastatic prostate cancer were compared with those of 52 healthy men in Southeast Texas (Chang *et al.*, 2005). The results showed that the cancer risk for men with high levels of α-carotene, trans-β-carotene, β-cryptoxanthin, lutein, and zeaxanthin in their plasma was less than half that of those with low levels of these compounds. When cancer aggressiveness was examined in these patients, no correlation was found between carotenoid plasma levels and aggressiveness of disease. This study suggests that high plasma levels of carotenoids may help reduce prostate cancer risk, but not its progression.

Role of lycopene in prostate cancer risk
A compound that has been suggested to reduce prostate cancer risk and its progression is lycopene. Lycopene is a compound mainly found in tomato products, and it exerts its antioxidant effects through the scavenging of free radicals and induction of apoptosis. Lycopene has been found to be associated with a lower prostate cancer risk in a number of studies. Studies conducted in 1995 and 2004 supported the claim that lycopene intake through tomato products resulted in a decreased risk of prostate cancer (Giovannucci *et al.*, 1995; Wu *et al.*, 2004), whereas another study did not find this inverse correlation (Kirsh *et al.*, 2006).

In another case–control study, it was shown that a daily intake of commercial spaghetti sauce containing 30 mg of lycopene in 200 g of sauce, 3 weeks before a radical prostatectomy, resulted in decreased oxidative damage in prostate cells compared with controls, and an increased apoptosis in prostate cancer cells (Kim *et al.*, 2003).

Similarly, another study demonstrated that the combination of lycopene and vitamin E inhibited the progression of prostate cancer and extended survival in the ortho-tropic model of prostate cancer in nude mice (Trion *et al.*, 2007). In a different study, transgenic adenocarcinoma of the prostate mice were fed with 10% whole tomato versus a tomato-free diet to observe its effects on prostate cancer tumor incidence and overall survival rate (Pannellini *et al.*, 2010). Results showed that the tomato-rich diet increased the overall survival rate and delayed the progression of prostatic intraepithelial neoplasia to adenocarcinoma. Lycopene, therefore, is an antioxidant that warrant further study. Overall results have shown that the presence of antioxidants, whether found in the body naturally or increased by dietary supplements, have antitumor and chemopreventive properties, and may therefore be useful in preventing or delaying the initiation of prostate cancer.

Conclusion
Prostate cancer is significant in terms of both morbidity and mortality. Increased research on its prevention and treatment is sorely needed. Two major conditions that contribute to the malignant phenotype of prostate cancer are when oxygen is lacking or when reactive forms of oxygen or nitrogen are prevalent. The lack of oxygen creates a hypoxic microenvironment, which leads to the initiation of angiogenesis, which leads to a more aggressive phenotype, whereas the generation of ROS contributes to that phenotype through the activation of oncogenes.

The body has several defenses to fight ROS, including antioxidants that are either produced by the body or acquired through diet. Research has shown that an antioxidant-rich diet aids in the delay of prostate cancer progression and helps reduce prostate cancer risk. By ingesting an antioxidant-rich diet, the probability of prostate cancer is reduced.

Prostate cancer prevention, although important, is not the complete answer to this problem. More research should be focused on finding novel therapeutic targets for future prostate cancer drugs and treatments. Prostate cancer diagnosis and prognosis techniques also require further study. Although much knowledge has been gained relative to different aspects of prostate cancer progression, the more we understand about the molecular mechanisms that operate in prostate cancer cells, the easier it will be to find novel methods to treat this disease.

References
Ahonen MH, Tenkanen L, Teppo L, Hakama M, Tuohimaa P (2000). Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland). *Cancer Causes Control* **11**:847–852.

Anastasiadis AG, Bemis DL, Stisser BC, Salomon L, Ghafar MA, Buttyan R (2003). Tumor cell hypoxia and the hypoxia-response signaling system as a target for prostate cancer therapy. *Current Drug Targets* **4**:191–196.

Arsova-Sarafinovska Z, Eken A, Matevska N, Erdem O, Sayal A, Savaser A, *et al.* (2009). Increased oxidative/nitrosative stress and decreased antioxidant enzyme activities in prostate cancer. *Clin Biochem* **42**:1228–1235.

Aydin A, Eken A, Erdem O, Arsova-Sarafinovska Z, Sayal A, Dimovski A, *et al.* (2009). Glutathione peroxidase 1 (GPX1) genetic polymorphism, erythrocyte GPX activity, and prostate cancer risk. *Toxicol Lett* **189**:S132–S132.

Bataller M, Portugal J (2005). Apoptosis and cell recovery in response to oxidative stress in p53-deficient prostate carcinoma cells. *Arch Biochem Biophys* **437**:151–158.

Borgermann C, vom Dorp F, Breuer G, Kliner S, Rubben H (2010). Early detection of prostate cancer: is serum PSA testing alone sufficient? *Urologe A* **49**:1351–1355.

Bostwick D (2000). Prostatic intraepithelial neoplasia. *Current Urology Reports* **1**:65–70.

Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, *et al.* (1991). Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science (New York, NY)* **251**:802.

Burkitt MJ, Lawrence A, Jones CM, Wardman P (2005). Activation of cytochrome c to a peroxidase compound I-type intermediate by H_2O_2: relevance to redox signalling in apoptosis. *Free Radical Res* **39**:S16–S16.

Chang S, Erdman JW, Clinton SK, Vadiveloo M, Strom SS, Yamamura Y, *et al.* (2005). Relationship between plasma carotenoids and prostate cancer. *Nutrition and Cancer* **53**:127.

Cheema U, Alekseeva T, Abou-Neel EA, Brown RA (2010). Switching off angiogenic signalling: creating channelled constructs for adequate oxygen delivery in tissue engeneered constructs. *Eur Cells Materials* **20**:274–281.

Cheung E, Wadhera P, Dorff T, Pinski J (2008). Diet and prostate cancer risk reduction. *Exp Rev Anticancer Therap* **8**:43–50.

Church DR, Lee E, Thompson TA, Basu HS, Ripple MO, Ariazi EA, *et al.* (2005). Induction of AP-1 activity by androgen activation of the androgen receptor in LNCaP human prostate carcinoma cells. *The Prostate* **63**:155–168.

Coulter JA, Page NL, Worthington J, Robson T, Hirst DG, McCarthy HO (2010). Transcriptional regulation of inducible nitric oxide synthase gene therapy: targeting early stage and advanced prostate cancer. *J Gene Med* **12**:755–765.

Drew BL (2002). Aging and the role of reactive nitrogen species. *Annals of the New York Academy of Sciences* **959**:66–81.

Fedewa SA, Etzioni R, Flanders WD, Jemal A, Ward EM (2010). Association of insurance and race/ethnicity with disease severity among men diagnosed with prostate cancer, National Cancer Database 2004–2006. *Cancer Epidemiol Biomark Prevent* **19**:2437–2444.

Frohlich DA, McCabe MT, Arnold RS, Day ML (2008). The role of Nrf2 in increased reactive oxygen species and DNA damage in prostate tumorigenesis. *Oncogene* **27**:4353–4362.

Ghafar MA, Anastasiadis AG, Chen MW, Burchardt M, Olsson LE, Xie H, *et al.* (2003). Acute hypoxia increases the aggressive characteristics and survival properties of prostate cancer cells. *Prostate* **54**:58–67.

Giovannucci E, Ascherio A, Rimm EB, Stampfer MJ, Colditz GA, Willett WC (1995). Intake of carotenoids and retinol in relation to risk of prostate cancer. *J Natl Cancer Inst* **87**:1767–1776.

Haddad JJ (2004). Redox and oxidant-mediated regulation of apoptosis signaling pathways: immuno- pharmaco- redox conception of oxidative siege versus cell death commitment. *International Immunopharmacology* **4**:475–493.

Harman D (2006). Free radical theory of aging: an update increasing the functional life span. In: Rattan S, Kristensen P, Clark BFC, editors. *Understanding and modulating aging.* pp. 10–21.

Hayes JD, Pulford DJ (2003). The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol* **30**:445.

Hendrickson WK, Julie L, Flavin R, Fiorentino M, Lis R, Fiore C, Penney K (2010). Vitamin D receptor expression is inversely associated with prostate cancer progression. *Cancer Prev Res* **3 (Suppl 1)**.

Heymach JV, Terrry JS, Tran H, Yoo S-Y, Do K-A, Wergin M, Saintigny P (2010). Low-fat diet reduces NF-{kappa}B regulated inflammatory cytokines and angiogenic factors in plasma of men with prostate cancer. *Cancer Prev Res* **3 (Suppl 2)**.

Ishikawa K, Takenaga K, Akimoto M, Koshikawa N, Yamaguchi A, Imanishi H, *et al.* (2008). ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. *Science (New York, NY)* **320**:661–664.

Jain A, Lamark T, Sjottem E, Larsen KB, Awuh JA, Overvatn A, *et al.* (2010). p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J Biol Chem* **285**:22576–22591.

Jemal A, Siegel R, Xu JQ, Ward E (2010). Cancer statistics, 2010. *Cancer Journal for Clinicians* **60**:277–300.

Kim H-S, Bowen P, Chen L, Duncan C, Ghosh L, Sharifi R, *et al.* (2003). Effects of tomato sauce consumption on apoptotic cell death in prostate benign hyperplasia and carcinoma. *Nutrition and Cancer* **47**:40–47.

Kirsh VA, Mayne ST, Peters U, Chatterjee N, Leitzmann MF, Dixon LB, *et al.* (2006). A prospective study of lycopene and tomato product intake and risk of prostate cancer. *Cancer Epidemiol Biomark Prev* **15**:92–98.

Kong Q, Beel JA, Lillehei KO (2000). A threshold concept for cancer therapy. *Medical Hypotheses* **55**:29–35.

Kumar B, Koul S, Khandrika L, Meacham RB, Koul HK (2008). Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. *Cancer Res* **68**:1777.

Lonn E, Bosch J, Yusuf S, Sheridan P, Pogue J, Arnold JMO, *et al.* (2005). Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *JAMA: the Journal of the American Medical Association* **293**:1338–1347.

Lu CC, Yang JS, Huang AC, Hsia TC, Chou ST, Kuo CL, *et al.* (2010). Chrysophanol induces necrosis through the production of ROS and alteration of ATP levels in J5 human liver cancer cells. *Mol Nut Food Res* **54**:967–976.

Mehraein-Ghomi F, Lee E, Church DR, Thompson TA, Basu HS, Wilding G (2008). JunD mediates androgen-induced oxidative stress in androgen dependent LNCaP human prostate cancer cells. *Prostate* **68**:924–934.

Mehraein-Ghomi FB, Basu HS, Church D, Hoffmann M, Wilding G (2010). Androgen receptor requires JunD as a coactivator to switch on an oxidative stress generation pathway in prostate cancer cells. *Cancer Res* **70**:4560–4568.

Melino G, Bernassola F, Knight RA, Corasaniti MT, Nistico G, Finazzi-Agro A (1997). S-nitrosylation regulates apoptosis. *Nature* **388**:432–433.

Miner M, Rosenberg MT, Perelman MA (2006). Treatment of lower urinary tract symptoms in benign prostatic hyperplasia and its impact on sexual function. *Clinical Therapeutics* **28**:13–25.

Miyoshi N, Oubrahim H, Chock PB, Stadtman ER (2006). Age-dependent cell death and the role of ATP in hydrogen peroxide-induced apoptosis and necrosis. *Proc Nat Acad Sci USA* **103**:1727–1731.

Nock NL, Bock C, Neslund-Dudas C, Beebe-Dimmer J, Rundle A, Tang DL, *et al.* (2009). Polymorphisms in glutathione S-transferase genes increase risk of prostate cancer biochemical recurrence differentially by ethnicity and disease severity. *Cancer Causes Control* **20**:1915–1926.

Ouyang X, DeWeese TL, Nelson WG, Abate-Shen C (2005). Loss-of-function of Nkx3.1 promotes increased oxidative damage in prostate carcinogenesis. *Cancer Res* **65**:6773–6779.

Pannellini TI, Lezzi M, Liberator M (2010). A dietary tomato supplement prevents prostate cancer in TRAMP mice. *Cancer Prev Res* **3**:1284–1291.

Potts JM, Pasqualotto FF (2003). Seminal oxidative stress in patients with chronic prostatitis. *Andrologia* **35**:304–308.

Rohrmann S, Genkinger JM, Burke A, Helzlsouer KJ, Comstock GW, Alberg AJ, *et al.* (2007). Smoking and risk of fatal prostate cancer in a prospective US study. *Urology* **69**:721–725.

Singh RK, Lokeshwar BL (2009). Depletion of intrinsic expression of interleukin-8 in prostate cancer cells causes cell cycle arrest, spontaneous apoptosis and increases the efficacy of chemotherapeutic drugs. *Mol Cancer* **8**:15.

Society American Cancer. 2010 What are the key statistics about prostate cancer? [cited; available from: *http://www.cancer.org/Cancer/Prostate Cancer/DetailedGuide/prostate-cancer-key-statistics*].

Surapaneni KM, Venkata GR (2006). Lipid peroxidation and antioxidant status in patients with carcinoma of prostate. *Indian J Physiol Pharmacol* **50**:350.

Terraneo L, Bianciardi P, Caretti A, Ronchi R, Samaja M (2010). Chronic systemic hypoxia promotes LNCaP prostate cancer growth *in vivo*. *Prostate* **70**:1243–1254.

Tong X, Li H (2004). eNOS protects prostate cancer cells from TRAIL induced apoptosis. *Cancer Lett* **210**:63–71.

Trion A, Van Hengel-Zonruiter D, Reneman S, De Ridder CMA, Schalch W, Van Weerden WM (2007). Combined treatment with lycopene and vitamin E. What is the optimal dose for inhibition of prostate cancer development in nude mice? *BJU Int* **100**:41–42.

Uotila P, Valve E, Martikainen P, Nevalainen M, Nurmi M, Härkönen P (2001). Increased expression of cyclooxygenase-2 and nitric oxide synthase-2 in human prostate cancer. *Urol Res* **29**:25–28.

Vaupel P (2004). Tumor microenvironmental physiology and its implications for radiation oncology. *Semin Radiat Oncol* **14**:198–206.

Villena J, Henriquez M, Torres V, Moraga F, Diaz-Elizondo J, Arredondo C, *et al.* (2008). Ceramide-induced formation of ROS and ATP depletion trigger necrosis in lymphoid cells. *Free Radical Biol Med* **44**: 1146–1160.

Wu K, Erdman JW, Schwartz SJ, Platz EA, Leitzmann M, Clinton SK, *et al.* (2004). Plasma and dietary carotenoids, and the risk of prostate cancer: a nested case-control study. *Cancer Epidemiol, Biomarkers Prev* **13**: 260–269.

Yu YP, Yu GY, Tseng G, Cieply K, Nelson J, Defrances M, *et al.* (2007). Glutathione peroxidase 3, deleted or methylated in prostate cancer, suppresses prostate cancer growth and metastasis. *Cancer Res* **67**: 8043–8050.

Zhao R, Xiang N, Domann FE, Zhong WX (2009). Effects of selenite and genistein on G(2)/M cell cycle arrest and apoptosis in human prostate cancer cells. *Nutr Cancer* **61**:397–407.

Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, *et al.* (1999). Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* **59**:5830–5835.

Zhong H, Semenza GL, Simons JW, De Marzo AM (2004). Up-regulation of hypoxia-inducible factor 1alpha is an early event in prostate carcinogenesis. *Cancer Detect Prev* **28**:88–93.

Zolochevska O, Figueiredo M (2009). Expression of cell cycle regulator cdk2ap1 suppresses tumor cell phenotype by non-cell-autonomous mechanisms. *Oral Oncology* **45**:106–112.