

**Do antioxidants and free radical scavengers still hold promise for the treatment of stroke, traumatic brain injury and aging?**

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**Abstract: [250 words max]**

The overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is a common underlying mechanism of many neuropathologies, as they have been shown to damage various cellular components, including proteins, lipids and DNA. Free radicals, especially superoxide ( $O_2^{\bullet-}$ ) and non-radicals, such as hydrogen peroxide ( $H_2O_2$ ), can be generated in quantities large enough to overwhelm endogenous protective enzyme systems, such as superoxide dismutase (SOD) and reduced glutathione (GSH). Here we review the mechanisms of ROS and RNS production, and their roles in ischemia, traumatic brain injury and aging. In particular, we discuss several acute and chronic pharmacological therapies, including changes in diet, which have been extensively studied in order to reduce ROS/RNS loads in cells and the subsequent oxidative stress. Although the overall aim has been to counteract the detrimental effects of ROS/RNS in these pathologies, success has been limited, especially in human clinical studies. This review highlights some of the successes in animal studies and the epidemiological data....

**Keywords [up to 8]:** anthocyanins; natural products; NXY-059; oxidative stress; oxyresveratrol; reactive nitrogen species; reactive oxygen species; superoxide

## **INTRODUCTION**

This review focuses on ways to mitigate the damaging effects of reactive oxygen species (ROS), such as superoxide ( $O_2^{\bullet-}$ ) and hydroxyl radicals ( $\bullet OH$ ), and reactive nitrogen species (RNS), such as nitric oxide ( $NO^{\bullet}$ ), in mammalian cells. A free radical can be defined as any chemical species containing one or more unpaired electrons. Other compounds, such as hydrogen peroxide ( $H_2O_2$ ) and peroxynitrite ( $ONOO^{\bullet}$ ), are non-radicals (i.e. do not contain unpaired electrons), but are still capable of causing extensive oxidative cellular damage. The cell attempts to maintain a delicate balance, or reduction/oxidation (redox) homeostasis, between the generation and the removal of ROS and RNS; indeed, a recent review has highlighted the beneficial effects of free radical species in cells [1].

Here, we focus on free radical damage in brain ischemia, traumatic brain injury (TBI) and aging. To date, the overall pharmacological treatment goal has been to reduce free radical loads in cells. These interventions have been met with varying success, and thus, we provide current evidence for the beneficial effects of natural products in the treatment of these diseases.

## **THE FORMATION AND FUNCTION OF ROS AND RNS IN MAMMALIAN CELLS**

A common underlying mechanism of many neuropathologies is the generation of ROS and RNS (for review, see [1, 2]). The overproduction of ROS and RNS is called oxidative and nitrosative stress, respectively, and both can lead to various forms of cellular damage and death. Oxidative stress, in particular, has attracted considerable attention in neuropathogenesis, and various signaling pathways have been investigated as targets for pharmacological intervention. In general, these therapeutic compounds have been termed “free radical scavengers” for their ability to neutralize ROS or RNS, and to readjust normal redox homeostasis.

Mitochondria utilize the vast majority of  $O_2$  taken up via inhalation in order to generate energy in the form of ATP. The electron transport chain is composed of several enzyme complexes embedded in the inner mitochondrial membrane, and it is the careful, controlled transfer of electrons from one complex to the next which completes the reduction of  $O_2$  to  $H_2O$ . In general, cellular respiration results in the leakage of 1-3% of total reduced  $O_2$ , which then forms the primary ROS  $O_2^{\bullet-}$ , particularly by the NAD(P)H oxidase complex. The major RNS in biological systems is  $NO^{\bullet}$ , a free radical with one unpaired electron. It is formed by various isoforms (e.g. endothelial, neuronal and induced) of nitric oxide synthase (NOS). In normal mammalian physiology,  $NO^{\bullet}$  is utilized as a signaling molecule with diverse functions.  $NO^{\bullet}$  can react with  $O_2^{\bullet-}$ , however, to form  $ONOO^-$ , particularly during inflammation.

The cell has evolved enzymatic systems to counteract the destructive effects of  $O_2^{\bullet-}$  and other secondary species. Most importantly, superoxide dismutase (SOD) catalyzes the conversion of  $O_2^{\bullet-}$  to  $H_2O_2$ , which is then converted to  $H_2O$  by catalase. The tripeptide glutathione (GSH), and its affiliated enzymes, glutathione peroxidase (GPx) and glutathione reductase (GR), also aid in the reduction of oxidative stress. In its reduced form, GSH contains a cysteine residue which acts as a nucleophilic scavenger, and two molecules of GSH are oxidized to reduce  $H_2O_2$  to  $H_2O$ . Oxidized GSH (GSSG) is formed by a disulfide bond between two molecules of GSH. GSH is found in millimolar concentrations in most cells, while less than 10% of the total glutathione pool is GSSG. The ratio of GSH to GSSG can be measured experimentally, and is considered to be a critical indicator of the redox state of the cell [3]. Endogenous enzymes, such as SOD and GSH, as well as exogenous compounds, such as vitamin C, have been defined as “antioxidants” due to their ability to delay or inhibit oxidation [4].

Free radical loads, which disturb normal redox homeostasis, can overwhelm endogenous protective systems, and are particularly harmful to DNA, lipids, cell membranes and proteins. As shown in Figure 1, although  $O_2^{\bullet-}$  can be dismutated to  $H_2O_2$ , in vitro experiments have shown that  $NO^{\bullet}$  reacts with  $O_2^{\bullet-}$  three times faster than the reaction rate of SOD [5]. RNS, and especially ROS, can markedly alter protein structure and induce protein cross-linking, thereby increase rates of proteolysis (for review, see [6]). They have been shown to cause single- and double-strand DNA breaks, and to damage both purine and pyrimidine bases, as well as the deoxyribose backbone [4]. Lipid membrane damage can be extensive:  $^{\bullet}OH$  radicals generate lipid radicals by abstracting an electron from a lipid molecule, which can then interact with  $O_2$  to form lipid peroxyl radicals. If these radicals are not reduced by antioxidants, lipid peroxidation can occur, leading to membrane breakdown. ROS can also generate a feedback-loop and irreparably damage both mitochondria and mitochondrial DNA, resulting in increased production of ROS and RNS (for review, see [7]).

## **THE ROLE OF ROS AND RNS IN STROKE**

### ***Epidemiology of stroke***

Stroke, a reduction in blood flow to the brain, is caused by blockage in a cerebral artery by a clot or embolus (ischemic stroke) or rupture of the blood vessel (hemorrhagic stroke). Both forms of stroke result in damage or death of neurons in the affected brain region, leading to loss of brain function. Stroke is a major problem in North America, and indeed the world, given the mortality and morbidity associated with cerebrovascular disease. There are over 40,000 new or recurrent strokes in Canada annually [8] and 700,000 in the US [9]. Estimates of worldwide stroke prevalence (number of individuals with the disease) range from 1.7 to 20 per 1000 individuals [10]. Thus, stroke accounts for 9.6% of all deaths in the world [9-11], making it the

fourth leading cause of death internationally [12], and the third highest cause of death in the US [9].

Increasing age is a major risk factor for stroke. The prevalence of silent cerebral infarction (a brain lesion that is assumed to be a result of cerebral ischemia, found incidentally in otherwise healthy individuals) is 11% for the age group 55-64, and increases to 43% of individuals older than 85 [9]. Older patients have less chance of surviving a stroke: 37% of patients 45-64 will die after a hemorrhagic stroke, whereas that number rises to 44% of patients over 65 years of age [9]. Half of all ischemic stroke patients who survive will have moderate to severe impairments, making a major impact of stroke the disabilities arising from brain damage. Stroke is a major cause of disabilities [8, 9, 12]; the severely disabled require long-term care, which has high costs (\$2.7 billion a year to the Canadian economy [8]; \$62.7 billion in the American economy [9]).

### ***ROS and RNS are produced during all phases of stroke***

During brain ischemia, acute oxygen and glucose deprivation leads to inability of neurons to synthesize sufficient ATP from oxidative metabolism. This energy failure causes neuronal death via several converging mechanisms, including oxidative stress (reviewed elsewhere, e.g. [13-15]): inefficient functioning of ionic pumps leads to accumulation of intracellular  $\text{Na}^+$  and  $\text{Ca}^{2+}$ , which in turns causes cellular swelling and necrosis. Also, these ions depolarize affected neurons, leading to excessive release of the excitotoxic neurotransmitter glutamate. Activation of glutamate receptors exacerbates metabolic failure and necrosis [15] and also enhances the production of ROS by stimulating pro-oxidant enzymes [16]. NOS1 (nNOS) is a  $\text{Ca}^{2+}$ -dependent enzyme, and is thus activated during  $\text{Ca}^{2+}$  overload seen in ischemia [16]. Neighboring neurons and non-neuronal cells also contain pro-oxidant enzymes that are  $\text{Ca}^{2+}$ -dependent (e.g. xanthine oxidase) or independent [COX1, COX2, NAD(P)H], leading to production of  $\text{O}_2^{\bullet -}$  in the ischemic brain [14, 16]. Superoxide,  $\text{NO}^{\bullet}$  and  $\text{ONOO}^-$  then oxidize lipids, proteins and DNA, killing surrounding neurons by necrosis, contributing to oxidative stress [13-18].

Even more ROS are produced after ischemia, when blood flow resumes (reperfusion) and brain oxygen tension increases. These large quantities of  $\text{O}_2^{\bullet -}$  and  $\text{NO}^{\bullet}$  are purported to be involved in the delayed necrosis of neurons in the area surrounding the infarct [13, 14, 16, 17, 19]. Even more important during reperfusion, ROS also trigger apoptosis via redox signaling [15, 17, 18, 20], contributing to delayed neuronal death occurring for hours or days after ischemic stroke. It is still debated whether mitochondrial damage leads to leakage of ROS from the electron transport chain into the cytosol, or whether the converse is more likely i.e. ROS generated in the cytosol causes oxidative stress to the mitochondria [17, 21, 22]. Both mechanisms are likely involved in neurodegeneration [22]. Oxidative stress is also evident after hemorrhagic stroke, the most prominent source of ROS being phagocytes which accompany brain inflammation [15, 19, 23, 24].

Knocking out nNOS decreases brain injury in various experimental models of stroke [16], supporting the role of  $\text{NO}^{\bullet}$  in ischemia-reperfusion brain injury. SOD knockout animals have larger ischemic lesions and SOD over-expression is protective [16]. Inhibiting NAD(P)H oxidase is neuroprotective against ischemia-reperfusion injury in gerbils, reducing lipid peroxidation in the hippocampus [25]. These results also support the notion that oxidative stress and  $\text{O}_2^{\bullet -}$  contribute to stroke brain damage. The damage by ROS and RNS can become more intense when endogenous antioxidant defenses are weakened. Therefore, treatment with antioxidants may prevent delayed neuronal death, thereby improving survival and reducing

morbidity. Thus, the relationship between oxidative stress and stroke has generated considerable interest in the development of antioxidant therapies for neuroprotection [15, 26-31].

### ***Antioxidants as therapeutic neuroprotectants for stroke***

Antioxidants reduce stroke *risk*. It has been well established in epidemiological trials that diets rich in fruits and vegetables help manage modifiable risk factors for stroke such as hypertension, thereby lowering stroke incidence [32-35]. Studies on individual antioxidants such as flavonoids and vitamins also show that stroke risk is reduced [33, 36, 37] by managing blood pressure [38] or atherosclerosis [39], although results are often contradictory [27]. Prevention is the key, as brain damage cannot easily be repaired. However, for many individuals, stroke will still occur especially if there is genetic predisposition to stroke, advancing age or a previous stroke has occurred [9]. During an ischemic stroke, the window of opportunity for dislodging the clot is only 3-6 hr [13, 30, 40]. At present, there is only one drug [recombinant tissue plasminogen activator (tPA)] that is licensed for use within 3 hr of ischemic stroke onset, and one other treatment (administering oral aspirin within 48 hours of ischemic stroke) that has been shown to have clinical benefit [30, 40]. Although tPA has neurologic benefits, it has the side effect of increasing intracerebral hemorrhage [41]. Given the high cost of tPA and the narrow window of opportunity, it is estimated that only 2% of patients suffering from a stroke would ever receive tPA [42, 43]. Thus, there is a real need for other approaches besides thrombolytic therapy, whose therapeutic strategy is to treat stroke by enhancing reperfusion (limiting the length of ischemia) [27]. Other therapeutic approaches would include slowing the molecular cascades leading to necrosis or apoptosis during and following ischemia [18, 27].

As described above, ROS and RNS are produced during ischemia, reperfusion and brain hemorrhage. Oxidative stress contributes to stroke-induced immediate and delayed neuronal death via both necrosis and apoptosis. Thus, several antioxidant agents of widely varying chemical structures have been investigated as neuroprotective therapeutic agents for brain injury. We used a unilateral model of brain hypoxia-ischemia in adult rats and demonstrated that feeding diets enriched with blueberries (*Vaccinium angustifolium*) for 6 weeks reduces damage to the hippocampus [44]. Similar results were seen in a transient middle cerebral artery occlusion model of stroke in rats fed blueberries for 4 weeks [45]. *Vaccinium* berries have a high antioxidant activity [46, 47] due to their high total polyphenol content [48-50] and not correlated with vitamin C content [47]. Thus, it is presumed that the neuroprotective effects of these blueberry diets are mediated, at least partially, by the antioxidant activity of their polyphenols. Indeed, direct application of polyphenolic fractions from blueberries [51] and cranberries [52] onto cultured neurons is neuroprotective against both necrosis and apoptosis induced by simulated stroke (hypoxia and oxidative stress) [49]. We are currently evaluating the molecular pathways and mechanisms involved in this neuroprotection.

Dietary polyphenols show a great diversity of structures, ranging from rather simple, non-flavonoid molecules (stilbenes and chlorogenic acid) to more complex flavonoids (anthocyanins, flavonols, flavones, and catechins) and finally to the most polymerized condensed tannins (proanthocyanidins) [49, 53, 54]. More than 4000 flavonoids have been identified in plants [53]. The concept that flavonoids are responsible for the health effects of plant-based foods is supported by *in vitro* studies, animal experiments and clinical research [55-58]. Specifically regarding the brain, anthocyanins appear to offer the best benefit against the effects of oxidative stress in the aging and ischemic brain [49, 51, 52, 52], and the best penetration of the blood-brain-barrier (BBB) [59]. The content of anthocyanins is 25-fold higher in blueberries than strawberries and raspberries [47], supporting the superior benefit of blueberry anthocyanins in

brain oxidative stress [59]. *Vaccinium* polyphenols decrease platelet aggregation [60] and reduce the release of inflammatory mediators [61]. In an *ex vivo* study, highbush blueberry polyphenols added to plasma collected from adults with normal blood lipid profiles inhibited oxidation of LDL [62]. All of these effects of blueberry flavonoids would be beneficial in the prevention and acute treatment of stroke. Consumption of quercetin, another flavonoid widely distributed in fruits, vegetables, tea and red wine, was reported to be inversely related to the incidence of stroke [33], although a reexamination of the data questioned this finding [63].

Resveratrol (chemical name 3,5,4'-trihydroxy-trans-stilbene) is another polyphenol abundantly found in the skin and seeds of grapes [54, 64] and many other plants including blueberries [49, 65, 66]. It is a non-flavonoid polyphenol (**Fig. 2**) found in relatively small amounts in foods, yet it has been suggested that it is one of the most bioactive polyphenolic compounds, e.g. responsible for the *French Paradox* [54]. Resveratrol inhibits platelet aggregation, promotes vasodilation, and exerts anti-atherosclerotic effects [67]. Thus, resveratrol is a beneficial chemical in stroke by preventing thrombosis and limiting ischemia. Moreover, resveratrol has been shown to have direct neuroprotective effects against ischemia/reperfusion [68-70] and other neurologic conditions [54] (see below). However, it was not neuroprotective in PPAR- $\alpha$  knockout mice, suggesting that it may not be working by directly scavenging ROS (at least in the mouse model of permanent ischemia) [70]. Like most antioxidants, levels of resveratrol in the plasma after moderate feeding or drinking are not likely adequate to completely neutralize ROS [54], further supporting the notion that the beneficial effects are due to mechanisms beyond direct antioxidant effects.

A possible theory for beneficial effects of antioxidants in the brain is that they activate genes involved in neuroprotection such as up-regulation of endogenous antioxidant systems [54, 58]. Feeding blueberry flavonoids has been shown to increase the level and activity of brain  $\gamma$ -glutamylcysteine synthetase, the rate limiting step in GSH synthesis [71, 72]. Treatments that increase GSH reduce oxidative stress [73, 74]. Thus, the polyphenol-mediated regulation of GSH would be one possible mechanism by which diet or natural products influence development of stroke and other diseases [58]. Another possible candidate gene altered by antioxidants is heme oxygenase [75], whose protein product degrades the pro-oxidant heme and leads to formation of a radical scavenger (bilirubin) [54]. Resveratrol induces heme oxidase in the brain [54, 75], as do other natural phenolic compounds such as curcumin, caffeic acid phenethyl ester and ethyl ferulate [76, 77]. Thus, antioxidant polyphenols may have a direct effect on neuronal genes, preconditioning neurons to better withstand hypoxia.

Although a few antioxidants showed some efficacy in preclinical trials such as animal models or in small clinical studies, these findings have not been supported in comprehensive, controlled trials in patients. The compound being discussed most right now is NXY-059 (chemical name:  $\alpha$ -(2,4-disulfophenyl)-N-tertbutylnitrone; drug named: Cerovive; Astra Zeneca). NXY-059 is a novel nitron free radical trapping (antioxidant) agent. Basically, this compound is a stable form of  $\text{NO}^\bullet$ , capable of inhibiting the reaction of  $\text{O}_2^\bullet$  and  $\text{NO}^\bullet$  to produce  $\text{ONOO}^-$ . Preclinical studies have shown that it reduces infarct size after transient and permanent ischemia in rats [78, 79] and in primate models of acute ischemic stroke [80-82]. NXY-059 also preserved brain function, such that monkeys and marmosets given this drug after permanent middle cerebral artery occlusion had better arm reaching abilities with the stroke-affected arm and less spatial perceptual neglect [80-82]. There was a 4 hr therapeutic window of opportunity; the effects lasted for 90 days, and NXY-059 appeared to reduce tPA-induced hemorrhage in animals [83].

Recently, NXY-059 became the first neuroprotectant to demonstrate a statistically significant reduction in disability in a clinical trial involving patients with acute ischemic stroke [31] but not acute intracerebral hemorrhage (unpublished); [84]. The phase III ischemic stroke

trial nick-named SAINT I (Stroke -Acute Ischemic – NXY-059 Treatment) assessed the neuroprotective efficacy of infusing NXY-059 i.v. (for 72 hr) within 6 hr of the onset of symptoms of acute ischemic stroke. Cerovive was well tolerated by the 1700 European subjects, and no safety issues were identified. It also significantly improved the overall neurologic scores using the modified Rankin scale, when compared with placebo, but had no effect on mortality rates or neurologic functioning measured according to the National Institutes of Health Stroke Scale (NIHSS) or the Barthel index [31]. When given in combination with tPA, NXY-059 reduced the risk of hemorrhagic transformation from 27.3% to 15.4% and of symptomatic intracranial hemorrhage from 6.4% to 2.5% [31].

SAINT I was followed up with a larger randomized multicenter clinical trial (SAINT II trial, 3400 subjects in North America). In contrast to SAINT I, NXY-059 did not demonstrate any treatment benefit in acute ischemic stroke [85-87]. The further development of this agent was suspended. It has been postulated that this failure is due to many reasons. From a chemical point of view, the use of old, and therefore unstable NXY-059, is a potential issue [8]. Because antioxidants (reducing agents) are inherently prone to oxidation, this creates a problem during storage. Furthermore, tert-butyl nitrones and derivatives (e.g. NXY-059) hydrolyze to make t-butylhydroxylamine (NtBHA) a powerful radical scavenger (**Fig. 3**). NtBHA further oxidizes to 2-methyl-2-nitrosopropane (MNP) which then (a) may be reduced back to NtBHA by vitamin C or mitochondria; (b) synthesizes NO<sup>•</sup>, which dilates blood vessels; or (c) forms a spin adduct with a free radical, neutralizing it. Thus, a putative neuroprotectant in SAINT I is NtBHA and/or its parent spintrap MNP, produced by hydrolysis of NXY-059 in storage. If the Cerovive used in SAINT II was older, it could explain the negative results. In support of this theory, the patent application from Astra Zeneca states “Standard aqueous formulations of  $\alpha$ -(2, 4-disulfophenyl)-N-tertbutylnitron and pharmaceutically acceptable salts thereof suffer from the problem that they readily undergo decomposition. In particular, the shelf life of such formulations is unacceptably short. The present invention discloses certain pharmaceutical formulations based upon concentrated aqueous solutions of  $\alpha$ -(2, 4-disulfophenyl)-N-tertbutylnitron disodium salt that solve the problems associated with decomposition and that are particularly suited for use in parenteral administrations.” [88].

Other reasons for the failure of SAINT II could have been a lack of sufficient pre-clinical studies in animals [87]. In support of this, NXY-059 had no protective effect on sodium nitroprusside- or H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in neuroblastoma cells in vitro [89]. Thus, it likely does not have a direct effect on neurons, rather it works as an NO<sup>•</sup>-mimetic on the cerebral vasculature [89]. It is not lipophilic, and thus would not easily cross an intact BBB. The lack of success of antioxidant compounds as neuroprotectants in clinical trials is not surprising. At least fifty agents that were neuroprotective in pre-clinical studies have been evaluated in over 100 human trials of stroke and traumatic brain injury, and none has yet proven successful [90, 91]. There are some inherent problems with translating animal studies that measure brain infarct sizes with human studies that typically evaluate neurologic functional outcomes. With specific regard to antioxidants, it is difficult to increase the antioxidant status of humans (blood, tissues) by single administration of an antioxidant (e.g. vitamin C), due to the high levels of endogenous antioxidants compared with other mammalian species [92]. Lastly, antioxidants are, by their chemical nature, prone to oxidation.

## **THE ROLE OF ROS AND RNS IN TRAUMATIC BRAIN INJURY**

### ***Epidemiology of traumatic brain injury***

Traumatic brain injury (TBI) is one of the leading causes of death and disability worldwide [93, 94]. Globally, the incidence of TBI is generally reported as approximately 200 in

100,000 individuals with a mortality rate of about 20 per 100,000 [94]. Automobile accidents are reported to be the major cause of TBI while falls and assaults also contribute greatly to the number of traumatically injured patients [93, 94]. In the United Kingdom, 200-300 per 100,000 people are hospitalized each year due to TBI [95] and the incidence is even higher in areas such as southern Australia and South Africa [96, 97]. In the United States, TBI is associated with the death of approximately 51,000 people each year and causes long-term disability that affects an estimated 70,000 to 90,000 individuals annually [98, 99]. TBI is particularly prevalent in young individuals, and is the leading cause of mortality in infants [100]. In the U.K., TBI may represent as much as 20% of all deaths of individuals in the range of 5-45 years of age [94]. In 1999, a National Institutes of Health Consensus Development Panel in the United States compiled a set of statistics on the occurrence of TBI and suggested that as many as 6.5 million individuals may be living with the consequences of TBI in the U.S. alone. In Europe, at least 11.5 million people suffer from disabilities related to a TBI [101]. The total financial burden of TBI is difficult to determine, however estimated costs of head injury per year in the United States is over one billion dollars [99, 102]. Given the global prevalence and enormous societal and economic costs of TBI, it is imperative to understand the mechanisms that contribute to dysfunction and death following trauma, so that we may devise appropriate therapeutic strategies to improve patient outcome.

### ***Pathophysiology of traumatic brain injury***

The pathophysiology of TBI is understood to consist of two main phases, a primary (mechanical) phase of damage and secondary (delayed) damage. Primary damage occurs at the moment of insult and includes contusion and laceration, diffuse axonal injury and intracranial hemorrhage [102-105]. Secondary damage includes processes that are initiated at the time of insult, but do not appear clinically for hours, or sometimes even days after the injury has occurred. Such processes include brain damage due to altered neurochemical mechanisms, activation of degradative enzymes, swelling (edema) and ischemia (for reviews see [106-112]).

Ischemia has been suggested as one of the most important mechanisms underlying secondary brain damage following TBI, especially in severely injured patients [113-115]. In fact, approximately 90% of traumatically injured patients who die demonstrate ischemia on histopathological examination of brain tissue [116]. Ischemia is caused by a reduction in cerebral blood flow (CBF) within the first few hours after a traumatic insult [117, 118]. Reduction of CBF causes damage to mitochondria, which is followed by a shift from aerobic to anaerobic metabolism in neurons. In addition, ionic homeostasis becomes compromised. Increases in intracellular  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  can lead to further cell swelling and an even greater reduction in CBF. Under these conditions, there is an elevated production of ROS and RNS, and the resulting pathology relating to these species is similar to that previously described for stroke.

Even in the absence of ischemia following TBI, for example after more mild to moderate insults, mechanical damage to mitochondria may lead to increased release of ROS such as  $\text{O}_2^{\bullet-}$  and  $\bullet\text{OH}$  radicals. These oxygen free radicals could subsequently damage cellular membranes by causing lipid peroxidation, and also feedback and further damage mitochondrial membranes leading to altered function [109, 119]. Damaged cell membranes can also lead to an increase in free fatty acids such as arachidonic acid (AA) in the cytosol. AA produces several additional metabolites such as leukotrienes, thromboxanes and prostaglandins, all of which can produce even more free radical species [106, 109].

Another toxic enzymatic pathway initiated in neurons after traumatic injury is the over-production of  $\text{NO}^{\bullet}$  [120]. Following trauma, there is elevated release of the neurotransmitter

glutamate, which causes over-activation of glutamatergic membrane receptors [121]. Excess activation of NMDA receptors in particular causes increased calcium influx, binding of  $\text{Ca}^{2+}$  to the enzyme calmodulin, which regulates NOS, and over-production of  $\text{NO}^{\bullet}$  from L-arginine. As previously mentioned, under physiological conditions the level of  $\text{NO}^{\bullet}$  is low and it contributes to several important biological processes including cerebral vasodilation, neurotransmission and synaptic plasticity [122]. However, following TBI excessive  $\text{NO}^{\bullet}$  can combine with superoxide to produce  $\text{ONOO}^-$ , which is highly toxic to neuronal proteins, membrane lipids and DNA [123, 124]. NOS itself produces  $\text{O}_2^{\bullet-}$ , but levels are generally kept in check by endogenous free radical scavengers like SOD. However following trauma, when there is over-activation of NOS and additional mechanisms producing  $\text{O}_2^{\bullet-}$  (i.e. metabolism of AA and damage to mitochondria), endogenous scavengers like SOD may become saturated, allowing high levels of  $\text{ONOO}^-$  to form, ultimately leading to neuronal damage. Given these scenarios, antioxidants and free radical scavengers have received immense attention as potential therapeutic agents for preventing damage and improving outcome following TBI.

### ***Antioxidant therapy for traumatic brain injury***

Overall, experimental studies of TBI evaluating antioxidants and free radical scavengers have yielded positive and promising results. Unfortunately, these findings have not directly translated to the clinical situation [125, 126]. For example, a large clinical study with polyethylene glycol (PEG)-conjugated SOD failed to elicit a significant effect on outcome following TBI [127]. PEG-SOD was administered within eight hours after injury, and the reason for a negative outcome was generally speculated that this administration was outside of the therapeutic window. Two other multicenter clinical trials evaluating Tirilazad Mesylate, a non-glucocorticoid, 21-aminosteroid that inhibits lipid peroxidation, also yielded no significant effects in moderately and severely injured patients [100, 128]. A clinical study in Europe evaluating the NOS inhibitor, lubeluzole, to treat TBI was stopped, mainly after the drug failed to significantly decrease mortality after ischemic stroke, despite evidence for an improvement in the outcome of surviving patients [129, 130].

Some of the reasons for the failure of antioxidants to improve the outcome of TBI patients may be due to flaws in the design of clinical studies [126]. For example, patients may have varying degrees of secondary insults such as ischemia, which could overshadow positive results in subgroups of patients. In addition, clinical studies most often contain severely injured patients, which may not respond to antioxidant treatment due to overriding mechanical damage, whereas these types of drugs may be useful in more moderately injured individuals. Other reasons for failure could be due to inappropriate timing of drug administration (as suggested for PEG-SOD), or due to a lack of therapeutic levels of the drug at the site of injury. In fact, despite positive experimental studies, which may eventually lead to the evaluation of compounds at the clinical level, pharmacokinetic studies are often not performed thoroughly.

Despite these failures, research with animal models continues to provide new hope. A promising class of drugs for the treatment of both stroke and TBI are nitroxides, which are new, low molecular weight, cell-permeable SOD mimics [131, 132]. These compounds readily cross the blood brain barrier, and one nitroxide in particular, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol), has already proven efficacious in animal models of ischemia and trauma [131, 133, 134]. Another potential neuroprotective compound is the naturally occurring hydroxystilbene oxyresveratrol (trans-2, 3', 4, 5'-tetrahydroxystilbene, OXY), which is a potent antioxidant and free radical scavenger isolated from mulberry wood (*Morus alba* L.). The structure of OXY is identical to that of resveratrol, with the addition of a hydroxyl group at

position 2 on the stilbene molecule (Fig. 3). OXY has increased scavenging ability over resveratrol, perhaps due to this additional hydroxyl group [135]. Analysis of blood brain barrier penetration has demonstrated that OXY enters the brain to a low extent in control animals [136]. However, OXY has much higher brain permeability in animals subjected to stroke, which explains the previously reported neuroprotective ability of this compound in cerebral ischemia [137]. These findings suggest that OXY may be useful in treating TBI. In a preliminary investigation, we have found that OXY protects cortical neurons from damage using an *in vitro* model of mechanical injury (J.T. Weber, unpublished results). Therefore, studies investigating the effects of OXY in animal models of TBI are warranted.

As previously mentioned, the pathology of TBI is quite complex and it is highly possible that treatment with a single drug will not prove efficacious for improving outcome. In this case, a drug such as OXY may be useful as a compliment to other types of drugs. We have found that the combination of SOD with NOS inhibitors provided protection against trauma *in vitro*, but this was no more significant than the drugs administered alone [138]. Therefore, the combination of drugs that target the same or similar, neurotoxic pathways may not prove as useful as combinations which inhibit considerably different mechanisms, such as antioxidants administered with various glutamate receptor antagonists. We also found that although SOD and NOS inhibitors provide some protection against trauma alone, they are relatively ineffective when simulated ischemia is superimposed on the trauma. This experimental finding again reiterates the importance of designing proper clinical studies where drug administration may prove useful in some subgroups of patients, but not others.

## **THE ROLE OF ROS AND RNS IN AGING**

### ***Epidemiology of aging***

With modern advances and general improvements in the quality of life, the average U.S. human life expectancy at birth has increased to about 77 years in 2003 [139] with a growing number of people living beyond 100 years of age. The aging process has been defined previously as the progressive, age-dependent accumulation of damaging changes to cells and tissues that ultimately increases the probability of disease and death [140]. It is well accepted that normal brain function declines with age, with such declines likely increasing the susceptibility for age-related neurodegenerative disease [141-144]. Increasing brain damage is likely to result from sustained exposure of brain tissue to oxidative insults, which is described in the following section. As such, many therapies have been tested and/or proposed in the hopes of attenuating age-related neuron loss while concomitantly preserving brain function.

### ***The free radical theory of brain aging***

For over fifty years, the Free Radical Theory of Aging has argued that the age-related decline in cell function is directly related to age-related increases in oxidative stress, leading to an increase in oxidized proteins, nucleic acids and lipids [145, 146]. Age-related increases in oxidative damage likely play an important role in the etiology of age-related neurodegenerative disease. In addition, intracellular oxidative damage may not only exacerbate damage resulting from brain injury but may also limit functional recovery following these events.

The brain appears particularly susceptible to oxidative damage, which may be attributed to a combination of reasons. First, neurons are post-mitotic or senescent, and by nature accumulate

oxidative damage with increasing age. Second, neurons have particularly high energy demands relative to other cell types [147]. As such, the generation of mitochondrial ATP by oxidative phosphorylation and the electron transport chain leads to the inherent production of ROS and RNS [148]. Age-related increases in mitochondrial H<sub>2</sub>O<sub>2</sub>, decreased activity of complexes I and IV of the electron transport chain with resultant increased electron leak, and increased oxidative damage to mitochondrial DNA have all been reported in rodent models [149]; reviewed in [150]. An increase in mitochondrial ROS and RNS in aging neurons may be a direct result of age-related mitochondrial dysfunction [151, 152] and may lead to the feed-forward oxidative damage of mitochondria or of other cell components, such as an increase in oxidatively damaged nuclear DNA [149] or proteins. It has been suggested that up to 40% of total cellular protein in neurons becomes oxidized with aging [153, 154]. Age-related increases in protein carbonyls and nitrated proteins have been documented previously in rats [155]. Third, the high content of lipid in the brain in the form of peroxidizable fatty acid increases the susceptibility for lipid-mediated oxidative damage [156, 157]. For instance, 4-hydroxynonenal (4-HNE) is a lipid oxidation product that has shown to increase in the brain with aging [158]. Fourth, iron levels are known to accumulate with brain aging [159], which increases the susceptibility of ROS generation. Fifth, age-related declines in reducing equivalents such as glutathione (GSH) have been shown in mouse brain [160], which contributes to age-related increases in oxidative stress.

Although it seems that they are exquisitely sensitive to oxidative damage, neurons appear to have adapted evolutionarily to prevent neuron death. For instance, species- and/or strain-specific decreases in caspase-3 have been indicated in aging brain [161, 162], which is important for activation of both the extrinsic and intrinsic apoptotic death pathways (reviewed in [163]). Age-related declines in other pro-apoptotic molecules such as Bax and Apaf-1 have also been reported [164, 165]. In contrast, oxidative damage may also play a role in age-related compromises in neuron function that may not correlate to neuron loss. Age-related declines in synaptic function, for instance, are thought to increase the susceptibility for age-related neurodegenerative disease [166].

One of the most reliable markers of brain aging is the age-related increase in ceroid/lipofuscin [167], an autofluorescent, peroxidated lipoprotein complex that accumulates in most every cell type proportionally to aging. It has been shown that up to 75% of neurons in the aging brain contain detectable lipofuscin material [168, 169]. Lipofuscin is considered to be undegradable and its accumulation may result from age-related declines in protein degradation pathways, in particular the autophagy-lysosome degradation pathway. Lysosome function is known to be altered or compromised with normal brain aging [170-173].

### ***Reducing age-related increases in brain oxidative damage***

Strategies that attenuate or prevent age-related declines in brain function may go hand in hand with a decreased susceptibility of age-related neurodegenerative disease and increased life span. Since it is well established that oxidative damage increases proportionally with brain aging, agents that are known antioxidants or induce antioxidant-like effects, or dietary regimens that result in decreased oxidative damage, hold great promise in preserving function in the aging brain. A healthy diet may play an important contributing role to increasing life span and preserving brain function. Epidemiological studies have indicated that specific diets that are rich in antioxidants or essential fatty acids may lead to a preservation of brain function with aging (reviewed in [174-176]). Since antioxidant-rich diets are suggested to have positive effects on brain function, a major focus of aging research has been to isolate and identify compounds present in such “healthy” foods that mediate neuroprotection, in the hopes of creating defined

supplements that mimic the effects of healthy dietary regimens. In the following sections, dietary supplements, including antioxidants and natural products, as well as dietary modification are described as potential therapies to reduce oxidative damage in brain aging.

### ***Agents that putatively reduce age-related declines in brain function***

Vitamin E ( $\alpha/\gamma$ -tocopherol) is a lipid-soluble antioxidant that acts in part by reducing membrane lipid peroxidation [177]. Vitamin E has been shown to preserve learning and memory upon chronic administration to aged rodents [178] and also enhance long-term potentiation (important in memory formation) in rat brain slices [179]. In aging humans, cognitive function was shown to be positively correlated to plasma levels of vitamin E [180] and that a diet rich in vitamin E improved cognitive function [181]. Clinical trials with vitamin E have offered some hope in the treatment of Alzheimer's disease (AD), suggesting at the very least that vitamin E may delay the onset of AD [182]. Interestingly, the absorption of vitamin E has shown to be greatly enhanced when taken during a high-fat vs. a low fat meal [183] and its pharmacokinetics have been shown to be altered significantly by cigarette smoking [184, 185]. In light of these recent findings, future clinical studies of vitamin E should assess population-specific differences in vitamin E absorption and carefully monitor the manner in which patients ingest vitamin E. In general, extensive pharmacokinetics analyses will remain a critical component of future pre-clinical and clinical studies, to verify that the neuroprotective functions of isolated compounds observed in the laboratory transcend to human use.

Nonsteroidal anti-inflammatory drugs (NSAIDs) including indomethacin and ibuprofen have been shown in clinical studies to hold great promise in reducing AD [186-188]. Curcumin, a non-traditional NSAID derived from curry, was shown to reduce oxidized proteins and A $\beta$  pathology in a rodent model of AD [189] and also promote antioxidant activity in aged rat brains [190]. A positive correlation has been suggested between treatment with NSAIDs and a reduction in microglial activation [191, 192]. A reduction in microglial activation would suggest a decrease in oxidative damage, since microglia are potent producers of O $_2^{\cdot-}$  and NO $^{\cdot}$  (reviewed in [193]). However, treatment of mice with indomethacin did not decrease age-related increases in oxidative damage although brain prostaglandin levels, a direct result of decreased cyclooxygenase activity, were significantly reduced [194]. Nevertheless, NSAIDs hold great promise in preventing the deleterious effects of brain aging and future studies are warranted to delineate mechanisms of neuroprotection and to design safe and effective compounds.

Epidemiological studies have shown that diets consisting of moderate wine consumption are associated with a preservation of age-related brain function [195]. In addition, neuroprotection and a decrease in cognitive decline were observed in aged rats administered a blueberry, spinach or strawberry-supplemented diet [196, 197]. Such fruits and vegetables are rich in polyphenolic compounds that possess potent antioxidant activity which are purported to play a major role in preserving age-related brain function. Of the fruit and vegetable polyphenols tested, those found in blueberry extracts appear to be particularly effective in attenuating both age-related cognitive and motor decline in rats [196, 197], and it has been suggested that in addition to serving as antioxidants, blueberry phenolics may be neuroprotective by activating select signal transduction cascades (reviewed in [198]). In addition, a blueberry-supplemented diet was shown to activate similar signal transduction events in a mouse model of AD concomitant with an attenuation of cognitive deficits [199]. Red wine polyphenols, including resveratrol, quercetin and catechin were shown to protect against A $\beta$  and oxidative stress-induced death of cultured neurons [200], suggesting their potential utility in the aging brain. Interestingly, resveratrol increased the lifespan of a short-lived fish species concomitant with

enhanced cognitive and motor function, and decreased biomarkers of aging [201]. A recent study showed that resveratrol stimulated AMP-activated kinase (AMPK), an enzyme that acts as a sensor for cellular energy levels, resulting in a preservation of cellular energy associated with formation of de novo mitochondria, [202], effects that could certainly lead to age-related decreased oxidative damage and an overall preservation of neuron function. Results from these findings suggest that polyphenolic compounds have great potential to combat against normal human brain aging and age-related neurodegenerative disease, and clearly warrant randomized clinical studies to verify the anti-aging potential of specific polyphenolic compounds in humans. Essential fatty acids, in particular the n-3 and n-6 long chain poly-unsaturated fatty acids, are important for brain function, but levels decrease with brain aging concomitant with an increased lipid peroxidation (reviewed in [203]). As such, increasing levels of essential fatty acids through dietary supplementation has been shown to be an effective means of attenuating markers of brain aging. Epidemiological studies have shown that a fish-rich diet, which provides n-3 poly-unsaturated fatty acids, is associated with reduced age-related cognitive decline [204, 205], whereas diets high in saturated and monounsaturated fatty acids, which are more susceptible to oxidative damage, are associated with increased age-related cognitive decline [204, 205]. In rodent models, age-related declines in brain function were shown to be significantly attenuated upon supplementation with n-3 fatty acids including docosahexaenoic acid (DHA) and eicosapentaenoic acid [206-209] or n-6 fatty acids such as AA [209-212], whereas a diet poor in n-3 fatty acids was shown to disrupt cognitive function [213]. Together these findings strongly support the benefit of dietary supplementation with n-3 and n-6 polyunsaturated fatty acids in preserving brain function with aging.

### ***Caloric Restriction and Brain Aging***

Caloric restriction (CR) is the practice of reducing dietary intake in the hopes of increasing life span and limiting susceptibility to age-related diseases. In laboratory animals, CR is achieved via a 40% reduction in *ad libitum* caloric intake. CR is associated with decreased serum glucose, insulin and amino acid levels that are normally observed during periods of fasting, and thus has direct consequences on energy metabolism that may contribute to a decrease in oxidative stress and an improvement of organellar function [155]; reviewed in [214]. In mice, age-related increases in 8-hydroxydeoxyguanosine (8-OHdG), a product of DNA oxidation, observed in the brain and other tissues (heart and skeletal muscle) rich in senescent, post-mitotic cells were lowered following the CR diet [215]. Levels of GSH and the ratio of GSH/GSSG were also increased in the brains of aged mice [160], and plasma membrane fractions obtained from CR-fed rats exhibited greater levels of  $\alpha$ -tocopherol and coenzyme Q<sub>10</sub> [216], suggesting a greater antioxidant defense in neuronal cells following CR. In brains of aged rats, a CR diet significantly attenuated age-related increases in mitochondrial H<sub>2</sub>O<sub>2</sub> production and oxidative damage to mitochondrial DNA and attenuated electron leak from complex I of the electron transport chain [149]. In addition, rats fed the CR diet exhibited significantly attenuated age-related increases in oxidatively modified proteins, including protein carbonyls and nitrated proteins [155, 216]. Other methods including intermittent fasting have been shown to increase life span and preserve brain function, possibly by reducing oxidative damage (reviewed in [214]).

CR has also been shown to attenuate age-related declines in macroautophagy in a variety of tissues and cells [217, 218], an intracellular process of lysosome-mediated degradation and recycling of macronutrients and organelles [219]. Age-related declines in macroautophagy are associated with an increase in oxidative stress, possibly due to the accumulation of damaged mitochondria and oxidatively modified protein including lipofuscin, and may result from age-

related declines in lysosome function [217, 218, 220]. Although the effects of CR on macroautophagy in the aging CNS have not been directly tested, it is possible that CR may enhance lysosome function, as suggested previously by the CR-induced decrease in brain lipofuscin accumulation [221] or may improve the clearance of mature autophagosomes. CR may also enhance the stimulation of nascent autophagosome formation due to a decrease in insulin signaling. Macroautophagy is known to be negatively regulated by the insulin-dependent activation of class I PI3-K/Akt/mTOR signaling pathway [217, 222]. Treatment with rapamycin, which directly inhibits mTOR signaling has been shown to stimulate macroautophagy [223, 224] and thus provides a potentially effective pharmacological means of mimicking CR and attenuating age-related declines in neuronal macroautophagy. In fact, a group of diverse agents defined as “calorie restriction mimetics” has now been identified [225], which suggests the potential for inducing the benefits of caloric restriction without strict dietary modification.

## CONCLUSIONS AND IMPLICATIONS

Our aim in this review was to answer the question: do antioxidants and free radical scavengers still hold promise for the treatment of stroke, TBI and aging? Clearly, these three pathologies are complex and interconnected, and both free radical production and the resultant cellular damage play key roles in the pathogenesis and progression of these diseases. Often, experimental and clinical data do not corroborate one another, and human clinical trials have produced mixed results. It is possible, however, that certain treatments which were attempted for acute conditions, such as TBI, may prove efficacious when administered chronically for the aging population.

## ABBREVIATIONS

4-HNE = 4-hydroxynonenal  
AA = arachidonic acid  
AD = Alzheimer’s disease  
ATP = adenosine triphosphate  
BBB = blood-brain-barrier  
CR = caloric restriction  
GSH = reduced glutathione  
GSSG = oxidized glutathione  
H<sub>2</sub>O<sub>2</sub> = hydrogen peroxide  
MNP = 2-methyl-2-nitrosopropane  
NAD(P)H = nicotinamide adenine dinucleotide (phosphate)  
NO<sup>•</sup> = nitric oxide  
nNOS = neuronal nitric oxide synthase  
NSAID = nonsteroidal anti-inflammatory drug  
NtBHA = t-butylhydroxylamine  
NXY-059 =  $\alpha$ -(2,4-disulfophenyl)-N-tertbutylnitron  
O<sub>2</sub><sup>•-</sup> = superoxide  
<sup>•</sup>OH = hydroxyl radical  
ONOO<sup>-</sup> = peroxynitrite  
OXY = oxyresveratrol

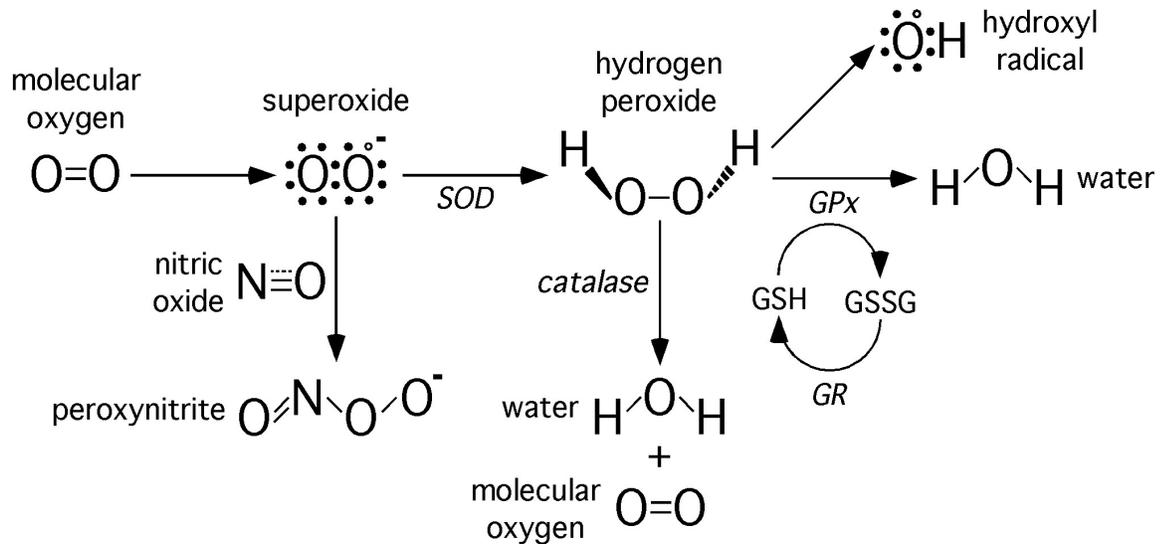
PEG-SOD = polyethylene glycol-conjugated superoxide dismutase  
ROS = reactive oxygen species  
RNS = reactive nitrogen species  
SAINT I / II = Stroke-Acute Ischemic – NXY-059 Treatment  
SOD = superoxide dismutase  
TBI = traumatic brain injury  
tPA = tissue plasminogen activator

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**Figure 1. Critical ROS and RNS pathways in mammalian cells.** Molecular oxygen can be transformed into superoxide ( $O_2^{\bullet-}$ ) radicals by various enzymes, particularly in mitochondria. Superoxide can combine with nitric oxide ( $NO^{\bullet}$ ) to form peroxynitrite ( $ONOO^-$ ), or can be dismutated by superoxide dismutase (SOD) to form hydrogen peroxide ( $H_2O_2$ ). Hydrogen peroxide can be neutralized by catalase or by glutathione peroxidase (GPx), or can be transformed to a hydroxyl radical ( $\bullet OH$ ) by the Fenton reaction (not shown). GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione.



**Figure 2. Proposed degradative pathway of  $\alpha$ -(2,4-disulfophenyl)-N-tertbutylnitron (NXY-059).** The efficacy of NXY-059 in SAINT I (but not in SAINT II; see text for details) may have been caused by its hydrolysis in storage to t-butylhydroxylamine (NtBHA), and/or by the oxidation of NtBHA to 2-methyl-2-nitrosopropane (MNP). MNP can be reduced back to NtBHA, can synthesize NO, or can neutralize ROS.

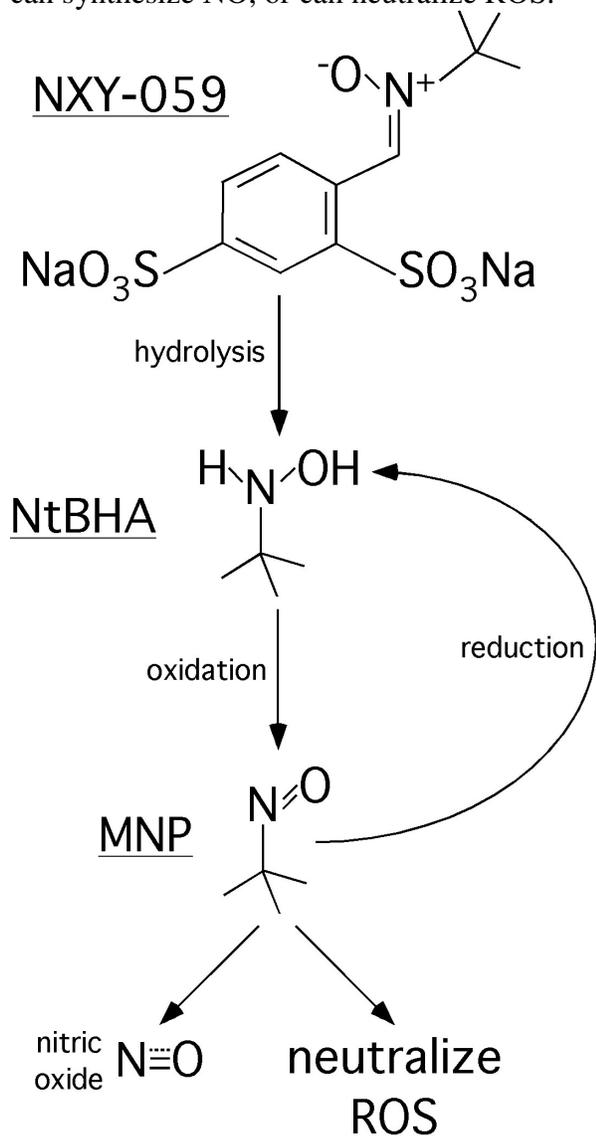
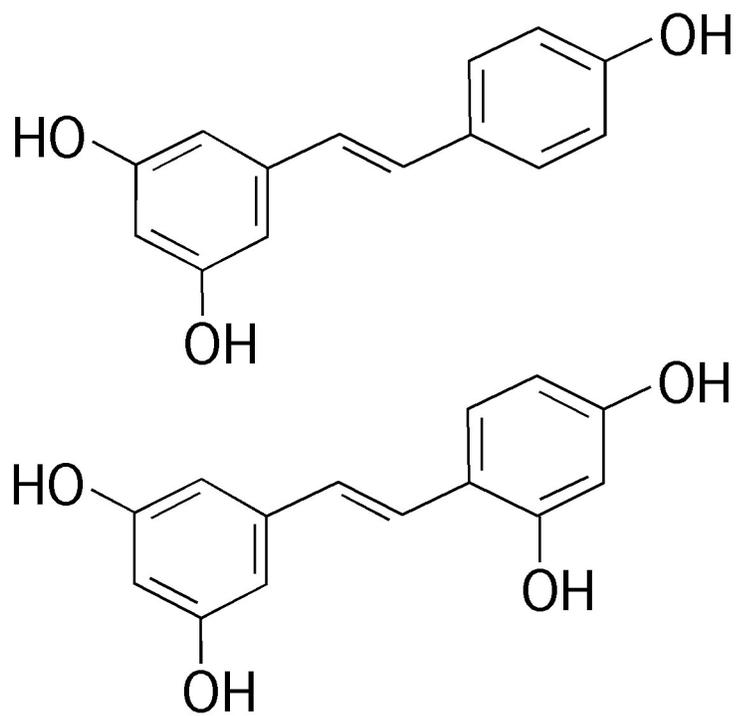


Figure 3. Structures of free radical scavengers derived from natural products.



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