John M. Pezzuto *Editor*

Grapes and Health

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Preface

Considering the current widespread use of traditional natural product remedies, and looking back throughout history at the dominant influence of natural products on drug design and development, there is no question whatsoever that nature produces chemical entities that can be exploited for the benefit of human health and well-being. A few obvious and indisputable examples include antitumor agents, antibiotics, statins, analgesics, anti-inflammatories, and antimalarials. Of course, not all natural products are beneficial, such as highly dangerous substances such as bacterial toxins and ricin, and even more generically, "Poison is in everything, and no thing is without poison. The dosage makes it either a poison or a remedy" (Paracelsus). But from a practical point of view, the diet of human beings has evolved to a point of not containing quantities of materials that lead to chronic or overt toxicity.

In fact, over the past decades, the precise opposite is true. The expectation is not only should the diet be nutritious, sanitary, and safe, but it should actually promote health and prevent disease. Perhaps this was the underlying message of Hippocrates in stating "Let food be thy medicine and medicine be thy food," but it is likely that even he would be flabbergasted to find a Google search yielding 432,000,000 hits in less than 1 s after entering the search term "diet and health." And so, what is the truth of the matter? What does scientific evidence have to offer, and when does common sense come into play?

This book focuses on one particular aspect of these much broader issues: the potential correlation of grapes and health. Of course the diet of an average human being needs to be viewed in a holistic manner. Mainstream science does not advocate *The Grape Cure*, a diet composed solely of grapes, as touted in the book written by Johanna Brandt. But largely spearheaded by the potential health benefits of resveratrol, a dietary stilbene found in the skin of grapes, a significant amount of research has been conducted over the past 15 years or so to investigate the possibility of the entire grape product mediating some beneficial response when included in the diet in a normal serving size. The chapters herein summarize our current state of knowledge with emphasis on a wide range of human health conditions.

As with any field of research, financial support is required to realize any progress. In the case of grape research, the California Table Grape Commission has played an instrumental role in advancing the field. Obviously, the Commission is hopeful that some positive response will be found to be mediated by the grape, but it is important to emphasize the sponsored work is elicited by an open call for proposals by independent investigators, most of whom are affiliated with world-renowned institutions. The proposals are judged for scientific merit by an independent review board comprised of experts in the field, and awards are made purely on the basis of merit. Once the awards are made, the investigators perform the work in a completely independent manner, they are free to publish their results in peer-reviewed scientific journals, and it is hoped they will be competitive in garnering additional extramural support to continue their work. This entire process was designed to avoid any perception of conflict of interest, and the approach has been successful.

In the beginning, following the advice of a team of experts, it was clear that a standardized product would be necessary to assure continuity and consistency of the work over the long haul. Thus, following a chapter by Creasy and Creasy describing grape anatomy, physiology, and overall chemical composition, it is fitting that the next chapter of this book by van Breemen et al. defines the preparation and analysis of a standard grape product. Subsequent chapters describe work that is potentially relevant to a variety of disease states. Many of these disease-specific studies were conducted with standardized grape product, but, importantly, many others were conducted with whole grapes or products derived from grapes (such as grape juice or pomace). Alcoholic beverages, such as wine and grappa, are not a major focal point of this book, although some results are described. The main subject is the grape itself, the consumption of which is relevant to all human beings, young or old.

From a chronological perspective, the influence of grapes on cardiovascular disease has been a key point of interest. Of course, as described in the chapter by Gross, the multifaceted etiology of cardiovascular is well known, and expansive investigations have been performed in areas such as oxidative damage/stress, inflammation, blood lipids and pressure, endothelial function, flow-mediated dilation, coronary calcification, plaque formation, fibrosis, and so on. Clearly, additional work is required, but some promising results have been reported. Notably, as summarized in the next chapter by Fernandez and Barona, there appears to be strong evidence that grape polyphenols exert protective effects against atherosclerosis.

Inflammation appears to play a critical role as an underlying factor in cardiovascular disease, as well as many other maladies. The influence of grapes on this process is addressed in the following chapter by Seymour and Bolling. We learn that the intake of grape products can modify both local and systemic inflammation through mechanisms well beyond direct scavenging of radicals or antioxidant effects. The ramifications of this type of response have far-reaching implications, as exemplified by the subsequent chapters.

Epidemiological evidence has long suggested that relatively high and routine consumption of fruits and vegetables may be associated with disease prevention. In

part this has led to public health campaigns by organizations such as the National Cancer Institute and the American Cancer Society. Grapes, of course, are included among the components that should be included in the healthy diet. As described in the chapter by Holcombe, many studies have been performed to evaluate the potential of grapes or grape constituents to inhibit the development of cancer, and especially intriguing results have been observed in the area Wnt signaling and colon cancer.

At first glance, the predicted response of orally consuming a dietary product such as the grape on a component of the alimentary tract such as the colon may be viewed as relatively straightforward, but the situation is much more complex. As cogently described in the chapter by McIntosh et al., polyphenols may influence nutrient digestion and absorption, and gut microbiota taxa and their fermentation products, in part, because they are poorly absorbed in the upper gastrointestinal tract and thus persist in the colon. But even at the colon, they come into direct contact with microbes, influencing microbial growth and metabolism, as well as undergoing enzymatic modification based on the available microbes. Relatively little is known in a definitive manner about the linkage between intestinal and holistic systems, but, clearly, the extent to which whole grapes alter features such as gut microbiota, inflammatory status, and barrier function may have a profound influence on outcomes.

Studies in the areas of cardiovascular disease and cancer are extremely important but not outside the realm of expectation. The subjects covered in the next few chapters are remarkable. As exemplified by the final collection of studies presented in this book, interest in the health benefits of grape consumption is indeed far-reaching. First, Maher provides a comprehensive overview of the beneficial effects of grape products on brain function. Studies have been performed to investigate a host of parameters relevant to cognitive function, posttraumatic stress disorder (PTSD), stroke, Alzheimer's disease (AD), etc. Various grape products have been found to reduce multiple symptoms of PTSD including anxiety, depression, and memory loss, provide a positive difference in learning and memory, improve both learning and memory in the AD mice, and improve brain function and specifically learning and memory in animals exposed to stress, aging, and disease. Human clinical trials are limited but clearly warranted.

The chapter by Juma et al. describes the effect of grapes on arthritic disease. Osteoarthritis is a complex chronic disease that reflects age-related degeneration of joint tissues in response to mechanical stress or injury. Normal repair and inflammatory responses follow and contribute to a cycle of further stress and damage, leading to inflammation, chronic pain, and impairment of mobility. Since grape constituents are associated with anti-inflammatory, antioxidant, cardioprotective, and chemopreventive properties, the grape is an ideal subject of research for arthritis and joint health. Evidence is presented suggesting benefit for both osteo- and rheumatoid arthritis or other forms of inflammatory arthritis.

Indicative of the breadth of work being conducted with grapes, Levin et al. describe effects on urinary bladder function. Using an in vivo model of ischemia and ischemia followed by reperfusion, it has been demonstrated that

oral ingestion of a grape suspension protects the bladder from the physiological, biochemical, and morphological dysfunctions mediated by ischemia and ischemia followed by reperfusion. These results are important since more than 80 % of males older than 50 years of age have varying degrees of bladder outlet obstruction secondary to benign prostatic hyperplasia (BPH), and incontinence is a common problem in women.

In the final chapter of this book, Bulloj and Finnemann describe recent experimental, clinical, and epidemiological studies that support the beneficial properties of grape components on vision. Cataract, glaucoma, and age-related macular degeneration are frequent causes of blindness worldwide, particularly in the elderly population. Again, this intriguing topic accentuates the versatility of grape action in a manner that is applicable to wide-ranging aspects of human health.

As illustrated by many examples described in this book, the open request for research proposals and the peer review system established by the California Table Grape Commission have been highly successful in facilitating basic and applied research as well as promoting intellectual curiosity. Although numerous investigations have focused on single components found in the grape, it is important to remember the entire grape product contains over 1600 different phytochemicals. Thus, it is especially notable that under conditions that are deemed to be physiologically relevant (e.g., no more than the equivalent of 2–4 servings per day), positive responses are beginning to emerge with applicability to a host of disease states.

Importantly, in studies performed with grape powder, the dosage in each experimental case has been limited to that which can easily be found in the human diet. Thus, the crucial subject matter of this book should not be viewed in the same context as reports describing dietary supplements or megadoses of specific natural products such as resveratrol that could never be achieved through normal dietary consumption. The goal is to focus on studies that are explicitly designed to investigate the potential benefits of normal grape consumption as one part of a normal diet. Since chronic or acute toxicity is not a rational consideration, from a holistic point of view, many of the results described in this book are very promising and thereby encourage further exploration of the potential health benefits of grape consumption.

Brooklyn, NY John M. Pezzuto

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Grape Anatomy and Physiology

Leroy L. Creasy and Min T. Creasy

Contents

Abstract Grapes are vines that are cultivated in temperate climates around the world. There are an estimated 8000 grape varieties grown for consumption. The latest data showed table grape production of about 20 million tons. Vine growth and floral initiation result in the berry formation of the subsequent year. The growth and maturation of the grape berry and/or seeds affect changes in sugars, acids, and phenolic compounds. Pests and diseases are numerous as are the continuous searches for better chemical control. Natural resistance such as the grape plants'

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ability to escape infection through anatomical attributes and the production of phytoalexins such as resveratrol also play important roles in control of diseases. Grapes are a multiuse crop with nutritional and pharmaceutical applications.

1 Introduction

Grapes (Winkler et al. 1974; Creasy and Creasy 2009) are fleshy fruits produced from a single flower, containing one ovary (the definition of a berry), and produced by a woody vine in the genus *Vitis*. Grape berries are produced in clusters of varying numbers of individual berries. Grapes are usually found in temperate climates. *Vitis vinifera* is the major commercial species native to the Middle East but has been introduced all around the world. However, the largest number of species is found in North America (Weaver 1976). *Vitis labrusca* is one North American species; the varieties Concord and Niagara are mainly used to make grape juice. Other North American species, e.g., *V. riparia* and *V. rupestris*, have been crossed with each other and with *V. vinifera* and used as wine, table grapes, or as rootstocks to protect vinifera varieties from phylloxera, a root insect that almost destroyed the European grape industry in the nineteenth century. *Vitis rotundifolia* is a species native to the Southeastern USA. Varieties have been selected for juice and wine production.

2 Uses

Grapes can be eaten raw; canned; used to make wine, jam, juice, raisins, grape seed oil, and sugar for food additives (in foods or juices usually called "all natural" or "no sugar added"); extracted for pigments and fermented/distilled into grape neutral spirits; and sometimes utilized to make fortified wines.

3 Varieties

There are estimates of 8000 grape varieties differing in size, color, shape, flavor, and disease and insect resistance (Winkler et al. 1974). Europe is the region with the largest production of grapes, but China produces the greatest amount followed by Italy, the USA, Spain, and France (www.FAO.STAT3). The classic wine varieties are Cabernet Sauvignon, Chardonnay, Chenin blanc, Merlot, Pinot noir, Riesling, Sauvignon blanc, Semillon, and Syrah.

Major table grape varieties are Sultana (Thompson Seedless), Flame, Muscat, Almeria, and Emperor. World table grape production is about 20 million tons; China is the largest producer at 9 million tons; Turkey is second with almost 2 million tons. The USA produces about 1 million tons (USDA 2015).

4 Seedless Grapes

The modern table grape consumer prefers seedless grapes although some seeded cultivars remain popular, e.g., Red Globe. Black Corinth is the only truly seedless grape (a parthenocarpic fruit that grows without fertilization of the flower). It is occasionally sold as a table grape and has very small berries (frequently called the Champagne grape). The commercially available dried currant is actually a dried Black Corinth grape berry. Other "seedless" grapes abort their embryos soon after fertilization (Pratt 1971). The aborted seeds are called seed remnants and the size and hardness of the remnant are a quality factor in table grapes.

Most raisins are made from the Thompson Seedless variety. California and Turkey are the major raisin producers (www.FAS.USDA.gov/data/raisins-world-markets-and-trade, accessed on 25 Oct 2015).

5 Berry Anatomy

A berry consists of skin, flesh, and seeds or seed remnants. The skin is composed of the epidermis plus variety-dependent layers of thick-walled cells called the hypodermis. Grape epidermis contains fewer stomata than do leaves and therefore berry temperature is poorly regulated. The hypodermis contains most of the skin phenolic compounds. The flesh (mesocarp) is composed of large cells with large vacuoles. The seeds number 0–4 per berry and are made up of a seed coat, endosperm, and embryo. Seedless berries have a seed remnant, resulting from the abortion of the embryo early in the growing season.

6 Chemical Composition

Grape components are easily divided into two groups. Primary components are those associated with basic life processes, those common to all plants. These include water, proteins, amino acids, nucleic acids, cellulose, and many others. Of course the largest component of grapes is water, 75–85 % of their weight. Secondary components have roles in enhancing life processes or protecting the plant from damage. Presumably the biosynthetic pathways to produce these chemicals were mutations of primary metabolism. Some enabled plants to live upright out of water or to withstand dry conditions, avoid damage from light or UV radiation, and ward off attack by grazing animals, insects, or diseases. A mutation may have resulted in a survival advantage perpetuated in the genetics of the plant. There are so many secondary components that we might assume that not all of the challenges that resulted in a survival mechanism still exist. All plants are resistant to most microorganisms (probably billions) and disease is the exception. Grapes

contain thousands of compounds that are classified as secondary components (Pezzuto 2008), and there are probably thousands more as yet unidentified.

7 Grape Berry Chemistry

7.1 Sugars

Grapes contain the highest concentration of sugars of any fresh fruit, 15–25 % of their weight. Accumulation of glucose and fructose in the vacuoles of berry flesh mesocarp cells occurs after veraison (start of ripening). Twenty days after the start of veraison, the hexose content of the berry is close to 1 M, with a glucose/fructose ratio of 1. Sucrose produced through photosynthesis in leaves is the main carbohydrate used for long-distance transport (Swanson and El-Shishiny 1958). Sucrose is loaded into the phloem by either a symplastic (via plasmodesmata) or apoplastic mechanism (Boss and Davies 2001). Because sucrose is the major translocated sugar in grapevine, the rapid accumulation of hexoses during berry ripening must involve the activity of invertases (Fillion et al. 1999). Invertases catalyze the hydrolysis of sucrose transported by the phloem into glucose and fructose. Hydrolysis of sucrose by cell wall invertase may promote unloading by preventing its retrieval by the phloem and by maintaining the sucrose concentration gradient. Both expression and activity of cell wall invertase increase around the onset of ripening and reach a high level in the late stage (Zhang et al. 2006).

7.2 Organic Acids

Rapid changes in the acid/sugar balance occur at the onset of berry ripening (veraison). Tartaric and malic acids generally account for 69–92 % of all organic acids in grape berries and leaves (Kliewer 1966). Minor amounts of citric, succinic, lactic, and acetic acids are also present in ripe grapes. The decrease of total organic acid content that begins at the onset of ripening is associated with a sudden induction of malate oxidation.

7.3 Simple Phenolics

Volatile phenolics, such as benzaldehyde, phenylacetaldehyde, benzyl alcohol, 2-phenylethanol, and vanillin, are found mainly in berry skin and are responsible for the primary aromas that develop during ripening (García et al. 2003). Benzoic

acids with a simple C6–C structure (benzoic protocatechuic and gallic acid) are found in grapes, but they are present at low concentrations (Kennedy et al. 2006).

7.4 Phenylpropanoids

Cinnamic acids (C6–C3) are products of the phenylpropanoid synthesis pathway. Cinnamic acid is produced from phenylalanine by the enzyme phenylalanine ammonia lyase. This is the entry point into phenolic and lignin biosynthesis.

The hydroxycinnamates coutaric acid and caftaric acid are the third most abundant class of soluble phenolics in grape berries, after tannins and anthocyanins. They are present in hypodermal cells along with tannins and anthocyanins and in the mesocarp and placental cells of the pulp. On a per berry basis, total hydroxycinnamates in mesocarp tissues peak prior to veraison and then decline, leading to a constant amount (per berry) as the fruit ripens. Genetics is apparently more important than exposure or climate (Clifford 2000). Singleton et al. (1986) showed that the level of hydroxycinnamates in the juice of different vinifera varieties is highly variable, ranging from 16 to 430 mg/L.

Several reviews summarize the advances on the understanding of phenolic structures and diversity and enlighten the synthesis and distribution of these compounds in grape berry tissue types (Kennedy et al. 2006; Adams 2006).

7.5 Flavonoids

Flavonoids (C3–C6–C3) are formed by the condensation of 4-hydroxycinnamoyl-CoA with 3 malonyl-CoAs controlled by the enzyme chalcone synthase (Fig. 1) (Rupprich and Kindl 1978).

The berry skin contains tannins and pigments, the pulp contains juice but usually no pigments, and the seeds contain tannins (Fig. 2). From a biological perspective, the insoluble cutin of the epidermis and the insoluble lignin of the hard seed coat are phenolics, which are as important as the skin tannins and pigments and seed tannins.

Flavonoids make up a significant portion of the phenolic material in grapes and include several classes, such as proanthocyanidins (tannins), anthocyanins, flavan-3-ol monomers, and flavonols. Flavonols and anthocyanins occur as glycosides in grape skins. Tannins or proanthocyanidins are polymers of flavan-3-ols and are the most abundant class of soluble polyphenolics in grape berries. Tannins confer astringency to fruits and are found in the hypodermal layers of the skin and the soft parenchyma of the seed between the cuticle and the hard seed coat. They are a

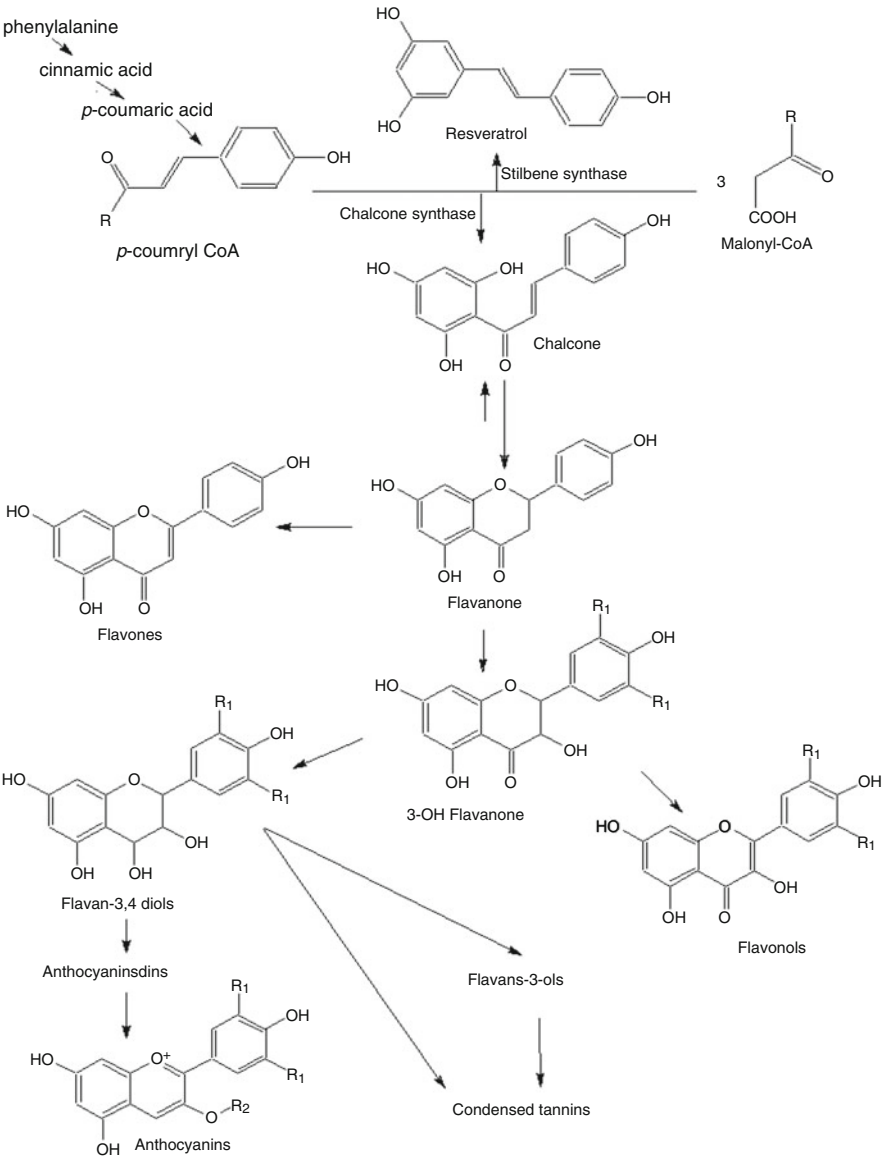


Fig. 1 Biosynthesis of grape resveratrol and flavonoids

very diverse set of biomolecules varying in size from dimers and trimers up to oligomers with more than 30 subunits (Kennedy et al. 2006; Adams 2006).

Table 1 shows the content of major phenolics in seeded and seedless grape berry skins, flesh, and seeds or seed remnants. Skin resveratrol is not constant in grapes so the values are transitory. In seeds and remnants, resveratrol is possibly correlated

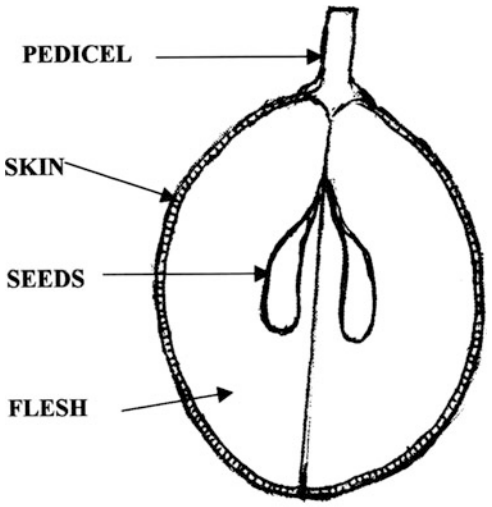


Fig. 2 Mature grape berry

Table 1 Phenolic components of grape berries

	% of berry weight	Resveratrol (μmol/kg)	Flavans (μmol/g)	Total phenol (mg/g)
Globe = 9.5 g/bry				
Skin	7.6	0.16	22	1.6
Seeds	1.4	3.4	238	8.4
Flesh	90.9	0	0	0.2
Flame = 5.0 g/bry				
Skin	15.9	1.25	19	1.6
Remnants	5.2	0.79	11	1.2
Flesh	78.8	0	0	1.1

Resveratrol analyzed by HPLC, flavans by vanillin reaction, and total phenols by Folin reaction

with the degree of woodiness of the seed coat like the constitutive concentration in grape wood. No resveratrol was found in the grape flesh samples. Flavans are principally flavan-3-ols (catechins) and flavan-3,4-diols (leucoanthocyanidins). They are major components of seeds and not in remnants. The total phenols are largely hydroxycinnamic acids plus flavans and occur in all three parts of the berry including seed remnants. Remnants are chemically not seeds but not flesh either.

Grape anthocyanins and flavonols are found mainly in the skin as assorted glycosides; major ones are shown in Fig. 3. Many others occur in small amounts.

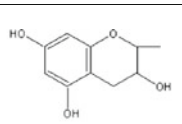
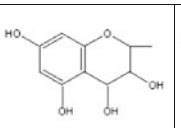
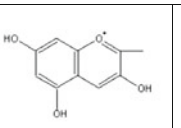
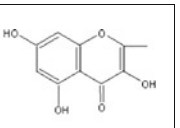
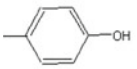
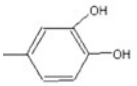
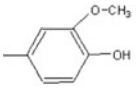
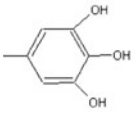
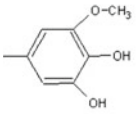
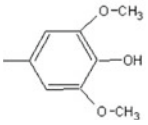
A ring and heterocyclic ring > B ring V	 Flavan-3-ols	 Flavan-3,4-diols	 Anthocyanins	 Flavonols
				Kaempferol-3-glucoside
	Catechin, Epicatechin	Leucocyanidin (as dimers and polymers terminated with a catechin)	Cyanidin-3-glucoside	Quercetin −3-glucoside
			Peonidin-3-glucoside	Isorhamnetin −3-glucoside
	Gallocatechin, Epigallocatechin		Delphinidin-3-glucoside	Myricetin-3-glucoside
			Petunidin-3-glucoside	
			Malvidin-3-glucoside	

Fig. 3 Names of major grape flavonoids

8 Flower Formation

Grape flowers are formed the year before bloom in the axils of leaves. By winter, the buds contain all of next year's flower parts in the axils of next year's shoots. Cool weather and hormonal restrictions assure that the buds will remain dormant until the next growing season. Growth begins with favorable weather and the enlarging shoots produce clusters of flowers. The flowers are self-fertile and not showy. The fruit starts growth immediately after fertilization (Pratt 1971).

9 Berry Growth

Berry growth follows a double sigmoid curve. Following flower fertilization, there is a stage of rapid cell division and the increase in berry size is largely due to an increase in cell number. This is followed by a period of slow berry growth and finally a third stage initiating rapid berry growth due to cell enlargement accompanied by ripening (veraison) (Pratt 1971).

10 Phytoalexins

Most grape component concentrations are controlled by variety, growth stage, or weather with modest variation provided by light exposure (Price et al. 1995) or cultural practices.

The major exception is the phytoalexin resveratrol. By definition, a phytoalexin is a small-molecular-weight antimicrobial substance not normally accumulated by a plant but induced by an interaction with a microorganism. Resveratrol is a phytoalexin of grape leaves (Langcake and Pryce 1976) and berries (Creasy and Coffee 1988). Resveratrol is also a major component of grape woody tissues (700 μg/g, about 3000 μmol/kg) where it may function to suppress disease as a constitutive component (Langcake and Pryce 1976).

11 Grape Disease Resistance

There are numerous and inevitable challenges in the production of a marketable product in large-scale cultivation of grapes. Even in an established vineyard with proven crop yield potential, control of pests is an ongoing process. Since the 1800s, grape diseases have historically resulted in economical losses for the industry (Pearson and Goheen 1988). The control measures used were toxic compounds such as copper, lime, and sulfur which also served as protectants and by changing cultivars to ones with natural resistance. Since that time, attention has focused to understanding of the etiology of the disease organism and the mechanisms of the plants' own defenses.

Studying the biology of the disease organisms, notably fungi, resulted in improved cultural practices for the plants (Pearson and Goheen 1988). Grapes, being vines, are amenable to manipulations for regulating size and shape. Training systems include the ornamental arbors to the refined systems to accommodate production requirements. Support structures such as posts and wires can train the vines to maximize sun exposure, allow air circulation within the canopy, and facilitate mechanization of cultural and harvesting procedures. Grafting to a disease-resistant rootstock is common practice to avoid injury and infection or to

promote growth and fruiting. Understanding the growth, development, and reproductive parameters of the pathogens facilitated the development of a multitude of fungicides that meet the Environmental Protection Agency (EPA) registry parameters. Low mammalian toxicity, environmental friendliness, and target organism specificity have gained importance in the consideration of new pesticide introduction. Except for physical protectants such as clay coating of the plant tissue, chemical control has faced increasing challenges. Over the past decade, more weed, insect, and microorganisms have developed resistance to pesticides, thus limiting the application of existing pesticides, resulting in a vigorous and continuous search for new chemistries and formulations for control. Recommendations for pesticide applications change rapidly as the race of chemical control against the pest's abilities to escape injury surges on.

11.1 Pests and Diseases of Grapes

The development of pest-resistant cultivars through plant improvement and enhancing the plant's own defense mechanisms became more relevant and critical as a solution to disease suppression or avoidance. There are many economically significant pests in grape culture such as the root aphid phylloxera, sharpshooter aphids transmitting Pierce's disease, mealybugs transmitting leaf roll virus, and the bacterium *Agrobacterium tumefaciens* producing crown gall, but fungal pathogens receive more attention due to the availability of chemical control. Rating of commercial grape cultivars for their resistance to various fungal diseases (Weigle and Muza 2015) can be one of the tools in helping the grower with planting choices.

Several fungi causing diseases in grape production are economically significant, especially in humid climates. Of those, two pathogens require special attention since they affect berry development and can possibly result in total crop failure, thus requiring stringent vigilance and control throughout the production cycle. Managing air circulation within the canopy through training systems and removing leaves to allow sun exposure are some of the cultural means to combat the infections.

Powdery mildew is caused by the fungus *Uncinula necator*. The fungus attacks green, living tissue and establishes on the epidermis of the plant. The white spores give the affected tissue a powdery appearance. The spores are disseminated by rain, wind, or mechanical means. Severe infections reduce photosynthesis and debilitate the plant and its subsequent ability to produce fruit. When fruit is infected, it is discolored and distorted and has a bitter taste, which is not desirable for processing or the fresh market. Botrytis bunch rot, caused by the organism *Botrytis cinerea*, is a major disease of grape berries before and after harvest. This organism attacks many plant species and is present in living as well as dead tissue. The conidia (spores) can be disseminated by wind, water, as well as mechanical means, and the plant tissue attacking hyphae can grow under humid conditions in a wide range of temperatures.

The morphology of the epidermal tissue of the leaves and berries is the first line of defense against hyphal intrusion. The epicuticular waxy layer and/or the presence of trichomes can prevent the accumulation of water and the adhesion of the conidia, rendering the conditions not conducive for fungal growth. However, breaks in the epidermis of the plant will allow the establishment of the spores and result in hyphal penetration of the skin. Fungal colonization of the berries liquefies the cells and renders the fruit useless for processing or for the fresh market, and postharvest fungicidal treatment for fresh market grapes has regulatory mandates. The resiliency of the berry skin against cracking and the amount of room on the rachis to allow for berry expansion can prevent cracks in the berry skin. Therefore, berry anatomy and cluster architecture are also characteristics to consider in disease resistance. Control of animal and insect feeding is essential for preventing damage to the berries. New production health standards such as Good Agricultural Practices (GAPs) certification regulate human and animal contact to produce sold for human consumption. Postharvest handling practices have strived to minimize physical injury to the skins and prevent infection during storage and transit.

11.2 Resveratrol

Antifungal compounds, notably phytoalexins, are produced by the grape plants to counteract the establishment of fungal colonies on and inside the plant tissues (Langcake and Pryce 1976).

Resveratrol, a stilbene, was reported in medicinal plants as early as the 1930s (Takaoka 1940). In the compendium of traditional Chinese herbs and their uses *A Barefoot Doctor's Manual* (The Revolutionary Health Committee of Hunan Province 1977), poultices of the leaves and roots of *Polygonum cuspidatum* were used to treat inflammation and to improve blood flow. Later studies of this plant showed it to be a rich source of resveratrol. Biochemists in Japan and Korea isolated and studied the structure of resveratrol and its mode of action (Kimura et al. 1983). Resveratrol in wood was extensively studied by Hillis and his group (Hart and Hillis 1974) as benefiting the lumber industry. Until the onset of DNA fingerprinting, stilbenes, including resveratrol, were used for chemotaxonomy of plant species and cultivars (Hathaway 1962).

In 1976, Langcake and Pryce (1976) found a major antifungal component of grape woody tissue and described it to be a phytoalexin in leaves, the structure of which is identical to the resveratrol studied in the 1930s by Takaoka (1940). Resveratrol was utilized in leaves as a marker for identifying resistance potential of grape cultivars.

Resveratrol was found to be rapidly produced in berry skins by Creasy and Coffee (1988). This discovery was not only important in the postharvest quality of the fruit but also resulted in a new look at disease resistance of detached fruit tissue. In 1992, this study of resveratrol in berries led to the discovery of berry skin resveratrol in wines (Siemann and Creasy 1992) and links to the medicinal results

of Asian studies (Arichi et al. 1982). This was eventually followed by biological investigations (Jang et al. 1997) resulting in a cascade of research efforts and a supercharged wine industry and made resveratrol a household name (Better Homes and Gardens 1992).

Interest in the presence of resveratrol in all fruits flourished. Value-added grape products, raisins, and grape juices were also analyzed (Creasy and Creasy 1998). Although resveratrol was found to be unstable in light (*cis–trans*), it is resistant to heat evidenced by its presence in heat-processed grape products. Raisin analysis revealed the affect of drying method on resveratrol content; sunlight isomerization is a factor. Extensive analysis of the production of Concord grape juice showed that resveratrol content was unchanged after heat extraction of the berries (Creasy, unpublished). Grape seed extracts have been used for human consumption and pomace from the pressing of wine grapes as animal feeds. Analyses of these products reflect that the original content of resveratrol and the handling and processing procedures affect the concentration of resveratrol in the products.

Field studies of resveratrol production showed that this phytoalexin can be induced in berries by fungal attack as well as by fungicide sprays.

Resveratrol synthesis does not continue after the disease event. Excess resveratrol is subject to turn over, thus protecting the cell from toxic concentrations (Langcake and Pryce 1976).

Other defense mechanisms by plant tissues exist, but none sparked the interest or has been as studied as completely as resveratrol (Park and Pezzuto 2015).

11.3 Transgenic Crops

New studies in genomics may assist in the revelation of the mechanisms of the plant cell vs. diseases interactions. Insertion of the patented gene for resveratrol synthesis into non-grape plant cultivars has already taken place (Hain et al. 1992). Genetically modified field crops have changed the productivity and profitability for the commodities crop as well as the pharmaceutical/pesticide industry. Incidents of resistance of weeds and insects to pesticides have become more prevalent, resulting in the need for new cultural and chemical strategies for production. However, fruit and vegetables will face more stringent government scrutiny and consumer resistance. In the USA thus far, only sweet corn and squash have received approval and are sold commercially. Transgenic tomatoes and potatoes made only a fleeting appearance on the market (Dias and Ortiz 2012). The endgame between genetically modified organism (GMO) labeling and successful promotion for consumer acceptance of transgenic produce may not be in the near horizon as evidenced by the upsurge of consumer products touting no GMOs. Growers of perennial crops such as grapes with several years of lag time between planting and harvesting of a marketable crop may need to wait to invest until the game plays out.

One characteristic of phytoalexins is that they are synthesized very rapidly following interaction with microorganisms only at the site of stimulation (Dercks

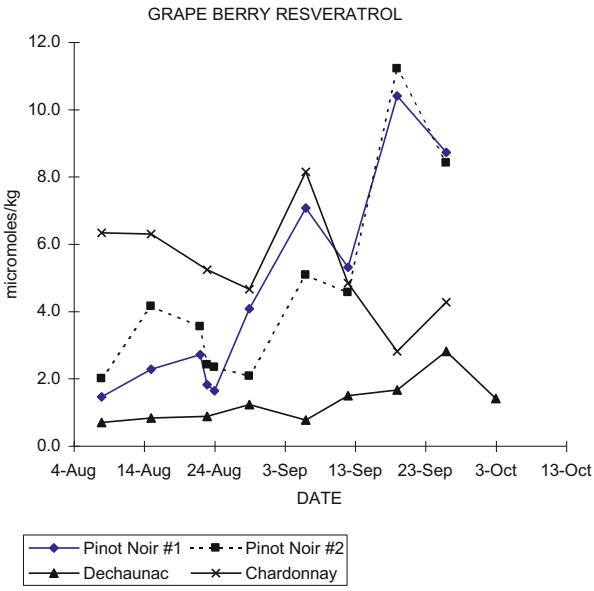


Fig. 4 Whole berry resveratrol content in the vineyard

and Creasy 1989). Resveratrol is toxic to grape cells at the localized concentration and breaks down rapidly. Therefore, the amount of resveratrol in a grape berry at any time is ephemeral. Grape analyses through a season in the vineyard show peaks of production but nothing constant (Fig. 4).

The time of harvest is the major factor in harvested grape resveratrol concentration. Since there is a reasonably frequent challenge of berries by microorganisms, there are always some berries with appreciable concentrations.

12 Stimulation of Resveratrol Production in Berries

There have been attempts to stimulate resveratrol production in berries. Stimulation must occur close to harvest. UV irradiation is the most common since it induces many cells at a time. Microorganisms or their extracts also work but have practical limitations. Experiments irradiating grapes in the vineyard with UV stimulated resveratrol production but only for a limited number of irradiation cycles (Creasy, unpublished). Pesticides have been applied near harvest with occasional success. The superior oil used for powdery mildew control showed promise although the stimulated concentrations in resveratrol only followed rain events (Fig. 5) (Creasy and Creasy, unpublished). Rain fell Sept. 3–4, Sept. 8–9, Sept. 11–12, Sept. 14–15, and Sept. 21–24. The rain dates correlated with increases in berry resveratrol. Oil sprays are legal in many states up to the day of harvest, but in subsequent years of experiments with no rain, there was no stimulation in resveratrol concentration. We

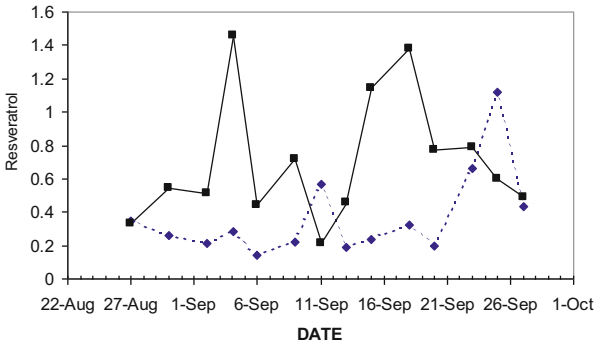


Fig. 5 Berry resveratrol content in the vineyard. *Dotted line* is control (sprayed with water on the 27th of August); *solid line* was sprayed with 1.5 % superior horticultural oil (JMS Stylet Oil) on the same day

did not detect the reported reduction in sugar accumulation due to the oil sprays (Baudoin et al. 2006).

13 Conclusions

Grapes are a multiuse horticultural crop with challenging requirements in disease control. The unique chemical composition of the fruits provides many avenues of potential use in nutritional and pharmaceutical applications.

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Standardized Grape Powder for Basic and Clinical Research

Richard B. van Breemen, Brian Wright, Yongchao Li, Daniel Nosal, and Tristesse Burton

Contents

Abstract To facilitate basic, preclinical, and clinical research regarding the health benefits of grapes, a freeze-dried grape powder has been developed that blends seeded and unseeded varieties of green, red, and blue–black California table grapes in proportions representative of an entire annual crop. Using good manufacturing practice, ~500 kg has been produced from a single vintage and analyzed for content and nutritional value. Chemical standardization is carried out that includes quantitative analysis of the major polyphenols and antioxidants catechin and epicatechin; the flavonols quercetin, kaempferol, and isorhamnetin; the anthocyanidins malvidin, peonidin, and cyanidin; and the chemoprevention agent resveratrol. For human clinical trials, a placebo powder has also been developed that is similar in caloric value, color, texture, and flavor. For animal studies, a sugar-matched diet is employed as the control. The powders can be blended with food or mixed with water and consumed as a beverage. As a result, long-term animal feeding studies or clinical trials can be carried out to assess the health benefits of grapes using a stable, reproducible, and chemically and nutritionally standardized freeze-dried grape product with an appropriate placebo powder as a control.

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1 Introduction

Fresh grapes contain ~82 % water, 12–18 % sugars, and 0.2–0.8 % organic acids, mainly tartaric acid and malic acid. Grapes also contain numerous antioxidant phenolic compounds, including phenols, cinnamic acids, stilbenes, flavonoids, flavans, flavonols, and anthocyanins (Yilmaz and Toledo 2004). The major phenolic compound in grapes is the flavonol catechin (Fig. 1), and related compounds include epicatechin, gallocatechin, and epigallocatechin. Grapes are good sources of flavonols, especially quercetin, isorhamnetin, and kaempferol (Fig. 1). Red and black grapes contain high amounts of the anthocyanins, which are glucosides of anthocyanidins that impart red and purple grape pigmentation to grape skins. Grape anthocyanidins include cyanidin, malvidin, and peonidin (Fig. 1). Also occurring in

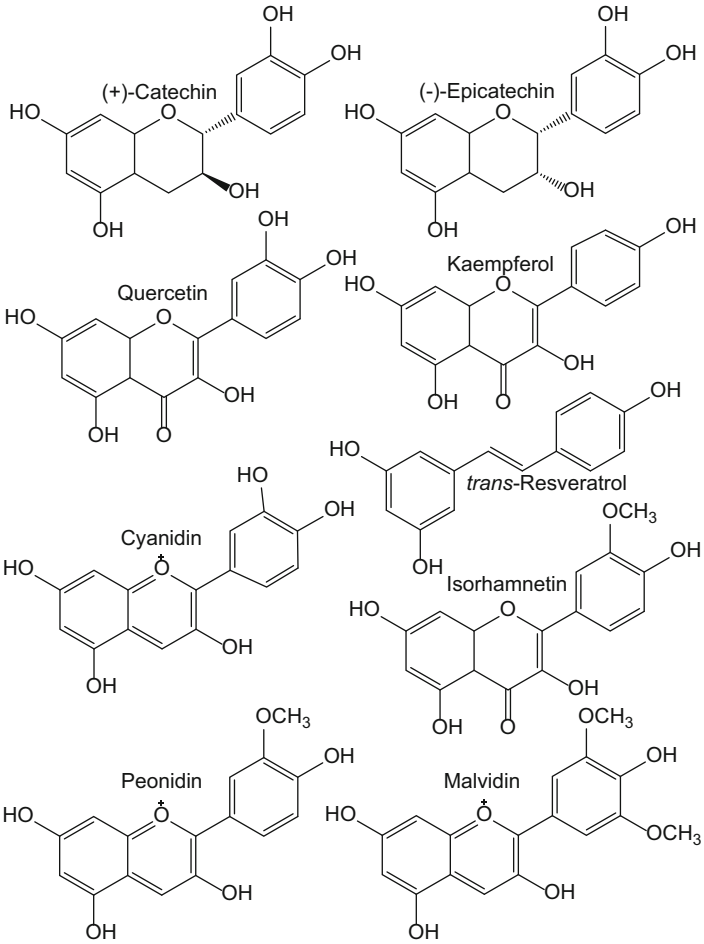

Fig. 1 Chemical structures of some major phenolic constituents of grapes

grape skins is the stilbene resveratrol (Pezzuto 2008), which has been linked to a wide variety of health benefits including cancer prevention (Park and Pezzuto 2015).

Table grapes include a wide range of varietals with colors from light to dark, in green, red and dark purple/blue-black hues. Although some varietals contain seeds, seedless table grapes are also available. For assessing the heath benefits of grapes, it must be borne in mind that the phenolic constituents and antioxidant properties of grapes vary by varietal (Kedage et al. 2007). This issue is further complicated by the seasonal nature of grapes, which limits the availability of each grape varietal throughout the year. These variables can impact the reproducibility of biomedical research with grapes and restrict the ability to carry out long-term in vivo laboratory studies and clinical studies of the health benefits of grapes.

Since 1999, the California Table Grape Commission Research has sponsored research on the health benefits of grapes (California Table Grape Commission Research Grant Program 2016). In response to the need for a uniform and reproducible source of grapes for scientific study, the Commission provides researchers with freeze-dried grape powder (Xu et al. 2009). Produced in bulk and frozen in small portions until needed, freeze-dried grape powder provides a reproducible source of material for both laboratory-based biomedical research as well as clinical studies. For clinical trials, a placebo powder is also available.

2 Preparation and Nutrient Analysis of Freeze-Dried Grape Powder

Freeze-dried grape powder is produced from fresh red, green, and blue–black seeded and seedless table grapes (*Vitis vinifera* L.) that are blended in proportion to their annual production in California. Using good manufacturing practices for food products, several tons of grapes are frozen, ground with food-quality dry ice, freeze-dried, and reground (National Food Laboratory; Livermore, CA). Silicon dioxide is added as a flow conditioner/anticaking agent during processing of the grape powder.

To ensure that the grape powder is safe for human consumption, the finished product is tested for microbial content by contract research laboratories using standard procedures. To preserve as much of the polyphenols and nutrients as possible, no pasteurization or irradiation is used, and as evident in the microbiological testing results shown in Table 1, no sterilization has been required. Because the powder is hygroscopic, it must be stored in moisture-proof containers. Storage at $\leq -70\ ^{\circ}C$ helps preserve the polyphenols as well as prevents microbial growth.

Fresh grapes are ~81.8 % water, while freeze-dried grape powder contains ~1 % water. Note that a standard serving size of fresh grapes is 125 g (~3/4 cup) which corresponds to 22.8 g of freeze-dried grape powder. For clinical studies, freeze-dried grape powder (or placebo powder, see next section) is typically mixed with

Table 1 Microbiological analysis of freeze-dried California grape powder (2011 crop)

Microorganism	Result	Units
Aerobic plate count	270	CFU/g
Anaerobic plate count	120	CFU/g
Total coliforms	<3	MPN
E. coli	<10	CFU/g
Clostridium sp.	<10	CFU/g
Listeria	Negative/25 g	±
Salmonella	Negative/25 g	±
Staphylococcus aureus	<10	CFU/g

Table 2 Nutrient analysis of freeze-dried table grape powder (2011 crop) (Source California Table Grape Commission 2016)

Nutrient	Amount (per 100 g powder)	Units
Calories	371	kcals
Total fat, acid hydrolysis	0.299	g
Total carbohydrate	88.6	g
Protein ($N \times 6.25$)	3.58	g
β-Carotene	0.127	mg
Vitamin A from carotene	212	IU
Vitamin C	2.7	mg
Calcium	50	mg
Iron	1.43	mg
Sodium	11.8	mg
Potassium	973	mg
Thiamine HCl	0.17	mg
Folic acid	49.0	mcg
Phosphorus	104	mg
Magnesium	33.3	mg
Zinc	0.416	mg
Copper	0.450	mg
Manganese	0.379	mg
Moisture	4.52	g
Ash	3.02	g

water for drinking (Zern et al. 2005), while animal studies usually mix grape powder [or an equivalent amount of glucose/fructose (1/1)] with animal feed (Hohman and Weaver 2015). Although the dosage may vary depending on the study design, the typical amount of grape powder recommended for clinical trials is 80 g/day, which is equivalent to 0.45 kg of fresh grapes or three USDA servings of grapes/day. The nutritional value of freeze-dried grape powder, essentially identical to that of grapes, is determined for each vintage using standard food analyses as indicated in Table 2.

3 Placebo Powder for Clinical Research

For use in human clinical trials, a placebo powder is also produced by the California Table Grape Commission that is formulated to closely match the freeze-dried grape powder in terms of dietary fiber, sugar profile, organic acid profile, as well as for sensory characteristics of sweetness, tartness, mouthfeel, and viscosity. The placebo contains fructose and glucose (the most abundant sugars in grapes) so that the placebo is isocaloric with the freeze-dried grape powder (Table 2). Because fresh grapes contain 0.2–0.8 % organic acids, tartaric, malic, and citric acids are added to the placebo, and FD&C artificial colors are included to simulate the color of a grape mixture. The placebo also contains modified food starch and tapioca maltodextrin for texture, dipotassium phosphate, potassium citrate (0.6 and 0.8 % by weight, respectively), and silicon dioxide at the same level as used in the grape powder.

4 Selection of Grape Compounds for Chemical Standardization

To ensure the reproducibility of the preclinical and clinical studies using freeze-dried grape powder, chemical standardization is essential. As has been described for botanical dietary supplements (Farnsworth et al. 2008), chemical standardization usually utilizes chromatography, such as high-performance liquid chromatography (HPLC) with ultraviolet absorbance detection, evaporative light scattering detection, or mass spectrometric detection, to measure levels of active compounds and any marker compounds that might be helpful for the preparation of a reproducible product. Such standardization also facilitates the interpretation of biomedical data by providing levels of compounds known to produce biological effects through specific mechanisms of action.

Catechin and epicatechin (Fig. 1), which are the monomeric constituents of the proanthocyanidins, are the most abundant antioxidant polyphenols in grapes, constituting ~50 mg/100 g of grape skin (dry weight) and even higher proportions of grape seed (Yilmaz and Toledo 2004). The most abundant flavonols in white or green grapes are quercetin (81.4 %), kaempferol (16.9 %), and isorhamnetin (1.7 %) (Fig. 1), whereas red grapes contain a greater variety of flavonols including not only quercetin (44.0 %), kaempferol (6.4 %), and isorhamnetin (3.9 %) but also the delphinidin-like flavonols such as myricetin (36.8 %) (Mattivi et al. 2006). Occurring primarily as glucosides, the major anthocyanins in grapes are malvidin (malvidin 3-*O*-glucoside is the most abundant at 42.0 % of total anthocyanins) followed by peonidin, cyanidin, delphinidin, and petunidin (Mulero et al. 2010).

For the purpose of freeze-dried grape powder standardization, the most abundant polyphenols of each class were selected for quantitative analysis including catechin and epicatechin; the flavonols quercetin, kaempferol, and isorhamnetin; and the

anthocyanins malvidin, peonidin, and cyanidin (Fig. 1). Due to its multiple mechanisms of action in chemoprevention (Park and Pezzuto 2015), the stilbene resveratrol was also measured.

5 Chemical Standardization Using UHPLC–MS/MS

5.1 Catechin, Epicatechin, and Resveratrol

For the measurement of catechin, epicatechin, and resveratrol (Fig. 1), freeze-dried grape powder (20.0 mg) was extracted three times with 2 mL portions of methanol for 30 min using sonication in ice-cold water. The extract was centrifuged at 4 °C and filtered to remove particulates. After concentration in vacuo, the residue was diluted to 10 mL in a volumetric flask using methanol containing naringenin (50 ng/mL) as an internal standard before analysis. Standard solutions (1 mg/mL) were prepared in methanol, and working solutions containing 0.1 mg/mL of each standard were prepared by dilution using methanol/water (1:1; v/v). Serial dilution of the working solutions was carried out to obtain ten standard solutions ranging from 1.56 to 1000 ng/mL. Naringenin (50 ng/mL final concentration) was added to each solution before analysis using ultrahigh-pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS).

Quantitative analyses of catechin, epicatechin, and resveratrol were carried out using a Shimadzu LCMS-8040 triple quadrupole mass spectrometer equipped with a Shimadzu Nexera UHPLC system and a Shim-pack XR-ODS C_{18} column (2.0 mm × 50 mm, 1.6 μm particle size). The mobile phase consisted of a 1.0 min linear gradient from 20 to 90 % methanol at 0.5 mL/min, and the injection volume was 5 μL. Negative ion electrospray, collision-induced dissociation, and selected reaction monitoring (SRM) were used during tandem mass spectrometry to monitor the transitions of deprotonated molecules of each analyte to abundant product ions as follows: *m*/*z* 289 to *m*/*z* 245 for catechin and its isomer, epicatechin, *m*/*z* 227 to *m*/*z* 185 for resveratrol, and *m*/*z* 271 to *m*/*z* 151 for naringenin. Collision energies for each SRM were optimized at 21 V, 20 V, 25 V, and 29 V for catechin, epicatechin, resveratrol, and naringenin, respectively.

All three analytes and the internal standard were separated in less than 2 min as shown in Fig. 2. This fast separation was facilitated by the use of UHPLC, which was five-fold faster than comparable HPLC separations (data not shown). The standard curves were linear over the entire range (up to 1000 ng/mL) and showed coefficients of determination (r^2) > 0.997. The levels of catechin, epicatechin, and resveratrol in the freeze-dried grape powder (in ppm) are shown in Table 3.

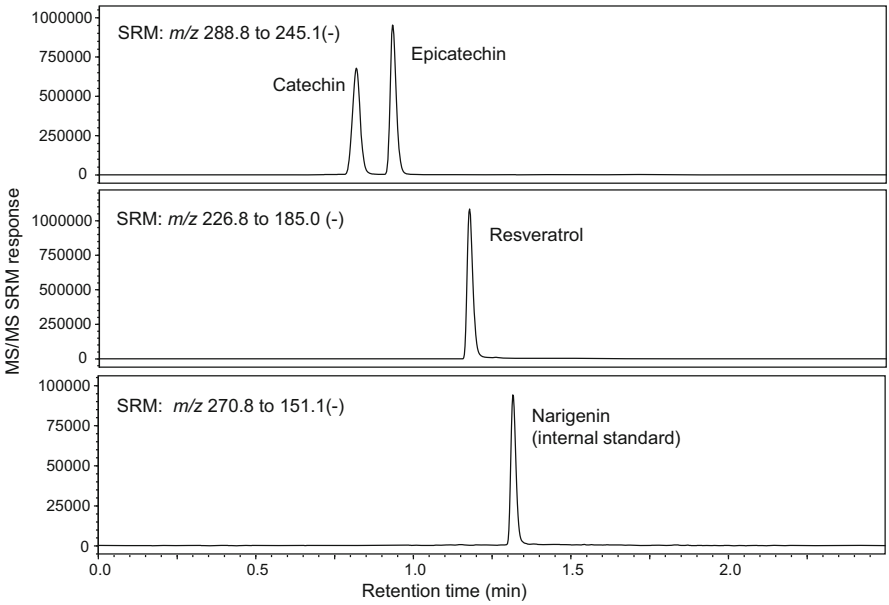

Fig. 2 Quantitative analysis of antioxidants catechin and epicatechin and the chemoprevention agent *trans*-resveratrol in freeze-dried grape powder using UHPLC–MS/MS with negative ion electrospray, collision-induced dissociation, and selected reaction monitoring (SRM)

Table 3 Results of chemical standardization of freeze-dried grape powder prepared from California table grapes harvested in 2011

Class	Compound	Level (mg/kg grape powder) ppm ± Std Dev
Phenols		
	Catechin	77.4 ± 12.5
	Epicatechin	58.9 ± 10.2
Anthocyanins		
	Cyanidin	266.7 ± 27.1
	Malvidin	219.3 ± 31.3
	Peonidin	47.63 ± 8.62
Flavonols		
	Isorhamnetin	13.95 ± 1.84
	Kaempferol	7.38 ± 0.62
	Quercetin	148.7 ± 10.5
Stilbenes		
	Resveratrol	13.6 ± 1.1

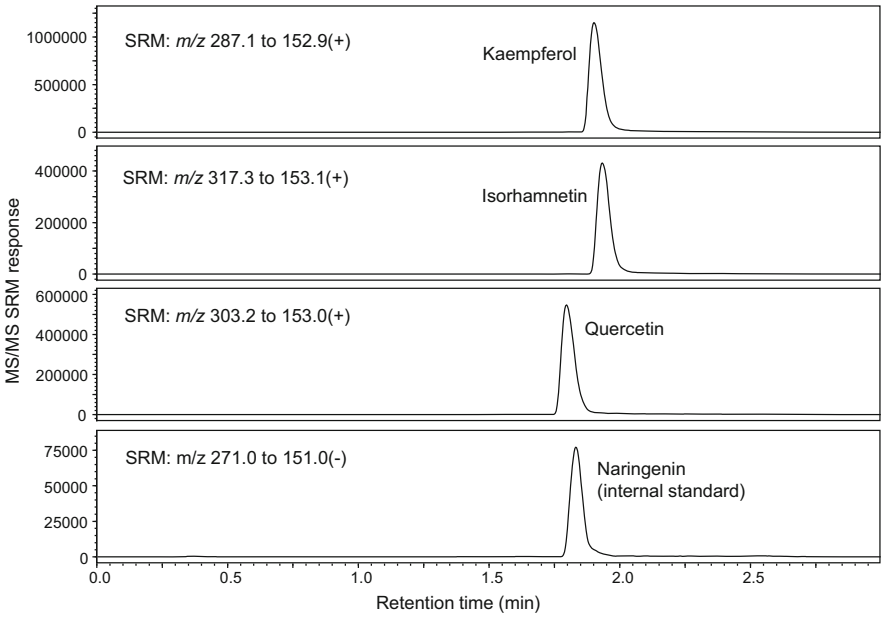

Fig. 3 UHPLC–MS/MS analysis of the flavonols isorhamnetin, kaempferol, and quercetin extracted from freeze-dried grape powder using positive ion electrospray, collision-induced dissociation, and SRM

5.2 Flavonols: Isorhamnetin, Kaempferol, and Quercetin

Hydrolysis of the various flavonol conjugates to the corresponding aglycones was carried out prior to UHPLC–MS/MS analysis. For example, the abundant glycoside quercitrin was hydrolyzed to form quercetin (Fig. 1). Freeze-dried grape powder (2.50 g) was hydrolyzed by refluxing for 15 min in 20 mL of methanol/HCl (4 M) (4:1; v/v). The solution was cooled and made up to 100 mL with methanol using a volumetric flask. Naringenin (50 ng/mL) was added as an internal standard (Fig. 3).

UHPLC–MS/MS analysis of flavonols was carried out as described in the previous section except that positive ion electrospray was used with different mobile phase and SRM parameters. Separations were carried out using two 0.5 min linear gradients, first from 20 to 70 % acetonitrile and then from 70 to 90 % acetonitrile containing 0.1 formic acid in water. SRM was used to monitor the transitions from protonated molecules to abundant product ions as follows: *m*/*z* 317 to 153 for isorhamnetin (collision energy, CE, 37 V), *m*/*z* 287 to 153 for kaempferol (CE 34 V), *m*/*z* 303 to 153 for quercetin (CE 43 V), and *m*/*z* 271 to 151 for the internal standard naringenin (CE 29) (Fig. 3).

The standard curves were linear with r^2 values $\geq$0.992. Consistent with previously reported flavonol levels in grapes (Mattivi et al. 2006), quercetin was the most abundant flavonol in the grape powder at 148.7 ppm followed at much lower levels by isorhamnetin and kaempferol at 13.95 ppm and 7.38 ppm, respectively (Table 3).

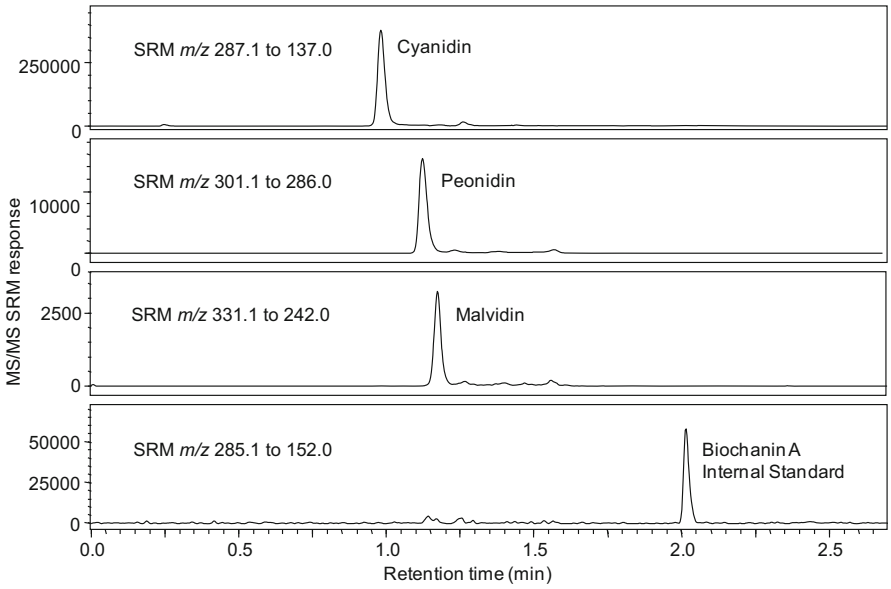

Fig. 4 Quantitative analysis of the abundant grape powder anthocyanidins cyanidin, malvidin, and peonidin using UHPLC–MS/MS with positive ion electrospray, collision-induced dissociation, and SRM

5.3 Anthocyanins: Cyanidin, Malvidin, and Peonidin

Because anthocyanins occur as a mixture of 3-*O*-glucosides, they were measured as their aglycones following chemical hydrolysis as described above for the flavonols. Biochanin A (50 ng/mL) was used instead of naringenin as an internal standard (Fig. 4). UHPLC–MS/MS was used as described above except that a linear gradient was used from 10 to 80 % acetonitrile over 1.8 min followed by a 0.01 min step to 90 % acetonitrile containing 0.1 % formic acid. Collision-induced dissociation of the deprotonated molecules was followed by SRM to record signals of abundant product ions for quantitative analysis as follows: *m*/*z* 287 to 137 for cyanidin (CE 31 V), *m*/*z* 301 to 286 for peonidin (CE 30 V), *m*/*z* 331 to 284 for malvidin (CE 29 V), and *m*/*z* 285 to 152 for biochanin A (CE 27 V). The standard curves were linear with r^2 values >0.992.

Although malvidin has been reported to be the most abundant anthocyanin in grapes (Mulero et al. 2010; Liazid et al. 2014), we found that cyanidin was slightly more abundant than malvidin followed lastly by peonidin (Table 3). Note that the glucosides of malvidin and peonidin have been reported to be more stable than those of cyanidin (Liazid et al. 2014). Therefore, it is unlikely that malvidin degraded more extensively than cyanidin during chemical hydrolysis. Instead, cyanidin was simply more abundant than malvidin in this mixture of California table grapes (Fig. 4).

6 Conclusions

A freeze-dried grape powder representative of an annual crop of California table grapes has been prepared to support research on the health benefits of grapes. Safety is ensured by preparing the grape powder using GMP and testing it for microbial contamination, while chemical standardization to the most abundant polyphenols and antioxidants as well as determination of nutritional value enables reproducible scientific studies. Since 1999, over 65 scientific studies have been carried out using freeze-dried grape powder (California Table Grape Commission Research Grant Program 2016).

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Grape Polyphenols in the Prevention of Cardiovascular Disease

Myron Gross

Contents

Abstract The consumption of grapes has been considered part of a prudent diet and a healthy lifestyle for many years. It contributes toward compliance with the recommendation of consuming five servings of fruits and vegetables per day. Compliance with this recommendation has been associated with cardioprotective effects and low levels of cardiovascular disease mortality and morbidity. The association of grape intake with low cardiovascular disease risk is supported by various epidemiologic, clinical, and experimental studies. In particular, grapes contain numerous compounds with bioactivities relevant to the prevention of cardiovascular disease. Grapes contain simple phenols, simple phenolic acids, cinnamic acids, stilbenes, proanthocyanidins, anthocyanins, flavonoids, flavans, anthocyanins, resveratrol, and carotenoids. Many of the compounds have multiple

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bioactivities. For instance, resveratrol has numerous bioactivities and is a potent cardioprotective agent. The bioactivities of resveratrol and other grape components include antioxidative, lipid-lowering, and anti-inflammatory effects. Clinically, grapes and grape products have anti-atherosclerotic, anti-arrhythmic, vasorelaxation activities, and possibly anti-hyperglycemic effects. In addition, grapes and its products have been associated with low platelet activity and low thrombosis, promoting normal endothelial function, blocking cellular adhesion molecule activity, and preventing the oxidation of LDL particles. We describe the association of grapes, its products, and compounds with specified bioactivities, pinpointing their sites of action. In addition, it is recognized that the bioactivity of several flavonoids in grapes may depend upon the context of their consumption, food versus supplement, and overall dietary composition. Diets high in fruits and vegetables are also generally low in saturated fat, low in calories, and high in omega-3 fatty acids: characteristics associated with low body weight and blood pressure. Many of these effects will require additional detailed studies for their recognition. Thus, understanding the effects of grape intake and the interactions of its components and overall dietary composition will require extensive additional research.

1 Introduction

Vitis vinifera, the common grape vine, originated throughout the Mediterranean region, Central Europe, and Southwestern Asia. It evolved with the spread of human populations and is currently grown on every continent of the world except Antarctica ("Vitis vinifera," Euro + Med PlantBase 2015; Robinson 2001, 2012). There are now more than 5000 varieties of *Vitis vinifera*, only a few of which have commercial significance as table grapes and wine. Nonetheless, grapes have been a significant part of the food supply in civilized nations for over 5000 years. Grapes have many beneficial characteristics as a food source and few limitations; both of these aspects of grapes have been studied extensively and are well known. These observations attest to the adaptability of grapes and their evolution as a food source for humans.

Beyond being a good food source, grapes have additional beneficial characteristics. Among these are the health benefits of grapes, including a role in the prevention of heart disease. These health benefits have been recognized relatively recently but have motivated substantial research in this area. This chapter will focus on the effect of grapes and their products on the risk factors and pathogenesis of cardiovascular disease. This information is highly relevant in view of recent developments in the field. Within the last 10 years, the entire genome of *Vitis vinifera* was sequenced and has provided new insights into the evolution of grapes and their use in the food supply (Jaillon et al. 2007; Commonwealth Scientific and

Industrial Research Organization 2007). This information will be invaluable in the development and adaptation of grapes for various uses in the future, including possible health benefits.

2 Composition of Grapes

Grapes have substantial nutritional value. One serving of grapes can be defined as ¾ cup or 126 g. This amount of grapes will contain about 90 cal, 23 g of total carbohydrates, 1 g of dietary fiber, 20 g of sugar, and significant amounts of various vitamins and secondary metabolites, including various phenolic compounds. The main constituents of grapes consist of water 82 %, sugar 12–18 %, and acids 0.2–0.8 % (Freeze-Dried Table Grape Powder Analysis Report 2011). Vitamins and phenolic compounds account for less than 0.05 % of the overall composition of grapes. Nonetheless, they account for most of the biological activity of grapes.

A freeze-dried grape powder preparation (FDGPP) is made by the California Table Grape Commission on a routine basis (generally every few years). The preparation is made from a large sample of California table grapes, including green, red, and black grapes. It is frozen and processed on dry ice, freeze-dried, and stored at −80 °C. Composition of the grapes is measured, and the preparation is provided for research purposes. It provides a common pool of grapes with a constant composition. It is acceptable for human consumption and allows the comparison of research results with the replicate use of this grape material.

Grapes contain many phenolic compounds including simple phenols, simple phenolic acids, cinnamic acids, stilbenes, flavonoids, flavans, and anthocyanins. They have a high content of flavonoids and are good sources of flavans. Well-recognized active phenolic compounds in grapes include resveratrol, anthocyanins, leucoanthocyanidin, catechins, and their derivatives. There are many structural variations of phenolic compounds based on the degree of hydrogenation, hydroxylation, and the formation of monosaccharide and disaccharide variants as well as complexes with oligosaccharides, lipids, amines, carboxylic acids, and organic acids. Classifications of these compounds and their structures are described in detail elsewhere in this volume and in the literature (Giada 2013; Duthie et al. 2003).

Grapes are consumed worldwide in the form of fresh or dried fruit, preserves, juice, or wine. Anthocyanins, proanthocyanidins, and tartrate esters of hydroxycinnamic acids account for most of the phenolic compounds in grapes. The remainder of the phenolic compounds includes monomeric flavan-3-ols, flavonols, hydroxybenzoic acids, free hydroxycinnamic acids, and stilbenes (Stalmach et al. 2011). The most common class of compounds in grapes is flavonoids. There are six common subclasses of the flavonoids. These subclasses and their basic structures are shown in Fig. 1 (Giada 2013).

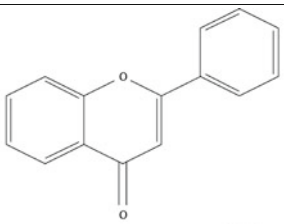
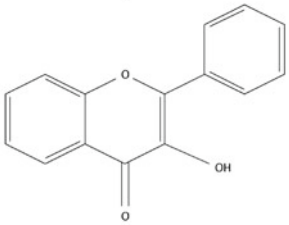
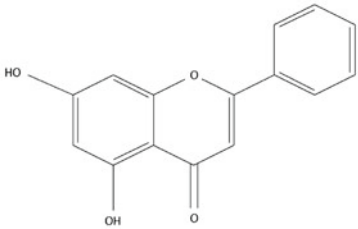
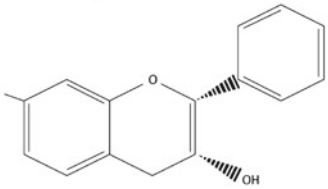
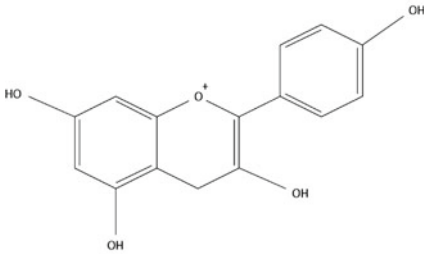
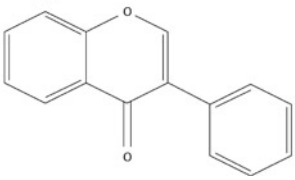
Flavonoid	Basic Structure
Flavones	
Flavonols	
Flavanones	
Flavanols	
anthocyanins	
Isoflavones	

Fig. 1 Flavonoid subclass structures

3 Bioavailability and Metabolism of Polyphenols from Food and Wine

Anthocyanidins (ACs) are a good model for bioavailability of the polyphenols as similar levels of response can be expected by other subclasses and radioisotope studies have been performed with the anthocyanidins. Anthocyanidins occur in their glycosylated form (Paganga and Rice-Evans 1997; Cao and Prior 1999; Tsuda et al. 1999; Matsumoto et al. 2001; Cao et al. 2001; Nielsen et al. 2003; McGhie et al. 2003) in foods. The bioavailability of ACs is low with 1.5–5.0 % of intake being excreted in the urine within 12 h (Morazzoni et al. 1991; Lapidot et al. 1998; Bub et al. 2001; Milbury et al. 2002; Passamonti et al. 2003) and some studies showing much lower amounts (0.04–0.10 %) of ingested dosage (Nielsen et al. 2003; McGhie et al. 2003; Netzel et al. 2002; Frank et al. 2003). A large amount of the ACs may be metabolized through several pathways or excreted in the feces. Levels in plasma are low, on the order of 0.1 μM or less (Matsumoto et al. 2001; Cao et al. 2001). The ACs can be methylated, glucuronidated, or sulfated in the stomach or small and large intestine. Very few studies have been performed regarding the absorption, distribution, metabolism, and excretion of (PAs). However, studies with radioisotope-labeled PAs indicated the distribution of PAs or their metabolites in most tissues of rats and mice (Laparra et al. 1977; Harmand and Blanquet 1978). Differences may exist between animal models. For instance, procyanidin polymers were not absorbed by chickens and sheep (Jimenez-Ramsey et al. 1994; Terrill et al. 1994) but were degraded by colonic microflora into various phenolic acids (Groenewoud and Hundt 1986; Déprez et al. 2000).

4 Biological Activities of Grape Phenolics

Phenolic compounds in grapes, especially the polyphenolic flavonoids, have numerous biological activities. The antioxidant polyphenols in grapes have been associated with antiaging and anti-neurodegenerative effects, reduction in atherosclerosis, and improved endothelial function (Dohadwala and Vita 2009). Grape polyphenols have also been reported to reduce obesity-induced chronic inflammation and aid in the prevention of metabolic syndrome and type II diabetes (Chuang and McIntosh 2011).

A number of these biological activities may play a larger role in the prevention and/or treatment of cardiovascular disease. Humans are exposed to a wide range of phenolic compounds, described above in *Composition of Grapes*, as part of normal dietary intakes. In particular, grapes are a good source of bioactive compounds relevant to cardiovascular disease. Grape phenolics can decrease oxidative damage/stress, decrease inflammation, lower blood lipids, improve endothelial function, lower blood pressure, improve flow-mediated dilation, prevent coronary calcification, prevent plaque formation, and prevent the formation of fibrosis. Some of the

recent developments will be described for these areas. Each of these activities aids in the prevention of cardiovascular disease events. Interestingly, individual polyphenols in grapes may have multiple independent activities, which act synergistically in the prevention of cardiovascular disease activities.

5 Cardiovascular Disease: The Development of Atherosclerosis

Grapes and their constituents have a wide range of biological activities which may aid in the prevention of cardiovascular disease. Before describing these activities, the pathogenesis of atherosclerosis and cardiovascular disease will be described as a basis for understanding the activities of phenolic compounds and their possible sites of action in cardiovascular disease. Atherosclerosis is the fundamental cause of most cardiovascular disease. It is a lipid-driven disease that leads to the formation of plaque at focal areas in the arterial blood vessels. Blood vessels consist of three major layers, including the intima (a thin layer of endothelial cells which form the lumen of the vessel), the media (the thickest layer), and the adventitia (mainly connective tissue). The subendothelial space is immediately below the endothelial cells and is the site of particle retention and initiation of atherosclerosis. Key steps in the development of atherosclerosis include retention of apoB-containing lipoproteins (e.g., low-density lipoproteins) by blood vessels (subendothelial space), intimal inflammation, cellular recruitment and propagation, adventitial and intimal angiogenesis, plaque development, calcification, and fibrosis (see Fig. 2 for response-to-retention hypothesis). These developments can be followed by plaque rupture and a thrombotic event or lumen narrowing and an ischemic event, leading to a heart attack or myocardial infarction. This pathogenesis underlies several vascular diseases, all classified as cardiovascular disease. These include myocardial infarction, angina pectoris, and death due to coronary heart disease (CHD), stroke, transient ischemic attack, heart failure (HF), or peripheral artery disease. Thus, atherosclerosis is the primary cause of these diseases, albeit each disease may have additional pathogenic features unique to the specific disease.

Atheromatous plaque has a core containing lipids and cellular debris called the lipid core or lipid-rich necrotic core. It is covered by a fibrous layer, which can be

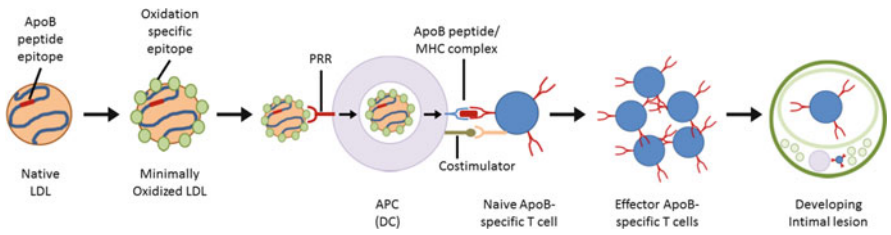


Fig. 2 Response to retention hypothesis

thick or thin and relates to more or less risk for plaque rupture. This fibrous cap contains smooth muscle cells and collagen fibers which stabilize the plaque. Immune cells including monocytes, T cells, and mast cells are present in the plaque, mostly in an activated state. These cells produce various cytokines, proteases, pro-thrombotic molecules, and vasoactive substances. Each of these molecules has a role in inflammation and vascular function. The formation of atheromatous plaque begins with endothelial dysfunction and increases further with advanced plaque development. Until complications occur, an intact endothelium covers the atheromatous plaque. The plaque causes luminal narrowing, aggravating thrombi production, and interference with circulation in the heart and brain. Denudation of the arterial blood vessels leads to advanced plaque through further intimal inflammation, necrosis, fibrosis, and calcification.

The retention of apoB 100 containing apolipoproteins in the arterial intima is sufficient for the causation of atherosclerosis (Nakashima et al. 2008). In the intima, the particles may be modified by oxidation and aggregation, which can modify their antigenicity (Öörni et al. 1998). Modification by risk factors can also contribute toward susceptibility, including age, sex, smoking, hypertension, diabetes mellitus, and genetic factors. For instance, causation can be achieved with lower levels of LDL in diabetics than nondiabetics (Leeper et al. 2007; Williams and Tabas 1995). Risk factors may influence the characteristics of LDL particles, which may vary in their size, charge, and composition and characteristics of the endothelial layer. These characteristics can influence the retention and the immune response to the lipoprotein particles.

The formation of plaque can be promoted by interactions of lipoproteins with the extracellular matrix and cellular components (Nakashima et al. 2007). Lipoproteins interact with the extracellular matrix in the arterial wall (Camejo et al. 1975, 1980; Vijayagopal et al. 1981). The main determinants are apolipoprotein characteristics (size, charge, and composition) and their ability to pass through the vessel wall due to a small size. Secretory phospholipase A2 can modify lipoprotein in a manner that promotes binding to the extracellular matrix. Binding of LDL with proteoglycans may make them more susceptible to oxidation, aggregation, and uptake by macrophages. Also, oxidized HDL may be pro-atherogenic and contribute to the formation of plaque, once it is retained (Thorne et al. 2007). Lipoprotein retention may be a self-propagating process in which retained LDL promotes cellular responses that leads to additional LDL entrapment. First, oxidized LDL and cytokines released from the inflammatory cells stimulate smooth muscle cells to proliferate. This activity can result in proteoglycans with elongated proteoglycan chains, which increases their affinity for LDL. Secondly, infiltrated macrophages produce bridging molecules, mostly lipoprotein lipase (LPL) which further enhances the lipoprotein retention. LPL has binding sites for both proteoglycans and lipoproteins. This increases the vessel wall affinity for lipoprotein lipase which is serving as a bridge between proteoglycans and lipoproteins (Babaev et al. 1999; Wilson et al. 2001).

Angiogenesis and neovascularization contribute to advanced plaque development. Angiogenesis contributes to the progression of intimal hyperplasia and is active in development of the necrotic core in plaque development. While this

activity occurs at the more advanced stages of plaque formation, it can be a significant factor in the progression of plaque development and rupture. These activities involve the recruitment of monocytes and macrophages which secrete various pro-angiogenesis factors, including monocytes chemotactic protein 1, and vascular endothelial growth factor (Nakano et al. 2005).

6 Oxidative Damage

The oxidative modification hypothesis of atherosclerosis was initiated approximately 30–40 years ago. It suggested that the modification of LDL particles by oxidation induced a scavenger receptor activity, which recognized oxidized LDL, but not unmodified LDL, and allowed the cellular uptake of modified LDL. It stated that oxidative damage was a primary cause of atherosclerosis. A corollary hypothesis is that antioxidants and antioxidant status can prevent the formation of oxidized LDL. Numerous studies have been performed as a test of this hypothesis, the most well known being the β-carotene trials. Those trials resulted in negative findings. Nonetheless, alternative antioxidants, dietary sources, or combinations of antioxidants could prove more effective. Also, many of the phenolics in grapes have antioxidant activity, which are higher than β-carotene. In a study of anthocyanins (AC), human subjects were given anthocyanin-rich beverages for 14 days, and its effect was measured on oxidative stress, antioxidants, and antioxidant enzymes. The beverages (330 mL) contained 8.9 (placebo), 984 (juice), and 840 (smoothie) mg/L of anthocyanins. The study design was a randomized crossover clinical trial with 30 women. Ingestion of the anthocyanin beverages resulted in an improvement of several but not all components of antioxidant activity. Exposure to anthocyanins increased plasma superoxide dismutase and catalase activity as well as improved Trolox antioxidant activity and lowered malondialdehyde concentrations. Plasma glutathione peroxidase and erythrocyte superoxide dismutase activity as well as an indicator of DNA oxidation were unchanged. Thus, ACN supplementation may enhance protection against some forms of oxidative stress including superoxide anions and lipid peroxidation, but not all.

These activities were tested further in various cell and animal studies, which have confirmed the high potential of anthocyanins to act as direct reactive oxygen species scavengers or act indirectly in the prevention of oxidative damage by influencing expression of antioxidant enzyme system activities (Siasos et al. 2013; Kim et al. 2012; Alvarez-Suarez et al. 2011). Further insight into these activities has come from structure-function studies. Some of the anthocyanins exhibit higher reactive oxygen species scavenging activities than other flavonoids. Recently, structural characteristics of ACs have been associated with their antioxidant activities and with inhibitory effects on endothelial dysfunction.

7 Oxidative Damage/Antioxidant Activity

Grapes are significant contributors of antioxidant capacity in the diet (Giada 2013). Antioxidant capacity of foods has been measured by several assays, wherein response to an oxidative challenge is measured and expressed as the utilization of an antioxidant, such as Trolox or its equivalents. These assays provide an overall measure of antioxidant capacity in foods. The assays include the ABTS (2,2-azino-*bis*-3-ethylbenzothiazoline-6-sulfonic acid radical assay), DPPH (2,2-diphenyl-picrylhydrazyl radical assay), FRAP (ferric reducing/antioxidant power assay), and ORAC (oxygen radical absorbance capacity assay). The antioxidant capacity has been correlated with the phenolic content of foods. High levels of phenolic compounds and antioxidant capacity are found in grapes and a number of other foods that are considered heart healthy foods, including yams, potatoes, tomatoes, kale, Brussels sprouts, broccoli, dark green leafy and brightly colored vegetables, legumes, cereals, spices, and fruits (Giada 2013). In a similar manner, red wine has been a rich source of phenolic compounds and has a high antioxidant capacity (Baroni et al. 2012; Rivero-Pérez et al. 2007). Red wine is also a rich source of anthocyanin (Morton et al. 2000; Pellegrini et al. 2003). Tea and coffee are also good sources of phenolic compounds (Yang et al. 2001; Thiagarajan et al. 2001). The antioxidant capacity was measured in a wide variety of Spanish red wines, from different varieties of grapes, vintages, and aging processes (Baroni et al. 2012; Rivero-Pérez et al. 2007). Red wine, in moderation, has been associated with a low risk for cardiovascular disease. These findings suggest a role for phenolics, acting as antioxidants or through other mechanisms in the prevention of cardiovascular disease.

However, a very limited number of studies have analyzed circulating phenolics, and none had found an association between specific phenolics and cardiovascular disease events. Antioxidant capacity assays have been measured in blood samples of individuals fed foods with high phenolic content and generally indicated an increase following the intake of foods with a high antioxidant capacity. The presence of a high antioxidant capacity in the blood is consistent with the antioxidant hypothesis for cardiovascular disease, which indicates a lower risk of cardiovascular disease with higher antioxidant levels. It is also consistent with the foods being consumed by the population. Nonetheless, as indicated by clinical trials, specific antioxidants alone may not be effective. In the case of antioxidant capacity of blood, the antioxidants present were unknown. Thus, interpretation of the results was difficult. More detailed studies of profiles may be warranted for a better understanding of the relationship between the phenolic compounds and the prevention of cardiovascular disease.

8 Inflammation

In atherosclerosis, apolipoprotein B-containing particles may be modified by oxidation, enzymatic reactions, and binding and retention in the intimal space. These modifications can be recognized as "foreign" substances, which can induce an immune response. While several immune cells participate in this activity, a key cellular component is monocytes/macrophages. Signals from the macrophages act in the induction of inflammation, which is a complex process involving several types of cells and the production of a wide range of cytokines. Inflammation is generally beneficial as it involves removing the substance seen as "foreign." However, excessive inflammation can cause cellular damage and initiate the pathogenesis of cardiovascular disease. Inflammation has a key role in many diseases, including cardiovascular disease and several cancers.

Many of the products of inflammation have been linked with cardiovascular disease. These include the activities of immune cells, such as T and B cells, macrophages, and dendritic cells. Proteins involved in these activities include cytokines, chemotactic factors, interleukins (immune regulators), and cellular adhesion molecules (CAMs). CAMs have a key role in the development of plaque as these molecules provide an anchor for cellular components, removing cells from the circulating and providing a focal site for accumulation. All of these activities are essential steps for inflammation and the development of plaque.

The sequestration of lipoproteins increases their susceptibility to modifications from oxidation, enzymatic and nonenzymatic cleavage, and aggregation. These modifications make the protein particles more pro-inflammatory and induce the activation of an overlying endothelial layer. A major effect is the activation of overlying endothelial cells in a manner that leads to the recruitment of more blood-borne monocytes into subendothelial space (Glass and Witztum 2001; Mestas and Ley 2008). The activated cells secrete chemoattractant or chemokines that interact with chemokine receptors on monocytes and promote their directional migration. Monocyte development may be regulated by cellular cholesterol content in a manner that affects atherogenic activity (Yvan-Charvet et al. 2010). The endothelium is activated and releases chemokines and their adherents of monocytes and endothelial lesions through their interaction with foam cell ligands. An interaction occurs through deposition of platelet-derived chemokines on activated endothelial (Koenen et al. 2009). This leads to the entry of monocytes into subendothelial space. Within the intima, the monocytes secrete proteoglycans, leading to the accumulation modified LDL, which sustains inflammation (Williams and Tabas 1995; Mestas and Ley 2008).

Once in the intima, monocytes under the influence of monocyte colony-stimulating factor change either to macrophages or dendritic cells. When the uptake of macrophages exceeds a certain level, the accumulation of macrophages results in the formation of foam cells. Foam cells promote further growth in the development of plaque, secretion of cytokines and chemokines, generation of reactive oxygen species, presentation of new activation markers to macrophages scavenger receptor,

production of matrix-degrading enzymes, and induction of apoptosis. Muscle cells migrate into the intima and promote formation of a local core of macrophages congregated in a central core, and cells can undergo apoptosis, hence producing the so-called necrotic core. Both antibody and cell-mediated immunity are involved in the development and progression of the atherosclerotic plaque (Hansson 1997). Activated CD4 plus T lymphocytes contribute to plaque vulnerability by producing pro-inflammatory cytokines including interferon-γ and tumor necrosis factor-α. Some secretions of macrophages and T cells may have a role in disruption of atherosclerotic plaque.

Polyphenols can influence the expression of inflammation and the related activity of oxidative stress. Several studies have identified specific foods and polyphenols which influenced these activities. Consumption of freeze-dried grape powder equal to 1.25 cups of fresh grapes for 21 days increased plasma total antioxidant capacity (Chaves et al. 2009). This treatment was also reported to decrease soluble intercellular adhesion molecule 1. Consumption of grape juice for 5 days has been reported to increase urinary total antioxidant capacity (González-Flores et al. 2012). Consumption of Concord grape juice for 8 weeks reduced DNA damage in healthy subjects (Park et al. 2009). Grape seed extract increased plasma-reduced glutathione concentrations and decreased C-reactive protein concentration (Kar et al. 2009). Two additional human trials found that grape seed extract and resveratrol decreased C-reactive protein, tumor necrosis factor-α, plasminogen activator inhibitor-1, and interleukin-6/interleukin-10 ratio as well as interleukin-10. Supplementation also increased anti-inflammation serum adiponectin and decreased thrombogenic factors. Both ethanol and phenolic compounds of red wine down-regulated serum concentrations of CD 40 and monocyte chemotactic protein 1 and vascular cell adhesion molecule-1 (Tomé-Carneiro et al. 2012a, b). A 200 mg per day dosing of grape preparation for 4 weeks increased the concentration of reduced glutathione in erythrocytes (Weseler et al. 2011). Consumption of red wine and dealcoholized red wine decreased serum concentrations of intercellular adhesion molecule 1, e-selectin, and IL-6 and inhibited the growth site function associated antigen-1 in T lymphocytes. Both ethanol and phenolic compounds of red wine down-regulated CD 40 antigen, CD 40, interleukin-16, monocyte chemotactic protein-1, and vascular cell adhesion molecule-1 (Chiva-Blanch et al. 2012). Alternatively, two human trials did not report changes in antioxidant or inflammatory markers with the consumption of grape product. This included the consumption of Concord grape juice for 12 weeks which had no effect and consumption of mustard wine-grape seed extract for 4 weeks which did not show an effect on C-reactive protein, interleukin-6, and plasma total antioxidant capacity (Hollis et al. 2009; Mellen et al. 2010). Also, a broad range of cytokines were analyzed in further experiments. Anthocyanin ingestion did not influence any of several markers of inflammation, including C-reactive protein; IL-2, IL-6, IL-8, or IL-10; TNF-α; MCP; differentiation 40 ligand; and cellular adhesion molecules (Dohadwala et al. 2011; Triebel et al. 2012; Hassimotto et al. 2008; Kolehmainen et al. 2012).

Studies of mechanisms indicate inhibitory effects of polyphenols. This effect has been demonstrated in animals as well as human studies. Freeze-dried grape powder preparation (FDGPP) treatment reduced interleukin-6 and tumor necrosis factor-α, biomarkers of inflammation, in pre- and postmenopausal women (Zern et al. 2005). Adhesion molecules and monocyte adhesion to endothelial cells were significantly reduced with red wine consumption in men (Estruch et al. 2004). Four weeks of red wine consumption also resulted in reductions of very late activation antigen, lymphocyte-associated function antigen, Mac-1, and monocyte chemoattractant protein (MCP) on monocytes and T lymphocytes. In addition, soluble cellular adhesion molecules were reduced with 4 weeks of wine intake and included ICAM-1, VCAM, and MCP (Feng et al. 1999). The mechanisms for these flavonoid effects are incompletely understood. However, it may involve the regulation of nuclear factor−kappaB (NFκB), a transcription factor involved in the activation of procoagulation proteins, adhesion molecules, and cytokines (Jialal et al. 2001). In a developing and progressive cycle, these proteins can induce NFκB, which in turn amplifies these proteins again. Consumption of red wine decreased NFκB expression. Additional mechanisms may be involved in the developing pathology. One of which is formation of reactive oxygen species, which also induces NFκB (Blanco-Colio et al. 2000), but can be blocked by the antioxidant activity of the flavonoids. Also, resveratrol, a stilbene found primarily in grapes, inhibits CAM and cytokine expression through the tyrosine kinase second messenger system (Ferrero et al. 1998) and provides another possible mechanism that is independent or synergistic with other mechanisms.

Overall, the effects on inflammation are mixed, and there are many possible explanations for experimental limitations.

9 Flavonoids and Cholesterol Absorption

Several cellular and animal studies have found decreases in cholesterol absorption and other alterations in lipid metabolism with flavonoid intake (Löest et al. 2002). Flavonoids may interact with cholesterol carriers and transporters at the brush border membrane and reduce cholesterol absorption (Conseil et al. 1998; Leslie et al. 2001). Green tea polyphenols lowered lymphatic absorption of cholesterol and lowered absorption of fatty acids by ovariectomized rats (Löest et al. 2002). This effect can lower cholesterol uptake by the liver from chylomicrons. An important lipoprotein for chylomicron remnant formation is the apolipoprotein B48. Caco2 cells produced and secreted less apolipoprotein B48 when treated with dealcoholized red wine, a rich source of flavonoids. This treatment resulted in lower intracellular concentrations of free and total cholesterol. These reductions of cholesterol absorption by flavonoids can alter cholesterol homeostasis in the liver, including changes in removal rate of lipoproteins from plasma and the secretion of apolipoproteins. Thus, flavonoids can alter key steps in cholesterol metabolism and total cholesterol levels in the blood of animals.

Several studies have examined the effects of flavonoids on lipoprotein production by hepatocytes. Naringenin and hesperetin, the citrus flavonoids, reduce apolipoprotein B secretion by hepatocytes (Borradaile et al. 2002; Pal et al. 2003). This effect was accompanied by a reduction in cholesterol ester mass, Acyl-CoA cholesterol acyltransferase, and microsomal transfer protein mRNA and activity. A similar effect was seen with dealcoholized red wine treatment (Pal et al. 2003). Lyophilized grape powder lowered hepatic cholesterol ester concentrations and ACAP activity, while increasing free cholesterol concentrations in guinea pigs as well as miniature pigs and African green monkeys (Carr et al. 1992).

Modifications in the packaging of VLDL through alterations in hepatic enzyme activity and apo secretion are associated with a decrease in plasma triglycerides and apo protein concentrations. A second possible mechanism for the decrease in cholesterol levels with the treatment of grape polyphenols is an induction of the LDL receptor.

Numerous experiments of polyphenols and lipid have been completed in cell culture/animal models, but only a small number have been completed with human subjects. A 200 mg per day dose of grape monomeric and oligomeric flavanols were given for 8 weeks in a clinical trial. It reduced total cholesterol and LDL cholesterol and improved a measure of vascular health, the Vascular Health Index (Weseler et al. 2011). Consumption of 640 mg per day of grape anthocyanin for 4 weeks in a clinical trial of prehypertensive men increased high-density lipoprotein (Hassellund et al. 2013). Consumption of high dosage resveratrol for 2 weeks reduced intestinal apolipoprotein-48 and apolipoprotein-100 in overweight and obese men (Dash et al. 2013). The source of the polyphenols in these studies was wine or a wine-grape mix. These effects were not universal for all polyphenols. Tea, a major source of polyphenols in the form of catechins, did not influence cholesterol concentrations in any of three clinical trials (Dower et al. 2015; Troup et al. 2015; Davies et al. 2003).

10 Cardioprotection by Vasorelaxation

Grapes and red wine exhibit endothelium-dependent relaxation of blood vessels with the enhanced generation and increased biological activity of nitric oxide, leading to the elevation of GMP levels (Fitzpatrick et al. 1993, 1995, 2000; Zenebe et al. 2003). This response has been demonstrated in both animals and patients (Mizutani et al. 1999; Diebolt et al. 2001; Bernátová et al. 2002). The dependence of this mechanism on the endothelium was demonstrated when the endothelium had been removed (Fitzpatrick et al. 1993). Subsequently, it was found that there were changes in nitric oxide production which were induced by cyclic GMP.

Moderate consumption of red wine has been associated with a lowering of the risk of coronary heart disease (Frankel et al. 1993; Maxwell et al. 1994; Aviram and Fuhrman 2002). Red wine is a rich source of anthocyanosides, catechins,

proanthocyanidins, and stilbenes. Red wine polyphenols possess several biological properties including the inhibition of platelet aggregation, vasorelaxation activity, modulation of lipid metabolism, and inhibition of low-density lipoprotein oxidation (Frankel et al. 1993; Maxwell et al. 1994; Santos-Buelga and Scalbert 2000; Bravo 1998; Demrow et al. 1995; Tedesco et al. 2000). Red wine polyphenols aid in the maintenance of vascular endothelial function and reduce endothelial dysfunction, smooth muscle cell proliferation and migration, and platelet aggregation. They also reduce blood pressure and increase vasodilation. Red wine polyphenols also affected the formation of endothelium-derived hyperpolarizing factor, prostacyclin, and endothelium 1. This action took effect through the inhibition of tyrosine kinases (Ndiaye et al. 2003; Schramm et al. 1997; Corder et al. 2001). The critical step in the production of nitric oxide involves an increase of cellular calcium. The changes in cellular calcium could mediate their affect through G proteins, phospholipid AC, and tyrosine kinase. The red wine polyphenols could also increase the bioavailability of nitric oxide and prolong its half-life by preventing its degradation by reactive oxygen species (Ndiaye et al. 2003; Schramm et al. 1997; Corder et al. 2001).

Red wine polyphenolics can have a long-term effect through their modulation of nitric oxide synthetase (Leikert et al. 2002) and cellular adhesion genes through blocking expression of genes of inflammation, which can induce the cellular adhesion genes. Proanthocyanidin treatment can reduce TNF-α expression and TNF-α-induced cellular adhesion molecule expression. These results were also found in one human study (Kalin et al. 2002). Anthocyanidin treatment of the carrageenan model for lung inflammation suppressed ICAM expression in lung tissue (Rossi et al. 2003).

Red wine polyphenols are active in the inhibition of several aspects of cardiovascular disease pathology. These include the reduction of smooth muscle cell proliferation and migration as they contribute toward the progression of intimal thickening and development of artery sclerosis. The inhibition of these activities appears to involve an interaction between red wine polyphenols or their metabolites and expression of platelet-derived growth factor (PDGF). PDGF is a potent mitogen and chemotactic agent for vascular smooth muscle cells, which is released by platelets, smooth muscle cells, and endothelial cells. PDGF exerts its effect through transmembrane receptor tyrosine kinases which are part of alpha- and beta-PDGF receptors. These pathways involve the activation of phosphatidylinositol 3′-kinase (PI3K) and mitogen-activated protein kinase (MAPK) (Knall et al. 1997; Imai and Clemmons 1999; Hedges et al. 1999; Iijima et al. 2000, 2002). A range of polyphenols, including those with molecular weights of 200–400 including monomers of the ACs, flavonoids and catechins, and oligomeric PAs (MW of 1200–1600), had similar antiproliferative effects. These effects may involve cyclin A gene expression and the transcription factors ATF-1 and cAMP-responsive element (CREB). A second pathway may involve PI3K and its interaction with $p27^{kip1}$.

Vascular endothelial growth factor released from smooth muscle cells is a mitogen and stimulates expression of adhesion molecules as well as monocyte chemotactic protein-1 (Ferrara 1999). VEGF can be stimulated by platelet-derived growth factors and alpha-thrombin and transforming growth factor-β1. The

inhibitory effect of red wine polyphenols was due to a reduced phosphorylation of $p38^{MAPK}$. Interestingly, VEGF expression in vascular smooth muscle cells was stimulated by the presence of reactive oxygen species.

11 Blood Pressure

The dietary intake of grapes has been shown to lower blood pressure in some subjects. In two studies, individuals receiving a high-fat meal experienced endothelial dysfunction as measured by flow-mediated dilation of the brachial artery. This acute effect was completely abolished by the concurrent consumption of the freeze-dried grape powder preparation (FDGPP) and resveratrol (Chaves et al. 2009). In a third study, consumption of Concord grape juice decreased blood pressure in subjects with borderline hypertension (Park et al. 2009). It did not improve blood pressure in healthy subjects (Dohadwala et al. 2010). Supplementation with FDGPP for 30 days decreased systolic blood pressure and increased endothelial nitric oxide (relaxation factor) production in subjects with metabolic syndrome (Kar et al. 2009; Razavi et al. 2013; Tomé-Carneiro et al. 2012c; Barona et al. 2012). In these studies, grapes or their products were effective in lowering blood pressure in individuals who were stressed by dietary manipulation or disease. Alternatively, two human trials of polyphenols reported no significant change in cardiovascular endpoints including flow-mediated dilation, blood pressure, platelet function, or blood lipids (van Mierlo et al. 2010; Botden et al. 2012). Also, two trials with polyphenol extracts reported negative results (van Mierlo et al. 2010; Botden et al. 2012). In clinical studies of wine, the consumption of red wine and dealcoholized wine for 4 weeks decreased systolic and diastolic blood pressure and increased plasma nitric oxide (Chiva-Blanch et al. 2012). An acute dose of quercetin decreased blood pressure in men with stage I hypertension (Larson et al. 2012). Dosages of 150 mg per day and 300 mg per day of grape procyanidins for a period of 4 months lowered systolic and diastolic blood pressure and heart rate in healthy prehypertensive and mildly hypertensive adults (Belcaro et al. 2013).

Based on these data, the relationship between grapes and prevention of high blood pressure in healthy subjects is unclear. Utilization as an adjunct treatment may be useful. Nonetheless, epidemiologic studies have indicated a relationship between polyphenols and blood pressure and cardiovascular risk. Two cross-sectional studies in PREDIMED analyzed the association of total and specific polyphenols with blood pressure and risk of cardiovascular disease. In the first study, subjects in the highest quartile of total urinary polyphenols had a reduced prevalence of hypertension compared to those in the lowest quartile (Medina-Remón et al. 2011). In a similar analysis, an association was found in the study of cardiovascular disease risk. The results indicated a 46 % reduction in CVD risk between the highest and lowest quartiles. There was a 33 % reduction in CVD risk for highest intake of anthocyanins (Tresserra-Rimbau et al. 2014). These studies

suggest that polyphenols may be active in the reduction of blood pressure in the general population.

12 Platelet Aggregation

The modulation of platelet aggregation activity is an important method of cardiovascular disease prevention. The most common method is the use of aspirin. Grapes have been associated with the modulation of platelet aggregation and contain several compounds with anti-aggregation activity. Various experiments have shown the presence of the anti-aggregation factor in grapes and its products, including wine. Flavonoid intake in the form of purple grape juice has been associated with anti-aggregation activity in human studies. Cellular, biochemical, and clinical studies have confirmed the presence of a platelet anti-aggregation factor in purple grape juice. Identification of the anti-aggregation factor is an ongoing project. The contribution of anthocyanidins (ACs) and proanthocyanidins (PAs) in the inhibition of platelet aggregation is controversial. While several studies show an anti-aggregating affect, others including human studies do not show any effect. Studies of white wine versus red wine and dealcoholized preparations find the anti-aggregating effect is associated with red wine polyphenols (Demrow et al. 1995; Tedesco et al. 2000; Frémont 2000; Landolfi et al. 1984; Gryglewski et al. 1987; Beretz et al. 1982; Rein et al. 2000; Russo et al. 2001; Pellegrini et al. 1996; Lavy et al. 1994; Pace-Asciak et al. 1996).

13 Resveratrol

Several of the polyphenols have a wide range of activities. Resveratrol possesses diverse biochemical and physiological actions including estrogenic, antiplatelet, and anti-inflammatory properties. These activities are described in separate reviews (Frémont 2000; Bhat et al. 2001; Kimura 2003). Resveratrol also attenuates myocardial ischemia-reperfusion injury and atherosclerosis and reduces ventricular arrhythmias. Resveratrol was identified in the 1940s as a medicinal component of grapes which was useful in a treatment for hyperlipidemia disease (Vastano et al. 2000). It was rediscovered in recent years as an antiproliferative agent for cancer. The first evidence for cardioprotective activities came from studies of alcohol consumption wherein its use decreased morbidity and mortality coronary heart disease (Renaud and de Lorgeril 1992). Direct evidence came from the study of isolated hearts from ischemia-reperfusion injury. The protective mechanisms are antioxidant, anti-inflammatory, and its ability to reduce nitrates oxide synthetase and ability to induce angiogenesis (Hattori et al. 2002). Red wine contains approximately 0.2–7 mg/L. It is also found in numerous fruits and vegetables, not common to the United States diet.

Table 1 Some molecular targets of resveratrol

Adenosine A_1/A_3 receptors	MSK	SIRT1
K_{ATP} channel	MAPKs	Bcl-2/Bax/Bad
COX-2	PKC/PKD/CKII	Akt
TNFα/IL-1β/TGFβ	CREB	iNOS/eNOS
VEGF/KDR	DR4/DR5	TRAIL
$P53/p21^{Cip1}$	Sir2	NK-κB/AP-1
IGF-1	ICAM/VCAM/E-Selectin	NO

Resveratrol has several activities. The first of these is functioning as an antioxidant. However, antioxidant activity is not very potent, although it can scavenge hydroxyl radicals, at rates slower than ascorbic acid (Leonard et al. 2003). Nonetheless, it may be a more potent antioxidant in vivo as it increases nitric oxide synthesis which scavenges superoxide radicals and lowers oxidative stress (Hattori et al. 2002; Cadenas and Barja 1999). This activity was found in several cell lines (Lee et al. 1998). Resveratrol inhibits the formation of reactive oxygen species that have been induced by tumor necrosis factor in a wide variety of cells (Manna et al. 2000). Resveratrol also scavenges peroxyl and hydroxyl radicals in the postischemic heart reperfusion myocardium. Resveratrol induced the levels of several antioxidant enzymes including glutathione reductase and also in the maintenance of glutathione levels in blood cells.

Another activity of resveratrol is actions of a phytoestrogen. Resveratrol can bind to the estrogen receptor and induce the expression of estrogen-dependent genes. However, some controversy exists regarding concentration dependence and whether it has a positive or negative affect (Ashby et al. 1999; Basly et al. 2000; Turner et al. 1999; Freyberger et al. 2001). A number of signaling pathways are modulated by resveratrol. The activities of resveratrol are given as an example and are listed in Table 1. These pathways are related to inflammation and gene expression. Resveratrol is active in the prevention of cancer as it acts on decreasing cell proliferation, increasing apoptotic cells by an increase expression of Bax protein. Thus, it acts on the three major stages of cancer initiation promotion and progression and inhibits the formation of preneoplastic lesions in mouse models (Caltagirone et al. 2000; Tessitore et al. 2000).

14 Conclusions

The dietary intake of grapes and its products has been associated with the prevention of cardiovascular disease. The association has been attributed to the phenolic compounds in grapes. Grapes contain numerous phenolic compounds, which have been shown to have bioactivities relevant to the prevention of cardiovascular disease. These activities include a reduction of blood pressure, oxidative stress, inflammation, platelet aggregation, and endothelial dysfunction, among others.

These effects may result from the activities of individual phenolic compounds or the simultaneous effect of multiple compounds. Whether these effects are the result of individual compounds or whether synergy exists between certain phenolic compounds as is suggested by some experiments is unknown and should remain an active area of investigation. Individual phenolic compounds do have multiple effects as has been demonstrated for resveratrol and other phenolics. The cumulative effect may be a significant lowering of the risk for cardiovascular disease. However, further research is needed for the quantification of these simultaneous multiple effects on cardiovascular disease risk. Substantial progress has been made in defining the mechanisms involved in several of the effects including platelet aggregation, vasodilation, oxidative stress, and inflammation. Identification of these mechanisms has strengthened the associations with cardiovascular disease and defined possible sites of action. While recent years have provided more information on the bioavailability and metabolism of the phenolics, further efforts are needed to define the biologically active metabolites and their effective in vivo concentrations in humans for the biologic effects seen in animal and cell culture experiments. Nonetheless, the biologic basis for phenolic compounds in the prevention of cardiovascular disease is becoming well defined in some instances and is progressing in several areas.

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Grapes and Atherosclerosis

Maria Luz Fernandez and Jacqueline Barona

Contents

Abstract Grapes are a good source of polyphenols, compounds characterized by their antioxidant properties, which may protect against atherosclerosis. Atherosclerosis is defined as the narrowing of the lumen in main arteries resulting in decreased blood flow leading to thrombosis or myocardial infarction. The formation of the atherosclerotic plaque can be accelerated by oxidative stress; thus, it is not surprising that antioxidants in blood can ameliorate this process. Evidence from a number of clinical interventions and animal studies has demonstrated that grape polyphenols affect several metabolic processes that lead to reductions in atherosclerosis including decreases in LDL oxidation and platelet aggregation, increases in flow-mediated vasodilation, reduction in inflammatory cytokines, as well as reduced concentration of cholesterol in the aorta. Grape polyphenols have also been shown to decrease atherosclerotic plaques in animal studies. Polyphenols also exhibit their effects at the cellular level including reduced nuclear factor-kappaβ (NF-κβ) production in mononuclear cells, upregulation of endothelial nitric oxide synthetase (eNOS), inhibition of platelet-derived growth factor receptor, decreased

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cyclooxygenase (COX) activity, and induction of endothelin-1 inhibition, among others. Overall, there appears to be strong evidence that grape polyphenols exert protective effects against atherosclerosis.

1 Introduction

Cardiovascular diseases (CVDs) are the first cause of death worldwide, with an estimation of 17.5 million deaths in 2012 (World Health 2015). Atherosclerosis is the underlying cause of CVD, which is a complex and multifactorial process associated with atherogenic dyslipidemia, increased oxidative stress, and inflammation (Sessa et al. 2014; Back et al. 2015). Interaction between modified lipoproteins (e.g., by oxidation), monocyte-derived macrophages, T cells, and cells of the arterial wall results in a chronic inflammatory process, that leads to formation of atherosclerotic plaques (Back et al. 2015). Several risk factors contribute to atherosclerosis development; most of them are related to lifestyle (tobacco use, unhealthy diet, and physical inactivity) and can be modified (Rafieian-Kopaei et al. 2014). Bioactive compounds from the diet targeting the multifactorial atherosclerotic process are of great interest, and grapes contain numerous polyphenols (reviewed in this chapter) with demonstrated potential to modulate positively multiple key players (pro-inflammatory cytokines, enzymes, transcription factors, lipoproteins, reactive oxygen species) to prevent or reduce this disease. There are many publications regarding the beneficial effects of grapes for human health; however, since atherosclerosis is for the most part an irreversible process, it is important to identify the best dietary approaches with grapes/grape products that have been successful for further guidelines.

This chapter will consider evidence from epidemiological and clinical studies to animal models and cell studies (in vitro and ex vivo) supporting the effects of grapes or grape products to ameliorate atherosclerosis or surrogate markers of this process. Most human studies presented here are randomized, double or triple bind, and placebo controlled to provide the most reliable evidence about grape supplementation effects. Besides the study design, also the dose, time of supplementation, matrix used, and/or the type of grape product (grape juice, dealcoholized wine, and grape-based extracts from grape seed, grape skin, whole grape, and grape pomace) are described as they may impact the bioavailability and success of a given study. In addition, information about the type of population studied (healthy patients, pre- and postmenopausal women, high-risk patients, or patients with cardiovascular disease) is analyzed, as it is important when designing adequate dietary recommendations for grapes.

The animal and cell studies provide a more direct evidence of the mechanism of action of the different polyphenols present in grapes and may help explain the beneficial effects observed in humans. In this regard, it is important to note that

researchers nowadays are using concentrations of grape/grape products more appropriate for supplementation, which are equivalent to human consumption rather than pharmacological doses. Also, we present information from animal models such as guinea pigs, which have shown to present similar cardiovascular risk factors, lipid metabolism, and atherosclerotic process to humans, as well as similar responses to diet and hypocholesterolemic drugs (West and Fernandez 2004).

2 Grape Composition

Grapes belong to the genus *Vitis*, which comprises more than 70 species growing widely in distinct geographical areas (Teixeira et al. 2013), from which over 70 % species are native to North America (Yang and Xiao 2013). The most recognized species is *Vitis vinifera,* with more than 10,000 cultivars, due to its high morphological and genetic diversity. In addition, climatic conditions determine, to a large degree, the grape varieties that can be cultivated. Heat, drought, and light/UV intensity severely affect phenolic metabolism and, thus, grape composition and development (Teixeira et al. 2013). For example, when ten different varieties of white table grape were compared, Rolle et al. (2011) reported that those with higher total phenols in the skin had lower total phenols in the flesh. Therefore, the differences in phenolic composition observed across grape varieties might impact their respective health benefits (Teixeira et al. 2013).

All phenolic compounds possess an aromatic ring bearing one or more hydroxyl group (Shi et al. 2003). Phenolic compounds of the grape are divided into non-flavonoid characterized by having a simple C6 backbone including hydroxybenzoic acids, hydroxycinnamic acids, volatile phenols, and stilbenes and flavonoid compounds composed of 15 carbon atoms (Teixeira et al. 2014): flavones, flavonols, flavanones, flavan-3-ols, and anthocyanins (Teixeira et al. 2013). These flavonoid compounds naturally occur as mostly conjugated in glycosylated or esterified forms, but can also occur as aglycones (Yang and Xiao 2013). The generic structure of flavonoids consists of two aromatic rings (A and B rings) linked by three carbons that are usually in an oxygenated heterocycle ring called a C ring; differences in the latter give rise to the flavonoid compound classification (Yang and Xiao 2013).

Among flavonoids, flavan-3-ols or flavanols are the most abundant phenolics in grapes, which are found in similar concentrations in skins and in seeds (Teixeira et al. 2014). Flavan-3-ols include catechins, the monomeric units for the proanthocyanidin biosynthesis (Teixeira et al. 2013). Proanthocyanidins (also known as tannins) are mainly present in seed, skin, and stem tissues of the grapes as both oligomeric and polymeric compounds. In grape seeds, proanthocyanidins represent the major fraction of the total polyphenol extract (Iriti and Faoro 2009), and their composition differs between seeds and skin with the seeds having shorter polymers comprised of similar amounts of catechin and epicatechin subunits. In the skin,

proanthocyanidin polymers tend to be much longer and comprised mainly of epicatechin subunits (Downey et al. 2006). Anthocyanins, which are conjugated derivatives of anthocyanidins, mainly bound to sugars, hydroxycinnamates, or organic acids, are water-soluble pigments conferring the red and blue colors found in the skins of blue, red, or black grapes (Teixeira et al. 2013; Shi et al. 2003; Iriti and Faoro 2009). Anthocyanins of *Vitis* are structurally based on five aglycones/anthocyanidins, malvidin, cyanidin, delphinidin, peonidin, and petunidin, which are classified depending on the number and position of their hydroxyl groups and methylation degree (Iriti and Faoro 2009). Red grapes can produce anthocyanins, while white grapes cannot; therefore, the total phenolic level of red grape skins is higher than that of white grapes (Yang and Xiao 2013).

Flavonols are another class of flavonoids that differ by the number and type of substituents on the B ring and are normally glycosylated at the C-3 position of the C ring giving glucosides, galactosides, rhamnosides, and glucuronides forms (Teixeira et al. 2013; Flamini et al. 2013). Grapes synthesize flavonols such as kaempferol, quercetin, and myricetin, and the methylated forms isorhamnetin (from quercetin), laricitrin, and syringetin (both from myricetin) (Teixeira et al. 2013; Flamini et al. 2013). The analysis of the flavonol profile conducted in 91 grape varieties presented several differences. In red grapes, the main flavonol was quercetin (mean = 43.99 %), followed by myricetin (36.81 %), kaempferol (6.43 %), laricitrin (5.65 %), isorhamnetin (3.89 %), and syringetin (3.22 %). In white grapes, the main flavonol was quercetin (mean = 81.35 %), followed by kaempferol (16.91 %) and isorhamnetin (1.74 %). The delphinidin-like flavonols myricetin, laricitrin, and syringetin were missing in all white varieties, indicating that the enzyme flavonoid 3′,5′-hydroxylase is not expressed in white grape varieties (Mattivi et al. 2006). Flavones differ from flavonols by the absence of the hydroxyl group in carbon 3 (Teixeira et al. 2014). Apigenin and luteolin are the main flavones in grapes (Yang and Xiao 2013; Teixeira et al. 2014).

Among non-flavonoid compounds, the hydroxycinnamates or hydroxycinnamic acids are the third most abundant class of soluble phenolics in grape berries, after proanthocyanidins and anthocyanins. These compounds accumulate mainly in the flesh; however, they are also present in other grape tissues (Teixeira et al. 2013). The predominant hydroxycinnamic acids in grapes are *p*-coumaric, caffeic, and ferulic acids, which differ by the type and number of substituents on the aromatic ring (Teixeira et al. 2013). The hydroxybenzoic acids are present in lower quantities and include gentisic acid, salicylic acid, gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, tannic acid, vanillic acid, and syringic acid, mainly found in their free form (Teixeira et al. 2013, 2014). Gallic acid is the most abundant hydroxybenzoic acid derivative in grape stems, skins, and seeds, followed by syringic acid in grape stems and protocatechuic acid in grape seeds and skins (Teixeira et al. 2014).

Other non-flavonoid compounds present in grapes are stilbenes, which structure comprises two aromatic rings linked by an ethane bridge (Yang and Xiao 2013). The main grape stilbenes are *cis*- and *trans*-resveratrol (3,5,4′-trihydroxystilbene), resveratrol-3-*O*-β-D-glucopyranoside (piceid), piceatannol (3,4,3′,5′-tetrahydroxy-

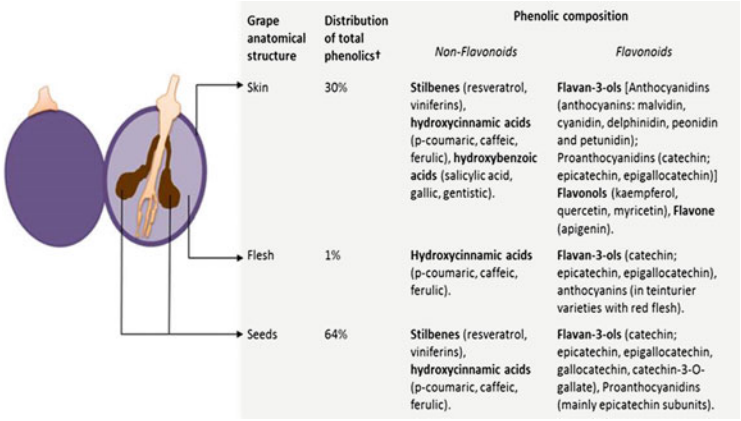

Fig. 1 Anatomical localization of the main phenolics in grapes. Data is taken from Yang and Xiao (2013), Teixeira et al. (2014), Iriti and Faoro (2009), and Flamini et al. (2013). Five percent of total phenolics in grape juice

trans-stilbene), and resveratrol oligomers (viniferins) (Flamini et al. 2013). Molecules belonging to the stilbene family possess the skeleton based on the *trans*-resveratrol structure (Teixeira et al. 2013; Iriti and Faoro 2009). The *trans*-resveratrol is distributed mostly in the skin, even though the concentration of this stilbene in grape skins and seeds varies considerably within *Vitis* germplasm (Teixeira et al. 2014). Although present in trace quantities, the synthesis of these compounds can increase in response to pathogenic attack and environmental stress such as injury, UV irradiation, or fungal infection (Teixeira et al. 2013, 2014; Yang and Xiao 2013).

It is important to note that the most powerful antioxidants, resveratrol, quercetin, rutin, catechin, epicatechin, and epicatechin gallate, are located in the skin of grapes (Sadovoy et al. 2011). Figure 1 presents the phenolic composition commonly described in grapes. In addition to phenolics, grape phytochemicals from the skin, seed, and juice also include other bioactive compounds like carotenoids and melatonin (Yang and Xiao 2013; Iriti and Faoro 2009). Soluble and insoluble dietary fiber, vitamin C, minerals (calcium, magnesium, iron, zinc, and manganese), lipids, proteins, and carbohydrate (fructose, glucose, and sucrose) are also important components of grapes (Sadovoy et al. 2011).

3 Atherosclerosis

Coronary heart disease is the leading cause of death in industrialized countries, and this trend continues to increase with a predicted 12 % increase by the year 2020 (Huffman et al. 2013). The main underlying cause of heart disease is atherosclerosis, a complex and multifactorial condition associated with oxidative stress and

inflammation (Huffman et al. 2013). Atherosclerosis or hardening of the arteries leads to the narrowing of the vessel lumen and the impairment of blood flow. It is a complicated process involving cholesterol accumulation in macrophages via modified LDL or remnant lipoproteins, which incites secretion of inflammatory cytokines released by macrophages trapped into the endothelium (Raines and Ferri 2005). These inflammatory conditions lead to smooth muscle cell proliferation and matrix synthesis, major contributors to atherosclerosis (Raines and Ferri 2005).

The first step in this process is the accumulation of LDL in the intima of the vessel as a primary event, which leads to the formation of minimally oxidized LDL (oxLDL). This modified lipoprotein promotes the differentiation of monocytes into macrophages with the concomitant expression of scavenger receptors (SR) including SR-A and cluster of differentiation (CD) 36 (Glass and Witztum 2001). This leads to unregulated uptake of LDL and cell foam formation, the first step in the atherosclerosis process. The continuous accumulation of cholesterol further leads to the formation of a necrotic center containing cholesteryl esters, cell debris, and macrophages. Advanced atherosclerosis results in a progressive narrowing of the vessels leading to ischemia. However, acute myocardial infarction largely results from plaque rupture and thrombosis (Sessa et al. 2014).

Atherosclerosis, for the most part, is an irreversible process. Thus, prevention of this condition, especially by dietary factors, mainly antioxidants, will be the focus of this chapter. There are several risk factors contributing to atherosclerosis, which can be classified in two major categories, behavioral and metabolic (Aggarwal et al. 2012). Behavioral refers to lifestyle and could be potentially modified including smoking, unhealthy diets, and lack of exercise. The metabolic factors can be a result of lifestyle or genetic predisposition (Montagnana et al. 2014), such as high blood pressure, elevated blood cholesterol, diabetes, and obesity.

Vascular function in the endothelium is regulated by the release of vasoconstrictors and vasodilators, of which nitric oxide has a central role (Vogel 2001). Endothelial function and how it is affected by diet can be measured by flow-mediated vasodilation (FMD) by assessing changes in brachial artery diameter after a 5 min constriction with a blood pressure cuff (Harris et al. 2010). FMD can be measured by high-frequency ultrasound providing key information on endothelial health and atherosclerosis risk (Ghiadoni et al. 2008). Antioxidants have been shown to prevent reductions in FMD in diabetic individuals, in hypercholesterolemic patients, and in those at risk for heart disease (Schini-Kerth et al. 2010).

There are several lines of evidence derived from epidemiological observations, clinical studies, and mechanistic studies with animal models and cells that support that the anti-oxidative effects of grape polyphenols can protect against atherosclerosis. These will be reviewed in the following paragraphs.

4 Epidemiological Information

Epidemiological studies have shown that a diet rich in fruits and vegetables prevents coronary heart disease and other conditions including type 2 diabetes, which eventually can lead to atherosclerosis and heart disease (Dalen and Devries 2014). For example, an evaluation of type 2 diabetes risk and fruit consumption was carried out in three longitudinal studies, The Nurses' Health Study (1984–2008), Nurses' Health Study II (1991–2009), and Health Professionals Follow-up Study (1986–2008) for a total of 187,382 individuals (Muraki et al. 2013). Investigators concluded that greater consumption of whole fruits including grapes, berries, and apples was significantly associated with lower risk of type 2 diabetes. In another study, conducted with 667 individuals, it was demonstrated that a healthy diet rich in fruits and vegetables decreased systemic inflammation (Dias et al. 2015) as determined by measurements of inflammatory cytokines and expression of CD14 and CD16, two monocyte subpopulations characterized for higher potency in antigen presentation and for being pro-inflammatory (Ziegler-Heitbrock 2007). These healthy effects of fruits and vegetables have been attributed in part to the antioxidant and anti-inflammatory properties of polyphenols. It has also been reported that proanthocyanidins, a class of polyphenols found in grapes, have a greater antioxidant effect compared to vitamin C or vitamin E in aqueous media, and proanthocyanidin-rich grape extract has been demonstrated to lower atherosclerosis, cataracts, and diabetes (Ariga 2004).

Grape polyphenols are one of the most studied for their documented protection against heart disease (Howes and Simmonds 2014; Vislocky and Fernandez 2010), and epidemiological studies acknowledge that consumption of grapes is associated with decreased cardiovascular risk (Dohadwala and Vita 2009). A review of epidemiological studies leads to the conclusion that in addition to the recognized antioxidant and anti-inflammatory properties of grape polyphenols, emerging data suggest protection against atherosclerosis through modulation of cellular lipid metabolism (Zanotti et al. 2015). Grape polyphenols have been shown to have other mechanisms by which grapes protect against atherosclerosis including decreases in platelet aggregation and in LDL oxidation (Wightman and Heuberger 2014). Endothelial dysfunction is considered an early feature in the progression of atherosclerosis (Hansson 2005), and flow-mediated vasodilation has been used to measure this function. Studies have demonstrated that polyphenols enhance the production of nitric oxide (NO) thus increasing vasodilation (Stoclet et al. 2004). A meta-analysis that included nine studies demonstrated that grape polyphenols increase flow-mediated vasodilation compared to controls (Li et al. 2013).

Resveratrol, a stilbene present in grapes, has also shown to exert a number of protective effects against cardiovascular disease risk (Delmas et al. 2005). In contrast, a study measuring urinary resveratrol, in an older population in the Chianti region, was not associated with cardiovascular disease or inflammatory markers suggesting that the amount of resveratrol consumed in the Western diet might not be sufficient to prevent heart disease risk (Semba et al. 2014). However, for the

most part, epidemiological data supports the role of grape polyphenols as potent antioxidants that protect against atherosclerosis development.

5 Clinical Studies

Several studies have demonstrated anti-atherogenic effects after consuming whole grapes, grape products (juice, extracts, dealcoholized wine, and wine), and individual components from grapes such as resveratrol. These protective effects are related to the improvement of endothelial dysfunction (increased FMD response, enhanced NO bioavailability, lowering blood pressure), positive modulation of the atherogenic lipid profile, prevention of LDL oxidation, and anti-inflammatory activities of the grape phytochemicals.

Endothelial dysfunction is one of the first clinically detectable alterations in atherosclerosis development (Sankatsing et al. 2005). Grapes have shown beneficial effects on endothelial function measured by FMD in healthy subjects as well as in people with cardiovascular risk factors. In 30 male patients with coronary heart disease, Lekakis et al. (2005) evaluated the acute intake effects of a red grape polyphenol extract (RGPE) on FMD, which was measured after fasting and 30, 60, and 120 min after the intake of the RGPE or placebo (water). They found a significant increase on the FMD response peaking at 60 min, compare to the placebo group. However, the authors pointed out that the RGPE that was given for each subject was derived from 1 kg of red grapes, a quantity that is rather unusual to be consumed even for a person following a Mediterranean diet (Lekakis et al. 2005). However, other studies using more practical doses (corresponding to 1.25 g to 2 cups fresh grapes) with standardized grape products have found similar results, increasing the FMD response after both acute and chronic intakes, compared to placebo (Chaves et al. 2009; Barona et al. 2012). Interestingly, a meta-analysis (described in the previous section) presenting the results of nine controlled trials on the acute effects of grape polyphenol consumption on FMD found that the FMD response significantly increased in the initial 2 h after intake of grape polyphenols as compared with controls, with a peak effect at 30 min in healthy participants compared to a more pronounce but delayed peak at 60 min in volunteers with cardiovascular risk factors (Li et al. 2013). The authors proposed that in these subjects with high cardiovascular risk factors, endothelial cells have been impaired and might require 60 min after the ingestion of grape polyphenols to produce adequate nitric oxide to improve endothelial function, resulting in the delayed effect. However, they also noted that exact mechanisms are still unclear and need to be explored in the future (Li et al. 2013).

Studies have also demonstrated acute and chronic effects of individual compounds from grapes on FMD. A significant dose-dependent increase on FMD, compared to placebo, and a linear relationship to the plasma resveratrol concentration, was found in a double-blind randomized crossover study testing the acute effects of three doses (30, 90, 270 mg) of resveratrol and placebo in 19 overweight/

obese (BMI 25–35 kg/m^2) men or postmenopausal women with untreated borderline hypertension (systolic blood pressure of 130–160 mmHg or diastolic blood pressure equal to 85–100 mmHg) (Wong et al. 2011). Using the same study design, these authors also evaluated the effects of chronic consumption (during 6 weeks) of 75 mg of resveratrol on a daily basis, in 28 obese but otherwise healthy adults with a BMI of 33.3 ± 0.6 kg/m^2. The authors reported a significant 23 % increase in the FMD response, compared to placebo (Wong et al. 2013). These and other authors have suggested that the improvements in FMD response after consumption of grape polyphenols and some individual components such as resveratrol may be related to an increase in endothelium-derived NO bioavailability. NO acts as an anti-atherosclerotic molecule in the vasculature; it induces vasodilation and inhibits platelet aggregation, leukocyte adhesion to the endothelium, and proliferation of vascular smooth muscle cells in response to injury. Some studies with grape juice, resveratrol, and nonalcoholic wine extracts have demonstrated increases in NO production by human platelets (Freedman et al. 2001; Gresele et al. 2008) and endothelial cells (Simoncini et al. 2011).

Hyperlipidemia, especially elevated LDL cholesterol, can lead to atherosclerosis by lipoprotein deposition inside the vessel wall, and oxidative stress induction and oxLDL are key players in the formation of atherosclerotic plaques (Back et al. 2015). These atherogenic markers have been shown to be modulated by the consumption of grapes/grape products. For example, supplementation studies up to 2 or 4 weeks with red grape juice or lyophilized grape powder, respectively, have demonstrated, in hemodialysis patients (Castilla et al. 2006) and in postmenopausal women (Zern et al. 2005), significant reductions on plasma concentrations of triglycerides, oxLDL levels, LDL cholesterol, and apolipoprotein B100, a marker of atherogenic particles, while increasing the concentrations of HDL cholesterol and apolipoprotein A-I and reducing urinary F2-isoprostanes, a marker of whole-body oxidative stress. A randomized double-blind placebo-controlled crossover study with 52 mildly hyperlipidemic individuals who received a capsule containing either 200 mg/day of a red grape seed extract (RGSE) or placebo for 8 weeks showed that RGSE consumption reduced significantly total cholesterol, LDL cholesterol, and oxLDL, compared to placebo (Razavi et al. 2013). Another study with a more challenging population needed more time of supplementation to observe similar effects. For example, a randomized, triple-blind, and placebo-controlled study with statin-treated patients ($n = 75$) undergoing primary prevention of cardiovascular disease with at least one risk factor including active tobacco use, arterial hypertension, and/or overweight/obesity evaluated the effects of consuming 350 mg/day (in a capsule) of either a resveratrol-enriched grape extract (RE-GE), a grape extract (GE), or placebo during 6 months and a double dose for the next 6 months. After 1-year follow-up, the RE-GE induced a significant decrease in LDL cholesterol, ApoB100, and oxLDL, whereas the ratio non-HDLc (total atherogenic cholesterol load)/ApoB increased (Tomé-Carneiro et al. 2012a). It is interesting to note from these studies that although the cardiovascular risk may differ among the populations studied, the presentation of the grape product may also have an impact

in its bioavailability and hence more time of supplementation to achieve beneficial effects (Ortuño et al. 2010).

Inflammation has a significant role in the atherosclerotic lesion development, which is driven by the release of local cytokines acting upon the endothelium and smooth muscle cells. After 1-year follow-up in the same cohort of subjects of the previous study (Tomé-Carneiro et al. 2012a), the authors further reported improvements of their inflammatory profile mainly by decreasing high-sensitivity C-reactive protein (CRP), tumor necrosis factor-α, and interleukin-6/interleukin-10 ratio and fibrinolytic profile by decreasing plasminogen activator inhibitor type 1 (PAI-1) status after consumption of RE-GE (Tomé-Carneiro et al. 2012b). Importantly, using this same study design and grape products (RE-GE, GE) in a high-risk population ($n = 75$) with stable coronary artery disease and receiving a higher dose of statins, Tomé-Carneiro et al. (Tomé-Carneiro et al. 2013) reported an increase of the anti-inflammatory serum adiponectin and a decrease of the thrombogenic PAI-1 in the RE-GE group, in contrast to GE and placebo groups, after 12 months of intervention. Additionally, RE-GE downregulated pro-inflammatory gene expression in peripheral blood mononuclear cells (PBMC) extracted from the participants.

Although the previous studies have shown beneficial effects after grape supplementation, using a similar study design (double blind, randomized, crossover, and placebo controlled), a dietary supplementation for 4 weeks with muscadine grape seed (2 capsules = 1300 mg daily) in 50 subjects with or at high risk for cardiovascular disease did not produce a statistically significant increase in FMD or a significant change in CRP, lipid peroxidation, or antioxidant capacity, compared to placebo (Mellen et al. 2010). Another crossover study evaluated the consumption effects of polyphenol-rich solids derived from either a wine grape mix or grape seed or placebo given as six capsules per day each of 500 mg during 2-week periods in healthy participants ($n = 35$). At the end of each treatment period, participants took three capsules with a low-fat breakfast and three capsules with a high-fat lunch (van Mierlo et al. 2010). The authors did not find significant differences in FMD response, platelet function, or serum lipids between the grape treatments and placebo (van Mierlo et al. 2010). These two studies have in common the presentation of the grape product in capsules and maybe a short supplementation period for a high-risk population as in the first study (Mellen et al. 2010). These authors argued that the muscadine grape (*Vitis rotundifolia*) has different phytochemical composition than other grape varieties (e.g., *Vitis vinifera*, *Vitis labrusca*) used in clinical studies with positive results (Lekakis et al. 2005). Adding to this, Carrieri et al. (2013) reported different efficacies of the antithrombotic activity of 12 table grape varieties showing different polyphenolic profiles. A summary of the beneficial effects of grapes or grape products on atherosclerosis or surrogate markers from clinical studies is presented in Table 1.

Table 1 Clinical studies of grape consumption beneficial effects on atherosclerosis and/or surrogate markers

Atherosclerotic surrogate marker	Population studied	Trial design/grape product used/ dose and time of intervention	Main results	References
Flow-mediated vasodilation (FMD)	Patients with coronary heart disease ($n = 30$, males); mean age 61 year	Random assignment either to a red grape polyphenol extract (RGPE) (600 mg from 1 kg of red grapes) dissolved in 20 mL of water ($n = 15$) or 20 mL of water (placebo) ($n = 15$). FMD measured after fasting, 30, 60, and 120 min	RGPE induced a significant increase on the FMD response peaking at 60 min, compared to the placebo group	Lekakis et al. (2005)
	Healthy normal young subjects ($n = 5$, males); mean age 24 year	Subjects received a single dose of a freeze-dried standardized grape product (GP) (equivalent to 1.25 cup fresh grapes) Placebo-matched sugar content of GP Chronic consumption: GP twice daily for 3 weeks Consumption of a high-fat (HF) meal alone and in conjunction with GP HF meal: 900 total cal, 49 g total fat, 13 g saturated fat, and 245 mg cholesterol	Acute consumption: improvement in FMD within 3 h when compared to control (sugar solution) Chronic consumption: FMD was improved and total anti-oxidant capacity in plasma was increased Concomitant consumption of HF meal prevented 50 % reduction in FMD after 45 min (induced by this HF meal) after 45 min	Chaves et al. (2009)
	Patients with metabolic syndrome ($n = 24$, males); mean age 51.3 ± 9.6 years	Randomized, double-blind, placebo-controlled, crossover design. Patients consumed either a freeze-dried grape polyphenol powder (GRAPE) (equivalent to two cups of fresh grapes) or a placebo (matching all macronutrients except grape polyphenols) for 30 day periods, separated by a 3-week washout period	FMD response increased systolic blood pressure and plasma sICAM-1 decreased during the GRAPE compared with the placebo period	Barona et al. (2012)

(continued)

Table 1 (continued)

Atherosclerotic surrogate marker	Population studied	Trial design/grape product used/ dose and time of intervention	Main results	References
	Healthy participants ($n = 94$), mean age 22–35 year; patients with CHD ($n = 30$), mean age 61 year; smokers ($n = 20$), mean age 29 year	Randomized, double-blind, placebo-controlled, crossover design Dose of grape polyphenols between 600 and 1200 mg. Source: red wine, red wine dealcoholized, red grape polyphenol extract, organic red grape juice with and without alcohol FMD measured at 30, 60, 120, and 180 min after polyphenol consumption	FMD significantly increased in the initial 2 h after intake of grape polyphenols as compared with controls. Peak at 30 min in healthy participants compared to a more pronounced but delayed peak at 60 min in volunteers with CHD	Li et al. (2013)
	Overweight/obese (BMI 28.7 ± 0.5 kg/m^2) men ($n = 14$) or postmenopausal women ($n = 5$) with untreated borderline hypertension (BP $141 \pm 2/89 \pm 1$ mmHg); aged 55 ± 2 years	Randomized, double-blind, crossover design. Acute effects of 30, 90, and 270 mg of synthetic *trans*-resveratrol (resVida™) in a single dose and placebo with 1-week washout between doses Plasma resveratrol and FMD measured 1 h after resveratrol consumption	Significant dose-dependent increase on FMD, compared to placebo, and a linear relationship to the plasma resveratrol concentration	Wong et al. (2011)
	Obese but otherwise healthy adults (BMI: 33.3 ± 0.6 kg/m^2) [$n = 28$ (12 men, 16 women)]; mean age 61 ± 1.3 years	Randomized, double-blind, crossover design. Chronic (6 weeks) effects of 75 mg of resveratrol (capsule) on a daily basis. On test day, FMD was assessed after at least 18 h of last capsule consumption. Following this, supplement was consumed again and FMD was measured after 1 h	Chronic supplementation resulted in a significant (relative 23 %) increase in FMD response, compared to placebo A single dose of resveratrol (75 mg) following chronic resveratrol supplementation resulted in a 35 % greater acute FMD response compared to placebo	Wong et al. (2013)

Nitric oxide (NO) bioavailability	Healthy (normal) subjects ($n = 20$, 12 male/8 female); mean age 30.6 ± 1.8 years	All participants received a supplementation with 7 mL/kg/day of purple grape juice (Welch's) for 14 days. Measurements were done before and after supplementation	Increase in platelet-derived NO release, inhibition of platelet aggregation, and decreased superoxide production	Freedman et al. (2001)
	Healthy volunteers ($n = 20$, 9 males/11 females); age ranged from 37 to 51 year	Random allocation to drink 300 mL of red ($n = 10$) (total polyphenolic concentration, 1.8 g/L) or white ($n = 10$) (total polyphenolic concentration 0.25 g/L) wine for 15 days. Blood was taken in fasting conditions and platelets were obtained. Measurements were done at baseline and after 15 days of wine consumption	Increases in plasma resveratrol concentration and release of NO by stimulated platelets, after wine intake Resveratrol enhanced significantly NO production, stimulated platelet NO synthase, and inhibited reactive oxygen species (ROS) production and platelet function	Gresele et al. (2008)
Lipid-lipoprotein profile/LDL oxidation/oxidative stress	Hemodialysis patients with cardiovascular risk factors ($n = 38$, 19 men/19 women); aged 55.4–65.4 year Healthy participants ($n = 15$, 10 women/5 men); aged 34.4 ± 3.3 years	Hemodialysis patients were randomly assigned to the supplementation ($n = 26$) (100 mL of concentrated red grape juice (RGJ) for 14 days. Total polyphenols: 0.64 g/100 mL) or control group ($n = 12$) Healthy group consumed the RGJ for 14 days Measurements were done at different time points [baseline, during intervention (7 days, 14 days), and follow-up after withdrawal of RGJ supplementation (4 weeks and 6 months)]	Decreases in LDL-C and ApoB concentrations, while increasing in HDL-C and ApoA-I. OxLDL also decreased after RGJ intake, with greater changes at 14 days, both in hemodialysis and healthy groups No changes were observed in the control group	Castilla et al. (2006)

(continued)

Table 1 (continued)

Atherosclerotic surrogate marker	Population studied	Trial design/grape product used/ dose and time of intervention	Main results	References
	Pre($n = 24$) (39.7 ± 8.5 years)- and post($n = 20$) (58.5 ± 7.5 years)-menopausal women	Randomized, single-blind, placebo-controlled, crossover design. Subjects consumed a lyophilized grape powder (LGP) (equivalent to 1.5 cups/day of grapes, total phenols: 5.8 g/kg) or placebo (fructose/glucose; 1:1) for 4-week periods, separated by a 3-week washout period	LPG supplementation lowered plasma TG, LDL-C, ApoB, ApoE, oxidative stress (urinary isoprostanes), and pro-inflammatory TNF-α concentration, in both pre- and postmenopausal women	Zern et al. (2005)
	Mildly hyperlipidemic (TG, 151–300 mg/dL; TC, 201–250 mg/dL) patients ($n = 48$, 20 males/28 females); mean age 48.22 ± 9.07 years	Randomized, double-blind, placebo-controlled crossover study. Individuals received two capsules containing either 200 mg/day of the red grape seed extract (RGSE) (equivalent to 5–8 grape seeds) or placebo (starch) for 8-week periods, separated by an 8-week washout period	RGSE consumption reduced significantly TC, LDL-C, and oxLDL	Razavi et al. (2013)
	Patients on statin treatment and at high risk of CVD (diabetes or hypercholesterolemia with at least one of the following: active tobacco smoking, hypertension, overweight/obesity) ($n = 75$, 34 males/41 females); age ranged from 45 to 72 years	Three parallel arms, randomized, triple-blind, placebo-controlled trial. Follow-up: 6 months. Daily ingestion of one capsule containing either 350 mg of placebo (maltodextrin) ($n = 25$), resveratrol-containing grape extract (RE-GE, Stilvid®, grape phenolics + 8 mg resveratrol) ($n = 25$), or conventional grape extract lacking resveratrol (GE) ($n = 25$)	RE-GE decreased LDL-C, ApoB, and oxLDL in patients beyond their treatment according to guidelines for primary prevention of CVD, oxLDL/ApoB ratio decreased non-HDLc/ApoB increased in this group after 6 months. GE supplementation reduced only LDL-C No changes were observed in the placebo group	Tomé-Carneiro et al. (2012a)

		Measurements were done at baseline and after 6 months		
Inflammatory/fibrinolytic markers	Patients on statin treatment and at high risk of CVD ($n = 75$) [same cohort as in Tomé-Carneiro et al. (2012a)]	Three parallel arms, randomized, triple-blind, dose-response, placebo-controlled trial. Follow-up: 12 months. Daily ingestion of a capsule containing either 350 mg of placebo (maltodextrin) ($n = 25$), resveratrol-containing grape extract (RE-GE, grape phenolics + 8 mg resveratrol) ($n = 25$), or conventional grape extract lacking resveratrol (GE) ($n = 25$) for 6 months and the double dose for the following 6 months. Measurements were done at baseline, 6 and 12 months after intervention	RE-GE significantly decreased hs-CRP, TNF-α, PAI-1, and IL-6/IL-10 ratio, and increased IL-10 after 12 months of intervention No change was observed in any marker in the GE group The placebo group increased hs-CRP after 12 months	Tomé-Carneiro et al. (2012b)
	Patients with stable coronary artery disease (CAD) treated according to current accepted guidelines for secondary prevention of CVD ($n = 75$); age ranged from 49 to 72 years	Three parallel arms, randomized, triple-blind, dose-response, placebo-controlled trial. Follow-up: 12 months. Daily ingestion of a capsule containing either 350 mg of placebo (maltodextrin) ($n = 25$), resveratrol-containing grape extract (RE-GE, grape phenolics + 8 mg resveratrol) ($n = 25$), or conventional grape extract lacking resveratrol (GE) for 6 months and the double dose for the following 6 months Measurements were done at baseline, 6 and 12 months	RE-GE significantly increased adiponectin levels and decreased PAI-1 levels after 12 months. Downregulation of pro-inflammatory gene expression in PBMCs isolated from RE-GE group patients was observed In the GE group, no marker was significantly affected In the placebo group, the anti-inflammatory IL-10 and adiponectin significantly decreased	Tomé-Carneiro et al. (2013)

Abbreviations: *TC* total cholesterol, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *ApoB* apolipoprotein B, *ApoA-I* apolipoprotein A-I, *ApoE* apolipoprotein E, *oxLDL* oxidized LDL, *TG* triglycerides, *CRP* C-reactive protein, *TNF-α* tumor necrosis factor alpha, *PAI-1* plasminogen activator inhibitor type 1, *IL* interleukin, *CVD* cardiovascular disease, *PBMC* peripheral blood mononuclear cells, *RE-GE* resveratrol-enriched grape extract, *GE* grape extract

6 Animal Studies

There are several animal studies carried out using freeze-dried powder made from fresh grapes or specific polyphenols found in grapes such as resveratrol and anthocyanins. Using a grape powder (GP) preparation, a study was conducted in guinea pigs to evaluate whether grape polyphenols could reduce early atherosclerosis development in these animals after being fed a hypercholesterolemic diet (Zern et al. 2003). Guinea pigs were divided into two groups, those consuming the control diet with 0.25 % dietary cholesterol and those who consumed the hypercholesterolemic diet in combination with 10 % GP. The composition of this GP was as follows: total phenols 0.58 g/100 g, flavans 0.41 g/100 g, anthocyanins 0.077 g/100 g, quercetin 10.2 μmol/100 g, myricetin 0.8 μmol/100 g, kaempferol 1.1 μmol/100 g, and resveratrol 0.7 μmol/100 g. Guinea pigs fed the GP presented lower concentration of cholesterol in aortas plus lower activity of acyl CoA acyltransferase in the liver suggesting less incorporation of cholesteryl ester into the secreted very low-density lipoprotein (VLDL) (Zern and Fernandez 2005), leading to lower concentrations of plasma triglycerides (Zern and Fernandez 2005). A study in apolipoprotein E (ApoE) knockout mice showed a 41 % reduction in atherosclerotic lesions compared to control animals after consumption of GP for 10 weeks (Fuhrman et al. 2005). The mice consuming the GP also had 33 % reduction in macrophage uptake of oxidized LDL plus 22 % increase in antioxidant capacity (Fuhrman et al. 2005). Studies in Dahl-salt-sensitive hypertensive rats have shown that consumption of whole grapes as lyophilized powder for 18 weeks reduced blood pressure, cardiac hypertrophy, and diastolic dysfunction (Seymour et al. 2008). The mechanisms involved in these cardiac improvements were related to enhanced peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ DNA-binding activity, while nuclear factor (NF)-κB DNA-binding capacity was reduced as a result of GP intake (Seymour et al. 2010). Polyphenolic compounds present in yellow wine have also shown reductions in the expression and activity of matrix metalloproteinase (MMP) 2 and decreased atherosclerosis in LDL-receptor knockout mice at doses of 10, 30, and 50 mg/day compared to controls (Zhai et al. 2014). Grape polyphenols have also shown effects on blood pressure in a rat hypertensive model (Cui et al. 2012) by increasing eNOS expression and the production of NO.

Other studies have evaluated the effects of resveratrol, a stilbene present in grapes (Vilanova et al. 2015), mainly in the skin of these fruits (Bertelli and Das 2009). Resveratrol has been recognized as having pleiotropic effects on chronic disease including cancer, diabetes, and atherosclerosis (Ramprasath and Jones 2010). The role of resveratrol in preventing the initiation and progression of atherosclerosis as well as its anti-inflammatory and antioxidant effects is well documented (Fan et al. 2008). It is recognized that resveratrol is present in negligible amounts in the diet. However, a recent study used a resveratrol-rich grape extract (GE-RES) to prevent early aortic lesions in pigs fed an atherogenic diet (Azorin-Ortuño et al. 2012) with concentrations more appropriate for

supplementation (10 mg equivalent for a 70 kg human) than pharmacological doses. Pigs were fed a control diet, grape extract lacking resveratrol (GE), GE-RES, or resveratrol alone. GE-RES was the most effective dietary supplement to prevent disruption of aortic elastic fibers and reduce intima thickness and accumulation of fatty cells (Azorin-Ortuño et al. 2012).

Studies were conducted in a hamster model of atherosclerosis comparing the effects of red wine, dealcoholized wine, and grape juice as antioxidants and lipid-lowering agents (Vinson 2001). The authors reported that grape juice had a more beneficial effect in inhibiting atherosclerosis and improving lipid parameter than either red wine or dealcoholized red wine. In another study using rabbits, risk factors for atherosclerosis were measured in plasma in the postprandial state with 0, 5, or 10 mL of verjuice, an acidic juice obtained by the pressing of unripe grapes (Setorki et al. 2010). The rabbits consuming the verjuice exhibited significant decreases in fibrinogen and plasma glucose compared to controls. The highest concentration of the juice was more effective as demonstrated by decreases in oxLDL malonaldehyde and nitrite (Lekakis et al. 2005). Other animal studies using grape or wine polyphenols have demonstrated decreases in atherosclerosis in hamsters (Suh et al. 2011; Décordé et al. 2008), ApoE knockout mice (Cui et al. 2012; Peluzio Mdo et al. 2011), hypercholesterolemic rabbits (Shanmuganayagam et al. 2007), male WHHL rabbits (Frederiksen et al. 2007), and zebra fish, a model for atherosclerosis (Kim et al. 2012). A recent study also reported decreases in atherosclerosis, alanine aminotransferase activity, and reduced oxidative stress in New Zealand white rabbits fed a hypercholesterolemic diet with Corinthian currants (Yanni et al. 2015), dried grapes, which have been part of the Mediterranean diet since ancient times. In another study with hamsters, phenolic compounds derived from grape powder not only decreased atherosclerosis but also had indirect effects on this pathology by reducing plasma cholesterol and inducing endothelium relaxation (Auger et al. 2004). Inhibition of platelet aggregation in dogs and monkeys by grape polyphenols has also been reported (Osman et al. 1998). Thus, overall studies in a variety of animal models are in agreement that grape polyphenols decrease atherosclerosis and affect the mechanisms leading to atherosclerosis development.

7 Cell Studies

Cell studies have been used to clarify specific mechanisms by which grape polyphenols decrease atherosclerosis. In a study with a primary culture of human monocytes, serum of postmenopausal women after 2, 4, and 6 h of intake of grape components prevented cholesterol accumulation in these cells (Nikitina et al. 2006). In another study, a wild grape extract was used to evaluate its effects on proliferation and migration of smooth muscle cells, an early event in atherosclerosis development (Kant et al. 2011). The grape extract attenuated cellular expression of fibrinogenic connective tissue growth factor and matrix

metalloproteinase in cells exposed to macrophage-conditioned media and ameliorated the migration of smooth muscle cells promoted by neighboring macrophages confirming a retardation in the thickening of the atherosclerotic plaque (Shafiee et al. 2003). An extract from grapes high in procyanidins was proven to be very effective in protecting LDL against oxidation and impairing superoxide production, two events that can lead to atherosclerosis (Shafiee et al. 2003). Similarly, Alvarez et al. (2012) determined that grape procyanidins inhibited NAPH oxidase, implicated in increased ROS production, in human umbilical vein endothelial cells. The authors concluded that grape procyanidins could be used as therapeutic targets for cardiovascular diseases. Grape polyphenols have also been shown to prevent endothelial dysfunction in endothelial progenitor cells by reducing ROS production induced by hyperglycemia (Felice et al. 2012) emphasizing the role of these compounds in preventing the development of atherosclerosis. Overman et al. (2010) examined a grape powder preparation on its ability to prevent inflammation induced by lipopolysaccharide (LPS) on macrophages. The authors reported that the grape powder was effective in decreasing inflammatory cytokines as well as the production of cyclooxygenase and attenuated the activation of protein kinases and NF-κB supporting the efficacy of grape polyphenols in ameliorating the events leading to atherosclerosis.

In a recent report, Hien et al. (2012) determined that among the six stilbenes present in grapes, amurensin G most potently relaxed endothelial aortic rings and increased nitric oxide production and also activated AMP-activated protein kinase suggesting that this stilbene might have a protective role against atherosclerosis. In other studies, resveratrol has been shown to decrease the pathology associated with atherosclerosis in animal and cell studies. For example, resveratrol suppressed cytokine signaling 1 (SOCS1) and SOCS3, key regulators of vascular cell responses in peripheral mononuclear cells isolated from pigs (Azorin-Ortuño et al. 2012). Resveratrol has also been shown to induce LDL-receptor expression through proteolytic activation of sterol regulatory binding protein (SRBP) 1, which resulted in LDL uptake in a model of human hepatocytes (Yashiro et al. 2012). The mechanisms by which resveratrol may protect against atherosclerosis were also examined in comparison with ε-viniferin, a hydromer of resveratrol (Zghonda et al. 2011). Although both resveratrol and the hydromer were effective in inhibiting cell proliferation and ROS production, ε-viniferin functioned more effectively. Resveratrol has also been shown to inhibit leukocyte adhesion to tumor necrosis factor-α-activated endothelium (Kim et al. 2007) and to downregulate tissue factor expression (Di Santo et al. 2003), a cellular receptor that plays a primary role in predisposing to thrombosis, emphasizing once more the role of resveratrol in the prevention of atherosclerosis. A summary of the proposed mechanism of action by which grape polyphenols can prevent atherosclerosis is illustrated in Fig. 2.

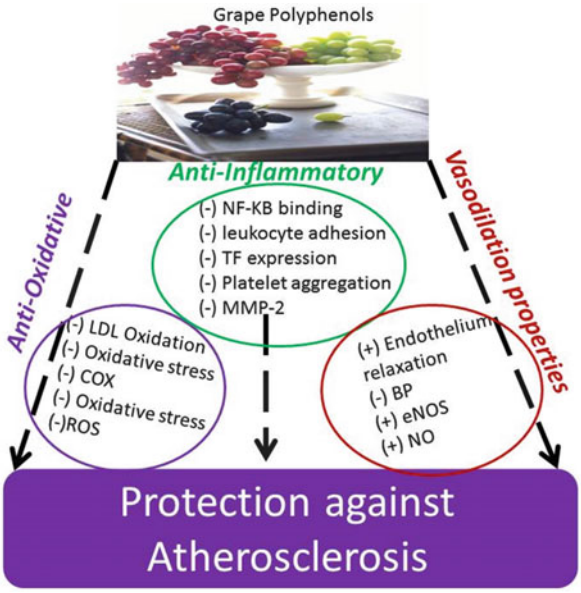

Fig. 2 Grape polyphenols have been shown to have anti-oxidative, anti-inflammatory, and vasodilation properties using animal models and cell studies. For the anti-oxidative properties, they have been shown to decrease LDL oxidation (Castilla et al. 2006; Vilanova et al. 2015) and oxidative stress (Fuhrman et al. 2005), reduce reactive oxygen species (ROS) (Ramprasath and Jones 2010; Vinson 2001), and reduce cyclooxygenase (COX) activity (Fan et al. 2008). The anti-inflammatory properties that have been reported are decreased NF-κB binding to DNA (Sankatsing et al. 2005; Fan et al. 2008), decreases in leukocyte adhesion (Suh et al. 2011), reduction in tissue factor (TF) expression (Décordé et al. 2008), reduction in platelet aggregation (Seymour et al. 2010), and reduced metalloproteinase-2 (MM2) (Lekakis et al. 2005; Cui et al. 2012). The vasodilation properties that have been reported are increases in endothelium relaxation (Seymour et al. 2008), decreases in blood pressure (Chaves et al. 2009), increases in the synthesis of nitric oxide synthetase (eNOS) (Ramprasath and Jones 2010), and increases in nitric oxide (NO) (Seymour et al. 2008; Suh et al. 2011)

8 Conclusion

There is a wealth of evidence derived from epidemiological data, clinical interventions, and animal and cell studies, which document the protection of grape polyphenols against atherosclerosis. This protection stems from various effects of grape polyphenols or other components in grapes on signaling pathways and metabolic routes associated with oxidative stress, inflammation, and dyslipidemias, all of which are associated with the pathology that leads to atherosclerosis. Further studies are required to determine what would be adequate dietary recommendations for grapes to result in the prevention and amelioration of metabolic dysregulations leading to atherosclerosis.

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Grapes and Inflammation

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Contents

Abstract Diet may play a role in the regulation of inflammation and inflammation-related pathologies. Inflammation is correlated with oxidative stress, and grape and grape phytochemicals can affect both inflammation and oxidative stress in cell, animal, and clinical models. The most effective constituents in grape are unknown, but grape, grape extracts, and grape phytochemicals can affect cell signaling, transcription, and translation related to inflammation. These effects are likely more impactful than any direct oxidative species scavenging effects of antioxidant phytochemicals in grape. As discussed here, in vivo preclinical and clinical studies demonstrate that intake of grape products can modify both the mechanisms and degree of local and systemic inflammation. Studies discussed here include grape effects upon inflammation in the organs of metabolism and elimination as well as those involved in obesity, type 2 diabetes, and cardiovascular diseases. This chapter will discuss some potential mechanisms of effect as well as future challenges and potential research directions for grapes and inflammation.

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1 Introduction

Inflammation participates in varied pathology and impacts both morbidity and mortality. Diet may play a role in the regulation of inflammation and inflammation-related pathologies. Observational studies indicate that cardiac mortality is inversely associated with higher wine consumption. The protective constituents in wine are unknown, but studies suggest that the phytochemical compounds in grapes impact mediators of inflammation. As discussed here, in vivo preclinical and clinical studies demonstrate that intake of grape products can modify both the mechanisms and degree of local and systemic inflammation. This chapter will discuss some potential mechanisms of effect as well as future challenges and potential research directions for grapes and inflammation.

2 Anti-inflammatory Effects of Phytochemical-Rich Foods: Current Questions

Throughout the body, inflammation is intimately tied with oxidative stress. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can be damaging to macromolecules like proteins, lipids, and nucleic acids. As discussed in this chapter, ROS/RNS can also stimulate redox-sensitive transcription factors and the translation of pro-inflammatory mediators. Our cells and tissues can handle these radicals through endogenous antioxidant mechanisms like enzyme activity (superoxide dismutase, catalase, glutathione peroxidase, etc.) or direct scavenging. However, stressors (like local injury and infection) can exceed our endogenous defenses. Diet-derived antioxidants can supplement our own efforts to limit ROS/RNS damage.

There is much speculation about the ability of diet-derived phytochemicals to directly impart antioxidant or anti-inflammatory activity in cells and tissues. Some of these chemicals are large and can be chemically incompatible with the intracellular milieu, so what gets to tissues is unknown. As discussed later, tissue bioavailability will continue to be a hot topic for critically testing the "function" of functional foods. Bioavailability is of particular concern for tissues that are distant to the organs of metabolism and excretion. Compared to remote tissues, organs involved in metabolism and excretion are exposed to higher levels of phytochemicals over the lifetime of the organism. Tissues like the heart and brain rely upon plasma delivery of absorbed phytochemical metabolites. The phytochemical concentrations measured in these tissues are typically a picogram or less, perhaps nanogram levels for discrete periods after ingestion. Therefore, it is unlikely that phytochemicals taken up by these tissues will outcompete endogenous antioxidant mechanisms for direct radical scavenging capability. Endogenous antioxidant compounds like glutathione are in the microgram range within cells—100- to 1000-fold higher than any phytochemical metabolite. Therefore, the primary effects of

phytochemical-rich foods upon inflammation are unknown as are the participating phytochemical metabolites.

The activity of redox-sensitive transcription factors is of considerable interest for phytochemical-rich foods. The activation of the transcription factor NF-κB occurs first through activation of IKK kinase by oxidative stress or by kinases impacted by growth factors or cytokines (Kawamura et al. 2005; Jones et al. 2003). Activated IKK kinase cleaves the p50/p65 subunits from IκBα, and the p50/p65 complex is now called NF-κB. NF-κB enters the nucleus where it binds to κB motifs in the genome to initiate the transcription of mRNAs related to fibrosis and inflammation, such as TNF-α, TGF-β, IL-1, ICAM-1, VCAM-1, COX-2, iNOS, and IL-6 (Gupta et al. 2005; Chandrasekar et al. 2004; Saito and Giaid 1999; Wong et al. 1998). Several in vitro and in vivo studies with polyphenols indicate that bioavailable polyphenols can impact the intracellular environment and/or gene transcription through reduced oxidative stress and by altered kinase activation (Rahman et al. 2006). Specifically, several of these studies also show altered NF-κB activity (Feng et al. 2005; Singh et al. 2003; Nomura et al. 2000; Kim et al. 2005; Lee et al. 2005; Park and Dong 2003; Yoshizumi et al. 2002; Chen et al. 1999, 2000; Chung et al. 1999; Dong et al. 1997). Bioavailable grape phytochemicals may act directly as antioxidants and thereby reduce oxidative stress and NF-κB activity.

3 Anti-inflammatory Effects of Grape and Grape Phytochemicals: Current Evidence

Instead of relying upon adequate tissue bioavailability and direct scavenging of ROS/RNS, the anti-inflammatory effects of functional foods can be obtained indirectly—by stimulating the production and maintenance of endogenous antioxidant defenses, thereby reducing oxidative damage and subsequent inflammation. Reduced oxidative stress can reduce the activity of transcription factors like NF-κB. For example, we previously showed that in hypertensive rats, consuming whole grape powder increased the concentration of cardiac glutathione and related antioxidant proteins while reducing cardiac NF-κB activity and the expression of NF-κB transcripts (Seymour et al. 2010, 2013a).

In vitro studies and animal studies suggest that grape polyphenols have the potential to modulate NF-κB targets. In particular, the 5-lipoxygenase pathway is an important target because it is involved in the synthesis of leukotrienes which contribute to inflammation (Li et al. 2001). In vitro, the polyphenols quercetin and resveratrol prove to be effective inhibitors of pro-inflammatory lipoxygenase pathways (Laughton et al. 1991). In addition, red wine extracts reduce adhesion of monocytes to the endothelial surface and block cytokine-induced expression of endothelial adhesion molecules (Carluccio et al. 2003). Red wine and select grape phytochemicals inhibit activation of NF-κB activity and production of

pro-inflammatory factors in endothelial cells and immune cells (Carluccio et al. 2003; Blanco-Colio et al. 2000). Incubation of monocytes with catechin decreased their adhesion to endothelial cells (Koga and Meydani 2001). Relevant polyphenols also inhibit NF-κB activity in T lymphocytes (Mackenzie et al. 2004, 2009). Resveratrol has also demonstrated anti-inflammatory effects, including inhibition of adhesion molecule expression and reduced responses to cytokines (Pellegatta et al. 2003; Bertelli et al. 2001; Fulgenzi et al. 2001; Ferrero et al. 1998a, b). Also, resveratrol inhibits the release of degradative enzymes by neutrophils (Birrell et al. 2005). Therefore, many phytochemicals in grape show effects upon inflammation mediators.

In vivo studies examining anti-inflammatory effects of grape products show that diverse grape products can lower systemic and local markers of inflammation. The intent of this chapter is to highlight grape product or grape phytochemical effects upon inflammation and its regulation in diverse tissues. A more detailed analysis of grape effects in these organ systems is provided throughout the chapters of this book. To focus, this chapter highlights select studies with the organs of elimination (large/small bowel, kidney, and liver), because they are exposed to the highest levels of phytochemical metabolites and would likely show anti-inflammatory effects, if present. Next, we highlight select studies of grape/grape phytochemical effect upon multi-organ, systemic pathologies involving inflammation—obesity, metabolic syndrome, type 2 diabetes, cardiovascular disease, and heart failure.

3.1 Grape and Intestinal Inflammation

3.1.1 Colitis

Colitis is an inflammatory disease of the bowels with relapsing-remitting nature. Piceatannol (3,5,3′,4′-tetrahydroxy-*trans*-stilbene; PIC) is a polyphenol found in grapes. PIC is a protein kinase inhibitor that modifies multiple cellular targets, exerting immunosuppressive activities in vitro. Kim et al. evaluated the anti-inflammatory effect of PIC on dextran sulfate sodium (DSS)-induced colitis (Kim et al. 2008). Experimental colitis was induced in BALB/c mice by dissolving 5 % DSS in their drinking water for 7 days. PIC (1, 2.5, 5, or 10 mg/kg body weight) was also administrated daily for 7 days. Piceatannol treatment showed a significant blunting of weight loss and clinical signs when compared to vehicle-treated mice. Treated mice also had greater preservation of colonic architecture, reduced colonic myeloperoxidase activity, and decreased production of inflammatory mediators such as nitric oxide, prostaglandin PGE_2, and pro-inflammatory cytokines.

Oxidative stress, neutrophil infiltration, pro-inflammatory cytokines, and eicosanoid generation are involved in the pathogenesis of intestinal bowel disease. Martin et al. investigated the effects of resveratrol on the colon injury caused by intracolonic instillation of trinitrobenzenesulfonic (TNBS) acid in rats (Martin et al. 2004). Resveratrol (5–10 mg/kg/day) significantly reduced the degree of

colonic injury, the index of neutrophil infiltration and the inflammatory cytokines. Resveratrol reduced COX-2 expression and enhanced apoptosis. In summary, resveratrol reduced the damage in experimentally induced colitis, alleviates the oxidative events, and stimulates apoptosis.

Wang et al. investigated the therapeutic effect of grape seed (GSPE) on recurrent ulcerative colitis in rats (Wang et al. 2010). To induce recurrent colitis, rats were instilled with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (80 mg/kg) into the colon through the cannula in the first induced phase, and then the rats were instilled a second time with TNBS (30 mg/kg) into the colon on the 16th day after the first induction ulcerative colitis. In one study, rats were intragastrically administered 200 mg/kg GSPE/day for 7 days after recurrent colitis was twice induced by TNBS (Wang et al. 2010). GSPE treatment facilitated recovery of pathologic changes in the colon as demonstrated by reduced colonic weight/length ratio and macroscopic and microscopic damage scores. GSE also reduced myeloperoxidase and iNOS activities and malondialdehyde and nitric oxide levels in serum and colon tissue. In addition, GSPE increased superoxide dismutase, glutathione peroxidase activities, and glutathione levels of colon tissues and serum. In a subsequent study, Wang et al. orally administered GSPE in doses of 100, 200, and 400 mg/kg daily for 7 days after recurrent colitis was twice induced by TNBS (Wang et al. 2011). In colon tissue, GSPE treatment increased GSH-Px and SOD activity and GSH levels. GSPE also reduced the expression of TNF-α, p-IKKβ, and p-IκBα and nuclear translocation of NF-κB in the colon mucosa.

In another study with ulcerative colitis, male Sprague-Dawley rats were gavaged daily (days 0–10) with GSE (400 mg/kg) (Cheah et al. 2013). Ulcerative colitis was induced by substituting DSS (2 % w/v) for drinking water from days 5 to 10. Compared to DSS-treated controls, GSE significantly decreased ileal villus height and mucosal thickness. GSE reduced qualitative histological severity score in the proximal colon, although no significant effect was evident in the distal colon. However, GSE did not prevent DSS-induced damage to the crypts of both colonic regions.

Intestinal mucositis is a common side effect of high-dose chemotherapy regimens. Cheah et al. evaluated GSE for its capacity to decrease the severity of chemotherapy-induced mucositis in vivo (Cheah et al. 2009). Female Dark Agouti rats (130–180 g) were gavaged with 1 mL GSE (400 mg/kg) daily (days 3–11) and received fluorouracil (150 mg/kg) by intraperitoneal (i.p.) injection on day 9 to induce mucositis. Compared with 5-FU controls, GSE significantly reduced myeloperoxidase activity in the proximal jejunum and distal ileum and decreased qualitative histological scores of damage in the proximal jejunum. GSE increased villus height in the proximal jejunum and distal ileum and attenuated fluorouracil-induced reduction of mucosal thickness in the jejunum and ileum. They concluded that GSE ameliorated intestinal damage induced by fluorouracil in rats and may represent a promising prophylactic adjunct to conventional chemotherapy for preventing intestinal mucositis.

3.1.2 Inflammatory Bowel Disease

Inflammatory bowel disease is a common chronic gastrointestinal disorder characterized by relapsing-remitting intestinal inflammation. Marchi et al. hypothesized that grape juice (1 or 2 %) could reduce the inflammatory effects induced by experimental colitis in rats (Marchi et al. 2014). Both 1 and 2 % juice reduced TNF-α immunoexpression and iNOS expression after drinking grape juice 24 h or after 7 days. Interestingly, COX-2 was similarly reduced, but only with the 1 % dose.

IL-10-deficient mice are a common model for studying inflammatory bowel disease. Using IL-10-deficient mice, Wang et al. showed that GSE (1 % of dry feed weight) ameliorated inflammatory bowel disease indices, increased colonic goblet cell numbers, and decreased myeloperoxidase levels in the large intestine (Wang et al. 2013). GSE supplementation attenuated inflammation. In another study, wild-type and IL-10-deficient mice were fed GSE at 0 or 1 % (based on dry feed weight) for 16 weeks (Yang et al. 2014). GSE supplementation increased the ratio of villus/crypt length in the terminal ileum. GSE decreased proliferation and enhanced differentiation of epithelial cells. These changes in gut epithelium were associated with suppressed NF-κB signaling.

3.2 Grape and Liver Inflammation

3.2.1 Liver Fibrosis

Li et al. examined the effect of the grape seed proanthocyanidin extract (GSPE) on developing hepatic fibrosis that was induced by thioacetamide (TAA) in mice (Li et al. 2012). Administration of TAA for 9 weeks led to necrosis and apoptosis of the parenchymal cells and liver fibrosis. In addition, the mRNA expression of transforming growth factor beta1 (TGF-β1) and α1-(I)-collagen was all upregulated. However, GSPE intake (at 100 mg/kg) suppressed hepatic mRNA expression of TGF-β1 and decreased collagen accumulation. Also, GSPE reduced mRNA expression of pro-inflammatory factors like iNOS and COX-2.

3.2.2 Liver Inflammation

Nishiumi et al. investigated whether an intake of grape pomace could suppress chronic inflammation induced by lipopolysaccharide (LPS) and galactosamine (GalN) in vivo (Nishiumi et al. 2012). Sprague-Dawley rats were fed an AIN93M-based diet containing 5 % red grape pomace for 7 days, followed by the intraperitoneal injection of LPS and GalN. The intake of the red grape pomace-supplemented diet suppressed the LPS/GalN-induced activation of NF-κB and expression of iNOS and COX-2 proteins.

3.3 Grape and Renal Inflammation

3.3.1 Renal Protection During Dialysis

Activation of neutrophils is a well-recognized feature in dialysis patients, and superoxide anion production by neutrophil NADPH oxidase may a critical role. Castilla et al. examined the effects of dietary supplementation with concentrated red grape juice (RGJ) on neutrophil NADPH oxidase activity and other cardiovascular risk factors in hemodialysis patients (Castilla et al. 2008). From 32 patients undergoing hemodialysis, blood was obtained at baseline and on days 7 and 14 of treatment. RGJ consumption altered several parameters and reduced plasma inflammatory biomarkers ICAM-1 and monocyte chemoattractant protein 1, an inflammatory biomarker associated with cardiovascular disease risk. In another study by Castilla in both healthy subjects and hemodialysis patients (Castilla et al. 2006), RGJ consumption increased plasma antioxidant capacity and reduced oxidized LDL. In hemodialysis patients, RGJ supplementation for 3 weeks significantly reduced plasma monocyte chemoattractant protein 1. Finally, in another study in dialysis patients (Janiques et al. 2014), those receiving grape powder (500 mg of polyphenols/day) showed increased serum GSH-Px activity and reduced CRP levels.

3.3.2 Renal Ischemia-Reperfusion Injury

Activation of reactive oxygen species and inflammation are implicated in renal ischemia/reperfusion (I/R) injury. Wei et al. investigated whether grape seed proanthocyanidin extract (GSPE) protects against renal I/R injury by its effect on ROS and inflammation (Wei et al. 2012). Wistar rats were administered GSPE before renal ischemia, followed by reperfusion for 24 h. GSPE significantly reduced increases in urea, creatinine, and cystatin C, increased kidney SOD activity and glutathione peroxidase levels, and reduced malondialdehyde levels. GSPE also reduced histological renal damage and NF-κB activity.

3.4 Grape and Obesity, Metabolic Dysregulation, and Type 2 Diabetes

Chuang et al. studied the impact of GP and GE on glucose tolerance and inflammation in obese mice (Chuang et al. 2012). Mice were fed high-fat diets supplemented with 3 % GP or 0.02 % GE for 18 weeks. GP supplementation decreased markers of inflammation approximately 20–50 % in both serum and adipose tissue.

Hogan et al. investigated whether 3-month GSE supplementation could improve oxidative stress, inflammation, and hyperglycemia associated with a Western diet-induced obesity (Hogan et al. 2011). Young diet-induced obese (DIO) mice were randomly divided to three treatment groups: a standard diet (S group), a Western high-fat diet (W group), and the Western diet plus GSE (2.4 g GSE/kg diet, WGSE group). By week 12, DIO mice in the WGSE group gained significantly more weight than the W and S groups; the high-fat diet groups gained 80 % more weight than the standard diet group. Eight of 12 mice in the W group, compared to only 1 of 12 mice in the WGSE group, had fasting blood glucose levels above 140 mg/dL. Mice in the WGSE group also had 21 % lower fasting blood glucose and 17.1 % lower C-reactive protein levels than mice in the W group. However, the GSE supplementation did not affect oxidative stress in diet-induced obesity as determined by plasma oxygen radical absorbance capacity, glutathione peroxidase, and liver lipid peroxidation.

In a study by Gourineni, C57BL/6J mice were given a low-fat diet (LF, 10 % kcal fat), high-fat diet (HF, 60 % kcal fat), HF + 0.4 % muscadine grape phytochemicals (HF + MGP), or HF + 0.4 % muscadine wine phytochemicals (HF + MWP) for 15 weeks (Gourineni et al. 2012). At 7 weeks, mice fed HF + MGP had significantly decreased body weights by 12 % compared to HF controls. Dietary MGP or MWP supplementation reduced plasma content of free fatty acids, triglycerides, and cholesterol in obese mice. Serum inflammation was attenuated, while the activity of serum glutathione peroxidase was enhanced.

Hogan et al. characterized the effects of grape pomace extract (GPE) in male diet-induced obese (DIO) mice (Hogan et al. 2010). Mice were randomly divided to three treatment groups: a normal diet (ND group), a high-fat diet (HF group), and the high-fat diet supplemented with GPE (HFGPE group). After 12-week treatment, mice in the high-fat diet groups gained 29 % more weight than the ND group. The GPE supplementation (estimated 250 mg/kg bw/day) lowered plasma CRP levels by 15.5 % in the high-fat diet-fed mice, suggesting a potential anti-inflammatory effect by dietary GPE. However, dietary GPE did not improve oxidative stress in DIO mice as determined by plasma ORAC, glutathione peroxidase, and liver lipid peroxidation.

Kim et al. investigated whether resveratrol attenuates high-fat diet (HFD)-induced adipogenesis and inflammation in the epididymal fat tissues of mice (Kim et al. 2011). In comparison with HFD-fed mice, mice fed with a 0.4 % resveratrol-supplemented diet showed significantly lower body weight gain (−48 %), visceral fat pad weights (−58 %), and plasma levels of triglyceride, FFA, total cholesterol, glucose, TNF-α, and MCP1. Resveratrol significantly attenuated the HFD-induced upregulation of pro-inflammatory cytokines (TNF-α, IFNα, IFNβ, and IL-6) and their upstream signaling molecules (TLR2/4, MyD88, TIRAP, TRIF, TRAF6, IRF5, p-IRF3, and NF-κB) in the adipose tissue.

Terra et al. evaluated the effect of procyanidin intake on inflammatory mediators in obesity-prone rats fed a hyperlipemic diet (Terra et al. 2008). Male Zucker fa/fa rats were randomly grouped to receive a low-fat (LF) diet, a high-fat (HF) diet, or a high-fat diet supplemented with procyanidins from grape seed (HFPE) (3.45 mg/kg

feed) for 19 weeks. HFPE diet decreased rat plasma CRP levels but not IL-6 levels. The decrease in plasma CRP in HFPE rats was related to reduced CRP mRNA expression in the liver and mesenteric white adipose tissue (WAT) and decreased expression of the pro-inflammatory cytokines TNF-α and IL-6 in mesenteric WAT. In contrast, adiponectin mRNA increased in these tissues. Plasma CRP levels correlated positively with CRP expression in the mesenteric WAT, suggesting that procyanidin extract (PE) modulates CRP transcription and translation. Expression of the anti-inflammatory cytokine adiponectin correlated negatively with TNF-α and IL-6 in the mesenteric WAT.

In a subsequent study, Terra tested the hypothesis that grape seed procyanidin extract (PE) would improve local and systemic inflammation in diet-induced obesity (Terra et al. 2011). Rats were fed a 60 % kcal fat diet with or without grape seed procyanidins (30 mg/kg/day) for 19 weeks. Then, rats were provided a "cafeteria diet" for 13 weeks to investigate the corrective effects of two PE doses (25 and 50 mg/kg/day) for 10 and 30 days. In the preventive model fed 60 % fat diet, PE reduced body weight, reduced plasma TNF-α and CRP, increased adiponectin expression and decreased TNF-α, interleukin-6 and CRP expression in mesenteric WAT, and decreased muscle TNF-α. PE also reduced liver NF-κB activity. Finally, PE dietary supplementation was linked to reduced expression of Emr1, a marker of macrophage F4/80, which suggests reduced macrophage infiltration of WAT. In the corrective model, however, only the high dose of PE reduced plasma CRP plasma and had no effect on plasma TNF-α.

Barona et al. evaluated the effects of grape consumption on inflammation and oxidation in the presence or absence of dyslipidemias in metabolic syndrome (Barona et al. 2012). Men with metabolic syndrome, 11 with high triglycerides and low HDL and 13 with no dyslipidemia, were randomly allocated to consume daily either 46 g of lyophilized grape powder (GRAPE), equivalent to 252 g fresh grapes, or placebo with an identical macronutrient composition and caloric value as GRAPE for 4 weeks. After a 3-week washout, participants followed the alternate treatment. Plasma adiponectin and interleukin (IL-10) were increased in the GRAPE compared to the placebo only in those individuals without dyslipidemia. Additionally, plasma IL-10 was negatively correlated with NOX2 expression, a marker of oxidative stress, while iNOS expression was positively correlated with the expression of SOD 2, a key antioxidant enzyme.

Kelishadi et al. determined the short- and long-term effects of consumption of grape juice on inflammation in adolescents with metabolic syndrome (Kelishadi et al. 2011). Fifteen individuals drank natural grape or juice for 1 month. Measurements of inflammatory factors (Hs-CRP, sE-selectin, sICAM-1, sVCAM, and IL-6) and flow-mediated dilation were made at baseline, 4 h after first juice consumption and after 1 month of juice consumption. The percent changes of FMD were significant in the short and long term. sE-selectin and IL-6 were significantly decreased after 1 month. Significant negative correlation of change in IL-6 with change in FMD was found at 1 month of supplementation.

In the obesity-prone Zucker fatty rat, we tested the effect of an American-style diet with added whole table grape powder (3 % w:w) (Seymour et al. 2013b). Rats

were fed for 90 days. Compared to a macronutrient and calorie-matched control group, grape intake significantly reduced serum CRP, TNF-α, and IL-6. Grape also reduced liver, kidney, and abdominal fat weight among several other phenotypes. Pharmacodynamic effect was measured by changes in NF-κB-related and antioxidant-related mRNA/proteins in the heart, abdominal fat, skeletal muscle, liver, brain, renal cortex, and renal medulla. NF-κB-related mRNA/proteins appear most significantly reduced in liver and abdominal fat. Antioxidant defense mRNA/proteins were most significantly increased in liver and renal cortex.

3.4.1 Grape and Cardiovascular Disease

Tome-Carneiro et al. investigated the effects of a resveratrol-rich grape supplement on the inflammatory and fibrinolytic status of subjects at high risk of CVD and treated with statins (Tome-Carneiro et al. 2012). Seventy-five patients consumed either a placebo, a resveratrol-rich grape supplement (resveratrol 8 mg), or a conventional grape supplement lacking resveratrol for the first 6 months, and a double dose for the next 6 months. In contrast to placebo and conventional grape supplement, the resveratrol-rich grape supplement significantly decreased high-sensitivity CRP, TNF-α, plasminogen activator inhibitor type 1, and IL-6/IL-10 ratio but increased anti-inflammatory IL-10.

Tome-Carneiro et al. examined the effect of resveratrol-containing grape extracts on transcriptional profiles of inflammatory genes in PBMCs in patients with stable coronary artery disease (Tome-Carneiro et al. 2013a). After 1 year of supplementation, in contrast to the placebo and conventional grape extract groups, the resveratrol-containing grape extract group showed an increase of the anti-inflammatory serum adiponectin. In addition, six key inflammation-related transcription factors were predicted to be significantly activated or inhibited. Twenty-seven extracellular-space acting genes involved in inflammation, cell migration, and T-cell interaction signals were downregulated by grape extract.

Tome-Carneiro investigated the molecular changes in PBMCs associated to the 1-year intake of a resveratrol-enriched grape extract (8 mg) in hypertensive male patients with type 2 diabetes (Tome-Carneiro et al. 2013b). Supplementation reduced serum IL-6. PBMC expression of the pro-inflammatory cytokines CCL3, IL-1β, and TNF-α was significantly reduced, and expression of the transcriptional repressor LRRFIP-1 was increased in PBMCs. Also, many microRNAs involved in the regulation of inflammation (miR-21, miR-181b, miR-663, miR-30c2, miR-155, and miR-34a) were highly correlated and altered by grape extract.

3.4.2 Vascular Function

Weseler et al. supplemented 28 male smokers with 200 mg/day of monomeric and oligomeric flavanols from grape seeds (Weseler et al. 2011). In the supplemented group, 8 weeks elevated the ratio of glutathione to glutathione disulfide in

erythrocytes compared to baseline. Supplementation exerted anti-inflammatory effects in blood toward ex vivo added bacterial endotoxin and significantly reduced expression of inflammatory genes in leukocytes. Conversely, alterations in CRP, fibrinogen, prostaglandin F2α, plasma antioxidant capacity, and gene expression levels of antioxidant defense enzymes did not reach statistical significance.

3.4.3 Hypertension-Associated Heart Failure

In addition to oxidative stress, heart failure pathogenesis also involves progressive local and systemic inflammation and local fibrosis. Increased fibrosis in the heart muscle reduces its compliance, which leads to contractile insufficiency. Our group investigated the effects of whole grape powder in a rat model of salt-sensitive hypertension and diastolic heart failure (Seymour et al. 2008). For 18 weeks, male Dahl-SS rats were fed one of five diets: low salt (LS), a low salt + grape powder (LSG), high salt (HS), a high salt + grape powder (HSG), or high salt + vasodilator hydralazine (HSH). Compared to the HS diet, the HSG diet lowered blood pressure and improved cardiac function; reduced systemic inflammation; reduced cardiac hypertrophy, fibrosis, and oxidative damage; and increased cardiac glutathione. The HSH diet similarly reduced blood pressure, but did not reduce cardiac pathogenesis. The LSG diet reduced cardiac oxidative damage and increased cardiac glutathione.

Prolonged hypertension is the leading cause of heart failure. Failing hearts show reduced peroxisome proliferator-activating receptor (PPAR) activity and enhanced NF-κB activity, which together modify cardiac inflammation and fibrosis. In Dahl-SS rats, we showed that dietary provision of whole table grape powder (3 % w:w) for 18 weeks enhanced cardiac PPAR-α and PPAR-δ DNA binding activity but reduced NF-κB DNA binding activity (Seymour et al. 2010). RT-PCR revealed that grape-fed rats showed upregulated mRNA for PPAR-α, PPAR-δ coactivator-1α, PPAR-δ, and the cytosolic NF-κB inhibitor, inhibitor-κBα. By contrast, grape-fed rats showed downregulated mRNA for TNF-α and TGF-β1. Finally, grape-fed rats showed significantly reduced cardiac TNF-α and TGF-β1 protein expression, increased inhibitor-κBα expression, and reduced cardiac fibrosis.

Charradi et al. used an experimental model of high-fat-diet (HFD)-induced obesity to analyze the effect of grape seed and skin extract (GSE) on oxidative stress and heart dysfunction (Charradi et al. 2011). Exposure of rats to HFD for 45 days induced heart hypertrophy, elevated plasma CRP, and cardiac contractile dysfunction as measured in ex vivo-perfused hearts undergoing ischemia/reperfusion injury. HFD also induced cardiac steatosis and lipotoxicity which were linked to oxidative stress status. Importantly, GSE alleviated all the deleterious effects of HFD treatment.

4 Grape Phytochemical Metabolite Bioavailability: What Could Impact Inflammation?

Grape phytochemicals involved in anti-inflammatory effects of grape products are unknown. The most common polyphenols in grape skin and pomace include phenolic acids, anthocyanidins, flavonols, and large molecular weight poly-galloyl polyflavan-3-ols. These components are found in fresh grapes, grape juice, wine, and grape skin extract. Grape seed extract contains mainly larger molecular weight compounds of repeating flavan-3-ol units esterified to gallic acid, with larger chains then those found in grape skin. Relative to grape skin, grape seed extract contains higher molecular weight flavonols called proanthocyanidins. The chemical constituents of grape seeds and stems are often found in wine due to prolonged exposure to macerated grapes during vinification. It remains unknown which grape constituents offer greatest anti-inflammatory effect or if these components act synergistically.

Research on tissue bioavailability is critical to the advancement of research in bioactive components from foods. For many years, little was known about the tissue bioavailability of phytochemicals. This was mostly due to difficulties in reliable quantification of the various food-derived phytochemicals and their metabolites in both biological fluids and tissues. The metabolism of several common phytochemicals is now reasonably well understood. Phytochemicals are extensively altered during metabolism, so that the molecular forms reaching the peripheral circulation and tissues are different from those present in whole foods. Here, the term "metabolism" describes the typical modifications that occur during or after absorption, which includes modifications made to intracellular metabolites. In general, the resulting metabolites are conjugates (e.g., sulfates and glucuronates) of the parent aglycone or conjugates of methylated parent aglycones.

Catabolism of phytochemicals in mammals generally occurs as a result of microbial activity in the large intestine (Gonthier et al. 2003). Most phytochemical glucosides are deglycosylated by β-glucosidases in the small intestine, namely, the broad-specificity cytosolic β-glucosidase and lactase phlorizin hydrolase; this step is requisite for the absorption of many of these phytochemicals (Scalbert et al. 2002). The small intestine appears to be the primary site responsible for glucuronidation; the major small intestine metabolites in the hepatic portal vein are glucuronides. After absorption, the conjugates may then travel to the liver and may be further methylated, glucuronidated, or sulfated. In the liver, polyphenols and their conjugates are metabolized by the phase II drug—metabolizing enzymes, the glucuronosyltransferases, sulfotransferases, and catechol-*O*-methyltransferases. The resulting molecules are glucuronate and sulfate conjugates, with or without methylation across the catechol functional group, and many are conjugated at multiple sites.

The predominance of phytochemical metabolites and conjugates over parent compounds has important consequences for biomedical research in this area. Phytochemical metabolites are chemically distinct from their parent compounds, differing in size, polarity, and ionic form. Consequently, their physiologic behavior

is likely to be different from that of the parent compounds. This is a critical factor to keep in mind when interpreting the possible clinical significance of in vitro studies using grape phytochemicals. Conversely, studies with physiologically relevant whole foods or supplements are viral for studying the link between grape product intake and disease.

For anti-inflammatory effects of grape in tissues, it is likely that tissue bioavailability of one of more grape-derived phytochemicals is required. The half-life of plasma phytochemical constituents and their metabolites is within hours, but constituents taken into tissues and cells can extend the time frame for exerting biologic effects. In support of this finding, many dietary supplementation studies demonstrate a biologic effect while not showing significant plasma presence of the predicted constituents. Therefore, enterohepatic conjugates, colonic metabolites, and intracellular metabolites of the parent compounds are actually responsible for the observed biologic effects (Silberberg et al. 2006).

For grape, many studies confirm the plasma and urine kinetics of flavonoids like catechins (Vinson et al. 2001; Prasain et al. 2009; Ferruzzi et al. 2009; Mata-Bilbao Mde et al. 2007; Tsang et al. 2005; Natsume et al. 2003), anthocyanins (Bub et al. 2001; Borges et al. 2007; He et al. 2006; El Mohsen et al. 2006; Bitsch et al. 2004; Frank et al. 2003; Tsuda et al. 1999), flavones (Egert et al. 2008; Davalos et al. 2006; Erlund et al. 2006; Meng et al. 2004; Mullen et al. 2002), and the stilbene resveratrol (Meng et al. 2004; Bertelli et al. 1996; Juan et al. 2009). Grape proanthocyanidins of high molecular weight like tannins may have questionable bioavailability (Rasmussen et al. 2005). From these larger molecules, it is likely that only the lowest molecular weight constituents (monomers, dimers) can be absorbed directly. An alternative hypothesis is that the proanthocyanidin polymers are metabolized by colonic microflora to alternate, absorbable phenolic acid compounds (Ward et al. 2004) that can be absorbed, distributed in plasma, and exert biologic activity in varied tissues.

Future research directions for anti-inflammation from grape would include identification of the grape constituents present in different tissues after prolonged feeding with grape product. Results could then be correlated with molecular and phenotypic effects, allowing hypothesis of causation. The tissue availability of individual grape phytochemicals like anthocyanins (El Mohsen et al. 2006; Tsuda et al. 1999; Vanzo et al. 2008; Talavera et al. 2005; Kalt et al. 2008), quercetin (Mullen et al. 2002, 2003; Bieger et al. 2008; de Boer et al. 2005; Morrice et al. 2000; Bugianesi et al. 2000), catechins (Ferruzzi et al. 2009; Lin et al. 2007; Garcia-Ramirez et al. 2006; Meng et al. 2002; Piskula and Terao 1998), and resveratrol (Juan et al. 2009; Gester et al. 2005; Wang et al. 2008; Sabolovic et al. 2007; Abd El-Mohsen et al. 2006) has been demonstrated using chromatography coupled with mass spectroscopy. The availability of authentic chemical standards and effective extraction techniques enables the identification and quantification of select phytochemicals in tissues.

An effect of grape phytochemicals upon transcription or cell signaling would likely require tissue availability, and the distribution among tissues is not even. For example, in similar long-term feeding studies, anthocyanins have demonstrated

bioavailability in several tissues as measured by LC-MS/MS (Tsuda et al. 1999; Talavera et al. 2005; Passamonti et al. 2005), including studies using whole food models (Kalt et al. 2008; Andres-Lacueva et al. 2005) rather than supraphysiologic levels of anthocyanin extracts. As expected, phytochemical levels are highest in the organs of metabolism and elimination. Importantly, these animals are typically fasted at sacrifice and show no detectable plasma anthocyanins, while showing diverse tissue deposition. We recently showed that tart cherry anthocyanins are present in the brain and heart after only 3 weeks of feeding a 1 % tart cherry diet, but were present at 2–5× higher concentrations in the bladder, kidneys, and liver (Kirakosyan et al. 2015).

Regarding inflammation-related cell signaling or posttranslational modification, bioavailable grape phytochemical metabolites may modulate cell signaling pathways including cascades such as phosphoinositide 3-kinase, Akt/PKB, tyrosine kinases, protein kinase C, and MAP kinases. Bioavailable phytochemicals and/or their metabolites can bind to the ATP-binding sites of a large number of proteins (Williams et al. 2004); this binding causes three-dimensional structural changes and altered protein activity. In addition, bioavailable grape phytochemicals, their enterohepatic metabolites, and their intracellular metabolites may interact with sulfhydryl moieties on kinase proteins and alter secondary protein structure and activity. The exact kinase signaling pathways involved in the observed grape-related effects are unknown and require further investigation in various tissues.

Regarding inflammation-related gene transcription, bioavailable grape phytochemical effect upon NFκB activity may be indirect rather than direct, though altered kinase signaling and gene transcription/translation. In addition to our results in hypertensive rats, we previously showed in obese rats with metabolic syndrome that grape intake reduced NF-κB activity and related mRNA and protein. As expected, grape effects were greatest in the kidney and liver but also were present in the heart, brain, and skeletal muscle. These results could support many interesting hypotheses for further study in models of metabolic syndrome and type 2 diabetes.

5 Clinical and Translational Challenges

Using physiologically relevant levels of grape phytochemicals, assessment of changes in systemic inflammation (like serum CRP) may be insufficient to assess the physiologic response to grape products. Commonly, clinical studies rely upon these serum biomarkers to show efficacy, but these approaches may underestimate physiologic impact of grape intake. At levels relevant to the human diet, effects of grape intake may be primarily nutrigenomic and mediated by very small quantities of polyphenolic compounds. Accordingly, obtaining relevant cells to assess gene expression profiles will be a critical step in understanding the potential anti-inflammatory effects of grape products in humans. Tissue may be obtained during surgery or via biopsy, but these invasive approaches are not indicated in most

patients and are particularly poorly suited to investigating differences pre- and posttreatment. Investigating gene profiles in easily harvested circulating immune cells obtained via recently developed, low-risk biopsy techniques may provide an appropriate avenue to assess the effects of grape products on oxidative stress, inflammation, and disease pathogenesis. The anti-inflammatory effects of grape products in diverse tissues are very promising for a range of chronic diseases and deserve further preclinical and clinical investigation.

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Grapes and Cancer

Randall F. Holcombe

Contents

Abstract Grape consumption has been linked to beneficial effects in the prevention or treatment of various different cancers in laboratory animals and humans. Several phytochemicals in the skin and seeds of grapes have been implicated in having anticancer activity including resveratrol, quercetin, and proanthocyanidins, among others. These components promote tumor cell apoptosis, have antiproliferative effects through cell cycle arrest, disrupt intracellular signaling including Wnt and PI3K/AKT pathways, suppress inflammatory responses, and display antiangiogenic activity. The best studied individual component of grapes with anticancer properties is resveratrol which can inhibit intestinal tumorigenesis, hepatocellular cancer, and skin cancer in rodent models. Only a handful of human studies have been performed with resveratrol, but these have demonstrated activity on key proliferative pathways in target organs despite questions about bioavailability and attainable serum concentrations of specific grape-derived phytochemicals. Overall, based on epidemiologic evidence, laboratory studies in vitro and in model organisms, and direct human studies, grapes or specific components of grapes hold promise for both cancer prevention and treatment.

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1 Introduction

Up to one-third of all cancers in industrial societies are believed to be attributable to lack of physical activity and dietary factors (Wiseman 2008; Glade 1999). From a dietary perspective, there has been increasing interest in cancer prevention and cancer treatment with natural compounds (Gullett et al. 2010). As far back as 1993, a case control study on oral cancer in Beijing demonstrated that increased consumption of grapes resulted in a reduced risk of oral cancers (Zheng et al. 1993). Several components have been implicated as having active antitumor activity ranging from grape seed extract, shown to reduce the risk of squamous cell carcinoma of the skin (Asgari et al. 2011), to resveratrol, as well as several other phytochemicals in the skin and seeds of grapes. Resveratrol is a key component of grapes with a myriad of actions on cellular processes and possible cancer preventive and treatment activity (Gescher et al. 2013; Gescher 2008). For example, resveratrol from grapes has been associated with reduced risk of breast cancer with an odds ratio of 0.39 for the highest tertile of grape ingestion (Levi et al. 2005). The various active components of grapes, their effects on cellular processes in vitro, and the evidence for anticancer activity in laboratory animal models and in human studies are summarized in this chapter.

2 Components of Grapes with Potential Anticancer Activity

Grapes contain over 100 distinct phytochemicals that may have metabolic activity (Pezzuto 2008). Phytochemicals are defined as nonnutritive chemicals found in plants. The phytochemicals found in grapes that are believed to have significant activity in humans or have been shown to affect cellular processes in vitro include phenolic acids, flavonols, flavan-3-ols, myricetin, peonidin, flavonoids, resveratrol, quercetin, tannins, anthocyanins, kaempferol, cyanidin, ellagic acid, and proanthocyanidins (Yang and Xiao 2013). Flavonoids such as quercetin and stilbenes such as resveratrol and piceatannol (Gullett et al. 2010) are principally localized in the skins of grapes, while flavan-3-ols such as catechin are found in the skins and seeds. Anthocyanins are primarily concentrated in the seeds, though some species of red grapes also contain significant amounts in the skin. Phytochemicals are found in grape products in addition to whole grapes. For example, resveratrol is at its highest in red wine, but white wines and dark red (unprocessed or minimally processed) grape juice also contains some of this compound. The compounds that have been most extensively studied for their anticancer or cancer preventive activity include resveratrol and anthocyanins in grape seed extracts though antiproliferative activity has been reported for many other components. Piceatannol, a stilbenoid, has antiproliferative activity against hepatocellular cancer cells and suppresses metastases of these cells in rats (Kita et al. 2012). Pterostilbene inhibits proliferation of

pancreatic cancer cells in vitro (Mannal et al. 2010). Ellagic acid-containing polyphenolic extracts from grapes are potent inhibitors of proliferation (Mertens-Talcott et al. 2006). Liofenol, a natural red wine lyophilized extract, promotes differentiation and reduces proliferation of HCT116 colon cancer cells (Signorelli et al. 2015).

There has been debate as to which part of the grape has the most anticancer activity. In one study, inhibition of HeLa and 4T1 growth in cell culture and in transplanted tumors in mice was much more effective utilizing a grape skin extract compared to grape pulp, juice, or seeds (Morre and Morre 2006). Grape skin polyphenols also have antimetastatic activities in a murine model of breast cancer (Sun et al. 2012), and numerous studies have been reported with resveratrol (Pirola and Frojdo 2008). However, grape seed extracts demonstrate activity as well, inducing apoptosis in human prostate cancer cells (Agarwal et al. 2002). Grape seed proanthocyanidins induce apoptosis and inhibit metastases in breast cancer cells (Mantena et al. 2006), and gallic acid in grape seed has antiproliferative and proapoptotic activity against pancreatic cancer cells (Cedo et al. 2014). Stilbenes and oligostilbenes found in the leaves and stems of grape plants display antiproliferative effects against cancer cell lines in vitro (Ha do et al. 2009) as do polyphenolic extracts from grape stems (Sahpazidou et al. 2014). Whole grape preparations such as grape powder, containing quercetin, epicatechin, and cyanidin, have been found to have antiapoptotic and antioxidant effects (Jing et al. 2011).

3 In Vitro Mechanisms of Action

Components in grapes, and particularly resveratrol, have a myriad of activities on cellular processes that may translate into anticancer or cancer prevention activity (see reviews: Pirola and Frojdo 2008; Borriello et al. 2014; Aluyen et al. 2012). Resveratrol has been studied extensively and has apoptosis-inducing activity, cell cycle effects, and anti-inflammatory effects and can modulate protein kinase signaling pathways (Shankar et al. 2007). Additional anticancer mechanisms include dysregulation of intracellular signaling including the MAP kinase/Akt pathway, inhibition of Wnt pathway signaling, anti-angiogenic effects, and effects on sirtuin-mediated processes.

Phytochemicals in Grapes Induce Tumor Cell Apoptosis Resveratrol induces apoptosis in liver cancer cells (Choi et al. 2009) and promotes apoptosis by enhancing CD95L expression in HL60 and T47D breast cancer cells (Clement et al. 1998). In T47D breast cancer cells, it also induces apoptosis through activation of p53 (Alkhalaf 2007). Low to moderate concentrations of resveratrol lead to Bax co-localization with mitochondria, activation of caspase-3 and caspase-9, and apoptosis in HCT116 colorectal cancer cells (Mahyar-Roemer et al. 2002; Juan et al. 2008). Resveratrol also activates caspase-2 triggering mitochondrial apoptotic events by inducing conformational changes in Bax/Bak (Mohan et al. 2006). In

multiple myeloma cells, resveratrol downregulates STAT3 and NFκB, leading to apoptosis, BAX release, and activation of caspase-3 (Bhardwaj et al. 2007). It induces apoptosis and inhibits angiogenesis in breast cancer xenografts (Garvin et al. 2006) and induces downregulation of survivin and apoptosis in adult T-cell leukemia (Hayashibara et al. 2002). In HepG2 cells, resveratrol reduces PTEN and increases bcl-xl mRNA expression, inhibiting HepG2 proliferation (Zheng et al. 2012). Pterostilbene, an analog of resveratrol mostly found in blueberries, induces caspase-dependent apoptosis through mitochondrial depolarization (Alosi et al. 2010), and piceatannol induces apoptosis in DU145 prostate cancer cells (Kim et al. 2009).

Grape seed extract has antiproliferative and proapoptotic effects on human colon cancer cell lines (Aghbali et al. 2013; Dinicola et al. 2010, 2012). It leads to caspase activation, mitochondrial membrane potential dissipation, inhibition of NFκB, cytochrome c release, and apoptosis in prostate carcinoma DU145 cells (Agarwal et al. 2002; Dhanalakshmi et al. 2003). Grape seed proanthocyanidin induces apoptosis through activation of p53 (Hu and Qin 2006; Huang et al. 1999). Anthocyanins also have anti-invasive and apoptosis-inducing activity through suppression of matrix metalloproteinases and activation of p38-MAPK, respectively (Shin et al. 2009, 2011).

Antiproliferative Activity Through Cell Cycle Arrest Resveratrol (Park et al. 2001) and grape seed extract induce cell cycle arrest in human colon cancer cells (Kaur et al. 2008). The growth inhibitory effects of resveratrol may be mediated through cell cycle arrest, with upregulation of p21Cip1/WAF1, p53, and BAX (Aggarwal et al. 2004). Various cyclins are downregulated, and caspases are activated. In addition, expression of several transcription factors such as NFκB is suppressed, and JNK, MAPK, and Akt protein kinases are inhibited. Resveratrol causes WAF-1/p21 G(1) arrest of cell cycle in A431 cancer cells, with decreased expression of cyclin D1 and cyclin D2 (Ahmad et al. 2001). Irreversible cell cycle arrest then leads to apoptosis. Resveratrol also inhibits cell cycle progression in U937 cells, blocking cells at the S phase checkpoint (Castello and Tessitore 2005), inhibits SW480 colorectal cancer proliferation by modulating cyclin and cyclin-dependent kinase activities (Delmas et al. 2002), and leads to cell cycle arrest and upregulation of cyclins A, E, and B1 (Larrosa et al. 2003).

Disruption of Intracellular Signaling A myriad of intracellular signaling pathways are inhibited by grape components, including resveratrol and anthocyanidins. Grape polyphenols inhibit Akt/mTOR signaling in breast cancer cells (Castillo-Pichardo and Dharmawardhane 2012). Resveratrol leads to the suppression of NFκB and promotion of differentiation in an in vitro leukemia model (Asou et al. 2002) and inhibits protein kinase C, suppressing proliferation of gastric adenocarcinoma cells (Atten et al. 2001). Resveratrol also regulates the PTEN/Akt pathway due to inactivation by MTA1 (Dhar et al. 2015), inhibits IκB kinase activation (Holmes-McNary and Baldwin 2000), and inhibits proliferation of A431 cells by inhibiting MEK1 and suppressing activating protein (AO)-1 activity (Kim et al. 2006). Resveratrol inhibits mTOR signaling via PI3K/PDK1/Akt (Brito et al. 2009) as do

grape seeds that have been reported to increase phosphorylation of MAPK and kinases in the PI3K/Akt pathway, promoting the activity of detoxifying and antioxidant enzymes (Bak et al. 2012). Proanthocyanidin from grape seeds can inactivate the PI3K pathway and induce apoptosis in colon cancer cell lines (Engelbrecht et al. 2007). Grape proanthocyanidin also inhibits pancreatic cancer cell growth via decreased expression of PI3K and p-Akt in tumor xenografts (Prasad et al. 2012) and mouse skin tumors (Roy et al. 2009). Other activities of resveratrol include inhibition of IL-6-dependent transcription of STAT3 in LNCaP cells (Lee et al. 2014); inhibition of EMT in pancreatic cancer cells by suppression of PI3K/Akt/NFκB signaling (Li et al. 2013); inhibition of TGF-β1-induced EMT, suppressing lung cancer invasion and metastases (Wang et al. 2013); and suppression of IGF-1-induced colon cancer cell proliferation by activating p53 and suppressing IGF-1R and Wnt signaling (Vanamala et al. 2010).

Resveratrol Has Inhibitory Effects on Wnt Signaling One of the many other activities of resveratrol is inhibition of Wnt signaling (Hope et al. 2008) (Fig. 1). We have shown that relatively low concentrations of resveratrol can inhibit Wnt signal throughput in colon cancer cell lines. Wnt signaling is central to the development of colon and many other types of cancer (Giles et al. 2003). Specific alterations in the components of the Wnt pathway have been noted by our group in colon cancers arising in the setting of inflammatory bowel disease, providing

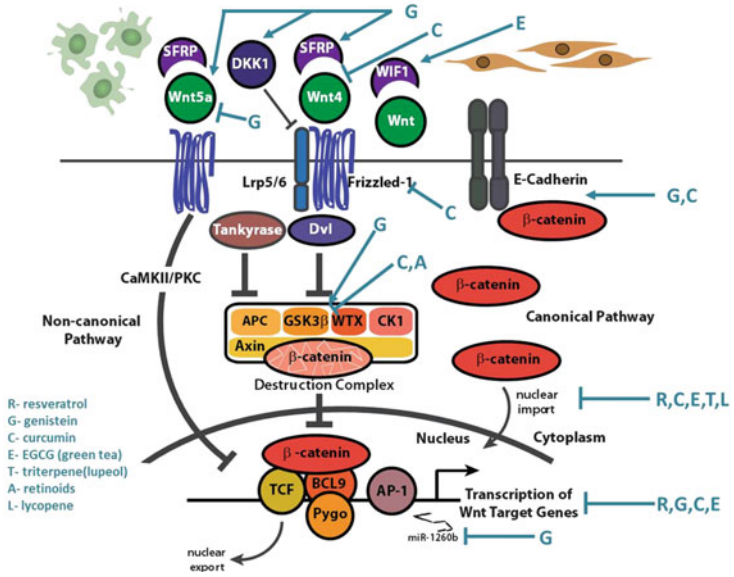

Fig. 1 Wnt pathway schematic, demonstrating roles of extracellular Wnt ligands, cell surface frizzled receptors, the APC-containing complex involved in phosphorylation of β-catenin, and the central role of β-catenin which binds to members of the LEF/TCF transcription factor family to regulate the expression of growth promoting target genes. The various effects of natural products, including resveratrol, are indicated

additional evidence of the confluence of Wnt signaling, inflammation, and colon cancer (You et al. 2007, 2008). In the Wnt pathway, evidence suggests that resveratrol acts downstream of GSK3β, possibly by disrupting the binding between β-catenin and TCF4 (Chen et al. 2012).

Resveratrol Suppresses Inflammatory Responses Resveratrol is a stilbene which has multiple activities in vitro (Pirola and Frojdo 2008) and is purported to have cardioprotective, cancer preventive, and antiaging properties in vivo (Baur and Sinclair 2006). Resveratrol has direct effects on mediators of inflammation. It is an inhibitor of both 5-lipooxygenase (LOX) and cyclooxygenase (COX) (Kimura et al. 1985), enzymes critical for the synthesis of proinflammatory mediators. Resveratrol inhibits COX2 at both a transcriptional and protein expression level, possibly through inhibition of PKCα and Erk1 (Subbaramaiah et al. 1998). Resveratrol also suppresses IKK-mediated phosphorylation of IκB (Kundu et al. 2006a) thereby inhibiting NFκB, an important signal transducer linking inflammation with tumorigenesis (Karin and Greten 2005). IKK is one of the targets most potently inhibited in vivo (Kundu et al. 2006b). Anti-inflammatory effects of resveratrol have also been tied to inhibition of LPS-induced NFκB activation in colon cancer cells (Panaro et al. 2012). Resveratrol has additionally been shown to attenuate the inflammatory response of peripheral blood leukocytes via reduced expression of IL-8 and TNF-α (Richard et al. 2005) and to downregulate iNOS through suppression of NFκB (Surh et al. 2001). Since resveratrol appears to have multiple intracellular targets, global effects such as suppression of inflammation need to be evaluated critically and interpreted within specific experimental contexts.

Anti-angiogenic Properties of Grape Components In vitro, four grape varieties have been tested for anti-angiogenic activity—Concord, Niagara, Chardonnay, and Pinot noir. Those with highest total phenolics and flavonoids displayed the most anti-angiogenic activity (Liu et al. 2010). High dosages of red wine polyphenols decrease VEGF expression and inhibit angiogenesis (Baron-Menguy et al. 2007). Resveratrol has been shown to have anti-angiogenic activity (Cao et al. 2005); it can inhibit angiogenesis in breast cancer xenografts (Garvin et al. 2006) and inhibits VEGF expression in liver cancer cells (Yu et al. 2010). Grape procyanidins have been shown to block tumor angiogenesis in a liver cancer xenograft model (Feng et al. 2014), and grape seed extract inhibits VEGF expression by inhibiting HIF-1α protein (Lu et al. 2009).

Resveratrol and Sirtuins Many reports have linked resveratrol to improved life expectancy through its effects on sirtuins (Baur et al. 2006). Sirtuin-activating capacity by resveratrol may explain the beneficial effects of the Mediterranean diet (Russo et al. 2014). This activity may also play a role in cancer treatment or prevention (Kelly 2010a, b). Sirt7 is implicated in cancer due to its effects on chromatin signaling (Paredes et al. 2014), sirt3 is implicated in cancer as regulator of mitochondrial adaptive responses to stress (Chen et al. 2014), and sirt3 is associated with survival in esophageal cancer (Zhao et al. 2013). More research is necessary to define whether this activity of resveratrol can be linked directly to cancer prevention or treatment.

Other Mechanisms of Action of Grape Components Relevant to Cancer Several other activities of grape components suggest a role in cancer prevention and treatment. Matrix metalloproteinases promote tumor growth, invasion, and metastases and are inhibited by grape seed proanthocyanidins (Katiyar 2006). Grape seed extract can function as an aromatase inhibitor (Kijima et al. 2006) so may be useful in the treatment of estrogen-responsive tumors. Resveratrol induces DNA double-strand breaks through interaction with topoisomerase II (Leone et al. 2010). Topoisomerase II inhibitors are frequently utilized chemotherapy agents. Finally, resveratrol inhibits ornithine decarboxylase (ODC) activity (Wolter et al. 2004). ODC is the rate-limiting step in polyamine synthesis which is closely linked to colon carcinogenesis (Zell et al. 2007).

4 Anticancer Activity in Laboratory Animals

Systemic administration of resveratrol has been shown to inhibit the growth of tumors in several different rodent cancer models (Baur and Sinclair 2006) and for multiple different tumor types (Carter et al. 2014). For colon cancer prevention, effects are seen over a wide variety of dose ranges depending on individual studies. Tessitore demonstrated activity of very low-dose resveratrol of 0.2 mg/kg/day in reducing aberrant crypt foci (ACF) in the colon in an azoxymethane-induced tumor model (Tessitore et al. 2000). In another carcinogen-based model, utilizing 1,2-dimethylhydrazine, resveratrol at 8 mg/kg/day reduced both ACF and colonic tumors (Sengottuvelan and Nalini 2006; Sengottuvelan et al. 2006). In genetic models utilizing the $APC^{min/+}$ mouse, which harbors a single allele mutation in *apc* and therefore has intrinsically activated Wnt signaling, Schneider demonstrated profound activity at dosages as low as 0.3 mg/mouse/day in reducing intestinal tumors (Schneider et al. 2001). In this study, expression of Wnt target gene *cyclinD1* as well as other markers of cell cycling was reduced. However, Ziegler (Ziegler et al. 2004) found resveratrol up to 90 mg/kg ineffective, and Gignac and Bourquin (2001) demonstrated an effect only at 500 mg/kg and, in this case, only in male mice. Sale utilized dosages of 60 and 240 mg/kg and found the former ineffective but the latter effective in inducing a more modest reduction in intestinal tumorigenesis (Sale et al. 2005). In DMH-treated Sprague-Dawley rats, administration of 60 mg/kg *trans*-resveratrol orally for 49 days decreased aberrant crypt foci by 52 % (Alfaras et al. 2010). Utilizing a SCID xenograft model implanted with HCT-116 colon cancer cells, Majumdar found that the combination of curcumin with resveratrol led to a reduction in proliferation accompanied by attenuation of NFκB activity (Majumdar et al. 2009).

Overall, these studies indicate that resveratrol has activity in both carcinogen-induced tumor models and in the Wnt-activated $APC^{min/+}$ mouse, but that the effective dose is unclear, with activity reported utilizing dosages ranging from <1 to 500 mg/kg/day. Results of animal model studies related to resveratrol and intestinal/colorectal cancer are summarized in Table 1. Proanthocyanidins have

Table 1 Summary of studies of resveratrol in rodent models on intestinal tumorigenesis

Author (year)	Animal model	Carcinogen	Outcome measure	Resveratrol concentration	Route	Duration (weeks)	Result
Tessitore et al. (2000)	F334 rat	AOM	ACF	0.2 mg/kg/day	Water	12	Significant decrease in # and multiplicity of ACFs
Schneider et al. (2001)	$APC^{min/+}$ mouse	Genetic	Tumors	0.01 % in H_2O (0.3–0.4 mg/mouse/day)	Water	7	Reduction by 70 %. ↓cyclinD1, ↑immune response genes
Gignac and Bourquin (2001)[a]	$APC^{min/+}$ mouse	Genetic	Tumors	500 mg/kg	Diet	2	Reduction by 50 %, males only
Ziegler et al. (2004)	$APC^{min/+}$ mouse	Genetic	Tumors	0, 4, 20 or 90 mg/kg	Diet	7	No change
Sale et al. (2005)	$APC^{min/+}$ mouse	Genetic	Adenomas (tumors)	0.05 % (60 mg/kg)	Diet	10–14	No change
				0.2 % (240 mg/kg)	Diet	10–14	Reduction by 27 %. Conc. ~36 nmol/g intestinal tissue
Sengottuvelan and Nalini (2006)[b]	Wistar rats	DMH	ACF	8 mg/kg/day	GG	15–30	50–75 % reduction
			Tumors	8 mg/kg/day	GG	15–30	35–70 % reduction
Sengottuvelan et al. (2006)[b]	Wistar rats	DMH	ACF	8 mg/kg/day	GG	15–30	50–75 % reduction
			Tumors	8 mg/kg/day	GG	15–30	35–70 % reduction
Majumdar et al. (2009)	SCID mice	HCT-116 cells	Tumor growth	150 mg/kg daily	GG	3	40 % inhibition with curcumin
Alfaras et al. (2010)	Sprague-Dawley rats	DMH	ACF	60 mg/kg	Oral	7	52 % reduction

[a]Information based on abstract only
[b]These two reports do not appear to describe independent data sets
AOM azoxymethane, *ACF* aberrant crypt foci, *DMH* 1,2-dimethylhydrazine, *GG* gastric gavages

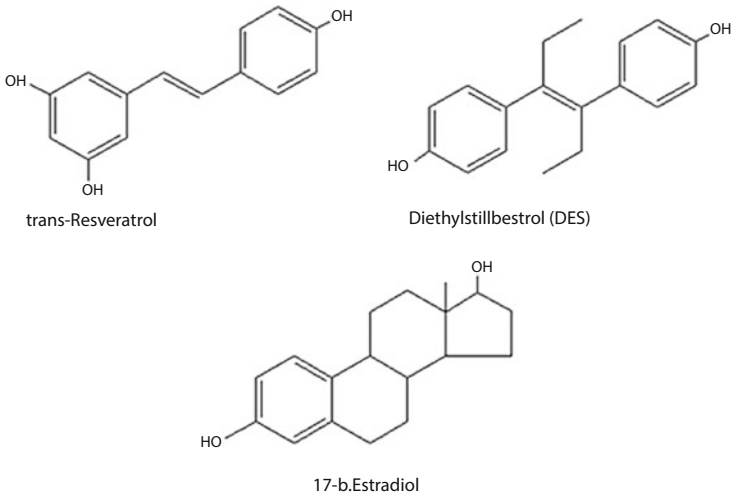

Fig. 2 Chemical structure of *trans*-resveratrol in comparison to diethylstilbestrol (DES) and estradiol

also been demonstrated to have anti-colon cancer activity. In an azoxymethane cancer model in F344 rats, a decreased frequency of aberrant crypt foci was demonstrated (Nomoto et al. 2004; Singletary and Meline 2001).

Resveratrol is considered a phytoestrogen (Fig. 2) and appears to have both antagonistic and agonistic effects through the estrogen receptor (Bowers et al. 2000; Bhat et al. 2001). Therefore, it is not surprising that studies in laboratory animals related to breast cancer have been contradictory. Several studies have demonstrated a reduction in breast cancer tumors or a delay in their initiation following DMBA [7,12-dimethylbenz(*a*)anthracene] administration (Banerjee et al. 2002; Whitsett et al. 2006; Chatterjee et al. 2011) or NMU (*N*-nitroso-*N*-methylurea) administration (Bhat et al. 2001). Other studies have not demonstrated benefit or even suggested that resveratrol may increase tumor formation when given to prepubescent rats (Sato et al. 2003; Castillo-Pichardo et al. 2013). These studies suggest that the phytoestrogen effects of resveratrol may counteract any cancer inhibitory activity under certain circumstances and that caution should be used prior to extrapolating to human use.

Induction of hepatocellular carcinoma with diethylnitrosamine (DENA) followed by phenobarbital can be inhibited by resveratrol, often associated with increased apoptosis in liver cancer cells (Bishayee 2009; Luther et al. 2011; Rajasekaran et al. 2011). Interestingly, a reduction in hepatocellular cancer development was also seen in a hepatitis B virus X protein model (Lin et al. 2012) suggesting a potential preventive role in virally mediated liver cancer. An activity of resveratrol for pancreatic cancer prevention in immune-deficient mice has also been reported (Harikumar et al. 2010; Oi et al. 2010; Roy et al. 2011). Resveratrol appears to inhibit prostate cancer growth in TRAP rats and TRAMP mice (Harper

et al. 2007; Seeni et al. 2008) but has no effect in a nude mouse xerograph model (Seeni et al. 2008; Wang et al. 2008).

Several different skin cancer models have suggested that resveratrol may be beneficial in prevention of this disease. Its administration inhibits skin cancer in a two-stage mouse model (Kapadia et al. 2002). Similar activity has been reported in UV-induced skin cancer as well (Aziz et al. 2005; Reagan-Shaw et al. 2004; Adhami et al. 2003; Afaq et al. 2003). A beneficial effect has also been seen in a Lewis lung carcinoma murine model (Kimura and Okuda 2001), possibly through inhibition of angiogenesis, an effect seen in other tumor model systems as well (Garvin et al. 2006; Chen et al. 2006; Mousa and Mousa 2005).

5 Human Studies for Cancer Treatment and Prevention

Multiple dietary agents have been purported to possess anticancer or cancer preventive activity (Aggarwal and Shishodia 2006) though there have been few controlled trials in humans on which to form conclusions regarding efficacy. Most of the trials relevant to grapes have focused on the activities of resveratrol, though a few have examined whole grapes or combinations of grape components. Several clinical trials have focused on the pharmacokinetics, pharmacodynamics, and safety of moderate to large oral dosages of resveratrol (Patel et al. 2011). Safety is always a concern when administering high dosages. At 5 g/day, nausea, diarrhea, fatigue, and renal insufficiency were noted in a patient with multiple myeloma (Popat et al. 2013). Others have found resveratrol to be well tolerated in high dosages for short periods of time (29 days of resveratrol supplementation at 2.5 g/day) and to reduce IGF-1 and IGFBP3 levels (Brown et al. 2010). Thus, resveratrol may have effects on energy metabolism and metabolic profiles similar to caloric restriction (Timmers et al. 2011), processes suggested as important for both primary and secondary cancer prevention (Voskuil et al. 2005). Howells et al. (2011) showed that 5 g resveratrol daily for 10–21 days increased apoptosis in colorectal cancer metastases in the liver. Of note, resveratrol may increase the activity levels of cancer-detoxifying enzymes such as glucuronosyltransferase (Chow et al. 2010). The authors of this study caution that a similar effect on inhibition of cytochrome P450 activity might counterbalance an antineoplastic effect.

A pilot study was performed in colon cancer patients who received either resveratrol or freeze-dried grape powder orally (Nguyen et al. 2009). Normal colonic mucosa and colon tumors were evaluated before and after the intervention. A reduction in Wnt pathway target genes was noted, primarily on the normal mucosa with minimal effect on Wnt signaling in tumor tissue. The most significant effects were observed with low-dose grape powder. These data suggest that the primary clinical efficacy may be in cancer prevention and not treatment of established colon cancer. A more recent study involved the administration of 1/3 to 1 pound of whole red grapes per day for 2 weeks to normal volunteers' diet

(Martinez et al. 2010). Colorectal mucosal biopsies were obtained pre- and post-grape supplementation and evaluated for markers of proliferation and Wnt signaling. Following grape ingestion, the mucosal proliferation rate was significantly reduced as measured by Ki67 staining at the base of crypts. In addition, Wnt target gene expression was reduced similar to the prior study with grape powder. The reduction in Wnt signaling and proliferation was seen primarily in individuals over the age of 50 and those on a high-arginine diet (Holcombe et al. 2014), two groups that are at increased risk for the development of colon cancer.

6 Bioavailability

While resveratrol effects multiple molecular targets, the concentrations attained in vivo are significantly lower than the concentrations required for activity in vitro, raising the question as to how resveratrol exhibits this activity in animal models and perhaps in humans (Gescher and Steward 2003). Selection of the correct dose is problematic as the concentrations noted in vitro to exhibit activity are much higher than can be achieved in humans (Scott et al. 2012). A recent pharmacokinetic study of single-dose resveratrol confirmed that peak plasma concentrations of the parent compound following a single large 5 g ingestion reached only 539 ng/ml (2.4 μM), significantly lower than the 5.0 μM expected to be necessary for cancer prevention activity (Boocock et al. 2007). The peak levels of conjugated metabolites resveratrol-3-sulfate and two monoglucuronides were 3–8× higher, raising the possibility that cancer prevention activity may be, at least in part, attributable to resveratrol's glucuronide and sulfate metabolites (Walle 2011). Sulfated metabolites have been shown to have antitumor activity against breast cancer cells in vitro (Miksits et al. 2009).

While well absorbed, resveratrol has low bioavailability (Walle et al. 2004). Lower-dose ingestions of resveratrol in the range of 25 mg yield systemic levels of only 7.5–40 nM (Soleas et al. 2001; Goldberg et al. 1995). One explanation of the in vivo activity of resveratrol may be that, even though serum concentrations are low, local concentrations in the gut are sufficient to provide activity (Patel et al. 2010). Still, it appears that the effective dose of the parent compound required to attain sufficient concentrations for activity is large and achieving this is not straightforward. Alternatively, the suspected mechanisms of action requiring high micromolar concentrations in vitro may not be those operative in vivo. Even low concentrations of resveratrol have been shown to affect signaling pathways in vitro and in the human GI tract (Hope et al. 2008; Nguyen et al. 2009).

7 Whole Food vs. Component Considerations

A significant issue when considering the benefits of dietary interventions is whether single components provided as supplements are as effective as consuming phytochemicals from whole food sources. For example, Burton-Freeman and Sesso found that ingestion of tomatoes had a superior effect on cardiovascular risk endpoints compared to lycopene supplementation (Burton-Freeman and Sesso 2014). In a study comparing freeze-dried grape powder or resveratrol on Wnt signaling endpoints in the colonic mucosa, Nguyen et al. reported a greater effect by the whole food source product, suggesting that other compounds found in grapes might be synergistic with resveratrol resulting in greater effectiveness than the isolated compound (Nguyen et al. 2009). Other studies have demonstrated synergy of resveratrol and curcumin in inhibition of colon cancer cell growth (Majumdar et al. 2009), and it is reasonable to assume that synergy exists among the myriad of phytochemicals present in grapes.

Grape juice prepared from whole grapes has been shown to increase plasma total antioxidant capacity (Yuan et al. 2011) and to have chemopreventive activity in a two-stage mouse skin cancer model, possibly by blocking activation of COX2 (Arimoto-Kobayashi et al. 2013). Similarly, consumption of grape powder increases plasma antioxidant activity (Prior et al. 2007). Finally, in a recent study looking at dietary supplementation of up to a pound daily of whole red grapes which is reported (Holcombe et al. 2015), significant effects were reported showing a reduction in the proliferation rate and in the extent of Wnt signaling in colonic mucosa. More research on the utility of whole grapes on cancer prevention and cancer treatment endpoints, in addition to further investigations with individual components and defined combinations of components, is needed.

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Grapes and Gastrointestinal Health: Implications with Intestinal and Systemic Diseases

Brian Collins, Jessie Baldwin, Kristina Martinez, Mary Ann Lila, and Michael McIntosh

Contents

Abstract The anti-inflammatory, antioxidant, or antimicrobial properties of phytochemicals found in fruits and vegetables are well documented. Phytoactive compounds and their metabolites have typically been monitored in blood or non-intestinal tissues of animals or human subjects consuming whole foods, extracts, or individual phytochemicals or examined after phytochemical treatment of cells in culture. Much less is known about the influence of polyphenols, in particular those found in grapes (e.g., anthocyanins), on intestinal health and how these polyphenols indirectly influence systemic metabolism. Notably, polyphenols may influence nutrient digestion and absorption, and gut microbiota taxa and their fermentation products, in part, because they are poorly absorbed in the upper gut and thus persist in the colon. Here, they come in direct contact with microbes, influencing microbial growth and metabolism, as well as undergoing enzymatic modification based on the available microbes. Whereas a great deal is known about

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the fermentation of fiber, there are gaps in the literature concerning how polyphenols influence microbial metabolism and vice versa. Therefore, this paper will focus on studies examining the influence of polyphenols in general and grape polyphenols in particular, on intestinal health, and subsequent metabolic consequences.

1 Nutrient Content of Grapes

Macronutrient Content Table grapes contain approximately 82 % water, 12–18 % simple sugars (primarily glucose and fructose), and 0.2–0.8 % acid, primarily tartaric and malic acid (California Table Grape Commission website 2015). One serving of table grapes (3/4 cup or 126 g) contains approximately 85 kcals, 20 g carbohydrate, 0.8 g protein, and 0.07 g fat.

Micronutrient One serving of table grapes contains approximately 0.7 g of minerals (ash; e.g., 224 mg potassium, 24 mg phosphorus, 12 mg calcium) and small amounts of vitamins (e.g., 49 IU vitamin A, 0.6 mg vitamin C, 11 μg folic acid) (California Table Grape Commission website 2015).

Phytochemical Content Table grapes are rich in polyphenols including flavonol glycosides (e.g., quercetin, kaempferol, myricetin, laricitrin, isorhamnetin, syringetin), anthocyanins (e.g., malvidin, peonidin, petunidin, cyanidin, delphinidin, pelargonidin), flavan-3-ols (e.g., catechin, epicatechin), phenolic acids (e.g., protocatechuic acid, gallic acid), hydroxycinnamates (e.g., caftaric acid, coutaric acid, fertaric acid), and stilbenes (e.g., *trans*-resveratrol) (Cantos et al. 2002; Castillo-Munoz et al. 2009; Nicoletti et al. 2008; Chuang et al. 2012).

2 Impact of Polyphenols on Nutrient Digestion and Absorption

Influence on Carbohydrate, Fat, or Protein Absorption Polyphenols have been reported to reduce carbohydrate absorption, possibly by interfering with amylase activity, thereby reducing starch digestion and glycemic index (Thompson et al. 1984; Forester et al. 2012). In so doing, they provide carbohydrate for microbial growth in the lower gastrointestinal (GI) tract, particularly saccharolytic bacteria in the human GI tract such as *Bacteroides, Bifidobacterium, Clostridium, Eubacterium, Lactobacillus,* and *Ruminococcus* [reviewed in Maukonen and Saarela (2015a)]. Such an effect would enhance microbial fermentation in the lower GI tract, thereby increasing short-chain fatty acid (SCFA) synthesis and energy harvest and decreasing intestinal pH. Thus, polyphenols have the potential

to influence bacterial diversity and enteric and systemic health status. Polyphenols may also interfere with lipases or proteases, decreasing fat and protein digestion, respectively, thereby increasing their potential to be fermented by intestinal microbes [reviewed in Jakobek (2015)].

Influence on Vitamin or Mineral Absorption Based on their robust antioxidant properties, dietary polyphenols may prevent the oxidation of macro- and micronutrients, thereby preserving their quality. Some polyphenols, however, can interfere with mineral absorption. For example, gallic acid, chlorogenic acids, monomeric flavonoids, and polyphenolic polymerization products inhibit nonheme iron absorption by as much as 50 % [reviewed in Monsen (1988), Smith et al. (2005)]. Moreover, tannins and gallic acid have been reported to bind to zinc and impair its absorption [reviewed in Monsen (1988)].

3 Intestinal Bioaccessibility, Bioavailability, and Metabolism of Polyphenols

Food Components Impact Polyphenol Bioaccessibility and Bioavailability Polyphenol bioaccessibility (i.e., the amount available for absorption in the intestine) and bioavailability (i.e., the rate and extent of absorption and availability for metabolism) are influenced by their own structure (e.g., glycosides versus aglycones), degree of polymerization (e.g., monomers versus polymers), and types of interactions (e.g., covalent or hydrophobic bonding) with food matrices they are associated with (e.g., sugars, fiber, and proteins in the specific berry), dietary status (e.g., fed versus fasting) and diet composition (e.g., protein, fat, carbohydrate, and fiber content), and the intestinal pH and abundance of digestive enzymes, which are influenced mainly by other dietary ingredients [Lila et al. 2012, reviewed in Bohn (2014)]. Conjugated polyphenols require deconjugation in order to diffuse into the enterocyte [reviewed in Rein et al. (2013)]. The brush border of the small intestine contains membrane-bound β-glucosidases which facilitate the process for hydrolyzing gluconated polyphenols into more readily absorbable aglycones [reviewed in van Duynhoven et al. (2001)]. Once within the enterocyte, the aglycone will undergo phase I (e.g., reduction, oxidation, or hydrolysis) or phase II (e.g., conjugation) metabolism, converting them into methyl esters, glucuronides, and sulfates, or be transported as aglycones via the portal system to the liver for similar metabolism [reviewed in Chiou et al. (2014)]. Conjugating aglycones reduces their potential microbial toxicity while also making them easier to transport as biotransformed polyphenols. Additionally, the type and amount of dietary macronutrients can alter the composition of intestinal microbes, which in turn influences polyphenol biotransformation in the GI tract (Fava et al. 2012).

For example, a high-fat meal increases the bioaccessibility of multiple berry anthocyanins, and protein-rich matrices protect berry anthocyanins from degradation in the upper GI tract, thus making them available to the lower GI tract for

microbial metabolism (Ribnicky et al. 2014). In order to demonstrate the potential beneficial effects of protein protection of polyphenols, defatted soybean flour was used to adsorb, concentrate, and stabilize Concord grape juice-derived polyphenols (e.g., particularly anthocyanins, hydroxycinnamic acids, and proanthocyanidins) and exclude polar sugars. Using this method to enhance polyphenol bioavailability, the authors tested the acute, antidiabetic properties of Concord grape juice in C57BL/6J mice. Notably, fasted mice gavaged with a bolus of defatted soybean flour enriched with grape juice polyphenols had lower blood glucose levels compared to control mice (Roopchand et al. 2012). Similarly, fasted mice receiving a single bolus of the polyphenol-rich grape pomace complexed to soy protein isolate had lower blood glucose compared to controls (Roopchand et al. 2013). It is noteworthy that the grape pomace polyphenols in the soy protein isolate complex had a much greater stability compared to those in the non-complexed extract.

In vitro GI systems have provided unique insights into the bioaccessibility and bioavailability of plant polyphenols. For example, using a model mimicking the human GI tract (TIM-1) from the mouth to the ileum, it was demonstrated that most berry anthocyanins were bioaccessible within the 2–3 h post-ingestion, primarily in the jejunum, and thus were potentially available for absorption (Lila et al. 2012). These authors further demonstrated using radiolabeled polyphenols generated in grape cell cultures fed ^{13}C- or ^{14}C-labeled carbohydrate sources and gavaged to rats that grape polyphenols enriched the blood system within 15 min to 4 h post-administration and reached systemic tissues including the brain. Interestingly, grape anthocyanin glycosides (e.g., cyanidins and peonidins) were better absorbed than less polar, grape proanthocyanidins. Consistent with these data, several polyphenols in California table grapes (e.g., quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, and rutin) appeared in the blood stream within the first hour post-gavage (Chuang et al. 2012).

Bacterial Metabolism or Biotransformation of Polyphenols Dietary polyphenols that escape absorption in the upper GI tract are exposed to microbes and intestinal enzymes of the lower GI tract and undergo further metabolism or biotransformations [e.g., deconjugation of the glycosyl or glucuronosyl component on the phenol backbone, cleavage of polymeric proanthocyanidins, hydrolysis of esterified phenolic acids; reviewed in Selma et al. (2009), Kemperman et al. (2010)]. Microbial transformation of polyphenols to more or less bioaccessible and bioactive metabolites can directly affect their influence on intestinal microbial growth and metabolism. Alternatively, biotransformed polyphenols (e.g., aglycones, phenolic acids, monomeric proanthocyanidins) may be directly absorbed into the mucosa or bloodstream, where they may activate local or systemic receptors influencing metabolism. In general, gut microbe enzymes (e.g., glucosidases, glucuronidases, esterases, hydrogenases, dehydroxylases, decarboxylases, demethylases, and isomerases) convert a diverse group of dietary polyphenols into a relatively small group of aromatic metabolites [reviewed in Selma et al. (2009)].

For example, benzoic, hippuric, and vanillic acids are the main microbial metabolites of green tea polyphenols [reviewed in Fang et al. (2008)]. These

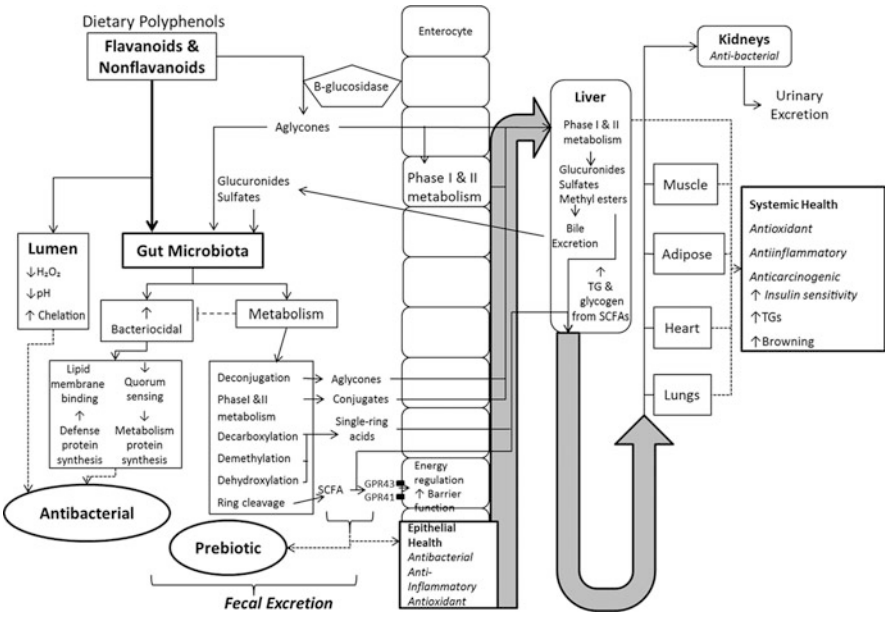

Fig. 1 Polyphenol metabolism and potential intestinal and systemic health benefits. Polyphenols are poorly absorbed and thus come in direct contact with gut microbes in the lower GI tract. Although some polyphenols are deconjugated in the small intestine into aglycones, which may passively diffuse into the enterocyte, the majority travel to the distal small intestine and colon. Within the intestinal lumen, polyphenols can indirectly influence microbial populations by reducing the pH and hydrogen peroxide (H_2O_2) levels. They can also chelate with unabsorbed metal ions, thus negatively influencing the growth of pathogenic bacteria and some Gram-positive bacteria. Polyphenols can be toxic to bacterial cells by disrupting normal cell properties (e.g., binding to cell membranes, proteins, or DNA) or otherwise be metabolized, thereby reducing their potential toxic effects on certain gut microbes. Some microbial metabolites may be used as energy sources (e.g., SCFAs like butyrate), promoting beneficial microbial growth and improving gut barrier function, whereas others interact within the enterocyte or diffuse through enterocytes. Absorbed polyphenol metabolites and aglycones are delivered to the liver via the portal vein before entering the systemic circulation or the bile. Aglycones undergo further metabolism by hepatic phase I and II enzymes, once again becoming conjugated to facilitate their transport systemically or for secretion into the bile for transport back into the intestinal lumen upon release from the gall bladder. As polyphenols and metabolites travel through systemic circulation, they may interact with various tissues or be excreted in the urine [adapted from Kemperman et al. (2010)]

metabolites, in turn, may be absorbed across the intestinal mucosa into the portal blood and sent to the liver for further metabolism (e.g., phase II conjugates such as glucuronidated and sulfated metabolites that can enter the circulation or bile acid pool) by the host, used by intestinal microbes, or excreted in the feces (Fig. 1). For instance, several intestinally derived polyphenol metabolites (i.e., hydrocaffeic, dihydroxyphenylacetic, and hydroferulic acids) suppressed inflammatory prostaglandin production in colon cancer cells and in rodents (Larrosa et al. 2009a). In addition, hydrocaffeic reduced inflammation and DNA damage in a chemically

induced model of ulcerative colitis [i.e., dextran sulfate sodium (DSS) treated; Larrosa et al. 2009a]. Similarly, a microbial metabolite of curcumin (i.e., ferulaldehyde) reduced inflammation and extended lifespan in endotoxin-treated rodents (Radnai et al. 2009). Polyphenols may influence the biotransformation of other phytonutrients [e.g., depolymerization, (de)glycosylation or glucuronidation, (de)methylations, (de)sulfation, or (de)hydroxylation] by selectively altering microbial populations that influence these enzymatic modifications, thereby impacting their solubility and potential for absorption.

4 Prebiotic Properties of Polyphenols

Promicrobial Properties Obesity is associated with intestinal dysbiosis, with an increased ratio of *Firmicutes* to *Bacteroidetes* (Ley et al. 2006). Grape products, extracts, and polyphenols including quercetin, fructo-oligosaccharides, and grape juice, on the other hand, have been shown to positively influence the intestinal microbiota. Fructo-oligosaccharide has also been shown to enhance the growth of health-promoting, butyrate-producing bacteria from *Firmicute* and *Bifidobacterium* families (Scott et al. 2013). Several grape juice varieties have also demonstrated promicrobial effects by increasing the growth of *Lactobacillus acidophilus* and *L. delbrueckii*, two probiotic bacteria, while attenuating growth of *E. coli* in vivo (Agte et al. 2010). Notably, dietary polyphenols have been reported to increase the abundance and diversity of microbial populations [reviewed in Tuohy et al. (2012)], including populations of healthy gut bacteria (e.g., decrease ratio of *Firmicutes*/*Bacteroidetes*; increase *Lactobacilli* and *Bifidobacteria*; increase *Akkermansia muciniphila*, *Roseburia* spp., *Bacteroides* and *Prevotella* spp.) (Selma et al. 2009; Neyrinck et al. 2013; Anhê et al. 2014).

In regard to grape polyphenols, DSS-treated rats consuming the phytoalexin resveratrol had higher levels of *Lactobacilli* and *Bifidobacteria* and improved colon mucosa architecture and inflammatory profile compared to controls (Larrosa et al. 2009b). Inoculation of *L. acidophilus* and *L. plantarum*, two probiotic bacteria, with quercetin plus fructo-oligosaccharides increased their growth in culture compared to normal growth conditions (Yadav et al. 2011). Additionally, grape anthocyanins such as malvidin-3-glucosides increased the growth of *Bifidobacterium* and *Lactobacillus-Enterococcus* bacteria (Hidalgo et al. 2012). Additionally, feeding grape antioxidant dietary fiber, containing the fiber and antioxidant components from grapes, significantly increased *Lactobacillus* spp. within in the cecum of rats compared to controls (Pozuelo et al. 2012). Grape pomace juice given to rats increased fecal abundance of *Lactobacillus* and *Bifidobacterium* and consequently resulted in an increase in the concentration of primary bile acids, cholesterol, and cholesterol metabolites, while decreasing the concentration of secondary bile acids in feces (Sembries et al. 2006). Such alterations in bile acids are associated with a reduced risk of intestinal cancers. Consistent with these data, rats supplemented with red wine polyphenols had lower levels

of *Clostridium* spp. and increased levels of *Lactobacillus* spp. (Dolara et al. 2005). Furthermore, healthy adults consuming red wine had a greater abundance of *Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis, Eggerthella lenta,* and *Blautia coccoides-Eubacterium rectale* groups compared to baseline. Moreover, the wine consumers had improved blood pressure, lower blood cholesterol, and C-reactive protein (CRP) levels, which were positively correlated with *Bifidobacteria* (Queipo-Ortuño et al. 2012). Another potential prebiotic benefit of wine polyphenols is their growth enhancement of specific strains of *L. plantarum* (Barrosa et al. 2014). Adult males given a proanthocyanidin-rich extract had a dramatic shift in fecal microbial populations from *Bacteroides*, *Clostridium*, and *Propionibacterium* phyla to *Bacteroides*, *Lactobacillus*, and *Bifidobacterium* predominance (Cardona et al. 2013).

As such, these phytochemical-mediated alterations in intestinal microbial populations can be considered prebiotic actions, as they can result in an improved health status for the host. Notably, mice consuming a high-fat, high-sugar diet supplemented with two polyphenols found in grapes, *trans*-resveratrol (15 mg/kg BW/day) and/or quercetin (30 mg/kg BW/day), had lower body weights and insulin resistance compared to controls (Etxeberria et al. 2015). Quercetin-mediated improvements in systemic health were associated with a decreased ratio of *Firmicutes*/*Bacteroidetes* and decreased abundance of bacteria induced by the high-fat, high-sucrose diet (e.g., *Erysipelotrichaceae, Bacillus, Eubacterium cylindroides*), thereby attenuating diet-induced dysbiosis. Within the quercetin-mediated increase in the *Bacteroidetes* phylum, the abundance of *Bacteroidaceae* and *Prevotellaceae* families was increased (Etxeberria et al. 2015), which has been previously found to be reduced in high-fat-fed mice (Hildebrandt et al. 2009). Although mice-fed *trans*-resveratrol suppressed intestinal markers of inflammation and enhanced markers of barrier function, it had only a minimal impact on gut microbial profiles.

Antimicrobial Properties Polyphenols have been reported to decrease populations of coliforms and other unhealthy gut bacteria, indicating that they have bacteriostatic or bactericidal activity or prevent adhesion of disease-causing bacteria (Selma et al. 2009). Other antibacterial properties include inhibiting quorum sensing [reviewed in Gonzalez and Keshavan (2006)], disrupting lipid membrane integrity (Kemperman et al. 2010), and DNA polymerase activity [reviewed in Cushnie and Lamb (2005)]. For example, anthocyanin-rich berries have been shown to prevent the growth of several infectious microbial strains (Lee et al. 2003, 2006). Tea polyphenols decreased the growth of *Candida albicans* (Evensen and Braun 2009). Microbial metabolites derived from exposure to phenolic compounds in berries attenuated salmonella growth (Alakomi et al. 2007). Grape seed extract, an oligomeric-rich fraction from grape seed extract, and a grape polyphenol (e.g., gallic acid) demonstrated robust antimicrobial actions against pathogens associated with respiratory diseases (Cueva et al. 2012). Several wine and grape seed polyphenols, specifically flavan-3-ol, exhibited antibacterial activity directed toward specific bacterial strains (e.g., Gram-positive bacteria such as the

Staphylococcus) in human fecal samples cultured with various wine or grape extracts (Cueva et al. 2015). Lastly, resveratrol was shown to be a candidate for decreasing the growth of drug-resistant strains of *Mycobacterium smegmatis* (Lechner et al. 2008).

Influence of Polyphenols on Microbial Fermentation Products By differentially impacting populations of gut microbes, polyphenols can alter the production of the SCFAs acetate, propionate, and butyrate, which are at molar ratios of 60:23:17 under normal feeding conditions (Blaut 2014) and represent approximately 10 % of energy intake in humans (Bergman 1990). These SCFAs attenuated high-fat diet-induced obesity and insulin resistance via increasing AMP kinase (AMPK) activity and oxidative metabolism and inhibiting peroxisome proliferator-activated receptor (PPAR)-γ. Such outcomes demonstrate the ability of SCFAs to decrease body fat by increasing fatty acid and glucose oxidation and decreasing adipogenesis or lipogenesis, respectively (den Besten et al. 2015). In a separate report, SCFAs produced from fructo-oligosaccharides, as well as butyrate and propionate alone, decreased body weight and improved glucose tolerance in mice (De Vadder et al. 2014). This was attributed to SCFA-induced intestinal gluconeogenesis. While butyrate directly induced gluconeogenic gene expression in the small intestine, propionate stimulated intestinal gluconeogenesis through a free fatty acid receptor (FFAR)3-mediated mechanism involving neural circuits between the gut and brain. Interestingly, the beneficial effects of fructo-oligosaccharides and SCFAs were lost in mice deficient in intestinal glucose-6-phosphatase (De Vadder et al. 2014). Other fermentation products include lactate, succinate, isobutyrate, 2-methyl propionate, valerate, isovalerate, hexanoate, and ethanol [reviewed in Blaut (2014), Samuel et al. (2008), Wong et al. (2006)].

Collectively, these fermentation products can regulate energy harvest, depending on the energy density and composition of the diet and subsequent products formed. For example, propionate is a precursor for hepatic gluconeogenesis, propionate and acetate are precursors of cholesterol synthesis, and acetate and butyrate are substrates for hepatic and white adipose tissue (WAT) triglyceride (TG) synthesis. However, butyrate is unique in that it is the preferred energy substrate for colonocyte growth and differentiation, accounting for approximately 70 % of the oxidation of SCFAs (Roediger 1980). Notably, butyrate has been shown to reduce the growth of and stimulate apoptosis in colorectal cancer cells via upregulation of wnt/β-catenin signaling due to butyrate's inhibition of specific histone deacetylases (Lazarova et al. 2014). Butyrate also increases the localization of tight junction proteins on the apical surface of epithelial cells, thereby impeding the translocation of endotoxins (e.g., LPS, bacterial DNA, or peptidoglycans) into the systemic circulation [reviewed in Cox and Blaser (2013)]. Systemically, butyrate has been shown to increase leptin secretion from adipocytes (Samuel et al. 2008). Therefore, phytochemicals that increase butyrate production and proportionately decrease acetate and propionate production decrease energy harvest and vice versa.

Polyphenol-mediated increases in butyrate production could also reduce endotoxemia and subsequent metabolic dysfunction via enhancing goblet cell-

mediated barrier function (Hatayama et al. 2007). Butyrate can also inhibit nuclear factor-κB (NFκB) signaling pathways, thereby reducing inflammatory cytokine synthesis in conjunction with ulcerative colitis or Crohn's disease (Segain et al. 2000; Lührs et al. 2002).

Fermentation Products Influence Intestinal pH Polyphenol-mediated changes in fermentation products by gut microbes influence intestinal pH, which in turn impacts the growth of specific bacteria. The acidic nature of SCFAs reduces luminal pH throughout the lower GI tract, potentially preventing the growth of pathogenic bacteria (i.e., *Enterobacteriaceae*) (Roe et al. 2002; Hirshfield et al. 2003). This effect on pH may also be a determining factor on which a class of fermenters predominates. At more neutral pH (6.5), acetate producers predominate, whereas in a more acidic environment (pH 5.5), butyrate producers predominate (Walker et al. 2005). Indigestible oligosaccharides facilitate a lower pH, allowing butyrate producers to compete for substrates more efficiently than acetate or propionate producers that have slower growth rates (El Oufir et al. 1996). The ring cleavage of flavonoids into SCFAs similar to fermentation of fiber has the same beneficial effects on energy intake, metabolism regulation, and improvements to epithelial health and integrity (Czank et al. 2013).

Fermentation Products May Influence Bile Acid Metabolites Polyphenols may also influence bile acid metabolism, thereby altering the types and abundance of primary and secondary bile acids that have local and systemic effects [reviewed in Maukonen and Saarela (2015b)]. This is particularly relevant during high-fat feeding, because fat increases the secretion of bile acids into the GI tract and fat type and amount influence microbial diversity. For example, consuming a milk/butter fat diet rich in saturated fat (e.g., 37 % kcals from fat, primarily milk/butter fat) for 24 weeks has been shown to increase biliary secretion of taurocholic acid that is metabolized by sulfidogenic bacteria, causing the production of proinflammatory metabolites that impair gut health (Devkota et al. 2012). Consistent with these data, feeding a diet rich in saturated fat (i.e., 60 % kcals from lard) increased the abundance of sulfidogenic bacteria (Zhang et al. 2010; Shen et al. 2014) and compromised gut barrier function (Shen et al. 2014). Consumption of low-carbohydrate, high-fat diets by adult subjects decreased butyrate concentrations and total SCFAs and the abundance of *Bifidobacteria* (Brinkworth et al. 2009).

Fermentation Products Activate Endocrine Cell Signals Polyphenols, their metabolites, or SCFAs may activate intestinal enteroendocrine cells (e.g., L cells) via activation of G-protein receptors (GPRs) including GPR41, GPR43, or GPR119 (aka FFARs). The GPRs are coupled to the secretion of peptides that influence host metabolism. For example, butyrate has been reported to increase glucagon-like-peptide (GLP)-1 secretion (Samuel et al. 2008), and GRP-mediated secretion of GLP-1 and 2 inhibits gastric emptying, enhances insulin secretion and sensitivity, and increases satiety [reviewed in Holst (2007)]. GLP-1 secretion is also influenced by the energy content of the diet and the presence of gut microbes (Wichmann et al. 2013). Similarly, GPR-mediated polypeptide YY (PYY) secretion can protect

against obesity via increasing satiety or energy expenditure, possibly via increasing thermogenesis in BAT or in WAT with beige adipocytes via direct activation of WAT or indirectly via activation of the sympathetic nervous system (Mestdagh et al. 2012). However, GPR41-mediated activation of PYY can paradoxically contribute to obesity via increasing intestinal transit time and thus energy harvest [reviewed in Cox and Blaser (2013)]. Additionally, germ-free mice that lack gut microbiota and thus SCFAs have increased GLP-1 levels, which was associated with decreased intestinal transit time. However, upon monoassociation with the propionate and acetate producer, *Bacteroides thetaiotaomicron*, intestinal transit time, and GLP-1 levels were restored (Wichmann et al. 2013). In spite of these findings, the influence of whole grape consumption on changes in intestinal microbial populations, energy harvest, and barrier function and associations with systemic health has not yet been reported.

5 Antioxidant Properties of Polyphenols and Their Roles in Health Promotion

Polyphenols Quench Prooxidants Polyphenols have been shown to scavenge reactive oxygen (ROS), nitrogen species (RNS), and nitric oxide (NO) radicals that trigger oxidative stress, cytotoxicity, apoptosis, or inflammation due to the their abundance of hydroxyl groups that readily donate a hydrogen atom to or stabilize an unshared electron in electrophiles (Fig. 2). Attenuation of free radicals prevents the activation of proenzymes [e.g., NAPDH oxidase, nitric oxide synthase (NOS)] that generate ROS and NO radicals, respectively, that trigger inflammatory mitogen-activated protein kinases (MAPKs) (e.g., ASK1, JNK, p38, ERK) and transcription factors (e.g., NFκB, AP-1) that induce inflammatory gene expression. Polyphenols also protect against oxidative damage via upregulating the expression of antioxidant genes [e.g., heme oxygenase (HO)-1, glutathione peroxidase (GPX), superoxide dismutase (SOD)-1/2, and γ-glutamate-cysteine ligase catalytic subunit (GCLC)] via activation of the nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2). By directly neutralizing free radicals and upregulating antioxidant enzymes, polyphenols may enhance glutathione levels and the need for GCLC, the rate-determining enzyme for glutathione synthesis.

Whole grapes, grape products, and grape components have been shown to decrease markers of oxidative stress systemically [reviewed in Chuang et al. (2012)]. However, less is known about the efficacy of grapes in attenuating intestinal prooxidants and disease risk. Red wine polyphenols fed to rats for 16 weeks blocked colon carcinogenesis, which was associated with a decrease in intestinal markers of oxidative stress and an abundance of *Bacteroides, Clostridium,* and *Propionibacterium* spp. (Dolara et al. 2005). Resveratrol supplementation of rats treated with the chemical colon carcinogen 1,2-dimethylhydrazine (DMH) decreased colonic tumor burden, which was associated with reductions in microbial

biotransforming enzymes linked with the development of cancer (e.g., β-glucuronidase, β-glucosidase, β-galactosidase, mucinase, nitroreductase, or sulfatase; Sengottuvelan and Nalinin 2006). Similarly, resveratrol supplementation of rats treated with DMH had decreased colonic DNA damage that correlated with increased activities of antioxidant enzymes (i.e., SOD, catalase, GPX, glutathione reductase) and levels of glutathione *S*-transferase and antioxidants (i.e., reduced glutathione, vitamin C, vitamin E, and β-carotene) and decreased markers of lipid peroxidation compared to non-resveratrol-supplemented mice (Sengottuvelan and Nalini 2009). However, the impact of whole grape consumption of intestinal markers of oxidative stress is unknown.

6 Anti-inflammatory Properties of Polyphenols

Activating PPARγ, an Anti-inflammatory Transcription Factor PPARγ is a transcription factor best known for its role in promoting adipogenesis and glucose uptake. It also has anti-inflammatory properties (Fig. 2). For example, increasing PPARγ activation has been demonstrated to antagonize NFκB and activator protein (AP)-1-mediated inflammatory gene expression, thereby reducing inflammation [reviewed in Ricote and Glass (2007)]. In addition, anti-inflammatory, alternatively activated (i.e., M2) macrophages require PPARγ for their activation (Bouhiel et al. 2007; Odegaard et al. 2007). Consistent with these data, grape feeding increased the expression of PPARγ and δ mRNA, subsequently increasing their DNA-binding activity, and decreased the activity of NFκB in hearts and reduced systemic markers of inflammation in rats (Seymour et al. 2010). Additionally, grape seed procyanidin supplementation reduced WAT mRNA levels of TNFα, IL-6, and CRP and reduced plasma levels of CRP in Zucker rats fed a high-fat diet (Terra et al. 2009). Quercetin and *trans*-resveratrol increased the abundance and activity of PPARγ and the mRNA levels of several PPARγ target genes and quercetin decreased inflammation and insulin resistance in primary cultures of human adipocytes treated with TNFα (Chuang et al. 2010). Similarly, quercetin and kaempferol increased PPARγ activity and decreased LPS-mediated nitric oxide levels and insulin resistance in murine 3T3-L1 (pre)adipocytes (Fang et al. 2008). However, the impact of grape consumption on intestinal PPARγ activity is currently unknown.

Activating Histone Deacetylases that Inhibit NFκB or Activate PGC1 Sirtuins (SIRTs) consist of a family of class III histone deacetylases (HDACs) that removes acetyl groups from lysine residues in histones and nonhistone proteins including transcription factors. This causes an increase or decrease in the activity of the targeted protein, depending on the role that the acetyl group plays in regulating the activity of the respective protein. For example, activation of SIRT1 causes NFκB deacetylation, thereby decreasing NFκB activity and inflammatory signaling. In contrast, deacetylation of PGC1α increases its activity, thereby enhancing mitochondrial biogenesis, activity, substrate oxidation, and energy expenditure.

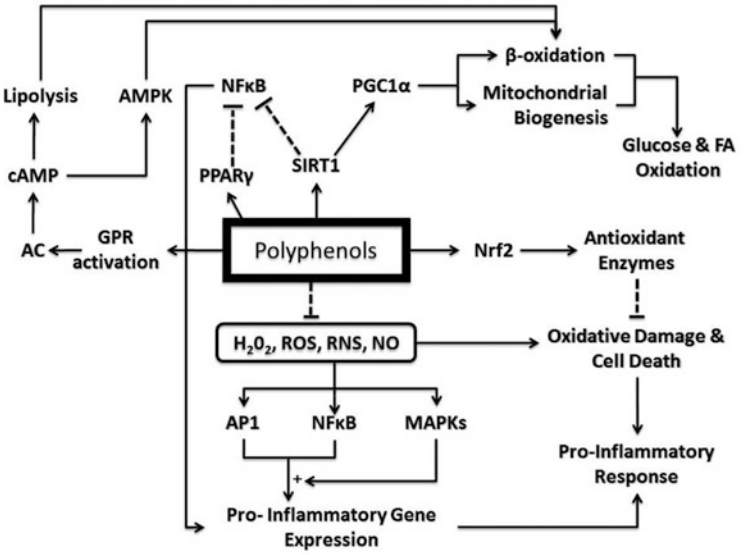

Fig. 2 Anti-inflammatory and antioxidant effects of polyphenols that impact glucose and fatty acid metabolism. Polyphenols have been shown to inhibit the production of prooxidant compounds including hydrogen peroxide (H_2O_2), reactive oxygen species (ROS), reactive nitrogen species (RNS), and nitric oxide (NO), thereby preventing activation of the mitogen-activated protein kinases (MAPKs), nuclear factor κB (NFκB), and activator protein (AP)1 pathways and subsequent proinflammatory response. Additionally, polyphenols have been shown to activate nuclear factor-erythroid 2-related factor 2 (Nrf2), which increases the expression of antioxidant enzymes that inhibit oxidative damage and cell death associated with prooxidants. Polyphenols also may activate peroxisome proliferator-activated receptor gamma (PPARγ) and sirtuin 1 (Sirt1), which also inhibit NFκB and proinflammatory responses via deacetylation. Sirt1 also can activate peroxisome proliferator-activated receptor γ coactivator 1-alpha (PGC1α) which stimulates β-oxidation and mitochondrial biogenesis, leading to glucose and fatty acid (FA) oxidation. Additionally, polyphenols may activate G-protein receptors (GPR) stimulating adenylate cyclase (AC), cyclic adenosine monophosphate (cAMP), and 5′-adenosine monophosphate-activated protein kinase (AMPK) which stimulate lipolysis, β-oxidation, mitochondrial biogenesis, and subsequent glucose and fatty acid oxidation

Notably, several polyphenols found in grapes (i.e., *trans*-resveratrol, quercetin) have been shown to activate SIRT1 (Howitz et al. 2003). Consistent with these data, in vitro or in vivo studies have demonstrated that resveratrol reduced inflammatory signaling and improved insulin sensitivity in a SIRT1-dependent manner by deacetylating NFκB (Fischer-Posovszky et al. 2010; Olholm et al. 2010; Yang et al. 2010; Zhu et al. 2008) and PGC1α, (Lagouge et al. 2006; Sun et al. 2007), leading to an increase in mitochondrial biogenesis, the expression of genes associated with oxidative phosphorylation, and aerobic capacity (Lagouge et al. 2006).

Suppressing Immune Cell Infiltration or Inflammatory Signaling in the Intestines Grape polyphenols such as rutin, glycosides of quercetin, and resveratrol have been reported to reduce intestinal inflammation in rodents (Galvez

et al. 1997; Kwon et al. 2005; Martin et al. 2004, 2006). Similarly, resveratrol attenuated nitric oxide synthase activity and mucosal damage in an experimental necrotizing enterocolitis rat model (Ergun et al. 2007). Intestinal colitis was attenuated by concentrated grape juice in Wistar rats, with the flavonoids being the proposed facilitators of these beneficial changes in gut health (Paiotti et al. 2013). In addition, grape seed extract given to IL-10 deficient mice reduced inflammatory bowel disease inflammatory markers, increased goblet cell number, and decreased myeloperoxidase activity, a marker of neutrophil infiltration (Suwannaphet et al. 2010). Notwithstanding, the impact of whole grape consumption of intestinal markers of inflammation is unknown.

7 Conclusions and Implications

Grapes and their by-products are rich in nutrients and phytochemicals that have anti-inflammatory, antioxidant, and antimicrobial properties that potentially influence intestinal and systemic health. They can have positive and negative effects on nutrient absorption. For example, polyphenols found in grapes have antioxidant properties that protect micro- and macronutrients from oxidative damage, thereby preserving their biological value. However, they can interfere with the digestion and absorption of macro- and micronutrients by interfering with hydrolytic enzymes necessary for digestion and by binding to micronutrients, thereby preventing their absorption. Notably, impaired absorption of nutrients in the upper GI tract allows microbes in the lower GI tract to metabolize them, thereby impacting their growth and the metabolic by-products they produce. Furthermore, the structure of polyphenols, the food matrices they are associated with, and the composition of the diet they are ingested with influence their bioaccessibility and bioavailability.

Dietary polyphenols that escape absorption in the upper GI tract come in contact with microbes in the lower GI tract. These interactions may favorably influence microbial growth (e.g., increased abundance of *Lactobacilli* and *Bifidobacteria*, two types of bacteria positively associated with intestinal health) and their metabolic products (e.g., the SCFA butyrate which enhances colonocyte growth and integrity or GLP-1/2 which enhances systemic insulin secretion and sensitivity and satiety). Furthermore, microbial biotransformation of polyphenols can enhance their absorption into the portal vein, delivering them to the liver, systemic tissues, or back to the gut via bile acid secretion from the gall bladder.

As antimicrobial agents, several polyphenols found in grapes or wine decrease the growth or adherence of disease-causing bacteria, thereby attenuating their virulence. As anticancer or anti-inflammatory bowel disease agents, they may alter bile acid metabolites (e.g., reduce cholic acid metabolism by sulfidogenic bacterial) or neutralize prooxidants (e.g., ROS, NO, RNS, H_2O_2) that damage DNA and proteins. They may also reduce populations of gut microbes that produce these electrophiles. Lastly, polyphenols found in grapes may directly promote local and systemic health of the host by activating HDACs like SIRT1 that activate the anti-

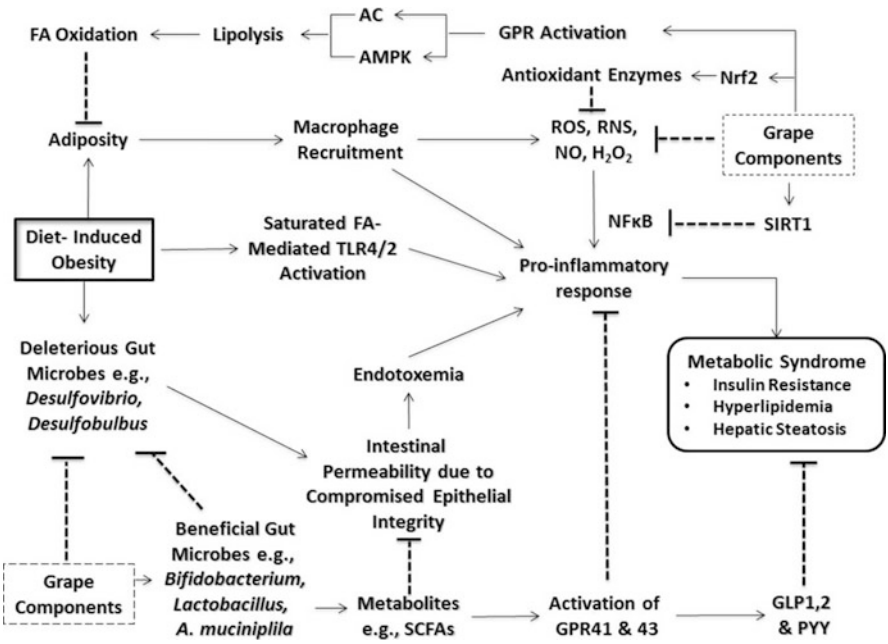

Fig. 3 Proposed working model on how table grapes attenuate intestinal microbes and metabolism, potentially contributing to reductions in diet-induced obesity, inflammatory signaling, and insulin resistance. As prebiotics, grape polyphenols may enhance the abundance of specific types of healthy bacteria (e.g., *Bifidobacterium, Lactobacillus, Akkermansia muciniphila*) that contribute to short-chain fatty acid (SCFA) production and activation of G-protein receptor (GPR)s 41 and 43 resulting in suppression of complications associated with consuming a high-fat diet (i.e., inflammation and metabolic syndrome). Grape polyphenols also decrease the abundance of noxious bacteria (e.g., sulfidogenic bacteria) that impair gut barrier functions (i.e., cause leaky gut). These improvements in barrier function prevent systemic endotoxemia (i.e., increase LPS, peptidoglycan, or bacterial DNA in the bloodstream)-mediated inflammatory gene or protein expression. Alternatively, grape polyphenols reaching the blood stream may directly attenuate saturated fatty acid (SFA)-mediated nutritional toxemia (e.g., SFA-mediated TLR4/2 activation) that triggers white adipose tissue inflammation leading to metabolic syndrome. Grape polyphenols may also activate G-protein receptors (GPRs), thereby activating adenylate cyclase (AC) and 5′-adenosine monophosphate kinase (AMPK), which increase lipolysis and fatty acid (FA) oxidation, thereby attenuating adiposity, subsequent macrophage recruitment and reactive oxidant species (ROS), reactive nitrogen species (RNS), nitric oxide (NO), and hydrogen peroxide (H_2O_2) production associated with high-fat-induced obesity. Alternatively, they may activate nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2), a transcription factor that induces the expression of antioxidant genes [e.g., heme oxygenase (HO)-1, glutathione peroxidase (GPX), superoxide dismutase (SOD)-1/2, and γ-glutamate-cysteine ligase catalytic subunit (GCLC)] that neutralize free radicals

inflammatory transcription factor PPARγ and deactivate the proinflammatory transcription factor NFκB.

A working model of these potential health-promoting properties of grape phytochemicals is shown in Fig. 3, including the proposed linkage between intestinal

and holistic systems. However, the extent to which whole grapes alter gut microbiota, inflammatory status, and barrier function is hypothetical, as are their contributions to systemic health. Research is needed to determine if feeding whole grapes at levels that are achievable in humans can indeed improve intestinal health and if these proposed beneficial effects in the GI tract are associated with systemic benefits.

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Grapes and the Brain

Pamela Maher

Contents

Abstract Since deficits in brain function can have a tremendous impact on overall physiology and greatly reduce the quality of life, much effort has gone into identifying treatments that can prevent the loss of brain function and/or restore normal brain function following disease or injury. Given that losses in brain function can involve multiple targets, perhaps the best therapeutic approach is to identify treatments that inherently contain multiple biological activities that can impact the different targets that are associated with the loss of brain function.

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Grapes provide one such treatment. Freeze-dried grape powder, grape seed extract and grape juice have all been shown to positively impact brain function in a variety of different in vivo models including stress, aging, ischemia, and Alzheimer's disease. In the brain, consumption of grape products has been shown to reduce oxidative stress, enhance trophic factor signaling, increase neurotransmitter release, reduce inflammation, improve vascular function, and reduce protein aggregation. These beneficial activities combined with the positive effects on cognitive function seen in many studies strongly suggest that further studies on the impact of grape products on brain function are highly worthwhile.

1 Introduction

In mammals, the brain acts as the core of the nervous system. As such, it exerts control over the other organs in the body. Moreover, it mediates information processing, perception, motor control, arousal, homeostasis, motivation, and learning and memory. Thus, deficits in brain function can have tremendous impacts on overall physiology and greatly decrease the quality of life.

Because of its importance to overall bodily function, much effort has gone into identifying treatments that can prevent the loss of brain function and/or restore normal brain function following disease or injury. Given that losses in brain function can involve multiple targets and the strong possibility that the relative importance of each of those targets will vary among individuals, it may be necessary to use combinations of drugs directed against different targets. However, this approach is subject to a number of potential problems including pharmacokinetic and bioavailability challenges which in the central nervous system (CNS) is exacerbated by the blood–brain barrier (BBB) and the potential for adverse drug–drug interactions. An alternative approach is to identify treatments that inherently contain multiple biological activities that can impact the different targets that are associated with the loss of brain function. One excellent source for these types of treatments is the original pharmacopeia, plants. The goal of this review is to provide an overview of the beneficial effects of grape products on brain function.

Almost all of the studies on grapes and the CNS have used one of three nonalcoholic grape products: grape juice, freeze-dried grape powder (FDGP) made from whole grapes including grape seeds, or grape seed extract (GSE) (Table 1). In all cases, FDGP was from the California Table Grape Commission and has been characterized with respect to polyphenol content. Since FDGP contains ~90 % (~50 % glucose, 50 % fructose) sugar, ideally all studies with this material should include controls given the same amount of sugars alone. The grape

Table 1 Summary of grape products used in the studies

Grape product	Source	Major components	References
FDGP	CTGC	Anthocyanins, catechins, flavonols	Solanki et al. (2015), Allam et al. (2013), Patki et al. (2013), Wang et al. (2005)
GSE	In house (H_2O extract)	NS	Sreemantula et al. (2005)
GSE	Kikkoman	Proanthocyanidins, flavanols	Peng et al. (2005), Devi et al. (2011)
GSE	In house (EtOH extract)	NS	Sarkari et al. (2013)
GSE	In house (mixed extract)	NS	Balu et al. (2005a, b)
GSE	Polyphenolics Inc.	Catechins, proanthocyanidins, gallic acid	Wang et al. (2008), Pasinetti et al. (2010)
GSE	Supplement Warehouse	NS	Wang et al. (2014)
GSE	Tarac Technologies	Catechins, proanthocyanidins, gallic acid	Wang et al. (2009)
Concord grape juice	Welch's	Anthocyanins, proanthocyanidins	Shukitt-Hale et al. (2006), Smith and Stouffer (2014), Krikorian et al. (2010), Krikorian et al. (2012)
Concentrated Concord grape juice	Welch's	Anthocyanins, proanthocyanidins, quercetin	Wang et al. (2014)

NS not specified

juice was generally from Concord grapes with white grape juice and/or a placebo drink designed to smell, look, and taste like grape juice usually employed as a caloric control. GSE was either a commercial preparation (e.g., Peng et al. 2005; Wang et al. 2008, 2009; Devi et al. 2011) or made "in house" (e.g., Sreemantula et al. 2005; Balu et al. 2005a; Sarkari et al. 2013). While the commercial preparations have all been characterized with respect to polyphenol content (see references in cited papers), the preparations made "in house" generally were not.

Although the vast majority of the studies on grape products and brain function have looked at the effects of grape intake on learning and memory, a few studies have addressed other behaviors such as anxiety. In this review, the effects of grape products in animal and human models of brain function will be addressed first (Table 2), and then the mechanisms that are likely to contribute to their efficacy in these models will be discussed (Fig. 1).

Table 2 Summary of studies on the effects of grape products on brain function

Model	Species	Grape product	Mode of administration	Dosage	Results	References
Physical stress	Rats	GSE	Oral/food	100–300 mg/kg bw	↑ Learning and memory	Sreemantula et al. (2005)
Physical stress	Rats	FDGP	Oral/water	330 mg/day	↓ Anxiety and ↑memory	Solanki et al. (2015)
Oxidative stress	Rats	FDGP	Oral/water	330 mg/day	↓ Anxiety and ↑ memory	Allam et al. (2013)
Ovariectomy	Rats	GSE	Oral/food	5 g/kg food	↑ Memory	Peng et al. (2005)
Ovariectomy	Rats	FDGP	Oral/water	330 mg/day	↓ Anxiety and ↑ memory	Patki et al. (2013)
Ischemia	Gerbils	FDGP	Oral/food	5 or 50 g/kg food	↓ Nerve cell death	Wang et al. (2005)
Ischemia	Rats	GSE	Oral/gavage	100 mg/kg bw	↑ Memory	Sarkari et al. (2013)
Aging	Rats	GSE	Oral	75 mg/kg bw	↑ Learning and memory	Devi et al. (2011)
Aging	Rats	Juice	Oral	10 mL/kg bw	↑ Learning	Smith and Stouffer (2014)
Aging	Rats	GSE	Oral/gavage	100 mg/kg bw	↑ Memory	Balu et al. (2005a)
Aging	Rats	Juice	Oral	2.5 or 12.5 ml/day	↑ Memory (2.5 only)	Shukitt-Hale et al. (2006)
Aging	Humans	Juice	Oral	6–9 mL/kg/day	↑ Memory	Krikorian et al. (2010)
Aging	Humans	Juice	Oral	6–8 mL/kg/day	↑ Memory	Krikorian et al. (2010)
AD	Mice	GSE	Oral/water	200 mg/kg bw/day	↑ Learning and memory	Wang et al. (2008)
AD	Mice	GSE	Oral/food	200 mg/kg bw/day	↑ Learning and memory	Wang et al. (2014)
AD	Mice	Juice	Oral	183 mg/kg bw/day	No effects	Wang et al. (2014)
Tauopathy	Mice	GSE	Oral/food	150 mg/kg bw/day	↓ Motor impairment	Pasinetti et al. (2010)

2 In Vivo Effects of Grapes on the CNS

2.1 Grapes and Stress in Healthy Young Animals

Most studies on grapes and learning and memory have focused on preventing or reversing losses associated with aging or disease (see below). However, there has also been interest in determining if grapes could maintain cognitive function in young, healthy subjects exposed to various types of stress. Numerous studies have shown that stress is a major modulator of memory [for review see Sandi and Pinelo-Nava (2007)]. In one study (Sreemantula et al. 2005), young rats were exposed to multiple episodes of forced swimming over several weeks. They were then untreated or orally administered 100, 200, or 300 mg/kg body weight (bw) of GSE 1 h before each day's training in a learning and memory test called the conditioned avoidance response test. For this test, animals are placed in a clear plastic chamber containing a pole. After 5 min of acclimation, a buzzer is sounded and the animals are given a shock through the grid floor. The animals have to jump onto the pole in order to avoid the shock. Jumping onto the pole after the shock is scored as an escape, while jumping onto the pole prior to the shock is scored as an avoidance. In this study, the animals were given multiple trials per day, and this was repeated daily until all of the groups reached 95 % avoidance. The acquisition time to achieve 95 % avoidance was 10 days for the control rats but decreased dose dependently in the rats given GSE: 9 days for 100 mg/kg bw, 8 days for 200 mg/kg bw, and 7 days for 300 mg/kg bw. Once the rats had reached 95 % avoidance, they were given scopolamine 30 min before the daily dosing of GSE. Scopolamine-induced memory impairment in rodents is a well-established model of memory dysfunction based upon acetylcholine metabolism (Klinkenborg and Blokland 2010), and reversal of scopolamine-induced cognitive impairment is a model for predicting pharmacodynamic signals of cognitive enhancing compounds in animals (Lenz et al. 2012). The training schedule was continued until the animals again achieved 95 % avoidance. GSE dose dependently both promoted memory retention and accelerated memory recovery following scopolamine treatment. The authors attributed these effects of GSE on the performance of the rats in the conditioned avoidance response test to the ability of GSE to reduce serum markers of stress (Sreemantula et al. 2005).

2.2 Grapes and Post-traumatic Stress Disorder (PTSD)

Further evidence for a beneficial effect of grape products on stress was obtained in a recent study that used freeze-dried grape powder (FDGP) in combination with the single-prolonged stress procedure, an animal model of post-traumatic stress disorder (PTSD) (Solanki et al. 2015). In this procedure, male rats are sequentially

immobilized for 2 h followed immediately by forced swimming for 20 min and then exposure to ether anesthesia.

For this study (Solanki et al. 2015), the animals were either untreated or pretreated for 3 weeks prior to the stress procedure with 15 g of FDGP/liter of drinking water (resulting in the consumption of ~330 mg FDGP/day). A control for the sugar content of the FDGP was not included. After 1 week of recovery from the single-prolonged stress procedure during which time the rats continued to have FDGP in their drinking water, they were tested in multiple tests for anxiety-like behavior including the light–dark exploration test, the elevated plus maze, and the open field test. In all three tests, the less time spent in the light and/or open spaces is considered as a measure of anxiety-like behavior. The animals exposed to the single-prolonged stress procedure showed increased anxiety-like behavior in all three tests, and this was prevented by FDGP. FDGP had no effect on the behavior of control animals not exposed to stress.

The animals were also analyzed for depression-like behavior using the forced swim test. In this test, the amount of time that an animal remains immobile in a water tank during a 5 min test session is indicative of depression-like behavior. The animals exposed to the single-prolonged stress procedure spent considerably more time immobile than the control rats, and this was prevented by the FDGP. FDGP had no effect on the behavior of control animals not exposed to stress in this test.

The animals were also tested for memory using the radial arm water maze. The radial arm water maze used by these investigators consists of a pool filled with water containing six swim paths. In this test, the animals have to locate a goal arm which contains a hidden platform at the end of the arm. The animals have 1 min to locate the goal arm. If they fail to locate the goal arm, they are guided to it. For this study (Solanki et al. 2015), each rat was given two sets of six learning trials followed 30 min later by the short-term memory test and 24 h later by the long-term memory test. The number of errors made in locating the goal arm was taken as an indication of memory. The rats exposed to the single-prolonged stress procedure produced many more errors in both the short- and long-term memory tests, and this was prevented by FDGP. In summary, administration of FDGP prior to exposure to an animal model of PTSD significantly reduced multiple symptoms of PTSD including anxiety, depression, and memory loss.

2.3 Grapes and Oxidative Stress

To determine if oxidative stress can directly impair cognitive function and whether this can be alleviated by grape products, male Sprague–Dawley rats were treated with buthionine sulfoximine (BSO), an irreversible inhibitor of glutamate cysteine ligase, the rate-limiting enzyme for the synthesis of glutathione (GSH), a major endogenous antioxidant (Allam et al. 2013). FDGP was dissolved in tap water at 15 g/L (resulting in the consumption of ~330 mg FDGP/day) and provided to the rats for 3 weeks prior to the administration of BSO for 7 days. The animals were

then tested for anxiety-like behavior and cognitive function. These investigators used two tests for anxiety-like behavior: the light–dark exploration test and the open field test. In both tests of anxiety-like behavior, the BSO-treated animals showed increased anxiety-like behavior, and this was prevented by FDGP. FDGP had no effect on the behavior of control animals not treated with BSO. The animals were tested for memory using the radial arm water maze. Each rat was given two sets of six learning trials followed 30 min later by the short-term memory test and 24 h later by the long-term memory test. The number of errors made in locating the goal arm was taken as an indication of memory. The rats treated with BSO produced many more errors in both the short- and long-term memory tests than untreated rats, and these errors were reduced by FDGP. The effects of BSO on memory seen in this study are similar to those seen with other compounds that can cause a loss of GSH [for review see Currais and Maher (2013)] although the authors did not directly assay brain GSH levels in this study. Importantly, GSH levels decline in the brain with age and may play a role in the age-dependent decreases in cognitive function (Currais and Maher 2013). Whether FDGP directly increased GSH levels or acted downstream of GSH loss was not investigated in this study but is an important question that should be addressed in the future.

2.4 Grapes and Animal Models of Menopause

Hypertension can impair cognitive function in an age-dependent manner. In females, this is accelerated following menopause. To test whether GSE could reduce hypertension and improve cognitive function in postmenopausal females, a strain of spontaneously hypertensive rats was used (Peng et al. 2005). Female rats were estrogen depleted by ovariectomy and then fed either a basal or high-salt diet that was either unsupplemented or supplemented with 5 g/kg GSE (Kikkoman). After 10 weeks on the diets, the rats given the basal salt diet were tested in the radial arm water maze. The rats given the high-salt diet without GSE were too sick to be tested for behavior, so neither group on the high-salt diet was tested. The animals were tested until they reached the criterion specified by the investigators. The format of the assay also allowed distinction between working and reference memory which correlate with short- and long-term memory, respectively. The rats receiving GSE in their food reached criterion in significantly fewer days and with fewer reference and working memory errors indicating that GSE supplementation improved both learning and memory in this model. GSE treatment also reduced hypertension in these rats.

Another study used ovariectomized normal rats (Wistar) to look at the effects of FDGP on learning and memory, anxiety, and blood pressure (Patki et al. 2013). The rats were given FDGP in their drinking water at 15 g/L (resulting in the consumption of ~330 mg FDGP/day) for 3 weeks following ovariectomy and then tested in the different assays. Similar to the study with the spontaneously hypertensive rats given GSE, this study with FDGP found that it decreased the blood pressure of

ovariectomized rats. Although this study with FDGP used a slightly different version of the radial arm water maze to test memory as compared with the GSE study, the authors also found that ovariectomy decreased short-term memory, and this decrease was prevented by FDGP. FDGP had no effect on short-term memory in sham-operated rats. However, in contrast to the study with GSE in spontaneously hypertensive rats, long-term memory was not significantly affected by ovariectomy and/or FDGP. As a complement to these behavioral assays for memory, the authors looked at long-term potentiation (LTP) in the dentate gyrus region of the hippocampus using in vivo electrophysiological recording. LTP is considered to be a good model of how memory is formed at the cellular level (Bliss and Collingridge 1993). Ovariectomized rats showed impairment in the induction of LTP that was prevented in the animals given FDGP. FDGP in sham-operated rats had no effect on LTP. Thus, these authors found physiological support for their behavioral data (Patki et al. 2013).

In addition, these investigators tested the rats in multiple assays for anxiety-like behavior including the light–dark exploration test and the open field test (Patki et al. 2013). In both tests of anxiety-like behavior, the ovariectomized animals showed increased anxiety-like behavior, and this was prevented by FDGP. FDGP had no effect on sham-operated animals.

In summary, two completely independent studies using two different grape product preparations found that grapes could make a significant, positive difference in learning and memory as well as blood pressure in ovariectomized rats. Since menopause is associated with both hypertension and subtle cognitive deficits (Greendale et al. 2011), these results strongly suggest that further investigation into these observations is warranted as daily grape consumption might provide a simple, inexpensive approach to reducing multiple symptoms of menopause and improving the quality of life.

2.5 Grapes and Age-Related Cognitive Decline

Similar to other organs, brain function declines with age. Indeed, a decline in both cognitive and motor functions is one of the characteristics of normal aging, resulting in changes in learning and memory as well as deficits in balance and coordination. Furthermore, age is the single greatest risk factor for a variety of neurological disorders including Alzheimer's disease (AD). Since the average age in many Western countries is increasing, identifying approaches for reducing the effects of aging on brain function is taking on a new urgency. Several studies have looked at the effects of grape products on brain cognitive function during various stages of normal aging, and, as described below, all saw a beneficial effect.

In one of the first studies on the impact of grape products on cognitive function during aging, 19-month-old male Fischer rats were given 0, 10, or 50 % Concord grape juice as their sole source of liquid for 8 weeks before cognitive testing (Shukitt-Hale et al. 2006). All three juice products were made to appear identical

by the use of a placebo formulated to match the grape juice for calories, sugar content, acidity, taste, color, and aroma. There were no differences in either food or juice intake over the course of the study. Learning and memory were tested using the Morris water maze (MWM), a test of spatial learning that is strongly correlated with hippocampal synaptic plasticity (Vorhees and Williams 2006). In this test, rodents are required to find a submerged platform in a circular pool of opaque liquid (usually water with nontoxic white paint added) by relying on distal visual cues. In this study with Concord grape juice, the rats were given two sets of two trials per day over a period of 4 days. There was a 10 min interval between each trial and the second trial of each set was used as a measure of short-term working memory. Interestingly, only supplementation with 10 % grape juice improved short-term memory as determined by a decrease in both the time taken to find the hidden platform (latency) and the distance traveled to reach it.

A more recent study investigated more subtle cognitive deficits that first appear during middle age (Smith and Stouffer 2014). These investigators first determined the age at which latent learning abilities begin to decline in rats and then, beginning at that age, treated the rats daily for 5 weeks with Concord grape juice, white grape juice, or a sugar solution at a dose equivalent to 10 mL/kg bw of 100 % grape juice prior to testing in the latent cue preference task (Smith and Stouffer 2014). The latent cue preference task can measure subtle memory deficits and involves exposing an animal to an irrelevant stimulus when it is in a non-deprived state, in this case exposure to water when water replete, and then testing recall of information about the stimulus when the animal is in a deprived state. In this study, water-replete rats were exposed to water in one compartment of a three-compartment box during the 2 days of training. Each of the compartments contained a single, unique visual cue, and the water became latently associated with its visual cue. Following the training period, the rats were made water deprived for 23 h and then tested for compartment preference with the water removed from the box. Latent learning is demonstrated if the rats spend significantly more time in the compartment that previously contained water relative to the other compartments. Latent learning abilities begin to decline in rats in middle age before other learning deficits appear. Rats given Concord grape juice but not white grape juice or sugar solution spent significantly more time in the water-paired compartment during the compartment preference test, indicating that the Concord grape juice could reverse the latent learning impairment in middle-aged rats. Since white grape juice had no effect, this suggests that the reversal of the latent learning impairment was due to the polyphenols that are specific to red grape juice.

In a study using a commercial GSE preparation (Kikkoman), adult (3-month-old) and middle-aged (12-month-old) female Wistar rats were given daily oral supplementation of 75 mg/kg bw GSE or vehicle for 12 weeks (Devi et al. 2011) and then tested for memory. For this study, the animals were trained in a T-maze apparatus using a food pellet as the reward for selecting the correct arm of the two arms of the T-maze. The animals were trained until they reached a predetermined criterion (80–90 % correct arm choice), and then memory of the correct arm choice was tested after 7, 15, 21, and 30 days with continued administration of GSE. In

both the adult and middle-aged rats, GSE both increased the rate of acquisition of the task and promoted memory retention of the task as compared to control unsupplemented animals.

A study using GSE prepared "in house" tested both adult (3–4-month-old) and old (24–26-month-old) Wistar rats in a similar T-maze test following treatment for 30 days with GSE (100 mg/kg bw) or vehicle by oral gavage (Balu et al. 2005a). In this study, only the acquisition of the task (correct arm choice of the T-maze) was examined, and this was significantly higher in the adult rats as compared to the old rats. The deficit in task acquisition was partially reversed by GSE in the old rats, but it had no effect on acquisition in the adult rats. However, unlike the study by Devi et al. (2011), the acquisition of the task did not improve over the trials generating some concern about the testing procedure. Nevertheless, the data show a clear deficit in the ability of the old rats to choose the correct arm, and this deficit was significantly reversed by administration of GSE.

Given the positive effects of grape juice on cognitive function in aging rats, these studies were extended to humans. In the first study (Krikorian et al. 2010), 12 older adults (average age 78 years) with early memory decline but not dementia were randomly assigned to receive a placebo beverage or 100 % Concord grape juice. The placebo beverage was formulated to look and taste like grape juice and to have the same carbohydrate composition and energy load but no polyphenols. The dosing schedule was determined by body weight so that participants received between 6 and 9 mL/kg bw/day which was similar to that which had been used in other human studies with grape juice. The treatment lasted for 12 weeks. The subjects were tested at the beginning and end of the treatment on the California Verbal Learning Test, a list learning and recall task that measures verbal learning and retention, and the Spatial Paired Associate Learning Test, a nonverbal memory task that measures memory for visual–spatial information that is not amenable to verbal encoding. The subjects given the Concord grape juice showed a significant 20 % improvement in item acquisition in the California Verbal Learning Test as compared to the subjects given the placebo. There were also nonsignificant trends toward improved performance in the subjects given grape juice in the delayed verbal recall and spatial memory tests. Although there were no significant differences in the effects of the two treatments on weight, waist circumference, or fasting blood glucose concentrations, the subjects given the grape juice did show a significant increase in fasting insulin levels relative to the subjects given placebo. Given that insulin has been suggested to play a positive role in cognition (Shemesh et al. 2012), this observation may be important to the interpretation of the results.

This study was followed up by a slightly larger study using 21 older adults (average age 76 years old) with mild cognitive impairment (Krikorian et al. 2012). The subjects were randomly assigned to either the placebo drink (same as in the earlier study) or Concord grape juice at 6–8 mL/kg bw/day for 16 weeks, 4 weeks longer than the earlier study (Krikorian et al. 2010). The subjects were tested at the beginning and end of the treatment on the California Verbal Learning Test. The brains of eight of the subjects were also imaged by fMRI while performing a working memory task. In contrast to the previous study, there were no differences

in performance on the learning portion of the California Verbal Learning Test. However, the subjects consuming the placebo committed significantly more interference errors on the recognition memory task. Thus, in this study, the subjects who consumed grape juice acquired new information similarly to the subjects who consumed the placebo, but they were better able to make discriminations when retrieving previously presented material. Furthermore, the subjects that consumed the Concord grape juice showed large increases in activation on fMRI in the anterior and posterior cortical regions during performance of a working memory task. An increase in regional fMRI activation represents a greater hemodynamic response which is strongly associated with increased neuronal activity and is consistent with the evidence for vascular benefits of grape juice consumption (Vislocky and Fernandez 2010). Interestingly, in contrast to the earlier study with a slightly shorter treatment period (12 vs. 16 weeks) and an improvement in learning (Krikorian et al. 2010), no differences in plasma insulin levels between the two groups of subjects were seen in this study.

2.6 Grapes and Stroke

Ischemic stroke is a devastating disease representing the second leading cause of death in the Western world and the leading cause of disability in adults (Ingall 2004). Ischemic stroke occurs when the normal blood supply to the brain is disrupted, usually due to artery blockage by a blood clot, thereby depriving the brain of oxygen and metabolic substrates and hindering the removal of waste products [for review see Lapchak and Araujo (2007)]. Ischemic stroke is believed to evolve in distinct phases. The initial ischemia results in immediate nerve cell death followed by an inflammatory response leading to secondary tissue damage after reperfusion (Gelderblom et al. 2009). There is good evidence that poststroke inflammation contributes to secondary tissue damage. The nerve cell damage and death caused by cerebral ischemia results in functional impairment including cognitive deficits or death (Cumming et al. 2013).

One study looked at the ability of grape products to reduce the impact of global forebrain ischemia as a model of stroke in gerbils (Wang et al. 2005). In this study (Wang et al. 2005), gerbils were fed control diet or diet supplemented with a low (5 g/kg diet) or high (50 g/kg diet) level of FDGF for 2 months prior to induction of global cerebral ischemia for 5 min. The animals were sacrificed 4 days after the ischemia and markers of cell survival and inflammation were examined by histochemistry/immunohistochemistry in the CA1 region of the hippocampus. Ischemia caused a marked reduction in neuronal survival that was significantly prevented by FDGP. Ischemia also caused large increases in two markers of inflammation, activated astrocytes and activated microglia. In both cases, the gerbils given FDGP showed much less glial activation. Interestingly, both the low and high doses of FDGP showed the same effects on all markers of cerebral damage.

A distinct disorder of the cerebral circulation that also contributes to brain dysfunction, especially with aging, is chronic cerebral hypoperfusion (Farkas et al. 2007). The standard model for studying this is permanent bilateral common carotid artery occlusion (PCAO) in rats (Farkas et al. 2007). Wistar rats were orally administered either vehicle or GSE (100 mg/kg bw) prepared "in house" and dissolved in saline for 28 days after the induction of PCAO (Sarkari et al. 2013). The animals were then tested for memory in the passive avoidance test. In this test, animals learn to associate an aversive stimulus with a specific environmental context. In this case, a chamber with a platform and a floor grid was used. During training, the animal is placed on the platform, and once it steps down onto the floor of the chamber, it is exposed to a mild foot shock. Later (1, 3, 7, and 14 days), the rats are returned to the platform, and the latency to step down onto the floor is measured. Animals that remember the task show a much higher latency to step down. At all the time points tested, PCAO significantly decreased the step down latency as compared to the sham-operated controls, while treatment with GSE significantly increased the step down latency in both the animals with PCAO and sham-operated rats at all time points. Thus, while GSE improved memory in this model, the effect was not specific to the injured animals so that GSE-treated rats with PCAO still showed deficits relative to GSE-treated sham-operated rats.

As a complement to the behavioral assay for memory, the authors (Sarkari et al. 2013) also looked at LTP in the dentate gyrus region of the hippocampus using in vivo electrophysiological recording. Rats with PCAO showed impairment in the induction of LTP that was prevented in the animals given GSE but, in contrast to the behavioral results, GSE in sham-operated rats had no effect on LTP.

2.7 Grapes and Alzheimer's Disease

Alzheimer's disease is the most common type of dementia. It is characterized pathologically by the presence of both extracellular neuritic plaques containing amyloid beta (Aβ) peptide and intracellular neurofibrillary tangles containing tau (Goedert 2006). Clinically, AD results in a progressive loss of cognitive ability and eventually daily function activities (McKhann et al. 1984; McKeith and Cummings 2005). Current approved therapies are only symptomatic, providing moderate improvements in memory without altering the progression of the disease pathology (Haas 2012; Rafii and Aisen 2009).

The group of Giulio Pasinetti has published a number of papers on the effects of a commercial GSE preparation (MegaNatural GSPE) on AD-dependent cognitive deficits as well as investigated its mechanisms of action [for review see Pasinetti (2012)]. In their initial studies (Wang et al. 2008), they used a transgenic mouse model of AD that is engineered to express a mutant human amyloid precursor protein (APP) that is the cause of a familial, genetic form of AD. The mice were given 200 mg/kg bw/day GSE in their drinking water for 5 months beginning at 6 months of age and then tested for learning and memory in the MWM. In contrast

to the study with Concord grape juice and aging rats (Shukitt-Hale et al. 2006), the version of the MWM used by these investigators allowed assessment of both learning and long-term memory. For this study, mice were trained over 8 days to find the submerged platform and the time required for the mouse to find the platform (latency) was recorded. A decrease in the latency to find the platform over the training period is an indication of learning. 24 h after the end of this acquisition phase, the mice were placed back in the pool, but in this case the platform was no longer present. They were given a single, 45 s trial (probe test), and the amount of time that they spent in the quadrant in which the platform was previously located relative to the time that they spent in the other three quadrants and the number of times that they crossed the area where the platform was previously located were recorded. Differences in the percent of time in the platform quadrant and number of platform area crossings between the conditions are indicative of differences in long-term memory. GSE administration significantly improved both learning and memory in the AD mice relative to untreated controls.

Since the accumulation of the Aβ peptide and the development of amyloid plaques are characteristic of these transgenic animals and are associated with the development of cognitive deficits, the investigators also looked at the effects of GSE administration on the accumulation of both soluble and insoluble Aβ peptides using specific ELISAs and Western blots of brain homogenates and amyloid plaques using thioflavin-S staining of brain sections (Wang et al. 2008). GSE reduced the levels of soluble Aβ peptides as well as the plaque burden.

Based on these results, the same investigators went on to compare the effects of GSE (200 mg/kg bw/day in the food), concentrated Concord grape juice (183 mg/kg bw/day in the drinking water), resveratrol (400 mg/kg bw/day in the food), and the combination of all three on the development of AD-mediated cognitive dysfunction (Wang et al. 2014). They used a different strain of transgenic AD mice that expresses two different familial human APP mutations for these studies and treated the mice for 7 months beginning at 3 months of age. Using the MWM to assess both learning and memory as described above, they found that only the GSE-treated and combination-treated mice performed significantly better than untreated mice in the learning phase. In the probe trial, which measures memory, GSE, resveratrol, and the combination treatment all had a significant positive effect on the ability of the mice to recall the platform quadrant as compared to the untreated controls. In addition, both GSE and the combination treatment reduced the levels of Aβ peptides and the amyloid plaque burden in the brains of the mice as compared to untreated mice while neither resveratrol nor grape juice had any effects on these parameters.

A different group of investigators (Wang et al. 2009) used a double transgenic AD mouse model that expresses both a familial human APP mutation and a familial human presenilin-1 mutation to investigate the effects of a commercial preparation of GSE (Vinlife) on Aβ peptide and amyloid accumulation and markers of inflammation in the brains of the mice. The animals were given GSE in their food at 20 g/kg for 9 months beginning at 3 months of age. Similar to the study from the Pasinetti lab (Wang et al. 2008), these investigators found that GSE administration

reduced the levels of soluble Aβ peptides as well as the amyloid plaque burden. In addition, GSE administration greatly reduced microglial activation in the brains of the AD mice. Microglia (brain macrophages) are the resident immune cell population of the CNS, comprising 5–10 % of the total cell population [for reviews see Rock and Peterson (2006), Garden and Moller (2006), Dringen (2005)]. In the context of the AD brain, there are thought to be multiple stimuli that generate an inflammatory response in the microglia including Aβ (Wyss-Coray and Rogers 2012). The activation of both microglia and astrocytes in AD is implicated in the loss of nerve cell function (Rao et al. 2011). However, in contrast to the robust effect of GSE on microglial activation in the AD mouse brains, it had no effect on astrocyte activation. These investigators did not look at the effects of GSE on AD-mediated cognitive deficits.

2.8 Grape Products and Tauopathies

Tauopathies are characterized by progressive, age-dependent intracellular formations of misfolded aggregates of the microtubule-associated protein tau. These diseases include not only AD but also progressive nuclear palsy, corticobasal degeneration, argyrophilic grain disease, Pick's disease, and a number of familial frontotemporal dementias. Since GSE showed beneficial results in a mouse model of AD (Wang et al. 2008), the same research group also tested it in a mouse tauopathy model (Pasinetti et al. 2010). The JNPL3 mouse model is engineered to express human familial P301L mutant tau that leads to age-dependent neurodegeneration and motor dysfunction. Mice were untreated or given GSE (150 mg/kg bw/day) in their drinking water beginning at 7 months of age prior to the initiation of motor dysfunction which begins at 12 months. GSE treatment significantly reduced motor dysfunction measured at 13 months by assessing the impairment in the natural tendency of mice to extend their hind limbs laterally when they are hung inverted by their tails.

3 Mechanism of Action

These in vivo studies raise the question of how the different grape products are acting to promote brain function. It is also important to know if the different grape products appear to be working via similar or distinct mechanisms. A number of the studies described above included experiments designed to identify possible mechanisms of action for the grape products. Among the mechanisms identified include inhibition of brain oxidative stress, maintenance of trophic factor signaling, vascular effects, inhibition of protein aggregation, maintenance of neurotransmitter signaling, and anti-inflammatory effects (Fig. 1). The evidence supporting each of these mechanisms is described in detail below.

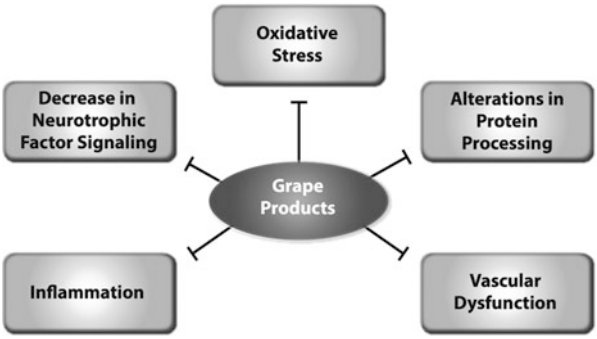

Fig. 1 Mechanisms of action of grape products on the brain

3.1 Inhibition of Oxidative Stress

Although it has been argued that the antioxidant activity of flavonoids and other polyphenols as measured in test tube assays may have little relevance to their effects in vivo (Schewe et al. 2008; Halliwell 2011), a number of the studies with the grape products showed effects on measures of oxidative stress both in the blood and the brain of the treated animals. In the study using FDGP in ovariectomized rats (Patki et al. 2013), 8-isoprostane, a marker of lipid peroxidation and oxidative stress, was increased by ovariectomy approximately two-fold in serum and approximately three-fold in urine, and these increases were prevented by FDGP. Consistent with this observation, in the study using GSE in ovariectomized spontaneously hypertensive rats, measurement of superoxide formation in aortic rings taken from control and GSE-treated animals demonstrated a significant inhibitory effect of the GSE treatment (Peng et al. 2005). In the study looking at the effects of FDGP on BSO-induced oxidative stress and cognitive impairment (Allam et al. 2013), the rats treated with BSO showed an ~2.5-fold increase in serum 8-isoprostane and an ~50 % increase in urine 8-isoprostane, and these increases were also prevented by FDGP. Similarly, an approximately three-fold increase in 8-isoprostane was also found in the plasma of the rats exposed to the single-prolonged stress paradigm (Solanki et al. 2015), and this increase was prevented by FDGP treatment. Together, these studies show that both FDGP and GSE can reduce markers of oxidative stress in the blood/blood vessels of animals exposed to a number of distinct insults. Does this translate to effects on brain oxidative stress?

In the study using FDGP in ovariectomized rats (Patki et al. 2013), two markers of brain oxidative stress, protein carbonylation and protein nitrotyrosinylation, were examined in three regions of the brain, the amygdala, hippocampus, and cortex. Only the hippocampus showed an increase in protein carbonylation, and this was prevented by treatment with the FDGP. Interestingly, while the hippocampus also displayed an increase in protein nitrotyrosinylation, this was not prevented by FDGP. Several antioxidant enzymes were also examined in the brains of these rats. Mn-SOD and Cu-Zn SOD were not affected by ovariectomy. While glyoxalase

1 (Glo-1) levels were decreased ~25 % by ovariectomy, FDGP did not prevent this decrease. Glo-1 is the rate-limiting enzyme for the removal of the reactive dicarbonyl and potent protein-glycating agent methylglyoxal. Methylglyoxal is a major source of advanced glycation end products which are implicated in the induction of oxidative stress (Desai et al. 2010).

Not surprisingly, BSO had a much greater effect than ovariectomy on markers of oxidative stress in the brain (Allam et al. 2013). First, malondialdehyde (MDA), a measure of lipid peroxidation and oxidative stress, was increased approximately twofold by BSO in the amygdala, hippocampus, and cortex and these increases were prevented by FDGP. Second, BSO decreased Glo-1 levels by two- to three-fold in the amygdala, hippocampus, and cortex and these decreases were prevented by FDGP. BSO treatment also decreased the levels of glutathione reductase in the same three regions of the brain and this decrease was also prevented by FDGP.

In contrast to the study with BSO, in the study using the single-prolonged stress paradigm and FDGP (Solanki et al. 2015), no changes were seen in the amygdala, hippocampus, or cortex in Glo-1, glutathione reductase, Cu-Zn SOD or Mn-SOD and other markers of oxidative stress were not evaluated.

In one of the studies on GSE in aging rats (Balu et al. 2005a, b), multiple markers of oxidative stress were measured in the spinal cord, cortex, striatum, and hippocampus. Reactive oxygen species, protein carbonyls, and lipid peroxidation were all increased significantly in all four regions of the CNS in the old rats as compared with the young rats and these increases were partially reversed by the 30-day treatment with GSE. On the other hand, total thiols, nonprotein thiols (includes GSH) and protein thiols were all significantly decreased in all four regions in the CNS of the old rats as compared to the young rats and these decreases were partially reversed by the treatment with GSE. The activities of several antioxidant enzymes including SOD, catalase, and glutathione peroxidase were also measured in the young and old rats. All three enzyme activities were significantly decreased in the four regions of the CNS in the old rats relative to the young rats and these decreases were partially reversed by GSE. GSE had no effect on any of these markers in the young rats consistent with its lack of effect on cognitive function in the young rats.

In the study testing the effect of GSE on cognitive function in young and middle-aged rats, quite a different picture was seen (Devi et al. 2011). While these investigators found an age-dependent increase in brain MDA, they also saw an age-dependent increase in brain catalase activity and no changes in protein thiols. GSE treatment reduced MDA levels and catalase activity and increased protein thiol levels in both the young and middle-aged rats. These results are consistent with GSE treatment improving cognitive function in both the young and middle-aged rats in this study.

In summary, all of the models that showed increases in markers of oxidative stress in the brain in response to physiological, physical, or chemical insults also demonstrated that grape products can at least partially decrease these markers in a manner consistent with their effects on cognitive function and other brain activities.

3.2 Trophic Factor Signaling

Neurotrophic factors play critical roles in promoting the differentiation, survival, and functional maintenance of nerve cells. They are also key players in synaptic plasticity (Chao et al. 2006), cognition, and memory formation (Korte et al. 1995; Figurov et al. 1996; Kelly et al. 1998). Changes in the levels of neurotrophic factors and/or their receptors are implicated in the pathophysiology of a variety of neurodegenerative diseases including AD, Huntington's disease, Parkinson's disease and amyotrophic lateral sclerosis [for reviews, see Price et al. (2007), Chen and Le (2006), Levy et al. (2005), Zuccato and Cattaneo (2007)].

Brain-derived neurotrophic factor (BDNF) is one of the neurotrophic factors that is involved in promoting memory. It is dramatically reduced in the brain with age and in AD, as well as in other neurological and neuropsychiatric disorders (Prior et al. 2014). The BDNF pathway has long been considered a drug target for multiple neurological disorders (Prior et al. 2014). In two of the studies discussed above, the investigators studied the effects of both the stress and FDGP on BDNF levels and activation of BDNF signaling pathways. In the study using BSO to induce oxidative stress (Allam et al. 2013), BSO decreased the levels of BDNF as well as two downstream targets of the BDNF receptor, Ca^{+2}-calmodulin-dependent protein kinase IV (CaM kinase IV) and phospho-CREB in the amygdala, cortex, and hippocampus. However, curiously, another target of BDNF receptor signaling, phospho-ERK, was increased by BSO treatment. In all three brain regions, treatment with FDGP prevented the loss of BDNF and CaM kinase IV and the decrease in CREB phosphorylation. FDGP also prevented the increase in ERK phosphorylation in the amygdala and cortex but potentiated the increase in the hippocampus. The latter observation is consistent with data showing that CREB phosphorylation is positively regulated by the ERK signaling pathway. In the study using the single-prolonged stress paradigm, BDNF was also found to be decreased but only in the amygdala (Solanki et al. 2015). This decrease in BDNF was prevented by FDGP.

In summary, some of the protective effects of FDGP and perhaps other grape products on cognitive function may be through maintenance of the levels of the neurotrophic factor BDNF and/or activation of its downstream signaling pathways. Further investigation into these mechanisms is clearly warranted especially as BDNF does not cross the BBB. Therefore, compounds that can cross the BBB and activate the receptor and/or its downstream signaling pathways have significant therapeutic potential.

3.3 Neurotransmitter Release

Changes in signal transduction in the brain can alter cognitive function and other behavioral parameters. Muscarinic enhancement of dopamine release from striatal slices is an indicator of muscarinic receptor sensitivity and striatal function.

Age-related alterations in muscarinic control of striatal dopamine release have been related to spatial memory and motor function (Joseph et al. 1999). In the study on the effects of Concord grape juice on cognitive function in rats, the investigators found that dopamine release was significantly greater in the rats given 10 % Concord grape juice as compared with either the control group or the group given 50 % Concord grape juice (Shukitt-Hale et al. 2006). This result correlated well with the beneficial effects on learning seen with 10 % but not 50 % Concord grape juice supplementation.

3.4 Vascular Effects

Impairments in vascular function can affect brain function directly and indirectly (Cohen et al. 2009). Thus, treatments that reduce hypertension might indirectly impact cognitive function. Several of the studies described above investigated the effects of grape products on blood pressure. Both studies in ovariectomized rats found modest increases in blood pressure due to ovariectomy that were reduced by either GSE (Peng et al. 2005) or FDGP (Patki et al. 2013). BSO-induced oxidative stress also increased blood pressure and this increase was prevented by FDGP (Allam et al. 2013). However, the more recent study that investigated the effects of Concord grape juice on cognitive function in older humans found no effect of grape juice supplementation on blood pressure (Krikorian et al. 2012). Although this study did not show a major effect of grape juice supplementation on learning and memory, it did show a very robust effect on regional fMRI activation in response to a working memory task. Since increased regional fMRI activation is indicative of a greater hemodynamic response, this suggests that the effects of grape products on brain vascular function may generally be more subtle than those that can be measured by changes in blood pressure and require more specific assays such as fMRI to monitor.

3.5 Protein Aggregation

A number of age-related neurodegenerative diseases, including AD, are associated with inappropriate and/or excessive protein aggregation. Thus, identifying treatments that can reduce this protein aggregation is another approach to therapy. In the studies using GSE in AD models, it was found that the primary effect of GSE was to prevent Aβ peptide oligomerization into soluble high molecular weight species (Wang et al. 2008). In the case of tauopathies, GSE also reduced protein aggregation by interfering with the generation and/or stability of neurotoxic tau protofibrils (Pasinetti et al. 2010).

3.6 Anti-inflammatory Effects

One of the studies on GSE and AD (Wang et al. 2009) and the study on FDGP in global ischemia in gerbils (Wang et al. 2005) provided evidence that grape products may have anti-inflammatory effects on the brain. Brain immune dysregulation is implicated in neurodegenerative diseases as well as stroke and may contribute to the development of cognitive deficits (Czirr and Wyss-Coray 2012; Cribbs et al. 2012; Heneka et al. 2015). Both the above studies suggest that grape products are able to reduce the activation of pro-inflammatory cells in the brain. In the case of ischemia, the activation of microglia and astrocytes was reduced by FDGP while in the AD model, only the activation of microglial cells was reduced by GSE. How grape products reduce brain inflammation remains to be determined but may be partially mediated by the effects of grape products on peripheral inflammation as suggested by the study on GSE and AD (Wang et al. 2009) since there is cross-talk between peripheral inflammation and brain inflammation (Perry et al. 2007).

4 Brain Bioavailability of Grape Polyphenols

Grapes contain a unique combination of phytochemicals including simple phenolics (derivatives of hydroxycinnamic and hydroxybenzoic acid) and polyphenols such as stilbenoids (e.g., resveratrol) and, by far the biggest group, flavonoids (anthocyanins, flavonols, flavanols, and proanthocyanidins) (Georgiev et al. 2014). The bioavailability of polyphenols is a complex process that is influenced by multiple factors including the food composition, dietary patterns, the dose and dose regimen as well as the nutritional and physiological state of the individual (Pasinetti 2012). Polyphenols are often poorly absorbed and then extensively metabolized first in the intestine and then in the liver (Cherniak 2012). Furthermore, to directly affect brain cells, they need to cross the BBB, and it is not clear how well any of them do so. However, if their primary effect is on the vasculature and/or peripheral inflammation then this becomes much less of a problem. In addition, many grape-derived polyphenols in the blood as well as the brain are in a derivatized form yet almost all in vitro studies on activity have used the aglycone forms (Pasinetti 2012). As noted in the studies cited above, multiple grape products have been used to improve brain function, but which product is best for delivering active grape phytochemicals to the brain is not yet clear. However, this is an active and growing area of research and some information is available.

Janle et al. (2010) used grape polyphenol fractions extracted from radiolabeled cell suspension cultures of grape to assess their pharmacokinetics and tissue distribution in rats. They found that the brain levels of all fractions were 2–3 % of the serum levels indicating that the potential for the polyphenols from these fractions for crossing the BBB was similar and that the limiting factor was rather intestinal absorption. Indeed, there was a large difference in intestinal absorption

between the fractions with small, polar anthocyanin glycosides and their metabolites much better absorbed than the less polar proanthocyanidins and their metabolites.

More specific to the studies described in this review, the pharmacokinetics and brain distribution of the GSE used by Pasinetti's group (Pasinetti et al. 2010) has been extensively characterized. This product consists entirely of catechin and epicatechin in monomeric (8 %), oligomeric (75 %), and polymeric (17 %) forms. It is treated to remove gallic acid from the flavanols so it also contains a high level of free gallic acid. Following a single oral dose of 150 mg/kg bw in rats, only the monomeric components were detected in the blood and no components were detected in the brain (Pasinetti et al. 2010). However, long-term (120 days) oral administration at 10 mg/kg bw/day resulted in readily detectable picogram levels of catechin and epicatechin in the rat brains. Moreover, in the study from the same lab that used a combination of GSE, Concord grape juice extract, and resveratrol (Wang et al. 2014), 10 days of twice daily gavage led to the accumulation of high picomolar concentrations of catechin, epicatechin, and resveratrol glucuronides and lower levels of quercetin and various anthocyanin glucuronides in the brain. These results strongly suggest that short-term pharmacokinetic studies may not accurately reflect the quantities of grape polyphenols that can accumulate in the brain following longer-term administration. This is an extremely important point that deserves further investigation.

5 Conclusions/Future Directions

Together, the various papers cited in this review strongly support the idea that grape products can improve brain function and specifically learning and memory in animals exposed to stress, aging, and disease. Positive effects have been seen with multiple types of grape products including FDGP, which contains both grape polyphenols and high levels of grape sugars; GSE, which contains mostly grape polyphenols (although these may vary depending on the source of the product); and Concord grape juice. The one study that compared several grape products (Wang et al. 2014) found that GSE was more effective at improving learning and memory than Concord grape juice. However, this result should be interpreted with caution since the study with aging rats and Concord grape juice (Shukitt-Hale et al. 2006) found that 10 % Concord grape juice improved short-term memory while 50 % Concord grape did not indicating that the effect of Concord grape juice is very dose dependent. One problem is that there is no standardized way to describe the administration of the grape products. Some are put in drinking water and others in food. In some cases, a daily intake is estimated but in others only the level in the food is given. Thus, it is almost impossible to compare dosing between studies. A more standardized way of describing the dosing would greatly facilitate the comparison of results.

Grape products appear to work through multiple mechanisms to improve brain function. These include preventing increases in oxidative stress and inflammation and decreases in neurotrophic factor signaling, vascular function, and cognitive function as well as reducing alterations in protein processing (Fig. 1). Further investigation into the details of how grape products impact each of these mechanisms would go a long way toward a better understanding of how grape products work as well as further validating their use for the treatment of brain disorders.

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Grapes and Joint Health

Casey Tiernan, Shanil Juma, Jacquelynn Lucero, Victorine Imrhan, Chandan Prasad, and Parakat Vijayagopal

Contents

Abstract Osteoarthritis (OA) is the most common arthritic disease worldwide. It is a complex chronic disease that reflects age-related degeneration of joint tissues in response to mechanical stress or injury. Normal repair and inflammatory responses follow and contribute to a cycle of further stress and damage, leading to inflammation, chronic pain, and impairment of mobility. The inflammation seen in OA is directly responsible for much of the structural degeneration and many of the clinical symptoms such as joint swelling, synovitis, and associated pain and stiffness. Because traditional therapy can be expensive and invasive and carries significant

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adverse effects, people are increasingly opting for alternative and naturally derived treatments. Polyphenols are phytochemicals present in fruits and vegetables that are associated with anti-inflammatory, antioxidant, cardioprotective, and chemopreventive properties, making them an ideal subject of research for arthritis and joint health applications. There is evidence that dietary polyphenols provide benefit for both OA and rheumatoid or other forms of inflammatory arthritis. The mechanism of action for the observed effects appears to be downregulation of the inflammatory cytokines, influencing antioxidant or anti-inflammatory pathway signaling, and/or estrogen receptor activation. Study results have shown many positive effects of polyphenols in both in vitro in chondrocytes and tissue explants, as well as in vivo in animal models. Two human studies suggest that grape consumption with its bioactive constituents may also reduce inflammation and influence OA outcomes. Specifically, daily consumption of a freeze-dried grape powder (FDGP) with its bioactive constituents may reduce symptoms of pain and possibly impact a biomarker of cartilage metabolism in individuals with self-reported knee OA. This is in keeping with the literature that has shown decreases in self-reported OA symptoms with soy isoflavones and tart cherry polyphenols. Other studies are currently underway that may confirm an effect of FDGP on arthritis and other chronic diseases in humans.

1 Overview of Osteoarthritis

Osteoarthritis (OA) is the most common arthritic disease worldwide (Arden and Nevitt 2006). It is a complex chronic disease that reflects age-related degeneration of joint tissues in response to mechanical stress or injury. Normal repair and inflammatory responses follow and contribute to a cycle of further stress and damage, leading to chronic pain and impairment of mobility (Arden and Nevitt 2006; Shen et al. 2012). OA can be broadly classified by (1) the site of joint involvement, (2) whether it is primary (idiopathic) or secondary in nature, or (3) less commonly by the presence of distinctive features that are inflammatory, erosive, atrophic, or destructive in nature (Arden and Nevitt 2006).

2 Osteoarthritis Pathogenesis and Changes to the Diseased Joint

Mature articular cartilage is divided into two major components: a network of cells (chondrocytes) and their surrounding gel-like extracellular matrix of collagen and elastic fibers set in chondroitin sulfate (Moskowitz et al. 2001). Synovial fluid in the

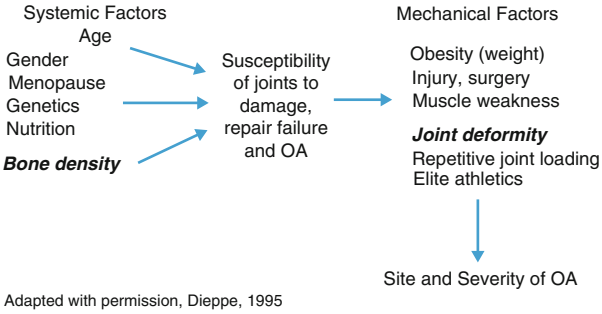

Fig. 1 Conceptual model for the pathogenesis of osteoarthritis (OA). Reprinted from *Best Pract. Res. Clin. Rheumatol.* **20**(1), Arden N, Nevitt, MC, Osteoarthritis: Epidemiology, Copyright (2005), with permission from Elsevier

joint cavity assists in the exchange of nutrients, oxygen, and waste products in the articular chondrocytes (Moskowitz et al. 2001; Tortora and Derrickson 2006). OA pathology occurs in three major phases: (1) the irreversible breakdown of cartilage matrix by proteases and other catabolic enzymes, (2) the fibrillation and deterioration of articular cartilage with the resulting release of eroded fragments into synovial fluid, and (3) phagocytic ingestion of the fragments by synoviocytes, producing more proteases and pro-inflammatory cytokines that create a continued cycle of synovial inflammation (Lotz and Kraus 2010; Martel-Pelletier 2004).

The etiology of OA is not fully understood but is thought to stem from the interaction of numerous systemic and mechanical risk factors that increase the susceptibility of the joint to injury from a variety of trigger events (Fig. 1) (Arden and Nevitt 2006; Hunter 2011). Age is a major systemic risk factor, possibly due to normal declines in neurological responses, as well as joint and muscle structure and function (Tortora and Derrickson 2006; Felson et al. 1987). This risk factor is primarily attributed to the higher proportion of women who have OA in older age groups (Felson et al. 1987). Most OA cases appear to be instigated by mechanical stress associated with obesity (Arden and Nevitt 2006). Resulting injury triggers normal repair processes that promote bone growth in an attempt to stabilize the joint, leading to in the formation of bony projections called osteophytes (Arden and Nevitt 2006; Hunter 2011; Das and Farooqi 2008; Felson 2013).

2.1 Cartilage Degradation

The pro-inflammatory enzymes called metalloproteinases (MMPs) are heavily involved in the cartilage matrix degradation seen in OA. Members of the MMP family include collagenases (MMP-1 and MMP-13) and stromelysin (MMP-3) that degrade collagen and proteoglycans, respectively (Martel-Pelletier 2004; Vincenti and Brinckerhoff 2002). Initially the proteoglycan coating surrounding collagen

offers some protection from MMP-1 and MMP-13, but once set in motion, collagen degradation is irreversible (Goldring 2012). Extensive loss of cartilage eventually exposes the surface of bone, creating the friction of bone against bone (Tortora and Derrickson 2006; Das and Farooqi 2008). MMP members called aggrecanases (ADAMTS-1, ADAMTS-4, and ADAMTS-5) present in synovial fluid can attack cartilage matrix and cause proteoglycan fragmentation (Martel-Pelletier 2004; Nagase and Kashiwagi 2003). Enzymes such as plasminogen activator/plasmin and cathepsin B are also involved in OA pathogenesis, but generally as activators of MMPs (Martel-Pelletier 2004).

2.2 Inflammatory Mediators and Pain

OA disrupts the normal balance of both degradation and synthesis of joint tissue through increased stimulation of the pro-inflammatory pathway (Hunter 2011). Pro-inflammatory cytokines, primarily interleukin (IL)-1β and tumor necrosis factor alpha (TNF-α), mediate the inflammation process triggered by proteases (Martel-Pelletier 2004; Das and Farooqi 2008). These cytokines promote chondrocyte apoptosis and synthesis of degradative enzymes, furthering the cartilage degradation process (Shen et al. 2012; Martel-Pelletier 2004). TNF-α is thought to promote the inflammatory process and IL-1β to augment the enzyme system. The number of receptors that mediate TNF-α and IL-1β signals are significantly increased during OA (Martel-Pelletier 2004). MMP activity is also stimulated by IL-1β and TNF-α, causing the chondrocyte and synovium to release IL-6, which perpetuates the inflammatory response (Lauder et al. 2007).

Both reactive oxygen species (ROS) and nitric oxide (NO) are required for normal chondrocyte activity. During OA the balance between their synthesis and degradation is also disrupted, leading to accumulation of oxidative stress products that promote inflammation (Shen et al. 2012; Ziskoven et al. 2011). This imbalance is suggested to be a primary driver of OA progression and cartilage degradation. Upon exposure to inflammatory cytokines such as IL-1β and TNF-α, chondrocytes induce production of NO via the NO synthase (NOS) enzyme with L-arginine being utilized as a substrate. Several isoforms of NOS have been found in OA joint tissue (Ziskoven et al. 2011). Oxidative stress promotes sclerosis and bone resorption in subchondral bone, cartilage thinning in chondrocytes and the matrix, inflammation in synovial fluid, and fibrosis in the joint capsule (Ziskoven et al. 2011).

Inflammatory changes are milder in OA when compared to other arthritic conditions such as rheumatoid arthritis (RA) (Hochberg et al. 1995). Nevertheless, the inflammation seen in OA is directly responsible for much of the structural degeneration and many of the clinical symptoms such as joint swelling, synovitis, and associated pain and stiffness (Das and Farooqi 2008; Ziskoven et al. 2011). Symptomatic pain can be caused by osteophytes irritating sensory nerve endings, as well as pro-inflammatory compounds like prostaglandins, proteinases, and cytokines (Hunter 2011). The synovial cavity of the joint contains densely packed

sensory nerves, and inflammatory mediators such as IL-1β and TNF-α can stimulate the nerve fibers, causing an inflated pain response (Hunter 2011; Das and Farooqi 2008). Circulating cytokines may also promote release of prostaglandin E_2 (PGE_2) and histamine from chondrocytes and increase the sensitivity of pain-receiving nociceptors (Hunter 2011). The cyclooxygenase (COX) enzyme mediates this prostaglandin synthesis from arachidonic acid. There are two isoforms of the COX enzyme, COX-1 being a constitutive enzyme and COX-2 inducible in inflammatory conditions (Subbaramaiah and Dannenberg 2001).

3 OA Symptoms

Progression of OA occurs slowly across years or even decades, yet there is a weak relationship between pathological structural changes and the presence of OA symptoms (Arden and Nevitt 2006; Duncan et al. 2006; Lawrence et al. 2008; Pereira et al. 2011). This may be partly explained by how differently people both experience and report pain; the population age studied; whether studies use mild, moderate, or severe evidence of structural changes in their definition of OA; and the radiographic methods used to define that evidence (Hunter 2011; Duncan et al. 2006; Lawrence et al. 2008; Pereira et al. 2011; Mogil 2012). Radiographic criteria for defining OA are based on the Kellgren-Lawrence scale, which emphasizes the presence osteophytes. Changes visible through radiographic assessment of the knee joint include joint space narrowing, osteophyte and cysts formation, subchondral bone sclerosis, and other alterations in bone shape (Fig. 2) (Arden and Nevitt 2006; Hunter 2011).

Symptomatic OA is defined as having frequent joint pain on most days of the previous month, accompanied by radiographic evidence of OA in the affected joint (Lawrence et al. 2008). The American College of Rheumatology (ACR) has developed updated diagnostic criteria by comparing site-specific joint pain in clinically diagnosed OA to different arthritic or musculoskeletal diseases versus healthy controls. This criterion is based on the presence of joint pain in the preceding month and is widely used in clinical studies (Arden and Nevitt 2006). Symptomatic OA is thought to be more clinically relevant than radiographic OA due to the disabling effects of pain and other symptoms on mobility and functionality (Hunter 2011; Lee et al. 2013). Novel therapies that take into account the molecular mechanisms behind OA pain may be useful for decreasing symptoms and increasing functionality (Lee et al. 2013).

4 Conventional Treatments and Therapies for OA

Non-pharmacological OA therapies are first-line approaches that include lifestyle changes, physical and occupational therapy, the use of assistive and supportive devices, water-based and strengthening exercises, and other measures (Zhang and

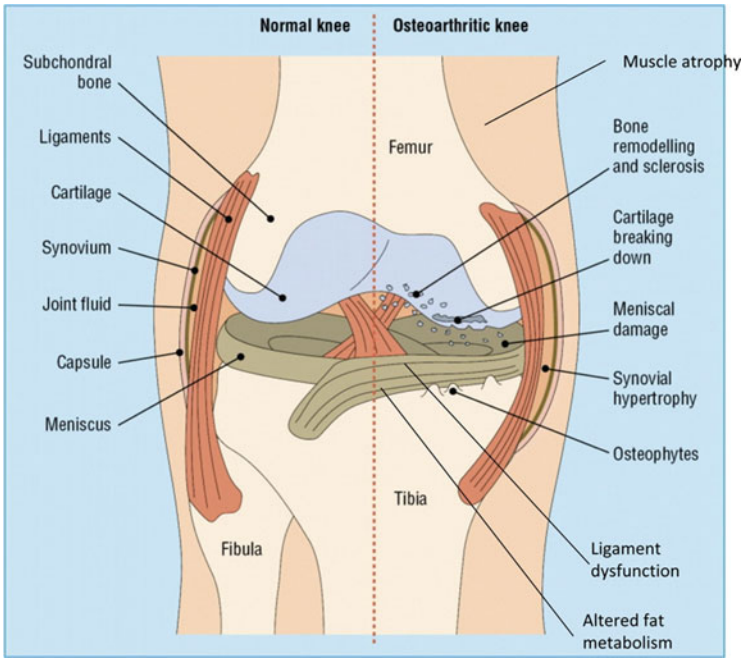

Fig. 2 Schematic of the knee joint depicting the synovial joint tissues affected in OA. Reprinted from *Best Pract. Res. Clin. Rheumatol.* **25**(6):801 = 814, Hunter DJ, Osteoarthritis, Copyright (2011), with permission from Elsevier

Jordan 2008). If symptom relief is not achieved then pharmacotherapy is initiated. Oral analgesics such as acetaminophen may be used for mild or moderate OA cases and nonsteroidal anti-inflammatory drugs (NSAIDs) like ibuprofen and naproxen for more severe pain (Zhang and Jordan 2008; Laine et al. 2008). Recently, the efficacy and safety of acetaminophen has been called into question, particularly with long-term use, with studies showing potential cardiovascular events, and gastrointestinal, liver, and renal toxicity at recommended or higher doses (Das and Farooqi 2008; Zhang and Jordan 2008; Laine et al. 2008; Bannuru et al. 2015; Roberts et al. 2015). NSAID use (whether selective for COX or nonselective) is also linked to a greater risk of adverse events such as gastrointestinal discomfort and bleeding and cardiovascular risks (Zhang and Jordan 2008; Laine et al. 2008). Surgical joint replacement or arthroplasties are effective and appropriate, yet invasive and expensive, treatment options when all other OA therapies have failed. Other procedures such as arthroscopic debridement may provide short-term symptom relief in those where arthroplasty is contraindicated or has failed (Das and Farooqi 2008; Zhang and Jordan 2008).

5 Nutraceuticals as Alternative Treatments for OA

5.1 Glucosamine and Chondroitin

Because traditional therapy can be expensive and invasive and carries significant adverse effects, people are increasingly opting for alternative and naturally derived treatments. The use of biological compounds such as glucosamine and chondroitin supplements has gained popularity as an OA treatment in recent years (Zhang and Jordan 2008; Clegg et al. 2006). Glucosamine is an amino sugar that is naturally present in cartilage, so is thought to stimulate proteoglycan synthesis (Huskisson 2008). Treatment effect is slow to appear, generally taking several weeks (Huskisson 2008; Lopez 2012). Chondroitin is a glycosaminoglycan (GAG) that is also found in cartilage proteoglycans. Its mechanism of action is thought to be through stimulation of cartilage repair and/or inhibiting degradation, as well as promoting joint viscosity (Huskisson 2008; Mazieres et al. 2001).

Research demonstrating the efficacy of these treatments has yielded conflicting results. Evaluating clinical trials of glucosamine treatment is challenging due to the many confounding factors present. Differences in efficacy may be due to differences in preparations used, bioavailability, a large placebo effect, and use of pain medication, among others (Huskisson 2008; Vlad et al. 2007). Overall, the literature supports the short-term use of glucosamine sulfate at 1500 mg daily and chondroitin sulfate at 800 mg daily, with treatment to be discontinued if no benefit is apparent within 6 months (Zhang and Jordan 2008).

5.2 Polyphenols

Nutrients present in the foods we eat may represent a promising approach to prevention and treatment of chronic diseases such as OA and one that is the least expensive, free of potential adverse effects, and least questionable in content compared to medications and nutrient supplements (Shen et al. 2012; Lopez 2012; Ameye and Chee 2006; Barnes 2008). Fruits and vegetables are rich sources of polyphenolic and other natural compounds with bioactive properties that may alter human health (Shen et al. 2012; Lopez 2012; Barnes 2008). Polyphenols are phytochemicals present in fruits and vegetables that consist of multiple hydroxyl groups bound to aromatic rings, divided into flavonoid and non-flavonoid classes dependent upon the number and interaction of the ring structures (Fig. 3) (Vauzour 2012). Polyphenols are associated with anti-inflammatory, antioxidant, cardioprotective, and chemopreventive properties, making them an ideal subject of research for arthritis and joint health applications (Lopez 2012; Barnes 2008; Bhat and Pezzuto 2002; Castillo et al. 2000; Fang et al. 2007; Frémont 2000; Hsieh and Wu 1999).

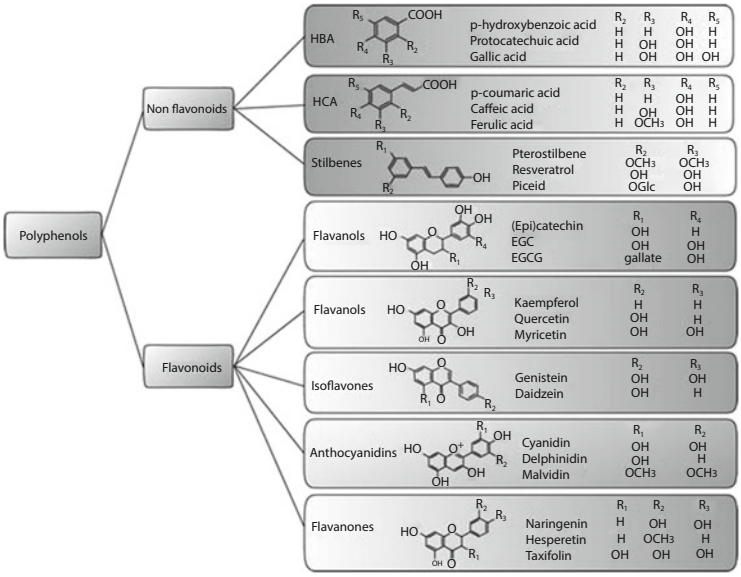

Fig. 3 Chemical structure of polyphenols. Vauzour D (2012) *Oxid. Med. Cell. Longev.* 2012:1–16. With permission

Approximately two-thirds of dietary polyphenols in humans are attributed to consumption of flavonoids, especially flavan-3-ols such as catechins, proanthocyanidins, and anthocyanidins (Dell'Agli et al. 2004). Flavonoids are a subclass of polyphenols present in spices, fruits, and vegetables. Examples include curcumin in turmeric; isoflavones in soy; proanthocyanidins in apples, grapes, and berries; and catechins in green and other teas (Shen et al. 2012; Lopez 2012; Barnes 2008). Normal mixed diets of adults typically contain about 200–400 mg of flavonoids daily, while clinical trials are usually set at 150–1500 mg daily dosages (Lopez 2012). However, the bioavailability and metabolic pathways of polyphenols such as resveratrol need to be better understood before firm conclusions can be drawn about the link between dietary consumption and health benefits (Frémont 2000; Dell'Agli et al. 2004; Elmali et al. 2005; Rennie et al. 2003). Dietary polyphenols should also be considered as adjuncts to current treatments, possibly with a goal of boosting efficacy and lowering treatment doses to decrease the risk of side effects and toxicity of drugs such as NSAIDs (Shen et al. 2012).

There is evidence that dietary polyphenols provide benefit for both OA and rheumatoid or other forms of inflammatory arthritis (Shen et al. 2012; Elmali et al. 2005, 2007; Aini et al. 2012; Wang et al. 2012; Woo et al. 2011). Study results have shown many positive effects of polyphenols in both in vitro in chondrocytes and tissue explants, as well as in vivo in animal models, and a limited number of human studies (Shen et al. 2012; Elmali et al. 2005, 2007; Aini et al. 2012; Wang et al. 2012; Woo et al. 2011; Ahmad et al. 2013; Cenesiz

et al. 2012; Chen et al. 2013, 2014; Cho et al. 2009; Decendit et al. 2013; Jhun et al. 2013; Li et al. 2001; Mossalayia et al. 2014; Park et al. 2011, 2012; Small et al. 2014; Tiernan et al. 2014; Xuzhu et al. 2012; Lei et al. 2012; Lucero et al. 2014; Liu et al. 2010).

6 Resveratrol and Grape Polyphenols: Overview

Resveratrol is a stilbene (i.e., non-flavonoid) polyphenol found in several fruits and plants. In grapes it is synthesized upon exposure to ultraviolet (UV) light or in response to fungal infection. The *trans* isomer is the major form found in red wine, present in amounts of about 0.1–15 mg/L. While the bioavailability of resveratrol in humans is uncertain, it has been shown in rat models to be absorbed rapidly and is easily detectable in tissue and plasma, particularly the heart, liver, and kidney (Frémont 2000; Ray et al. 1999). Plasma resveratrol levels peak at 30 min post-consumption and return to baseline at 1 h (Williams et al. 2004). Average wine consumption in humans is thought to allow for resveratrol concentrations linked to health benefits in red wine, particularly over the long term (Frémont 2000). Resveratrol has an excellent safety profile and is not toxic, even at high doses of 3000 mg/kg in a rat model (Bhat and Pezzuto 2002).

7 Resveratrol and Grape Polyphenols: In vitro OA Studies

Resveratrol has demonstrated anticarcinogenic, antiapoptotic, antioxidant, and anti-inflammatory properties (Shen et al. 2012; Csaki et al. 2008; Gehm et al. 1997; Henrotin et al. 2011). In human primary articular cartilage stimulated by IL-1, resveratrol inhibits apoptosis by (1) decreasing caspase-3 activity followed by cleavage of the DNA repair enzyme poly(ACP-ribose) polymerase or PARP (Shen et al. 2012; Csaki et al. 2008) and (2) suppressing the mitochondrial ROS and p53 tumor suppressor protein that activate caspase-3 and apoptosis. Resveratrol also decreases production of IL-1 and TNF-α, thus preventing activation of NF-κB, a regulatory agent in apoptosis. In vitro studies have demonstrated that resveratrol reduces gene expression of vascular endothelial growth factor and COX-2 and downregulates the MMPs associated with matrix degradation. In addition, resveratrol has been shown to protect major matrix proteins, proteoglycan, type II collagen, and aggrecan from MMPs or inflammatory compounds such as inducible NOS (iNOS) and COX-2 (Shen et al. 2012; Csaki et al. 2008; Henrotin et al. 2011).

Gene-regulating transcription factors that are affected by resveratrol may be important for understanding the OA inflammatory response. Nuclear factor kappa beta (NF-κB) is a ubiquitous cytoplasmic transcription factor, i.e., it responds to environmental triggers, and is important in regulating more than 150 genes. It is critical to the regulation of genes involved in inflammatory and immune responses,

such as inducible NOS, COX-2, and adhesion molecules (Barnes and Karin 1997; Roman-Blas and Jimenez 2006; Shakibaei et al. 2008). It is stimulated by antioxidants, cytokines, viruses, certain proteins, and other factors (Barnes and Karin 1997). Lauder et al. determined in three different OA cell models that inhibition of NF-κB was associated with reductions of IL-6, MMP-1, and MMP-3 (Lauder et al. 2007). It is also weakly inhibited by resveratrol, IL-10, aspirin, and antioxidants (Elmali et al. 2005, 2007; Barnes and Karin 1997; Shakibaei et al. 2008, 2011; Blanco-Colio et al. 2000). Subbaramaiah and Dannenberg studied whether resveratrol could block synthesis of the NF-κB-regulated COX-2 enzyme, thus reducing circulating levels. The authors treated a previously described cancer cell line with resveratrol concentrations ranging from 0 to 20 μM and found suppression of detectable COX-2 mRNA and synthesis (Subbaramaiah and Dannenberg 2001).

An important study by Shakibaei et al. found that treatment with 100 μM resveratrol suppressed inflammatory signaling and apoptosis in IL-1β-stimulated human articular chondrocytes, possibly through the NF-κB pathway. Resveratrol inhibited nuclear translocation of and activation of NF-κB, and gene expression of vascular endothelial growth factor, MMP-3, MMP-9 and COX-2, all of which are regulated by NF-κB. To further test this relationship, they examined the NF-κB signaling pathway and found resveratrol increased phosphorylation and suppressed the degradation of the NF-κB inhibitor IκBα. Therefore, the inhibitory effect of resveratrol appears to be mediated by phosphorylation and degradation of IκBα (Shakibaei et al. 2008). A follow-up study with bone-derived cells also resulted in inhibition of NF-κB, suppression of IκBα kinase, and phosphorylation and degradation of IκBα (Shakibaei et al. 2011).

In addition to inhibiting NF-κB through the suppression of the degradation of IκBα, resveratrol has also been shown to suppress the activation of NF-κB through the deacetylation of transcription factor p65 via sirtuin 1 (SIRT1) (Lei et al. 2012; Yeung et al. 2004). SIRT1 belongs to silent information regulator 2 family, and its induction has been shown to inhibit apoptosis in metabolically stressed articular chondrocytes (Takayama et al. 2009). In a study performed by Lei et al. in 2011, resveratrol demonstrated the ability to directly suppress the activation of the NF-κB pathway in primary rat articular chondrocytes stimulated with IL-1β via two different mechanisms: (1) increasing the expression of SIRT1, and (2) decreasing the expression of p65. This effectively decreases the ability of p65 to activate the NF-κB pathway by reducing the number of p65 subunits that can be acetylated and by increasing the enzymatic activity of the p65 specific deacetylases (Lei et al. 2012).

A majority of the studies exploring complementary and alternative treatment modalities for OA have focused on the effect of a single polyphenol isolated from grapes, resveratrol. There has been limited research regarding the effects of a polyphenol treatment derived from whole grapes. Human chondrocytes stimulated with the inflammatory compound *tert*-butyl hydroperoxide exhibited a dose-dependent increase in cell proliferation when pretreated with whole grape polyphenols (WGP) at 20 μg/mL (147 % vs. control). Staining of the cells revealed that the presence of two important cartilage proteins, type II collagen and proteoglycan,

increased in the cells treated with WGP. A marker of cartilage degradation, glycoprotein-39, was significantly decreased in the cells treated with 10, 15, and 20 μg/mL WGP when compared against the control and the stimulated control. However, the In-Cell ELISA revealed no significant changes in the expression of COX-2, indicating that proliferative capabilities of WGP cannot be attributed to the inhibition of COX-2 (Lucero et al. 2014).

Another signaling pathway of interest in OA is the activator protein-1 (AP-1) signaling pathway, as it can induce the expression of iNOS and COX-2 genes through its activation (Allport et al. 2000; Cho et al. 2002). The transcription factor is activated through the phosphorylation of AP-1 by a class of mitogen-activated protein kinases (MAPKs). The most notable of the MAPKs are the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (Mengshol et al. 2001; Karin 1995).

Chondrocytes stimulated with advanced glycation end products (AGEs) have been shown to activate both AP-1 and NF-κB signaling pathways (Huang et al. 2009; Nah et al. 2008). In a study performed by Liu et al. in 2010, pig chondrocytes were pretreated with varying doses of resveratrol and then stimulated with AGEs to induce the AP-1 and NF-κB signaling pathways. This study verified and expanded upon previous findings, as the cells treated with resveratrol demonstrated a significant decrease in the expression of iNOS and COX-2, as well as the subsequent decreases in the production and activity of PGE_2, NO, and MMP-13. The inhibition of the AP-1 and NF-κB pathways by the resveratrol was attributed to the suppression of the signaling molecules ERK/JNK and IκBα kinase, respectively (Liu et al. 2010). AGE-stimulated porcine chondrocytes cannot induce AP-1 signaling via p38 MAPK, so the ability of resveratrol to module this particular kinase is still unknown (Huang et al. 2009).

Explants derived from the articular cartilage of pigs were used to examine the proposed chondroprotective role of resveratrol in AGE-stimulated cartilage tissue. Explants that were pretreated with 50 and 100 μM resveratrol for 24 h before being stimulated with 100 μg/mL retained significantly higher levels of proteoglycan and exhibited significantly less end products of aggrecan degradation when compared to experimental controls (Liu et al. 2010). These results can be partially attributed to the ability of resveratrol to suppress COX-2 and iNOS expression, thereby reducing AGE-mediated proteoglycan degradation and subsequent cartilage damage (Huang et al. 2009). An additional chondroprotective role of resveratrol can be attributed to its ability to suppress the expression and enzymatic activity of MMP-13, as the destruction of type II collagen is accompanied by an abrupt increase in the expression of MMP-13. This increase in MMP-13 coincides with cartilage degradation seen in active OA lesions (Mitchell et al. 1996). In addition to degrading type II collagen, MMP-13 has also been known to degrade proteoglycan and aggrecan (Burrage et al. 2006; Miwa et al. 2006). This makes MMP-13 a prime target for interventional methods targeted at preventing the development of OA and or delaying the progression of the disease.

8 Resveratrol and Grape Polyphenols: In vivo Animal OA Models

A limited number of in vivo studies have demonstrated a protective effect of grape compounds on cartilage in animals with induced OA (Elmali et al. 2005; Aini et al. 2012; Wang et al. 2012; Woo et al. 2011). Daily intra-articular (IA) injections of resveratrol were administered into the cartilage and synovium of an experimental OA rabbit model at 10 μMol/kg for 2 weeks. Resveratrol-treated rabbits had significantly reduced cartilage degradation scores (1.7 vs. 2.8, $p = 0.016$) and loss of proteoglycans (1.2 vs. 2.3, $p = 0.016$), but no differences in synovial inflammation. The results suggest that, at least in early stage OA, resveratrol applied via IA injections may have a protective effect in cartilage (Elmali et al. 2005). IA resveratrol injections ranging from 10 to 50 μMol have been tested in a similar rabbit model in comparison to both normal and untreated control groups. After 4 weeks of daily treatment, cartilage destruction, loss of matrix proteoglycan, chondrocyte apoptosis, and synovial NO were decreased in a dose-dependent manner in the resveratrol groups compared to the arthritic control but not the normal control (Wang et al. 2012).

Oral consumption of grape extracts has also been shown to reduce clinical signs of OA in two studies of induced knee arthritis in mice (Aini et al. 2012; Woo et al. 2011). Three doses weekly of an oral grape seed proanthocyanidin extract (GSPE) was shown to decrease measures of inflammation, pain, and joint damage in a monosodium iodoacetate (MIA)-induced knee arthritis mouse model. This 4-week study demonstrated significant reductions in animal reactions to mechanically induced pain, which promoted regain of hind paw weight distribution compared to saline-treated controls when GSPE was administered at 100 and 300 mg/kg ($p < 0.05$). At the 300 mg/kg dosage, there was an absence of osteophyte formation and subchondral bone fractures, and the decrease in cartilage damage was comparable to non-arthritic rats (Woo et al. 2011). A similar protective effect on joint health was found after 4 weeks of treatment with an oral extracted grape seed procyanidin B3 (B3). Five daily doses of 1 mg B3/g were administered to C57BL/6 J mice. In surgically induced OA joints, the articular cartilage was protected from surface damage and proteoglycan loss in the treated mice. B3 treatment also led to prevention of chondrocyte apoptosis and cartilage formation secondary to inflammation near the surgical regions (Aini et al. 2012).

9 Resveratrol and Grape Polyphenols: In vivo Animal Inflammatory Arthritis Models

More research on the relationship between grape extracts and joint health has been done using animal models that mimic inflammatory forms of arthritis, which may experience more severe inflammation, oxidative stress, and clinical impairment

than OA (Park et al. 2012). However, study results do align with those using in vivo OA animals, showing reduction in joint inflammation and damage accompanied by in vitro evidence of antioxidative and anti-inflammatory effects (Elmali et al. 2007; Ahmad et al. 2013; Cenesiz et al. 2012; Chen et al. 2013, 2014; Cho et al. 2009; Decendit et al. 2013; Jhun et al. 2013; Mossalayia et al. 2014; Park et al. 2011, 2012; Xuzhu et al. 2012).

9.1 Injected Treatments and Clinical Outcomes

Intraperitoneally (IP) injected grape compounds have shown potential as a novel treatment or adjunct in induced inflammatory arthritis models (Elmali et al. 2007; Cenesiz et al. 2012; Chen et al. 2014; Cho et al. 2009; Li et al. 2001; Park et al. 2012; Xuzhu et al. 2012). Injected GSPE has led to reduced joint inflammation, cartilage and bone damage, and arthritis incidence in some studies (Cho et al. 2009; Li et al. 2001; Park et al. 2012). A dose-dependent reduction was observed in croton oil-induced ear swelling in mice and collagen-induced arthritic (CIA) paw edema in rats given 10–40 mg/kg of intraperitoneal GSPE at inducement. Further, the effect of GSPE was greater than that of a dexamethasone treatment control and was longer lasting (Li et al. 2001). Five daily doses of 10, 50, and 100 μg/mL of GSPE given every other day significantly decreased OA severity in a dose-dependent manner in CIA mice. In this 2-week study, mean arthritis scores and histology scores were reduced in GSPE-treated compared to control mice ($p < 0.01$). In addition, osteoclastogenesis, synovial inflammation, and erosion of the cartilage and bone were reduced in treated versus control groups (Cho et al. 2009). Twice-weekly (IP) GSPE injections of 100 mg/kg were administered for 18 days in a CIA mouse model, resulting in reduced severity of mean arthritis symptom scores compared to saline-treated arthritic controls (Park et al. 2012).

A similar improvement in synovial inflammation and cartilage and bone damage has been observed in in vivo animal models given (IP) injected resveratrol (Cenesiz et al. 2012; Chen et al. 2014; Xuzhu et al. 2012). Cenesiz et al. (2012) compared 21 days of 10 mg/kg/d of (IP)-injected resveratrol to *N*-acetyl cysteine (NAC) in induced arthritic rats. NAC and resveratrol groups both demonstrated greater recovery from synovial inflammation compared to arthritic controls ($p < 0.01$), as evidenced by decreases in edema, regional inflammation, joint area heat, pain, and loss of function. The control and NAC groups also had more chondrocyte loss and pannus formation and synovial lymphocyte and neutrophil infiltration than the resveratrol group (Cenesiz et al. 2012). In DBA1 mice, (IP)-injected resveratrol was shown to have preventive and therapeutic effects in a dose-dependent manner. At 20 mg/kg, (IP) resveratrol caused a significant decrease in clinical disease parameters, including disease incidence, footpad thickness, and clinical index scores. Histologically, there were decreases in mono- and polymorphonuclear cell infiltration into the joint, synovial hyperplasia, and adjacent cartilage and bone

erosion for up to 40 days posttreatment (Xuzhu et al. 2012). As a follow-up to their study of IA resveratrol treatment in an induced OA rabbit model (Elmali et al. 2005), Elmali and colleagues conducted a similar study using an experimental inflammatory arthritis rabbit model. A daily treatment of 10 μMol/kg resulted in reduced cartilage destruction scores (1.8 vs. 2.5, $p = 0.04$) and loss of proteoglycans as determined by safranin O staining (1.7 vs. 2.5, $p = 0.03$) compared to controls. While noticeable synovial inflammation was present after inducement in the control group knees, this was not the case for the rabbits treated with resveratrol, which had a milder inflammatory reaction (1.8 vs. 2.7, $p = 0.01$) (Elmali et al. 2007). Intraperitoneal resveratrol has also been administered to an acute gouty arthritis mouse model, showing that pretreatment (but not co-treatment) alleviated clinical signs of footpad inflammation (Chen et al. 2014).

9.2 Oral Treatments and Clinical Outcomes

Grape preparations administered in oral forms have also demonstrated a protective effect in induced arthritis animal models (Ahmad et al. 2013; Chen et al. 2013; Decendit et al. 2013; Jhun et al. 2013; Mossalayia et al. 2014; Park et al. 2011). Oral GSPE has been tested at doses ranging from 25 to 300 mg/kg/d without reports of toxicity (Ahmad et al. 2013; Jhun et al. 2013; Park et al. 2011). CIA rats that were treated with 300 mg/kg GSPE three times weekly for 2 weeks had significantly reduced clinical signs of arthritis severity, including decreased inflammation scores, mono- and multinuclear cell infiltration of the joint compartment, synovial hyperplasia, cartilage destruction, and bone erosion compared to saline-treated controls ($p < 0.05$) (Park et al. 2011). Two-week oral administration of increasing doses of GSPE (25, 50, or 100 mg/kg/d) in an adjuvant-induced RA mouse model resulted in significantly decreased paw edema and joint damage compared to saline and non-arthritic controls. The effect was dose dependent, with more marked improvement in joint inflammation, joint space changes, synoviocyte proliferation, cellular infiltration, and articular cartilage erosion at the 100 mg/kg dose (Ahmad et al. 2013). Seven weeks of oral GSPE treatment was shown to influence not only arthritis outcomes but also obesity parameters in both obese CIA and diet-induced obese mouse models. Arthritis severity was diminished in the CIA mice treated with 300 mg/kg GSPE three times weekly, with reductions seen in mean arthritis scores, inflammation scores, and cartilage loss compared to controls (Jhun et al. 2013).

Two studies have used oral grape extracts other than GSPE, alone or in combination, in female Lewis rats with adjuvant-induced arthritis (AIA). This rat model mimics chronic human Th1-dependent arthritis (Decendit et al. 2013; Mossalayia et al. 2014). The results indicate a potential for grape extracts in both treatment and prevention of inflammatory arthritis pain-related symptoms. Five oral doses of 25 mg/kg malvidin-3-*O*-β-glucoside (Malβg), the primary anthocyanin in grapes, were given every 2 days for 10 days following evidence of paw inflammation.

Inflammatory cachexia was reversed with treatment compared to controls ($p < 0.0001$), and clinical arthritis severity was decreased compared to control and hydrocortisone-treated rats ($p < 0.003$). Prophylactic treatment at 125 mg/kg Malβg every 2 days in the 5 days leading to induced inflammation also significantly decreased cachexia and arthritis severity ($p < 0.0009$) (Decendit et al. 2013). A grape polyphenol extract (GPE) mix combined with propolis from bee sources also significantly reduce cachexia ($p < 0.002$) and arthritis severity ($p < 0.0004$) in treated rats compared to untreated and hydrocortisone-treated controls. Interestingly, the effect was more pronounced with a continual low dose (50 mg/kg GPE every 2 days) compared to separate high doses (five 250 mg/kg doses GPE every 2 days) ($p < 0.001$) (Mossalayia et al. 2014).

As with injected resveratrol treatments, oral administration also appears to reduce clinical inflammatory arthritis in AIA (Chen et al. 2013; Bauerova et al. 2011). An intragastric gavage of 10, 50, and 100 mg/kg resveratrol was administered daily in an AIA rat model to examine the effect on the secondary inflammation response. Two weeks of treatment alleviated synovial hyperplasia, inflammatory cell infiltration, and pannus formation. At the 100 mg/kg dose, decreased paw edema and inflammatory polyarthritis were comparable to the animals treated with chelerythrine, a protein kinase C (PKC) inhibitor ($p < 0.01$ vs. untreated rats) (Chen et al. 2013). Bauerova and colleagues tested a 30 mg/kg oral dose of pinosylvin, a resveratrol analogue, for 2 weeks in AIA Lewis rats. When compared to other natural monotherapy alternatives, such as coenzyme Q10 (CoQ10) and carnosine, pinosylvin was the only compound that effected a significant reduction in hind paw volume relative to baseline ($p < 0.05$). All tested compounds in this study significantly improved hind paw volume when combined with methotrexate and compared to methotrexate alone, including pinosylvin at a 50 mg/kg dose (Bauerova et al. 2011).

10 Potential Mechanisms for Grape and Arthritis Symptom Relief

The mechanism of action for the effects observed appears to be downregulation of the inflammatory cytokines or influencing antioxidant or anti-inflammatory pathway signaling (Shen et al. 2012). In addition, cartilage tissue is estrogen receptor positive. Polyphenols may bind and activate the estrogen receptor (Gehm et al. 1997), which may influence perceptions of pain (Coulombe et al. 2011). The in vivo animal studies showing improvement of clinical parameters and structural modification of joint tissue have been accompanied by evidence of in vitro anti-inflammatory and antioxidant effects. These included decreases in synovial NO, nitrotyrosine in cartilage and synovium, and inhibition of iNOS in the synovium and pseudocapsule surrounding surgical areas (Aini et al. 2012; Woo et al. 2011; Li et al. 2001; Park et al. 2012). Nitrotyrosine is a marker of

NOS-mediated oxidative stress, inflammation, and cell damage (Woo et al. 2011; Park et al. 2012).

While one study that combined resveratrol injections with NAC did not find significant changes in serum inflammatory cytokines, many others consistently observed decreases in serum and cellular IL-1, IL-4, IL-6, IL-8, IL-17, and TNF-α (Woo et al. 2011; Cenesiz et al. 2012; Cho et al. 2009; Mossalayia et al. 2014; Park et al. 2011; Xuzhu et al. 2012). This was particularly the case for IL-1β expression in cartilage, serum IL, and IL-1β in human peripheral leukocytes (Woo et al. 2011; Chen et al. 2013; Li et al. 2001; Mossalayia et al. 2014; Xuzhu et al. 2012). IL-1 induces MMPs, which may have been the mechanism responsible for observed decreases in MMP-13 and MMP-9, both of which contribute to cartilage degradation (Woo et al. 2011; Park et al. 2012). Additionally, reduced production of IL-17 in synoviocytes and splenocytes was observed with a near eradication achieved in serum levels of IL-17 (Cho et al. 2009; Park et al. 2011; Xuzhu et al. 2012). Combined with evidence of TNF-α inhibition, this suggests a decreased activation of NF-κB, which contributes to IL and TNF-α secretion (Chen et al. 2013; Cho et al. 2009; Li et al. 2001; Mossalayia et al. 2014; Xuzhu et al. 2012). Other concurrent in vitro effects demonstrated in these studies include inhibition of COX-2, decreased serum type-II-collagen-specific immunoglobulin G2a (IgG2a) and uric acid, inhibition and modulation of the T-cell immune response, and decreased differentiation of osteoclasts coupled with increased differentiation of osteoblasts (Ahmad et al. 2013; Chen et al. 2014; Cho et al. 2009; Jhun et al. 2013; Li et al. 2001; Park et al. 2011, 2012; Xuzhu et al. 2012).

The literature highlights four different target areas that may positively impact progression of arthritis such as OA through the use of dietary polyphenols found in grapes and other foods (Fig. 4) (Shen et al. 2012). The first approach is through decreased matrix degradation. This occurs via increased matrix synthesis of type II collagen, GAGs, aggrecans, and anabolic cytokines, as well as increases in the MMP inhibitor called tissue inhibitor of metalloproteinase (TMP)-1. The second approach is by decreased inflammatory activity. Polyphenols may reduce stimuli from inflammatory mediators such as COX-2 and PGE_2, as well as downregulate inflammatory cytokines like IL-1β, IL-6, IL-8, and TNF-α (Shen et al. 2012; Lopez 2012), and decrease the synthesis of inflammatory proteins such as C-reactive protein (CRP). CRP also contributes to decreased inflammatory activity. The third area of impact is from decreased oxidative activity and damage. Polyphenols may decrease activity of iNOS and NO and increase activity of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase. A fourth area for therapy is by decreasing cell apoptosis of caspases, thus increasing chondrocyte proliferation. The resulting reduction in joint stiffness, pain, and inflammation from these actions could be a mechanism for improving joint mobility and overall OA management (Shen et al. 2012).

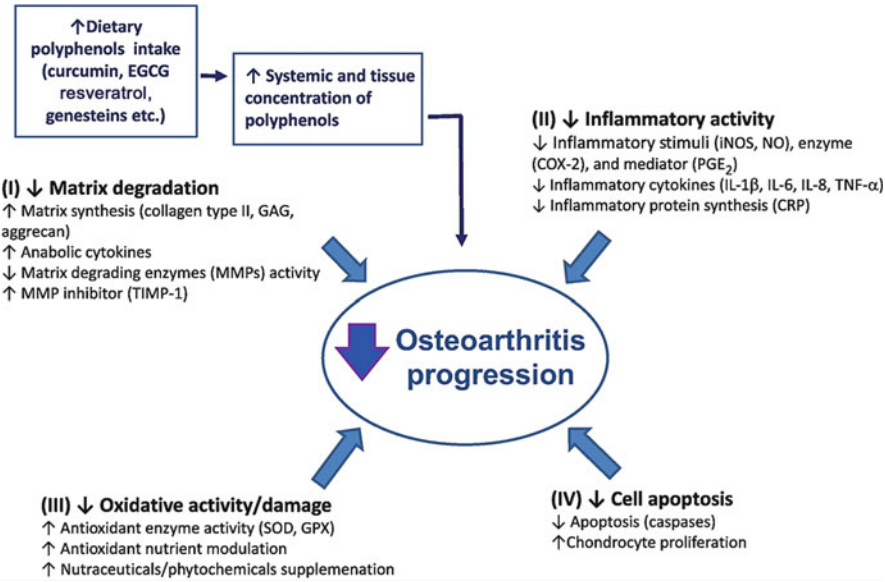

Fig. 4 The potential therapeutic approach to inhibit the progression of OA by dietary polyphenols. Reprinted from *J. Nutr. Biochem.* **23**(11):1367–1377, Shen C et al., Dietary Polyphenols and Mechanisms of Osteoarthritis, Copyright (2012), with permission from Elsevier

11 Resveratrol and Grape Polyphenols: Human OA Studies

Very few studies have been conducted on the effects of grapes in human joint conditions such as OA (Small et al. 2014; Tiernan et al. 2014; Welker 2011). A small case study of nine female patients suffering from gonarthrosis followed a daily treatment plan which included supplementation with ascorbic acid, L-lysine, a broad-spectrum nutrient mix, and GSE at 500 mg. After 3–4 weeks, five of the patients reported being pain-free and four reported significant pain relief using a 4-point Likert scale ($p < 0.05$). While some of the women eventually reduced their dosages of the treatment or discontinued it all together, no analgesic use or surgery tied to gonarthrosis was reported in the follow-up period of 3 years or longer in some cases (Welker 2011).

A very recent study examined the effect of grape consumption on self-reported knee pain, joint range of motion (ROM), and biochemical markers of inflammation (CRP) and cartilage metabolism (insulin-like growth factor-1 (IGF-1), human cartilage glycoprotein 30 (YKL-40)) in individuals with knee OA. Qualified participants ($n = 72$) were randomly assigned to consume 47 g of freeze-dried grape powder (FDGP) ($n = 35$, 27 female, 8 male) or a comparable placebo ($n = 37$, 28 female, 9 male) daily for 4 months. The FDGP group had a significant decrease in activity-related pain from baseline to end of treatment compared to placebo

(−5.3 vs. −2.1, $p < 0.05$). At midpoint, both groups had a significant reduction in total knee symptoms and impact on quality of life (QOL). Further, this effect was greater in females. FDGP resulted in gender-specific changes in IGF-1 compared to placebo group. Males in the FDGP group had a significant increase in IGF-1 from baseline compared to males in the placebo group (1.6 and 19.9 ng/mL in FDGP; 6.8 and 2.0 ng/mL in placebo for baseline and final, respectively, $p < 0.05$). There was no change in ROM, CRP, or YKL-40 between FDGP and placebo groups (Tiernan et al. 2014). Additional components of this study showed that very hard activity increased 70 % for participants ≤64 years of age in the FDGP group but decreased in the placebo group. Those >65 years old reported a significant decline in moderate and hard activities ($p < 0.05$) in both groups. IL-1β increased significantly in both treatment groups and more so in males. However, this increase was 637.3 % in the placebo group and 194.6 % in the FDGP group, suggesting that FDGP may have had some anti-inflammatory activity (Small et al. 2014). Longer treatment durations with FDGP may be needed to see benefits on ROM and physical activity in this population.

12 Conclusions

Unlike other chronic diseases such as hyperlipidemia and hypertension, there is no reliable biomarker or clinical measure for diagnosing OA (Hunter 2011). Prevention of joint damage is therefore a critical component of therapy and one that may be enhanced with grape intake (Aini et al. 2012; Chen et al. 2014; Decendit et al. 2013; Xuzhu et al. 2012). Relying on the use of radiography to assess joint space width does not take into account metabolic aspects of OA pathology that occur prior to structural damage and the weak correlation between radiographic and symptomatic arthritis (Duncan et al. 2006; Lawrence et al. 2008; Pereira et al. 2011; Henrotin 2012).

Grape polyphenols have anti-inflammatory and antioxidative properties in vitro that may influence joint structure and OA outcomes related to pain and mobility (Elmali et al. 2005, 2007; Aini et al. 2012; Wang et al. 2012; Woo et al. 2011; Ahmad et al. 2013; Cenesiz et al. 2012; Chen et al. 2013, 2014; Cho et al. 2009; Decendit et al. 2013; Jhun et al. 2013; Mossalayia et al. 2014; Park et al. 2011, 2012; Xuzhu et al. 2012; Henrotin et al. 2011; Lei et al. 2012; Lucero et al. 2014; Liu et al. 2010). Two human studies suggest that grape consumption with its bioactive constituents may also reduce inflammation and influence OA outcomes (Small et al. 2014; Tiernan et al. 2014; Welker 2011). In particular, daily consumption of FDGP may reduce symptoms of pain and possibly impact a biomarker of cartilage metabolism in individuals with self-reported knee OA (Tiernan et al. 2014). This is in keeping with the literature that has shown decreases in self-reported OA symptoms with soy isoflavones and tart cherry polyphenols (Arjmandi et al. 2004; Schumacher et al. 2013). Other studies are currently

underway that may confirm an effect of FDGP on arthritis and other chronic diseases in humans (Pezzuto 2008).

Nutritional research related to OA is a budding field with many unknowns. Most of the available literature is focused on pharmacokinetics or single nutrient and target interventions (Ameye and Chee 2006). OA research examining the intake of bioactive components in whole foods is lacking. Many plant and nutrient extracts may be harmful in therapeutic doses, so whole foods are a more desirable approach. The literature suggests consumption of whole grapes with their bioactive constituents may be a natural alternative to reducing pain and improving symptoms associated with OA. In addition, consumption of diets rich in polyphenol sources is consistent with dietary recommendations that promote 8–10 daily servings of colorful fruits and vegetables (Barnes 2008).

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Grapes and Urinary Bladder Function

Robert M. Levin, Robert E. Leggett, and Catherine Schuler

Contents

Abstract The function of the urinary bladder is to collect and store urine at low intravesical pressure and then, periodically, to expel the urine via a highly coordinated and sustained contraction. Bladder function depends on several factors including state of innervation, vascularization, structure of the organ as a whole, contractile response of the smooth muscle (SM) elements to autonomic stimulation, availability of metabolic energy (cytosolic adenosine triphosphate [ATP] and

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mitochondrial oxidative metabolites), and the density and distribution of the connective tissue in the detrusor. These factors are intimately connected, and an alteration in one factor can induce substantial adaptive changes in the others.

Urinary bladder outlet obstruction is a common medical problem. More than 80 % of males older than 50 years of age have varying degrees of bladder outlet obstruction secondary to benign prostatic hyperplasia (BPH). Our current studies are focused on: (1) correlating obstructive bladder dysfunction with ischemia, ischemia followed by reperfusion (I/R), and oxidative stress (generation of free radicals and protein, lipid and phospholipid oxidation and peroxidation), and (2) demonstrating that natural products that have a high antioxidant and membrane-protective activities, such as grapes, are effective in reducing the progression and severity of partial bladder outlet obstruction, ischemia, ischemia followed by reperfusion, and oxidative stress.

In a series of studies, we demonstrated that oral administration of a standardized grape suspension (equal to 3–8 oz glasses of mixed grape juice per day) can significantly protect the urinary bladder from obstructive and ischemic damage. As mentioned above, partial outlet obstruction secondary to BPH is the most common dysfunction men over 50 develop. It can result in bladder smooth muscle hypertrophy, mucosal hyperplasia, nocturia, incomplete emptying, reduced intravesical pressure, and significantly decreased compliance. The progression from a compliant bladder to severe obstructive bladder dysfunction is significantly reduced in both severity and progressive dysfunction by oral administration of the grape suspension. Using an in vivo model of ischemia and ischemia followed by reperfusion, we clearly demonstrated that oral ingestion of the grape suspension protected the bladder from the physiological, biochemical, and morphological dysfunctions mediated by ischemia and ischemia followed by reperfusion.

Ischemia, ischemia followed by reperfusion, and oxygen stress are also directly involved in the etiology of several bladder dysfunctions in women including interstitial cystitis and incontinence. Although we did not test the effects of oral ingestion of the grape suspension on female rabbit models of these bladder dysfunctions, based on the studies published, we would expect that the grape suspension would be effective in reducing the level and progression of these two models of female bladder dysfunction.

1 Introduction

The function of the urinary bladder is to collect and store urine at low intravesical pressure and then, periodically, to expel the urine via a highly coordinated and sustained contraction (Steers 1992; Zderic et al. 1996a). Bladder function depends upon several factors including state of innervation, vascularization, structure of the organ as a whole, contractile response of the smooth muscle (SM) elements to

autonomic stimulation, availability of metabolic energy (cytosolic adenosine triphosphate [ATP] and mitochondrial oxidative metabolites), and the density and distribution of connective tissue in the detrusor. These factors are intimately connected, and an alteration in one factor can induce substantial adaptive changes in the others (Steers 1992; Zderic et al. 1996a).

Urinary bladder outlet obstruction is a common medical problem. More than 80 % of males older than 50 years of age have varying degrees of bladder outlet obstruction secondary to benign prostatic hyperplasia (BPH) (Barry and Meigs 2000). Our current studies are focused on: (1) correlating obstructive bladder dysfunction with ischemia/reperfusion (I/R) and oxidative stress (generation of free radicals and protein, lipid and phospholipid oxidation and peroxidation), and (2) demonstrating that natural products that have a high antioxidant and membrane-protective activities, such as grapes, are effective in reducing the progression and severity of partial bladder outlet obstruction.

2 Relevance of the Rabbit Model of Partial Outlet Obstruction to the Study of Human Obstructive Bladder Dysfunction Secondary to BPH (Levin et al. 1999a, 2000)

In humans, it is difficult to investigate the cellular mechanisms by which progressive bladder dysfunction occurs secondary to BPH. However, many of the functional changes associated with human bladder pathology can be induced in experimental animal models including the rabbit (see reviews Levin et al. 2000, 1999b). Rabbit bladder capacity is between 50 and 100 mL, and compliance can be evaluated cystometrically using an 8 Fr. Foley catheter to catheterize the bladder. The cystometric curve of the rabbit is similar in shape to that of humans. Similar to humans, bladder emptying occurs during the tonic phase of contraction. The ability of the bladder to sustain increased pressure in response to stimulation is significantly reduced by partial outlet obstruction before any change in maximal pressure generation occurs.

Major characteristics of the rabbit's response to partial outlet obstruction include (1) an increase in bladder mass to a stable level, (2) reduced compliance during bladder filling, (3) increase micturition frequency and decreased volume per micturition (urgency and frequency), and (4) development of overactive bladder dysfunction. These dysfunctions are very similar to those secondary to BPH observed in men.

Ultrasound studies have confirmed that not only do men with obstructive uropathies exhibit an increase in bladder mass (Manieri et al. 1998; Bright et al. 2010), but bladder wall thickness has been shown to be the most accurate noninvasive way to identify men with obstructive bladder dysfunction. Another common feature of obstruction in both rabbits and man is denervation which has been demonstrated immunohistochemically and biochemically in both rabbits and in men (Levin et al. 2000; Gosling et al. 2000). In addition, obstructed rabbits and

men both show an increase in the density and distribution of connective tissue (CT) within the bladder wall resulting, functionally, in decreased compliance and higher pressures during filling (Malmqvist et al. 1991; Yang et al. 2013).

In two major studies performed with Dr. John Gosling (expert in electron microscopy of the lower urinary tract), we clearly demonstrated that the level of contractile dysfunction in rabbits subjected to partial outlet obstruction correlated with the degree of ultrastructural damage to nerve, synaptic, mitochondrial, and sarcoplasmic reticulum (SR) membranes (Gosling et al. 2000; Levin et al. 2002) which in turn correlated with similar findings in men with obstructive bladder dysfunction.

3 Stages of Rabbit Bladder Response to Obstruction (Levin et al. 1994, 1999b)

The progressive response to partial outlet obstruction can be divided into three phases:

(a) An initial response to surgical induction of partial outlet obstruction lasting 1–14 days and characterized by bladder distension followed by a progressive increase in mass to a stable level.
(b) Compensated bladder function immediately follows the "initial phase" and lasts a variable length of time. This period is characterized by relatively stable bladder mass and normal or even increased contractile responses to field stimulation (FS), carbachol, and potassium chloride (KCl) stimulation.
(c) Decompensated bladder function. At some point, the functional ability of the bladder to contract and empty begins to degenerate, and the organ becomes "decompensated." Decompensation is a process characterized by progressive deterioration in contractility and function (i.e., ability to generate pressure and empty), a further increase in bladder mass, and a progressive decrease in the volume fraction of SM elements in the bladder wall. The end result is either an organ with a thick fibrous wall, low capacity, poor compliance, and little or no contractile function or a dilated bladder with a thin fibrous wall, high capacity, and little or no contractile function (end-stage decompensation).

4 Compensation Versus Decompensation

Results of experiments in which mild partial outlet obstruction was studied longitudinally (up to 6 months) showed that the level of bladder decompensation was related to both the magnitude of the increase in bladder mass and the level of contractile dysfunction but not directly to the duration of obstruction. Therefore, we

differentiate the status of the obstructed rabbit bladder (state of compensation/decompensation) by bladder mass and by the comparative contractile responses of isolated bladder strips to various forms of contractile stimulation (Kato et al. 1990; Nigro et al. 1999).

Although it is clearly true that the longer rabbits are obstructed, the greater proportion of them shift to decompensation and then progress to severe decompensation, individual rabbits may remain compensated or at mild decompensation for prolonged periods of time (Levin et al. 2013; Callaghan et al. 2013a, b; Jock et al. 2014). This is very similar to progressive decompensation in men with obstructive bladder dysfunction secondary to BPH. That is to say, the level of obstructive dysfunction is not directly related to size of the prostate or how long individual men have had BPH. Some men with extremely large prostates have no urological problems, while some men with small prostates have severe dysfunctions.

5 Criteria for Defining the Level of Compensation–Decompensation

In *compensated* bladders, the response to all forms of in vitro stimulation averages >80 % of control. *Mild decompensation* is defined as the average response to all forms of stimulation which is >60 % and <80 % of the control responses. *Intermediate decompensation* is defined as having an average contractile function of >20 % and <60 % of control. *Severely decompensated* bladders exhibit maximal responses averaging <20 % of control.

In reversal studies, we categorize the severity of dysfunction by the bladder mass at the time of reversal. We empty the bladder after removing the ligature, and with a caliper, we measure the length and width of the bladder and calculate the mass as a solid cylinder or sphere depending on the shape of the empty bladder. We take the same measurements on the sham-operated control bladders in order to have control bladder mass. Compensated bladders have up to 2 times the bladder mass of controls; mildly decompensated bladders have a bladder mass between 2 and 5 times control; intermediate decompensated bladders have between 5 and 10 times the mass of controls, and severely decompensated bladders have over 10 times the control bladder mass.

6 Partial Bladder Outlet Obstruction and Oxidative Stress

Partial bladder outlet obstruction (PBOO) results in elevations of reactive oxygen species (ROS) and reactive nitrogen species RNS) of free radicals and alterations in cellular antioxidant mechanisms (Levin et al. 2013; Juan et al. 2007). Oxidative

injury occurs when ROS and RNS react with endogenous macromolecules (lipids, proteins, and nucleic acids) and disrupt normal cellular function. The reaction of specific ROS and RNS with lipids, proteins, and nucleic acids results in the formation of characteristic products that can be used to monitor oxidative stress (Levin et al. 2013; Juan et al. 2007). Measurement of these characteristic products, along with the status of various antioxidant mechanisms, provides a thorough evaluation of the oxidative injury that occurs in the urinary bladder during partial outlet obstruction. We use nitrotyrosine (NT), dinitrophenol (DNP), thiobarbituric acid reactive substances (TBARSs), and malondialdehyde (MDA) (product of lipid peroxidation) as markers for ROS and RNS damage.

Lin et al. have provided direct evidence that partial outlet obstruction in rabbits induces a rapid lipid peroxidation of mitochondrial membranes resulting in significant decreases in high-energy phosphate synthesis (Lin et al. 2005a). Our research group has completed a number of studies that present both direct and indirect evidence for the importance of free radical damage to bladder decompensation. We have directly demonstrated that partial outlet obstruction stimulates the oxidation and nitration of proteins within both the bladder smooth muscle and mucosa (Juan et al. 2007; Kalorin et al. 2008; Lin et al. 2008; Hydery et al. 2009).

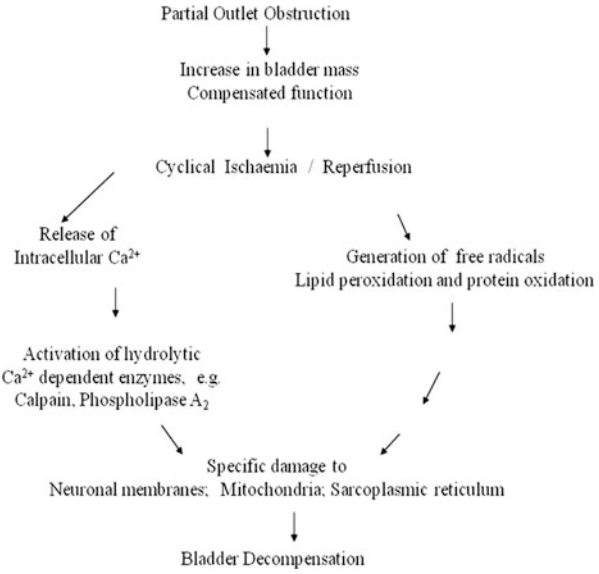
7 Importance of Studying the Bladder Muscle and Mucosa Separately

Bladder mucosa has very different metabolic requirements and responses to stress (e.g., partial outlet obstruction and ischemia) than the SM compartment (Hypolite et al. 1993; Levin et al. 1996a). If these tissue compartments are not separated from one another, the results from metabolic or biochemical analyses of the detrusor SM may be misleading. Briefly, the mucosa exhibits a significantly higher rate of oxidative metabolism than the muscle, a significantly greater rate of oxidative ATP synthesis, and significantly lower concentrations of creatine phosphate and creatine kinase. The mucosa has significantly different activities of the enzymes citrate synthase (CS), choline acetyltransferase (ChAT), and sarcoendoplasmic reticular calcium ATPase (SERCA) than SM (Hypolite et al. 1993; Levin et al. 1996a). We recently demonstrated that the mucosa exhibits a four-fold greater blood flow (BF) than the detrusor (Lin et al. 2011; Schroder et al. 2001). These findings are consistent with our demonstration that the mucosa is more sensitive to anoxia than the detrusor SM (Levin et al. 1996a).

8 Biomarkers for Determining the Severity of Obstructive Bladder Dysfunction (Levin et al. 1990, 1995)

In summary, obstructive bladder dysfunction is initiated by four specific pathological processes: (1) selective postsynaptic denervation and defective neurohumoral transmission (Levin et al. 1993; Harrison et al. 1990), (2) mitochondrial damage and intracellular metabolic dysfunction (Hsu et al. 1994; Wang et al. 2001), (3) SR damage and calcium dysregulation resulting in an increase in basal intracellular free calcium (Rohrmann et al. 1996; Zderic et al. 1996b), and (4) increased connective tissue synthesis and distribution within and between smooth muscle cells (Kato et al. 1990; Levin et al. 2005a).

The schematic shown below demonstrates the sequence of events leading to bladder decompensation. The common link in this sequence is the generation of free radicals and oxidative stress. Neuronal membranes and neuronal mitochondria are extremely sensitive to oxidative stress (Luque-Contreras et al. 2014; Areti et al. 2014), as are all mitochondria (Dai et al. 2014; Peinado et al. 2014), and the sarcoplasmic reticulum (resulting in increased intracellular free calcium) (Grim et al. 2014; Dhalla et al. 2012).



9 Evidence that Ischemia/Reperfusion Is an Etiological Factor for Bladder Dysfunction After Partial Outlet Obstruction (Schroder et al. 2001; Chichester et al. 2000, 2001; Lin et al. 2007a)

Blood flow (BF) was measured using a quantitative fluorescent microsphere method (Schroder et al. 2001; Lieb et al. 2000). The vascular endothelium was visualized by immunohistochemistry (IHC) using an antibody to CD31, while the vascular density and vessel circumference were quantitated using digital morphometry (Chichester et al. 2000, 2001).

We examined the effect of 4 weeks of PBOO on bladder BF and correlated it with the severity of bladder contractile dysfunction. Compensated bladders had normal BF. In decompensated bladders, a direct correlation was observed between the severity of contractile dysfunction and the magnitude of the decrease of BF to the muscle compartment.

In these same rabbits, we evaluated the effect of obstruction on vascular distribution, density, and circumference using an endothelial cell antibody to CD31 (Chichester et al. 2000, 2001). Vascular density remained relatively constant and independent of bladder weight. However, the distribution of vessel circumferences shifted to the left indicating a significantly greater number of small-size vessels (microvessels) in the hypertrophied bladder. In addition, the blood vessels continued to run in between the hypertrophied smooth muscle bundles and not within the bundles or between the cells which caused central hypoxia within the smooth muscle bundles even though the net number of blood vessels did not significantly decrease (Chichester et al. 2000, 2001). Additional evidence for angiogenesis comes from our studies showing that partial outlet obstruction stimulates a significant increase in vascular endothelial growth factor (VEGF) and angiopoietin 1 and 2 at both the RNA and protein level (Walker et al. 2009).

The density and distribution of tissue hypoxia parallels the severity of the decreased blood flow and decreased contractile function (Levin et al. 2003).

Similar to the rabbit, neovascularization of the obstructed rat bladder increases with increased hypertrophy (Ghafar et al. 2002a, b). The distribution of new vessels in the hypertrophied rabbit bladder is similar to that shown in rats by Boels et al. (1996) with the increased number of blood vessels being within the interstitial spaces around muscle bundles and not between cells. Gabella and Uvelius (1999) demonstrated the presence of capillaries between muscle bundles, but not within them, in normal rat bladders and an increase in cross-sectional area of the muscle bundles in obstructed bladders. This would make oxygen perfusion throughout the entire hypertrophied muscle bundle more difficult than in normal muscle and relates directly to the increased focal areas of hypoxia observed as the bladder continues to hypertrophy (Levin et al. 2003).

10 Nitrotyrosine and Carbonyl Groups as Biomarkers for Oxidative Damage (Juan et al. 2007; Kalorin et al. 2008)

Twenty New Zealand white (NZW) rabbits were subjected to 1–28 days of partial outlet obstruction. Sham-operated rabbits served as controls. At each time point, isolated strips of bladder body were mounted in individual baths, and the contractile response to field stimulation (FS), carbachol, and KCl is determined. Western blotting was used to determine both the level of nitrotyrosine and carbonyl groups at the protein level.

The results can be summarized as follows: Bladder weight increased rapidly during the first 7 days and then increased slowly thereafter. Contractile responses to FS decreased progressively over the study period. The contractile responses to carbachol and KCl were significantly less dysfunctional than the response to FS. There was a four-fold increase in the expression of nitrotyrosine in the 7-day-obstructed groups when compared to sham controls which remained consistently elevated at two-fold higher than control at 14 and 28 days of obstruction. Protein oxidation progressively increased over the 28-day study to approximately fivefold higher than control. The present study clearly demonstrates that both RNS and ROS actively participate in bladder decompensation following PBOO and can be used as markers for reperfusion-based injury. These results have been corroborated in other labs and in other animal models (Griebling 2013; Bisogni et al. 2012; Matsumoto et al. 2010).

11 Effect of Antioxidants on Obstructive Bladder Dysfunction

If our hypothesis concerning the etiology of obstructive bladder dysfunction is correct, then natural products having high antioxidant potential and membrane protection against oxidative stress publications on grape products clearly indicate that these suspensions have potent antioxidant and membrane-protective actions (Lamuela-Raventos and de la Torre-Boronat 1999; Stein et al. 1999; Mosca and Cingolani 2002; Wu et al. 2001; Das et al. 1999) that should protect bladder function even more effectively than other products tested. The advantages of grape products are availability, palatability, diversity of preparations, and self-motivation in terms of patient compliance. Because of the potential benefits, the effects of grapes were tested in our rabbit models of bladder outlet obstruction, in vivo ischemia/reperfusion, and in vitro ischemia/reperfusion.

12 Effect of Grapes on the Response of Rabbits to Partial Outlet Obstruction (PBOO)

In our first study (Agartan et al. 2004), a total of 24 male New Zealand white rabbits (3–4 kg) were separated into four groups of six each. Rabbits in groups 1 and 3 were treated by gavage with 10 mL aqueous grape suspension daily (20 mg/mL standardized grape powder obtained from the California Table Grape Commission). Those in groups 3 and 4 were given 10 mL sugar–water vehicle by gavage (10 mg sucrose + 10 mg fructose/mL). This volume of grape suspension was calculated to equal 3–8 oz glasses of grape juice per day for a man. After 3 weeks of daily oral administration, each rabbit was sedated with ketamine–xylazine (25–10 mg/kg). The bladder was catheterized with an 8 Fr Foley catheter and emptied, and the volume of urine was measured. Cystometry was then performed at a filling rate of 1.5 mL/min. At the completion of cystometry, the rabbits were immediately anesthetized with pentobarbital, and moderate outlet obstruction was created in groups 1 and 3, while sham operations were performed in groups 2 and 4. Treatment was continued for an additional 3 weeks. At the end of the 3-week-obstructed period, each rabbit was sedated with ketamine–xylazine, and a final cystometry was performed. Each rabbit was then anesthetized with pentobarbital and the bladder excised and weighed, and four full-thickness longitudinal strips of detrusor were prepared for contractility studies. Two additional full-thickness strips were taken for immunohistochemistry, and the remaining bladder body was separated between muscle and mucosa, frozen in liquid nitrogen, and stored at −70 °C for biochemical analyses.

The components in the grape powder supplied by the California Table Grape Suspension

Compound	Total	Individual
Total phenols	5.8 mg/g	
Flavans	4.1 mg/g (as catechin)	
Anthocyanins	770 mg/kg (as malvidin glucoside)	
Flavonols		
Quercetin		102 μmol/kg
Myricetin		8 μmol/kg
Kaempferol		11 μmol/kg
Stilbenes		
Resveratrol		7 μmol/kg

The results of the study are as follows: The bladder weight of the obstructed grape group was significantly greater than the bladder weight of the sham-grape group but was significantly lower than the bladder weight of the obstructed sham group showing significant protection against hypertrophy of the bladder (Fig. 1). Similarly, the compliance of the bladder (the stiffness of the bladder wall) of the obstructed grape group was significantly lower (stiffer) than the sham-grape group but was significantly higher than the obstructed vehicle group (Fig. 2), indicating that the obstructed grape group showed significant protection against the increased

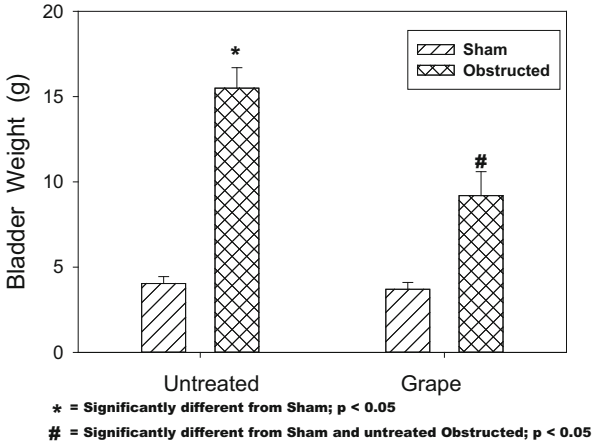


Fig. 1 Effect of grape suspension on the bladder weight following partial outlet obstruction. Each *bar* is the mean ± SEM of six individual rabbits

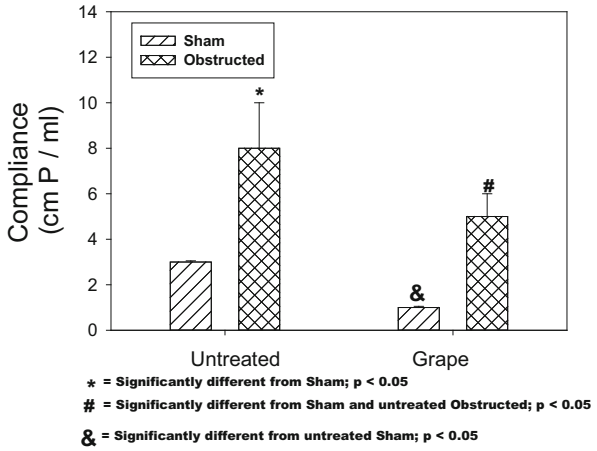


Fig. 2 Effect of grape suspension on the bladder compliance following partial outlet obstruction. Each *bar* is the mean ± SEM of six individual rabbits

bladder wall tension mediated by obstruction. Interestingly, the compliance of the sham-grape group was significantly higher than that of the sham-untreated group showing better elasticity of the wall (Fig. 2).

The contractile responses to all frequencies of field stimulation were significantly reduced in both obstructed groups, although the responses of the obstructed vehicle group were significantly lower than the responses of the obstructed grape group (Fig. 3). The contractile response to both carbachol and KCl of the sham-grape group was similar to that of the sham-vehicle group. Although the responses of both obstructed groups to carbachol and KCl were reduced, the contractile response to carbachol of the obstructed grape group was significantly higher than for the obstructed vehicle group (Fig. 4). Choline acetyltransferase is the enzyme that synthesizes acetylcholine and is a marker enzyme for cholinergic transmission. In the vehicle-treated rabbits, obstruction mediated a significant decrease in ChAT activity, whereas in the grape-treated rabbits, there was no decrease in ChAT

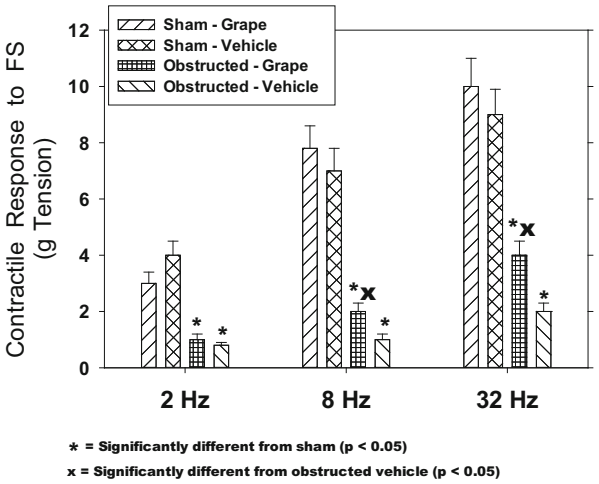


Fig. 3 Effect of grape suspension on the contractile response to field stimulation: maximal contractile response. Each *bar* is the mean ± SEM of six individual rabbits

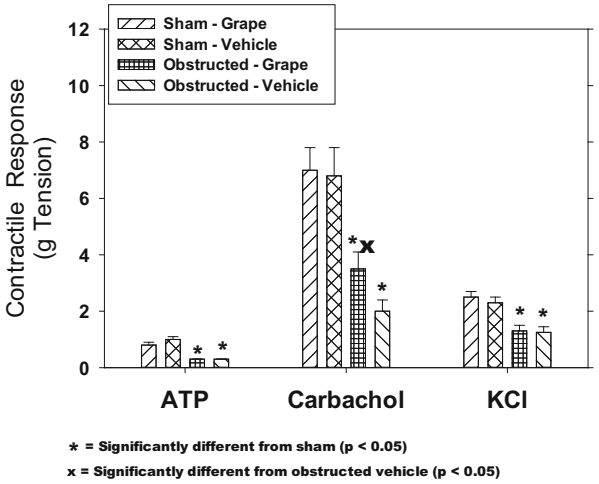


Fig. 4 Effect of grape suspension on the contractile responses to ATP, carbachol, and KCl: maximal contractile response. Each *bar* is the mean ± SEM of six individual rabbits

activity (Fig. 5). The density of neurofilaments within the detrusor smooth muscle is presented in Fig. 5. There was a significant reduction in the density of nerve tracks within the detrusor smooth muscle of both obstructed groups. However, the density of the nerve tracks in the grape-treated obstructed rabbits was significantly greater than in the vehicle-treated obstructed rabbits. These data match the contractile responses to field stimulation, which is also a measure of the integrity of the neuronal innervation of the smooth muscle.

The conclusion from this study was that feeding rabbits with physiologically relevant levels of this grape suspension significantly protected the bladder from obstructive bladder dysfunction. Specifically, the level of bladder hypertrophy was significantly reduced; the decreased compliance mediated by obstruction was significantly reduced; and the level of contractile dysfunction was significantly

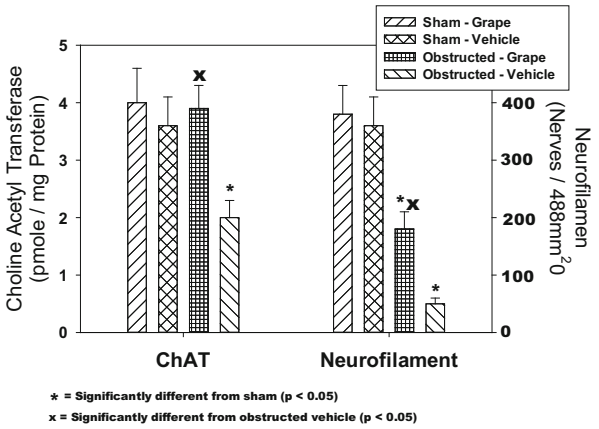


Fig. 5 Effect of grape suspension on the choline acetyl transferase activity and neurofilament density. Each *bar* is the mean ± SEM of six individual rabbits

reduced as was the level of neuronal damage. Thus, the bladder of the rabbits fed grape suspension showed a significantly better level of physiological and biochemical functions than rabbits fed sugar–water.

13 Effect of the Ethanol on the Effect of Grapes on the Response of Rabbits to PBOO

Our second study was directed to see if feeding rabbits grape suspension in 8 % ethanol would be more efficacious than feeding the rabbits with the grape suspension in water (Agartan et al. 2005). The idea was that it has been shown that ethanol can increase the absorption of various food products. Thus, we thought it might be of benefit in the absorption of the antioxidants and membrane-protective components in the grapes (Lyu et al. 2014; Feng et al. 2014; Fagerberg et al. 2012; Lennernas 2009). It was not to simulate wine in any way.

A total of 48 New Zealand white rabbits were separated into eight groups of six rabbits each. Groups 1 and 3 were pretreated by gavage for 3 weeks with a standardized grape suspension (obtained from the California Table Grape Commission) suspended in water (100 mg/kg/day); groups 2 and 4 were treated with vehicle (sugar–water). Groups 5 and 7 were pretreated with the grape suspension suspended in 8 % ethanol (100 mg/kg/day), and groups 6 and 8 were treated with ethanol vehicle. Groups 1, 2, 5, and 6 underwent a sham operation after 3 weeks of treatment, and groups 3, 4, 7, and 8 underwent partial outlet obstruction.

After 3 weeks of daily oral administration, each rabbit was sedated with ketamine–xylazine (25–10 mg/kg). The bladder was catheterized with an 8 Fr Foley catheter and emptied, and the volume of urine was measured. Cystometry was then performed at a filling rate of 1.5 mL/min. At the completion of cystometry, the rabbits were immediately anesthetized with pentobarbital, and moderate outlet obstruction was created in groups 1 and 3, while sham operations were performed in groups 2 and 4. Treatment was continued for an additional 3 weeks. At the end of

the 3-week-obstructed period, each rabbit was sedated with ketamine–xylazine, and a final cystometry was performed. Each rabbit was then anesthetized with pentobarbital and the bladder excised, weighed, and four full-thickness longitudinal strips of detrusor were prepared for contractility studies, and the remaining bladder body was separated between muscle and mucosa, frozen in liquid nitrogen, and stored at −70 °C for biochemical analyses.

The results were as follows: The contractile responses to all forms of stimulation were significantly lower in all obstructed rabbits than their sham counterparts. The grape-obstructed bladders showed significantly better responses than the vehicle-obstructed bladders. Ethanol had no effect on the contractile responses.

In conclusion, suspending the grape preparation in ethanol did not enhance the beneficial effects of the grapes. In fact, some parameters showed the aqueous suspension to be superior. One possible explanation for this result relates to a recent study that demonstrated that the presence of ethanol enhanced the severity of the effects of in vitro ischemia on the contractile responses of isolated strips of rabbit bladder smooth muscle but had no effect on the contractile responses in oxygenated buffer (Levin et al. 2005b).

14 Effect of Grapes on In Vivo Ischemia and Ischemia Followed by Reperfusion

Our third study was designed to determine if feeding rabbits grape suspension protected the bladder from a model of in vivo ischemia/reperfusion (Lin et al. 2005b). Six groups of four male New Zealand white rabbits were treated by gavage with 10 mL aqueous grape suspension (groups 1–3, 40 mg/mL standardized grape powder from the California Table Grape Commission) or 10 mL sugar–water (vehicle, groups 4–6; 20 mg sucrose + 20 mg fructose/mL). All rabbits were treated twice daily for 3 weeks before surgery, continuing until the end of the experiment. After 3 weeks, rabbits were sedated with ketamine–xylazine (25 mg/kg ketamine/5 mg/kg xylazine, i.m.). The bladder was catheterized with an 8 Fr Foley catheter and emptied. Cystometry was performed at a filling rate of 1.5 mL/min until the micturition reflex was stimulated or leakage around the catheter occurred. Bladder capacity was defined as the point of voiding or leakage. Cystometry was performed on all rabbits immediately before surgery and again before euthanasia. All experiments were approved by the Institutional Animal Care and Use Committee at the Stratton Veterans Affairs Medical Center, Albany, NY, USA.

After cystometry, each rabbit was anesthetized (inhalation) with isoflurane (1–3 %), and bilateral ischemia was induced in groups 1 and 4 by clamping the vesical arteries with microvascular clamps for 2 h (ischemia-only groups). In groups 2 and 5, bilateral ischemia was similarly induced for 2 h, at which time the clamps were removed and the rabbits allowed to recover (I/R groups) for 1 week. Groups 3 and 6 were controls (shams) and were not exposed to ischemia. Sham surgery consisted of isolating the vessels entering the bladder base and

closing the incision. Two sham rabbits from groups 3 and 6 were analyzed with the ischemia-only groups and the other two with the I/R groups. There were no differences between the sham groups, and so they were combined.

Two hours prior to euthanasia, each rabbit was injected i.p. with 2 mL hypoxyprobe-1 (for immunohistochemical studies). The bladder was excised and placed in an oxygenated physiological Tyrode's buffer solution equilibrated with 95 % O_2 and 5 % CO_2 at 37 °C for 2 h, to regenerate cellular ATP required for contractile responses. Separate studies showed that maximum concentrations of ATP and creatine phosphate were generated at 2 h. The excised bladders were opened longitudinally, and four full-thickness strips (~2 × 10 mm) were placed in separate organ baths containing 30 mL Tyrode's solution. A force-displacement transducer was connected to the end of each strip, and muscle tension was recorded on a Model 7D Polygraph. The signal was digitized using the analogue–digital Polyview conversion system and recorded on a Pentium computer. The software calculates peak contractile response and maximum rate of tension generation. The balance of the bladder was separated by blunt dissection into mucosa and muscle compartments, frozen under liquid nitrogen, and stored at −80 °C for biochemical analysis.

The results were as follows: Ischemia in the grape-fed rabbits had no significant effect on the contractile responses to field stimulation, ATP, carbachol, or KCl, whereas there were significant decreases in the contractile responses to all forms of stimulation except KCl. The contractile response to KCl was significantly higher in the grape-fed rabbits than either the control (nonischemic) or ischemic vehicle-fed rabbits (Fig. 6).

Ischemia followed by reperfusion resulted in significant decreases in the responses to field stimulation for both vehicle- and grape-fed rabbits, although there was a significantly greater decrease in the vehicle-fed rabbits than in the grape-fed rabbits. Ischemia followed by reperfusion had no effect on the contractile

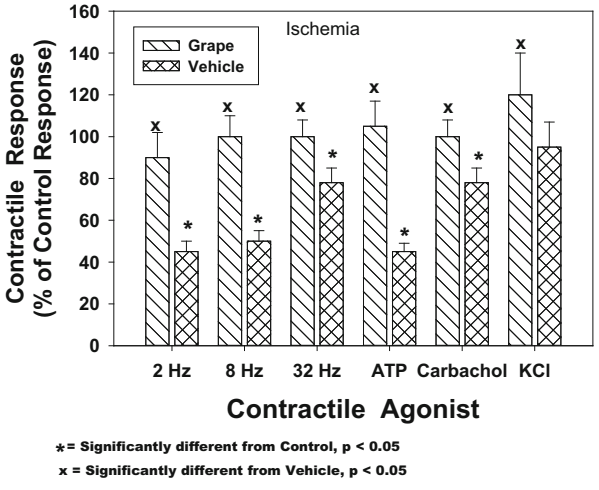


Fig. 6 The effect of ischemia on the contractile responses to a variety of agonists. Each *bar* is the mean ± SEM for four individual rabbits

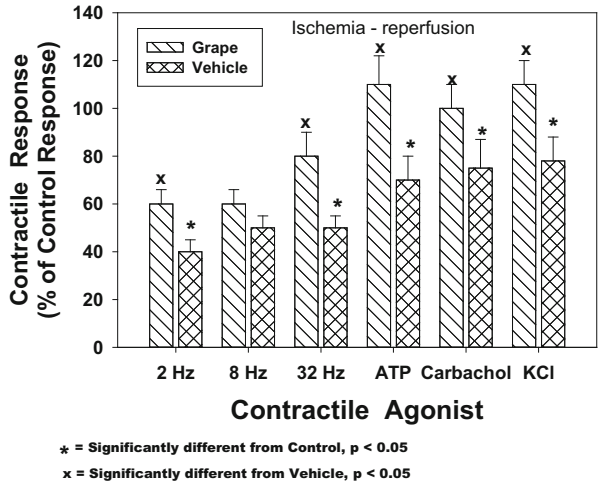


Fig. 7 The effect of ischemia/reperfusion on the contractile responses to a variety of agonists. Each *bar* is the mean ± SEM for four individual rabbits

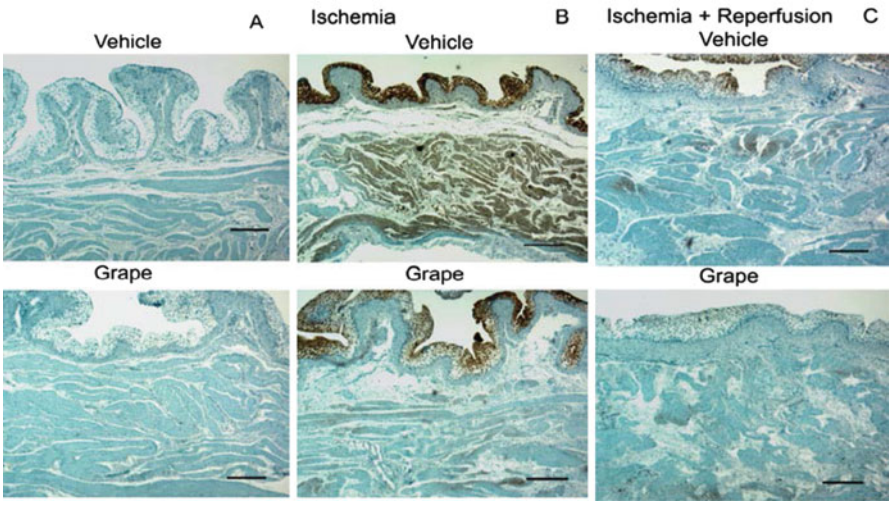


Fig. 8 Representative cross sections of control bladders and bladders following in vivo ischemia and ischemia followed by reperfusion on hypoxyprobe staining for hypoxia. The *dark areas* represent hypoxic areas in the bladder muscle and mucosa; the darker the staining, the greater the level of hypoxia

responses to ATP, carbachol, or KCl, in the grape-fed rabbits, whereas there were significant decreases in the responses to these three agents in the vehicle-fed rabbits (Fig. 7).

Figure 8 displays the immunohistochology of hypoxyprobe for the control (a), ischemic (b), and ischemia/reperfusion (c). Hypoxyprobe-1 is a chemical that binds

only to proteins that are hypoxic and not to proteins that are in a normally oxygenated state. As can be seen, the control vehicle and grape tissues show no staining in the mucosa, submucosa, or muscle. In the ischemia vehicle, there is heavy staining to all tissue layers: the mucosa, submucosa, and muscle. The ischemic grape tissues only showed moderate staining in the mucosa and only light focal staining within the submucosa and muscle. The vehicle ischemia/reperfusion tissues showed moderate staining in the mucosa and focal staining in the submucosa and muscle, whereas the grape ischemia/reperfusion tissues showed virtually no staining.

The frozen tissues were analyzed for the activities of superoxide dismutase (SOD) and catalase (CAT), two naturally occurring antioxidant mechanisms found in virtually all cells (Spettel et al. 2013). The standardized grape suspension upregulated both SOD and CAT activities of the bladder muscle and mucosa in control animals. There were few differences in the grape suspension-treated animals after ischemia, and in general the activities decreased following I/R.

15 Conclusions

Increases of SOD and CAT activity in control animals as a result of grape suspension suggest a greater antioxidant capacity. This increase in the antioxidant defense system may explain the increased protection of grape suspension in the face of ischemia and I/R. However, the activities of both enzyme systems decreased in the smooth muscle subjected to I/R showing that reperfusion damages these systems probably via oxidation damage to the enzymes themselves.

In conclusion, this study clearly demonstrated that ingestion of a standardized grape suspension protected the bladder from ischemia and ischemia/reperfusion injury, at the cellular, biochemical, and physiological levels. It is believed that grape products included in the diet might reduce the progression of ischemic bladder dysfunction, e.g., obstructive bladder dysfunction in men with BPH, women with incontinence, and bladder dysfunction in ageing men and women.

16 Comparison of Grapes and Pure Resveratrol on the In Vitro Response of Biomarkers to Hydrogen Peroxide

As mentioned previously, it is known that grape products contain a variety of antioxidant and membrane-protective compounds including resveratrol, quercetin, procyanidins, flavonoids, phenolics, and others (Agartan et al. 2004). Many investigators believe that resveratrol is the primary active ingredient responsible for antioxidant properties of the grape (Hung et al. 2000).

A number of our studies have shown that the mitochondria are one of the most sensitive subcellular organelles to develop oxidative stress and free radical damage from partial outlet obstruction and bilateral I/R (Levin et al. 2000; Gosling et al. 2000; Levin and Hudson 2004; Nevel-McGarvey et al. 1999). Citrate synthase, an enzyme that exists in nearly all living cells, is commonly used as a quantitative marker for the activity of intact mitochondria (Haugaard et al. 1992; Makeeva et al. 2008). It is involved in the first step of the citric acid cycle (or Krebs cycle) which is a series of chemical reactions used by all aerobic organisms to generate energy through the oxidization of acetate derived from carbohydrates, fats, and proteins into carbon dioxide. In addition, the cycle provides precursors including certain amino acids as well as the reducing agent that is used in numerous biochemical reactions. The key role of citrate synthase in the citric acid cycle is catalysis of the condensation reaction with acetyl coenzyme A (acetyl-CoA) and oxaloacetate. The reaction produces CoA along with citrate, both of which are used substantially throughout the cycle (Remington 1992; Wiegand and Remington 1986).

In our most current study, our objective was to determine the comparative effect of the grape suspension versus pure resveratrol on (a) the contractile response of isolated bladder strips to a field stimulation, and (b) citrate synthase activity to the oxidative effects of H_2O_2. The contractile studies are important because field stimulation requires the release of acetylcholine, and synaptic function has been demonstrated to be a major target for oxidative damage as is mitochondrial function (Kato et al. 1990; Levin et al. 1990; Liu et al. 2009; Lu et al. 2000a, b).

Three male New Zealand white rabbits were anesthetized with pentobarbital (25 mg/kg), and the bladder was exposed through a midline incision. Each bladder was then removed and sectioned between body and base at the level of the ureteral orifices. The bladder was opened longitudinally, and six full-thickness isolated strips were taken (1×0.3 mm) and mounted in individual baths containing oxygenated Tyrode's solution (15 mL) at 37 °C for contractile studies. Two of the six strips from each bladder were incubated in the presence of 1 mg/mL grape suspension for 30 min, two strips were incubated in the presence of 1 mg/mL resveratrol solution, and the last two strips were incubated in the presence of 1 mg/mL sugar composed of equal parts sucrose and fructose and used as the control. The balance of the bladder was separated by blunt dissection into muscle and mucosal compartments, and each compartment is frozen in liquid nitrogen and stored at −80 °C for biochemical evaluation.

The CUPRAC assay was utilized to determine the total antioxidant capacity of the grape and resveratrol solutions. This assay relies on the electron donating capabilities of antioxidants to reduce the copper ion. The CUPRAC working solution consisted of 10 mM copper (II) chloride dihydrate, 1 M ammonium acetate, and 7.5 mM neocuproine; 0.15 mL of the above three solutions were added to 0.15 mL of each sample and allowed to react for 30 min at room temperature, after which the absorbance was read at 450 nm in a Hitachi U-2001 spectrophotometer.

Contractile Studies (Hsu et al. 1994)
Each isolated bladder strip was allowed to equilibrate for 30 min at 37 °C in an oxygenated Tyrode's bath. Passive tension (2 g) was placed on each strip, and they were equilibrated for an additional 30 min. Preliminary studies demonstrated that at 2 g passive tension, maximal active tension is generated. Each strip was then stimulated by field stimulation (FS) at 2, 8, and 32 Hz; 1 ms; and 80 V. H_2O_2 was added to each 15 mL bath to give a final concentration of 0.1 %. The strips were incubated for 10 min, and a second set of stimulations were performed. This procedure was repeated at 0.2, 0.4, and 0.8 % H_2O_2. Both the maximal contractile response and the maximal rate of tension generation were recorded for each stimulation. No pharmacological or chemical contractile agents were utilized because of the need to wash between additions and the re-addition of the H_2O_2. With the FS only, there was no need to wash between increasing concentrations of H_2O_2.

Citrate Synthase Studies (Haugaard et al. 1992)
Samples of muscle and mucosa were homogenized in 0.05 M Tris buffer (100 mg/mL). Samples were then spun at 2500 rpm for 10 min; 0.9 mL of supernatant plus 0.1 mL of Triton X-100 were combined in a test tube for each sample. Samples (40 μL) were added to ten 0.5 cm cuvettes, along with 1.1 mL 0.05 M Tris buffer (pH 7.6), 30 μL 24.6 mM acetyl coenzyme A, and 100 μL 1 mM 5.5′-dithiobis-2-nitrobenzoic acid (DTNB). Grape powder (10 mg/mL) was added to all cuvettes to give a final concentration of 1 mg/mL. The final volume in each cuvette was 1400 mL excluding the 50 μL oxaloacetate (10 mM substrate) used to start the reaction. Before the oxaloacetate was added, 1.4 μL of the mixtures in cuvettes 1 and 2 are removed, and 1.4 μL of Tris buffer is added to serve as controls. Similarly, for the remainder of the cuvettes, 1.4 μL of the mixtures were removed, and 1.4 μL of H_2O_2 was added to give final concentrations of 0.005, 0.01, 0.02, and 0.04 % H_2O_2. A pair of cuvettes contained no sample served as additional controls. A magnetic stir bar was placed in each cuvette to mix the chemicals, and after 1 min, 50 μL of 10 mM oxaloacetate was added to each cuvette. The activity was read every 2 min for 30 min in a Hitachi U-2001 spectrophotometer, while the free coenzyme A generated by citrate synthase activity reacts with DTNB to form a colored compound that is quantified at 412 nm. This experiment was repeated with resveratrol at a final concentration of 1 mg/mL.

The results were as follows. Figure 9 presents a comparison of the total antioxidant capacity of resveratrol and the grape suspension both at 1 mg/mL. Chemically, resveratrol has about 100 times the antioxidant capacity of the grape suspension.

In the contractile studies, the results showed that increasing the concentration of H_2O_2 resulted in a progressive and significant decrease in the maximal contractile responses at all frequencies of stimulation. Neither the grape suspension nor resveratrol had any effect on the maximal contractile responses in the presence or absence of H_2O_2. However, the grape suspension had significant protective effects when the rate of tension was quantitated at all concentrations of H_2O_2 (Fig. 10).

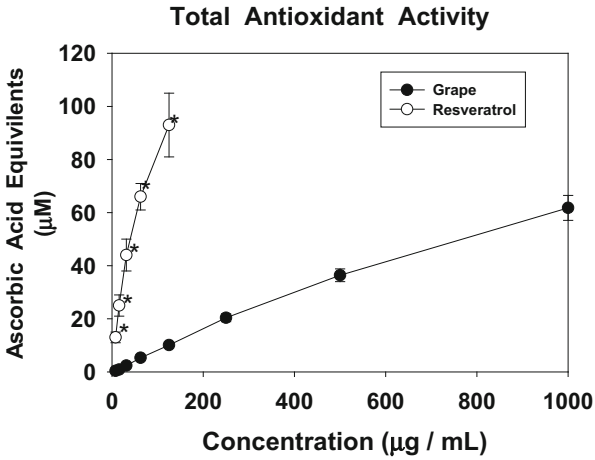


Fig. 9 The total antioxidant activity of resveratrol and the grape suspension. Each *point* represents the mean ± SEM of five independent curves. *Asterisk* significantly different from grape, $p < 0.05$

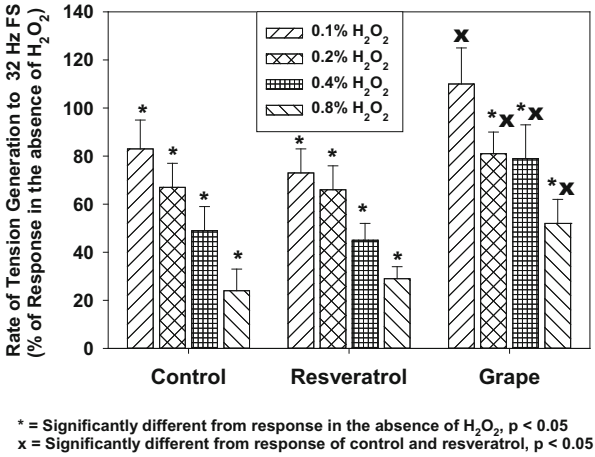


Fig. 10 The rate of tension generation to 32 Hz field stimulation in the presence and absence of hydrogen peroxide (0.1 – 0.8 % H_2O_2). Each *bar* is the mean ± SEM of three different pieces of bladder body from three different rabbits

Resveratrol had no protective effect. We show only the response to 32 Hz; the responses to 2 and 8 Hz were similar.

When comparing the citrate synthase activities of rabbit bladder muscle and mucosa, it was found that the activity of the citrate synthase is significantly higher in the mucosa than in the muscle (Francis et al. 2014). Neither the grape suspension nor the resveratrol had any effect on citrate synthase activity in the absence of H_2O_2. The citrate synthase activity of the muscle was protected by the grape suspension and not by the resveratrol (Fig. 11). The response of the mucosa to H_2O_2 was similar except that resveratrol was protective only at the 0.01 % H_2O_2.

Although not part of this published study, we found the enzyme choline acetyltransferase (Chat), the enzyme that synthesizes acetylcholine in nerve

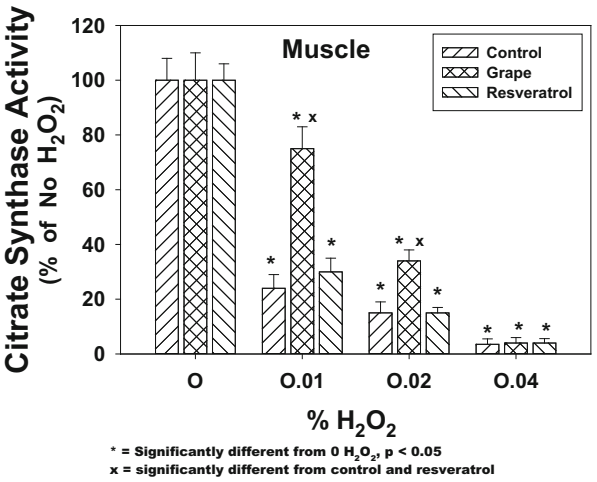


Fig. 11 Citrate synthase activity in the presence and absence of hydrogen peroxide (0.01 – 0.04 % H_2O_2). Each *bar* is the mean ± SEM of three different pieces of bladder body from three different rabbits

synapses, to be very sensitive to H_2O_2, and this was significantly protected from oxidative damage by the grape suspension but not by resveratrol.

It should be remembered that these were in vitro experiments where the tissues were exposed to either the grape suspension or the resveratrol, and solubility and permeability may have been a problem with resveratrol.

Our conclusions from these experiments can be summarized as follows: (1) Oral administration of a standardized grape suspension equal to 3–8 oz glasses of mixed grape juice per day can significantly protect the urinary bladder from obstructive and ischemic damage (Agartan et al. 2004; Lin et al. 2005b, 2007b). (2) Partial outlet obstruction secondary to BPH is the most common dysfunction men over 50 develop (Steers 1992; Zderic et al. 1996a). It can result in bladder smooth muscle hypertrophy, mucosal hyperplasia, nocturia, incomplete emptying, reduced intravesical pressure, and significantly decreased compliance (Barry and Meigs 2000). (3) The progression from compliant bladder to severe obstructive bladder dysfunction is significantly reduced in both severity and progressive dysfunction by oral administration of the grape suspension (Agartan et al. 2004). (4) The etiology of obstructive bladder dysfunction includes ischemia, reperfusion, and the generation of free radicals and oxidative stress (Callaghan et al. 2013c; Levin et al. 1997; Mannikarottu et al. 2005; Greenland and Brading 2001; Greenland et al. 2000; Brading 1997; Dixon et al. 1989). (5) Using an in vivo model of ischemia and ischemia followed by reperfusion, we clearly demonstrated that oral ingestion of the grape suspension protected the bladder from the physiological, biochemical, and morphological dysfunctions mediated by ischemia and ischemia followed by reperfusion (Lin et al. 2005b, 2007b). (6) Ischemia, ischemia followed by reperfusion, and oxygen stress are also directly involved in the etiology of several bladder dysfunctions in women including interstitial cystitis and incontinence (Levin et al. 1996b; Palmieri et al. 2007; Rehfuss et al. 2010). Although we did not test the effects of oral ingestion of the grape suspension on female rabbit models of these bladder dysfunctions, based on the studies published, we would expect that

the grape suspension would be effective in reducing the level and progression of these two models of female bladder dysfunction.

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Grapes and Vision

Ayelen Bulloj and Silvia C. Finnemann

Contents

Abstract Cataract, glaucoma, and age-related macular degeneration are frequent causes of blindness worldwide particularly in the elderly population. Continuous exposure to deleterious chemical agents in addition to an inadequate diet enhances oxidative stress in several tissues of the human eye and contributes to these and other ocular dysfunctions. Grapes contain a wide variety of phytochemicals that can function as cellular antioxidants and anti-inflammatory agents. Increased dietary intake of grapes or grape compounds may thus be effective in preventing or delaying the progression of these eye diseases. In this review, we focus on recent experimental, clinical, and epidemiological studies that support such beneficial properties of grape components on vision.

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1 The Anatomy of the Human Eye

Much of the surface of the eyeball consists of fibrous tissue termed the sclera, which corresponds to the white outer part of the eye that surrounds the iris. The sclera is continuous with the cornea, which is the transparent external surface that covers the pupil and the iris and that together with the lens focuses light on the retina. Just within the sclera lies the choroid, a layer of connective tissue rich in blood vessels. Finally, the retina overlies the choroid outlining the inner surface of the posterior eye (Wade 2007).

Light enters the eye through the pupil aperture on the front of the iris; it is focused by the cornea and the lens and then traverses the gelatinous body termed the vitreous humor that fills the eye cavity. The retina senses the light and converts it into electrochemical signals that are processed and ultimately transmitted to the brain via the optic nerve for further processing necessary for perception (O'Brien 1982).

The retina is organized in layers comprised of different cell types. Synapses are primarily found in the outer plexiform layer where photoreceptors make synaptic contacts with bipolar and horizontal cells, and in the thicker inner plexiform layer where bipolar cells, and amacrine cells make synaptic contacts with ganglion output cells.

The human retina contains two types of photoreceptors, rods and cones. Rods are extremely sensitive to light; they mediate night vision and they are achromatic. In contrast, cones are responsible for bright (day) light and color vision. Moderate loss of rods leads mainly to night vision blindness in human patients, but individuals with excessive rod deficiency or with significantly impaired cone functionality suffer severe loss of vision and are often legally blind. The fovea is the region of highest visual acuity located in the center of the human retina. This region almost exclusively host cones; it has the highest ratio of ganglion cells to photoreceptors; the cell bodies of the proximal retinal neurons are shifted peripherally enabling the foveal photoreceptors to receive light unobstructed (Kuffler 1953). The fovea is the center of the macula, a specialized retinal region characterized by its distinctive yellow pigmentation due to high concentration of xanthophyll carotenoids lutein and zeaxanthin. Macular pigment is thought to act as a short wavelength light filter in addition to the filtering provided by the lens. To ensure lifelong functionality of foveal photoreceptors for human daylight vision, such protective mechanisms are essential shields from excess blue light and ultraviolet irradiation damage.

The retinal pigment epithelium (RPE) forms the outermost layer of the retina. RPE cells form a pigmented monolayer with their apical membranes facing photoreceptors and their basolateral membranes facing the choroid. The RPE performs numerous functions in support of photoreceptors specifically and the neural retina in general (Ruggiero and Finnemann 2014). These include light scavenging, establishment and maintenance of the blood-retinal barrier, and directed transport of ions, water, and metabolic products from the subretinal space to the blood and of nutrients from the blood for delivery to photoreceptors (Hosoya and Tachikawa

2012). In addition, the RPE performs critical roles in support of photoreceptor maintenance with its contributions to the visual cycle and the continuous renewal of photoreceptor outer segments (Bibb and Young 1974; Bok 1993). Any disturbance of these functions could lead to accumulation of toxic components exacerbating RPE oxidative damage and inducing degeneration of the retina, loss of visual function, and blindness. Like retinal neurons, RPE cells are postmitotic in the human eye. They therefore acquire gradually increasing damage and dysfunction with age in response to even low-level environmental insults over time.

2 Cellular Production and Dissipation of Reactive Oxygen Species

Reactive oxygen species (ROS) are constantly formed in any cell type that utilizes molecular oxygen in aerobic metabolism. While mitochondrial respiration converts most oxygen to water, a small amount of oxygen radicals escapes the electron transport yielding superoxide radicals. Superoxide dismutase (SOD) catalyzes the formation of moderately reactive hydrogen peroxide from the radicals derived from this so-called electron leak. When hydrogen peroxide interacts with ions of transition metals such as iron and copper, hydroxyl radicals form, which are highly active ROS (Wink et al. 1994). Moderate levels of ROS are necessary for normal cell functionality, e.g., ROS act as short-lived signaling molecules in pathways maintaining cellular homeostasis. ROS may regulate many metabolic and cellular processes including proliferation, migration, gene expression, immunity, and wound healing (D'Autreaux and Toledano 2007). However, excess levels of ROS are cytotoxic. All cells thus possess efficient antioxidant mechanisms that can dissipate excess ROS.

2.1 Cellular Antioxidant Mechanisms

In all human cell types, antioxidant mechanisms are either accomplished by endogenous or exogenous antioxidants, which are acquired from dietary sources.

Endogenous antioxidants can be categorized into primary and secondary antioxidants. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) are the primary antioxidant enzymes which inactivate the ROS once formed. SODs are metalloproteins that accelerate the dismutation of superoxide to hydrogen peroxide. There are two main types of SOD in human tissues: cytosolic SOD1, which contains copper and zinc, and mitochondrial SOD2, which contains manganese. CAT is located in peroxisomes and mitochondria, and its function is to catalyze the dismutation of hydrogen peroxide to water and molecular oxygen. GSH-Px is located in the cytosol and in mitochondria. Its role is to eliminate

hydroperoxides by transforming them into water. These enzymes also require cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity. It has been suggested that an inadequate dietary intake of these trace minerals may compromise the effectiveness of these antioxidant defense mechanisms (Carocho and Ferreira 2013; Rahman 2007). Secondary antioxidant enzymes are glutathione reductase, glucose 6-phosphate dehydrogenase, glutathione *S*-transferase, and ubiquinone. These provide NADPH and glutathione for use by primary antioxidant enzymes.

Exogenous antioxidants are mainly derived from food and other dietary sources. Physiologically relevant examples are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, and catechins. They may be water soluble and cytosolic, such as vitamin C, or liposoluble located in the membrane like vitamin E and carotenoids (Birben et al. 2012; Rahman 2007).

3 Cellular Oxidative Stress in the Eye

Cells experience oxidative stress when the level of prooxidants exceeds the level of antioxidants resulting in oxidation of cellular components and consequent loss of cellular functions.

The eye is continuously exposed to different damaging agents including ionizing radiation, visible light, and high concentration of oxygen and environment chemicals from pollutants, industrial smoke, tobacco, and highway driving fumes. This harmful environment strongly promotes the generation of ROS at a level exceeding the dissipation capacity of many ocular cells. These excess ROS may react with lipids, proteins, and nucleic acids ultimately leading to dysfunction of ocular tissues (Beatty et al. 2000; Ohia et al. 2005). Of particular relevance to the eye, mitochondria from postmitotic retinal cells exposed to visible light produce ROS through interactions with mitochondrial photosensitizers like cytochrome c oxidase, which results in increasing damage to mitochondrial DNA (mtDNA) with age (Chen et al. 1992; Godley et al. 2005). Furthermore, specific tissues in the eye can also generate significant levels of ROS from non-mitochondrial sources. NADPH oxidase is considered to be a major source of superoxide in microvascular endothelial cells. These ROS can also contribute to exogenous oxidative damage of the mitochondria enhancing mitochondrial dysfunction and ocular disease (Newsholme et al. 2007; Whiteside 2005). In addition to photooxidation, it has been proposed that RPE cells may be subjected to excess oxidative stress due to their daily phagocytosis of photoreceptor outer segments, which may yield hydrogen peroxide as product of phagosomal NADPH oxidase activity or of peroxisomal β-oxidation of phagocytosed lipids (Miceli et al. 1994; Tate et al. 1995). The physiological relevance of these reactions for the functionality and oxidative damage of the RPE in vivo remains to be studied.

In contrast, clinical and experimental studies agree that incomplete digestion of phagocytosed photoreceptor compounds yields formation and accumulation of

lysosomal lipofuscin in RPE cells (Sparrow et al. 2012). Lipofuscin in the RPE is a complex mix of oxidized, indigestible compounds rich in oxidized lipids and retinoid derivatives (Jung et al. 2007; Ng et al. 2008; Sparrow and Yamamoto 2012). Lipofuscin accumulates in human RPE cells with age where it acts as potent photoinducible generator of ROS.

4 Ocular Pathologies Related to Oxidative Stress

4.1 Dry-Eye Disorder

Dry-eye disorder (DED) develops when tears are not able to provide adequate moisture to the anterior surface of the eye. It can be caused by insufficient tear production or abnormal tear composition. DED causes significant pain and discomfort. Yet, no cure exists for DED, and management is highly unsatisfactory for many patients. DED has high prevalence rates worldwide and is especially common among patients older than 65 years of age (Munoz et al. 2000; Perry 2008). It is more common in women than in men, with an estimated 3.23 million US American women afflicted today (Moss et al. 2008; Schaumberg et al. 2003). A significant increase in oxidative reactivity and a decrease in antioxidant defenses in ocular fluids and tissues have been associated with DED (Cejkova and Cejka 2015). Evidence from experimental animals suggests a causal link between increased oxidative stress and abnormal tear production: increasing activity in a conditional transgenic mice of the gene *mev-1*, which encodes the large subunit of cytochrome b_{560} of the succinate-ubiquinone oxidoreductase in mitochondrial complex II, yields excess oxidative stress that lowers tear volume and specifically damages ocular surface epithelium (Higuchi et al. 2010; Uchino et al. 2012).

4.2 Cataract

In an eye with a cataract, the lens becomes cloudy due to abnormal pigment deposition and formation of non-transparent protein aggregates. Cataracts prevent the light from being focused on the retina generating blurred or double vision, sensitivity to light and glare, as well as fading or yellowing of colors (Fig. 1).

Much of cataract formation in the developed world is related to impaired protein homeostasis with age. Oxidative stress plays a significant role in age-related cataract formation and is a major factor in formation of cataracts in response to environmental insult (Brennan and Kantorow 2009; Lou 2003). Laboratory studies have shown that human lens cells are highly susceptible to oxidative stress and that antioxidant activity of lens cells is inversely proportional to cataract severity (Spector 1995). Significantly, elevated oxidation of proteins, lipids, and DNA has

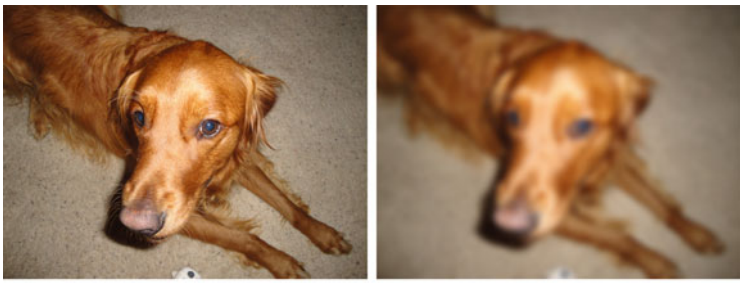

Fig. 1 Vision affected by cataracts is characterized by clouded, blurred, or dim vision

been detected in lens samples with cataracts (Hightower et al. 1999; Reddan et al. 1999; Yao et al. 2009). Proteins from cataractous lenses often lose sulfhydryl groups, have oxidized residues, and generate insoluble aggregates (Lou 2003). Altogether, the link between oxidative stress and cataract formation is strong.

4.3 Glaucoma

Glaucoma is the second leading cause of blindness worldwide. It is characterized by progressive optic neuropathy that is often but not always caused by elevated intraocular pressure. Much evidence suggests that elevated oxidative stress contributes to the etiology of at least some forms of glaucoma. For instance, oxidative damage is found in DNA in the trabecular meshwork from glaucoma patients, and there is a significant correlation between oxidized DNA and intraocular pressure (Sacca and Izzotti 2008). A subset of glaucoma patients may be genetically more susceptible to oxidative damage as deletion of the gene encoding for antioxidant GSH *S*-transferase is associated with glaucoma (Sacca and Izzotti 2008). Finally, experimentally increasing intraocular pressure can induce oxidative stress in the retina (Kong et al. 2009; Simpkins et al. 2005; Tezel 2006). However, the molecular and cellular mechanisms linking initial causes to oxidative stress and pathology in glaucoma remain to be unraveled.

4.4 Retinal Diseases

Compared to other ocular tissues, the retina is especially vulnerable to ROS damage. Retinal neurons contain higher concentrations of polyunsaturated fatty acids (PUFAs) than other cell types, making the retina highly susceptible to lipid oxidation (Doly et al. 1992). Moreover, photoreceptors and RPE cells are more sensitive to oxidative damage because they are nonproliferative postmitotic cells

and they lack of DNA damage detection systems. The RPE is also exposed to an extra source of oxidative stress coming from the formation of ROS during phagocytosis of shed photoreceptor outer segments (Cai et al. 2000).

4.4.1 Diabetic Retinopathy

Diabetic retinopathy is the most common diabetic eye disease and one of the leading causes of blindness in developed countries (Cheung et al. 2010). The molecular and cellular mechanisms causing diabetic retinopathy are complex and a subject of intense investigation. Uncontrolled hyperglycemia over time causes abnormalities in the inner retinal vasculature, with both existing and newly forming blood vessels leaking fluid generating blurred vision which may progress quickly to severe vision impairment (Fig. 2).

These complications of persistent hyperglycemia are increased by excess generation of ROS and nitrogen species (Arden and Sivaprasad 2011). Mitochondrial dysfunction has been shown to play an important role in the disease. Experimental mouse models of diabetes show increased superoxide levels in retinal mitochondria, decreased activity of mitochondrial complex III, and higher mitochondrial membrane permeability (Kanwar et al. 2007). Membrane lipid peroxidation and oxidative damage to mtDNA are elevated in the retina in diabetes (Kowluru and Chan 2007). Finally, endoplasmic reticulum (ER) stress is associated with diabetic retinopathy and other progressive retinal diseases, and it can be exacerbated by oxidative stress (Ma et al. 2014).

Taken together, clinical and experimental data agree that oxidative stress, while not an initial cause, is an important contributor to progression of diabetic retinopathy.

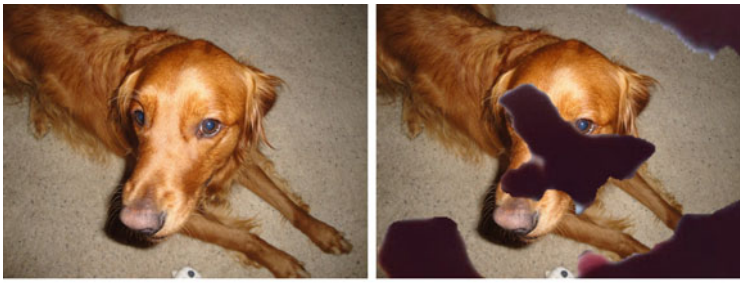

Fig. 2 Diabetic retinopathy is characterized by blurred vision, dark spots of flashing lights, or rings around lights

Fig. 3 AMD vision is characterized by center vision distortion, dark blurry areas, and diminished or changed color perception

4.4.2 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a disease that mostly affects people above the age of 60 (Gehrs et al. 2006). AMD is the leading cause of vision loss among the elderly (Congdon et al. 2004). At present, 1.75 million people in the United States have advanced AMD. Initial symptoms of AMD include loss of central visual acuity and a subjective impression of the curvature of straight lines. At later stages of the disease, a central scotoma forms and continues to grow eventually severely impairing vision (Fig. 3).

Late-stage AMD is classified into two forms, atrophic AMD often called dry AMD and neovascular AMD often called wet AMD. In neovascular AMD, abnormal new vessels grow in the choroid beneath the RPE and retina and especially its macula. Choroidal neovascularization is characterized by leaky vessels and penetrance of vessels through the RPE and into the retina. It causes rapid and severe vision loss. In the dry form, gradual vision impairment follows atrophy of patches of macular RPE followed by dysfunction and death of overlying photoreceptors deprived of RPE support. AMD is a complex disease that remains poorly understood and that is influenced significantly by many genetic and nongenetic or environmental factors. It is generally accepted that the primary cell type of injury is the RPE. The characteristic sign of early AMD before other severe form develops is the presence of large, soft drusen beneath macular RPE, and/or subretinal drusenoid deposits above the RPE and progressive RPE degeneration (Del Priore et al. 2002; Dunaief et al. 2002; Oak et al. 2014; Owsley et al. 2016). To date, aging and smoking remain the strongest nongenetic risk factors (Heiba et al. 1994; Vingerling et al. 1995). Other nongenetic risk factors that have been reported to affect risk for AMD or AMD progression include diet, higher body mass index, serum cholesterol, cataract surgery, cardiovascular disease, hypertension, and sunlight exposure (Clemons et al. 2005; Heiba et al. 1994; Vingerling et al. 1995).

An increase in oxidative burden of the RPE due to a reduction in antioxidative protective mechanisms or an increase in number and concentration of active photooxidative ROS have been linked to the pathogenesis of AMD (Boulton and Dayhaw-Barker 2001; Boulton et al. 1994). Many clinical studies and animal models support that mitochondrial genomic dysfunction of the RPE may be involved in AMD. With age, there is a significant decrease in the number of mitochondria in human RPE, and these age-related changes are significant higher in the eyes of AMD patients (Feher et al. 2006). Prevalence of AMD correlates with specific mtDNA haplogroups suggesting that the bioenergetics consequences of mtDNA modifications may contribute to the development of AMD (Jones et al. 2007; SanGiovanni et al. 2009; Udar et al. 2009). Increased mtDNA deletions have been documented in aged human and rodent retina (Wang et al. 2008), and increased mtDNA damage and decreased repair are associated with aging and AMD (Liang and Godley 2003). Finally, changes in select redox proteins and proteins involved in mitochondrial trafficking and a decrease in RPE mitochondrial respiration also correlate with AMD progression (Decanini et al. 2007; Nordgaard et al. 2006; Nordgaard et al. 2008).

Besides mitochondria, other organelles change with age and with oxidative stress in the RPE. With aging, there is a reduction of melanosomes accompanied by an increase of lipofuscin granules with its toxic compounds that are both a result of and a cause of increased oxidative stress (Feeney-Burns et al. 1984; Feeney 1978; Schmidt and Peisch 1986; Weiter et al. 1986). Both increased ROS and cytotoxic lipofuscin components destabilize internal cellular compartment such as lysosomes and mitochondria. Finally, this is accompanied by decreasing antioxidant defense mechanisms of the aging RPE such as decreased levels of vitamin E (Friedrichson et al. 1995).

Altogether, AMD is accompanied by excess oxidative stress and oxidative damage to RPE cells. While unlikely to be a sole or primary trigger, increased oxidative stress likely contributes to AMD progression or severity.

5 Grapes and Their Benefits in Vision

Grapes contain a large numbers of nutrients that have been associated with antioxidant or other health benefits. They are summarized below in Table 1.

Table 1 Categorized nutrients in grapes

Stilbenes	Flavanols	Flavonols	Phenolic acids	Carotenoids
Resveratrol	Catechins	Quercetin	Caffeic acid	β-Carotene
Piceatannol	Epicatechins	Kaempferol	Coumaric acid	Lutein
Pterostilbene	Procyanidins	Myricetin	Ferulic acid	Zeaxanthin
	Proanthocyanidins	Isorhamnetin	Gallic acid	
	Viniferones			

5.1 *Effects of Grape or Grape Extracts on Eye Health*

There are no clinical studies testing efficacy of grape consumption on onset, progression, or outcome of human eye disease or visual function. However, three laboratory investigations have recently been published that directly test effects of grape intake on ocular health.

Of relevance to aging of the human retina and atrophic AMD, effects of grape consumption on disease onset and progression were tested in a mouse model of age-related blindness that shares important aspects with atrophic AMD. Mice lacking the integrin phagocytosis receptor αvβ5 due to disruption of the *ITGB5* gene ($\beta5^{-/-}$ mice) show a constitutive impairment in diurnal clearance phagocytosis by RPE cells of spent photoreceptor outer segment fragments. In $\beta5^{-/-}$ mice, outer segment renewal is dysregulated such that basal levels of outer segment shedding and phagocytosis take place at all times of day as compared to a phagocytic burst that takes place only at light onset in wild-type mice (Ruggiero et al. 2012). Lack of phagocytosis rhythm in $\beta5^{-/-}$ mice results in phagolysosomal inefficiency and accumulation of lipofuscin and oxidative damage specifically of the F-actin cytoskeleton between 6 and 12 months of age (Nandrot et al. 2004; Yu et al. 2012). Visual function gradually declines between 6 and 12 months of age due to increasing dysfunction of photoreceptor cells (Nandrot and Finnemann 2008). Strikingly, supplementing the regular diet of $\beta5^{-/-}$ mice with grapes throughout adulthood (from 3 to 12 months of age) largely prevents lipofuscin accumulation, oxidative protein damage, and vision loss (Yu et al. 2012). Mice received food pellets enriched in freeze-dried grapes ad libitum leading to a human consumption rate equivalent of 3.5 servings of grapes per day. Notably, grape consumption did not rescue the primary phagocytic defect of $\beta5^{-/-}$ mice, and vision of wild-type mice did not improve above normal if mice consumed grapes. Moreover, grape consumption in early adulthood (only from 3 to 6 or from 6 to 9 months of age) was effective in maintaining vision until the high age of 12 months, i.e., the end point of the study (Fig. 4). In contrast, consuming grapes only from 9 to 12 months of age had no effect on progression of vision loss.

Of relevance to neovascular AMD, wild-type mice receiving drinking water ad libitum supplemented with dissolved grape powder for 12 days prior to laser injury of the posterior retina and disruption of the RPE's basement membrane, which is commonly used to acutely stimulate choroidal neovascularization in experimental animals. Grape supplementation was equally effective as resveratrol in this experiment, and both treatments significantly reduced the severity of abnormal vessel growth (Kanavi et al. 2014). Changing the mode treatment from ad libitum supplementation to a once-a-day gavage eliminated this beneficial effect. Reasons for this discrepancy were not established in this study. It is conceivable that continuous low-dose oral consumption of grapes in solution or mixed with food [as by Yu et al. (2012)] supplies phytonutrient more efficiently than period high doses as supplied here and as also provided by once-a-day nutritional supplements in tablet format.

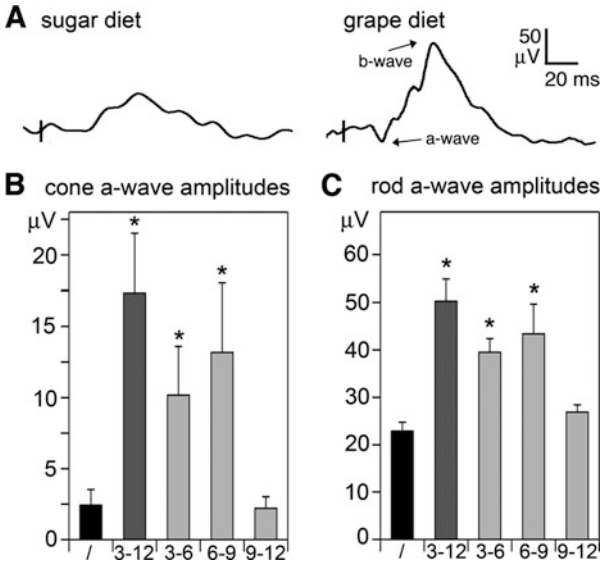

Fig. 4 Grape-enriched diet consumed throughout adulthood (3–12 months of age) or only at young or middle age (3–6 months of age or 6–9 months of age) but not only at old age (9–12 months of age) preserves cone and rod photoreceptor function in aging $\beta5^{-/-}$ mice. (**a**) shows example of cone light responses in control mice fed sugar control diet or in mice fed grape diet from 3 to 12 months of age, (**b**) and (**c**) show mean cone- and rod-derived responses, respectively. For details, see Yu et al. (2012). © Yu et al. 2012 *Free Rad Biol Med.* with permission

Of relevance to ocular inflammation, muscadine grape phytochemical extract at a dose of 100–200 mg/kg body weight was provided by gavage for 7 days to wild-type mice, which subsequently received an intravitreal injection of lipopolysaccharide to acutely induce ocular inflammation. Ocular inflammation including upregulation of pro-inflammatory cytokines and leukocyte infiltration as well as retinal tissue damage were largely prevented by pretreatment with grape polyphenols (Ha et al. 2014). In the same study, tests on the ARPE-19 human RPE cell line in culture suggested that incubation with grape polyphenols prevented chemically induced ER stress and increase in transcription of genes for pro-inflammatory cytokines in response to TNF-α.

We are aware of only the three studies discussed above to date that have directly tested the efficacy of consumption of grapes or of complex grape polyphenols in preventing or altering progression of ocular disease. However, other studies have been performed testing select-specific components also found at physiologically relevant concentration in grapes. It is tempting to speculate that grape consumption or administration of grape extracts will likely have similar or even higher beneficial effects on ocular cells and vision as determined for individual grape compounds. The best-studied bioactive compounds also found in grapes are quercetin, resveratrol, and lutein/zeaxanthin. We will briefly summarize studies reported thus far on their effects on ocular cells and vision below.

5.2 Effects of Quercetin on Eye Health

Quercetin belongs to the flavonoid family. Its beneficial effects are thought to be primarily as direct scavenger of ROS and reactive nitrogen species (Boots et al. 2008; Heijnen et al. 2001; van Acker et al. 1995). However, it is also known to exhibit strong anti-inflammatory effects (Read 1995). Like resveratrol, quercetin in purified form has been tested in models of ocular cells and disease in vitro and in vivo.

With respect to benefits of quercetin on ocular cells in culture, quercetin protects RPE cells in culture from oxidative damage. The ARPE-19 human RPE cell line or primary human RPE cells were treated with either hydrogen peroxide or *t*-butyl hydroperoxide in the presence or absence of quercetin. The accumulation of intracellular reactive oxygen species and cellular death was reduced in the presence of the flavonoid. Moreover, quercetin induced the expression of phase-2 antioxidant genes such as genes for the transcription factor Nrf2 and one of its downstream gene products heme oxygenase 1 (HO-1) that catalyze reactions to neutralize reactive oxygen (Hanneken et al. 2006). In another study using the same human cell line and hydrogen peroxide as source of oxidative stress, quercetin protected RPE cells enhancing the transcript levels of BCL-2 and suppressing the transcription of inflammatory factor such as tumor necrosis factor-α (TNF-α), COX-2, and iNOS. These in vitro effects are not specific to RPE cells. For instance, quercetin affects endothelial cells in culture. When the rhesus choroidal endothelial cell line (RF/6A) was treated with quercetin, cell migration and cell proliferation were reduced in a dose-dependent manner (Chen et al. 2008).

Of relevance to neovascular AMD, daily intraperitoneal injection of quercetin treatment (25 mg/kg) for 2 months was sufficient to recover systemic antioxidant defense capacity, suppress the systemic expression of nitric oxide and COX, and decrease levels of RPE lipofuscin in Ccl2/Cx3cr1 double knockout mice (Cao et al. 2010). These animals develop progressive retinal lesions mimicking AMD (Tuo et al. 2007). However, it had no effect on upregulation of expression of pro-inflammatory or apoptotic pathway genes in the eye (Kook et al. 2008). Altogether, quercetin has potent antioxidant properties when applied to ocular cells. Its in vivo benefits largely remain to be established.

5.3 Effects of Resveratrol on Eye Health

Resveratrol is a polyphenolic antioxidant found in grapes and many of their products. It has cell-protective benefits against inflammation, and it reduces reactive oxidative species with vitro and in vivo.

With respect to benefits of resveratrol on ocular cells in culture, a protective effect of resveratrol was reported in a human RPE cell line (D407) after hydrogen peroxide-induced oxidative stress. Pretreatment with resveratrol induced a

significant, dose-dependent increase in activities of superoxide dismutase, glutathione peroxidase, and catalase. Moreover, resveratrol significantly increased the level of reduced glutathione available under both basal and oxidative stress conditions. Its capacity to inhibit the generation of intracellular ROS supports the hypothesis that resveratrol can contribute to the antioxidant defense by directly scavenging ROS in RPE cells (Pintea et al. 2011).

In addition, emerging evidence with in vivo experiments indicates that resveratrol may combat different ocular pathologies and promote eye health.

Of relevance to cataracts, resveratrol prevents selenite-induced cataract in animal models. Rats were given 40 mg/kg of resveratrol for 4 days. Encapsulated lenses and erythrocytes were analyzed for reduced glutathione (GSH) and malondialdehyde (MDA), a marker of lipid peroxidation and MDA. Resveratrol suppressed selenite-induced oxidative stress and cataract formation in rats. This protective effect was supported by higher GSH and lower MDA in lens and erythrocytes (Doganay et al. 2006).

Of relevance to diabetic retinopathy, resveratrol (injected into the vitreous of mouse eyes) inhibits tunicamycin-induced ER stress and capillary degeneration in vivo (Li et al. 2012). Tunicamycin was injected into the vitreous of mouse eyes, and retinal injury was induced by elevating intraocular pressure followed by reperfusion. This is an important finding as ER stress may contribute to neurodegeneration in multiple ocular diseases (Surguchev and Surguchov 2010). In another study, mice rendered diabetic with streptozotocin injection received 20 mg/kg of resveratrol by oral gavage daily for 4 weeks. Diabetes increased vessel leakage, pericyte loss, and levels of pro-angiogenic VEGF protein in the mouse retina. Strikingly, these changes were effectively blocked by resveratrol treatment (Kim et al. 2012). Another study corroborated these results by showing that resveratrol inhibits angiogenesis and suppresses retinal neovascularization in mice lacking the very low-density lipoprotein receptor (*VLDLR*) (Hua et al. 2011). Diabetic hyperglycemia is associated with activation of nuclear factor-κB (NF-κB) (Ramana et al. 2004; Romeo et al. 2002). Four-month oral resveratrol administration (5 mg/kg per day) was able to reduce the elevated levels of NF-κB and apoptosis in retinas of diabetic rats. Moreover, resveratrol significantly alleviated hyperglycemia, upregulation of oxidative markers, and superoxide dismutase activity in both blood and retina tissue of diabetic rats (Soufi et al. 2012). Finally, it was also reported that inhibition of oxidative damage and NF-κB by resveratrol prevents ocular inflammation in a mouse model of endotoxin-induced uveitis (Kubota et al. 2009).

Of relevance to anterior segment health, resveratrol decreases corneal neovascularization. Mice received in drinking water 0.4 μg/mL resveratrol in 1 % ethanol (equivalent to the amount of resveratrol in approximately three glasses of red wine) or water with 1 % ethanol as control. The angiogenesis effect of FGF-2 and VEGF in corneal neovascularization was significantly reduced in the resveratrol-drinking group as compared with the control (Bråkenhielm et al. 2001).

Altogether, the significance of elevated oxidative stress, neovascularization, inflammatory processes, and cellular apoptosis in different ocular pathologies that

have already been shown to be prevented by resveratrol support the possibility that high natural intake of resveratrol, for instance, through grape consumption may benefit ocular health and vision.

5.4 *Effects of Lutein and Zeaxanthin on Eye Health*

Lutein and its isomer zeaxanthin belong to the xanthophyll family of carotenoids. In biological specimens, they usually occur together, and available in vitro preparations provide both isomers; we will thus refer to them together as L/Z unless they are specifically studied in isolation. In the primate eye, L/Z represents the primary pigments enriched in the macula (Bone et al. 1985). L/Z is not localized only in the macula but also broadly throughout the retina (Rapp et al. 2000). It is found in photoreceptor outer segments and in the RPE. However, L/Z is most abundant in the outer plexiform layer of the retina (Bhosale et al. 2009). One of the protective roles of L/Z is to be an oxygen-free radical scavenger in conditions of oxidative stress (Bone and Landrum 1984; Landrum et al. 2012; Qin et al. 2011).

In addition to and independent of its antioxidant role, L/Z selectively absorbs blue light with a peak of absorption at 446 nm. Light-induced retinal damage differs with wavelength, exposure time, and intensity of light. For instance, the blue light requires 100 times less intensity to cause damage than orange light. The presence of L/Z capable of absorbing light of the blue range wavelength strongly suggests that they play a light-protective role in the macula. L/Z in particular acts as photoprotective agent effectively filtering the blue light reducing excessive damage of photoreceptors (Nilsson et al. 2003).

L/Z has been widely studied, and numerous effects on ocular cells in culture, experimental animal models for ocular diseases, and human patient populations have been published.

With respect to benefits of L/Z on ocular cells in culture, addition of L/Z and other antioxidants together with photoreceptor outer segments to rabbit and bovine RPE cells under normobaric hyperoxia reduces formation of RPE lipofuscin (Sundelin and Nilsson 2001). Moreover, L/Z prevents photooxidative damage to lipofuscin-laden RPE cells in culture (Bian et al. 2012). In the same study, L/Z addition was also sufficient to suppress activation of pro-inflammatory genes by RPE cells.

Of relevance to AMD, consumption of lutein-enriched diet throughout adult life was similarly effective as grape consumption in preventing loss of visual function of the $\beta5^{-/-}$ mouse model of late-onset blindness (Yu et al. 2012). L/Z supplementation throughout adulthood had no adverse effects and reduced RPE oxidative stress and lipofuscin accumulation. Laser-induced choroidal neovascularization is widely used in animal models to study diseases associated with it including neovascular AMD. Choroidal vessels invade the subretinal space after photocoagulation in mice. In one study, L/Z was orally administered daily at different concentrations (1, 10, or 100 mg/kg body weight) from 3 days before laser

photocoagulation. L/Z treatment significantly suppressed extent of choroidal neovascularization and inflammatory processes including NF-κB activation providing experimental support for validity of L/Z supplementation as an anti-neovascular therapeutic strategy (Izumi-Nagai et al. 2007).

A large genetic study on French and North American patients with and without AMD analyzed single-nucleotide polymorphism rs5888 of the SCARB1 gene coding for SRB1, which is involved in the lipid and L/Z metabolic pathways. To investigate if this SNP polymorphism is associated with AMD, the study used control and AMD patients who did not carry two known genetic variations strongly associated with AMD (ARMS2 and CHF). They showed that there is a significant association in these patients between SCARB1 gene polymorphism and either atrophic or neovascular AMD (Zerbib et al. 2009).

Given the recognized importance of L/Z to the macula, numerous epidemiological studies have evaluated potential benefits of L/Z dietary intake or supplementation for AMD onset or progression. In 1992, the Eye Disease Case-Control Study Group showed that total serum carotenoids (L/Z, zeaxanthin, β-carotene, α-carotene, cryptoxanthin, and lycopene) were inversely related to AMD risk. Prevalence of AMD among those in this sample with highest total serum carotenoid concentration was 66 % lower than those with the lowest levels (Eye Disease Case-Control Study Group 1992). These results are consistent with a protective effect of serum and dietary L/Z against AMD (Seddon et al. 1994).

Another study analyzed levels of macular pigment density in AMD patients and age-matched controls. They observed that macular pigment density was significantly lower in AMD patients compared to controls if the subjects were not consuming high-dose L/Z supplements. Macular pigment density, however, was significantly higher in AMD patients consuming an L/Z supplement (>4 mg per day) relative to those not receiving it (Bernstein et al. 2002).

In the LISA study ("Lutein Intervention Study Austria"), 126 patients with AMD were randomized to receive L/Z supplements or placebo for 6 months. L/Z supplementation significantly increased macular pigment density, but it did not show effect on macular function assessed by microperimetry or visual acuity (Weigert et al. 2011).

In contrast, L/Z supplementation has been shown to improve retinal activity in a small trial of AMD patients. In this study, 108 AMD patients were randomized to receive daily 10 or 20 mg lutein and 10 mg lutein plus 10 mg zeaxanthin, or placebo, for 48 weeks. Multifocal electroretinogram responses significantly improved for the 20 mg lutein group and for the lutein plus zeaxanthin group. Macular pigment density improved in all treatments (Ma et al. 2012a, b).

Finally, the comprehensive, multiyear AREDS2 study recently concluded that L/Z supplementation may be beneficial in delaying the progression from early- to late-stage AMD (AREDS2 2013).

Altogether, these studies show that L/Z supplementation is associated with increased macular pigmentation and that supplementation may be beneficial to patients with AMD.

Of relevance to diabetic retinopathy, mice with streptozotocin-induced diabetes were constantly fed either an L/Z-supplemented diet or a control diet from the onset of diabetes. In 1-month old diabetic mice, ROS in the retina were measured, and visual function was evaluated by electroretinography. L/Z did not affect the metabolic status of the diabetic mice, but it prevented ROS generation in the retina and the visual impairment induced by diabetes (Sasaki et al. 2010). Importantly, L/Z protected retinal ganglion cells and inner retinal cells from diabetes-induced cell death for at least 4 months after induction of diabetes. A small epidemiological study recently tested effects of supplementation with 6 mg lutein and 0.5 mg zeaxanthin per day for 3 months on 30 patients with nonproliferative diabetic retinopathy and found that L/Z supplementation improved visual acuity and decreased foveal thickness (Hu et al. 2011).

Of relevance to cataracts, L/Z are the only carotenoids present in normal human lens (Yeum et al. 1995). Animal studies have shown that L/Z treatment prevents the development and progression of cataracts in diabetic rats. Lipid peroxidation is significantly increased in diabetic lens and is reduced by L/Z administration in this rat model (Arnal et al. 2009).

The Carotenoids in Age-Related Eye Disease Study, an ancillary study of the Women's Health Initiative Study, showed that women in the highest quintile category of diet or serum levels of L/Z were 32 % less likely to develop nuclear cataracts as compared to those in the lowest quintile category (Moeller et al. 2008). A cross-sectional study of 376 subjects further found an inverse relationship between lens optical density and macular pigment optical density suggesting that L/Z may retard aging of the lens in the human eye (Berendschot et al. 2002).

Of relevance to acute innate retinal inflammation, subcutaneous injection of lutein prevented decrease of rhodopsin expression, outer segment shortening, and vision loss in a mouse model of endotoxin/LPS-induced uveitis (EIU). Levels of ROS, activation of inflammatory signaling, and pathologic changes of Müller glial cells, all common to EIU, were also reduced by lutein in this model (Sasaki et al. 2009). In another study using the same EIU mouse model, oral administration of L/Z (125 and 500 mg/kg per day for 5 days) reduced the level of NO in the eye. L/Z increased oxygen radical absorbance capacity, levels of glutathione and vitamin C, and activities of total SOD and glutathione peroxidase. mRNA levels of SOD1 and SOD2 also increased (He et al. 2011). Finally, multiple animal studies on the neuroprotective effects of L/Z against retinal damage caused by inflammation in EIU have shown that L/Z has a dose-dependent anti-inflammatory effect. The possible mechanism may depend on the ability of L/Z ability to inhibit activation of NF-κB and subsequent inhibition of pro-inflammatory mediators (Jin et al. 2006; Sasaki et al. 2009). Despite these promising experimental results, no clinical administration of L/Z for prevention or treatment of uveal diseases has been reported to date.

6 Conclusions

Increased oxidative stress is associated with numerous common eye diseases, and all evidence suggests that it contributes to the progression of AMD, the most common cause of adult-onset blindness in the developed world. Daily intake of nutritional supplements with select antioxidants has shown only moderate beneficial effect in clinical trials when supplied to elderly patients already diagnosed with AMD. It remains to be studied if daily grape consumption with its high content of numerous natural antioxidants may have similar benefits on AMD patients. Yet, in mice, supplementation of the regular diet with grapes only at advanced age and when visual function has started to decline due to oxidative stress in the retina has no detectable benefit in rate of progression to blindness (Yu et al. 2012). In contrast, grape consumption by mice prior to old age and before visual function is due to decline is sufficient to significantly delay visual impairment even if grape intake is not continued in old age. This is in agreement with recent epidemiological studies suggesting that overall dietary habits affect the odds of AMD with dietary patterns high in vegetables being protective (Amirul Islam et al. 2014; Chiu et al. 2014). It is thus tempting to speculate that adding grapes to the daily diet may be especially beneficial at a young age and before the onset of eye disease.

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