EFFECT OF DIETARY SUPPLEMENTATION WITH GLUTATHIONE, GLUTATHIONE ESTER, AND N-ACETYLCYSTEINE ON REDUCED GLUTATHIONE (GSH) LEVELS IN MITOCHONDRIA FROM RAT KIDNEY CORTEX AND MEDULLA

A THESIS

SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIRMENTS

FOR THE DEGREE

MASTER OF PHYSIOLOGY

BY

STEVEN C. BERTRAND

Committee Approval:

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BALL STATE UNIVERSITY
MUNCIE, INDIANA
JULY 2011

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ABSTRACT

THESIS: Effect of Dietary Supplementation with Glutathione, Glutathione Ester, and N-Acetylcysteine on Reduced Glutathione (GSH) Levels in Mitochondria from Rat Kidney Cortex and Medulla

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The present study determined whether dietary supplementation with reduced glutathione (GSH), glutathione ester (GSHE) or N-acetylcysteine (NAC) increased the mitochondrial level of GSH, the major antioxidant inside cells, in rat kidney cortex and medulla. Nine month-old female Lewis rats were given daily intraperitoneal injections of isotonic saline (n=6), or saline containing GSH (250mg or 0.81mmol/Kg of body wt; n=7), GSHE (12mg or 0.03mmol/Kg; n=8), or NAC (200mg or 1.22mmol/Kg; n=8) for four weeks. At the end of the injection period, the rats were anesthetized and the kidneys removed. The kidneys were separated into cortical and medullary sections, weighed, and homogenized. The sections were separated into cytosolic and mitochondrial fractions by differential centrifugation. The GSH levels were determined by a colorimetric assay. Cortical and medullary mitochondrial GSH levels were significantly increased by all three supplements. Cytosolic GSH levels were also significantly increased in both cortical and medullary sections. Thus, dietary supplementation can significantly increase the mitochondrial pool of GSH in the rat kidney.

Table of Contents

Appendix

List of Tables and Figures

Tables:

Figures:

Introduction

The purpose of the present study was to investigate whether exogenous dietary supplementation can increase the level of reduced glutathione (GSH) in mitochondria from the rat kidney. GSH functions inside the cell as the major antioxidant, and maintaining adequate levels is important for protecting the cell against damage by free radicals, such as the hydroxyl radical (·OH), superoxide radical ($\cdot O_2^-$), and nitric oxide (·NO). Free radicals are generated in oxidative metabolism and by other cellular functions (Andreyev, 2005; Zachara, 2006). GSH also functions as a coenzyme in metabolic reactions, plays a role in neutralizing toxic compounds, and is considered one of the most important anticarcinogens inside cells (Zachara, 2006). Glutathione-S-transferases (GST) protect the cell from various toxins, including products of lipid peroxidation, by adding a GSH molecule to the toxin (Andreyev, 2005). They are located primarily in the cytosol, but may also be present in the mitochondrial matrix (Lash, 1996). Mitochondrial DNA (MtDNA) is particularly susceptible to damage from free radicals because it is located close to the respiratory chain, lacks the protection of histones or DNA-binding proteins, and has limited base excision repair mechanisms (Genova, 2004; Lim, 2002; Sastre, 2000; Szeto, 2006b). Cell damage and dysfunction due to oxidative stress caused by free radicals has been linked to the pathophysiological processes seen in numerous diseases (Ault, 2003; Dhanasekaran, 2004; Halliwell, 1994; Kowluru, 2007; Lluis, 2005; Mansfield, 2004; Panee, 2007; Sagara, 1998; Young, 2001; Zhan, 2004).

There is evidence that dietary supplementation with GSH, glutathione ethyl ester (GSHE), and N-acetylcysteine (NAC) will increase kidney tissue GSH levels. However,

it is not clear from the literature if the mitochondrial pool of GSH is increased with dietary supplementation. Indeed, within the cell, the GSH pool in the mitochondria is much smaller (i.e., ~ 5 % or less) than the cytosolic pool (i.e., ~ 95 %; see Results), and changes in whole kidney tissue GSH may not reflect changes in the mitochondrial pool. Thus, this study was undertaken to determine whether exogenous dietary supplementation with GSH, GSHE, or NAC will increase the mitochondrial GSH pool in the rat kidney.

Literature Background

Oxidative stress inside cells and protection by GSH

Oxidative stress occurs when there is an imbalance between pro-oxidants and antioxidants in a particular organelle, cell, tissue, or organism (Cocco, 2005; Sen, 2000). Pro-oxidants include free radicals, such as the hydroxyl radical (·OH), superoxide radical ($\cdot O_2^-$), and nitric oxide (·NO) (Andreyev, 2005). Continuous production of pro-oxidants occurs in the mitochondria from complexes I and III of the electron transport chain (ETC) during normal respiration (Choksi, 2007; Panee, 2007; Szeto, 2006). Free radicals are highly reactive molecules because of their unpaired electron (Young, 2001). Oxidative damage occurs when free radicals react with proteins inside the cell, such as DNA or RNA, as well as lipids in the cell or organelle membrane (Ames, 1993; Halliwell, 2000; Hayes, 2005; Szeto, 2006b). Estimates are that there are 100,000 oxidative hits a day to rat DNA, and 10,000 hits a day to human DNA. The oxidative hits cause damage by generating different base oxidations and modification products in DNA (Ames, 1993; Halliwell, 2000), and results in cell dysfunction. Physiological dysfunctions due to oxidative damage have been observed in Alzheimer's disease, diabetes mellitus, amyotrophic lateral sclerosis (ALS), Parkinson's and Huntington's Disease, cardiovascular disease, inflammation, arthrosclerosis, rheumatoid arthritis, cancer, hypoxia, hypertension, ischemia-reperfusion injury and aging (Ault, 2003; Dhanasekaran, 2004; Halliwell, 1994; Kowluru, 2007; Lluis, 2005; Mansfield, 2004; Panee, 2007; Scaduto, 1991; Sagara, 1998; Slusser, 1990; Young, 2001; Zhan, 2004). Free radicals have also been implicated in non age-related oxygen radical diseases of the newborn (Njalsson, 2005). The pathological effects of free radicals are also seen in kidney

diseases, such as acute and chronic renal failure, glomerulonephritis, rhabdomyolysis and obstructive nephropathy (Baud, 1993; Budisavljevic, 2003; Mashiach, 2001; Poovala, 1999; Rodrigo, 2002b, 2006).

The cell is protected from oxidative damage by antioxidants that are able to donate an electron to a free radical and inactivate it (Young, 2001). The principal antioxidant inside cells is GSH (Anderson, 1998; Sen, 2000). GSH is a water soluble tripeptide composed of glutamate, cysteine, and glycine, and is present in all mammalian cells as the most abundant intracellular thiol (Anderson 1998; Sen, 2000). Hopkins (1921) discovered and named glutathione, which he initially believed was a dipeptide containing glutamate and cysteine. Eight years later it was discovered that GSH was actually a tripeptide (see Figure 1) (Sen, 2000). GSH is present in many parts of the cell, and it has concentrations varying from 1-10mM depending on factors including age, nutritional status, synthesis, the rate of GSH efflux, and intracellular utilization of GSH (Chen, 1989; Hazelton, 1980; Leeuwenburgh, 1996; Liu, 2003; Nakata, 1996; Smith, 1996; Söderdahl, 2003; Sun, 1996). Maintenance of cellular GSH levels is important to protect the cell against oxidative damage. Indeed, decreases in the cell GSH level and the GSH redox ratio (GSH/GSSG), where GSSG is oxidized form of glutathione, have been used to assess the level of oxidative stress being experienced by cells (Andziak, 2006). GSSG, composed of two GSH molecules, is formed when GSH neutralizes a free radical. The reduction of GSSG back to GSH is catalyzed by glutathione reductase (Andreyev, 2005).

Other indicators of oxidative stress include an increase in lipid oxidation products (MDA or malondialdehyde and isoprostanes), an increase in protein oxidation products

Figure 1: Structure of Glutathione

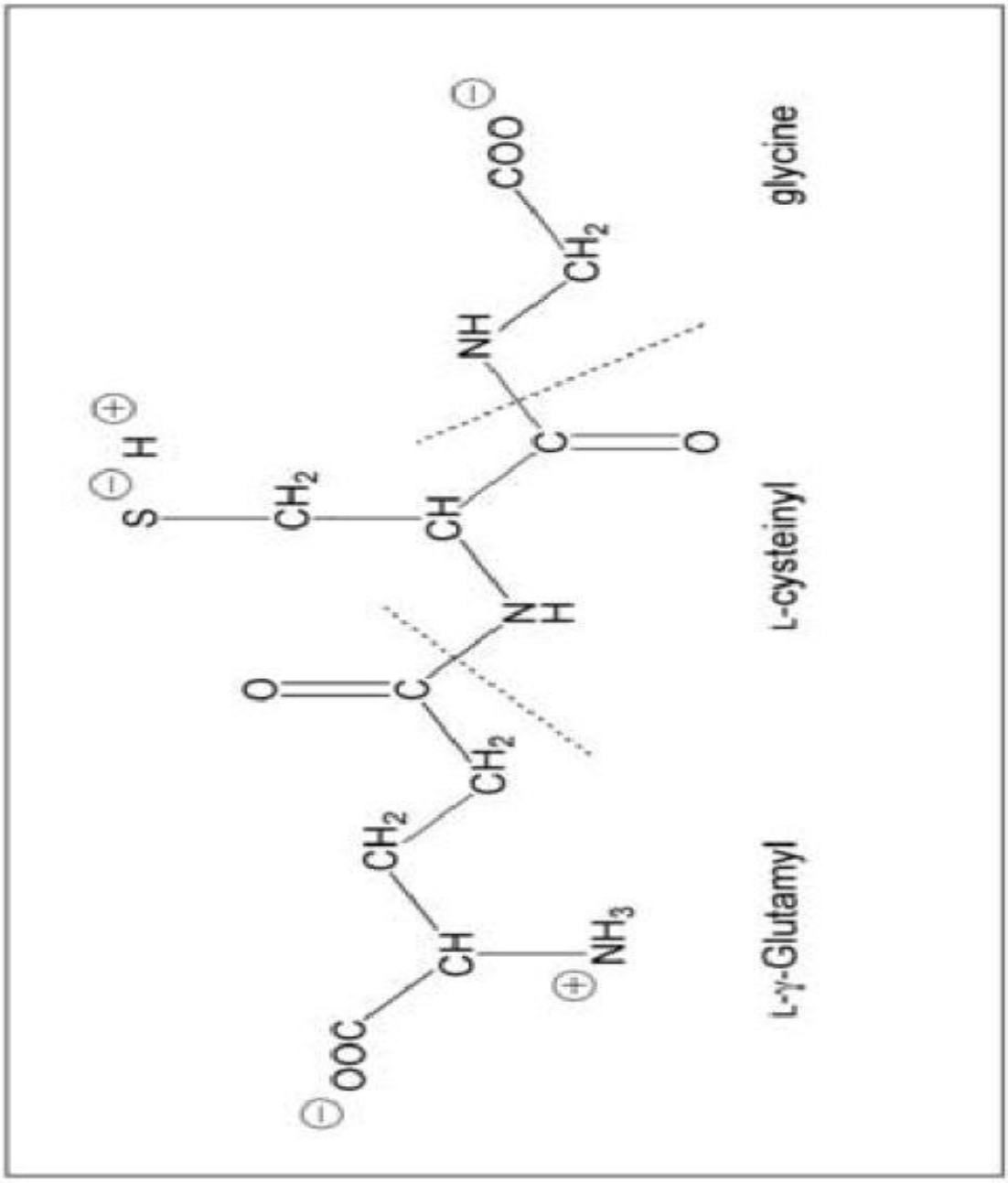


Figure 1: Structure of Glutathione

Reduced glutathione (GSH) is a water soluble tripeptide composed of glutamate, cysteine, and glycine. (Figure adapted from Lash, 2006)

(carbonyls), and an increase in DNA oxidation products (8-OHdG or 8-hydroxy-2'-deoxyguanisine) (Andziak, 2006). Peroxidation of polyunsaturated fatty acids results in lipid peroxides which are highly unstable and decompose to a variety of compounds, of which MDA is the most abundant (Lecomte, 1994; Ogawa, 2006). When DNA is damaged, 8-OHdG is formed upon the oxidation of deoxyguanosine by the hydroxyl radical (De La Asuncion, 1996; Ogawa, 2006). When DNA undergoes repair, 8-OHdG is excised and excreted in urine. The excretion rate of 8-OHdG has been used as an indicator of oxidative stress or damage (Chen, 2007; Fraga, 1990; Ogawa, 2006).

Role and maintenance of GSH inside cells

GSH has many roles inside the cell besides being an antioxidant. GSH functions as an electron-donating substrate to several enzymes involved in oxidant-detoxification and as a coenzyme in many metabolic reactions. It promotes formation of reduced forms of other antioxidants, such as ascorbate (vitamin C) from dehydroascorbate and α-tocopherol (vitamin E) (Andreyev, 2005; Mårtensson, 1991b; Ortolani, 2000), plays an important role in neutralizing toxic compounds, and maintains the redox state of the cell (Anderson, 1985, 1998; Godwin, 1992; Hagen, 1990; Markovic, 2007; Reichard, 1981; Sen, 1998; Smith, 1996; Valencia, 2002; Zachara, 2006). GSH synthesis takes place primarily in the cytosol, and requires the consecutive action of two-enzymes, gamma (γ)-glutamycysteine synthetase (γ-GCS) and GSH synthetase (GS) (Townsend, 2003; Wang, 1998). In the γ-GCS reaction, γ-glutamylcysteine is formed when the γ -carboxyl group of glutamate reacts with ATP to form γ-glutamylphosphate, which in turn reacts with the

amino group of cysteine (Griffith, 1999). The peptidic γ –linkage formed protects γ-glutamyl-cysteine from hydrolysis by intracellular peptidases (Griffith, 1999). GSH synthetase adds glycine to γ- glutamyl- cysteine, forming GSH in a mechanism similar to γ-GCS (Griffith, 1999; Valencia, 2001) (see Figure 2). The addition of glycine protects GSH from intracellular cleavage by γ-glutamylcyclotransferase (Lu, 1999). Regulation of γ-GCS is controlled through negative feedback from GSH with an inhibition constant of ~ 2.3mM in the kidney (Aebi, 1992; Richman, 1975; Wang, 1998). Richman (1975) reported the inhibition constant of GSH is close to whole kidney GSH concentrations of ~ 2.3 mM (~ 8.74 µmol/mg tissue protein). Negative feedback control of GSH levels is evidenced in studies that show the hepatic cellular concentration of GSH will not exceed a plateau of between 7.5 and 8 µmol/g wet tissue (~ 1.97 -2.10mM) (Grattagliano, 1995). De novo synthesis of GSH in the cell is regulated at the level of feedback inhibition of γ-GCS and the availability of substrates (Griffith, 1999). The synthesis of GSH requires two moles of ATP per mole of GSH produced, and any physiological or pathological process that limits ATP availability will compromise GSH synthesis (Shan, 1989). The rate limiting step in the synthesis of GSH is generally thought to be the availability of cysteine, but availability of the other two amino acids (i.e., glutamate or glycine) will be limiting as well (Deneke, 1989; Townsend, 2003; Wang, 1998; Wu, 2004). Cysteine can enter the cell in thiol, disulfide, mixed disulfide and γ-glutamyl amino acid forms (Banks, 1994; Bannai, 1988; Burdo, 2006; Chen Z, 2000; Lo, 2008b; Meier, 1995; Sen, 2000; Shan, 1989; Welbourne, 1979). Cystine can also be transported into cells and reduced to cysteine (Bannai, 1980; Burdo, 2006; States, 1974). Cystine is the oxidized dimer of cysteine and is the more prevalent (90%) extracellular form due to the instability of

Figure 2: Glutathione Synthesis

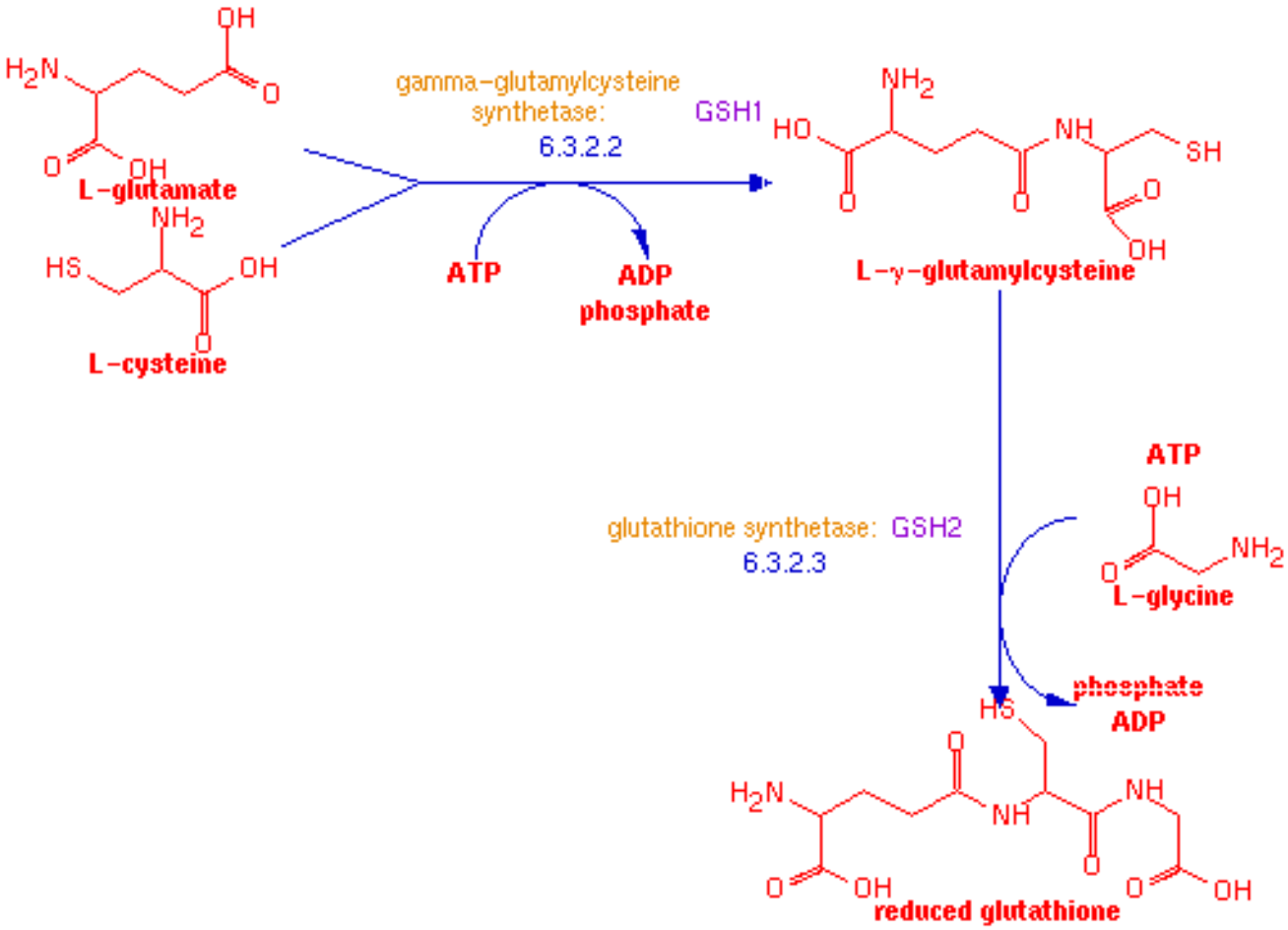


Figure 2: Glutathione Synthesis

GSH synthesis takes place in the cytosol and requires the consecutive action of two-enzymes, gamma (γ)-glutamycysteine synthetase (γ-GCS) and glutathione synthetase. In the γ-GCS reaction, γ-glutamyl-cysteine is formed when the γ -carboxyl group of glutamate reacts with ATP to form γ-glutamylphosphate, which in turn reacts with the amino group of cysteine. Glutathione synthetase adds glycine to γ-glutamyl-cysteine forming GSH in a mechanism similar to γ-GCS. (Figure adapted from Peuke, 2005).

cysteine that auto-oxidizes to cystine under aerobic conditions (Burdo, 2006; Sen, 2000; Shan, 1989; Welbourne, 1979).

There appears to be compartmentalization of GSH inside the cell so that its concentration is variable (Conour, 2004; Smith, 1996). Nuclear GSH is thought to make up approximately 5-10% of the cells total GSH and at concentrations below that of cytosol (Smith, 1996; Söderdahl, 2003). Transport into the nucleus is passive, with the nucleus also having synthetic capability (Markovic, 2007; Smith, 1996). As opposed to the nucleus, the endoplasmic reticulum (ER) is very oxidized with a redox state of 20-100 times greater (-170mV to -185mV) than cytosol due to an increased GSSG concentration (Bass, 2004; Hwang, 1992). GSH is present in the ER at a concentration of 6-10 mM (Hwang, 1992; Bass, 2004). Up to 50% of the GSH in the ER is in the form of mixed disulfides with protein (Jessop, 2004). Mitochondrial GSH makes up a much smaller portion of the cells GSH (Chen, 1998). Mitochondria have a limited capability to synthesize GSH (Fernandez-Checa, 1997; Green, 2006; Smith, 1996), but can reduce GSSG back to GSH via glutathione reductase (Taniguchi, 1986). In most physiological states, mitochondrial GSH uptake is an energy dependant process (Anderson, 2002; Lash, 2002, 2006; Mårtensson, 1990). However, it should be noted that in studies in liver mitochondria, GSH uptake was passive with high cytosolic levels of GSH (Meister, 1994, 1995). Kurosawa (1990) found that GSH in the liver is freely moveable across a proton-permeated mitochondrial membrane, and the movement is determined by its own gradient. This gradient is necessary to maintain GSH in the mitochondrial matrix. However, Lash (2002) suggests that GSH is not coupled to a proton gradient because changes in extra-mitochondrial pH have no affect on GSH uptake. What is agreed upon is

that neither a change in pH or membrane potential is required for GSH transport into mitochondria (Smith, 1996).

Uptake of GSH into mitochondria is thought to involve both a low capacity, high affinity transporter (dicarboxylate or DIC) and a high capacity, low affinity transporter (2-oxoglutarate or OGC) (see Figure 3) (Andreyev, 2005; Chen, 1998; Fernandez-Checa, 1997, 1998; Lash, 1998, 2007). The DIC carrier exchanges GSH for inorganic phosphate while the OGC transporter exchanges 2-oxogluterate (2-OG) for GSH (Lash, 2007). The DIC and OGC carriers are estimated to account for 70-80% of GSH uptake with the higher affinity DIC carrier accounting for more of the transport (Chen, 2000; Lash, 2002**;** Xu, 2006). The mechanism for both the OGC and DIC carriers is one of simultaneous transport, a mechanism that requires the carriers to form a complex with the two counter substrates prior to translocation (Capobianco, 1996; Palmieri, 1992; Stipani, 1996). The simultaneous transport mechanism allows for enhanced uptake when both substrates are present, but also creates an impediment if one of the substrates is limited (Palmieri, 1992; Stipani, 1996; Capobianco, 1996). GSH uptake by rat kidney mitochondria is saturable (K_m = 1.3 mM, V_{max} = 5.59 nmol/min per mg protein) (Lash, 2006). Mitochondrial GSH uptake is affected by the cell redox state, other amino acids, and membrane fluidity (Fernandez-Checa, 2005, Lash, 2006).

The different concentrations of GSH within the cell may be related to the regulation of the redox status of the various regions of the cell (see Figure 4). The redox status or state is related to the ratio of reduced to oxidized states of molecules, such as GSH/GSSG, NADPH/NADP, and NADP/NAD (Schafer, 2001). The redox state

Figure 3: Mitochondrial Glutathione Transport

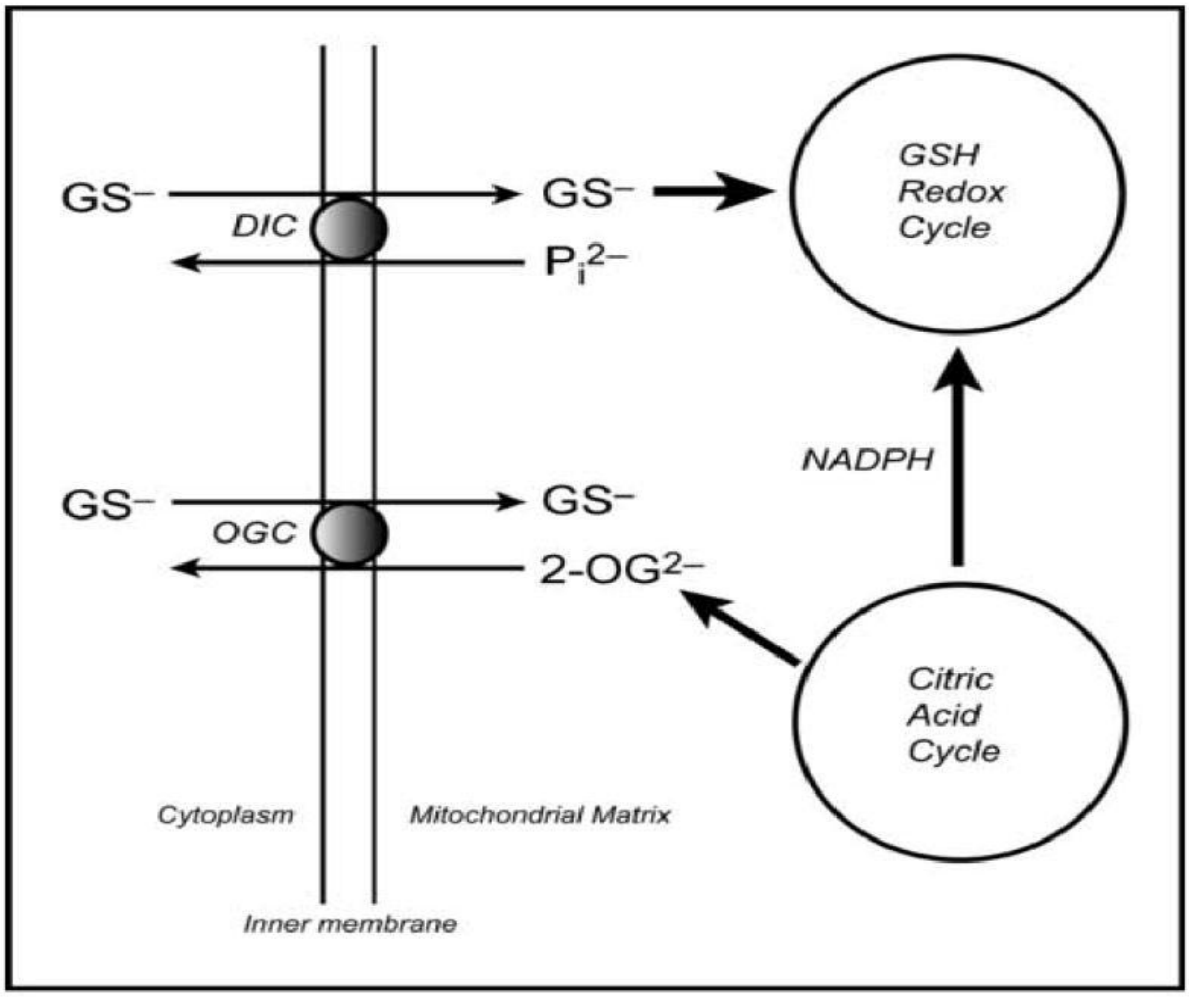
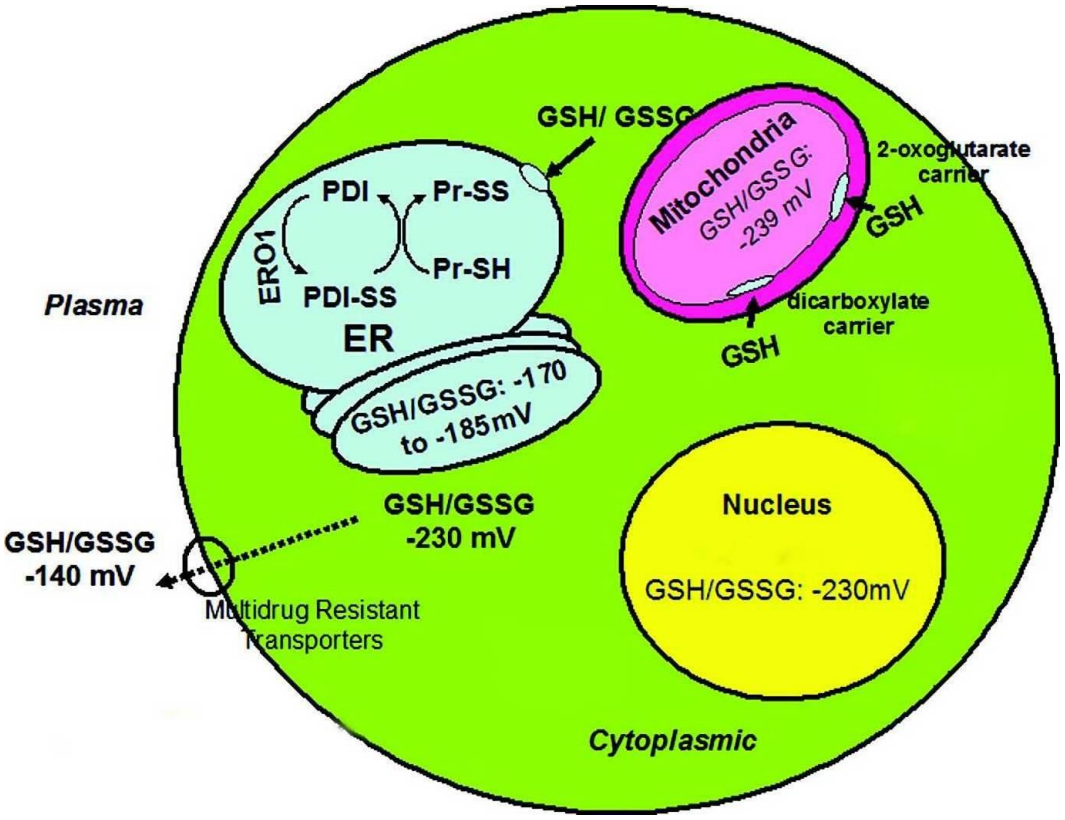


Figure 3: Mitochondrial Glutathione Transport

Uptake of GSH into mitochondria is through a low capacity, high affinity GSH transporter (dicarboxylate or DIC) and a high capacity, low affinity GSH transporter (2 oxoglutarate or OGC). The DIC carrier exchanges GSH for inorganic phosphate (P_i^{2-}), while the OGC transporter exchanges 2-oxogluterate (2-OG^{2-}) for GSH. The DIC and OGC carriers are estimated to account for 70-80% of GSH uptake.

(Figure from Lash, 2006)

Figure 4: Redox Environment in Cell



Thiol/disulfide redox states are independently controlled in the cytoplasm (*green*), nuclei (*yellow*), mitochondria (*red*), endoplasmic reticulum (ER) (*blue*), and plasma (*white area surrounding cytoplasm*). Known transporters of GSH are shown. Nuclear GSH makes up approximately 5-10% of the cells total GSH and at concentrations below that of cytosol. Cells that are in the proliferative stage are in a more reduced state (~-260mV to -230mV) as they progress from the G1 to G2/M phase of cell division. GSH in the ER has a concentration of 6-10 mM and is mostly present as GSSG. The redox state of the ER is 20-100 times greater (-170mV to -185mV) than the cytosol. Mitochondrial total glutathione (GSH+2GSSG) content is in the range from 2 to 14 mM with a majority in the reduced form.

(Figure adapted from Moriarty-Craige, 2004).

regulates various processes occurring in the different parts of the cell (Conour, 2004; Lash, 1996; Schafer, 2001). For example, the more reduced environment of the nucleus during proliferation allows certain transcription factors to operate efficiently, may protect genomic DNA from oxidative damage, and facilitates repair of DNA following oxidative damage (Conour, 2004; Smith, 1996). After cytokinesis or division of the cytoplasm, the change to an oxidized environment of the nucleus may be a signal to stop cell proliferation (Conour, 2004). As opposed to the nucleus and mitochondria, the endoplasmic reticulum (ER) is very oxidized due to an increased GSSG concentration (Bass, 2004; Hwang, 1992).

Changes in kidney tissue GSH with exogenous supplementation

GSH SUPPLEMENTATION: Previous studies have found that exogenous supplementation with GSH increased GSH levels in the kidney. Scaduto (1991) reported a significant increase in GSH, from 10$\pm$1 to 39$\pm$1 µmol/g kidney dry weight (X $\pm$ SEM; n=3-4), two hours following one intravenous (i.v.) injection of GSH of 1 mmol (307mg) per Kg of body weight in the rat. Sen (1994) reported a significant three-fold increase in total glutathione (i.e., GSH + 2GSSG) in kidney when rats were given GSH at 1g (3.25mmol) per Kg body weight by intraperitoneal (i.p) injection for three days. Abul-Ezz (1991) gave GSH at 2mmol (614mg) per Kg i.v. every three hours for five doses to rats. Kidney GSH levels were increased ~69% in two hours, returning to baseline after four hours. Aebi (1992) injected 1.67 or 8.35 mmol per Kg body weight of GSH i.v. into rats and found significant increases, from 2.46 $\pm$0.54 to 3.73 $\pm$ 0.65 and

6.70 ± 1.79 µmol/g wet kidney (X+ SEM, n= 9), 1 hour after the respective doses. Exogenous GSH has also been shown to increase the amount of GSH in other organs, such as the heart, intestine, nervous tissue, as well as in disease states, such as diabetes mellitus in rats and mice (Aw, 1991, 1992; Lash, 1986; Ramires, 2001; Ueno, 2002).

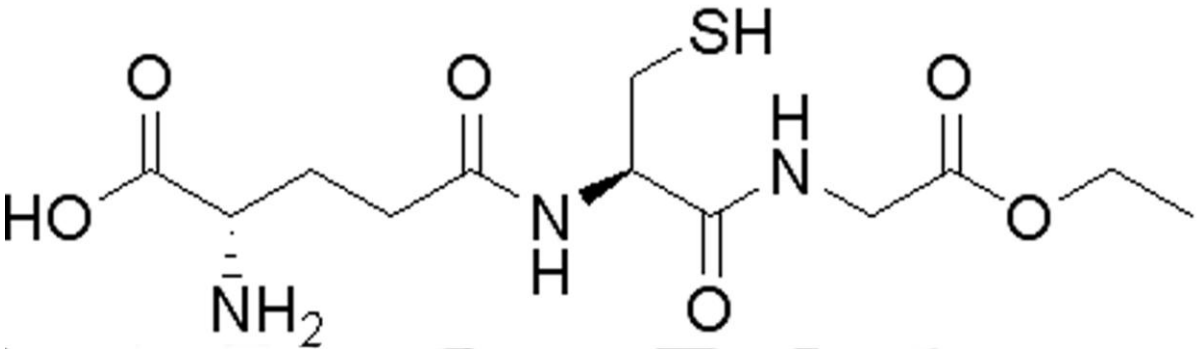
The mechanism by which exogenous GSH supplementation increases tissue GSH is thought to involve uptake of GSH or its precursors by cells from the blood. High concentrations of extracellular GSH will also reduce cystine to cysteine, thereby facilitating cysteine uptake and availability for GSH synthesis within the cell (Aebi, 1992; Bukowski, 1995). GSH reacts with cystine to form cysteine and a cysteine glutathione mixed disulfide through a transhydrogenation reaction (Bannai, 1986; Deneke, 1995). In addition, by reducing cystine to cysteine, more cysteine will be made available to the liver for synthesis of GSH. The GSH can then be released into blood for uptake by the kidney or other organs (Aebi, 1992; Banks, 1994; Hagen, 1990b; Lewerenz, 2006). Intraperitoneal injection in the rat results in the deposited fluid and substances, such as GSH, being taken up by the organs of the peritoneal cavity. Approximately 30-40% of the anatomic peritoneum is in contact with the peritoneal cavity, but changes in body position will alter the amount of contact with the cavity (Flessner, 1996). Uptake of the injection is directly related to the surface area of the organ in relation to the peritoneal cavity (Flessner, 2007). Solutes will be transported through the mesothelium, the interstitium, and finally through the capillary wall via diffusion (Flessner, 1991). All absorbed solutes enter the hepatic portal vein and eventually the liver through a plethora of veins. These veins include the anterior mesenteric that drains the ileum, caecum and colon, the posterior mesenteric that drains

the distal colon and rectum, and the posterior pancreatico-deudenal, pyloric and lineal veins (Coria-Avila, 2007; Sharp, 1998; Waynforth, 1980; Wells, 1964). Once in the liver, the injected substances are either metabolized or are carried with deoxygenated blood by the inferior vena cava into the right atrium of the heart. From the heart, the substances enter the pulmonary circulation, and then the systemic circulation (Coria-Avila, 2007). Distribution of exogenous GSH occurs rapidly and evenly throughout the extracellular space Aebi, 1992; Ammon, 1986). Hahn (1978) administered radiolabeled 10µmol or 30.7 mg of GSH i.v. to rats and found radioactivity first accumulated in the kidney, and then the liver five minutes after injection. Schumacher (2001) administered radiolabeled 1mmol or 307mg per Kg of GSH i.p. to mice and found 11% of ^{3}H labeled GSH in the kidney after 15 minutes. Eight percent of the labeled GSH was still present in the kidney after four hours. Nineteen percent of ^{35}S labeled glutathione was found in the kidney after 60 minutes, falling to 9% after four hours (Schumacher, 2001).

GLUTATHIONE MONOETHYL ESTER (GSHE) SUPPLEMENTATION:

Previous studies have reported that exogenous GSHE (Figure 5) supplementation increases GSH levels in rat kidney. Scaduto (1988) found a significant increase, from 13.6 + 0.9 to 32.8 + 10.1µmol/g dry wt. (X + SD; n=4), when GSHE was given i.p. at 2mmol (670 mg) per Kg of body wt two hours prior to harvest. GSHE also increased total glutathione (i.e., GSH + 2 GSSG) in rat kidney cortical mitochondria, from 3.3 to ~ 5.1 nmol/mg of mitochondrial protein. Robinson (1992) gave an intravenous bolus of 5mmol (1677mg) per Kg of GSHE to rats and found a three fold increase in kidney GSH levels, from ~4 to ~15 µmol/g kidney wet wt four hours after administration. Chen and Richie

Figure 5: Structure of GSHE



Glutathione ester contains an additional CH_2-CH_3 on the glycine portion of the glutathione molecule. (Figure from Sigma, 2011).

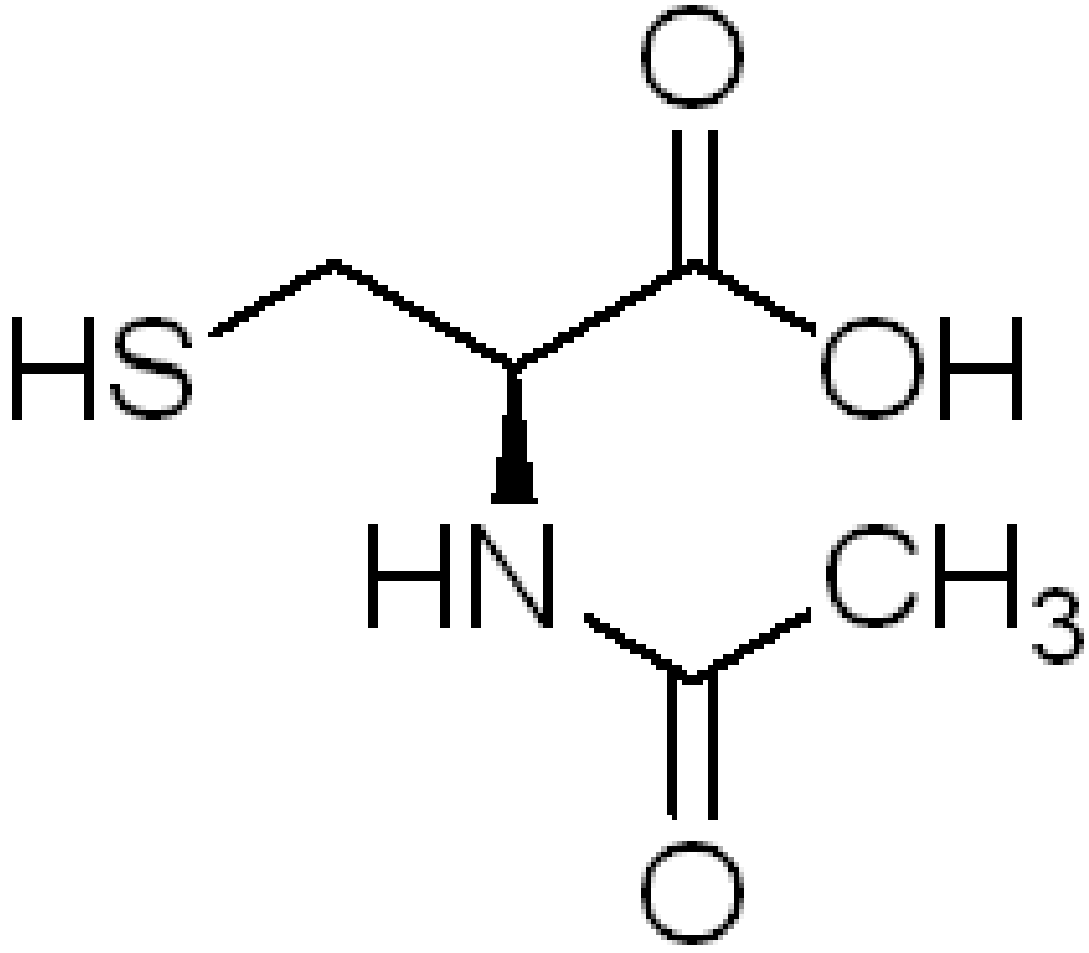
(2000) gave an i.p. injection of GSHE of 10mmol (3354mg) per Kg and showed significant increases in kidney GSH and cysteine levels in mice of different ages. Puri (1983) gave 10mmol (3354mg) per Kg i.p. of GSHE to mice and found an increase in GSH, from ~2 µmol/g to ~8µmol/g of wet wt, in kidney and liver after two hours. Anderson (1985) gave 7.5mmol (2515mg) per Kg of GSHE to mice two hours prior to sacrifice, and found GSHE increased the kidney GSH, from ~ 0.200µmol/g to 3.75µmol/g kidney wet wt, in animals depleted of GSH by buthionine (S,R)-sulfoximine (BSO). GSHE has been shown to raise cellular GSH levels in other organs such as the brain, liver, heart, spleen, lung, lymphoid and lens epithelial cells (Anderson, 1985, 1989; Mårtensson, 1989, 1989b; Murali, 2007; Rajasekaran, 2002; Wellner, 1984). GSHE has also been shown to raise mitochondrial GSH levels in rat lens epithelial cells (Mårtensson, 1989b), and in ischemic rat liver following reperfusion (Mårtensson, 1989c). In addition, GSHE raised GSH levels in the mitochondria of mouse heart (Mårtensson, 1989) and liver (Mårtensson, 1989c), but not in mitochondria from mouse skeletal muscle (Mårtensson, 1989).

GSHE administered via i.p. injection follows the same route as described previously for i.p. administration of GSH. GSHE can be taken up by the liver or enter the general circulation. GSHE can be converted to GSH in blood, and then the GSH can be taken up the kidney cells to increase tissue GSH levels. Mice given 10 mmol (3354 mg) per Kg of GSHE i.p. increased the blood plasma levels of GSH, from 15-35 uM to ~ 155 uM , one hour after administration (Anderson, 1985). GSHE can be hydrolyzed through the action of carboxylesterases that hydrolyze GSHE into GSH and the corresponding alcohol (i.e., ethanol) (Anderson, 1985; Grattagliano, 1995). GSHE is slowly hydrolyzed

by plasma carboxylesterases resulting in a longer persistence in the circulation than GSH (Grattagliano, 1995). GSHE may also be taken up by kidney cells intact (Anderson, 1985; 1989) and then converted to GSH inside the cell via carboxylesterases. Carboxylesterases are ubiquitously expressed inside cells, with the highest activities occurring in the liver, kidney and intestine (Hosokawa, 2008; Imai, 2006; Tsujita, 1962). Carboxylesterases are also thought to be the major determinant of pharmokinetics and pharmodynamics of ester drugs or ester prodrugs (Hosokawa, 2008; Lee, 2000; Satoh, 1998, 2002, 2006; Yan, 1994). Once inside the cell, conversion of GSHE to GSH is relatively rapid. Anderson (1985) found that two hours after $^{[35]}$S labeled glutathione ester was injected into mice (n=3-4), only 5-18 % of the radioactive label was present in cells as the ester. Anderson (1985) also suggested that GSHE may directly react with free radicals.

N-ACETYLCYSTEINE SUPPLEMENTATION: The glutathione precursor N-Acetylcysteine (NAC) (see Figure 6) has been found to increase tissue GSH levels in the kidney and other organs. Arfsten (2004) showed that when NAC was given by gavage or stomach tube for 30 days at 600mg/Kg/day, total glutathione (GSH + GSSG) in rat kidney increased significantly, from 2.6 $\pm$ 0.1 to 4.7 $\pm$0.2 µM/ µg total protein (X $\pm$ SEM; n=20). Nitescu (2006) reported that kidney total glutathione levels were increased significantly when NAC (200mg/Kg) was administered i.p. at 2, 12 and 24 hrs before induction of renal ischemia-reperfusion. The total glutathione level of the NAC group was 80$\pm$ 9 versus 38$\pm$ 10 nmol/g kidney wet wt (X $\pm$ SEM; n=10) for the control group.

Figure 6: Structure of NAC



The structure of NAC is a cysteine molecule connected to an acetyl group. The acetyl group contains a methyl group single-bonded to a carbonyl. The –SH group of NAC is responsible for its biological activity while the acetyl substitution makes the molecule less easily metabolized and oxidized. (Figure from Sigma, 2011).

Nitescu (2006) also found improved renal function as well as reduced oxidative stress. NAC has been found to increase GSH content in erythrocytes, liver, lung (De Flora, 1985; Nakata, 1996; Shattuck, 1998). However, the effects of NAC supplementation are not always consistent. Arfsten (2007) gave multiple doses of NAC (1200mg/Kg) at 4 hour intervals to rats and found kidney GSH levels were not increased significantly. McLellan (1995) found that i.v. and i.p. injected NAC (320 mg/Kg) in rats raised bladder and bone marrow GSH concentrations, but had no effect on the liver GSH concentration. Estrela (1983) gave large doses of NAC i.p. or orally and found that i.p. injection decreased GSH content in the liver. Rats given 0.125 g/Kg of NAC had a liver GSH content of 5.21+0.18 µmol/g, whereas rats receiving 1g/Kg had a liver GSH content of 2.34+0.37 µmol/g. The decrease in GSH with the large dose of NAC may be due to toxicity.

NAC has been shown to affect the mitochondrial GSH levels of tissues. Grattagliano (2004) fed rats a diet that contained 0.3% (mass of solute/ mass of solution) (w/w) NAC for 16 months. Liver mitochondrial GSH levels rose significantly, from ~4.5 to ~ 5.9$\pm$ 0.8 nmol/mg protein. Martinez (2000) and Banaclocha (1997) both fed mice a diet that contained 0.3% (w/w) NAC for 20 plus weeks and found insignificant increases in synaptic mitochondria. Cocco (2005) found age related decreases in GSH in brain and heart mitochondria, and reported a NAC supplemented diet resulted in partial recovery of heart mitochondrial GSH.

Exogenous NAC injected i.p. follows the same path as i.p. administration of GSH and GSHE. Whether i.p injected NAC is metabolized by the liver has not been resolved (Arfsten, 2007). It should be noted that only three percent of radioactively-labeled NAC

is excreted in the feces following oral administration, indicating an almost complete absorption of NAC and its metabolites (Kelly, 1998). NAC may form disulfides of N,N'-diacetylcysteine (NAC-NAC), or react with other low molecular weight thiols, such as cysteine and glutathione, to form mixed disulfides in the plasma following injection (Issels, 1989; Johansson, 1987; Meier, 1995). The exact mechanism for NAC or its disulfides entry into cells is not completely understood. NAC has a five carbon backbone and a net negative charge, and has been shown to be a substrate for the organic anion transporter 1 (OAT1) in the kidney, as well as the anion exchanger 1 (AE1) transporter in erythrocytes (Koh, 2002; Raftos, 2007). The AE1 transporter is also found in α-intercalated cells in the distal nephron of the kidney, where it transports bicarbonate in exchange for chloride across the basolateral membrane. Whether NAC is a substrate of AE1 transporter in the kidney is unknown (Walsh, 2008; Pang, 2008). NAC may also diffuse across the cell membrane (Aoyama, 2006; Holdiness, 1991; Moldéus, 1986), and has been shown to be taken up by cultured hepatocytes (Banks, 1994). After entering the cell, NAC may persist for an extended period of time (Arfsten, 2007; Borgström, 1986; McLennan, 1995). McLennan (1995) gave an i.v. injection of radioactive NAC (320mg/Kg) in mice and found NAC was localized to kidney, liver, and GI tract 45 min after injection and present up to five hours later. Twenty-four hours after injection, the renal cortex and facial glands were still highly radioactive (McLennan, 1995). Arfsten (2007) performed a similar study with a higher dose of radioactive NAC (600mmg/Kg) in the rat, and also found 51% of the total radioactivity being present 24 hours later.

Once NAC is inside kidney cells, it is converted to cysteine by acylases (McLennan, 1995; Yamauchi, 2001). Acylases, primarily acylase I (N-acyl-L-amino acid

amidohydrolase), are cytosolic enzymes that catalyze the deacetylation of *N*-acyl-L-amino acids, such as NAC (Newman, 2007; Uttamsingh, 2000; Yamauchi, 2002). Deacetylation of NAC to cysteine has been shown to occur in rat, mouse, and human tissues, with deacetylase activity highest in the kidney (De Vries, 1993; Sjödin, 1989; Yamauchi, 2002). Yamauchi (2002) localized acylase I to the renal proximal straight and convoluted tubules in primates, and Uttamsingh (2000) found acylase I in the glomeruli, proximal and distal convoluted tubules in rats. The cysteine from deacetylation can then be used for GSH synthesis by the cell (Banks, 1994; Bonanomi, 1980; Issels, 1989; Johansson, 1987; Meier, 1995; Sen, 1998).

In conclusion, few of the previous studies have investigated the effects of long term dietary supplementation on mitochondrial GSH levels in the rat kidney. The present study was undertaken to quantitate the changes in mitochondrial and cytosolic GSH levels in rat kidney cortex and medulla following exogenous supplementation with GSH, GSHE and NAC for four weeks.

Materials and Methods

Experimental Design

There were four groups with 7-9 rats in each group. Animals in the GSH-S Experimental group (n = 7) were given GSH (250 mg or 0.81mmol/Kg body weight) for one month by daily i.p. injection. Animals in the NAC-S Experimental group (n=8) were given NAC (200 mg or 1.22mmol/Kg body weight) for one month by daily i.p. injection. Animals in the GSHE-S Experimental group (n=8) were given glutathione monoethyl ester GSHE (12 mg or 0.03mmol/Kg body weight) for one month by daily i.p. injection. The Control group (n =6) was given sterile isotonic saline for one month by i.p. injection. The overall health and body weights of the rats were monitored during the study. The weight change of the rats were compared to a group of similar aged rats (n = 3) that received no treatment. At the end of one month, the kidneys were harvested from the rats. The levels of GSH, GSSG, total glutathione (i.e., GSH + 2 GSSG), and the glutathione redox ratio (i.e., GSH/GSSG) were determined in the mitochondria and cytosol from cortex and medulla. Statistical differences among the groups were assessed.

Experimental Methodology

Animals: Female Lewis rats, approximately eight to twelve months of age and weighing between 185-351g, were used in the study. The rats were bred in the Penthouse of Cooper Science Building in the Department of Physiology and Health Science. The rats were kept under controlled conditions (21-25°C) with a 12-hour light-dark cycle. The rats also had free access to food (i.e., 2018 Harlen Teklan Global 18% Protein Rodent Diet (see Appendix A) and water during the study. All procedures were approved by the Animal Care and Use Committee of Ball State University.

Preparation of the supplements for Injections: Reagent grade NAC, GSH, and GSHE were purchased from Sigma Biochemical (St. Louis, MO). For the GSH-S group, 2.94g of GSH was dissolved in 28ml of isotonic saline and titrated with saturated NaOH to a pH of 7.4. The solution was subsequently diluted to 30ml for a final concentration of 98 mg/ml or 0.81mM and filtered through a 0.22µm millipore sterile syringe filter. For the GSHE-S group, 100mg of GSHE was dissolved in 21.5ml of isotonic saline and titrated with saturated NaOH to a pH of 7.4. The final solution (4.65 mg/ml or 0.03mM) was filtered through a 0.22µm sterile millipore filter. For the NAC-S group, 2.1 grams of NAC was dissolved in 25ml of isotonic saline which and titrated with saturated NaOH to a pH of 7.4. The solution was subsequently diluted to 28ml for a final concentration of 75 mg/ml or 1.22mM and filtered through a 0.22µm sterile millipore filter. The Control group was given approximately 0.7 ml of pH adjusted isotonic saline that was also filtered through a 0.22µm sterile millipore filter. Care was taken to inject the rats on the right side of the peritoneal cavity as injection into the cecum can occur when rats are injected on the left side of the peritoneal cavity (Arioli, 1970; Coria-Avila, 2007; Miner, 1969; Steward, 1968).

Harvesting of Kidney Tissue: At the end of the injection period, the rats were anesthetized with Inactin (100mg/Kg of body weight). A midline abdominal incision was used to expose the left and right kidneys. The intestines were moved aside to expose the abdominal aorta and vena cava. A tie was placed just superior to the bifurcation of the left and right femoral arteries. A second tie was placed just above the first suture. The suture above the femoral arteries was tied off and the abdominal aorta was clamped

above the second tie. An incision was made in the abdominal aorta and a cannula filled with isotonic saline was inserted and threaded up to the level of the clamp. The cannula was tied in place and the clamp removed. The kidneys were flushed with approximately 30ml of cold isotonic saline. The kidneys were harvested, decapsulated and separated into cortical and medullary sections. The kidney sections were weighed and homogenized in 5% metaphosphoric acid (MPA) in isotonic saline. The sections were further separated into cytosolic and mitochondrial fractions by differential centrifugation. The samples were centrifuged for 10 minutes at 5° at 2400 rpm (650x g) to remove crude cellular debris. The supernatant was removed and further centrifuged at 5° for 15 minutes at 11,500 rpm (12,000x g) to separate the mitochondria from the cytosolic fractions (Paller, 1984). The supernatant (cytosol) was transferred to a newly tared tube and weighed. The mitochondrial pellet was also weighed and dissolved in 0.4ml of 5% MPA dissolved in distilled water.

Determination of Glutathione: The GSH and total glutathione levels (GSH + 2 GSSG) levels in the mitochondrial and cytosolic fractions were determined by a colorimetric assay purchased from Calbiochem (San Diego, CA). Turbidity was removed by filtering the supernates through a 0.22µm millipore filter prior to the assay. Dilutions of 1:36 for cytosol and 1:4.8 for mitochondria were made with 200mM potassium phosphate buffer. A BioTek Instruments µQuant Microplate Spectrophotometer was used to read the absorbance of six standards (i.e. 0, 11, 22, 44, 66, 88, and 108µmol/L) as well as each sample. Buffer (200mM potassium phosphate) was added to each sample to reach a final volume of 720 µl. Twenty microliter of the R1 proprietary solution was added to each of

the samples and the samples were mixed. The samples were incubated at 25° for ten minutes in the dark before measuring absorbance at a wavelength of 356 nm to determine the GSH concentration. Twenty microliters of 30% NaOH was then added to each sample, to convert GSSG to GSH. The samples were once again incubated for ten minutes in the dark at 25°C. The absorbance was then measured at 400 nm to determine the total glutathione concentration. The optical densities of the samples were plotted against the standards to determine concentrations. The GSSG concentrations were calculated from the difference between the absorbance readings at 400nm (i.e., total glutathione) and 356nm (i.e., GSH) and dividing the result by two. Concentrations of GSH, GSSG and total glutathione were expressed as umol or nmol per gram of kidney wet weight (see sample calculation in Appendix B).

Statistical Analysis of Data: ANOVA followed by the Fishers protected post hoc test was used to compare differences among the groups (Bluman, 2007). All data are expressed as $X \pm$ SEM and a $p < 0.05$ was used to indicate statistical significance.

Results

Effect of GSH, NAC and GSHE Supplementation on the body weights of Rats (see Figure 7)

All rats receiving injections underwent a small change (~5%) in body weight when compared to rats receiving no treatment. The weight loss in the rats receiving GSH-S, GSHE-S, or NAC-S was not different from the Control rats receiving only saline.

Effect of Supplementation on Mitochondrial Glutathione Levels (see Figures 8 and 9 and Table 1)

All three supplements significantly increased mitochondrial GSH levels in both the cortex and medulla. The increases in cortical mitochondria were ~72% for GSH-S, ~122% for GSHE-S, and ~168% for NAC. The increases in medullary mitochondria were ~48% for GSH-S, ~73% for GSHE-S, and ~177% for NAC. Total glutathione levels in the mitochondria were also increased within cortical and medullary mitochondria with NAC-S (~175% and ~142%, respectively) and GSHE-S (~100% and ~52%, respectively). However, GSH-S caused a significant increase in total glutathione levels only in cortical mitochondria (~58%). Although GSSG levels exhibited a tendency to increase with supplementation, the increases were not significant. There was no significant change in the redox ratio with supplementation.

Figure 7 – Weight Change in Rats

Rats (n=3) in the No Treatment group received no injections of sterile isotonic saline. Their weight was monitored for one month. The percent change was the difference in weight from the beginning of the treatment until the end of treatment divided by the weight at the beginning of treatment.

a- Significantly different from the No Treatment group

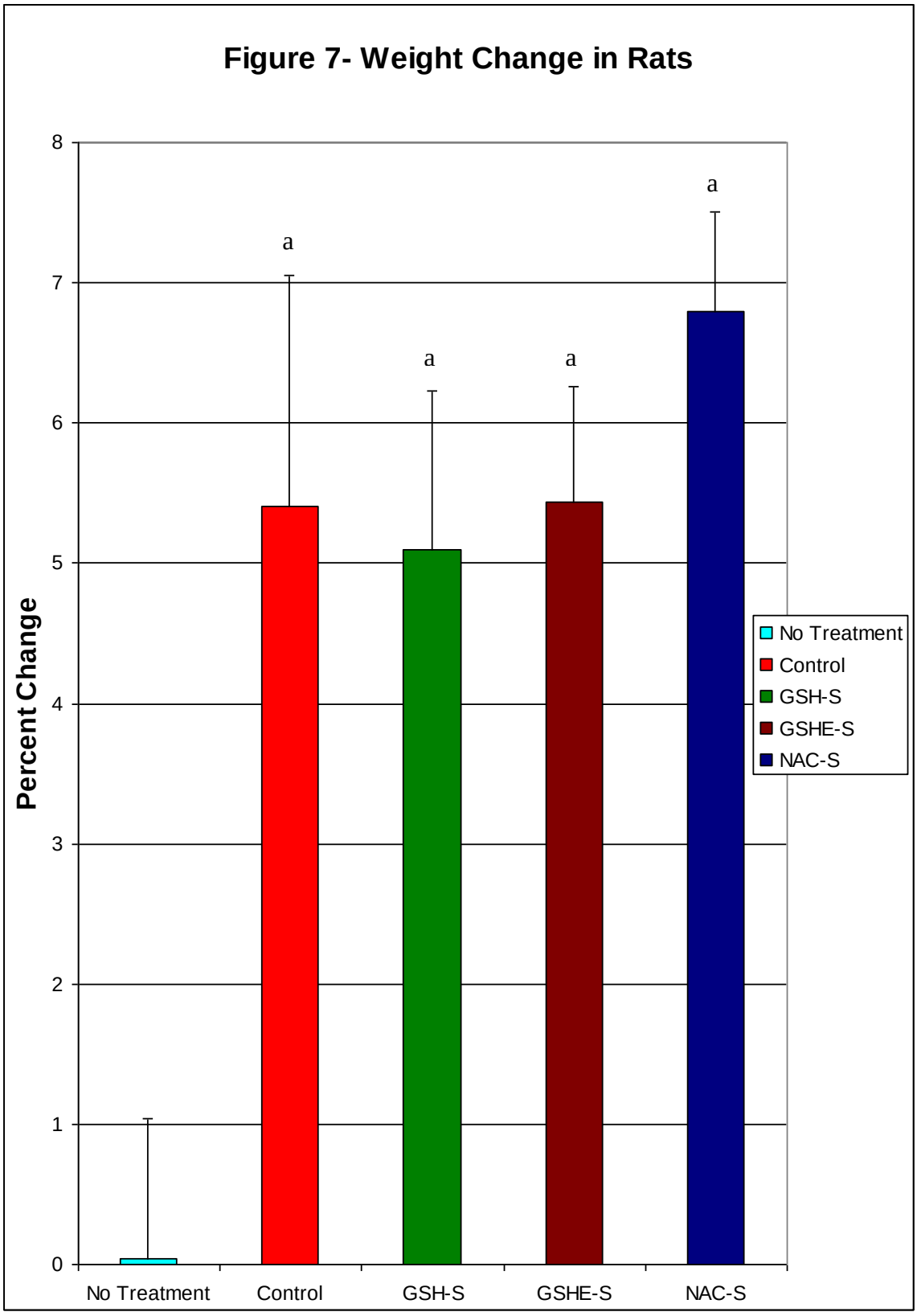
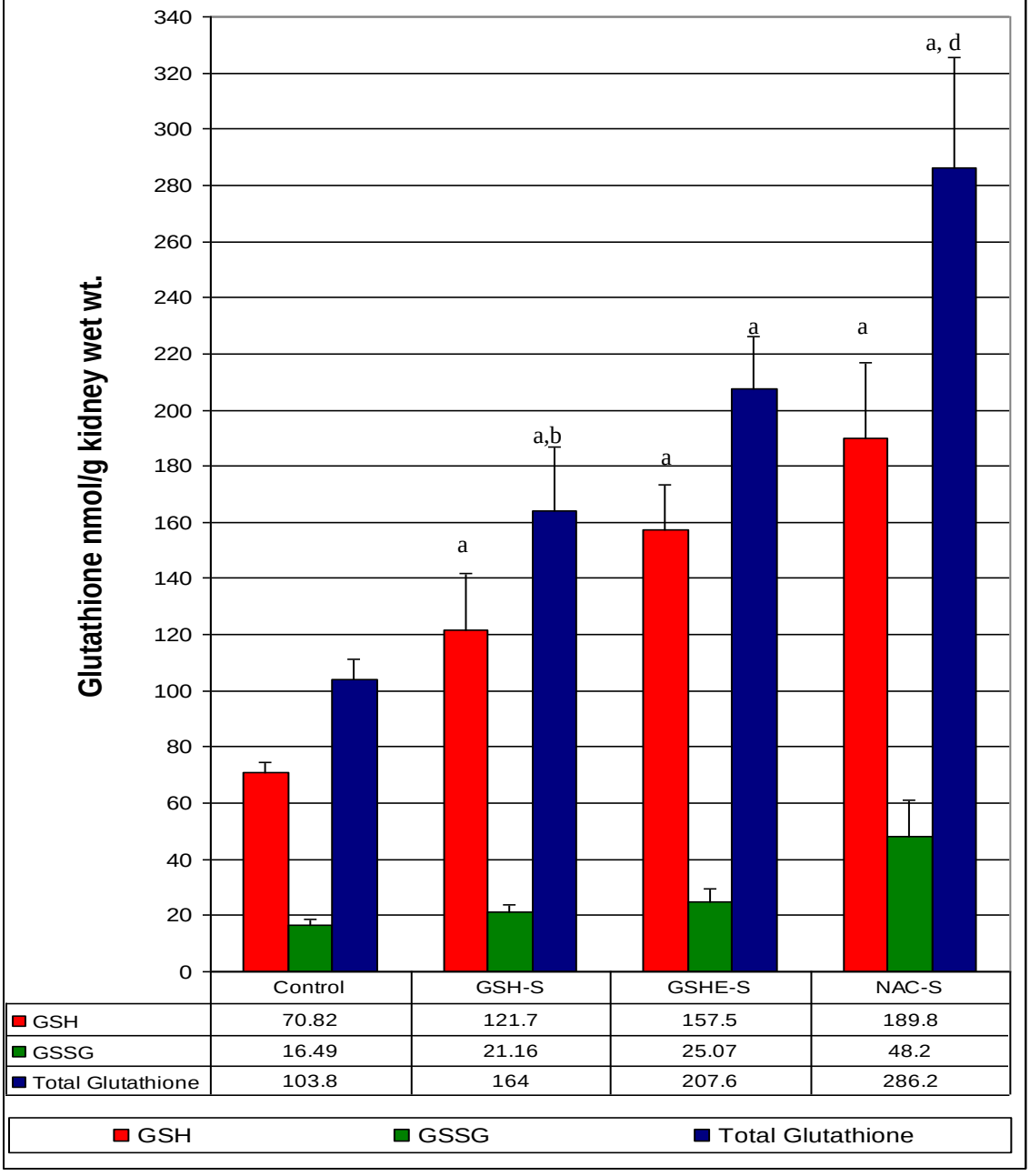


Figure 7- Weight Change in Rats
Percent Change
8
7
6
5
4
3
2
1
0
a
a
a
a
No Treatment
Control
GSH-S
GSHE-S
NAC-S
No Treatment
Control
GSH-S
GSHE-S
NAC-S

Figure 8 – Mitochondrial Glutathione Levels in the Kidney Cortex

a- Significantly different from Control (n = 6)
b- Significantly different from NAC-S (n = 7)
c- Significantly different from GSHE-S (n =8)
d- Significantly different from GSH-S (n = 7)

Figure 8- Mitochondrial Glutathione Levels in Kidney Cortex

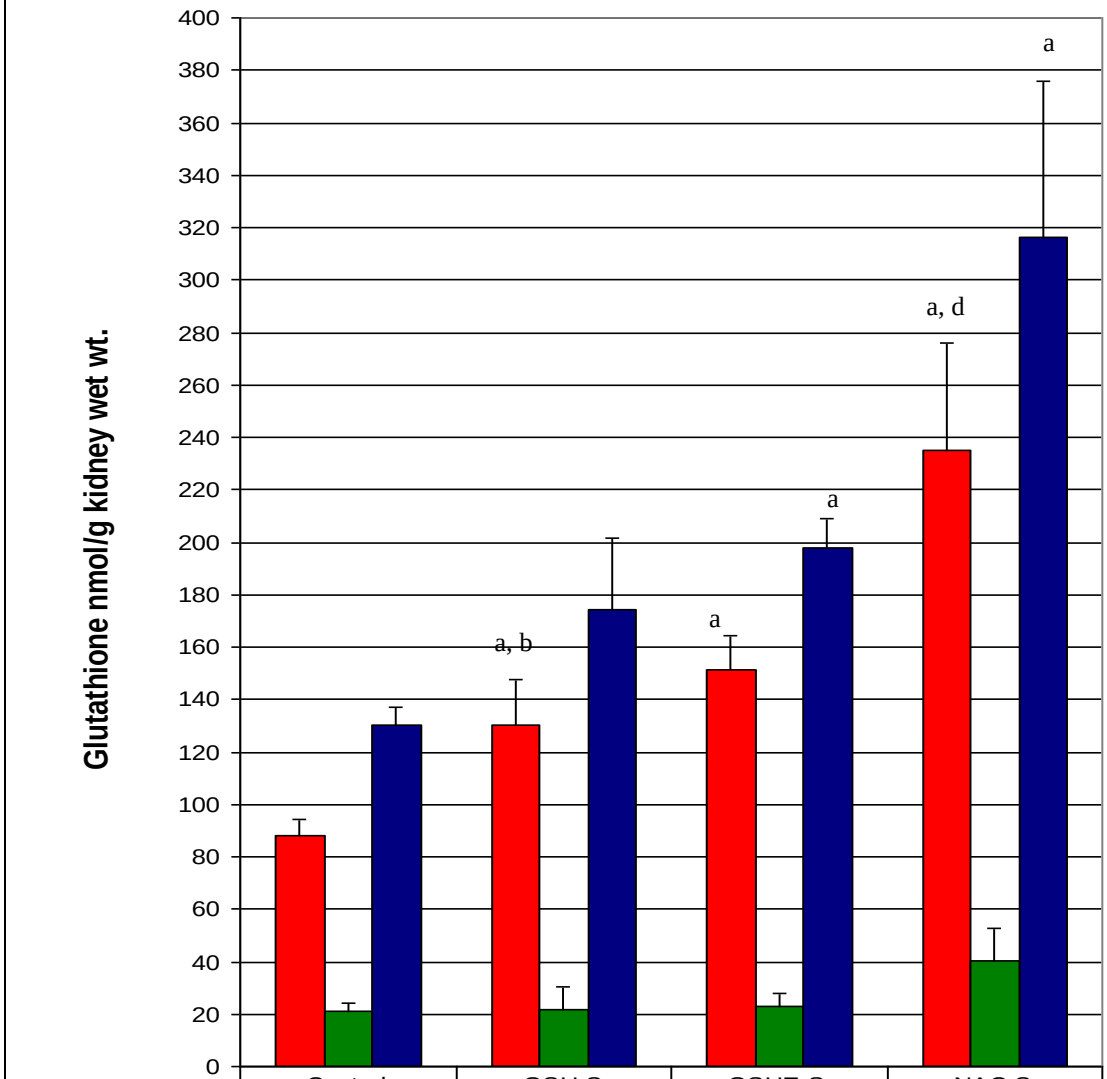


	Control	GSH-S	GSHE-S	NAC-S
GSH	70.82	121.7	157.5	189.8
GSSG	16.49	21.16	25.07	48.2
Total Glutathione	103.8	164	207.6	286.2

Figure 9 – Mitochondrial Glutathione Levels in Kidney Medulla

a- Significantly different from Control (n = 6)
b- Significantly different from NAC-S (n = 8)
c- Significantly different from GSHE-S (n = 8)
d- Significantly different from GSH-S (n = 7)

Figure 9- Mitochondrial Glutathione Levels in Kidney Medulla



	Control	GSH-S	GSHE-S	NAC-S
GSH	87.82	130.3	151.6	235
GSSG	21.3	21.9	22.96	40.6
Total Glutathione	130.41	174	197.6	316.1

Table 1: Glutathione Redox Ratio (GSH/GSSG) in Kidney Mitochondria

	Control n=6	**GSH-S** n=8	**GSHE-S** n=8	**NAC-S** n=7
Cortex	4.73±0.68	5.9±0.83	7.4±1.1	5.87±1.56
Medulla	4.5±0.61	10.65±4.2	11.34±4.4	12.73±5.4

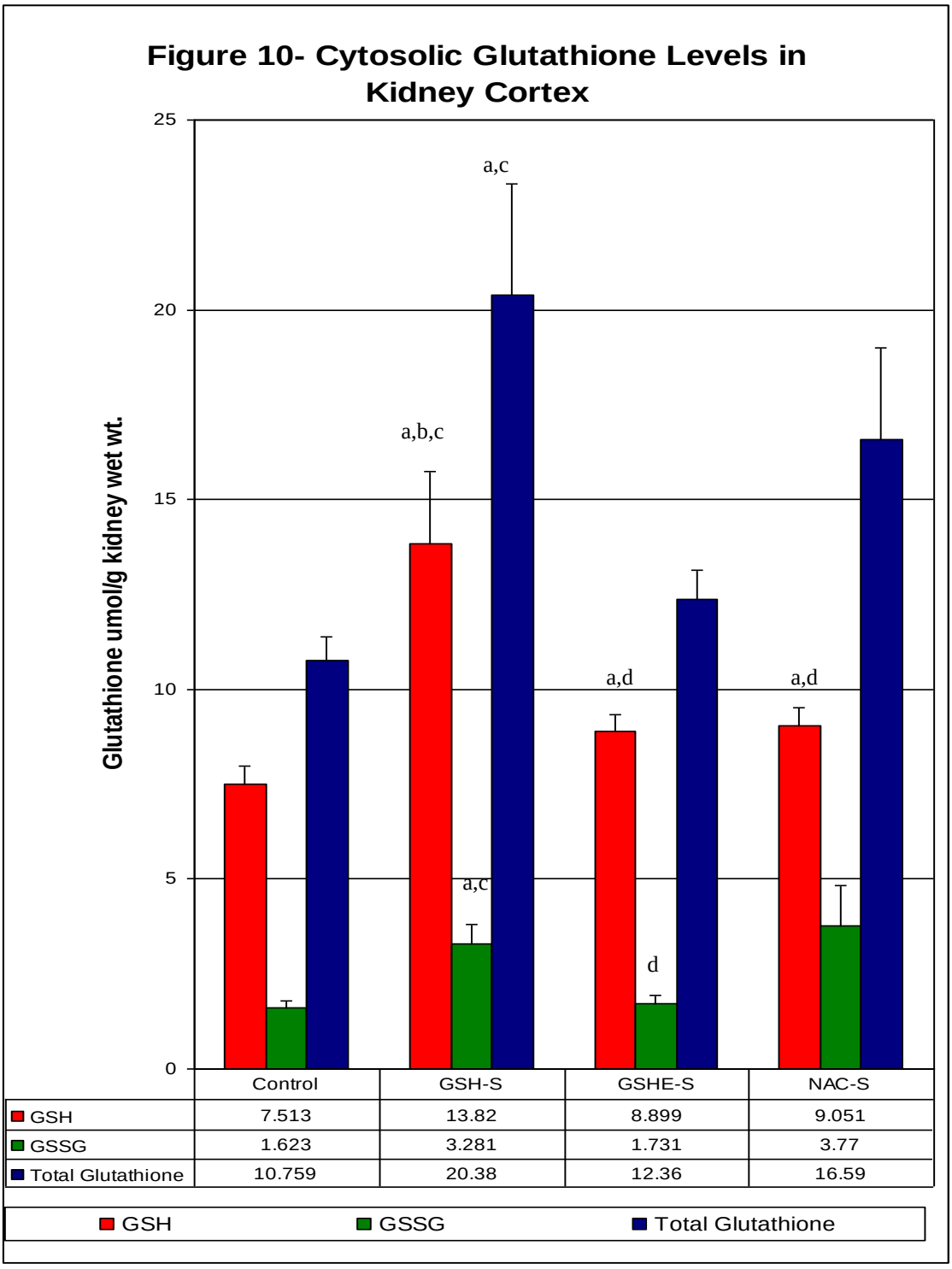
Effect of Supplementation on Cytosolic Glutathione Levels (see Figures 10 and 11 and Table 2)

All three supplements significantly increased cytosolic GSH levels in both the cortex and medulla. The increases in cortical cytosol were ~84% for GSH-S, ~18% for GSHE-S, and ~20% for NAC. The increases in medullary cytosol were ~77% for GSH-S, ~30% for GSHE-S, and ~77% for NAC. The increases in cytosolic GSH with GSH-S were two-fold higher than with either GSHE-S or NAC-S in the kidney cortex and medulla. The level of GSSG in the cytosol showed variable changes with supplementation. Total glutathione levels tended to increase with supplementation, but the increases were not always significant. Similar to the mitochondria, the redox ratios in the cytosol were not changed with supplementation.

Figure 10 – Cytosolic Glutathione Levels in Kidney Cortex

a- Significantly different from Control (n = 6)
b- Significantly different from NAC-S (n = 8)
c- Significantly different from GSHE-S (n = 8)
d- Significantly different from GSH-S (n = 7)

Figure 10- Cytosolic Glutathione Levels in Kidney Cortex



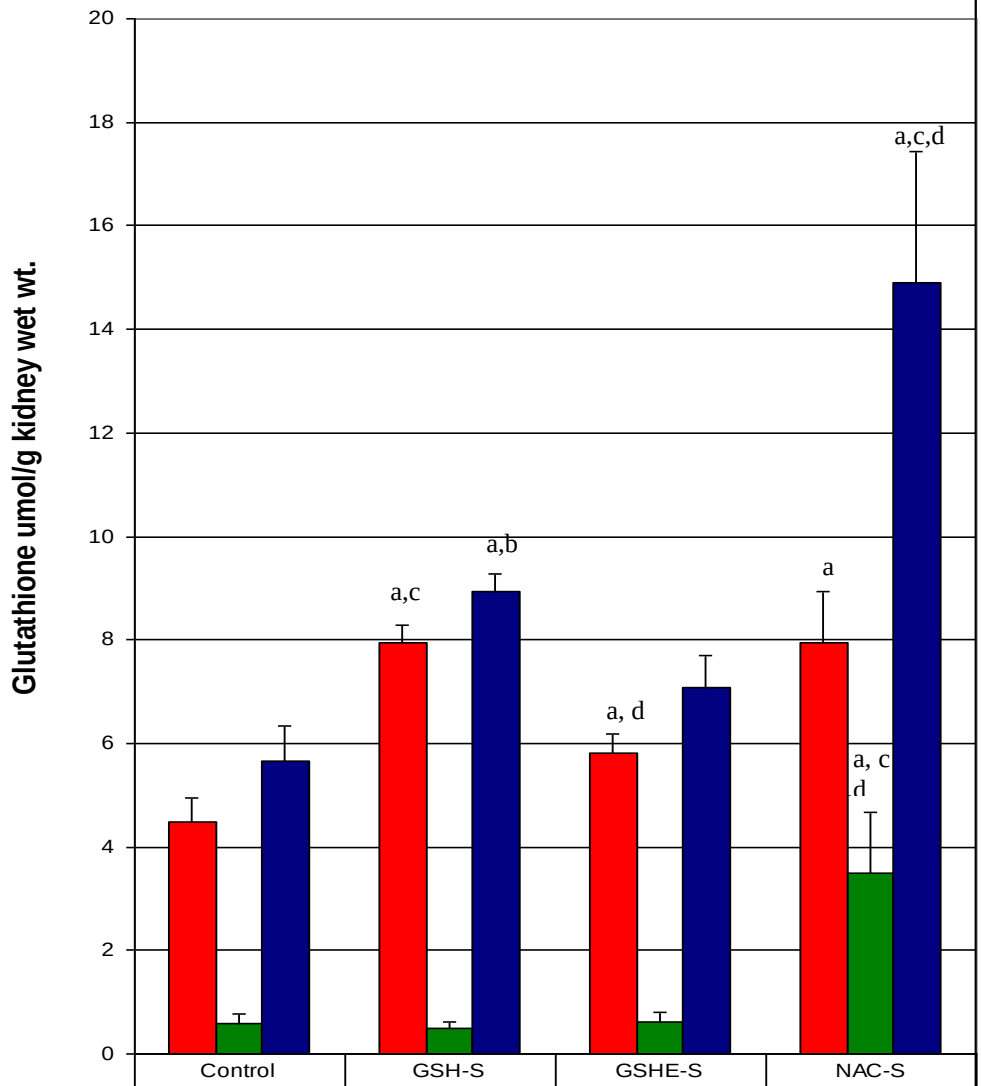
	Control	GSH-S	GSHE-S	NAC-S
GSH	7.513	13.82	8.899	9.051
GSSG	1.623	3.281	1.731	3.77
Total Glutathione	10.759	20.38	12.36	16.59

GSH GSSG Total Glutathione

Figure 11 – Cytosolic Glutathione Levels in Kidney Medulla

a- Significantly different from Control (n = 6)
b- Significantly different from NAC-S (n = 8)
c- Significantly different from GSHE-S (n = 8)
d- Significantly different from GSH-S (n = 7)

Figure 11- Cytosolic Glutathione Levels in Kidney Medulla



	Control	GSH-S	GSHE-S	NAC-S
GSH	4.487	7.931	5.825	7.929
GSSG	0.601	0.503	0.62	3.48
Total Glutathione	5.668	8.937	7.065	14.89

GSH GSSG Total Glutathione

Table 2
Glutathione Redox Ratio (GSH/GSSG) in Kidney Cytosol

	Control n=6	**GSH-S** n=8	**GSHE-S** n=8	**NAC-S** n=7
Cortex	4.94±0.71	4.36±0.33	5.75±0.7	3.48±0.67
Medulla	10.17±2.49	17.1±4.02	14.79±3.3	8.93±4.13

Discussion

The present study demonstrates that exogenous supplementation with the antioxidants GSH, GSHE and NAC are effective at increasing the mitochondrial as well as the cytosolic pool of GSH in the rat kidney. The increases in the cytosolic GSH pool with exogenous supplementation is not surprising since cytosolic GSH makes up most of the kidney tissue GSH, and increases in kidney GSH have been reported with supplementation (Abul-Ezz, 1991; Aebi, 1992; Arfsten 2004; Scaduto, 1991; Scaduto, 1988; Sen, 1994). Few of the previous studies investigated whether supplementation increases the mitochondrial GSH pool. Arivazhagan (2001) reported that alpha lipoic acid (100 mg/Kg of body weight) increased the mitochondrial GSH level in whole rat kidney of old animals (i.e., 22 months of age) when given the supplement via intraperitoneal injection for one or two weeks. Since the supplements in this study were given at different concentrations, with GSH at 250 mg/Kg body wt, GSHE at 25 mg/ Kg body wt and NAC at 200 mg/Kg body wt, it is difficult to make any conclusions on which supplement was more effective at increasing the cytosolic and/or mitochondrial GSH pools. The handling of each supplement by cells is also different. The dose of GSHE used was very low compared to GSH and NAC due to the cost of GSHE. Yet there were significant increases in mitochondrial and cytosolic GSH levels with GSHE despite the dose being one tenth of the dose for GSH or NAC.

The cortex and medulla of the kidney are structurally distinct areas of the kidney, and there is limited information on the effects of dietary supplementation on these regions of the kidney. The cortex of the kidney, containing glomeruli and proximal and distal tubules, has a high blood flow and high rate of aerobic metabolism (Lash, 1994;

Higgins, 2004). The generation of ATP by oxidative phosphorylation results in increased free radical production (Zhan, 2004), which has been shown to increase GSH content in a variety of tissues (Deneke, 1989; Woods, 1992, 1995, 1999). In contrast, the medulla of the kidney, containing the limbs of Henle and collecting duct, has a lower blood flow and a high rate of anaerobic metabolism (Jung, 1988; Kean, 1962; Mori, 2006). The medullary mitochondria may require less ATP. The activity of gamma (γ) - glutamylcysteine transferase, the enzyme that adds GSH to a toxin to neutralize it, has been shown to be two fold higher in the cortex of the rabbit kidney when compared to the outer medulla (Mohandes, 1984). The inner medulla of the rabbit kidney has about one eighth the activity of the outer medulla (Mohandes, 1984). The cortex of the kidney contains more mitochondria than the medulla (Abrahams, 1991; Bondi, 1972; Kean 1962), and there is a lot of heterogeneity in the size of mitochondria in the kidney (Lash, 1998). The mitochondrial GSH pool turnover is also much slower (i.e., 30 – 70 hrs) compared to the cytosolic GSH pool turnover (i.e., 2 hrs) (Lash, 1995; Petrushanko, 2006). In this study, mitochondrial GSH levels were similar in both the cortex and medulla before supplementation, and mitochondria in both the cortex and medulla showed significant increases in GSH with supplementation. The cytosolic GSH levels were higher in the cortex than the medulla in this study before supplementation, but similar magnitude increases were seen in cytosol in both cortex and medulla following supplementation.

The redox ratio (i.e., GSH/GSSG) has been used to determine oxidative stress in cells (Andziak, 2006). It was anticipated that with an increase in GSH levels with supplementation, the redox ratio (i.e., GSH/GSSG) would increase. However, there were

no significant changes in the redox ratio in either the cytosol or mitochondria from kidney cortex or medulla with supplementation. This may be due to increases in the GSSG level that were also seen with supplementation. The redox ratio is tightly coupled to the metabolic rate in the different parts of the cell (Andziak, 2006).

In summary, the present study confirms that exogenous dietary supplementation with antioxidants is effective at increasing both the mitochondrial and cytosolic GSH pools in the rat kidney. An increase in the mitochondrial GSH pool with supplementation may prove to be beneficial in protecting the mitochondria from damage related to increased oxidative stress seen in various diseases and conditions, such as ischemia-reperfusion injury following surgical trauma or transplantation.

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Appendix A- 2018 Teklad Global 18% Protein Rodent Diet

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Product Description—**2018 Teklad Global 18% Protein Rodent Diet** is designed and manufactured with high quality ingredients. 2018 is a **fixed formula**, non-autoclavable diet containing a minimum of 18% protein and 5% fat. Promotes gestation, lactation, and consistent growth of rodents. Absence of animal protein and fish meal eliminates the presence of nitrosamines. Does not contain alfalfa, thus lowering the occurrence of natural phytoestrogens. For autoclavable diet, please refer to 2018S Teklad Global 18% Protein Rodent Diet (Sterilizable). **Diet 2018 is available certified (2018C) and irradiated (2918).**

Ingredients—Ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, calcium carbonate, brewers dried yeast, dicalcium phosphate, iodized salt, L-lysine, DL-methionine, choline chloride, niacin, vitamin A acetate, biotin, pyridoxine hydrochloride, thiamine mononitrate, vitamin D_3 supplement, folic acid, menadione sodium bisulfite complex (source of vitamin K activity), vitamin E supplement, vitamin B_{12} supplement, riboflavin, calcium pantothenate, ferrous sulfate, magnesium oxide, manganous oxide, zinc oxide, copper sulfate, calcium iodate, cobalt carbonate, chromium potassium sulfate, kaolin.

Macronutrients◆

Crude Protein	%	18.8
Crude Oil (Fat)	%	6.0
Crude Fiber	%	3.8
Ash	%	5.9
Carbohydrate (available)	%	50.0
Starch	%	45.0
Sugar	%	5.0
Digestible Energy[†]	Kcal/g (MJ/kg)	3.4(14.0)
Metabolizable Energy[†]	Kcal/g (MJ/kg)	3.3(13.7)
Calories from Protein*	%	23
Calories from Fat*	%	17
Calories from Carbohydrate*	%	60

Minerals◆

Calcium	%	1.00
Phosphorus	%	0.65
Sodium	%	0.23
Potassium	%	0.68
Chloride	%	0.40
Magnesium	%	0.20
Zinc	mg/kg	77.0
Manganese	mg/kg	118.0
Copper	mg/kg	15.0
Iodine (added)	mg/kg	12.0
Iron	mg/kg	225.0
Selenium	ug/kg	200
Cobalt	ug/kg	600
Chromium	ug/kg	500

Amino Acids◆

Aspartic Acid	%	1.42
Glutamic Acid	%	3.70
Alanine	%	1.08
Glycine	%	0.79
Threonine	%	0.67
Proline	%	1.55
Serine	%	0.97
Leucine	%	1.94
Isoleucine	%	0.85
Valine	%	0.95
Phenylalanine	%	0.99
Tyrosine	%	0.61
Phe + Tyr	%	1.60
Methionine	%	0.35
Cystine	%	0.33
Met + Cyst	%	0.69
Lysine	%	0.92
Histidine	%	0.47
Arginine	%	1.06
Tryptophan	%	0.20
Available Lysine	%	0.83

Standard Product Form: ***PELLET***

Vitamins◆

Vitamin A	iu/g	15.4
Retinol	mg/kg	4.7
Vitamin D_3	iu/g	1.5
Cholecalciferol	ug/kg	38
Vitamin E (a-tocopherol)	mg/kg	100.0
Vitamin K_3 (menadione)	mg/kg	51.0
Vitamin B_1 (thiamine)	mg/kg	16.5
Vitamin B_2 (riboflavin)	mg/kg	14.9
Avail. Niacin (nicotinic acid)	mg/kg	41.2
Vitamin B_6 (pyridoxine)	mg/kg	18.5
Pantothenic Acid	mg/kg	33.0
Vitamin B_{12} (cyanocobalamin)	ug/kg	80
Avail. Biotin	mg/kg	0.3
Folate	mg/kg	3.3
Vitamin C	mg/kg	—
Choline	mg/kg	1120.0
B Carotene	mg/kg	2.5
Inositol	mg/kg	1450.0

Fatty Acids◆

C16:0 Palmitic	g/kg	7.6
C18:0 Stearic	g/kg	1.5
C18:1ω9 Oleic	g/kg	12.6
C18:2ω6 Linoleic	g/kg	31.3
C18:3ω3 Linolenic	g/kg	2.8
Total Saturated	g/kg	9.6
Total Monounsaturated	g/kg	12.8
Total Polyunsaturated	g/kg	34.1
Cholesterol	mg/kg	—

*Calculations performed using values of 4 kcal/g for protein and carbohydrate and 9 kcal/g for oil (fat).

[†]**Digestible Energy** (DE) is the total (gross) amount of energy in the diet minus the energy eliminated in the feces. It accounts for energy contained in the indigestible portion of the diet. Of the digestible energy, some is lost as a result of various metabolic processes in the urine and sometimes in gases from the digestive tract. The remaining energy in the body is available to support metabolism and is known as the **Metabolizable Energy** (ME). This value more accurately reflects the usable energy contained in the diet.

◆Nutrient levels are calculated from raw material data and are adjusted to 10% moisture level in the diet. Nutrient values may vary due to the inherent variability in the natural ingredients and from laboratory analysis.

Appendix B- Calculations of Cytosolic and Mitochondrial GSH

GSH Levels in the Cytosol

GSH levels in the cytosol were calculated as shown below

[GSH] from 356nm reading in µmol/L ⁄ g kidney/L homogenate = [GSH] in µmol/ g kidney (eq.1)

Sample Calculation:

$$\frac{1706.4}{233.8\ g/L} = 7.30\ \mu mol/g\ kidney$$

GSH in Mitochondria

[GSH] from 356nm reading in µmol/L X Pellet vol. in L = GSH umol (eq. 2)

$$\frac{\text{GSH umol}}{\text{Volume supernatant 1 from 900 X g spin}} = \text{GSH in µmol/ml (eq. 3)}$$

$$\frac{\text{GSH in µmol/ml}}{\text{g kidney/L homogenate}} = \text{[GSH] µmol/g kidney (eq. 4)}$$

Sample Calculation:

72.5 µmol/L X 0.000406L = 0.02943µmol

$$\frac{0.02943\ \mu mol}{0.00166\ L} = 17.7\ \mu mol/L$$

$$\frac{17.7\ \mu mol/L}{233.8 g/L} = 0.0758\ \mu mol/g\ kidney$$