

**Ascorbate and Flavonoids as Protectors against Mutant Cu/Zn
Superoxide Dismutase-Induced Oxidative Damage in a Mouse Model of
Amyotrophic Lateral Sclerosis**

A Thesis

Submitted to the College of Graduate Studies and Research

In Partial Fulfillment of the Requirements

for the Degree of Master of Science

in the College of Pharmacy and Nutrition

University of Saskatchewan

Saskatoon

By

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ABSTRACT

The experiments in this thesis tested *in vitro* and *in vivo* the proposal that zinc-deficient superoxide dismutase, resulting from mutations or oxidative damage to the enzyme, gains ascorbate oxidase activity that contributes to the pathology of amyotrophic lateral sclerosis (ALS). They also tested whether flavonoids can help protect against this activity.

The *in vitro* experiments showed that zinc-extracted Cu/Zn-SOD (Cu-SOD) as well as SOD treated with H₂O₂ or H₂O₂ plus ascorbate accelerated ascorbate oxidation 100 to 300 %, while native SOD had no effect. With Cu-SOD, the activity was unaffected by EDTA, EGTA, or catalase, showing that the catalytic copper was firmly bound and that the H₂O₂ product of SOD activity was not responsible. Catechin and uric acid slowed ascorbate oxidation by Cu-SOD by 72% and 67%, respectively.

The *in vivo* study investigated tissue levels of ascorbate and biomarkers of oxidative stress in a transgenic mice bearing a mutation in Cu/Zn-SOD as a model of familial ALS (FALS mice), and the effects of dietary ascorbate and quercetin. In FALS mice on control modified AIN93G diet for 10 weeks compared to the wild-type, liver thiobarbituric acid reactive substances (TBARS) were 47% higher and liver oxidized vitamin C was 2800% higher. These results support, in liver, that mutant SOD acquired ascorbate oxidase activity and increased oxidative stress. The only difference in other tissues was a 136% increase in GSH/GSSG ratio in thigh muscle of FALS mice.

In dietary treatments of FALS mice, spinal cord TBARS was 93 % higher with ascorbate-supplemented diet compared to control diet, suggesting that dietary ascorbate increased oxidative stress. Also in spinal cord, oxidized-vitamin C was 250% higher in ascorbate + quercetin-fed FALS mice, which suggests there is no protection by quercetin against ascorbate oxidation. In brain, protein thiols were 56% and 58% lower in quercetin-fed and ascorbate + quercetin-fed FALS mice, suggesting that quercetin worsened oxidative damage. In liver, quercetin feeding produced a 40% decrease in vitamin C, total vitamin C and oxidized-vitamin C, perhaps by down-regulating ascorbate biosynthesis.

Overall the results support a gain of ascorbate oxidase activity of mutant SOD in ALS, but do not support protection by dietary treatment with ascorbate or quercetin.

ACKNOWLEDGMENTS

I would like to thank and appreciate the people who helped me during the course of this research.

Initially I would like to express my deepest appreciation to my supervisor (Dr. Brian Bandy) for his continuous support and guidance throughout my graduate program.

Special thanks to the members of my advisory committee, Drs. Phyllis Paterson, Ed Krol, and Bernhard Juurlink for their helpful instructions and advices.

I also would like to thank my colleagues and fellow graduates in the College of Pharmacy and Nutrition who supported me throughout my graduate program. In particular, I express gratitude to Ester, Masoumeh, and Zohreh for their continuous help and support.

I acknowledge the research funding from CIHR and SHRF, and many thanks to the University of Saskatchewan, College of Pharmacy and Nutrition for offering the facilities to conduct this research.

Finally I would like to thank my lovely family in Canada who played a major role in my achievement. My husband (Fawzy) and my son (Moaaz) were always supporting me and creating the time and space for me to study and work. I express my gratitude to my family in Egypt who raised me and fostered me with the skills I needed to achieve this success.

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LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

ALS	Amyotrophic lateral sclerosis
ALS/PDC	ALS/parkinsonium-dementia complex
AIN-93G	American Institute of Nutrition-93 growth diet
BHT	Butylated hydroxytoluene
CNS	Central Nervous System
CSF	Cerebrospinal fluid
CCS	Copper chaperone for SOD1
DMSO	Dimethyl sulfoxide
DTNB	5,5-dithiobisnitrobenzoic acid
DTT	Dithiothreitol
EAAT2	Excitatory amino acid transporter
ECF	Extracellular fluid
EDTA	Ethylenediamine tetra acetic acid
EGCG	Epigallocatechin gallate
EGTA	Ethyleneglycol- <i>bis</i> (β -aminoethyl)-N,N,N',N'-tetraacetic acid
FALS	Familial amyotrophic lateral sclerosis
GSH	Reduced form of glutathione
GSSG	Oxidized form of glutathione
HEPES	N-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Pressure Liquid Chromatography
LMN	Lower motor neurons
MDA	Malodialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NADP ⁺	Nicotinamide adenine dinucleotide phosphate (oxidized)
NF-H	Heavy subunit of neurofilament
NF-L	Light subunit of neurofilament
NF-M	Medium subunit of neurofilament
NOS	Nitric oxide synthase
NRC	National Research Council
PD	Parkinson's disease
PBS	phosphate buffered saline
RIPA	Radioimmunoassay buffer
ROS	Reactive oxygen species
SALS	Sporadic amyotrophic lateral sclerosis
SDS	sodium dodecyl sulfate
SOD	Superoxide dismutase
tBHQ	Tert-butylhydroquinone
TBARS	Thiobarbituric acid reactive substances
TBA	Thiobarbituric acid
TNB	Thionitrobenzoic acid
UMN	Upper motor neurons

1. LITERATURE REVIEW

I. BACKGROUND AND RATIONALE

1. Epidemiology of Amyotrophic Lateral Sclerosis (ALS) (Charcot's disease, Lou Gehrig's disease)

Amyotrophic Lateral Sclerosis (ALS) is one of the most serious neurodegenerative diseases. The disease is characterized by the loss of upper motor neurons in the cerebral cortex and lower motor neurons in the brain stem and spinal cord (Julien JP, 2001). The loss of motor neurons leads to atrophy and degeneration of the skeletal muscles that are under the control of these motor neurons. The disease finally leads to paralysis of the respiratory muscles and death usually within 3-5 years after the clinical onset (Julien JP, 2001). The incidence of the disease all over the world ranges from 0.86 to 2.4 per 100,000 per year (McGuire and Nelson, 2006), and it increases with age. It often occurs between 40-70 years old, but it can occur in younger and older ages (Cwik, V.A., 2001). The prevalence of ALS is about 6 per 100,000 per year. In Canada, approximately 2,200 people currently have ALS (http://www.als.ca/als_manuals.aspx).

Three major forms of ALS have been identified: 1) sporadic ALS, 2) familial or hereditary ALS, and 3) the Western Pacific (Mariana Islands) form. The latter was identified among the Chamorro people of Guam (Armon C, 1994), and it was described pathologically as a complex of Parkinson's disease (PD) and Alzheimer's disease. It was referred to as ALS Parkinsonium-dementia complex (ASL/PDC) (McGuire and Nelson, 2006).

The Western Pacific form of ALS was discovered in the early 1950s, with incidence rates ranging from 50-100 per 100,000 per year. The male to female ratio was 2:1 and the average age at diagnosis was 44 years. ALS/PDC was identified in three

different areas (McGuire and Nelson, 2006): in Guam (Marianna Islands), the Kii Peninsula of Japan, and Irian Jaya (Western New Guinea). The cause of ALS/PDC in these areas remains unclear. The spouses of many patients were also affected which does not support the hereditary cause of ALS/PDC. There are two hypotheses on the pathogenesis of ALS/PSC : mineral deficiency, especially calcium and magnesium in soil and drinking water, and poisoning through ingestion of the cycad seed (McGuire and Nelson, 2006). The involvement of an environmental agent or toxicant as a cause for ALS/PDC needs further investigation.

The majority of the ALS cases (90%) are sporadic, while only about 10% are familial cases (Julien JP, 2001). The incidence of sporadic ALS is 20-60% higher in men than in women (McGuire and Nelson, 2006). Of familial cases, 20% are due to mutations at the SOD1 gene on chromosome 21 (McGuire and Nelson, 2006). Not all carriers of SOD1 mutations will develop ALS, suggesting that other unknown factors must be contributing to the development of the disease in familial cases.

2. Risk factors for ALS

There are many risk factors that have been cited in the literature for ALS, although the only clear risk factors until now are age and sex (Figure 1.1). Although age is a risk factor in ALS, with a maximum susceptibility between the ages of 55 and 70 years old, other environmental or exogenous factors may be implicated in ALS development (Kurtzke JF, 1991). As to sex, there is an increased male to female ratio by 1.5-2 to 1 (Kurtzke JF, 1991). Regarding race, there is a highly significant decrease in ALS for nonwhites in the U.S, which suggests protection for blacks; however there are no good surveys that either confirm or reject the protection for U.S. blacks (Kurtzke JF, 1991).

Physical trauma, whether mechanical, electrical, or surgical, has been identified as the strongest and most consistent risk factor in ALS (Kurtzke JF, 1991). The strong association between trauma and ALS may explain the increased incidence of sporadic ALS in males rather than females as injury is more frequent in men than in women (Kurtzke JF, 1991).

Physical activity was suggested as a risk factor in ALS by several mechanisms (Longstreth et al., 1991). Physical activity can increase the risk of exposure to a neurotoxin by either facilitating its transport to its target or it can increase the susceptibility of motor neurons to injury by the neurotoxin. Epidemiological studies found an association between vigorous physical activity at work and during leisure time, and ALS (Longstreth et al., 1991)

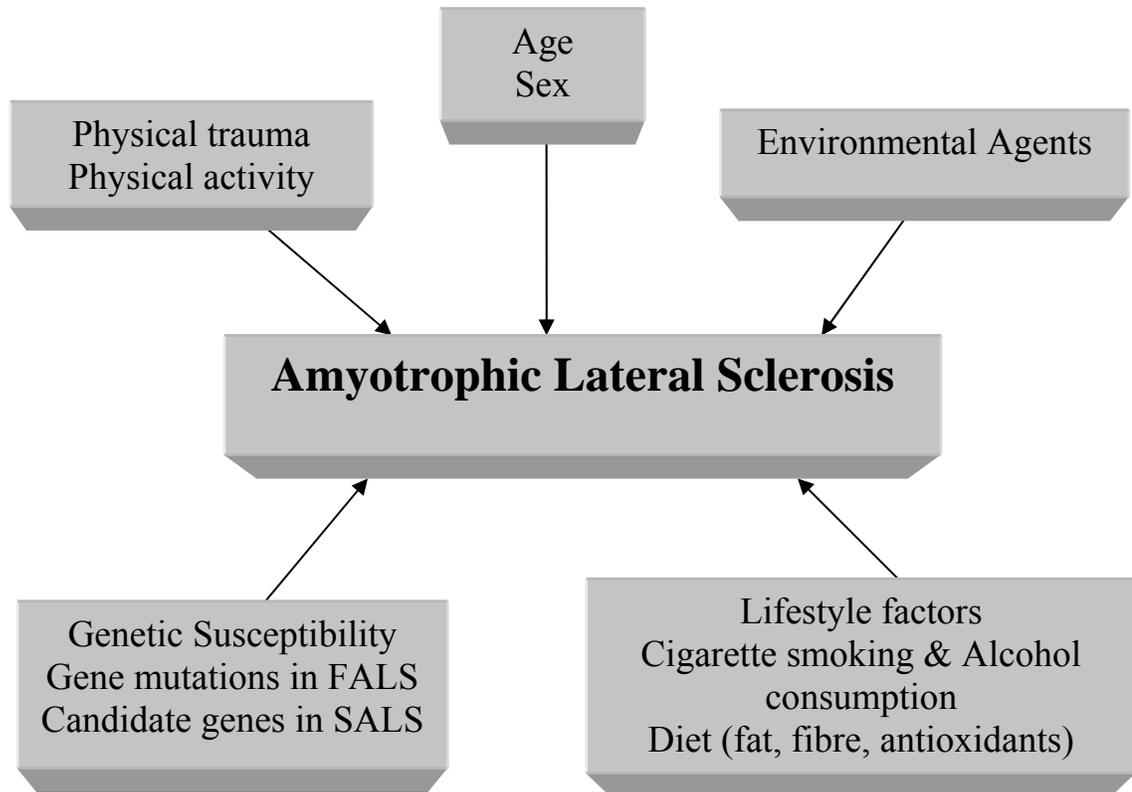


Figure 1.1 *Etiological theories and proposed risk factors in ALS*

Certain lifestyle factors have been linked to ALS such as diet, alcohol consumption, exercise, smoking tobacco, and other environmental factors such as residence in rural areas and job-related exposure to certain toxins. The role of cigarette smoking and alcohol consumption in the etiology of ALS has been investigated. Only three studies (Kamel et al., 1999, Nelson et al., 2000b, Weisskopf et al., 2004) have found an association with cigarette smoking, and none of the studies has found an association between ALS and alcohol consumption. The mechanisms by which cigarette

smoke could contribute to the risk of ALS were proposed by Nelson et al., 2000b. Nelson and colleagues suggested that cigarettes may directly cause toxic injury to motor neurons by its chemical constituents, or indirectly by the formation of free radicals during the metabolism of the chemical constituents of cigarette smoke. The hypothesis that cigarette smoking is associated with ALS needs further investigation.

There is a positive association between the increased risk of ALS and dietary fat intake, especially saturated fat and polyunsaturated fat such as linoleic acid (Nelson et al., 2000a). Fat intake (>93g/day compared with <42g/day) was associated with about a three-fold increased risk of ALS (Nelson et al., 2000a). The high concentration of polyunsaturated fatty acids in brain makes it vulnerable to oxidative damage (Penzes et al., 1993). As the dietary intake of polyunsaturated fatty acids affects their levels in brain, the increased consumption of polyunsaturated fatty acids could increase the risk of lipid peroxidation in brain (Bourre et al., 1993). On the other hand, dietary fiber intake (>18g/day compared with <10g/day) was found to decrease the risk of ALS by 70% (Nelson et al., 2000a). A mechanism by which dietary fiber could protect against the development of ALS is to cause luminal dilution of potential carcinogens (Hillemeier C, 1995), so it could reduce the absorption of a dietary toxin associated with ALS. Dietary fiber also was proven to shorten the transit time in the large intestine (Hillemeier C, 1995) and thereby could reduce the absorption of a dietary toxin.

The role of dietary antioxidants in ALS is inconclusive. A recent study investigated the relationship between the use of vitamin E, vitamin C, vitamin A, and multivitamin supplements and ALS mortality (Ascherio et al., 2005). In this study a group of one million people were surveyed and the investigators reported 525 deaths from ALS. The subjects were classified into 3 groups: nonusers of vitamin E, C, A, and multivitamin supplements, occasional users (less than 15 times per month), and regular users (15 times or more per month). It was found that regular use of vitamin E supplements for more than 10 years reduced the risk of death from ALS by 62%. On the other hand, vitamin C, vitamin A, and the use of multivitamins did not affect the risk of ALS. This agrees with the results from animal studies, as in one study it was found that dietary supplementation with vitamin E to transgenic mice over-expressing one of the mutated SOD1 genes delayed the onset of motor neuron disease (Gurney et al., 1996).

However, another study from Washington State university did not find any association of antioxidant and vitamin consumption, either in the diet or as a supplement, with the risk of ALS (Nelson et al., 2000a). Further studies need to be done to investigate more the role of diet in ALS.

Although there is still no clear evidence that a single environmental agent could directly cause ALS, epidemiological studies still suggest that environmental causes are important to study to clearly understand their roles in ALS.

3. Clinical motor signs and symptoms of ALS

ALS is a progressive degenerative disease of both upper motor neurons (UMN) and lower motor neurons (LMN). There is no specific test to confirm the diagnosis of ALS. The diagnosis is mainly dependent on the recognition of the disease signs and symptoms (Cwik, V.A., 2006). It is also dependent on the exclusion of other neurodegenerative diseases that might have similar signs and symptoms. The early symptoms of ALS are usually not clear enough and many people consider them as normal signs of aging. They include tripping, dropping things, slurred speech, and muscle weakening. Weakness of the respiratory muscles develops slowly over months or years (http://www.als.ca/als_manuals.aspx).

The diagnosis of ALS depends on the presence of both UMN and LMN abnormalities. The signs of UMN abnormalities are hard to quantify compared to LMN ones, as the UMN lesions in ALS are not responsible for the major disabilities that occur in the disease. The signs of UMN abnormalities include muscle stiffness or rigidity, emotional lability (decreased ability to control laughing or crying) and increased or hyperactive reflexes. The weakness due to UMN loss in ALS is usually masked by the weakness due to LMN lesions, and this is why it is hard to quantify clinically (Cwik, V.A., 2006). The signs of LMN loss include muscle weakness and atrophy. In early stages of ALS, LMN lesions are not disabling; however, the lesions accumulate over time and become more disabling.

4. The causes of sporadic ALS

Approximately 90% of ALS cases are sporadic cases (SALS) with no family history of ALS (Siddique et al., 1989). There is evidence that suggests that multiple genetic and environmental factors may be involved in ALS pathogenesis (Julien JP, 2001). We still do not know much about the genetic defects that cause or predispose to ALS. The only proven cause of ALS is missense mutations in the SOD1 gene. This accounts for about 20% of familial ALS cases (Rosen et al., 1993). Mutations in the neurofilament NF-H gene have been identified in a small number of sporadic ALS patients (~1% of cases) (Cleveland DW, 1999). The data suggest that NF-H mutations may represent risk factors for ALS disease (Julien JP, 2001).

For the majority of ALS cases, the main cause of sporadic ALS is still unclear. Various hypotheses have been suggested such as oxidative damage, excitotoxicity, mitochondrial defects and autoimmunity but these could be secondary to the neurodegeneration process and not the leading cause of ALS (Julien JP, 2001).

5. Familial ALS and genetic links to ALS

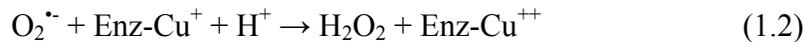
Approximately 10% of cases are familial ALS (FALS). Seven ALS-associated genes to date have been identified; however, only three of these genes have been precisely located (http://www.als.ca/als_manuals.aspx). ALS1 gene, which codes for the abnormal form of the enzyme copper-zinc superoxide dismutase (SOD1) on chromosome 21, causes the most common form of Familial ALS. While mutations in SOD1 account for ~20% of familial ALS (Rosen et al., 1993), identifying the remaining 80% of FALS cases will need further investigation.

5.1 Cu/Zn superoxide dismutase structure and activities

Superoxide dismutases are a group of enzymes that catalyze the conversion of the superoxide anion ($O_2^{\bullet -}$) into hydrogen peroxide and oxygen. SODs protect against

the toxic effects of $O_2^{\bullet -}$ and its derivatives (Siddique et al., 1997). In humans there are three types of SOD: cytosolic Cu, Zn superoxide dismutase (SOD1, CuZnSOD), mitochondrial Mn superoxide dismutase (SOD2, MnSOD), and extracellular superoxide dismutase (SOD3, ECSOD), which is also a CuZn enzyme. No mutations in SOD2 or SOD3 have been reported in FALS patients (Parboosingh et al., 1995).

SOD1 is present in the cytoplasm of most cells including red blood cells and is abundant in neurons (Pardo et al., 1995). Each SOD1 monomer has 153 amino acids and a mass of 16 KiloDaltons. Each monomer contains one atom each of Cu and Zn. The zinc is very important for pH stability of the reaction and for the rapid dissociation of the H_2O_2 produced, which can inactivate SOD1 (Hand and Rouleau, 2002). Access to the active site is limited by size and charge of the molecules. It usually favors small, negatively charged molecules such as the superoxide anion $O_2^{\bullet -}$ and excludes molecules with positive charges or large size (Siddique et al., 1997). The two-step dismutase reaction proceeds as follows:



The net reaction is:



Besides the dismutase activity, SOD1 has a marginal peroxidase activity (Hodgson and Fridovich, 1975). H_2O_2 , which is the product of the dismutase reaction, inactivates SOD1. The inactivation of SOD1 by its own H_2O_2 product and peroxidase activity would proceed as follows:



Enz is the enzyme and ImH63 is the imidazole moiety of the histidine residue at position 63 of the SOD1 polypeptide. In vivo, the peroxidase activity would be significant only in the presence of an excess production of H_2O_2 which may result from increased production of $O_2^{\bullet -}$ molecules as in oxidative stress conditions (Yim et al., 1993).

5.2 Cu/Zn SOD1 mutation in FALS

The first ALS-associated gene to be identified was the SOD1 gene, on human chromosome 21 (Rosen et al., 1993). *SOD1* gene is about 11 kb in size, and the SOD1 protein consists of 153 highly conserved amino acids with copper and zinc binding sites (Deng et al., 1993). Over 100 mutations, predominantly missense, have been found in 68 of the 153 positions in the protein (Siddique et al., 1996). For most people with SOD mutations, the time of death is typically three to five years after onset of symptoms, which is similar to the death in sporadic ALS. Certain SOD mutations, such as A4V and A4T, cause a more rapid progression of the disease, with death occurring within a year after diagnosis (Beckman and Estevez, 2006).

The discovery of SOD1 mutations associated with ALS shed light on the mechanism by which the mutated enzyme could cause the disease. It was first suggested that the disease was caused by loss of the scavenging activity of the mutant SOD1 enzyme, which leads to accumulation of the harmful superoxide anions (Rosen et al., 1993). Decreased SOD1 activity in the cerebrospinal fluid (CSF) of ALS patients has been reported (Bracco et al., 1991). Another mechanism was that mutant SOD might produce excessive hydrogen peroxide, which may be rapidly converted to the more toxic hydroxyl radical in the presence of ferrous ions (Imlay et al., 1988). The loss of function hypothesis was disproved by measurement of enzymatic activities and by transgenic mouse studies. Transgenic mice expressing familial ALS-linked mutant SOD1^{G93A} (i.e., glycine substituted to alanine at position 93) or SOD1^{G37R} developed motor neuron disease despite elevation in SOD1 activity levels (Cleveland DW, 1999). In addition, SOD1 knockout mice did not develop motor neuron disease (Reaume et al., 1996). Therefore, it is proposed that the mutations in SOD1 lead to a gain of new toxic properties rather than loss of the scavenging activity of SOD1 (Julien JP, 2001).

5.3 The mechanism of SOD1-induced oxidative damage to motor neurons

Although the toxic gain of function of mutant SOD1 has not yet been determined, there are various hypotheses of SOD1-mediated toxicity including: copper toxicity, improper metal binding, oxidative stress, abnormal protein aggregation, disorganization of neurofilaments, glutamate-mediated excitotoxicity, mitochondrial dysfunction and apoptosis (Kunst CB, 2004).

5.3.1 The copper-mediated oxidative damage hypothesis

One of the first hypotheses of SOD1-mediated toxicity was copper-mediated oxidative damage by the mutant SOD1 enzyme. In this hypothesis it is proposed that the misfolding of SOD1 caused by mutations can cause some undesirable substrates such as peroxynitrite to reach the catalytic site, hence lead to nitration of tyrosine residues (Beckman et al., 1993). Consistent with this hypothesis were findings of increased levels of free 3-nitrotyrosine in the spinal cord of human ALS patients (Beal et al., 1997) and free but not protein bound 3-nitrotyrosine in mouse models of ALS (Bruijn et al., 1997). Another hypothesis was that the mutant SOD1 enzyme can use the produced hydrogen peroxide as a substrate to generate toxic hydroxyl radicals that can cause cellular damage (Wiedau-Pazos et al., 1996). Elevated hydroxyl radical-like activity in mice expressing mutant SOD1 was found (Bogdanov et al., 1998). However there was an argument against the peroxidase activity hypothesis after the results of an activity study that was done in a mouse model of ALS (Bruijn et al., 1997). In that study they generated mutant SOD1^{G85R} mice either in SOD1 knockout mice or in transgenic mice overexpressing the wild type SOD1. It was expected that deleting the wild type-SOD1 in SOD1^{G85R} mutant mice would increase the levels of superoxide anion or the hydroxyl radicals while increasing the normal SOD1 activity would do the opposite. It was found that neither the elimination nor the excessive activity of the wild type SOD1 affected the disease progression in SOD1^{G85R} mice (Bruijn et al., 1997). This argues against the mechanism of disease progression involving superoxide anion or hydroxyl radicals. On the other hand there were some studies that tried to investigate the role of copper in the

pathogenesis of FALS. It was found in one study that deleting the gene for the copper chaperone for SOD (CCS), which is necessary for inserting copper in SOD1 did not affect the onset or the progression of the disease in mutant SOD1 mice, although it did decrease the copper content of the enzyme (Subramaniam et al., 2002). In another study they found that mutation of the four copper binding histidine residues, which results in a copper-less enzyme, also caused motor neuron disease in mice (Wang et al., 2003). These experiments suggest that the copper-mediated oxidative damage by the mutant SOD1 enzymes is not the primary cause of motor neuron degeneration in these models of FALS. However these experiments do not discount that increased levels of free (non-SOD bound) copper may have contributed to the motor neuron disease in these cases.

5.3.2 Improper metal binding hypothesis

Improper metal binding is another proposed hypothesis by which the mutant SOD1 enzymes could exert their new toxic function. ALS-related SOD mutants have decreased Zn affinity and when they are expressed *in vitro*, they accumulate in a Zn-deficient state (Crow et al., 1997). In SOD enzymes, Zn affinity is usually less than copper and Zn is held 7000-fold less strongly than copper (Crow et al., 1997). Mutation in SOD can weaken the enzyme structure and consequently cause Zn to be lost from the active site more easily than copper. The copper in the active site of the zinc-deficient SOD1 becomes more accessible, and it is suggested to participate in harmful reactions including transferring electrons to oxygen to produce superoxide (Estevez et al., 1999). Nitric oxide reacts very rapidly with superoxide to produce peroxynitrite.

In one experiment they found that the wild type zinc-depleted SOD1 and the mutant SOD1 lacking zinc were equally toxic to cultured motoneurons (Estevez et al., 1999). On the basis of these results, it is proposed that the mutations to the SOD enzyme could increase its susceptibility to lose Zn. Thus, wild type SOD can participate in sporadic ALS if it becomes Zn deficient (Beckman et al., 2001).

The question is what causes SOD to become Zn deficient in motoneurons? It was found that neurofilaments, a highly expressed protein in motoneurons, have high capacity for binding Zn and even compete with SOD for binding Zn *in vitro* (Crow et

al., 1997). The high concentration of neurofilaments combined with their high capacity for binding zinc could explain the tendency of SOD to become Zn deficient and consequently kill the motorneurons *in vivo*. Deletion of the light subunit of neurofilament (NF-L) subunit protects motorneurons against mutant SOD in ALS transgenic mice (Julien JP, 2001). So any abnormal upregulation of neurofilament proteins or other Zn-binding proteins such as metallothionein could result in the production of Zn-deficient SOD from even the wild type enzyme and thus could explain the genesis of sporadic ALS (Beckman et al., 2001).

5.3.3 Toxic protein aggregates

Another hypothesis to explain the toxicity of mutant SOD1 in FALS was the formation of toxic protein aggregates from the misfolded mutant proteins. The presence of protein aggregates is considered a pathological mark of the mutant SOD1 mouse model of ALS (Dal Canto and Gurney, 1995). Different hypotheses have been proposed to explain the toxicity of these aggregates. First it was proposed that mutant SOD1 aggregates may seize other protein components that are necessary for neuronal function. Second, the repetitive misfolding of the SOD1 mutants and their aggregates could result in reduced capacity of protein chaperones which catalyze the folding and unfolding of proteins inside the cell (Bruening et al., 1999). Third, the SOD1 mutant aggregates overwhelm the capacity of the ubiquitin-proteasome to degrade other protein components (Johnston et al., 2000).

It was found that aggregates of mutant SOD1 are found mainly within the nervous system of mutant SOD1 transgenic mice (Puttapparthi et al., 2003). In one study by Puttapparthi et al. (2003), they showed that proteasome-mediated protein degradation represents the major clearance mechanism for SOD1 aggregates in spinal cord. The proteasome activity was found to be decreased in spinal cord during aging, and this decrease correlates with accumulation and aggregation of mutant SOD1 *in vivo* (Puttapparthi et al., 2003). These observations could explain the selective vulnerability of motor neurons to mutant SOD1 with increased age.

5.3.4 Mitochondrial defects

There is some evidence that links mutation in SOD1 to mitochondrial dysfunction. First SOD1 was considered as a cytoplasmic protein, but recent studies have shown that ~1-2% of SOD1 is found in the intermembrane space of mitochondria (Mattiuzzi et al., 2002). One of the early pathological signs in G93A and G37R SOD1 transgenic mice is the presence of large membrane-bound vacuoles that came from degenerating mitochondria in motor neurons (Dal Canto and Gurney, 1995). Studies have shown increasing numbers of degenerating mitochondria within motor neurons immediately before the onset of symptoms in G93A transgenic mice (Kong and Xu, 1998). Damaged mitochondria can produce many toxic effects such as excess production of superoxide ion, and release of cytochrome *c* into the cytoplasm, which activates caspase-3, and affects Ca^{2+} homeostasis. In SOD^{G93A} transgenic mice, there is evidence of impaired calcium homeostasis in motor neurons which leads to motor neuron degeneration (Siklos et al., 1998).

5.3.5 Glutamate Excitotoxicity

Glutamate is considered the principal excitatory neurotransmitter in the brain. The receptor excitation that results from presynaptic glutamate release is usually terminated by reuptake of glutamate by specific transporters (Heath and Shaw, 2006). Failure of glutamate uptake by these transporters leads to excessive excitation to the receptors and neurotoxic effects. Glutamate excitotoxicity leads to calcium and sodium influx and activation of some enzymes such as nitric oxide synthase (NOS) (Leigh and Meldrum, 1996). This may lead to the formation of NO, which contributes to protein nitration and inactivation. For spinal motor neurons, the specific glutamate transporter is EAAT2 (excitatory amino acid transporter), and is present in astrocytes. It was found that the majority of sporadic ALS cases (~65%) have a decrease in the astroglial glutamate transporter EAAT2 in motor cortex and spinal cord (Rothstein et al., 1995). This decrease in EAAT2 levels may be due to RNA processing errors which lead to the formation of aberrant EAAT2 mRNA species.

In patients with familial ALS, mutant SOD1 could catalyze the inactivation of EAAT2, as it does in the presence of hydrogen peroxide (Trotti et al., 1999), and lead to excitotoxic injury. This process would represent another link between familial and sporadic ALS. To date, the only effective approved treatment for ALS is riluzole. It has a neuroprotective role by inhibiting glutamate release (Doble A, 1996). Treatment with riluzole significantly prolongs the survival by 13-15 days, an improvement of about 11% in the animal model of ALS (Gurney et al., 1996). Another potential treatment may be the use of carboxyfullerenes. These antioxidants exhibit neuroprotective properties by blocking glutamate receptor-mediated excitotoxicity (Dugan et al., 1997)

5.3.6 Neurofilaments

Neurofilaments are considered possible targets of SOD1-induced toxicity. The neurofilament proteins, which are composed of heavy (NF-H), medium (NF-M), and light (NF-L) subunits have an important role in axonal transport (Rowland and Shneider, 2001). Aggregation of neurofilaments was found in both sporadic and familial ALS patients (Hirano et al., 1984) as well as in SOD1-knockout mice (Rouleau et al., 1996). The overexpression in mice of any of the three wild type neurofilament subunits leads to the formation of perikaryal neurofilament aggregations. But, neurofilament aggregations in the cell body of motor neurons do not cause death of motor neurons. For example, NF-H protein overexpression caused severe motor dysfunction but did not cause motor neuron death even in two-year old mice (Beaulieu et al., 2000). It was found that overexpression of the wild type human NF-H, or to a lower extent mouse NF-H proteins, which causes perikaryal neurofilament inclusions or accumulation of NFs in the cell body and not in the axons of motor neurons, extended the longevity of mutant SOD1 mice by 65% and 15%, respectively (Kong and Xu, 2000). The most effective approach to increase the longevity of mutant SOD1 mice is overexpressing human NF-H proteins. The proposed protective mechanism might be that the accumulation of perikaryal neurofilament may protect the cell by binding excess Ca^{2+} or by acting as a sink for oxidative damage.

5.3.8 Oxidative stress

Oxidative stress occurs when there is imbalance between production of oxidants, such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals ($\cdot OH$), and hydrogen peroxide (H_2O_2), and the antioxidant defence mechanism inside the cell (Coyle and Puttfarcken, 1993). There are a number of defence mechanisms in the cell to reduce the levels of oxidants: SOD, catalase, ascorbic acid (vitamin C), and α -tocopherol (vitamin E). Studies have shown that reactive oxygen species (ROS) production is increased in ALS and is associated with the disease onset and progression (Said et al., 2000). The high ROS levels could cause motor neuron degeneration by either reacting with, and damaging the cellular macromolecules inside the cell, or by stimulating the expression of genes that are associated with inflammation (Xu and Kong, 2006). The benefit of antioxidants in ALS was demonstrated by vitamin E treatment of transgenic SOD1 mutant mice (Gurney et al., 1996). Treatment with vitamin E and selenium delays the onset of clinical disease in SOD1^{G93A} transgenic mice by 12-15 days (14%), although it did not affect disease progression (Gurney et al., 1996).

II. Therapeutic approaches in ALS

Different therapeutic agents have been tried in mutant SOD1 mice and they have proved to slow disease progression. Riluzole, an inhibitor of glutamate release, is the only drug approved by the Food and Drug Administration for the treatment of ALS. Riluzole was found to prolong the survival of SOD G93A mice by 10-15 days, without affecting the disease onset (Gurney et al., 1996). Similarly, gabapentine, an inhibitor of glutamate biosynthesis, was found to extend the survival in transgenic mice with mutant SOD1, but it did not significantly delay the disease onset (Gurney et al., 1996). In contrast, vitamin E was found to delay the onset and the progression of the disease without extending the survival of the transgenic mice. Despite being beneficial in mice, gabapentine plus vitamin E did not show any benefits in patients with ALS (Miller et al., 2001).

Other therapeutic targets have been tested in mice. The overexpression of Bcl-2 was found to extend the survival of SOD1^{G93A} mice by 5-6 weeks (Kostic et al., 1997). The highest reported effect on the survival of mutant SOD1 mice (~5 months) was obtained by overexpressing a human NF-H transgene (Couillard-Despres et al., 1998). Another approach in treating ALS is using zinc supplementation. If zinc-deficient SOD could cause motor neuron death in ALS, a zinc-deficient diet fed to transgenic animals should accelerate the disease progression and zinc supplements might be protective. Disagreeing with this hypothesis, it was found that the high dose of zinc that was added to drinking water accelerated the death of ALS-SOD transgenic mice (Groeneveld et al., 2003). The zinc dosages were 75 and 375 mg/kg/day of zinc, which were 15 to 75 times greater than current recommendations for rodents (Reeves et al., 1993b). Excess zinc was found to block copper absorption and it is used to treat copper overload in Wilson's disease. On the other hand, copper is important for ceruloplasmin which inserts iron into heme and its deficiency causes fatal anaemia (Fox PL, 2003). Another study found that using moderate dose of zinc (12 mg/kg/day) extended the mice survival by 11 days compared to the zinc-deficient group (Ermilova et al., 2005). Increasing the dose to 18mg/kg/day accelerated the mice death, which can be prevented by the addition of a low dosage of copper to the drinking water. These results indicate that zinc supplementation could be used to treat ALS in transgenic mice with moderate dosage and without affecting the balance between copper and zinc inside the body.

III. The role of ascorbic acid as antioxidant in ALS

Ascorbate is an important antioxidant, enzyme co-factor and neuromodulator in the brain (Rice ME, 2000). Ascorbate enters the CNS by active transport and it diffuses from the cerebrospinal fluid (CSF) to the brain extracellular fluid (ECF) (Schenk et al., 1982). From the ECF, ascorbate is transferred to brain cells, where its concentration is increased up to 20-fold. The brain, spinal cord and adrenal glands have the highest ascorbate concentrations of all the tissues in the body (Rice ME, 2000). Both SOD and ascorbate eliminate $O_2^{\cdot-}$, producing hydrogen peroxide as a product. The high levels of ascorbate in neurons enable it to compete with Cu/Zn-SOD for $O_2^{\cdot-}$

elimination (Rice and Russo-Menna, 1998). In this way, intracellular ascorbate levels may compete against the excessive formation of toxic product generated by the mutant SOD. A decrease in cytoplasmic ascorbate levels would increase $O_2^{\cdot-}$ metabolism by the mutant SOD, and consequently allow more toxic product to form (Kok AB, 1997). Ascorbate could play both a direct and an indirect antioxidant role in ALS. First, ascorbate can directly reduce the superoxide radicals and hence prevent their direct damage to the cell, and their indirect damage through superoxide reactions with H_2O_2 to form hydroxyl radicals or with nitric oxide to form peroxynitrite anions (Kok AB, 1997). In a second indirect mechanism, ascorbate can restore oxidized vitamin E, which has been reported to delay ALS onset in transgenic mice (Dal Canto and Gurney, 1995). It has been reported that long-term users of vitamin E supplements had less than half the risk of dying from ALS than nonusers, suggesting that vitamin E supplementation may have a role in ALS prevention (Ascherio et al., 2005).

A correlation has been found between physiological ascorbate levels and the characteristics of ALS with respect to age and gender. First, there is an age-dependent decrease in ascorbate levels in both serum and tissue levels, which is consistent with the delayed onset of ALS (Kok AB, 1997). Second, men show lower plasma ascorbate levels, and animal studies have shown increased demand for ascorbate in males under injury conditions. Males clearly develop the disease earlier than females (Rosen AD, 1978).

The efficacy of combined treatment with a copper chelator triethylenetetramine dihydrochloride (trientine) and ascorbate on the disease development in mutant SOD1 transgenic mice has been demonstrated (Nagano et al., 1999). In this study, a high dose of trientine in the distilled water (0.2% w/v) and ascorbate mixed in the standard AIN-93G diet at the concentration of 0.8% w/w was initiated before the onset of the disease at 45 days of age. This combined treatment extended the survival of the transgenic mice by 11 % and maintained the motor function of the mice longer than the diseased control. They determined the onset of the neurological signs by postural change of one limb and the end point by the total paralysis of hind limbs when the mouse was lifted by the tail. The onset of neurological symptoms in the treated group was significantly delayed compared with that in the control group, and the time to reach total paralysis in the

treated group was delayed as well. The efficacy of trientine or ascorbate alone on FALS mice was also investigated (Nagano et al., 2003) when administered before or after the onset of the disease. The mice with a high dose of trientine (0.5% w/v) or ascorbate (0.8% w/w) administered before disease onset survived significantly longer by 62% than the control. None of the treatments affected the mean age of the onset, and none of the agents administered after the onset prolonged the survival.

IV. The implication of flavonoids in protecting against ALS

Flavonoids are naturally occurring polyphenolic compounds (Figure 1.3). They have antioxidant activities through their reactions with superoxide anions and lipid peroxides (Bors et al., 1990). Flavonoids were found to stabilize ascorbate against oxidation by different mechanisms. They can either bind metals that might catalyze ascorbate oxidation, or they can scavenge reactive oxygen species (Kuhnau J, 1976). Flavonoids can partition between aqueous and lipid phases due to their amphiphilic nature (Saija et al., 1995). This nature enables the flavonoids to cooperate with ascorbate to protect cell membranes (Bandy and Bechara, 2001).

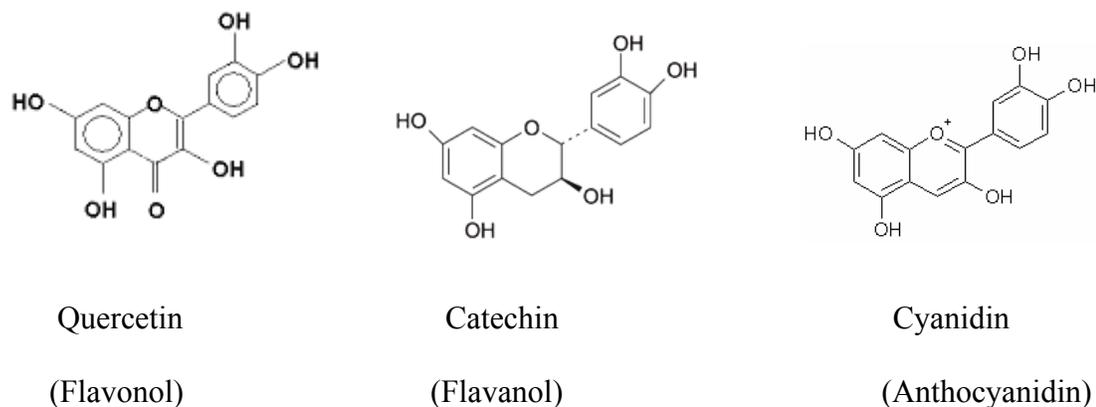


Figure 1.2 Different classes of flavonoids

In relation to the role of flavonoids in ALS, almost no research has been done in this area except for two studies. Genistein was suggested for its general neuroprotective effect. Genistein, a phytoestrogen, has both estrogen-dependent and estrogen-

independent neuroprotective activities, and the authors suggested that it should be investigated as a prophylactic agent against pathologic conditions such as ALS and stroke (Trieu and Uckun, 1999). In another study with epigallocatechin gallate (EGCG), it was found that EGCG protects motor neurons isolated from wild type (WT) and G93A SOD1 mutant mice from oxidative stress-induced cytotoxicity. EGCG protects against cell death in these motor neurons by affecting upstream signalling through the phosphoinositide 3' kinase/Akt and glycogen synthase kinase-3 pathway, as well as downstream signalling through cytochrome c release and caspase-3 activation (Koh et al., 2004). When differentiated WT and G93A cells pre-treated with EGCG were subsequently exposed to H₂O₂-induced oxidative stress, the viability of both cells was significantly increased by 77% and 78%, respectively, compared to the cells treated with H₂O₂ only (Koh et al., 2004).

2. HYPOTHESES

- 1- Zinc-deficient SOD (Cu-SOD), resulting from mutations or oxidative damage to the superoxide dismutase enzyme (SOD), gains function as an ascorbate oxidase that contributes to the pathology of ALS.
- 2- Ascorbate as an antioxidant can compete with mutant SOD for scavenging the superoxide anions and consequently decrease the oxidative damage induced by the mutant SOD in ALS
- 3- Flavonoids as antioxidants can act synergistically with ascorbate to decrease the oxidative damage induced by the mutant SOD in ALS, and at the same time protect against any pro-oxidant effects of ascorbate.

3. OBJECTIVES

3.1 *In vitro* study

The first objective in the *in vitro* study is to investigate the effect of zinc-extracted Cu/Zn SOD (Cu-SOD) and H₂O₂-damaged SOD on the rate of ascorbate oxidation by oxygen, to see if these enzyme preparations will accelerate ascorbate oxidation in comparison to the native SOD.

The second objective is to test the effect of selected flavonoids such as catechin or quercetin and selected antioxidants such as uric acid on the rate of ascorbate oxidation in the presence of Cu-SOD. If Cu-SOD does accelerate ascorbate oxidation, we need to see if the flavonoids or uric acid might have any protective effect *in vitro* against ascorbate oxidation by Cu-SOD

3.2 *In vivo* study

The first objective in the *in vivo* study is to investigate the protective role of ascorbate as an antioxidant against the oxidative damage that is caused by the mutant Cu/Zn SOD in ALS, and to see if supplementation with ascorbate during the course of the disease will ameliorate the disease. Conversely, supplementation with ascorbate may have no effect or even worsen disease progression by increasing $O_2^{\bullet-}$ and H_2O_2 production catalyzed by zinc-deficient SOD (Cu-SOD). Cu-SOD could increase $O_2^{\bullet-}$ and H_2O_2 by the following reactions:



The second objective is to investigate the protective role of one of the flavonols (quercetin) as an antioxidant against the oxidative damage induced by the mutant Cu/Zn SOD in ALS, and to see if increasing quercetin intake during the course of the disease could affect the fate of ascorbate. Also quercetin may protect against the pro-oxidant effect of ascorbate.

4. MATERIALS AND METHODS

4.1 Chemicals and Reagents

The chemicals purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada) were: Cu/Zn superoxide dismutase (bovine erythrocyte), catalase (bovine liver), copper sulphate, zinc chloride, ascorbic acid, hydrogen peroxide, catechin, quercetin, uric acid (sodium salt), DMSO, ferric chloride, chelex 100 (sodium form), sodium salt of ascorbic acid, bovine serum albumin, sodium dodecyl sulfate, HEPES, monobasic and dibasic potassium phosphate, sodium deoxycholate, dithiothreitol, EDTA, tripotassium EDTA, BHT, potassium chloride, TBA, MDA, sodium potassium tartrate, potassium iodide, DTNB (5,5-dithiobisnitrobenzoic acid or Ellman's reagent), glutathione reductase, GSH, and GSSG. Chemicals obtained from Alfa Aesar (Ward Hill, MA, USA) were: EGTA, and m-phosphoric acid. Chemicals purchased from EMD chemicals (Durham, NC) were: triton X-100, monobasic sodium phosphate, sodium chloride, glacial acetic acid, sulphuric acid, and HPLC grade methanol. Dibasic sodium phosphate was obtained from Spectrum Chemical Mfg. Corp. (Gardena, CA). Sodium acetate trihydrate, sodium hydroxide, pyridine, and n-butanol were obtained from EM Science (Gibbstown, N.J., USA). NADPH was obtained from Calbiochem (San Diego, CA, USA).

4.2 Methods for *in vitro* experiments

4.2.1 Reagent preparation

Buffers and other aqueous solutions were prepared in deionized, purified water (18 M Ω). The flavonoids catechin (20 mM) and quercetin (20 mM) were prepared by dissolving the powder in DMSO.

4.2.2 Preparation of Zn-deficient SOD by dialysis procedure

Spectrapor® membrane tubing was obtained from Spectrum Medical Industries (MW: 6-8,000 and diameter: 14.6 mm). To prepare the dialysis tubes, they were cut to the desired length, put into deionized, purified water, and let stand overnight.

Afterwards, the dialysis tubes were transferred into chelex-treated 0.1 M phosphate buffer (pH 3.5) and let stand for several hours before use. Stocks of 0.1 M monobasic potassium phosphate buffer and 5 mM sodium acetate buffer were treated with chelex beads contained in a dialysis tube. After removal of the dialysis tube with chelex, phosphate and acetate buffers were adjusted to pH 3.5 and 4.0 respectively. All glass wares were washed with 1 mM EDTA and rinsed exhaustively with deionized water.

The zinc was extracted from the enzyme according to previous procedures (Sampson and Beckman, 2001). The enzyme is made zinc deficient by dialysis against 2 changes of 4 L of 0.1 M potassium phosphate buffer at pH 3.5 overnight at 4°C and then dialyzed against 5 mM sodium acetate buffer pH 3.5-4.0. This resulted in partial copper deficiency, which is corrected by incubation with a 10% excess (per mol subunit of enzyme) of cupric sulfate overnight at 4°C and brief (1-2 h) dialysis against 5 mM sodium acetate, pH 3.5 – 4.0, to remove unbound copper.

4.2.3 Preparation of H₂O₂-damaged SOD preparations

Oxidative damage was induced in two SOD enzyme preparations by treating the SOD native enzyme with hydrogen peroxide alone or hydrogen peroxide plus ascorbate

(previously neutralized to pH 7.2) in equal concentration(s) to the SOD enzyme (357 mM) at physiological pH (50 mM potassium phosphate buffer pH 7.2). The treated enzyme in both preparations was incubated at room temperature (25°C) for 2 hours, and then it was dialyzed against 2 changes of 2L of 50 mM potassium phosphate buffer at pH 7.2, one overnight and the other for 8 hours at room temperature.

4.2.4 Effects of SOD enzyme preparations on ascorbate oxidation

The effect of different SOD enzyme preparations on the rate of ascorbate oxidation with comparison to the native SOD (nSOD) was tested. We tested the effect of three enzyme preparations; Zn-extracted SOD (Cu-SOD), H₂O₂-treated SOD (H₂O₂-SOD), and ascorbate plus H₂O₂-treated SOD (A-H₂O₂ SOD) on the rate of ascorbate oxidation, and the effect of the metal chelators EDTA and EGTA on this rate. Final concentrations in the quartz cuvette (containing 1 ml 50 mM potassium phosphate buffer, pH 7.2, at 37°C) were: 0.16 mM ascorbate, 10.68 mM nSOD, 21 mM H₂O₂-SOD, A-H₂O₂ SOD and Cu-SOD, 0.5 mM EDTA and EGTA,. The change in ascorbate absorbance was measured at λ_{max} 265 nm. The experiment was done in duplicate under the same conditions and on the same day.

4.3 Methods for *in vivo* experiments

4.3.1 Animals and diets

Twenty female transgenic mice (6-8 week old) carrying a mutated human SOD1 gene (JAX® Gemm® strain) weighing an average (\pm SEM) of 17.8g (\pm 0.3g) plus six female control wild type mice (6-8 week old) weighing an average (\pm SEM) of 18g (\pm 0.4g), were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The strain distinction for the transgenic mice is B6SJL-TgN (SOD1-G93A) 1Gur/J, often referred to as G1H. The stock number of the transgenic mice is 002726. The strain distinction for the control wild type is [B6SJL-Tg \(SOD1\)2Gur/J](#). The stock number of the control wild type mice is 002297. Transgenic mice were maintained as hemizygotes by crossing the

male transgenic mice with the female wild-type B6SJL. The transgene construct is composed of the human SOD1 gene carrying a glycine to alanine transition at position 93 (G93A). The G93A mutation does not alter the superoxide dismutase activity of the protein. These transgenic mice express high levels of the transgene with a 4-fold increase in SOD activity, and exhibit a phenotype similar to amyotrophic lateral sclerosis (ALS) in humans. Hemizygous transgenic mice become paralyzed in one or more limbs and have a life span of approximately 19-23 weeks. Paralysis is due to loss of motor neurons from the spinal cord.

The mice were housed under constant temperature ($22 \pm 2^{\circ}\text{C}$) and were maintained on a 12 hour light: dark cycle (0700 to 1900 hours). The transgenic mice were randomly assigned to four experimental groups plus the wild type group (total five groups) and housed individually in stainless steel cages. The control diet was the AIN-93G diet (Reeves et al., 1993a) updated to meet 1995 NRC growth guidelines (NRC, 1995). Tert-butylhydroquinone (tBHQ) was omitted to avoid the possibility that this antioxidant could mask the effect of the experimental treatment. The ingredients of the control diet are shown in Table 4.1.

Table 4.1 *The composition of the modified AIN-93G purified rodent diet with tBHQ omitted*¹

Ingredient	kcal/g	g/kg	kcal/kg
Casein	3.58	200	716
Sucrose	4	90	360
Cornstarch	3.6	397.5	1431
Dyetrose ²	3.8	132	501.6
L-Cystine	4	3	12
Cellulose	0	50	0
Soybean Oil	9	70	630
t-Butylhydroquinone	0	0	0
Mineral Mix # 210025 ³	0.88	35	30.8
Vitamin Mix # 310025 ⁴	3.87	10	38.7
# 410750 to bring to 95 values ⁵	3.34	10	33.4
Choline Bitartrate	0	2.5	0
		1000.00	3753.5

¹Diets were purchased from Dyets Inc. (Bethlehem, PA, USA).

²Dyetrose (Dyets, Bethlehem, PA, USA) is dextrinized cornstarch containing 90-94% tetrasaccharides.

³Mineral Mix # 210025 (AIN-93G mineral mix prepared in sucrose finely powdered and used at 35g/kg diet), is shown in Table 4.2.

⁴Vitamin Mix # 310025 (AIN-93VX vitamin mixture prepared in sucrose and used at 10g/kg diet), is shown in Table 4.3.

⁵# 410750 to bring to 95 values (Dyets AIN-93G rodent diet supplement to bring AIN-93G diet formulations into compliance with 1995 NRC rat/mouse values and used at 10g/kg diet), is shown in Table 4.4.

Table 4.2 *AIN-93G Mineral Mix*

Ingredient	g/kg
Calcium carbonate	357.00
Potassium Phosphate (monobasic)	196.00
Potassium Citrate H ₂ O	70.78
Sodium Chloride	74.00
Potassium Sulfate	46.60
Magnesium Oxide	24.00
Ferric Chloride, U.S.P.	6.06
Zinc Carbonate	1.65
Manganous Carbonate	0.63
Cupric Carbonate	0.30
Potassium Iodate	0.01
Sodium Selenate	0.01025
Ammonium Paramolybdate·4H ₂ O	0.00795
Sodium Metasilicate·9H ₂ O	1.45
Chromium Potassium Sulphate·12H ₂ O	0.275
Lithium Chloride	0.0174
Boric Acid	0.0815
Sodium Flouride	0.0635
Nickle Carbonate	0.0318
Ammonium Vanadate	0.0066
Sucrose finely powdered	221.026

Table 4.3 *AIN-93VX Vitamin Mixture**

Ingredient	g/kg
Niacin	3.00
Calcium Pantothenate	1.60
Pyridoxine HCl	0.70
Thiamine HCl	0.60
Riboflavin	0.60
Folic Acid	0.20
Biotin	0.02
Vitamin E Acetate (500 IU/g)	15.00
Vitamin B12 (0.1%)	2.50
Vitamin A Palmitate (500,000 IU/g)	0.80
Vitamin D3 (400,000 IU/g)	0.25
Vitamin K1/Dextrose Mix (10 mg/g)	7.50
Sucrose	967.23

Table 4.4 *Dyets Inc. AIN-93G rodent diet supplement to bring AIN-93G diet formulations into compliance with 1995 NRC rat/mouse values**

Ingredient	g/kg
L-Methionine	160.00
Vitamin B12 (0.1%)	2.5
Vitamin K1/Dextrose Mix (10 mg/g)	2.5
Sucrose	835.00

* Nutrient Requirements of Laboratory Animals. 4th Rev. Ed., National Research Council, 1995.

The mice arrived with no clinical signs of disease. The clinical signs for disease development were monitored once per week for the first week, increased to twice per week starting from the second week (7-9 weeks old), and finally increased to 3 times per week at the end of the feeding study (15-17 week old mice). Monitoring the disease progression was done by the following clinical signs (Nagano et al., 1999):

- 1- The ability to grasp a pencil with hind limb when suspended by the tail.
- 2- The number of the steps they miss in 2 minutes when they were put in a metabolic cage.
- 3- Observations of activity, gait, and cage-climbing.

The animals were sacrificed immediately if they reached any of the following endpoints:

- 1- Inability to right themselves within 5 seconds when placed on their side.
- 2- Inability to eat, drink or move toward food or water in a low dish.
- 3- Loss of >10% of body weight in 24 hours.
- 4- Notable lack of grooming, rough coat, or nasal/ocular discharge.
- 5- Notably increased or labored breathing compared to wild-type controls.

The animals were assigned into 5 experimental groups:

- 1) Control wild type mice (CW), fed the modified AIN-93G diet (n=6).
- 2) Control transgenic mice (CT), fed the modified AIN-93G diet (n=5).
- 3) Ascorbate supplemented transgenic mice (A), fed the modified AIN-93G diet plus ascorbic acid (45 mmol/kg diet) (n=5).
- 4) Quercetin supplemented transgenic mice (Q), fed the modified AIN-93G diet plus quercetin (29 mmol/kg diet) (n=5).
- 5) Ascorbate plus quercetin supplemented transgenic mice (AQ), fed the modified AIN-93G diet plus ascorbic acid (45 mmol/kg diet), and quercetin (29 mmol/kg diet) (n=5).

The AIN-93G diet was received in a powdered form, packed under vacuum by the manufacturing company, Dyets Inc. (Bethlehem, PA, USA). Ascorbic acid and quercetin were from the Sigma Chemical Company (St. Louis, MO, USA). The ascorbic acid and the quercetin were mixed with the powdered diet in a twin shell blender (“V blender”). For each kg of diet, it took 15 minutes of mixing and this was increased to 45 min for making 2 kg diet. Preparing the diet was done under N₂ gas and then the diet

was collected into Ziploc bags under N₂ gas and stored at -20°C until needed. The mice in each group had free access to the assigned experimental diet and distilled tap water for 10 weeks. Weekly body weight was monitored and food intake was calculated four times during the feeding study.

The mice were sacrificed on the same day at a point when several of the animals had developed paralysis in one of the hind limbs, a marker of disease development (Nagano et al., 1999). One animal in the AQ group (ascorbate plus quercetin-fed group) was sacrificed 4 days early due to limb paralysis and prospective inability to move towards food or drink. The animals were first anaesthetized with isoflurane and then were killed by transcardial perfusion with phosphate-buffered saline (PBS) to eliminate blood from the tissues. The organs (liver, kidney, brain, heart and thigh muscles) were collected rapidly and then they were flash frozen in liquid nitrogen and stored at -80°C until use. For the spinal cord removal, we were successful in separating the whole intact spinal cord by using pressure from a syringe with a blunted needle, injecting PBS to push the spinal cord out from the thinnest area of the cord towards the thick, wider cervical area (as described in <http://www.histosearch.com/histonet/Jun03/mousespinalcordremovalrev.html>). The spinal cords were collected in 2 ml cryotubes, flash frozen in liquid nitrogen and stored at -80°C until use. All procedures were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals and were approved by the Protocol Review Committee of the University Committee on Animal Care and Supply (Protocol # 20050134).

4.3.2 Crushing and homogenization of tissues

Prior to the biochemical analysis, all tissues were crushed into a fine powder under liquid nitrogen using Bio-Pulverizer model 59013N. The Bio-Pulverizer consists of a hole machined in a stainless steel base into which fits a special piston. In a typical procedure, up to 1g of animal tissue is hard-frozen in liquid nitrogen and placed in the pre-chilled Bio-Pulverizer, and the tissue is pulverized with one or two blows to the pestle with a hammer. The mass of the stainless steel preserves the cold long enough to

complete the pulverization without adding additional liquid N₂. The powdered tissues can be poured easily into cryotubes and stored at -80°C for subsequent homogenization or extraction procedures. The tissues afterwards were weighed in portions and homogenized in the appropriate homogenization media with a tissue grinder (Pellet Pestles® cordless motor, Kimble-Knotes # 749521-1590). The tissue grinder grinds soft tissues in Micro centrifuge tubes (1.5 ml) into homogenous solution.

4.3.3 Tissue measurement of oxidative stress

4.3.3.1 Thiobarbituric acid reactive substances assay (TBARS)

TBARS were measured in liver, brain, kidney, heart, thigh muscle and spinal cord as a marker of oxidative stress. The measurement of TBARS was adapted from the method of (Ohkawa et al., 1979). This method determines MDA and other reactive aldehyde levels by heating the samples in the presence of TBA at low pH, resulting in the formation of a pink chromophore with an absorption maximum near 532 nm (Abuja and Albertini, 2001). The procedure was scaled down for use with small samples (100µl of sample), and the TBA reaction products were extracted with an equal volume of the butanol/pyridine (15 butanol: 1 pyridine, v/v). BHT, and EGTA, previously neutralized to pH 7.2, were included in the tissue homogenization buffers to prevent artefactual formation of TBARS during sample preparation.

4.3.3.1. A. Preparation of reagents

Radioimmune precipitation assay buffer, pH 7.2 (RIPA buffer: 100 mM sodium phosphate buffer, 1.5 M sodium chloride, 1% triton X-100, 1% sodium deoxycholate, 2 mM EDTA, 0.1% SDS) was prepared in deionized water. KCl/EGTA/BHT mixture was prepared by adding the following components: 2 mM EGTA (previously neutralized to pH 7.2), 0.02% BHT (prepared in DMSO) and 1.15% KCl). 8.1% SDS was prepared in deionized water. 0.8% TBA was prepared in deionized water with mild heating to

dissolve in water. 20% Acetic acid was adjusted to pH 3.5 using NaOH or KOH, and 0.02% BHT was prepared in DMSO.

Preparation of 150 mM MDA in 1% sulphuric acid was done by diluting 10.4 ml of sulphuric acid (96.5%) with 90 ml water (slowly adding the sulphuric acid to the water in the fume hood), then adding 0.227 ml of 1,1,3,3-tetraethoxypropane (97%) which will be hydrolyzed by the sulphuric acid to MDA. The stock solution was diluted in water by 100 times to obtain a final concentration of 1.5 mM MDA. Preparation of (15:1 v/v n-butanol/pyridine) was done by mixing, in the fume hood, 375 ml of butanol (99.4%) with 25 ml of pyridine (99.5%).

4.3.3.1. B. Preparation of tissue homogenates for the TBARS assay

For liver, brain, and spinal cord tissues, the samples were homogenized in KCl/EGTA/BHT mixture in the dilution ratio (9:1 v/w). The frozen tissues were first weighed and homogenized in KCl mixture (4:1 v/w), and then 5 parts of KCl mixture were added. For thigh muscle, heart and kidney tissues, the sample was homogenized in KCl mixture in the dilution ratio (4:1 v/w), and then 5 parts of RIPA buffer were added. Afterwards the muscle, heart and kidney homogenates were centrifuged at 15,000 x g for 10 min at 4°C using an Allegra™ 25R Beckman Coulter centrifuge. For the liver, brain, and spinal cord tissues, the homogenates were assayed without prior centrifugation.

4.3.3.1. C. TBARS assay procedures

The measurements were done in duplicate for all tissue samples. Eppendorf tubes (2.0 mL) were prepared for incubation by adding the appropriate volumes of the following reagents: 8.1% SDS, 20% acetic acid, 0.8% TBA, and 0.02% BHT. Tissue homogenates were directly added to the prepared tubes (for assay blanks, the homogenization buffer was used without the sample) and the samples were heated at 95°C for 60 min; then cooled in cold water. In the fumehood, 15:1 v/v n-butanol/pyridine was added, and then the tubes were vortexed and centrifuged at 4000 x g for 10 min at 4°C using the Allegra™ 25R Beckman Coulter centrifuge. In the fume

hood, the organic layer was transferred to 1.5 ml cuvettes, and covered with Parafilm. The absorbance of this organic layer was read at 532 nm using a Beckman Coulter DU[®] 640B spectrophotometer.

4.3.3.2 Biuret protein assay

Biuret protein assay was used to measure the protein concentration in tissue homogenates in order to make other measurements comparable. In the biuret assay, protein samples were combined with biuret reagent (32 mM sodium potassium tartrate, 12 mM copper sulphate, 30 mM potassium iodide, 0.2 M NaOH). The copper ions form a complex with the amide groups in the proteins and create a blue color that was measured at 550 nm using a Beckman Coulter DU[®] 640B spectrophotometer. Protein concentrations of tissue homogenates were measured in duplicate by the biuret method (Layne E, 1957) using bovine serum albumin as a calibration standard.

4.3.3.3 The ratio of GSH/GSSG, and protein thiols assay

The tripeptide glutathione (γ -glutamylcysteinylglycine) is the major free thiol in most living cells and participates in diverse biological processes such as the detoxication of xenobiotics, removal of hydroperoxides, maintenance of the sulphhydryl status of proteins, and modulation of enzyme activity by disulfide interchange. The oxidized form of GSH is glutathione disulfide (GSSG), and intracellular glutathione is effectively maintained in the reduced state by glutathione reductase linked to the NADPH/NADP⁺ system. When mammalian cells are exposed to increased oxidative stress, the ratio of GSH/GSSG will decrease as a consequence of GSSG accumulation. Measurement of the GSSG level or determination of the GSH/GSSG ratio is a useful indicator of oxidative stress (Akerboom and Sies, 1981). In protein SH and GSH assays, the sulphhydryl group reacts with DTNB (5,5-dithiobisnitrobenzoic acid or Ellman's reagent) and produces a yellow colored 5-thionitrobenzoic acid (TNB), which is measured at 412 nm. In the GSSG assay, the GSSG is reduced by glutathione reductase using NADPH as a coenzyme, and the NADPH becomes oxidized to NADP⁺. The decrease of A₃₄₀ was measured to determine the change in NADPH.

4.3.3.3.1 Preparation of reagents

10 mM DTNB was prepared in methanol. The other reagents such as, 3% *m*-phosphoric acid, 10 mM NADPH, 250 U/ml glutathione reductase, 20 mM GSH, 10 mM GSSG, and 10% SDS, were prepared in deionized water. Potassium phosphate buffers with different pH (11, 8.5, and 7.6) were also prepared in deionized water.

4.3.3.3.2 Preparation of tissue homogenates for glutathione assay

Liver, thigh muscles and brain tissue samples were homogenized in 3% *m*-phosphoric acid in the dilution ratio (9:1 v/w). The frozen tissues were first weighed and homogenized at 4:1 v/w, and then 5 parts of 3% *m*-phosphoric acid were added. Afterwards, the homogenates were centrifuged at 15,000 x g for 10 min at 4°C using an Allegra™ 25R Beckman Coulter centrifuge. The supernatant was used for GSH/GSSG assay and the pellet was used for protein thiols and biuret protein measurements.

4.3.3.3.3 Reduced-glutathione (GSH) assay

The measurements were done in duplicate for all tissue samples. The supernatant was mixed with potassium phosphate buffer (pH 11), and the absorbance was measured at 412 nm (A1). DTNB was added to the cuvette (0.1 mM final concentration), the samples were incubated in the dark for 20 min at room temperature, and then the second absorbance was measured at 412 nm (A2). The difference in absorbance before and after the addition of DTNB was calculated (A2-A1) and the GSH concentration was calculated using a standard curve. For the blank, 3% *m*-phosphoric acid was used instead of the supernatant.

4.3.3.3.4 Oxidized-glutathione (GSSG) assay

The measurements were done in duplicate for all tissue samples. The supernatant was mixed with potassium phosphate buffer (pH 11), 0.2 mM NADPH was added and the absorbance was measured at 340 nm (A1). Glutathione reductase (0.2 U/ml) was added and the samples were incubated for 40 min at room temperature. Then, the second absorbance was measured at 340 nm (A2). The decrease in absorbance was calculated (A1-A2) and the GSSG concentration was calculated using a standard curve. For the blank, 3% *m*-phosphoric acid was used instead of the supernatant.

4.3.3.3.5 Protein thiols assay

The pellet was suspended in 26 μ l 10% SDS, diluted with 850 μ l potassium phosphate buffer (pH 8.5), mixed until complete dissolution, and then the absorbance was measured at 412 nm (A1). DTNB was added to the cuvette (0.1 mM final concentration), the samples were incubated in the dark for 20 min at room temperature, and then the second absorbance was measured at 412 nm (A2). The difference in absorbance before and after the addition of DTNB was calculated (A2-A1), and the protein thiols were calculated using reduced GSH as a calibration standard. For the blank, 10% SDS was used instead of the pellet and it was diluted with potassium phosphate buffer (pH 8.5) to the same volume as the samples. After dissolving the pellet and diluting with potassium phosphate buffer (pH 8.5), aliquots for the biuret protein assay were taken before doing the protein thiols assay.

4.3.3.4 HPLC method for ascorbic acid determination

Determination of total vitamin C (ascorbic acid plus dehydroascorbic acid) as well as ascorbic acid and dehydroascorbic acid in brain, spinal cord and liver tissues was done using a validated HPLC method (Romeu-Nadal et al., 2006). The HPLC system consisted of Waters Model 590 pump system, Model 481 LC spectrophotometer, WatersTM Model 717 Plus autosampler and a Millennium data module (Millipore-

Waters, Milford, MA, USA). All chromatographic separations were carried out on a reversed phase C₁₈ column (Nova-Pak[®] C18 4µm 150 x 3.9 mm) maintained at 25°C.

Brain, spinal cord, and liver tissue samples were homogenized in 0.56% *m*-phosphoric acid in the dilution ratio (5:1 v/w). The measurements of vitamin C and total vitamin C were done in duplicate for all tissue samples. For the determination of total vitamin C content, dehydroascorbic acid was reduced to ascorbic acid with DL- DTT. 100 µl of the tissue homogenates were mixed with 267 µl of DTT (100 mM), vortexed for 30 seconds, and then the mixture was incubated at room temperature (25°C) in the dark for 15 min. The homogenates were then centrifuged at 15,000 x g for 15 min at 4°C using an Allegra[™] 25R Beckman Coulter centrifuge, and the supernatant was used for HPLC analysis. To analyze ascorbic acid, 100 µl of the tissue homogenates were directly centrifuged at 15,000 x g for 15 min, and the supernatant was used for analysis.

Using the previous HPLC technique, ascorbic acid and total vitamin C were assayed by directly injecting 50 µl of the filtrate into the HPLC system. Isocratic chromatographic separation was done using a mobile phase composed of Milli-Q water with acetic acid (0.1%, v/v) and methanol in proportion of (80: 20) delivered at 0.5 ml/min. Calibration curves were constructed from known concentrations of ascorbic acid solutions in 0.56% (w/v) *m*-phosphoric acid. Ascorbic acid was identified by comparing the retention time of the sample peak with that of the ascorbic acid standard at 254 nm.

4.3.4 Statistical Analysis

Statistical analyses were performed using Excel software (Microsoft Office 2002). Results were expressed as the mean ± SEM. Differences among the four experimental groups were determined by one-way analysis of variance followed by Dunnett's post hoc test for pair-wise multiple comparisons to the control transgenic SOD G93A group using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). The level of significance was set at $p < 0.05$. Differences between the control-wild type group (CW) and the control transgenic SOD G93A (CT) group were determined by two-tailed unpaired t-test. A probability of less than 0.05 for the null hypothesis was considered significant.

5. RESULTS

5.1 *In vitro* study

5.1.1 The effect of zinc-extracted SOD (Cu-SOD) on ascorbate oxidation

The *in vitro* experiments were conducted to see the effect of Cu-SOD on the initial rate of ascorbate air oxidation and to compare it to the rate of ascorbate oxidation. The zinc was extracted from the enzyme according to previous procedures (Sampson and Beckman, 2001). The results showed that Cu-SOD enzyme accelerates the rate of ascorbate oxidation, while native SOD has no effect (Figures 5.1 and 5.2). In Cu-SOD, the acceleration of ascorbate oxidation was only partially removed after adding EDTA.

5.1.2 The effect of H₂O₂-treated SOD enzyme preparation on ascorbate oxidation

Different H₂O₂-treated preparations of SOD were made with the hypothesis that in sporadic ALS, rather than having a mutation to the enzyme, the enzyme suffers oxidative damage that weakens its affinity for zinc and changes the catalytic activity. The native SOD enzyme was treated with H₂O₂ and ascorbate or H₂O₂ alone in concentrations equal to that of the SOD as described in the materials and methods section. The effects of these SOD preparations on ascorbate oxidation are shown in Figure 5.3. The results have shown that the rate of ascorbate oxidation was most highly accelerated with the ascorbate plus H₂O₂-treated enzyme (A-H₂O₂ SOD), followed by H₂O₂ treated SOD (H₂O₂ SOD), then finally the Zn-extracted SOD (Cu-SOD), while native SOD (nSOD) had no effect (note that nSOD was in lower concentration due to a limited supply for these experiments).

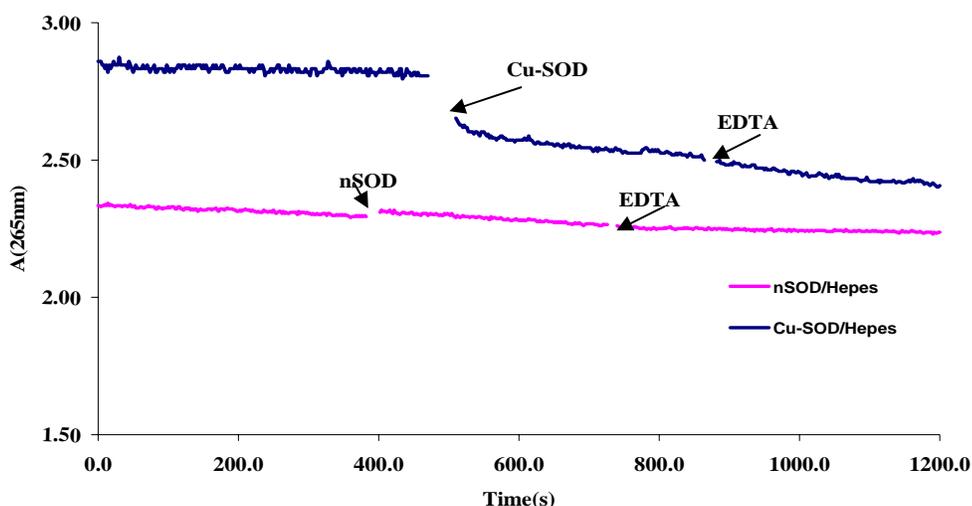


Figure 5.1 The change in ascorbate absorbance against time in the presence of Zn-extracted SOD (*Cu-SOD*) and native SOD (*nSOD*). The rate of ascorbate oxidation was measured at λ_{max} 265 nm in 10 mM HEPES buffer, pH 7.2, 37°C, and with subsequent additions of SOD enzyme preparations in equimolar amounts and EDTA (as indicated by arrows). Concentrations in the cuvette were: 0.16 mM ascorbate, 21 mM nSOD or 21 mM Cu-SOD, and 0.5 mM EDTA.

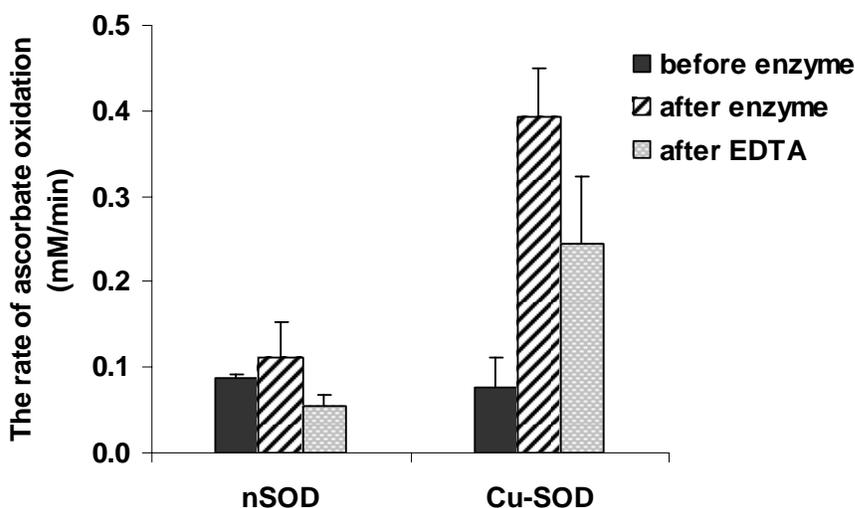


Figure 5.2 The rate of ascorbate oxidation in the presence of Zn-extracted SOD (*Cu-SOD*) and native SOD (*nSOD*). The rate of ascorbate oxidation was measured at λ_{max} 265 nm in 10 mM HEPES buffer, pH 7.2, 37°C as in Figure 5.1, with ascorbate alone (before enzyme) and with subsequent additions of SOD enzyme preparations (after enzyme) and EDTA (after EDTA). Concentrations in the cuvette were: 0.16 mM ascorbate, 21 mM nSOD or 21 mM Cu-SOD, and 0.5 mM EDTA. The rates were calculated from the portions of the progress curve before and after additions, and the values are the mean of two experiments \pm SEM.

The Cu-SOD preparation accelerated ascorbate oxidation less strongly in these experiments than in Figure 5.1 and 5.2, although this was with a separate Cu-SOD preparation and was in phosphate buffer rather than HEPES. Unlike with Zn-extracted SOD, the acceleration of ascorbate oxidation with the two H₂O₂ treated SOD was removed after adding EDTA.

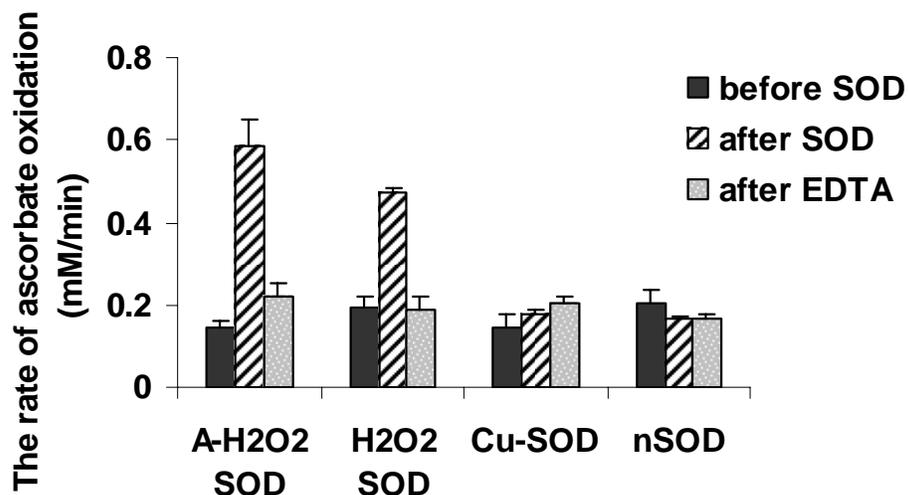


Figure 5.3 *The effect of different SOD preparations and EDTA on the rate of ascorbate oxidation.* The rate of ascorbate oxidation was measured at λ_{\max} 265 nm in 50 mM potassium phosphate buffer, pH 7.2, 37°C, with ascorbate alone (before SOD), and with subsequent additions of SOD enzyme preparations (after SOD) and EDTA (after EDTA): (nSOD) native SOD enzyme, (H₂O₂ SOD) H₂O₂-treated enzyme, (A- H₂O₂ SOD) H₂O₂ plus ascorbate treated enzyme, and (Cu-SOD) Zn-extracted SOD. Concentrations in the cuvette were: 0.16 mM ascorbate; 10.68 mM nSOD; 21 mM Cu-SOD, 21 mM H₂O₂ SOD or 21 mM A- H₂O₂ SOD; and 0.5 mM EDTA. The values are the mean rates of two experiments \pm SEM.

The effect of different SOD preparations on the rate of ascorbate oxidation was further tested by adding EDTA first to the reaction media before adding the different SOD enzyme preparations. It was found that EDTA removed the acceleration of ascorbate oxidation in all SOD enzyme preparations except for the Zn-extracted one. The results are shown in Figure 5.4, and confirm that only the Cu-SOD persisted in catalyzing ascorbate oxidation in the presence of EDTA.

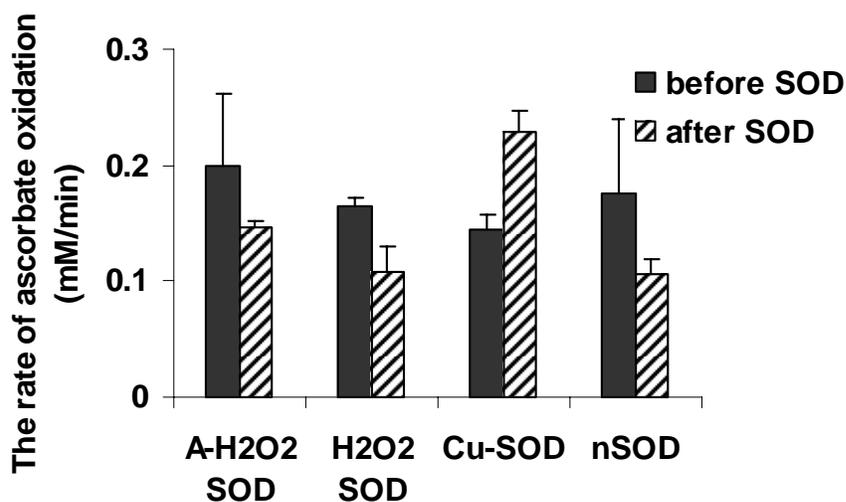


Figure 5.4 The effect of different SOD preparations on the rate of ascorbate oxidation in the presence of EDTA. The rate of ascorbate oxidation was measured at λ_{\max} 265 nm in 50 mM potassium phosphate buffer, pH 7.2, 37°C, containing EDTA, with ascorbate alone (before SOD), and with subsequent additions of SOD enzyme preparations (after SOD): (nSOD) native SOD enzyme, (H₂O₂ SOD) H₂O₂-treated enzyme, (A- H₂O₂ SOD) H₂O₂ plus ascorbate treated enzyme, and (Cu-SOD) Zn-extracted SOD. Concentrations in the cuvette were: 0.16 mM ascorbate; 10.68 mM nSOD; 21 mM Cu-SOD, 21 mM H₂O₂ SOD or 21 mM A- H₂O₂ SOD; and 0.5 mM EDTA. The values are the mean rates of two experiments \pm SEM.

The effect of another chelator (EGTA) on the rate of ascorbate oxidation with different SOD preparations was tested. The results are shown in Figure 5.5. The results have shown that EGTA worked better than EDTA as a chelator in preventing background autoxidation of ascorbate. Adding EGTA first to the reaction media before adding the different SOD enzyme preparations resulted in removing the acceleration of ascorbate oxidation in all SOD enzyme preparations except the Zn-extracted one. These results were consistent with what we have obtained with EDTA.

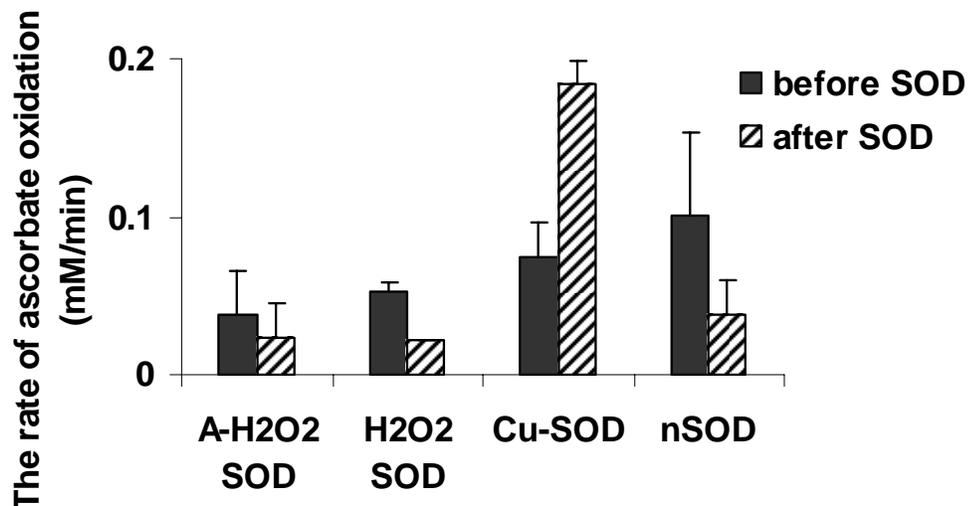


Figure 5.5 The effect of different SOD preparations on the rate of ascorbate oxidation in the presence of EGTA. The rate of ascorbate oxidation was measured at λ_{\max} 265 nm in 50 mM potassium phosphate buffer, pH 7.2, 37°C, containing EGTA, with ascorbate alone (before SOD), and with subsequent additions of SOD enzyme preparations (after SOD): (nSOD) native SOD enzyme, (H₂O₂ SOD) H₂O₂-treated enzyme, (A- H₂O₂ SOD) H₂O₂ plus ascorbate treated enzyme, and (Cu-SOD) Zn-extracted SOD. Concentrations in the cuvette were: 0.16 mM ascorbate; 10.68 mM nSOD; 21 mM Cu-SOD, 21 mM H₂O₂ SOD or 21 mM A- H₂O₂ SOD; and 0.5 mM EGTA. The values are the mean rates of two experiments \pm SEM.

Since H₂O₂ produced during ascorbate oxidation may contribute to the rate of ascorbate oxidation, the effect of adding catalase enzyme with EGTA to the reaction media before adding different SOD enzyme preparations was tested. The results are shown in Figure 5.6. Comparing Figures 5.6 and 5.5, it was found that the removal of H₂O₂ by catalase enzyme in the presence of EGTA did not decrease the rate of ascorbate oxidation either before or after adding the SOD enzyme preparations. With native SOD, the presence of catalase appeared to reverse the effect of SOD, from inhibiting to accelerating ascorbate oxidation.

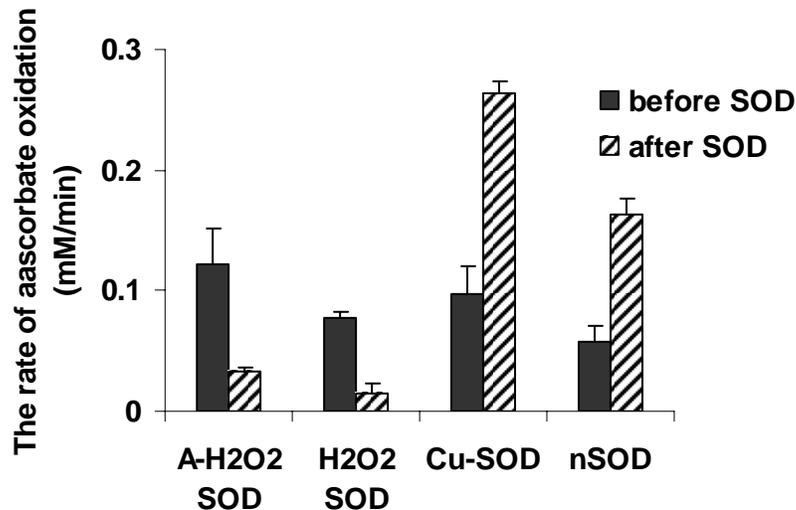


Figure 5.6 The effect of different SOD preparations on the rate of ascorbate consumption in presence of catalase and EGTA. The rate of ascorbate oxidation was measured at λ_{\max} 265 nm in 50 mM potassium phosphate buffer, pH 7.2, 37°C, containing EGTA and catalase, with ascorbate alone (before SOD), and with subsequent additions of SOD enzyme preparations (after SOD): (nSOD) native SOD enzyme, (H₂O₂ SOD) H₂O₂-treated enzyme, (A- H₂O₂ SOD) H₂O₂ plus ascorbate treated enzyme, and (Cu-SOD) Zn-extracted SOD. Concentrations in the cuvette were: 0.16 mM ascorbate; 10.68 mM nSOD; 21 mM Cu-SOD, 21 mM H₂O₂ SOD or 21 mM A-H₂O₂ SOD; 0.5 mM EGTA; and 10 U/ml catalase. The values are the mean rates of two experiments \pm SEM.

5.1.3 The effect of catechin and uric acid on ascorbate oxidation in the presence of Cu-SOD

Some experiments with the flavonoids catechin and quercetin and with uric acid were conducted. All are efficient antioxidants and observed to protect ascorbate from oxidation in different situations (Ames et al., 1981), (Kuhnau J, 1976). The effect of catechin and uric acid on ascorbate oxidation in the presence of the Zn-extracted SOD (Cu-SOD) is shown in Figure 5.7. Uric acid was found to be protective by 67% against ascorbate oxidation by Cu-SOD, and catechin was protective by 72%. The results with quercetin were inconclusive due to overlapping absorbance of quercetin and ascorbate so that the extent to which the Cu-SOD enzyme was oxidizing ascorbate or quercetin was not clear (not shown).

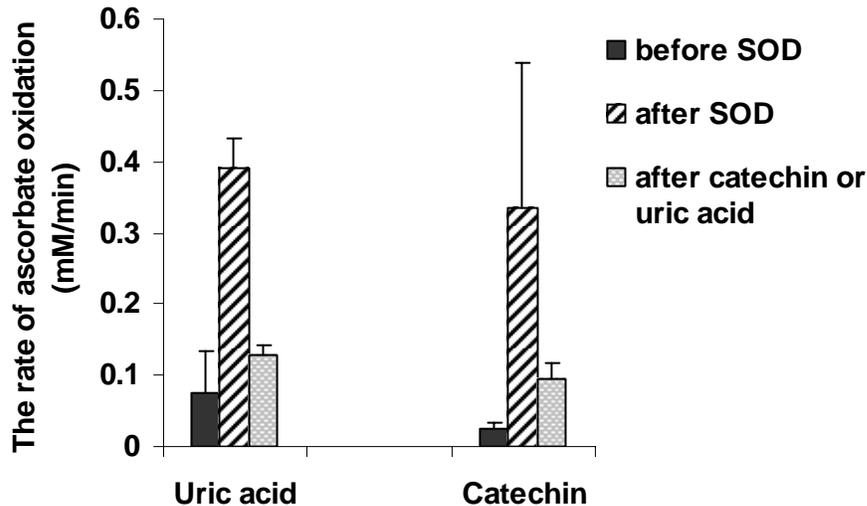


Figure 5.7 The effect of catechin and uric acid on the rate of ascorbate oxidation in presence of Cu-SOD. The rate of ascorbate oxidation was measured at λ_{\max} 265 nm in 10 mM HEPES buffer, pH 7.2, 37°C, containing EDTA, with ascorbate alone (before SOD), and with subsequent additions of Cu-SOD (after SOD) and catechin or uric acid (after catechin or uric acid). Concentrations in the cuvette were: 0.16 mM ascorbate, 15 mM Cu-SOD, 34 mM tri potassium EDTA previously neutralized to pH 7.2, and 0.1 mM catechin or 0.1 mM uric acid. The values are the mean rates of two experiments \pm SEM.

5.2 In vivo study

5.2.1 Food consumption

Food intake was calculated four times during the study, on weeks 3, 4, 8 and 9, and it was calculated for the whole group/day. The data are represented in Table 5.1. Food intake was increased in week 4 among all groups compared to other weeks. There was no apparent reason for this and I speculate that it might be because of the acclimatization of the animals to the new environment and the food. In comparing the food intakes of the different groups (average of the 4 measurements from each cage), there was no significant difference between them (Table 5.1). There were some

limitations in food intake calculation such as food spilling by the animals and estimating how much each mouse in a cage ate per day.

Table 5.1 *Food intake in different mice groups during the different four weeks**

	CW	CT	A	Q	AQ
Week 3	3.33	6.00	8.00	7.00	8.00
Week 4	10.50	10.60	10.60	10.00	10.60
Week 8	6.83	7.20	5.60	6.20	7.40
Week 9	6.83	5.00	6.40	6.80	7.20
Average	6.88±1.19	7.20±1.09	7.65±0.99	7.50±0.76	8.30±0.70

*Values are the amount of food consumed in grams divided by the number of animals in that cage. Statistical analysis on the average food intakes over the 4 weeks was done by one-way ANOVA followed by Dunnett's Multiple Comparison Test, which found no significant difference in food intake among the transgenic animals compared to control wild-type ($p>0.05$).

5.2.2 Body weight in mice

The weight of the animal was monitored weekly during the whole study. The body weight results are represented in Figure 5.8. There was a significant decrease in the body weight of (CT) control transgenic mice starting from the 7th week ($p<0.05$) compared to (CW) control wild type group (Figure 5.9). Also there was significant decrease in the body weight of all the transgenic mice groups starting from the 9th week ($p<0.05$) in comparison to (CW) control wild type group (Figure 5.10). The loss in body weight was not related to the food intake as there was not a significant decrease in food intake despite the significant decrease in body weight.

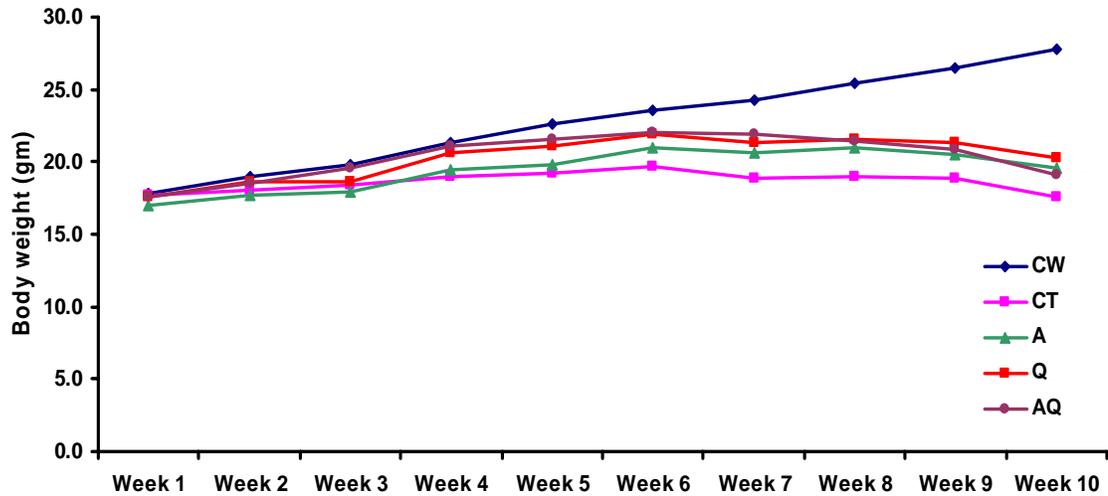


Figure 5.8 The mice body weights over the 10 weeks of the study. (CW) control wild type group, (CT) control transgenic SOD G93A mice, (A) ascorbate-fed transgenic mice, (Q) quercetin-fed transgenic, and (AQ) ascorbate + quercetin-fed transgenic. Body weight was calculated as the mean \pm SEM; n=5 for all groups except for the control-wild type (CW), n=6.

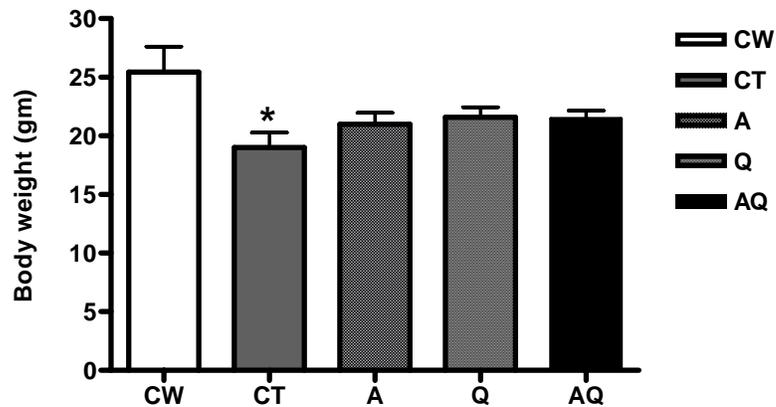


Figure 5.9 The body weight of mice in the 8th week of the study. (CW) control wild type group, (CT) control transgenic SOD G93A mice, (A) ascorbate-fed transgenic mice, (Q) quercetin-fed transgenic, and (AQ) ascorbate + quercetin-fed transgenic. Body weight was calculated as the mean \pm SEM; n=5 for all groups except for the control wild type (CW), n=6. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison tests which found significant decrease only in the body weight of (CT) control transgenic SOD G93A mice compared to the (CW) control-wild type group (*p<0.01).

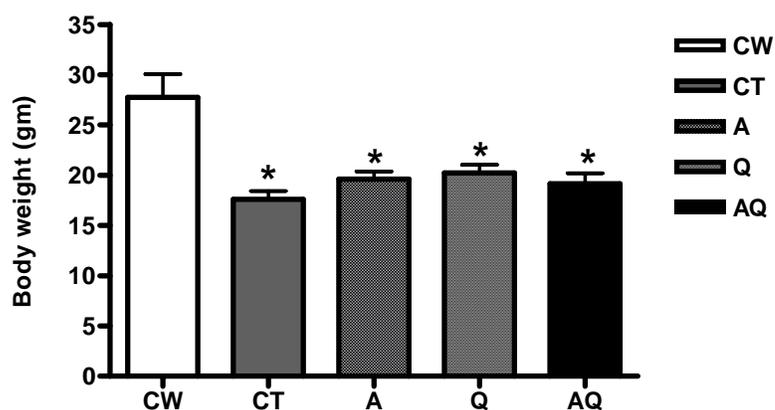


Figure 5.10 The body weight of mice in the 10th week of the study. (CW) control wild type group, (CT) control transgenic SOD G93A mice, (A) ascorbate-fed transgenic mice, (Q) quercetin-fed transgenic, and (AQ) ascorbate + quercetin-fed transgenic. Body weight was calculated as the mean \pm SEM; n=5 for all groups except for the control wild type (CW), n=6. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison tests which found a significant decrease in the body weight of all transgenic SOD G93A mice groups in comparison to (CW) control-wild type group (*p<0.01).

5.2.3 Monitoring disease development in mice

The mice were monitored for development of disease signs and symptoms twice per week starting from the third week until the 7th week, and then 3 times per week for the 8th and 9th week. In the last week, mice were monitored on a daily basis as they started to develop most of the signs and we did not want them to reach an end stage or to suffer. The test that we used to monitor the signs of the disease was their ability to grasp a pencil with their hind limb when they were suspended by the tail. We graded their response as good (having no problem in holding the pencil once we suspend them by their tail), having some difficulty (struggling for sometime, but finally could hold the pencil), or having much difficulty (struggling a lot and couldn't hold the pencil as their hind limb was too weak to do this movement).

Another test was used to monitor the mice activity level by counting the number of steps they miss over 2 minutes when they were put in a metabolic cage with a wire grid bottom. This test was started on the 6th week (twice per week) and then it was stopped at the 9th week as their activity level was very low at that time. The mice were

also watched closely every day while feeding them and monitoring their activity level by the way they moved and climbed over the cage lid. Upon subjective observation, the CW group had the highest activity level during the whole study followed by Q group, then AQ, then A, and finally CT with the lowest activity level. In the 10th week, one mouse of AQ group started to develop the disease more rapidly than the others and started to get paralyzed in one hind limb, so i had to sacrifice it with one of the CW group. This was done 4 days before sacrificing all groups. Also another mouse was sacrificed from the same group (AQ) one day early due to a bad wound in its tail and swelling in its leg. The next day, all the mice were sacrificed. Table 5.2 represents the number of animals in each group that became paralyzed in one limb (diseased) versus the animals that did not develop advanced signs or symptoms at the time of sacrifice (healthy).

Table 5.2 *The number of diseased versus healthy mice in each group at the time of sacrifice*

	CT	A	Q	AQ	Total
Diseased	3	2	1	3	9
Healthy	2	3	4	2	11
Total number of mice	5	5	5	5	20

5.2.4 Comparisons between transgenic and wild type mice

Different measurements of oxidative stress were compared between control wild type (CW) and control transgenic SOD G93A (CT) mice (Table 5.3). There was 47% increase ($p < 0.05$) in liver TBARS in control transgenic SOD G93A (CT) mice compared to control wild type (CW) mice. There was also 136% increase ($p < 0.05$) in GSH/GSSG ratio in the muscle of CT mice compared to CW mice.

Table 5.3 Comparison between different measurements in control wild type (CW) and control transgenic SOD G93A (CT) mice

	Brain		Liver		Thigh muscle	
	CW	CT	CW	CT	CW	CT
GSH (mmol/mg protein)	5.47±0.68	3.8±0.72	6.91±2.7	7.44±1.47	1.13±0.42	2.4±0.46
GSSG (mmol/mg protein)	1.53±0.50	1.85±0.61	2.95±1.38	1.26±0.35	0.88±0.22	0.85±0.22
Total GSH (GSH+2GSSG) (mmol/mg protein)	8.52±1.53	7.51±1.61	12.80±4.59	9.96±1.49	2.49±0.75	4.09±0.85
GSH/GSSG	6.89±3.01	4.16±2.47	4.30±1.34	9.39±3.73	1.43±0.36	3.37±0.84*
Protein thiols (mmol/mg protein)	12.10±2.95	16.5±3.37	8.67±4.25	5.65±2.04	4.79±1.76	5.03±1.00
TBARS (mmol/mg protein)	0.308±0.071	0.328±0.058	0.047±0.005	0.069±0.007*	0.010±0.001	0.016±0.004
	Kidney		Heart		Spinal cord	
TBARS (mmol/mg protein)	0.006±0.001	0.008±0.001	0.080±0.081	0.051±0.009	0.626±0.104	0.326±0.074

Each value is the mean ± SEM; n=6 for CW and n=5 for CT. Statistical analysis was done using an unpaired two-tailed t-test.

*Significant difference in CT (p<0.05) group in comparison to CW group.

The levels of vitamin C, oxidized-vitamin C and total vitamin C levels were compared in brain, spinal cord, and liver tissues between control wild type (CW) and control transgenic SOD G93A (CT) mice. The data are summarized in Table 5.4. The level of oxidized-vitamin C in spinal cord was found to be unexpectedly lower by 60% (p<0.05) in control transgenic SOD G93A (CT) mice compared to control wild type (CW). However, the level of oxidized-vitamin C in spinal cord was very low in comparison to other tissues. The most remarkable result was that oxidized-vitamin C in liver of control transgenic SOD G93A (CT) mice was 29-times that of (p<0.01) control wild type (CW). The levels of vitamin C and total vitamin C in liver were also

significantly increased (43%, $p < 0.05$ and 460%, $p < 0.01$) in control transgenic SOD G93A (CT) mice compared to control wild type (CW). It should be noted that prior to these measurements (but not any others), the liver tissue had thawed in a malfunction of the -80°C freezer. It could be that some of the ascorbate oxidation occurred during this period, but even so it shows a large difference between transgenic and wild type livers (both of which thawed).

Table 5.4 Comparison between vitamin C, oxidized-vitamin C and total vitamin C in control wild type (CW) and control transgenic SOD G93A (CT) mice

	Brain		Spinal cord		Liver	
	CW	CT	CW	CT	CW	CT
Vitamin C ($\mu\text{mol}/\text{mg}$ tissue)	7.9 \pm 0.3	7.8 \pm 0.3	3.4 \pm 0.4	3.1 \pm 0.1	6.1 \pm 0.6	8.7 \pm 1.0*
Total Vitamin C ($\mu\text{mol}/\text{mg}$ tissue)	11.1 \pm 0.5	10.6 \pm 0.6	4.1 \pm 0.4	3.4 \pm 1.5	7.2 \pm 0.7	40.4 \pm 4.5**
Dehydroascorbic acid ($\mu\text{mol}/\text{mg}$ tissue)	3.2 \pm 0.2	2.8 \pm 0.2	0.7 \pm 0.1	0.3 \pm 0.1*	1.1 \pm 0.2	31.7 \pm 3.5**

Each value is the mean \pm SEM; $n=6$ for CW and $n=5$ for CT. Statistical analysis was done using an unpaired two-tailed t-test. * Significant difference in CT ($p < 0.05$) group in comparison to CW group. ** Significant difference in CT ($p < 0.0001$) group in comparison to CW group.

5.2.5 Comparisons of Dietary Treatments

5.2.5.1 TBARS assay

TBARS assays were done on brain, liver, thigh muscle, kidney, heart and spinal cord from transgenic SOD G93A mice fed different diets (Figure 5.11). The highest TBARS level was found in spinal cord tissue, in which there was a significant 98% increase ($p < 0.05$) in TBARS in the ascorbate-fed group (A) in comparison to control transgenic mice (CT). Regarding other tissues there was no significant difference

between the different dietary treatment groups in comparison to (CT) control transgenic mice.

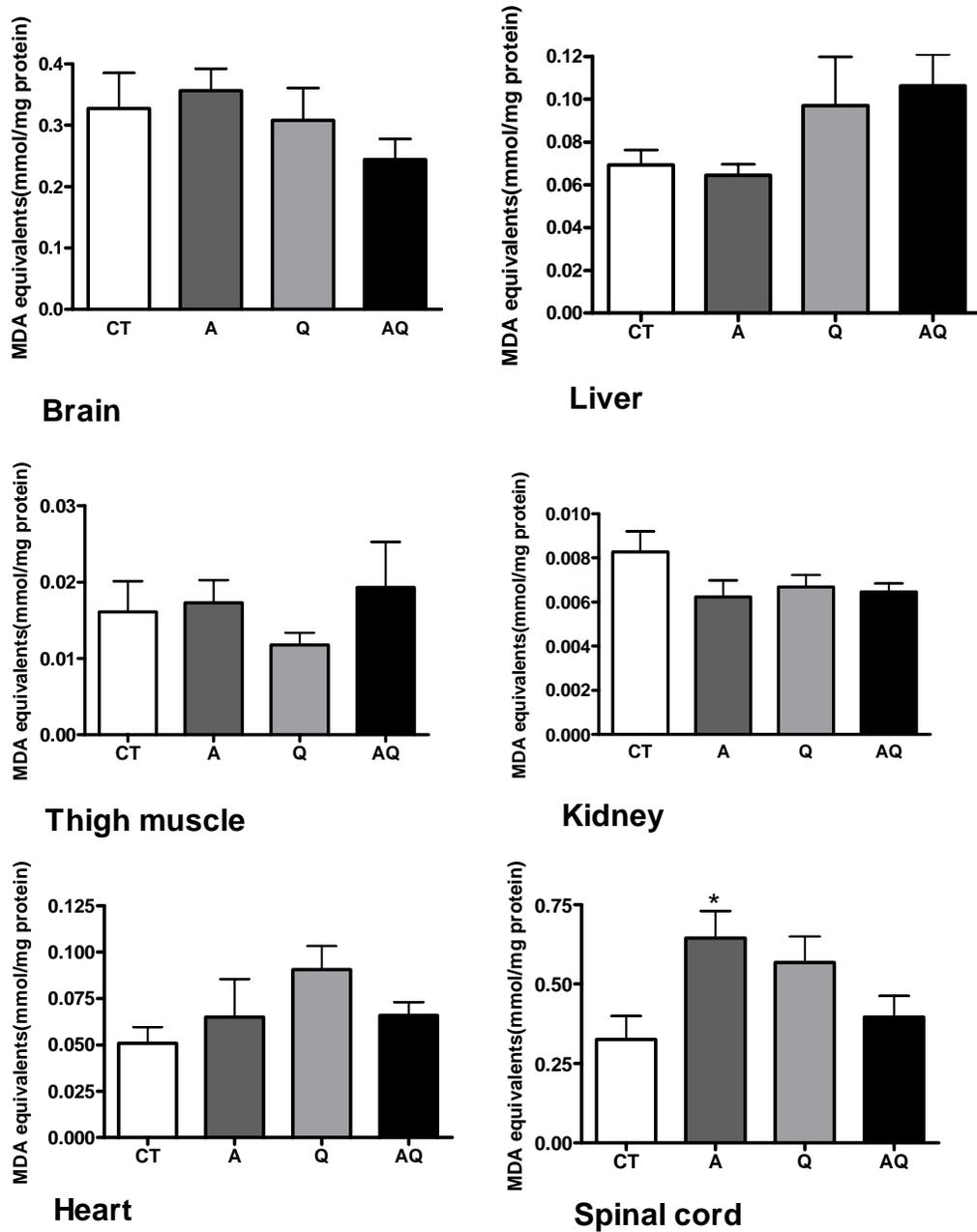


Figure 5.11 *TBARS assays in different tissues.* Assays of TBARS as MDA equivalents were done on different tissues from SOD G93A transgenic mice fed the following diets for 10 weeks: (CT) control-diet transgenic SOD G93A mice, (A) ascorbate-supplemented, (Q) quercetin-supplemented, and (AQ) ascorbate + quercetin-supplemented. Each value is the mean \pm SEM; n=5. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison tests (* p<0.05).

5.2.5.2 GSH assay

Reduced-glutathione was measured in brain, liver and thigh muscle. The data are represented in Figure 5.12. The highest level of GSH was found in liver tissue. There was no significant difference between the different dietary treatment groups in comparison to (CT) control transgenic mice SOD G93A group.

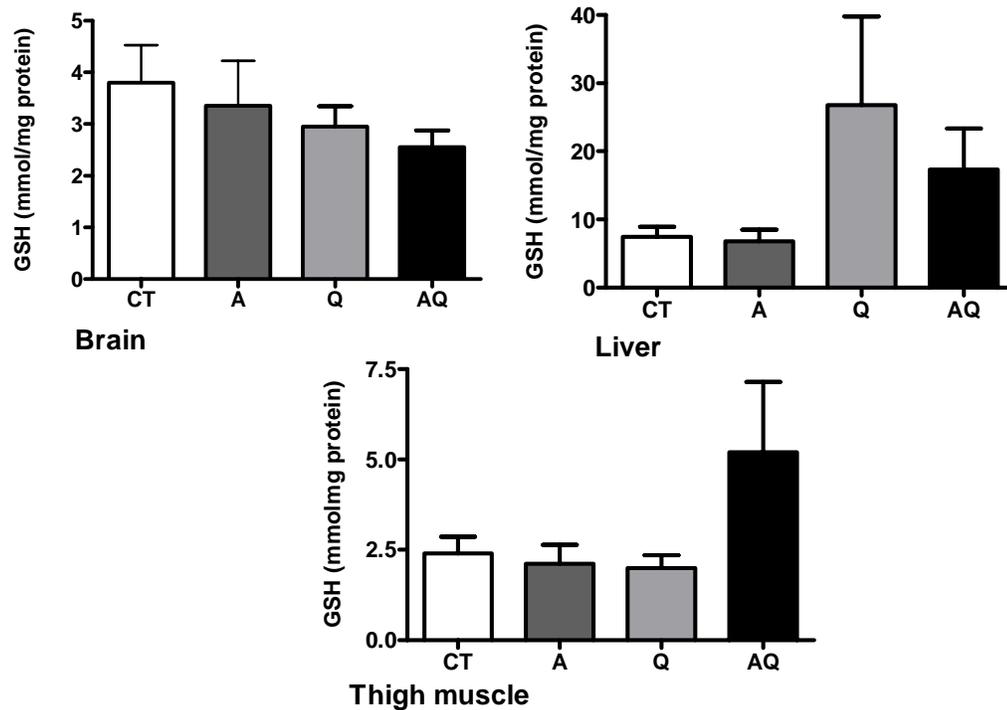


Figure 5.12 *GSH assays in different tissues.* Assays of GSH were done on different tissues from SOD G93A transgenic mice fed the following diets for 10 weeks: (CT) control-diet transgenic SOD G93A mice, (A) ascorbate-supplemented, (Q) quercetin-supplemented, and (AQ) ascorbate + quercetin-supplemented. Each value is the mean \pm SEM; n=5. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison tests.

5.2.5.3 GSSG assay

Oxidized-glutathione was measured in brain, liver, and thigh muscle. The data are represented in Figure 5.13. There was no significant difference between the different

dietary treatment groups in comparison to the (CT) control transgenic mice SOD G93A group.

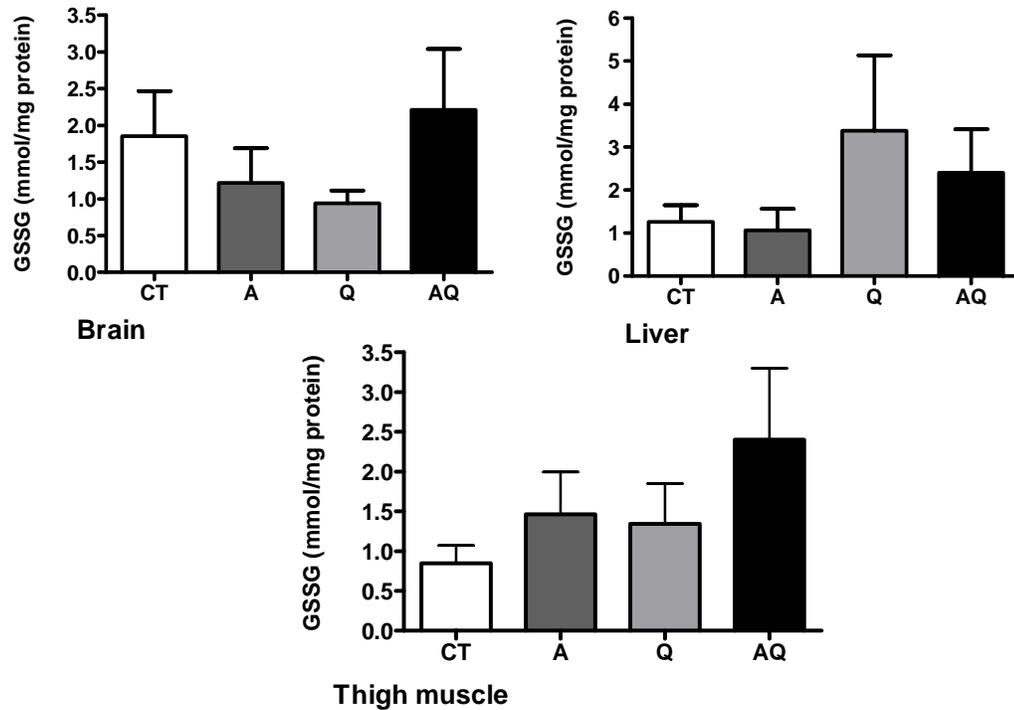


Figure 5.13 *GSSG assays in different tissues.* Assays of GSSG were done on different tissues from SOD G93A transgenic mice fed the following diets for 10 weeks: (CT) control-diet transgenic SOD G93A mice, (A) ascorbate-supplemented, (Q) quercetin-supplemented, and (AQ) ascorbate + quercetin-supplemented. Each value is the mean \pm SEM; n=5. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison tests.

5.2.5.4 The total glutathione (GSH+2GSSG)

Total glutathione was calculated in brain, liver, and thigh muscle. The data are represented in Figure 5.14. The highest level of total glutathione was found in liver tissue. There was no significant difference between the different dietary treatment groups in comparison to the (CT) control transgenic mice SOD G93A group.

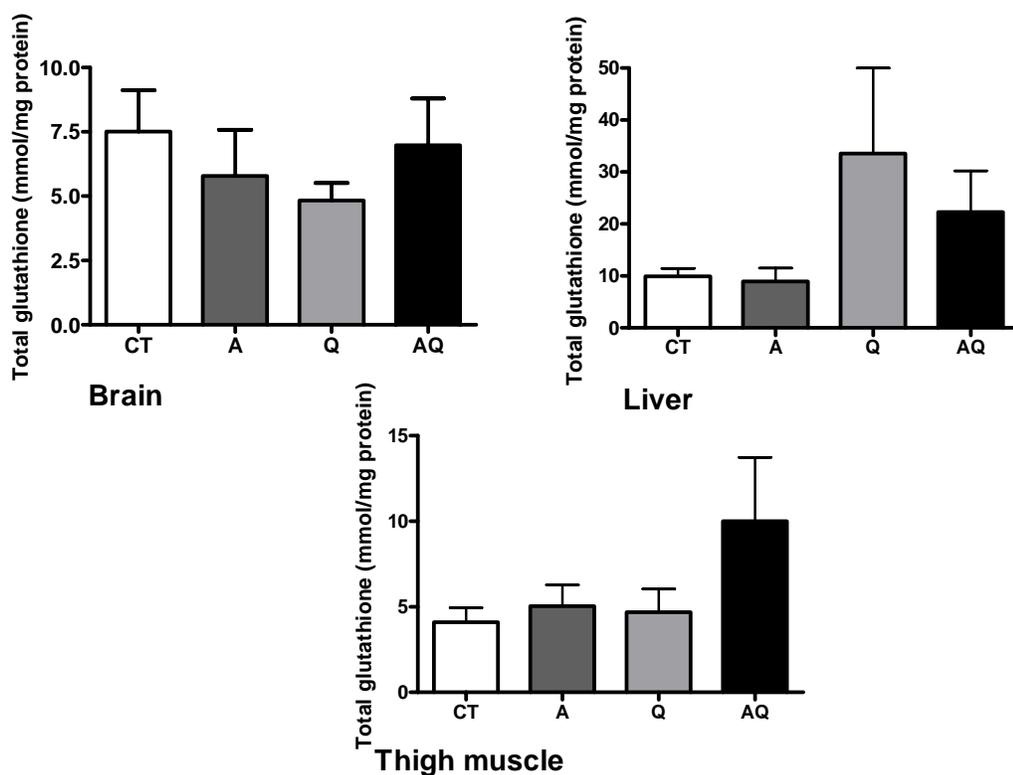


Figure 5.14 Total glutathione (GSH+2GSSG) in different tissues. (CT) control transgenic SOD G93A mice, (A) ascorbate-fed transgenic mice, (Q) quercetin-fed transgenic, and (AQ) ascorbate + quercetin-fed transgenic. Each value is the mean \pm SEM; n=5. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison tests.

5.2.5.5 The ratio between reduced and oxidized-glutathione (GSH/GSSG)

The ratio between reduced and oxidized-glutathione (GSH/GSSG) was calculated in brain, liver, and thigh muscle. The data are represented in Figure 5.15. The highest ratio of reduced to oxidized-glutathione (GSH/GSSG) was found in liver tissue. There was no significant difference between the different dietary treatment groups in comparison to the (CT) control transgenic mice SOD G93A group.

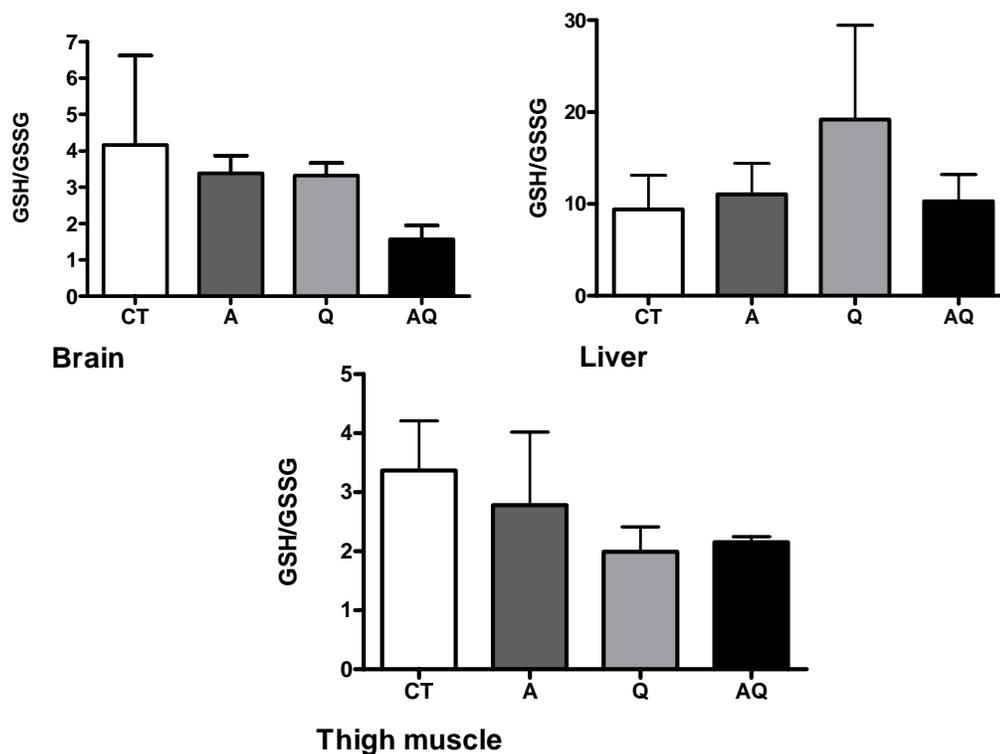


Figure 5.15 The ratio between reduced and oxidized-glutathione (GSH/GSSG) in different tissues. (CT) control transgenic SOD G93A mice, (A) ascorbate-fed transgenic mice, (Q) quercetin-fed transgenic, and (AQ) ascorbate + quercetin-fed transgenic. Each value is the mean \pm SEM; n=5. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison tests.

5.2.5.6 The measurement of protein thiols

The measurement of total protein thiols was done in brain, liver, and thigh muscle. The results are represented in Figure 5.16. In brain tissues, the total protein thiols were significantly decreased ($p < 0.05$) by 58% and 56% in both the (AQ) ascorbate + quercetin-fed transgenic mice group and in (Q) quercetin-fed transgenic mice group in comparison to (CT) control transgenic mice SOD G93A group.

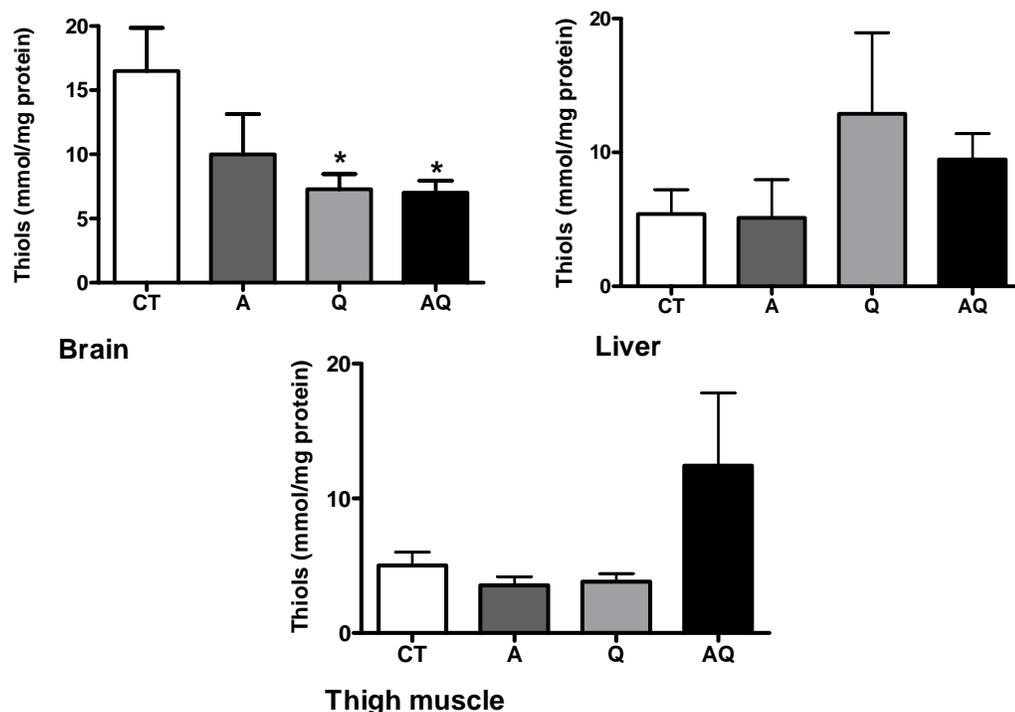


Figure 5.16 *The total protein thiols in different tissues.* Assays of total protein thiols were done on different tissues from SOD G93A transgenic mice fed the following diets for 10 weeks: (CT) control-diet transgenic SOD G93A mice, (A) ascorbate-supplemented, (Q) quercetin-supplemented, and (AQ) ascorbate + quercetin-supplemented. Each value is the mean \pm SEM; $n=5$. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison tests (* $p<0.05$).

5.2.5.7 The measurement of ascorbic acid and total vitamin C

The concentration of ascorbic acid and total vitamin C in brain, spinal cord, and liver from the different dietary groups was measured using HPLC. The highest concentration of vitamin C was found in liver tissue (Figure 5.17). In liver tissue the level of vitamin C was significantly lower ($p<0.05$) by 40% in (Q) quercetin-fed transgenic mice group compared to the (CT) control transgenic mice SOD G93A group. In brain and spinal cord, there was no significant difference in vitamin C concentrations between the different dietary treatment groups in comparison to the (CT) control transgenic mice SOD G93A group.

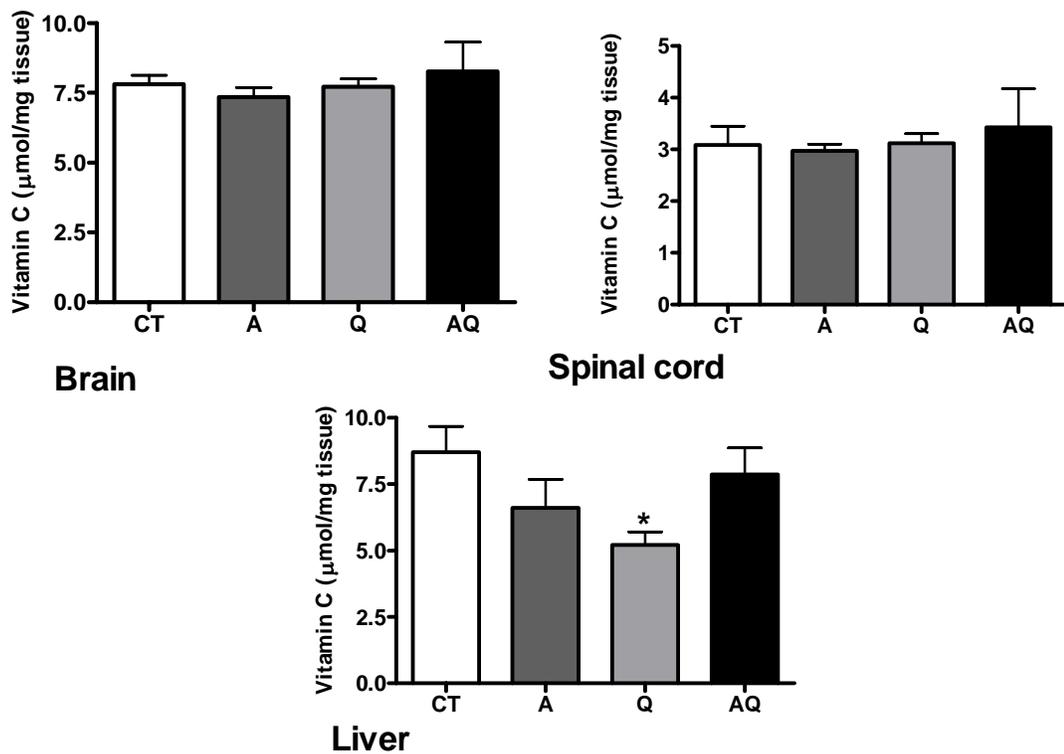


Figure 5.17 *Vitamin C concentrations in different tissues.* Assays of vitamin C were done on different tissues from SOD G93A transgenic mice fed the following diets for 10 weeks: (CT) control-diet transgenic SOD G93A mice, (A) ascorbate-supplemented, (Q) quercetin-supplemented, and (AQ) ascorbate + quercetin-supplemented. Each value is the mean \pm SEM; $n=5$. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison tests (* $p<0.05$).

The concentration of total vitamin C was also measured in brain, spinal cord, and liver homogenates (Figure 5.18). The highest level of total vitamin C was found in liver tissue. Also the level of total vitamin C in liver was found to be significantly lower ($p<0.05$) by 40% in (Q) quercetin-fed transgenic mice group compared to (CT) control transgenic mice SOD G93A group. In brain and spinal cord there was no significant difference in total vitamin C concentrations between the different dietary treatment groups in comparison to (CT) control transgenic mice SOD G93A group.

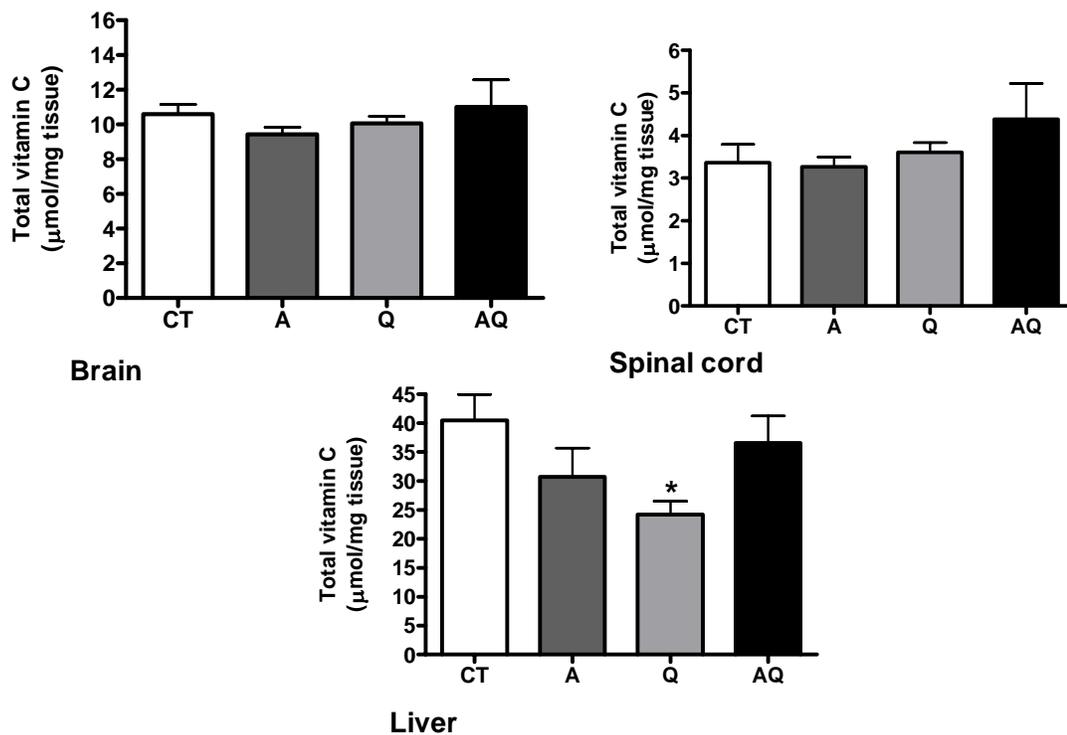


Figure 5.18 Total vitamin C concentrations in different tissues. Assays of total vitamin C were done on different tissues from SOD G93A transgenic mice fed the following diets for 10 weeks: (CT) control-diet transgenic SOD G93A mice, (A) ascorbate-supplemented, (Q) quercetin-supplemented, and (AQ) ascorbate + quercetin-supplemented.. Each value is the mean \pm SEM; n=5. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison tests (* $p < 0.05$).

The concentration of oxidized-vitamin C (dehydroascorbic acid) was calculated in brain, spinal cord and liver tissues (Figure 5.19). Much higher levels of oxidized-vitamin C were found in liver tissue. The level of oxidized-vitamin C in liver was found to be significantly lower ($p < 0.05$) by 40% in (Q) quercetin-fed transgenic mice compared to (CT) control transgenic mice. In spinal cord there was a 250% increase ($p < 0.05$) in oxidized-vitamin C in the (AQ) ascorbate + quercetin-fed transgenic mice group compared to the (CT) control transgenic mice SOD G93A group. In brain there was no significant difference in oxidized-vitamin C concentrations between the different dietary treatment groups in comparison to (CT) control transgenic mice SOD G93A group.

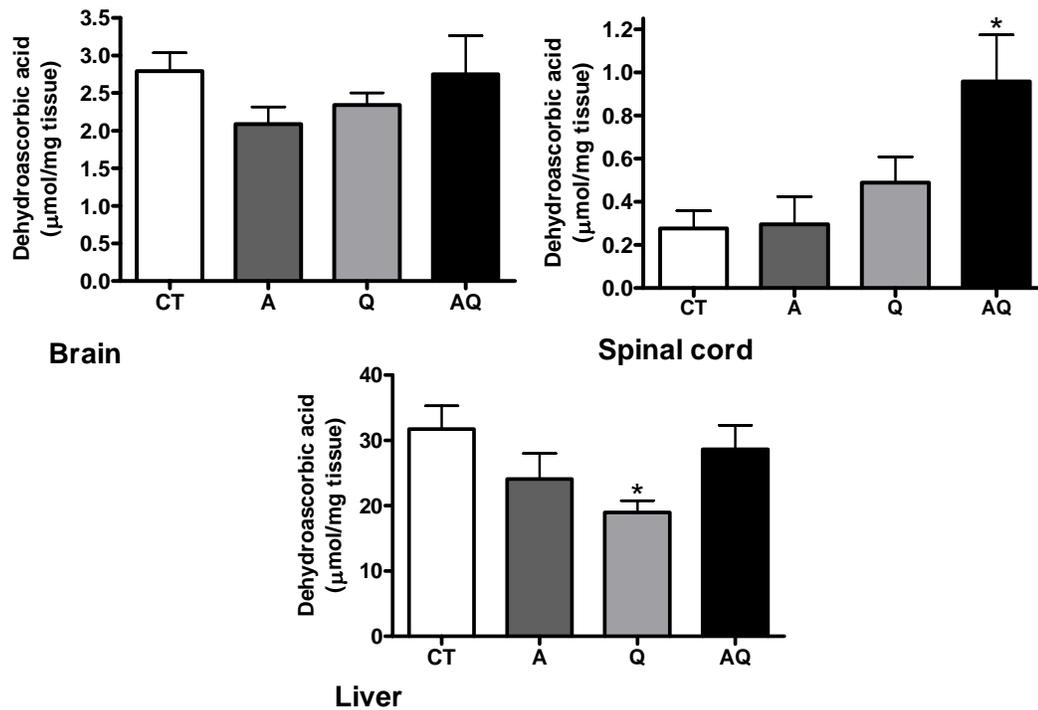


Figure 5.19 Oxidized-vitamin C concentrations in different tissues. (CT) control transgenic SOD G93A mice, (A) ascorbate-fed transgenic mice, (Q) quercetin-fed transgenic, and (AQ) ascorbate + quercetin-fed transgenic. Each value is the mean \pm SEM; n=5. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison tests (* p<0.05).

5.2.6 Comparisons of healthy versus diseased animals

5.2.6.1 TBARS assay

Different measurements of oxidative stress was compared between transgenic SOD G93A mice from all four treatment groups which developed advanced signs of disease at the time of sacrificing the animals (“diseased”) and the transgenic SOD G93A mice which had no advanced signs of disease at the time of sacrifice (“healthy”). For TBARS, the data are shown in Figure 5.20. There was no significant differences in TBARS levels between the diseased and the healthy transgenic SOD G93A mice in any of the tissues.

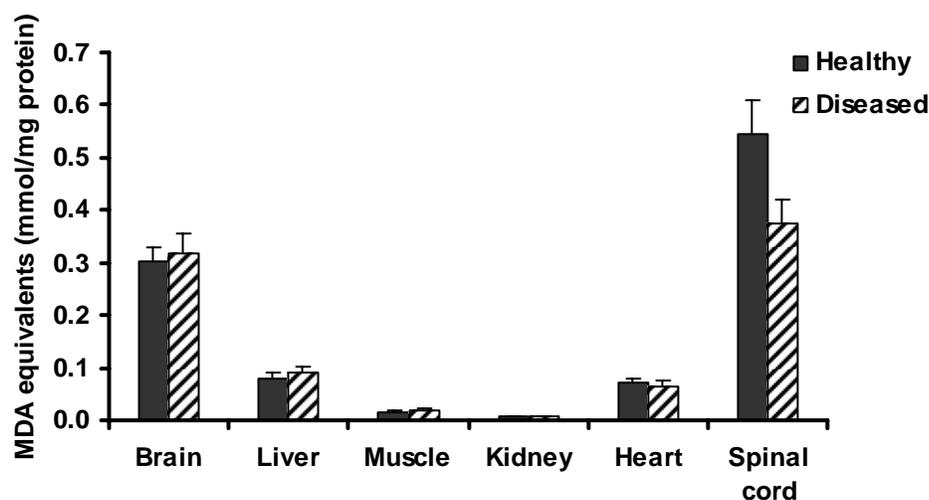


Figure 5.20 TBARS level in different tissues in healthy versus diseased transgenic SOD G93A mice. TBARS was measured as described in materials and methods. The data are represented as the mean \pm SEM; n=9 for the diseased mice and n=11 for the healthy mice. Statistical analysis was done using an unpaired two-tailed t-test.

5.2.6.2 GSH assay

For GSH measurement, the data are shown in Figure 5.21. In muscle tissue, there was a 120% increase ($p < 0.05$) in GSH level in the diseased transgenic SOD G93A mice compared to the healthy mice.

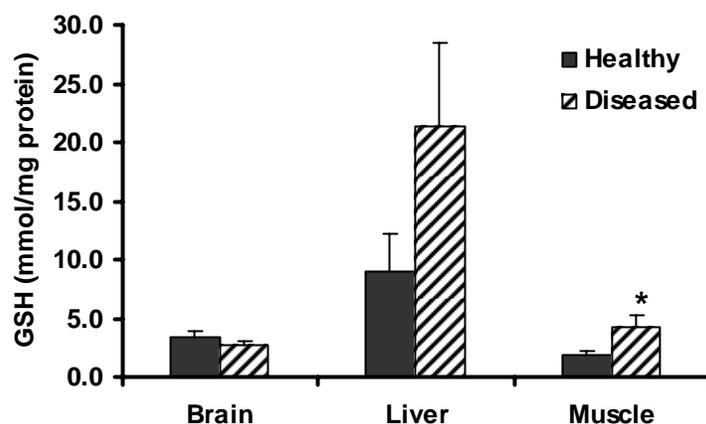


Figure 5.21 GSH measurements in different tissues in healthy versus diseased transgenic SOD G93A mice. The data are represented as the mean \pm SEM; n=9 for the diseased mice and n=11 for the healthy mice. Statistical analysis was done using an unpaired two-tailed t-test (* $p < 0.05$).

5.2.6.3 GSSG assay

For GSSG measurement, the data are shown in Figure 5.22. The GSSG level was generally higher in diseased transgenic SOD G93A mice compared to healthy transgenic SOD G93A mice in all tissues. The highest GSSG level was found in liver tissue but it was not significantly higher in diseased mice compared to the healthy transgenic SOD G93A mice. In muscle tissue there was a 130% increase ($p < 0.05$) in the GSSG level in diseased transgenic SOD G93A mice compared to the healthy mice.

5.2.6.4 The total glutathione (GSH+2GSSG)

The total glutathione (GSH+GSSG) was calculated in diseased transgenic SOD G93A mice and it was compared to the healthy transgenic SOD G93A mice. The data are shown in Figure 5.23. The total glutathione was the highest in liver tissue, but it was not significantly different between the diseased transgenic SOD G93A mice compared to the healthy mice. The total glutathione level was 130% higher ($p < 0.05$) in diseased transgenic SOD G93A mice compared to the healthy mice in muscle tissue.

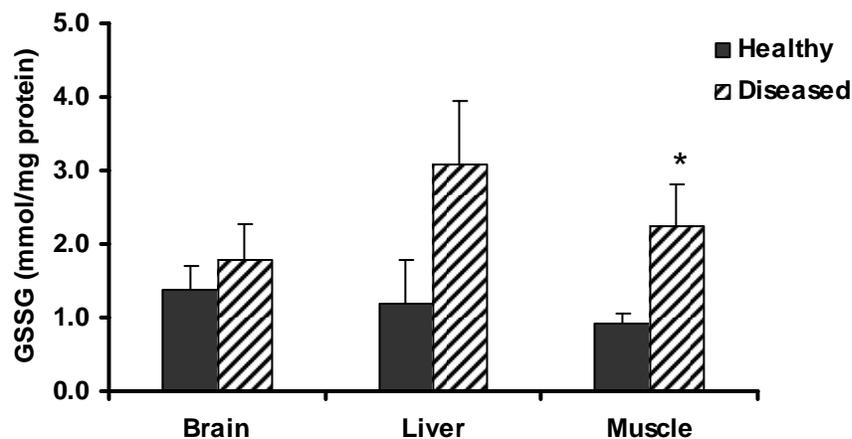


Figure 5.22 GSSG measurements in different tissues in healthy versus diseased transgenic SOD G93A mice. The data are represented as the mean \pm SEM; $n=9$ for the diseased mice and $n=11$ for the healthy mice. Statistical analysis was done using an unpaired two-tailed t-test ($*p < 0.05$).

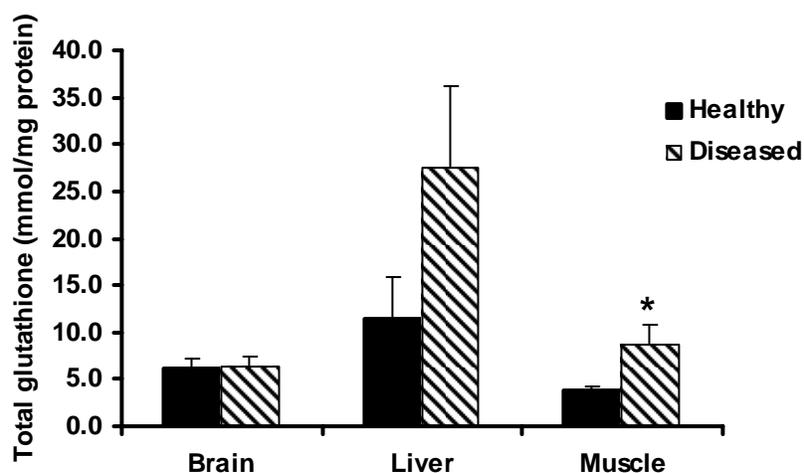


Figure 5.23 The total glutathione (GSH+2GSSG) in different tissues in healthy versus diseased transgenic SOD G93A mice. The data are represented as the mean \pm SEM; n=9 for the diseased mice and n=11 for the healthy mice. Statistical analysis was done using an unpaired two-tailed t-test (*p< 0.05).

5.2.6.5 The ratio between reduced and oxidized-glutathione (GSH/GSSG)

The ratio between reduced and oxidized glutathione (GSH/GSSG) was calculated in diseased transgenic SOD G93A mice and it was compared to the healthy mice. The data are shown in Figure 5.24. There was no significant difference in the ratio (GSH/GSSG) between diseased transgenic SOD G93A mice and healthy mice in all tissues.

5.2.6.6 The measurement of protein thiols

For the total protein thiols, the data are shown in Figure 5.25. There was no significant difference in the total protein thiols between diseased transgenic SOD G93A mice versus the healthy mice in all tissues.

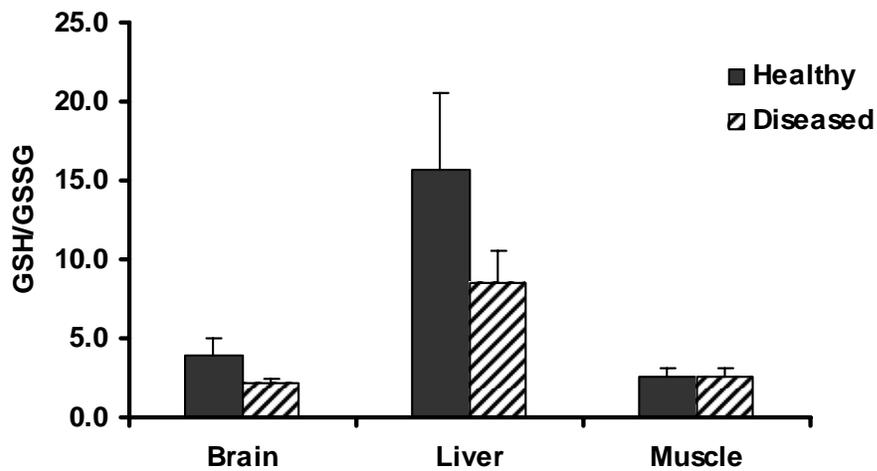


Figure 5.24 The ratio between reduced and oxidized-glutathione (GSH/GSSG) in different tissues in healthy versus diseased transgenic SOD G93A mice. The data are represented as the mean \pm SEM; n=9 for the diseased mice and n=11 for the healthy mice. Statistical analysis was done using an unpaired two-tailed t-test.

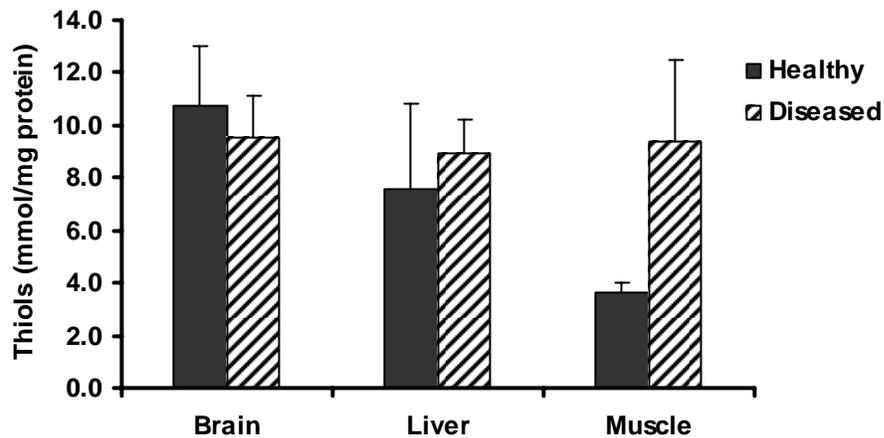


Figure 5.25 The total protein thiols in different tissues in healthy versus diseased transgenic SOD G93A mice. The data are represented as the mean \pm SEM; n=9 for the diseased mice and n=11 for the healthy mice. Statistical analysis was done using an unpaired two-tailed t-test.

5.2.6.7 The measurement of ascorbic acid and total vitamin C

The levels of vitamin C, total vitamin C and oxidized-vitamin C (dehydroascorbic acid) were compared between diseased and healthy transgenic SOD G93A mice. The data for vitamin C are represented in Figure 5.26. The level of vitamin

C was generally higher in diseased transgenic SOD G93A mice compared to healthy mice in all tissues, but not significantly in brain or liver tissues. In spinal cord tissue there was 40% increase ($p < 0.01$) in vitamin C level in diseased transgenic SOD G93A mice compared to the healthy mice.

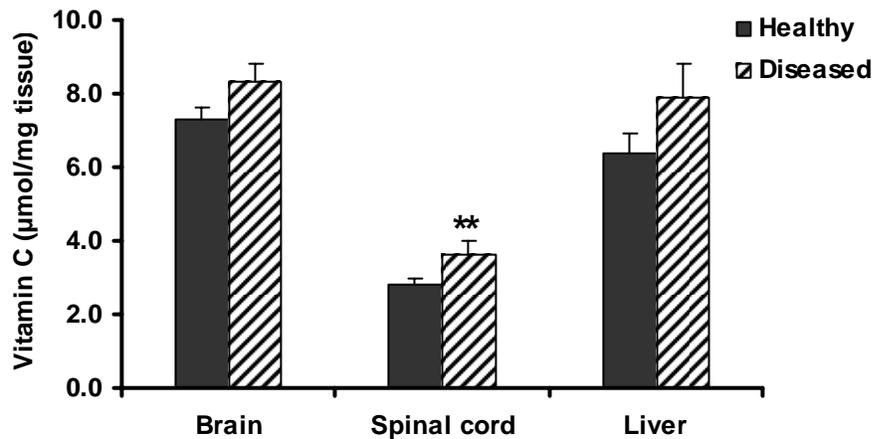


Figure 5.26 Vitamin C level in different tissues in healthy versus diseased transgenic SOD G93A mice. The data are represented as the mean \pm SEM; $n=9$ for the diseased mice and $n=11$ for the healthy mice. Statistical analysis was done using an unpaired two-tailed t-test (** $p < 0.01$).

The level of total vitamin C was compared between diseased and healthy transgenic SOD G93A mice. The data are represented in Figure 5.27. The level of total vitamin C was generally higher in diseased transgenic SOD G93A mice compared to the healthy mice in all tissues, but not significantly in liver. In both brain and spinal cord tissues, there were significant increases ($p < 0.01$) (16% and 23%) in total vitamin C in diseased transgenic SOD G93A mice compared to the healthy mice.

The level of oxidized-vitamin C (dehydroascorbic acid) was also compared in brain, spinal cord and liver tissues between diseased and healthy transgenic SOD G93A mice. The data are represented in Figure 5.28. There was no significant difference between diseased and healthy transgenic SOD G93A mice.

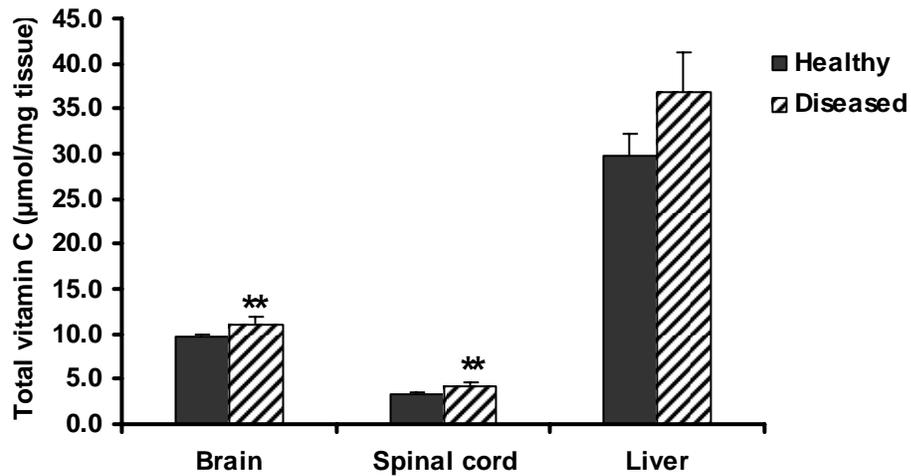


Figure 5.27 Total vitamin C levels in different tissues in healthy versus diseased transgenic SOD G93A mice. The data are represented as the mean \pm SEM; n=9 for the diseased mice and n=11 for the healthy mice. Statistical analysis was done using an unpaired two-tailed t-test (**p<0.01).

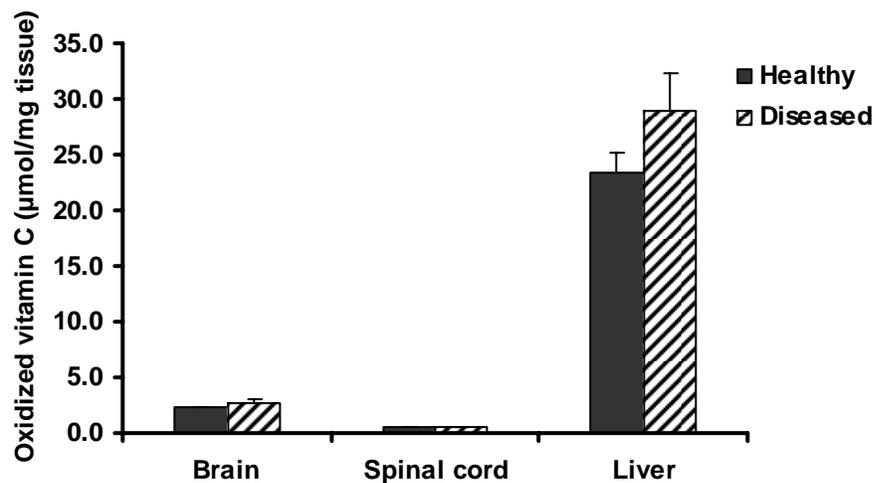


Figure 5.28 Oxidized-vitamin C level in different tissues in healthy versus diseased transgenic SOD G93A mice. The data are represented as the mean \pm SEM; n=9 for the diseased mice and n=11 for the healthy mice. Statistical analysis was done using an unpaired two-tailed t-test.

6. DISCUSSION

ALS is one of the most serious neurodegenerative diseases. It is characterized by loss of both upper and lower motor neurons (Julien JP, 2001). There are two distinct types of ALS: familial ALS which represents 10% of ALS cases, and sporadic ALS with the majority of cases (90%) (Julien JP, 2001). The exact cause of ALS is still unknown, but there have been different hypotheses trying to explain the mystery behind this serious illness. The only proven cause until now is the mutation in the gene expressing the superoxide dismutase enzyme (SOD1) (Rosen et al., 1993). This mutation occurs in only 20% of the familial cases. Different hypotheses have been proposed to explain the toxicity of the mutant SOD1 in ALS. One of these hypotheses is that the mutant enzyme can release zinc from its binding site, as the affinity of the mutant enzyme for zinc is decreased. Once the enzyme is zinc-deficient, it gains an ability to induce oxidative damage (Beckman et al., 2001).

Beckman's group suggested that zinc loss from mutant SOD enzyme leads to apoptosis of motor neurons by a mechanism involving peroxynitrite (Beckman et al., 2001). They hypothesized that zinc-deficient SOD can steal electrons from cellular reductants such as ascorbate or GSH and transfer them to oxygen to produce a bound superoxide intermediate. The superoxide intermediate in turn can react with nitric oxide to produce peroxynitrite, which induces cellular damage and provokes apoptosis. In the studies of this thesis, we investigated whether zinc-deficient SOD, resulting from mutations or oxidative damage to the superoxide dismutase enzyme (SOD), gains function as an ascorbate oxidase that contributes to the pathology of ALS. We proposed that the zinc-deficient enzyme can either catalyze the transfer of two electrons from ascorbate to oxygen to produce hydrogen peroxide as an end product, or in single electron steps, it can transfer electrons from ascorbate to oxygen and release free superoxide anion which then dismutates to hydrogen peroxide. By either

mechanism, the aerobic oxidation of ascorbate in the presence of zinc-deficient SOD enzyme leads to production of hydrogen peroxide which contributes to the toxicity of zinc-deficient SOD in ALS. This differs from the hypothesis of Beckman et al., 2001 in that it does not involve the need for nitric oxide.

6.1 *In vitro* objectives

6.1.1 The effect of zinc-extracted SOD (Cu-SOD) on ascorbate oxidation

The *in vitro* experiments supported the hypothesis that zinc-deficient SOD can act as an ascorbate oxidase, showing that Zn-extracted SOD (Cu-SOD) accelerated the rate of ascorbate aerobic oxidation 5-fold, while native SOD had no effect (Figures 5.1 and 5.2). The acceleration by Cu-SOD was greater in HEPES buffer than in phosphate buffer (Figure 5.2 versus Figure 5.3), partly due to greater background ascorbate autoxidation in phosphate buffer. With Cu-SOD, the acceleration of ascorbate oxidation was only partially removed after adding EDTA in HEPES buffer (Figure 5.2), and unaffected by EDTA in phosphate buffer (Figures 5.3 and 5.4). The lack of inhibition by EDTA means that it is copper that is tightly bound to the enzyme and not copper released to the solution that accelerates ascorbate oxidation. This means that after zinc loss from the active site of the enzyme, copper becomes more exposed and thus prone to reduction by ascorbate and re-oxidation by oxygen. These experiments also show that Cu-SOD is able to catalyze substantial ascorbate oxidation with oxygen alone, producing H₂O₂ as the presumed end product. These experiments suggest a different or alternative reaction that unlike the one suggested by Beckman et al 2001, does not need nitric oxide and produces H₂O₂ instead of peroxynitrite.

Part of our hypothesis was that the SOD enzymes in sporadic ALS, rather than having a mutation, suffer oxidative damage that weakens the zinc affinity and changes the catalytic activity. In order to test this hypothesis, different H₂O₂-treated preparations of SOD were made, and the effects of these SOD preparations on ascorbate oxidation were tested. The results showed that the rate of ascorbate oxidation was most highly accelerated with ascorbate plus H₂O₂-treated enzyme (A-H₂O₂ SOD), followed by H₂O₂

treated SOD (H_2O_2 SOD), then finally Zn-extracted SOD (Cu-SOD), while native SOD (nSOD) decreased the rate from that of the baseline (Figure 5.3). These findings with H_2O_2 -treated enzymes were not from any residual H_2O_2 since it was dialysed away (along with any released zinc or copper). Unlike with Cu-SOD, the acceleration of ascorbate oxidation with the two H_2O_2 -treated SODs was removed after adding EDTA. From these findings, we can say that the acceleration of ascorbate oxidation by H_2O_2 -treated SODs was due to copper that is still loosely bound to the enzyme but it was pulled away from the active site of the enzyme by the strong chelating effect of EDTA. This means that oxidative damage to the SOD enzyme in sporadic ALS may lead to ascorbate oxidation *in vivo*. This mechanism represents a link between sporadic and familial ALS.

The same experiment was repeated but with a different chelating agent, EGTA. Like EDTA, adding EGTA to the reaction media prevented the acceleration of ascorbate oxidation by all SOD enzyme preparations except the Zn-extracted one (Figure 5.5 versus Figure 5.3). Also, EGTA worked better than EDTA as a chelator in preventing background autoxidation of ascorbate. These results are interesting in that EGTA is normally used as a calcium chelator and not generally recognized to inhibit metal-catalyzed oxidations.

Since H_2O_2 produced during ascorbate oxidation may contribute to the rate of ascorbate oxidation, the effect of adding catalase (with EGTA) to the reaction media before adding different SOD enzyme preparations was tested. Comparing Figures 5.6 and 5.5, it was found that the removal of H_2O_2 by catalase did not decrease the rate of ascorbate oxidation either before or after adding the SOD enzyme preparations. This confirms our hypothesis that the acceleration of ascorbate oxidation was solely from the effect of zinc-extracted SOD enzyme on oxidation by oxygen. With native SOD, the presence of catalase appeared to reverse the effect of SOD from inhibiting to accelerating ascorbate oxidation. This is an unexpected result that might be due to the increased amount of oxygen from the catalase reaction or due to mass action in removing H_2O_2 product of SOD activity.

6.1.2 The effect of catechin and uric acid on ascorbate oxidation in presence of Cu-SOD

The second *in vitro* objective was to test the effect of selected flavonoids such as catechin or quercetin and an antioxidant, uric acid, on the rate of ascorbate oxidation in the presence of Cu-SOD, and to see if they might have any protective effect. Both uric acid and the flavonoid catechin were found to be protective against ascorbate oxidation by Cu-SOD by 67% and 72%, respectively. The results with quercetin were inconclusive due to overlapping absorbance of quercetin and ascorbate so that the extent to which the Cu-SOD enzyme was oxidizing ascorbate or quercetin was not clear (data not shown). This supports the possibility that flavonoids as antioxidants can act synergistically with ascorbate to decrease the oxidative damage induced by the mutant SOD in ALS, or that they may protect against the pro-oxidant effect of ascorbate. Since we previously found quercetin to be the most synergistic with ascorbate in protecting against oxidative damage (Bandy and Bechara, 2001), we chose to test whether dietary ascorbate with quercetin offer protection to FALS mice. Also, ascorbate and quercetin have been found to inhibit elevated ROS generation in lymphoblast cell lines from FALS patients (Said et al., 2000). In this study they found that L-nitroarginine, a nitric oxide synthase inhibitor had no effect on ROS levels, which argues against the hypothesis of Beckman et al., 2001, of the involvement of nitric oxide in the oxidative damage that results from Zn-deficient SOD in ALS.

6.2 *In vivo* objectives

6.2.1 Comparison between transgenic and wild type mice

To investigate the role of oxidative damage in ALS pathogenesis, markers of oxidative stress, such as TBARS, the ratio between reduced and oxidized glutathione (GSH/GSSG) and total protein thiols were measured. In TBARS measurements, there was a 47% increase in liver TBARS in transgenic mice compared to the wild type. This result supports, in liver, the theory of increased oxidative stress during disease

progression in ALS. In the literature there is support for this theory as well. One study has reported higher levels of MDA in the spinal cord of FALS transgenic mice compared to the wild type (Hall et al., 1998), and the intensity of lipid peroxidation, evidenced by increased immunocytochemical staining of MDA-modified proteins, increased with the disease progression.

Another marker of oxidative stress is the ratio between reduced and oxidized glutathione (GSH/GSSG). The role of altered glutathione metabolism in ALS is still unclear. The reports on GSH concentration in spinal cord or motor cortex of ALS patients are equivocal, and results from studies on GSH peroxidase activities in ALS are controversial (Schulz et al., 2000). In this study it was found that in muscle tissue, the ratio between reduced and oxidized-glutathione was unexpectedly 136% higher in control transgenic mice (CT) compared to the wild type (CW). This increase possibly reflected compensatory protective mechanisms in this tissue.

In order to investigate whether tissue ascorbate is depleted in ALS mutant mice, and to investigate the protective role of ascorbate as an antioxidant against the oxidative damage that is caused by the mutant Cu/Zn SOD in ALS, total vitamin C (ascorbic acid and dehydroascorbic acid) and vitamin C levels were measured in brain, liver and spinal cord tissues. In spinal cord tissue, there was a 60% decrease in oxidized vitamin C (dehydroascorbic acid) in the control transgenic group (CT) compared to the wild type (CW). This might be against our hypothesis that the mutant SOD enzyme acts as ascorbate oxidase and hence increases the concentration of oxidized ascorbic acid in the affected area such as brain and spinal cord. However in both transgenic and wild type mice, the amount of oxidized-vitamin C in spinal cord was relatively small, and the levels of vitamin C and total vitamin C in spinal cord were slightly but insignificantly lower in the transgenic mice. A possible explanation for this result is that mechanisms for removal of dehydroascorbic acid from spinal cord were upregulated in transgenic mice.

Most notable were the results in liver tissue, where the levels of oxidized-vitamin C (dehydroascorbic acid) in FALS transgenic mice were 29-times that of wild type mice (Table 5.4). This result strongly supports the hypothesis that mutant SOD can oxidize ascorbate which may in some way contribute to disease progression. That this increase

in oxidized-vitamin C is only evident in liver may be due to the liver being the site of ascorbate biosynthesis in animals such as mice expressing L-gulonolactone oxidase (Nishikimi et al., 1994). Thus, the higher level of reduced-vitamin C (43%), and much higher level of total vitamin C (460%) in liver of FALS transgenic mice compared to wild type mice might be due to induction of ascorbic acid synthesis upon an increase in its oxidation by the mutant SOD enzyme.

6.2.2 Comparison of dietary treatments on FALS mice

In order to investigate the effect of feeding ascorbate and quercetin on disease progression in transgenic SOD G93A mice, oxidative stress markers such as TBARS, and the ratio between reduced and oxidized-glutathione (GSH/GSSG), and total protein thiols were measured. In spinal cord tissue, there was a 98 % increase in TBARS in ascorbate-fed transgenic mice compared to control transgenic SOD G93A mice. This means feeding ascorbate did not confer protection against disease progression. Instead, it increased oxidative stress. This is contrary to the hypothesis that ascorbate can compete with mutant SOD for scavenging the superoxide anion and consequently decrease oxidative stress induced by the mutant enzyme. On the other hand, we considered that supplementation with ascorbate may worsen disease progression by increasing superoxide and H₂O₂ production catalyzed by Zn-deficient SOD. This finding is against what has been cited in the literature. Nagano et al., 2003 have found that feeding the same high dose of ascorbate as in the current studies (0.8% w/w) to FALS mice had no effect on disease onset, but extended the time to end-point (paralysis) of the mice by 8% compared to the control. I did not follow the mice to that end-point, but the current results suggest that feeding vitamin C does not diminish, and may increase, oxidative stress in the spinal cord.

Feeding quercetin also did not decrease markers of oxidative stress in the FALS mice in any of the tissues. In brain tissue, there was 56%, and 58% decrease in total protein thiols in quercetin-fed and ascorbate + quercetin-fed transgenic mice group compared to control transgenic mice (Figure 5.16), suggesting that quercetin increased oxidative stress. These results are consistent with our observations *in vitro* that quercetin

may be co-oxidized by zinc-extracted SOD. These findings also are supported in our observations on the extent of disease progression in the different mice groups. From our observation, ascorbate + quercetin-fed transgenic mice group was the fastest group to develop the disease compared to other treatment groups. Another interesting finding was in spinal cord tissue where the level of oxidized-vitamin C was 250% higher in ascorbate + quercetin-fed compared to control transgenic mice. This means that feeding quercetin with ascorbate did not protect spinal cord against the pro-oxidant effect of ascorbate; instead it increased ascorbate oxidation.

In liver, there were some results suggesting that quercetin may spare vitamin C. In quercetin-fed transgenic mice there was a 40% decrease in vitamin C, total vitamin C and oxidized-vitamin C compared to control transgenic mice (Figures 5.19 - 5.19). This result suggests that dietary quercetin might have led to downregulation of ascorbate synthesis in the liver, shown by the decreased vitamin C and total vitamin C levels. However dietary quercetin did not significantly decrease any of the liver indices of oxidative stress (Figures 5.11, 5.15, and 5.16).

6.2.3 Comparisons of healthy versus diseased animals

In order to see the effect of disease progression on the different parameters, the transgenic mice (regardless of diet) were divided into two groups: the diseased group (n=9) which had developed advanced signs of disease at the time of sacrificing the animals, and the healthy transgenic SOD G93A mice (n=11) which had no advanced signs of disease at the time of sacrifice. Then the different measurements were compared between these two groups. In muscle tissue, the levels of reduced, oxidized, and total glutathione were significantly higher (120%, 130%, and 130%) in diseased transgenic mice compared to the healthy transgenic mice. The finding that the level of oxidized glutathione (GSSG) in muscle tissue was 130% higher in diseased mice compared to the healthy ones suggests that there is increased oxidative stress in muscle tissue with disease progression. The increased levels of reduced and total glutathione may be due to compensatory upregulation of glutathione synthesis in this tissue with disease progression.

Comparing the levels of vitamin C, total vitamin C and oxidized-vitamin C between disease and healthy transgenic mice revealed some interesting results. In spinal cord tissue, the levels of vitamin C and total vitamin C were 40% and 23% higher in diseased transgenic mice compared to the healthy one that did not have advanced signs of disease at the time of sacrifice. In brain tissue the level of total vitamin C was 16% higher in diseased transgenic mice compared to the healthy one. This means vitamin C did not deplete with disease progression. Instead, it increased in the affected area (brain and spinal cord). This might be due to upregulation of ascorbate uptake by these tissues during disease progression. It should be noted that oxidized-vitamin C can be re-reduced by glutathione (Meister A, 1992), which may explain a lack of accumulation of oxidized vitamin C. We do not have glutathione measurements for spinal cord, but in brain, there tended to be a decrease in the GSH/GSSG ratio (Figure 5.24), which would be consistent with increased demand for recycling of oxidized-vitamin C.

7. CONCLUSIONS

From *in vitro* experiments we conclude that zinc-deficient SOD, which might arise *in vivo* either from mutation or oxidative damage to the SOD enzyme, can catalyze ascorbate oxidation by molecular oxygen. This can occur with both Zn-extracted or H₂O₂-damaged SOD, but only with Zn-extracted SOD does it persist in accelerating ascorbate oxidation in the presence of EDTA or EGTA. This means that after zinc loss from the active site of the enzyme, copper becomes more exposed and thus prone to reduction by ascorbate and re-oxidation by oxygen. Uric acid and the flavonoid catechin were also found to protect ascorbate against oxidation by Cu-SOD. This supports the possibility that flavonoids as antioxidants can help decrease the oxidative damage induced by the mutant SOD in ALS.

From *in vivo* study we found that ascorbate did not protect against the oxidative damage that is caused by the mutant Cu/Zn SOD in ALS. Instead, it increased oxidative stress. This was evidenced by the higher spinal cord TBARS level (93%) in ascorbate-fed mice compared to the control FALS mice. From our study, supplementation with ascorbate would appear to worsen disease progression by increasing superoxide and H₂O₂ production catalyzed by Zn-deficient SOD.

Supplementation with quercetin alone or together with ascorbate did not protect against the oxidative damage in FALS mice, even depleting protein thiols in brain and increasing the level of oxidized-vitamin C in spinal cord. So the combined treatment of quercetin plus ascorbate did not protect against the pro-oxidant effect of ascorbate; instead adding quercetin unexpectedly increased oxidative stress in ALS.

One interesting finding is the higher level of oxidized-vitamin C in liver of FALS mice (29-times that of wild-type mice). This result coincided with 47% higher liver TBARS in FALS mice. These findings strongly support an involvement of mutant

SOD in oxidation of vitamin C and production of reactive oxygen species in liver. This effect may be amplified in liver due to compensatory upregulation of vitamin C biosynthesis in this organ. Feeding quercetin may be able to spare ascorbate, as evidenced by 40% lower levels of oxidized, reduced and total vitamin C in liver of quercetin-fed FALS mice.

In summary, these results support the hypothesis that zinc-deficient and mutant SOD can accelerate ascorbate oxidation *in vitro* and *in vivo*. The role of ascorbate and quercetin as antioxidants in ALS is not supported by our *in vivo* results. A limitation of this study however was the small sample size (5 mice per group). Nevertheless, the very large increase in liver dehydroascorbate in FALS mice points to a substantial disturbance of ascorbate homeostasis. Further investigation needs to be done to determine the role of ascorbate or flavonoids in ALS.

8. FUTURE STUDIES

These are some suggestions for the future studies:

1- Consider using higher number of FALS mice (8-10 mice/group), although we are limited by the high price of the transgenic mice, and that the supplier could only supply 20 at the same age.

2- The flavonoids and ascorbic acid could be injected intraperitoneally instead of feeding them. In this way we can avoid the inhibitory effect of flavonoids, especially quercetin, on ascorbate absorption and can achieve higher bioavailability of both flavonoids and ascorbate in tissues.

3- Consider using catechin instead of quercetin as catechin showed some protective effect in the *in vitro* study and we did not get positive results *in vivo* with quercetin.

4- Searching for a source of the mutant SOD, such as purifying it from the liver of FALS mice, and test the effect of the mutant SOD on ascorbate oxidation *in vitro*.

5- Using red blood cells or tissue homogenates from FALS mice to test the effect of the mutant SOD on ascorbate oxidation *in vitro*.

By combining all these factors we could find out the effect of mutant SOD on ascorbate oxidation and if flavonoids could help with ascorbate in maintaining the antioxidant balance inside the body during the course of ALS disease.

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