Nutraceutical Antioxidants and Their Therapeutic Potential in Neurodegeneration

Erika Kristine Ross
University of Denver

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Abstract
Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disease that affects motor neurons of the brain and spinal cord. Many studies indicate that mitochondrial oxidative stress (MOS) is a principal mechanism underlying the pathophysiology of this and other devastating neurodegenerative diseases. Here, we investigated a unique whey protein supplement (Immunocal®) to determine its neuroprotective efficacy in several in vitro models of MOS and in an in vivo mouse model of ALS. This non-denatured whey supplement contains cystine which is an oxidized form of cysteine, an essential precursor for synthesis of the endogenous antioxidant, glutathione (GSH). In primary cultured rat cerebellar granule neurons (CGNs), pre-incubation with Immunocal® completely protected against MOS induced by HA14-1, an inhibitor of the pro-survival Bcl-2 protein. This effect was prevented by co-incubation with the gamma-glutamyl cysteine ligase inhibitor, buthionine sulfoximine, demonstrating that the de novo synthesis of GSH underlies the neuroprotective mechanism of Immunocal®. Additionally, Immunocal® displayed significant protection against an array of MOS-inducing agents, including sodium nitroprusside, copper, and aluminum, supporting its ability to upregulate mitochondrial antioxidant capacity. In accordance with these findings in CGNs, Immunocal® decreased cell death due to both H2O2 and glutamate toxicity in NSC34 motor neuron-like cells. Immunocal® also significantly protected CHO cells from MOS evoked by overexpression of amyloid precursor protein (APP). Immunocal® treatment in NSC34 motor neuron-like cells decreased cell death caused by both H2O2 and glutamate glycine. Most compelling are our findings in the hSOD1G93A mouse model of ALS. These mice were given Immunocal® (3.33% solution in drinking water) ad libitum, beginning at 60-days-old. Although no effect on overall survival was observed, Immunocal®-treated mice displayed a significant (7 ±1.08 day) delay in disease onset, compared to mutant control mice. Importantly, Immunocal®-treated mice showed a highly significant decrease in the rate of decline in grip strength. Finally, using HPLC-ECD we found that whole blood and lumbar spinal cord GSH levels were each depleted by nearly 50% in end-stage hSOD1G93A mice, and these reductions were essentially prevented in mutant mice receiving Immunocal®. These findings suggest that sustaining GSH by supplementation with Immunocal® may help to mitigate the progression of ALS through suppression of MOS.

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Second Advisor
David Patterson

Third Advisor
Keith Miller
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NUTRACEUTICAL ANTIOXIDANTS AND THEIR THERAPEUTIC POTENTIAL IN NEURODEGENERATION

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A Thesis
Presented to
the Faculty of Natural Science and Mathematics
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In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

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by
Erika K. Ross
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Advisor: Daniel A. Linseman
Author: Erika K. Ross  
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Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disease that affects motor neurons of the brain and spinal cord. Many studies indicate that mitochondrial oxidative stress (MOS) is a principal mechanism underlying the pathophysiology of this and other devastating neurodegenerative diseases. Here, we investigated a unique whey protein supplement (Immunocal®) to determine its neuroprotective efficacy in several in vitro models of MOS and in an in vivo mouse model of ALS. This non-denatured whey supplement contains cystine which is an oxidized form of cysteine, an essential precursor for synthesis of the endogenous antioxidant, glutathione (GSH). In primary cultured rat cerebellar granule neurons (CGNs), pre-incubation with Immunocal® completely protected against MOS induced by HA14-1, an inhibitor of the pro-survival Bcl-2 protein. This effect was prevented by co-incubation with the gamma-glutamyl cysteine ligase inhibitor, buthionine sulfoximine, demonstrating that the de novo synthesis of GSH underlies the neuroprotective mechanism of Immunocal®. Additionally, Immunocal® displayed significant protection against an array of MOS-inducing agents, including sodium nitroprusside, copper, and aluminum, supporting its ability to upregulate mitochondrial antioxidant capacity. In accordance with these findings in CGNs, Immunocal® decreased cell death due to both H$_2$O$_2$ and glutamate toxicity in NSC34 motor neuron-like cells. Immunocal® also significantly protected CHO cells
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Chapter 1: Introduction

1.1. Oxidative stress and neurodegenerative disease

Mitochondrial oxidative stress (MOS) and dysfunction are major factors underlying the pathophysiology of several devastating neurodegenerative disorders including Parkinson’s disease (PD), Alzheimer’s disease (AD), and amyotrophic lateral sclerosis (ALS) [1]. Reactive oxygen species (ROS) are generated in substantial quantities during normal cell metabolism, in particular, at the level of mitochondria as a byproduct of electron transport and ATP production. ROS can play essential roles in cell signaling; however, when these free radicals are produced in excess of antioxidant defense mechanisms, cell damage and death will follow [2,3]. The brain is especially vulnerable to oxidative damage because of its high lipid content and high rate of oxygen consumption. As a result of oxidative damage, region-specific neuronal cell death is an underlying cause of each of the above-mentioned neurodegenerative diseases [4].

There is mounting evidence suggesting that deficiencies in the electron transport chain (ETC) and subsequent oxidative stress at the level of the mitochondria are integral factors in a variety of neurodegenerative diseases [5,6]. ETC deficits lead to decreased ATP production and increases in ROS [7]. More specifically, oxidative damage causes complex I deficiencies in the ETC and further increases ROS.
production in PD [8]. For instance, complex I deficiency and the consequent increase in mitochondrial ROS play a critical role in the death of dopaminergic neurons in PD [9]. Consistent with this concept, mice heterozygous for deletion of the mitochondrial-specific, manganese-superoxide dismutase (SOD2) gene show markedly increased susceptibility to dopaminergic neuronal loss induced by the complex I inhibitor, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine [10]. Pathophysiologically relevant complex I inhibitors such as rotenone and N-methylpyridinium ion (MPP+) generate free radicals and induce dopaminergic pathology which is used to model PD in vitro and in vivo for studies into potential therapeutics [11,12]. Methods aimed at bolstering endogenous antioxidant defense mechanisms to scavenge free radicals generated by these toxic compounds during disease progression hold therapeutic potential for neurodegenerative diseases such as PD.

Additionally, amyloid-β precursor protein (AβPP) aggregates, which are a pathological hallmark in AD, have been shown to induce MOS and cytochrome c release in vitro and in vivo [13,14]. Aβ accumulation and oligomerization has been proposed to lead to mitochondrial dysfunction and caspase activation [15]. In either patients with AD or mutant AβPP transgenic mice, beta-amyloid associates with the mitochondrial Aβ-binding alcohol dehydrogenase (ABAD) and triggers an enhanced generation of mitochondrial ROS [16]. Moreover, overexpression of ABAD in mutant AβPP expressing mice increases neuronal oxidative stress and worsens spatial memory deficits. Conversely, crossing of Tg19959 mutant AβPP transgenic mice
with mice overexpressing SOD2 significantly reduces amyloid plaque burden and sustains spatial memory relative to Tg19959 control mice [17]. Healthy and functional mitochondria are imperative for defense against Aβ-induced oxidative stress; therefore, therapies that enhance intrinsic antioxidant activity have promise for this particular neurodegenerative disease [18]. More specifically, strategies that protect cells at the level of the mitochondria appear to have protective potential in these diseases [19].

1.2. GSH is a key endogenous antioxidant

One of the crucial modulators of cell survival, under both normal and pathological conditions, is the endogenous tri-peptide glutathione (GSH, γ-glutamyl-L-cysteinyl-glycine). GSH is involved in many essential cell processes including DNA synthesis and cell metabolism, but its primary function is to act as an antioxidant and scavenger of ROS [20]. GSH exists in a reduced and active state and is oxidized to glutathione disulfide (GSSG) upon reducing unstable molecules, such as ROS. The reduction of ROS by GSH is facilitated by the enzyme GSH peroxidase (GPx) [21]. The enzyme, glutathione reductase (GR), then returns GSSG to its reduced form GSH (Fig. 1) [22].
Biosynthesis of GSH happens in two distinct steps. The first occurs when γ-glutamylcysteine ligase (GCL) combines glutamate and cysteine together. This reaction is the rate-limiting step in GSH synthesis. The second step in GSH synthesis occurs when GSH synthetase adds glycine to the C-terminus of the end product of the first step, γ-glutamylcysteine. Cysteine is the rate-limiting precursor in GSH synthesis (Fig. 2) [22-24]. Because of its potent antioxidant activity, and its neuroprotective capacity demonstrated in many in vitro and in vivo systems (discussed below), strategies that enhance GSH synthesis have drawn attention as potentially novel therapies for neurodegenerative diseases [reviewed in 25].
1.3. Neuroprotective effects of GSH and GSH precursors *in vitro* and *in vivo*

Several strategies to increase levels of endogenous GSH have previously shown to be effective in disease treatment *in vitro* and *in vivo*. N-acetylcysteine (NAC) supplementation provides cysteine in a cell-permeable and non-toxic form, and has been studied for its efficacy in various neurodegenerative and progressive human diseases [26-28]. NAC supplementation decreased ROS levels and increased cell antioxidant potential against rotenone-induced apoptosis *in vitro* and in a rat model of PD *in vivo*. Cell survival was significantly increased in NAC-treated cells and animals in these models of PD, suggesting that NAC reduced toxicity with its ability to decrease oxidation of cellular GSH and subsequently decrease ROS [29]. NAC treatment in SHSY5Y cells has also been shown to decrease levels of Aβ 1-42, and to...
decrease the levels of phospho-tau protein, which are hallmarks in AD [30]. Finally, NAC has been investigated in a model of ALS, where it delayed clinical onset and prolonged survival in hSOD1<sup>G93A</sup> mice, a familial model of ALS [31].

In addition to NAC, other means of increasing GSH levels have been investigated for prevention and treatment of various neurodegenerative diseases, though the research is not as comprehensive as with NAC. Glutathione monoethylester (GSH-MEE) is a cell permeable derivative of GSH. Its efficacy has been investigated in several murine models of neurodegeneration. GSH-MEE has been shown to prevent mitochondrial GSH depletion and to provide neuroprotection following transient focal cerebral ischemia [32,33]. Additionally, GSH-MEE has been shown to improve recovery and survival of spinal cord neurons in a model of spinal cord injury in rats [34]. These approaches, among other methods of increasing intracellular GSH, are unique and deserve further investigation in the lab and in the clinic.

1.4. GSH depletion plays a central role in ALS pathogenesis

ALS is the most common adult-onset motor neuron disease. It is characterized clinically by progressive muscle weakness and atrophy, ultimately leading to respiratory failure and death, typically within 1 to 5 years of diagnosis. There is currently only one FDA approved drug for ALS, riluzole, which is an anti-glutamatergic compound that merely prolongs survival by 2 to 3 months and has little effect on quality of life for ALS patients [35,36]. ALS is characterized pathologically by a progressive loss of motor neurons in the cortex, brainstem, and spinal cord [37].
Though its underlying cause remains elusive, oxidative damage due to aberrant production of ROS and associated mitochondrial dysfunction play key roles in motor neuron death [38,39,40].

In the case of ALS, mutant forms of copper, zinc-superoxide dismutase (SOD1) which are collectively responsible for approximately 20% of cases of familial ALS, accumulate at mitochondria and trigger a shift in the redox state of these organelles [41]. G93A mutant SOD1 displays a gain-of-toxic-function which is characterized by an increased generation of mitochondrial ROS [42]. Finally, crossing of hSOD1G93A mutant mice with mice heterozygous for deletion of the SOD2 gene significantly exacerbates motor neuron pathology and reduces lifespan in this model of familial ALS, indicating a central role of MOS in disease pathogenesis [43].

Given the key role of MOS in neuronal cell death, identifying the major players that regulate the mitochondrial oxidant/antioxidant balance is essential to discover novel therapeutics for neurodegeneration. Glutathione (GSH) is an endogenous tripeptide antioxidant that plays a central role in preventing MOS and evading apoptosis [44]. GSH depletion and a decline in antioxidant enzyme activity have been observed in the erythrocytes of sporadic ALS patients with active disease [45]. This decrease in antioxidant defense enzyme activity and GSH metabolism has been correlated directly with the rate of disease progression [46]. Many characteristic pathological changes observed in ALS may be induced by intracellular GSH depletion. For example, the protein TAR DNA-binding protein 43 (TDP-43) forms cytoplasmic inclusions, which is one of the hallmark pathologies seen in sporadic
ALS patients. These TDP-43 inclusions can be recapitulated in cultured neurons subjected to GSH depletion [47]. Oxidative stress also causes accumulation and aggregation of mutant SOD1 in familial ALS, and these inclusions have been shown to decrease GSH content, which is attributed to disruption of mitochondrial function [48,49]. In agreement, SOD1 knockout causes oxidative stress and subsequent distal motor axonopathy, as well as significant GSH oxidation [50].

The familial ALS-associated mutant, hSOD1$^{G93A}$, has recently been shown to have significant effects on mitochondrial GSH in vitro. NSC34 motor neuronal cells stably expressing hSOD1$^{G93A}$ display a significant and selective decrease in mitochondrial GSH content when compared to parental NSC34 cells [51]. Furthermore, these NSC34/hSOD1$^{G93A}$ cells demonstrate significantly enhanced sensitivity to the GSH depleting agent, ethacrynic acid. In a similar manner, infection of NSC34 cells with adenoviral hSOD1$^{G93A}$ induces oxidative stress, mitochondrial dysfunction, and intrinsic apoptosis which are significantly alleviated by co-expression of the mitochondrial antioxidant enzymes SOD2 and GSH peroxidase-4 [52]. Finally, NSC34 cells expressing hSOD1$^{G93A}$ but not wild type SOD1, show marked morphological and functional alterations in mitochondria and exhibit a significant decrease in the reduced GSH to oxidized GSSG ratio in mitochondria following exposure to the inflammatory cytokines, tumor necrosis factor alpha and interferon gamma [53]. Thus, hSOD1$^{G93A}$ appears to sensitize motor neuronal cells to MOS and apoptosis by specifically diminishing the mitochondrial GSH pool. These cumulative data suggest a prominent role for GSH depletion in ALS pathogenesis and
therefore, there is great interest in developing a method to target this aspect of the
disease for ALS, other neurodegenerative diseases, as well as non-neuronal
progressive human diseases such as heart disease and cancer.

1.5. Nutraceutical modulation of GSH: Pre-clinical and clinical studies with

Immunocal®

Immunocal® is a novel undenatured whey protein supplement designed to
augment the available intracellular GSH pool. Cellular GSH concentrations are highly
dependent on the availability of cysteine, which is the limiting precursor in GSH
synthesis [54,21]. The cysteine precursor, cystine, occurs in high levels in
Immunocal® because the supplement is rich in serum albumin, alpha-lactalbumin,
and lactoferrin. These proteins have a significant number of cystine residues in this
undenatured preparation. In addition, the direct GSH precursor, glutamylcysteine, is
also present in the serum albumin fraction of the supplement. When cystine is
provided in this peptide form, it is readily cleaved and reduced to two cysteine
molecules within the target cell. This is significant, as cysteine supplementation alone
is cytotoxic [55]. Immunocal® was initially developed as a nutritional supplement to
increase immune system function after dietary amino acids were discovered to
increase immune reactivity [56]. It has been investigated in several human diseases,
and it is one of only a handful of nutritional supplements that are included in the
Physician’s Desk Reference (PDR). Immunocal® is comprised of natural food protein and is in the FDA category of generally recognized as safe (GRAS) [57].

Immunocal® was initially studied in various animal models or clinical disorders of immune system deficiency and cancer as an approach to increase the available GSH pool and increase cellular antioxidant and immune system defenses. Free radical generation is a key element of carcinogenesis and diets rich in antioxidants are believed to decrease one’s susceptibility to cancer [58,59]. Consistent with this, dietary whey protein decreased tumor burden in a mouse model of dimethylhydrazine-induced colon cancer [60]. Similarly, Immunocal® was shown to increase apoptosis in an in vitro model of human hepatoma cells by increasing cytotoxicity of specific chemotherapeutic agents such as baicalein, a flavone used for its anti-inflammatory and anti-proliferative properties in cancer cells [61]. In addition to its anti-tumor effects, this undenatured whey protein was shown to reverse weight loss in lung cancer patients receiving chemotherapy or radiotherapy [62]. Thus, Immunocal® provides anti-cancer activity while also preserving overall health, and its method of action may be applicable in many other human pathologies.

Immunocal® was also investigated as a potential antioxidant therapy in human immunodeficiency virus (HIV) infection [63]. Oxidative stress is a known activator of HIV replication and based on this, Immunocal® was tested as an approach to attenuate this stress by increasing intracellular GSH levels. In a small pilot study, Immunocal® administration over 3 months was shown to elevate blood GSH by 70%
in HIV-seropositive individuals back to normal values [64]. Moreover, this restorative effect on blood GSH was associated with parallel increases in bodyweight.

Oxidative stress plays a significant role in muscle weakness. Immunocal® administration for 3 months was shown to significantly increase muscle power and work capacity in comparison to individuals that received placebo treatment. Undenatured whey protein supplementation also significantly increased lymphocyte GSH levels compared to placebo (36% +/- 11%) [65]. Oxidative stress is also key in the pathogenesis of cystic fibrosis. It is well accepted that GSH is a major defense in lung diseases, such as cystic fibrosis, and treatment methods that augment the available pool of this antioxidant are continually investigated. In cystic fibrosis patients receiving Immunocal® for 3 months, there was a 46.6% increase in lymphocyte GSH levels compared to individuals receiving a placebo [66].

This dietary whey protein supplement was also investigated in diseases of aging, where it extended longevity by 6.3 months in mice that received the supplement late in life (specifically, over a 3 month period between 17 and 20 months of age). This lifespan extension in the mouse corresponds to approximately 25 human years. Although there was little investigation into the specific mechanism behind this effect, both heart and liver GSH levels were significantly increased in Immunocal®-fed mice [67]. To date, there has been minimal investigation into the efficacy of Immunocal® treatment in neurodegenerative diseases; however, with the wide array of human diseases that show promising results with this dietary supplement, it is encouraging for other progressive human diseases. Immunocal® holds potential to increase GSH
levels in many relevant disorders like neurodegeneration whose underlying pathology includes oxidative stress.

1.6. **Immunocal® patents**

Immunocal® has not been patented specifically for use in neurodegenerative diseases; however, it has been investigated in depth for several other human pathologies. Immunocal® appears to show beneficial effects in these diseases because of its capacity to act as a cysteine-donor, resulting in an increase of GSH levels in treated individuals. Initial studies with this undenatured whey protein isolate were conducted to investigate its efficacy in immune system deficiency. Immunocal® is highly effective in bolstering immune function, and was patented because of its ability to improve active systemic humoral immune response [68]. More recently, it was patented for use in HIV-seropositive individuals, in which it induces a decrease in viral load and an increase in immune function in affected individuals [69]. Finally, Immunocal® was patented as an anti-cancer therapy because of its ability to increase the efficacy of certain chemotherapy agents, to decrease the oxidative stress component of cancer, and to increase the body weight of patients who were receiving chemotherapy back to healthy levels [70]. This dietary supplement provides a unique approach to increase intracellular GSH levels, and there is much promise for Immunocal® in the treatment and prevention of neurodegenerative diseases.
1.7. Therapeutic approaches to modulate GSH in neurodegenerative disease

Based on extensive evidence that oxidative stress is a fundamental aspect of neurodegeneration, multiple strategies have been proposed to limit free radical damage to the nervous system. In particular, methods designed to augment intracellular GSH levels are of great interest, due to the evolving pool of literature indicating a key role of GSH depletion in neurodegenerative disease [reviewed in 71]. GSH does not penetrate the blood brain barrier (BBB) and therefore, therapies aimed at indirectly modulating GSH levels have been investigated, including the introduction of GSH precursors, analogs, and mimetics [72,73]. Other strategies, such as bolstering the expression of enzymes responsible for increases in GSH synthesis for example, are also gaining interest.

The NF-E2-related factor 2 (Nrf2) transcription factor regulates the expression of several prominent antioxidant defense proteins. Cellular GSH levels are dependent upon Nrf2 translocation into the nucleus to initiate the expression of key GSH synthesis enzymes including the catalytic and regulatory subunits of γ-glutamyl cysteine synthase (GCS) [74,75]. Nrf2 activation is therefore an interesting target to modulate GSH in an effort to decrease neuronal cell death in relevant neurodegenerative diseases. Several compounds that stimulate Nrf2 nuclear translocation and the subsequent increases in GSH synthesis have been investigated and have proven to be neuroprotective in vitro [76,77]. Consistent with these in vitro studies, Nrf2 overexpression in glial cells and subsequent GSH secretion from astrocytes increased survival and delayed disease onset in a familial mouse model of
ALS [78]. In a similar manner, stimulation of Nrf2-dependent GSH synthesis by synthetic triterpenoid compounds also delayed disease onset and enhanced survival in the SOD1<sup>G93A</sup> mouse model of ALS [79]. These studies suggest that a promising approach to Nrf2 activation is increasing GSH levels in the nervous system.

Glutamate transporter-associated protein 3-18 (GTRAP3-18) interacts with excitatory amino acid carrier 1 (EAAC1) at the plasma membrane. EAAC1 is a neuronal glutamate/cysteine transporter, responsible for regulating intracellular cysteine levels. It is suggested that GTRAP3-18 plays a dominant negative role in cysteine transport when it interacts with EAAC1 [80]. Accordingly, GTRAP3-18 deficient mice show increased intracellular GSH levels, and these mice have demonstrated a decreased sensitivity to oxidative stress and increased cognitive function [81]. Thus, inhibitors of GTRAP3-18 may be effective inducers of GSH accumulation in the nervous system.

Another technique to boost cellular GSH that has received attention is direct N-acetyl-L-cysteine (NAC) supplementation. This has been shown to increase the available cysteine pool and to decrease oxidative damage [82]. Wobbler mice that display lower motor neuron degeneration have shown reductions in neurodegeneration and retention of motor function when administered NAC. They also display increased GSH levels and increased muscle mass [83].
1.8. Modulation of GSH using Immunocal® as a neuroprotective and therapeutic strategy for ALS

Although the mitochondrial GSH content in particular, has not been measured in vivo in spinal cord of the hSOD1\textsuperscript{G93A} mouse, GSH depletion has been observed in the spinal cord of end-stage mice [84]. Moreover, crossing of hSOD1\textsuperscript{G93A} mice with mice lacking the glutamate-cysteine ligase modifier subunit significantly accelerated disease progression [85]. Conversely, a number of strategies that are predicted to increase GSH, such as supplementation with N-acetyl-L-cysteine or induction of the Nrf2 transcription factor, demonstrate significant benefits on disease onset, progression, and survival in this model of familial ALS [31,78,79]. In the current study, we investigated the neuroprotective effects of a undenatured whey protein supplement, Immunocal®, in vitro in several models of MOS and in vivo in the hSOD1\textsuperscript{G93A} mouse model of familial ALS [86].

1.9. Anthocyanins and neurodegeneration

Anthocyanins are a major sub-class of the extensive flavonoid family, and they are responsible for the distinctive red, blue, and purple pigments in many fruits and vegetables. Found in widely consumed foods, including berries, purple sweet potatoes, red rice, and grapes, they exist in relatively high concentrations compared to other dietary polyphenolic compounds [87]. Anthocyanins display a number of potential health benefits that warrant their investigation as potential clinical treatments for several human disorders. Due to their anti-inflammatory and
antioxidant properties, these novel nutraceuticals have proven effective in both *in vitro* and *in vivo* models of various chronic human conditions, including cardiovascular disease, obesity, atherosclerosis, ophthalmologic disorders, and type II diabetes [88-92].

The polyphenolic and cationic attributes of anthocyanins and their metabolites activate a variety of cellular responses. Anthocyanins consist of an aglycon component, or anthocyanidin, and a sugar moiety (glucose, galactose, or arabinose). This anthocyanin structure is more stable than its aglycon alone, specifically in acidic environments such as the stomach. The polyphenolic nature of anthocyanins is principally responsible for their strong antioxidant and free radical scavenging activities, and their overall metabolic benefits. There are many known anthocyanins varying in the anthocyanidin skeleton and the complexity of the attached glycoside (Fig. 3), implicating multiple pathways for absorption [93].
Recent bioavailability studies suggest that anthocyanins are absorbed rapidly from the stomach and small intestine, and they are highly metabolized resulting in very low to undetectable concentrations of the parent compound being observed in plasma within only a few hours of consumption [94,95]. The low concentrations of parent anthocyanins are seemingly paradoxical to the beneficial role displayed by anthocyanins in vivo. This issue has drawn interest to their specific mechanism of action, in particular which metabolites of anthocyanins produce their positive health effects [96,97].
1.9.1. Intrinsic antioxidant activity

Anthocyanins have a robust antioxidant capacity, which appears to be highly effective in several models of neurodegenerative disease [98-100]. They have a high oxygen radical absorption capacity (ORAC) value, which is partially responsible for this neuroprotective effect [101-102]. More specifically, anthocyanins act as antioxidants because of their ability to directly scavenge free radicals and to prevent ROS formation in affected cells. For example, anthocyanins decrease the formation of ROS in in vitro models of Aβ peptide-induced toxicity, as well as in H2O2 injury [99,103]. Additionally, using the highly accurate method of electron spin resonance (ESR) spectroscopy it has been shown that anthocyanins have a high affinity to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH), alkyl, and hydroxyl free radicals, and that this increases dose-dependently [103].

Anthocyanins activate the antioxidant response element (ARE), which controls expression of a large array of endogenous antioxidant and phase II detoxification genes [104]. This effect appears to be via activation of the Nrf2 transcription factor [103]. In addition, anthocyanins have been shown to enhance the activities of free radical scavenging enzymes including superoxide dismutase, catalase, and glutathione peroxidase [105-106]. Finally, levels of the endogenous antioxidant GSH have been shown to increase with anthocyanin treatment, suggesting that anthocyanins exert their protective effects in part, via modulation of endogenous antioxidant defenses, and not exclusively due to their intrinsic antioxidant activity [107].
1.9.2. Anti-inflammatory properties

Induction of inflammatory gene expression and subsequent production of interleukins and pro-inflammatory cytokines is a common feature in neurodegeneration. Methods that target these inflammatory processes may be beneficial in limiting neuronal apoptosis associated with disease. Anthocyanins exhibit significant anti-inflammatory properties, as they have been shown to inhibit various inflammatory biomarkers, including interleukin-8 (IL-8) [101]. In addition to decreased IL-8 production, pomegranate anthocyanins suppress activation of nuclear transcription factor kappaB (NFκB), which is responsible for the expression of several pro-inflammatory genes. They were also shown to inhibit mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), which are responsible for the expression of a number of pro-inflammatory cytokines [108]. Furthermore, cherry and blackberry anthocyanins act as potent cyclooxygenase-2 (COX-2) inhibitors; COX-2 is an important pro-inflammatory enzyme involved in the synthesis of prostacyclin [109,110]. Anthocyanins have been shown to inhibit up to 95% of COX activity at high concentrations (250 μg/mL) [111]. These cumulative data suggest that anthocyanins may be useful in attenuating inflammatory processes that are associated with neurodegenerative disease.
1.9.3. Anti-apoptotic properties

Anthocyanins modulate specific anti-apoptotic pathways to increase survival in neuronal cells. They have been demonstrated to regulate both caspase-dependent and caspase-independent cell death pathways [112]. It is mainly by acting on a variety of signaling proteins that they promote cell survival. Anthocyanins from purple sweet potato were shown to suppress JNK activity, release of cytochrome c from mitochondria, and cell apoptosis in D-galactose-treated mice. Additionally, it was demonstrated that this protective effect was dependent on phosphatidylinositol 3-kinase (PI3K) an upstream activator of pro-survival Akt [106,113]. Anthocyanins have also been shown to increase the expression of pro-survival proteins such as Bcl-2 and to decrease levels of the pro-apoptotic protein Bax, effects which play a significant role in prevention of 6-hydroxydopamine (6-OHDA)-induced toxicity, a model of PD [114].

1.10. Anthocyanin absorption and blood brain barrier permeability

Intact anthocyanins taken orally are absorbed rapidly into many relevant tissues [115,116]. There is considerable interest into the mechanism behind this rapid absorption, as revealing this process could lead to further development of these nutraceuticals for treatment of many pertinent diseases. It is likely that absorption initially occurs in the stomach, which accounts for the rapid kinetics of anthocyanins appearing in plasma [117]. There is accumulating data suggesting that anthocyanin transport occurs via a bilitranslocase transporter, primarily into the vascular
endothelium, and subsequently into target tissue [118]. Despite their rapid uptake into the systemic circulation, the overall bioavailability of anthocyanins is very low due to their rapid metabolism and excretion principally as glucuronidated, methylated, and glycosylated species [119].

Anthocyanins are present in brain endothelial cells and in brain parenchymal tissue of rodents after one treatment, suggesting that they are capable of crossing the blood brain barrier (BBB) [120]. They are rapidly transported to the brain after ingestion, and once there they may accumulate to concentrations up to 0.21 nmol/g of tissue [121]. It is posited that anthocyanins enter the brain in a similar fashion to their absorption in the stomach and GI tract. There is a bilirubin-binding motif in biliranslocase in the CNS, which is likely the transporter involved in transporting anthocyanins into the CNS [122]. This interaction occurs between the anthocyanin and the biliranslocase due to hydrogen bonds and not charge, which provides further selectivity for this transport mechanism [123]. Although they do penetrate the BBB, the concentrations of parent anthocyanins detected in brain tissue after oral administration fall well below the concentrations which display significant antioxidant and neuroprotective effects in vitro. This finding suggests that metabolites of anthocyanins may also play significant neuroprotective roles in vivo. Moreover, the ability of chronic anthocyanin administration to induce accumulation of anthocyanins in brain has not been studied. Ultimately, there is very little known about anthocyanin transport, specifically for BBB permeation, or the possible
accumulation of anthocyanins or their metabolites in brain after chronic oral dosing. Both of these areas warrant further investigation.

1.11. Anthocyanin metabolites in neurodegeneration

There are several anthocyanin metabolites that show promise for prevention and treatment in neurodegenerative disease. Anthocyanin metabolites may be responsible for the high bioactivity of anthocyanin compounds, despite their apparently low bioavailability. The various identified metabolites are more stable than the whole anthocyanins in a variety of conditions, which make them more likely candidates as the bioactive moieties in target tissues [96,97]. Recent research suggests that different anthocyanin conjugates are found in high concentrations in circulation, including sulfated, methylated, glucuronidated, and glycosylated conjugates [124].

Protocatechuic acid (PCA) and the anthocyanidin base are common anthocyanin metabolites (Fig. 4). During metabolism, the anthocyanidin in its aglycone form has a tendency to stay intact and retain its basic function. From there, conjugates of the anthocyanin are readily formed [125]. PCA concentrations have been detected up to eight times higher than the parent anthocyanin in rodent plasma. PCA has also been shown to remain in target tissues for longer periods of time than the parent anthocyanins [126].
Significantly, both PCA and the anthocyanidin base provided significant protection and decrease of ROS formation in SH-SY5Y neuronal cells treated with H₂O₂ [127]. Finally, PCA induced the Nrf2 transcription factor in murine macrophages; whether similar effects would be observed in neuronal or glial cells has not been investigated [128]. There are relatively few studies into the neuroprotective properties of isolated anthocyanin metabolites; however, with the pool of data suggesting their relevant concentrations and accumulation in tissue, it is important to further investigate their neuroprotective effects both *in vitro* and *in vivo* [129].
1.12. Hypothesis and rationale

Given the recent interesting research involving GSH modulation as a method to moderate neurodegeneration, we pursued nutraceuticals that could potentially provide neuroprotection by this mechanism. Nutraceutical intervention is an appealing option because of the relatively low side effects and high availability for patients; therefore, we decided to investigate the efficacy of the whey protein supplement, Immunocal®, which had yet to be studied in models of neurodegeneration. This protein isolate was interesting due to its high purity and undenatured conformation, yielding a greater concentration of active cystine that could be converted to cysteine, which is the limiting precursor in GSH synthesis. We tested its neuroprotective potential both in vitro, and eventually in vivo in the hSOD1<sup>G93A</sup> mouse model of ALS.

In addition to our interest in modulating the available GSH pool with nutraceutical intervention, we investigated natural compounds that could bolster cellular antioxidant response by different mechanisms. There is an increasing pool of literature (discussed above) that points to anthocyanins decreasing cellular apoptosis in disease models. This was novel, as anthocyanins have never been investigated for their potential in motor neuron disease. These two separate nutraceutical interventions had the potential to increase cellular antioxidant defenses through different mechanisms, and they were very appealing as new therapeutic strategies for neurodegenerative disease.
1.13. Summary of major findings

Using *in vitro* and *in vivo* models of neurodegeneration, we show here that Immunocal® protected CGNs from various sources of oxidative stress. Immunocal® decreased cell apoptosis in CGNs treated with the Bcl-2 inhibitor, HA14-1, and this protective effect was diminished by the inhibitor of *de novo* GSH synthesis, BSO. Additionally, Immunocal® treatment rescued CGNs from CuCl$_2$, AlCl$_3$, and sodium nitroprusside (SNP)-induced apoptosis. Furthermore, Immunocal® decreased active caspase-3 expression in CHO cells that were transfected with wild type APP. Immunocal® also decreased cell death caused by both H$_2$O$_2$ and glutamate glycine in the motor neuron-like cell line, NSC34s. Next, we evaluated its potential benefit in ALS by administering it to hSOD1$^{G93A}$ mice. Consistent with prior findings, in which Immunocal® protected neurons *in vitro* from MOS via a GSH-dependent mechanism, this supplement also preserved blood and spinal cord GSH levels in end-stage hSOD1$^{G93A}$ mice, delayed disease onset, and sustained grip strength in this model of familial ALS.

After isolating an anthocyanin-enriched extract from freeze dried strawberries (SAE), we tested its efficacy in the hSOD1$^{G93A}$ mouse model of ALS. SAE administration delayed disease onset, increased lifespan, and sustained grip strength in this model of familial ALS. In summary, both Immunocal® and SAE displayed neuroprotective effects, likely due to their different effects on bolstering both exogenous and endogenous antioxidant defenses.
Chapter 2: Materials and Methods

2.1. Materials

2-amino-6-bromo-α-cyano-3-(ethoxycarbonyl)-4H-1-benzopyran-4-acetic acid ethyl ester (HA14-1), and sodium nitroprusside (SNP) were obtained from Calbiochem (San Diego, CA). DL-buthionine-sulfoximine (BSO), 4,6-diamidino-2-phenylindole (DAPI), Hoescht dye 33258, and a monoclonal antibody against β-tubulin were from Sigma Aldrich Co. LLC (St Louis, MO). The polyclonal antibody for active caspase-3 was from Promega (Madison, WI). Cy3- and FITC-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). HPLC-grade methanol, acetonitrile, and ethyl acetate were obtained from Fisher Scientific (Pittsburg, PA).

2.2. Cell culture, transfection, and treatment

Rat cerebellar granule neurons (CGNs) were isolated as previously described from 7-day-old Sprague-Dawley rat pups of both sexes [130]. CGNs were seeded on 35-mm diameter plastic dishes coated with poly-L-lysine at an average density of 2.0 x 10^6 cells/mL in basal modified Eagle’s medium containing 10% fetal bovine serum, 25 mM KCl, 2 mM L-glutamine, and penicillin (100 units/mL)/streptomycin.
(100μg/mL). Cytosine arabinoside (10 μM) was added to the culture medium 24h after plating. Experiments were performed after 6 days in culture. Immunocal® (3.3% w/v) was added in serum-free medium for 24h before treatment with the specified cell insult (i.e., SNP, HA14-1, etc.) for a further 24h. NSC34 motor neuron-like cells were seeded in a 6-well plate at an approximate density of 1.0 x 10^6 cells/mL and maintained in DMEM high glucose medium containing 10% fetal bovine serum, 2 mM L-glutamine, and penicillin (100 units/mL)/ streptomycin (100μg/mL). For serum withdrawal, NSC34s were maintained in DMEM high glucose medium containing 1% fetal bovine serum, 2 mM L-glutamine, and penicillin (100 units/mL)/ streptomycin (100μg/mL) for 7 days prior to being seeded in a 6-well plate at an approximate density of 1.0 x 10^6 cells/mL and subsequent experimental treatment. Immunocal® (3.3, 1.6, 1.1 % w/v) was added in culture medium for 24h before treatment with the specified cell insult (i.e. H₂O₂, glutamate glycine) in NSC34s. For transient transfection, CHO cells were seeded in a 6-well plate at an approximate density of 1.0 x 10^6 cells/mL and then cultured for 24 hours in Ham’s F12 medium containing 10% fetal bovine serum, 2 mM L-glutamine, and penicillin (100 units/mL)/ streptomycin (100μg/mL). Lipofection (5 μg DNA, 5 μL lipofectamine) was performed in serum-free medium for 6h to express vectors encoding DsRed-tagged wild type APP or DsRed. Cells were incubated for 24h post-transfection in either the absence or presence of Immunocal®.
2.3. Cell viability, lipid peroxidation, and cellular GSH assay

All assays were performed according to commercially available kit instructions. GSH/GSSG assay kit was purchased from Oxford Biomedical Research (Oxford, MI). MTT cell viability assay was from BioAssay Systems (Hayward, CA). Malondialdehyde (MDA) lipid peroxidation assay was obtained from OXIS Research Inc. (Foster City, CA).

2.4. Immunocytochemistry

After treatment, CGNs were fixed in 4% paraformaldehyde for 1h, washed once in PBS, and then permeabilized and blocked in 0.2% Triton X-100 and 5% BSA. Primary antibodies were diluted in 2% BSA and 0.2% Triton X-100 in PBS, and cells were incubated with primary antibodies for 24h at 4 °C. They were then washed 5 times in PBS and then incubated for 1h with either Cy3- or FITC-conjugated secondary antibody diluted in 2% BSA and 0.2% Triton with DAPI. The cells were washed 5 times with PBS before the addition of anti-quench solution (0.1% p-phenylenediamine in PBS). Images were captured using a Zeiss Axiovert-200 incerted fluorescence microscope (Stuttgart, Germany).

2.5. Animal model of ALS

FVB-Tg(hSOD1-G93A) mice with the toxic gain of function hSOD1 Gly93 to Ala mutation (G93A) were obtained from The Jackson Laboratory (Bar Harbor, ME). The colony was maintained and bred in the animal facility at the University of Denver. Animal genotyping was done by the third party company, Transnetyx
Animals of both sexes were included in each group, with an age and sex-matched non-transgenic FVB (NonTG) mouse as control. Animals receiving supplementation had ad libitum access to Immunocal® (3.3%) in drinking water beginning at 60 days old. Animals receiving SAE were administered a 5% solution via oral gavage twice daily.

2.6. Clinical tests

PaGE (paw grip endurance) test was determined by the latency of the animal’s hind limbs to detatch from a conventional housing wire cage lid after inversion, and was recorded as the average of 3 separate attempts. PaGE test was carried out twice weekly [131]. Animals were weighed every other day starting on day 30. End-stage was determined by a failure of the animal to right itself to sternum in 20s. Onset was determined by the appearance of trembling in one or two hind limbs or failure to extend one hind limb when suspended by the tail. All animal experiments were performed according to the University of Denver and IACUC approved protocol.

2.7. Tissue processing

Lumbar spinal cord and whole trunk blood were obtained from end-stage hSOD1<sup>G93A</sup> or age and sex-matched NonTG mice following isoflurane overdose, and were immediately frozen in liquid nitrogen (-196 °C). For HPLC-EC analysis, trunk blood was weighed and 300 µL of HCl was added to the sample and vortexed rapidly. It was next centrifuged at 10,000 g for 20 minutes at 4 °C. The supernatant was removed and added to a new tube containing 10% perchloric acid (PCA), which was
centrifuged for 10 minutes at 10,000 g at 4 °C. A clear supernatant was removed and filtered. Lumbar spinal cord was weighed and added to 1 mL of 10% PCA and then vortexed 3 times for 15s each time. The supernatant was removed and filtered. Spinal cord GSH and GSSG were normalized to protein concentrations which were determined by a commercially available protein assay kit (BCA, Thermo Scientific, PA). Samples were analyzed in triplicate and an additional sample was spiked with GSH and GSSG to verify peak identification.

2.8. High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ECD)

GSH and GSSG in samples and known standards were separated by reversed-phase HPLC on a C18 bonded silica column at 35 °C (3µm, 3 x 150 mm for whole blood; 3µm, 3 x 250 mm for lumbar spinal cord) from Dionex, Inc. (Sunnyvale, CA). Analytes were detected using a CoulArray® detector (model 5600A, ESA) on three coulometric array cells in series; electrochemical detectors were set between 0 and 900 mV at increments of 100 mV. Concentrations were determined with a standard curve of each identified analyte. Mobile phase consisted of 125 mmol/L Na₃PO₄ and 1% methanol in water, pH 2.8 [132]. The flow rate was set to 0.4 mL/min for all samples. CoulArray® software was used for baseline correction and peak analysis.

2.9. Anthocyanin purification and isolation

Freeze dried strawberry powder was homogenized with methanol and HCl (0.01%) for 1h at 4 °C. The slurry was then filtered through Whatman no. 1 filter
paper under vacuum filtration at 40 °C. The filtrate was then applied to a Waters® C18 cartridge, rinsed with water and HCl (0.1%), and ethyl acetate. The final fraction left on the column was eluted using methanol and HCl (0.01%), and this final fraction was evaporated at 40 °C and re-suspended in water [133]. To confirm the purity of the final strawberry anthocyanin fraction, a sample was run on HPLC-UV with an Agilent model HP1100 series II with multi-wavelength detector (MWD) set to 520 nm (Avondale, PA), using a reversed phase C18 bonded silica column (3µm, 3 x 150 mm) from Dionex, Inc. (Sunnyvale, CA). 100 μL samples were injected and run at a flow rate of 0.6 mL/min, and the mobile phase consisted of 0.1% TFA in water and acetonitrile that were mixed as a gradient. The sample was compared to known anthocyanin standards.
2.10. Statistical analysis

Each in vitro experiment was performed in duplicate and repeated a minimum of three times; data are reported as mean ± standard error of the mean. Statistical significance was analyzed with a one-way analysis of variance (ANOVA) followed by post hoc Tukey’s test. Survival and onset data were analyzed with Kaplan-Meier curves and log rank test with an n value of 13 for each treatment group.
Chapter 3: Results

3.1. Immunocal® preserves cellular GSH and decreases apoptosis in CGNs exposed to the Bcl-2 inhibitor, HA14-1

Figure 6. Immunocal® preserves cellular GSH and reduces apoptosis in CGNs exposed to the Bcl-2 inhibitor, HA14-1. A. Representative images of CGNs left untreated (control), treated with HA14-1 (15 μM), or pre-incubated for 24h with Immunocal® before HA14-1 treatment for a further 24h. Panels from left to right, pseudocolored dapi, pseudocolored β-tubulin, merged images showing β-tubulin, (green) and DAPI, (blue). Scale bar, 10 μm. B. Quantification of apoptosis for 4 independent experiments performed as in A except some cultures were pre-incubated with 200 μM BSO as well. Apoptotic cells were those with condensed or fragmented nuclei. Results are shown as mean +/- SEM, n=4. *** indicates p<.001 compared to control, ††† indicates p<.001 compared to HA14-1, ‡‡‡ indicates p<.001 compared to ICAL + HA14-1. C. CGNs were treated exactly as described in B. Total cellular GSH was measured as described in Materials and Methods. Data shown represent the percent of control cellular GSH concentration, mean +/- SEM, n=4. *** indicates p<.001 compared to control, † indicates p<.05 compared to HA14-1, and ‡‡ indicates p<.01 compared to ICAL + HA14-1. Significant differences were determined by one way ANOVA with a post hoc Tukey’s test. Abbreviations used: Con, control; ICAL, Immunocal®; BSO, Buthionine sulfoximine.
Primary CGNs were initially incubated with Immunocal® for 24h, followed by a 24h exposure to the BH3 mimetic, HA14-1 (15μM) as we have previously reported [134-136]. HA14-1 induced marked nuclear condensation and microtubule disruption (Fig. 6A) indicative of apoptosis (Fig. 6B) while also causing significant depletion of GSH (Fig. 6C). Immunocal® significantly protected CGNs from apoptosis induced by HA14-1 and significantly preserved GSH levels. To confirm that the mechanism of protection was due at least in part, to enhanced GSH synthesis, CGNs were co-treated with Immunocal® and the γ-glutamyl cysteine synthetase-inhibitor, buthionine sulfoximine (BSO), which prevents GSH synthesis [137]. Co-incubation with Immunocal® and BSO for 24h before HA14-1 treatment completely prevented any protective effect that Immunocal® alone displayed against HA14-1 (Fig. 6B). Moreover, the capacity of Immunocal® to preserve cellular GSH levels upon HA14-1 exposure was eliminated by BSO co-treatment (Fig. 6C). These data indicate that Immunocal® protects CGNs from MOS induced by the inhibition of Bcl-2 via an enhanced *de novo* synthesis of GSH.

3.2. Immunocal® protects CGNs from CuCl₂-induced oxidative damage and decreases cellular lipid peroxidation

To further investigate the neuroprotective potential of Immunocal® in primary neurons, we used copper chloride (CuCl₂) as a model of MOS. Copper overload is associated with free radical-induced fragmentation of mitochondrial cardiolipin and reduction in mitochondrial dehydrogenase and electron transport
chain complex activities [138,139]. This transition metal is known to generate reactive oxygen species (ROS) such as hydrogen peroxide (H$_2$O$_2$) \textit{in vitro}, and has been reported in abnormally high concentrations in several neurodegenerative diseases [140-141]. In addition, a central role for copper in the motor neuron death underlying ALS has been suggested [142].

Figure 7. Immunocal® decreases CuCl$_2$-induced apoptosis and lipid peroxidation in CGNs. A. Representative images of CGNs left untreated (control), treated with CuCl$_2$ (50 μM), or pre-incubated with Immunocal® for 24h before CuCl$_2$ treatment for a further 24h. Immunocytochemistry shows β-tubulin (green), and DAPI (blue). Scale bar, 10 μm. B. Quantification of apoptosis for 4 independent experiments performed as in A. Results are shown as mean +/- SEM, n=4. ** indicates p<.01 compared to control, †† indicates p<.01 compared to CuCl$_2$. C. Cellular lipid peroxidation (malondialdehyde, MDA) was measured as described in Materials and Methods. Results are shown as mean +/- SEM, n=5. ** indicates p<.01 compared to control, ††† indicates p<.001 compared to CuCl$_2$. Abbreviations used: Con, control; ICAL, Immunocal®.
Fluorescence analysis of the microtubule network revealed robust protection from this transition metal in CGNs pre-treated with Immunocal® (Fig. 7A). Quantification of apoptotic cells revealed that there was a significant reduction in CGN apoptosis with Immunocal® pre-treatment compared to CGNs treated with CuCl₂ alone (Fig. 7B). The antioxidant effect of Immunocal® was confirmed with a lipid peroxidation assay which revealed a significant decrease in malondialdehyde content in CGNs pre-treated with Immunocal® (Fig. 7C).
3.3. Immunocal® protects CGNs exposed to SNP-generated nitric oxide species and from AlCl₃-induced neurotoxicity

Figure 8. Immunocal® preserves CGN viability and protects from apoptosis after treatment with SNP. A. Representative images of CGNs left untreated (control), treated with SNP (100 μM), or pre-incubated with Immunocal® for 24h before SNP treatment for a further 24h. Immunocytochemistry shows β-tubulin (green), and DAPI (blue). Scale bar, 10 μm. B. Quantification of apoptosis for 5 independent experiments performed as in A. Results are shown as mean +/- SEM, n=5. C. MTT cell viability was measured as described in Materials and Methods. Results are shown as mean +/- SEM, n=3. For B and C, *** indicates p<.001 compared to control, ††† indicates p<.001 compared to SNP. Abbreviations used: Con, control; ICAL, Immunocal®.

SNP is a nitric oxide donor that causes dissipation of the mitochondrial membrane potential and enhanced generation of mitochondrial ROS in cortical neurons and
CGNs [143-144]. As expected, nitric oxide species generated by SNP caused overt apoptotic cell death in CGNs which was significantly mitigated by pre-treatment with Immunocal® (Fig. 8A). Apoptotic cell counts confirmed that there was significant neuroprotection in CGNs pre-treated with Immunocal®, decreasing cell apoptosis by approximately 80% (Fig. 8B). An MTT cell viability assay demonstrated similar results and showed that mitochondrial viability was also significantly preserved in Immunocal®-pre-treated cells, compared to CGNs treated with SNP alone (Fig. 8C).

Figure 9. Immunocal® protects CGNs from AlCl₃-induced toxicity A. Representative images of CGNs left untreated (control), treated with AlCl₃ (10 μM), or pre-incubated with Immunocal® for 24h before AlCl₃ treatment for a further 48h. Panels from left to right, pseudocolored DAPI, pseudocolored β-tubulin, merged image showing β-tubulin (green), and DAPI (blue). Scale bar, 10 μm. B. Cell survival was quantified for 4 independent experiments as described in A. Results
are shown as mean +/- SEM, n=4. *** indicates p<.001 compared to control, † indicates p<.05 compared to AlCl₃. Abbreviations used: Con, control; ICAL, Immunocal®.

Aluminum is a neurotoxic metal that impairs mitochondrial structure and function in neural cells exposed in vitro and in vivo [145,146]. AlCl₃-induced toxicity in CGNs was characterized by nuclear condensation and marked disruption of the microtubule network; these effects were markedly decreased in CGNs pre-treated with Immunocal® (Fig. 9A). To confirm that this protection was due to cysteine supplementation, and not metal chelation, we removed the Immunocal® after the pre-treatment period and washed the CGNs with serum-free media before treating with AlCl₃. Under these conditions, we still observed a significant increase in cell survival compared to CGNs treated with AlCl₃ alone (Fig. 9B).

3.4. Immunocal® prevents caspase-3 cleavage in CHO cells transfected with WT APP

To explore the role of Immunocal® in a different cell model, we transiently transfected CHO cells with wild type (WT) APP. We have recently shown that overexpression of WT APP induces GSH-sensitive opening of the mitochondrial permeability transition pore and intrinsic apoptosis in CHO cells [13]. Both control cells transfected with an empty DsRed plasmid, as well as CHO cells overexpressing DSRed-WT APP that were incubated with Immunocal® post-transfection, expressed very low levels (10-20%) of active caspase-3. In contrast, untreated cells overexpressing WT APP showed a marked
increase in immunoactivity for active caspase-3 to approximately 50% (Fig. 10A). Quantification of active caspase-3 revealed that there was a significant decrease in CHO cell apoptosis in Immunocal®-treated cells compared to non-treated CHO cells overexpressing WT APP (Fig. 10B).

Figure 10. Caspase-3 activation induced by wild type APP overexpression in CHO cells is decreased with Immunocal® treatment A. Representative images of CHO cells subject to wild type DsRed-APP overexpression in the absence or presence of Immunocal® incubation. Panels from left to right, pseudocolored DAPI, active caspase-3 (green), merged image showing active caspase-3 (green), and DsRed (red). Scale bar, 10 μm. B. Quantification of transfected CHO cells expressing active caspase-3. Results are shown as mean +/- SEM, n=4. *** indicates p<.001 compared to DsRed, † indicates p<.05 compared to APP. Abbreviations used: APP, amyloid precursor protein; ICAL, Immunocal®.
3.5. Immunocal® protects NSC34 motor neuron-like cells from H₂O₂ and glutamate glycine-induced toxicity

Glutamate excitotoxicity is thought to play a role in ALS, leading to neuronal damage and apoptosis. NSC34 motor neuron-like cells are used as a model for studying motor neuron disease; however, cells do not express functional glutamate receptors until cells are exposed to serum withdrawal for 7 days in culture and allowed to reach a higher level of maturity under these conditions rather than normal growing conditions. After this point cells become sensitive to glutamate excitotoxicity, which is an interesting in vitro model for ALS [147]. We show here that glutamate glycine (1 mM/100 μM) caused apoptotic cell death in NSC34s after serum withdrawal, which was significantly mitigated by pre-treatment with Immunocal®. An MTT cell viability assay demonstrated that mitochondrial viability was preserved in Immunocal®-pre-treated cells in a dose-dependent manner, compared to NSC34 cells treated with glutamate glycine alone (Fig. 11A).
Figure 11. Immunocal® protects NSC34 cells from glutamate glycine and H2O2-induced toxicity

A. Cell survival was quantified for 2 independent experiments in NSC34 left untreated (con), treated with glutamate glycine (1 mM/100 μM), or pre-incubated with Immunocal® for 24h before glutamate glycine treatment for a further 24h. B. Cell survival was quantified for 2 independent experiments in NSC34 left untreated (con), treated with H2O2 (250 μM), or pre-incubated with Immunocal® for 24h before H2O2 treatment for a further 24h. Results are shown as mean +/- SEM, n=2. Abbreviations used: Con, control; ICAL, Immunocal®; glu. gly., glutamate glycine.

H2O2-mediated cell apoptosis is a classic model of ROS in neuronal systems, as it generates free radicals that are hallmark in neurodegeneration [148].

As expected, ROS generated by H2O2 caused overt apoptotic cell death in NSC34s which was significantly mitigated by pre-treatment with Immunocal®.
An MTT cell viability assay demonstrated that mitochondrial viability was preserved in Immunocal®-pre-treated cells in a dose-dependent manner, compared to NSC34 cells treated with H$_2$O$_2$ alone (Fig. 11B).

3.6. Immunocal® delays disease onset but does not extend lifespan in the hSOD1$^{G93A}$ mouse model of ALS

After finding that Immunocal® provided significant neuroprotection in vitro from multiple insults, we investigated the effects of Immunocal® supplementation in the hSOD1$^{G93A}$ mouse model of ALS. Beginning at 60 days-of-age, mice received Immunocal® ad libitum in drinking water. Although there was no measurable difference in lifespan (Fig. 12A), Immunocal®-supplemented mice displayed a significant delay in disease onset of approximately 7 days (7 ±1.1 days) (Fig. 12B).

Mice were visually assessed for deficits in motor function and coordination daily, and by PaGE hanging wire grip test twice a week. PaGE testing revealed a highly significant decrease in the rate of decline in grip strength between 100 days and 120 days of age when Immunocal®-treated mice were compared to hSOD1$^{G93A}$ mice that did not receive supplementation (Fig. 12C). Thus, Immunocal® preserved grip strength and delayed onset of motor dysfunction in this mouse model of familial ALS.
Figure 12. Immunocal® delays clinical onset and diminishes the rate of decline in grip strength in the hSOD1G93A mouse model of ALS A. Median survival is not significantly different between hSOD1G93A mice receiving Immunocal® ad libitum and untreated mutant mice (n=13). B. hSOD1G93A mice receiving Immunocal® ad libitum displayed a delay in disease onset and clinical decline compared to untreated mutant mice (n=13). Onset curves are significantly different (p<.001) determined by Gehan-Breslow-Wilcoxon Test. C. PaGE hanging wire test represented as (mean ± SEM) latency to fall at indicated ages (n=13). *** indicates p<.001 compared to NonTG, ** indicates p<.01 compared to NonTG, † indicates p<.05 compared to hSOD1G93A mice (one-way ANOVA with a post-hoc Tukey’s test).
3.7. Immunocal® prevents GSH depletion in whole blood and lumbar spinal cord of hSOD1G93A mice

Figure 13. HPLC-EC detection reveals whole blood GSH is depleted in the hSOD1G93A mouse model of ALS and is preserved at NonTG levels in hSOD1G93A mice receiving Immunocal®. A. Mean GSH and GSSG concentrations and GSH/GSSG ratios from the whole blood of end stage trial animals, n=13. ** indicates p<.01, * indicates p<.05. B. Representative HPLC-EC chromatograms from the whole blood of end stage animals, peaks of interest include GSH oxidizing at 600 and 800 mV.

The neuroprotective effects of Immunocal® observed in vitro are largely due to its capacity to enhance GSH synthesis by providing the key precursors cystine and cysteine. To investigate this potential mechanism of action in vivo, whole blood and lumbar spinal cord tissue was collected from end-stage hSOD1G93A mice and analyzed for reduced GSH and oxidized GSSG using HPLC-ECD. We found that hSOD1G93A
mice that did not receive supplementation displayed a significant decrease in whole blood GSH when compared to nontransgenic littermate controls (NonTG). Levels of GSSG in whole blood did not significantly change; however, the ratio of GSH to GSSG in whole blood was significantly decreased in hSOD1\textsuperscript{G93A} mice compared to NonTG controls. Both the decreases in GSH and the reduction in the GSH/GSSG ratio were prevented in hSOD1\textsuperscript{G93A} mice receiving Immunocal\textsuperscript{®} (Fig. 13A). Representative HPLC chromatograms from these animals are shown in Figure 13B.

Most interestingly, we found that a similar pattern also existed for GSH levels in the lumbar spinal cord of study animals. Spinal cord GSH levels were decreased by approximately 50\%, GSSG levels were elevated two-fold, and the ratio of GSH to GSSG was significantly decreased in hSOD1\textsuperscript{G93A} mice compared to NonTG controls. Immunocal\textsuperscript{®} treatment prevented these changes in GSH and GSSG and maintained the GSH/GSSG ratio at a level that was indistinguishable from NonTG controls (Fig. 14A). These results indicate that preservation of whole blood and spinal cord GSH levels correlates with the beneficial effects of Immunocal\textsuperscript{®} supplementation in the mutant hSOD1\textsuperscript{G93A} mouse model of ALS. Representative chromatograms from these animals are displayed in Figure 14B.
3.8. Strawberry anthocyanin extract delays disease onset and extends lifespan in the hSOD1\(^{G93A}\) mouse model of ALS

In addition to our studies with Immunocal®, we investigated a strawberry anthocyanin extract (SAE) for its efficacy in the SOD1\(^{G93A}\) mouse. After isolating the extract, we confirmed that the major anthocyanin in the final isolate was callistephin at 9.3 min retention time using HPLC-UV analysis (Fig.15). We next began oral dosing with a 5% solution of SAE twice daily in the hSOD1\(^{G93A}\) mouse model of ALS.
Figure 15. HPLC-UV identification of major anthocyanin in strawberry extract. A. Chromatogram of callistephin (Pelargonidin-3-\textit{O}-glucoside) standard. B. Chromatogram of strawberry anthocyanin extract.

We saw a significant delay in disease onset, and a significant increase in survival in the hSOD1\textsuperscript{G93A} mice receiving SAE (Fig. 16A&B). Additionally, there was a significant retention in motor function in the hSOD1\textsuperscript{G93A} mice receiving SAE (Fig. 16C). The antioxidant properties of these nutraceuticals, particularly at the level of the mitochondria, appear to underlie their neuroprotective effects; however, further investigation into the mechanism behind this neuroprotection is
necessary in further exploration of these compounds as natural therapies for
neurodegenerative disease.

Figure 16. SAE delays clinical onset, increases survival, and diminishes the rate of decline in grip strength in the hSOD1<sup>G93A</sup> mouse model of ALS. A. Median survival is different between hSOD1<sup>G93A</sup> mice receiving Immunocal® ad libitum and untreated mutant mice (n=2). B. hSOD1<sup>G93A</sup> mice receiving Immunocal® ad libitum displayed a delay in disease onset and clinical decline compared to untreated mutant mice (n=2). C. PaGE hanging wire test represented as (mean ± SEM) latency to fall at indicated ages (n=2). *** indicates p<.001 compared to NonTG, ** indicates p<.01 compared to NonTG, † indicates p<.05 compared to hSOD1<sup>G93A</sup> mice, †† indicates p<.01 compared to hSOD1<sup>G93A</sup> mice, ††† indicates p<.001 compared to hSOD1<sup>G93A</sup> mice (one-way ANOVA with a post-hoc Tukey’s test).
Chapter 4. Discussion

Here, we show that Immunocal® elevates cellular GSH levels and provides neuroprotection in vitro. We also show that it delays disease onset, attenuates decline in motor function, and sustains whole blood and spinal cord GSH at NonTG levels in the hSOD1\textsuperscript{G93A} mouse model of ALS. However, previous research using nutraceutical antioxidants in neurodegenerative disease has translated to relatively nominal clinical efficacy. For instance, vitamin E initially received a great deal of attention for its antioxidant activity in cellular and murine models of ALS, however its clinical translation was discouraging [149]. Yet there is a great deal of research that points to good nutrition and nutritional supplements as being clinically beneficial against neurodegeneration, arguing that there is promise within this field[150]. Nevertheless, there should be a greater selectivity of the nutritional supplements that make it to the clinic.

Studies that point to a readily available and non-prescription relief for a disease as devastating as ALS may be an appealing alternative for affected patients. For this reason, among others, we looked to a nutraceutical antioxidant for its neuroprotective activity in these disease models. Immunocal® was a unique option because of its undenatured conformation and high cystine concentration, as well as its potential to mitigate some aspects of ALS that are hallmark in the disease [55]. It has been shown that ALS patients who receive
whey protein supplement present weight gain and increased body mass compared to control group patients, suggesting that Immunocal® may have this benefit in addition to its effects on the available GSH pool [151].

Although researchers may disagree on the correct path to potential therapeutics, there is an understanding in the field that neurodegenerative diseases, such as ALS, have underlying mitochondrial dysfunction. Therefore, there is an emerging class of treatment modalities that target mitochondrial function as a potential therapy. For instance, dexpramipexole, a drug that is currently in phase III clinical trials for ALS is a mitochondrial modulator, and has had encouraging results in phase I and II trials. It is suspected to preserve mitochondrial function through its antioxidant activity, inhibition of pro-apoptotic proteins, and preserving mitochondrial structure [152, 153]. Effective treatments that target mitochondrial function such as dexpramipexole suggest that mitochondrial dysfunction is an underlying aspect of ALS, and therapies that target mitochondrial antioxidant defenses similarly hold promise in this field.

Mitochondrial GSH depletion is associated with increased apoptosis in NSC34 cells stably expressing the hSOD1\textsuperscript{G93A} mutation [51]. Additionally, GSH depletion in the hSOD1\textsuperscript{G93A} mouse is correlated with increased age-dependent motor degeneration [154]. Therefore, increasing the available cellular and mitochondrial GSH pool, and decreasing oxidative stress by promoting endogenous cellular defenses is an appealing treatment modality. As aforementioned, cysteine supplementation and providing the limiting precursor
for GSH synthesis holds promise for therapy in various neurodegenerative diseases, including ALS [155]. In accordance with these data, Immunocal® treatment and modulation of the GSH pool is a very promising therapy for ALS, particularly at the level of the mitochondria.

It is posited that the best exogenous precursor for GSH is the amino acid cystine [156]. Cystine is transported across the astroglial cell membrane in exchange for glutamate by the $X_c$ transport system in astroglial cells, and it is broken down into cysteine and used in GSH biosynthesis [157]. Although this is not the only mechanism behind the anti-apoptotic role of Immunocal®, this is an interesting aspect of the supplement and further investigation is necessary into this potential neuroprotective role.

In this study, we also showed that strawberry anthocyanins provided neuroprotection in the hSOD1$^{G93A}$ mice. These preliminary data are very promising and suggest that there may indeed be a role for these nutraceuticals in the clinic after further investigation into their mechanism of action. In addition to our findings, several recent in vivo studies have exhibited exciting results using anthocyanins, suggesting that these novel nutraceuticals could be beneficial in a clinical setting for neurodegenerative diseases. A central aim of these other studies was to examine the therapeutic potential of anthocyanins in the treatment of specific neurodegenerative diseases, specifically using animal models of PD. In the MPTP mouse, a neurotoxin model widely used to test potential therapeutic options for PD, anthocyanin treatment preserved dopamine neurons in the
substantia nigra pars compacta and dopaminergic innervation of the striatum [114]. Anthocyanins have also exhibited neuroprotection in rats subjected to unilateral striatal lesioning with 6-OHDA, reinforcing their potential in this particular neurodegenerative disease [158]. Anthocyanins have also been shown to have neuroprotective effects in in vivo models of acute neuronal injury such as middle cerebral artery occlusion and reperfusion in rats [159].

Anthocyanins have also been examined in models of aging, as research suggests that oxidative stress plays a large role in the etiology of aging. Recent studies investigating anthocyanins have revealed other potential benefits of these nutraceuticals. Anthocyanins appear to diminish age-related oxidative stress [106,160]. Treatment with anthocyanins significantly attenuated cytosolic cytochrome c release, and promoted neuronal survival in a model of D-galactose-induced brain aging in old mice [106]. Finally, in a recent clinical study, consumption of wild blueberry juice for 12 weeks enhanced memory performance in older adults with early symptoms of dementia [160]. These data are suggestive of a possible therapeutic role for anthocyanins in age-associated cognitive impairment.

An important element of anthocyanin treatment is the cell type-specific effects of these compounds exhibited both in vitro and in vivo. As they act in a pro-apoptotic fashion in cancer cells, and as anti-apoptotic agents in neurons, it is clear that their effects on cell physiology are complex.
Anthocyanins affect many different signaling pathways in different cell systems. They exhibit anti-proliferative, anti-inflammatory, and pro-apoptotic effects in cancer cells, supportive of their use as chemotherapeutic agents. They also exhibit strong antioxidant and anti-apoptotic effects in neuronal injury models, which provides evidence toward the potential of anthocyanins as a novel treatment for neurodegeneration. *In vivo* studies in animal models of neurodegeneration involving anthocyanins have delivered promising results that may lean towards their clinical development in neurodegenerative diseases. Studies in human subjects are limited, but are also promising for this treatment modality. These novel nutraceuticals displayed promising effects in preliminary clinical studies of memory impairment in older adults, again suggesting the potential benefit of developing anthocyanins for the treatment of neurodegenerative diseases [160]. Though the data in human models are limited and preliminary, they provide a basis for more comprehensive human trials of anthocyanins as neuroprotective agents. Seeing the clinical potential of anthocyanins, recent studies are directed towards delivery methods of these anthocyanins *in vivo*, even looking toward slow anthocyanin-releasing implants for a novel delivery method in disease prevention and treatment.

The unique properties of anthocyanins may be extremely beneficial for the clinical treatment of chronic human diseases, as diverse as cancer and neurodegeneration. Future development of these nutraceuticals as neuroprotective
agents will one day hopefully lead to their use in a clinical setting for the treatment of neurodegenerative diseases as well as highly invasive cancers.
Chapter 5: Conclusions and future directions

Experiments *in vitro* have convincingly demonstrated that cystine supplementation via Immunocal® may be promising against an array of neurodegenerative disease-relevant insults. Here, we show that Immunocal® protects CGNs against the Bcl-2 inhibitor, HA14-1, and that this effect is diminished when CGNs are co-incubated with BSO, which is a *de novo* GSH synthesis inhibitor. Furthermore, we show that Immunocal® protects CGNs from CuCl$_2$, AlCl$_3$, and SNP-induced toxicity. In CHO cells that are transiently transfected with wild type APP, Immunocal® decreases active caspase-3 expression. Moreover, in NSC34 motor neuron cells we saw that Immunocal® increased cell viability in a dose-dependent manner in cells treated with either H$_2$O$_2$ or glutamate glycine. These data are promising *in vitro*, and were encouraging enough to study the effects of Immunocal® in the hSOD1$^{G93A}$ mouse model of ALS. We found that Immunocal® administered orally *ad libitum* reduced age-associated decline in muscle function, as measured by the PaGE grip endurance test. We also show that Immunocal® increased healthspan by delaying disease onset in mutant mice. Finally, we show that Immunocal® prevented whole blood and spinal cord GSH depletion in the hSOD1$^{G93A}$ mice, and maintained GSH at NonTG levels in the mice receiving supplementation.
After isolating an anthocyanin-enriched fraction from freeze-dried strawberries (SAE) and testing their efficacy in the hSOD1<sup>G93A</sup> mouse model of ALS, we show a significant neuroprotective effect. SAE significantly delayed disease onset and extended survival in the hSOD1<sup>G93A</sup> mice receiving the extract orally. We also show a significant retention in motor function, as measured by the PaGE grip endurance test. These data are preliminary, and further investigation into the mechanism of action is necessary to further develop these compounds for their neuroprotective effects. These collective data suggest that nutraceutical intervention holds potential in neuroscience, and that compounds such as Immunocal® and SAE deserve further investigation in models of neurodegeneration.

Our future studies involving these compounds will model a clinical synergy approach to pharmacologically moderate cell death. For instance, if we combine Immunocal® with the glutamate inhibitor, Riluzole, we should see an
increase in survival in the hSOD1$^{G93A}$ mouse, as these compounds will affect
different aspects of neurodegeneration that are hallmark in these diseases (Fig.
16.) Furthermore, if we couple these compounds with an Nrf2-inducer, which will
increase GSH synthesis, we will theoretically see an increase in the resulting
neuroprotection. Future studies with Immunocal® are promising and they should
give rise to a novel approach using nutraceuticals to enhance pharmacological
intervention in ALS and other neurodegenerative disorders.
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