GLUTATHIONE IN PARKINSON'S DISEASE

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Abstract

Glutathione Deficiency in Parkinson's Disease

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Parkinson's Disease (PD) is one of several prevalent neurodegenerative diseases plaguing the aging population. To date, no therapies have been shown to slow, stop, or reverse disease progression; the disease is considered irreversible and progressive. Post mortem brain from individuals with premotor PD show a deficiency of reduced glutathione, GSH, and it has been hypothesized that deficiency of GSH contributes to PD neurodegeneration. The role of GSH in the healthy brain is described, and evidence of GSH deficiency in PD is reviewed. The pros and cons of various augmentation strategies are discussed. Subsequent chapters demonstrate intranasal GSH, (in)GSH, is safe and tolerable and provide evidence that 200 mg (in)GSH is capable of augmenting brain GSH by more than 200%. In congruence with intravenous GSH studies, (in)GSH intervention groups had a mild symptomatic improvement following three months of (in)GSH administration. In a cross-sectional analysis of 58 individuals with PD, low blood GSH was associated with

greater disease severity. Taken together, this body of research supports the hypothesis that GSH depletion contributes to PD and that (in)GSH has therapeutic potential as both a symptomatic treatment and a disease modification strategy. The final chapter describes an underlying GSH deficiency syndrome, with elderly, sick, and/or malnourished individuals at greatest risk. Sufficient data exists to warrant further investigation of GSH as a biomarker and (in)GSH as a disease-modifying therapy in PD.

Previously Published

The following manuscripts were first published in the following journals and are reprinted with permission.

Conditionally Essential Nutrients: The State of the Science

Mischley LK. Conditionally Essential Nutrients: The State of the Science. Journal of Food and Nutrition 2014:v1:e204:1-4.

Safety Survey of Intranasal Glutathione

Mischley LK, Vespignani M, Finnell J. Safety Survey of Intranasal Glutathione. Journal of Alternative and Complementary Medicine 2013 May;19(5):459-63. PMID: 23240940.

A Randomized Phase I/IIa Study of Intranasal Glutathione in Parkinson's Disease

Mischley LK, Leverenz JB, Lau RC, Polissar NL, Neradilek MB, Samii A, Standish LJ. *A Randomized, Double*-Blind Phase I/IIa Study of Intranasal Glutathione in Parkinson's Disease. Movement Disorders 2015 Oct;30(12)1696-701.

Central Nervous System Uptake of Intranasal Glutathione

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DEDICATED WITH GRATITUDE

My parents, Bill & Nancy Mischley
My best friend, Jason Allen
My children, Oliver and Evangeline

Each of you has sacrificed so that I may thrive.
My success is a result of the extra weight you've carried.

My patients

It has been my pleasure to serve you. Thank you for trusting me and teaching me.
You make my life intellectually, socially, and spiritually fulfilling.

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List of Abbreviations

CNS	central nervous system
GABA	gamma-aminobutyric acid
Glu	total glutathione, combined GSH and GSSG
GSH-Px	glutathione peroxidase
GSH-Rd	glutathione reductase
GSH	reduced GSH
GST	glutathione S-transferase
H202	hydrogen peroxide
(in)GSH	intranasal reduced GSH
(in)saline	intansal saline
(iv)GSH	intravenous reduced glutathione
(iv)NAC	intravenous N-acetyle cysteine
MoCA	Montreal Cognitive Assessment
MRS	magnetic resonance spectroscopy
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMDAR	N- methyl-D-aspartate receptor
NO	nitric oxide
O-	superoxide
PRO-PD	Patient Reported Outcomes in Parkinson's Disease
SOD	superoxide dismutase
UPDRS	Unified Parkinson's Disease Rating Scale

Glutathione Deficiency in Parkinson's Disease

Laurie K Mischley

1.1 Background & Significance

1.1.1. Parkinson's Disease: State of the Science

Parkinson's disease (PD) affects up to approximately 1% of individuals over the age of 60 in industrialized nations.[1] Already a physically, socially, and financially devastating disease, the aging population is likely to further increase the burden. Originally thought to be exclusively a motor disorder, PD is now recognized as a syndrome affecting non-motor and cognitive facilities, as well. First described almost 200 years ago, the most significant advance in PD therapeutics was born from the observation that affected brain regions were deficient in dopamine, and that exogenous provision of the dopamine precursor, levodopa (l-dopa), provided symptomatic relief.[2] Early fortification efforts used l-dopa extracted from fava beans and today pharmaceutical preparations of l-dopa remain the most commonly employed therapy for PD symptom management. Other medicines exist to treat PD symptoms, although most of them mask symptoms by targeting the dopaminergic system.

It is now known that the dopaminergic system is affected late in the course of the disease. While the provision of dopamine is essential for symptomatic relief, upstream prevention and disease-modification efforts may, or may not, involve dopaminergic pathways. On autopsy, individuals with PD have distinct cellular inclusions, called Lewy bodies; an increasing body of literature suggests these inclusions first appear in the intestinal tract up to a decade before the disease and spread in a prion-like fashion up the vagus nerve, into the midbrain, eventually reaching the

substantia nigra (SN), which produces dopamine, essential for movement and pleasure. When more than half of the SN has been destroyed, the patient begins to notice symptoms that eventually lead to the PD diagnosis.[3-5]

Mitochondrial dysfunction, impaired autophagy, and other hallmarks of PD pathophysiology are known to increase the free radical burden of the cell, and thus metabolic waste, which cannot be cleared, into a cycle of perpetually increasing oxidative stress and degeneration. There is wide agreement in the scientific community that reactive oxygen and nitogen species (ROS, RNS) are a hallmark of Parkinson's disease (PD), and that the redox disequilibrium contributes to the cascade of neurodegeneration.[6, 7] What is widely debated, however, is whether the radical stress is the cause of PD, or consequent to, the disease and whether it is possible to significantly influence oxidative stress using exogenously provided antioxidants.

The distinction between symptomatic therapies and disease-modifying therapies is an important one. For instance, a therapy that doesn't improve symptoms, but slows the rate of progression, patients and insurance companies may dismiss it because the efficacy cannot be observed in the short-term. Therapies that impact the rate, or slope, of progression are likely to have more value to younger patients, who will likely live with the disease longer than their elderly counterparts, and thus may be more willing to accept certain risks or side effects. Conversely, if a therapy exclusively conceals symptoms of the disease without altering the rate of progression, there is no reason for a patient to remain on the therapy if he/ she does not perceive benefit. These distinctions could reduce costs and improve prescribing practices, and thus public health. There

are currently dozens of biological therapies approved for the management of PD symptoms, and not a single therapy has demonstrated the capacity to affect disease progression.

1.1.2. The nutrient theory of disease

The concept of vitamins dates back to 1906, in which FG Hopkins stated, "…no animal can live upon a mixture of pure protein, fat, and carbohydrate, and even when the necessary inorganic material is carefully supplied the animal still cannot flourish. Scurvy and rickets are conditions so severe that they force themselves upon our attention; but many other nutritive errors affect the health of individuals to a degree most important to themselves.,…"[8, 9] During the early 1900s, many vitamins were isolated, described, and synthesized, which led to the development of dietary allowances and fortification during the second half of the century. In spite of great historical success, the era of nutrient discovery has become relatively dormant in recent decades.

GSH is essential for life [10] individuals with PD, as well as other conditions, appear unable to synthesize sufficient quantities to supply demand, GSH should be considered a conditionally essential vitamin. As both cause and consequence of the disease, the clinical manifestations of GSH have yet to be described, with increasing benefit from augmentation becoming apparent over time, with improved redox equilibrium and cellular detoxification processes. If GSH renders cells vulnerable to subsequent insult, diverse and numerous comorbidities would be anticipated.

The vast majority of GSH is endogenously synthesized from amino acids in the diet, although intact GSH is taken up by sodium dependent transporters in the intestinal epithelium and does

contribute to circulating plasma concentration.[11] There was a mean 34.8 mg of GSH consumed per day in 69 white participants in 1992, with intake ranging from 13.0 to 109.9 mg.[12] Among those deficient, it is unlikely that food alone will be able to adequately supply GSH needs, which are in excess of normal physiological demands.

1.1.3. The identification of GSH depletion in Parkinson's disease

"Parkinson's Disease: A disorder due to nigral glutathione deficiency?" was published in 1982, citing evidence of a deficiency of reduced glutathione, GSH, in the SN compared to age-matched controls.[13] The authors noted that, even in controls, the SN had a reduced GSH content compared to surrounding brain tissue, which led to the hypothesis that low GSH may render the SN especially susceptible to oxidative damage. In a follow-up study in 1996 that replicated these findings of GSH depletion in the PD SN, the authors concluded, "The most reasonable explanation for the GSH deficiency in the SN in PD is that a compound which can be conjugated with GSH by glutathione transferase, and which may be toxic to dopaminergic neurons, is accumulating in this brain region."[14] Subsequent analysis on post mortem SN tissue demonstrated a mean weight of 49.4 $\pm$ 4.6 μg GSH /g fresh weight $\pm$ S.E.M. in PD subjects, compared to 92.8 $\pm$ 12.6 μg GSH/g in control subjects.[15] As a result of these early studies, administration of buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, is being currently employed in PD models to mimic the pathophysiology of PD.[16]

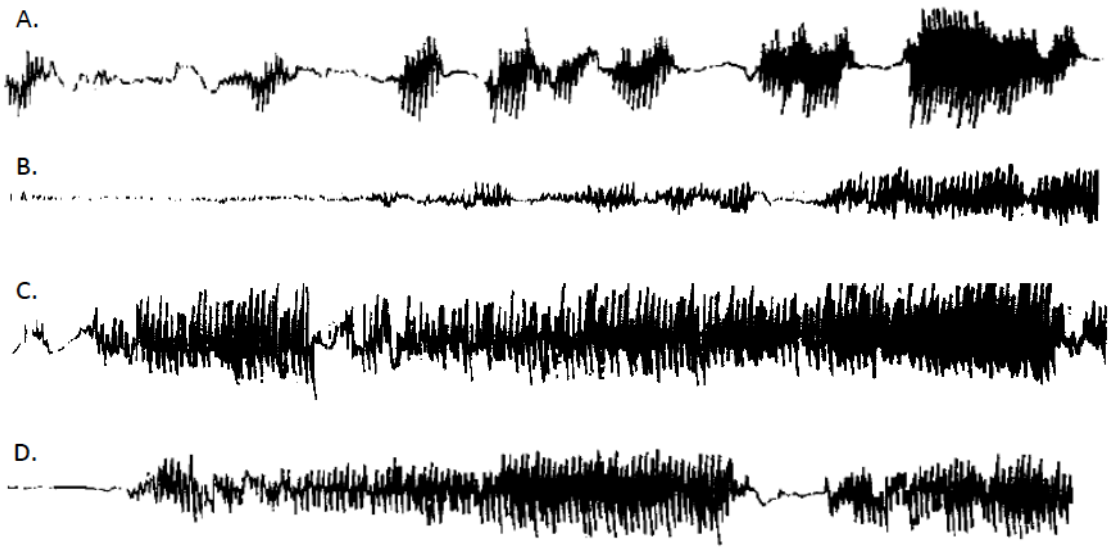
It is worth noting that GSH depletion does not, on its own, result in SN cell loss in rodent models of GSH depletion.[17] However, under conditions of normal aging or when neurons are exposed to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), reductions in

GSH exacerbate SN degeneration.[18] This, combined with evidence that reduced GSH is seen in numerous other diseases affecting the central nervous system [19, 20] suggests that GSH depletion is not likely the inciting incident in PD, but occurs early in the disease process secondary to an inciting environmental or metabolic insult.

1.1.4. Early attempts at GSH augmentation in PD

In spite of a substantial body of literature supporting the notion of GSH depletion in the PD brain, GSH repletion efforts have been hindered by a lack of oral bioavailability,[21] inability to quantify brain GSH concentrations *in vivo*, and lack of industry investment in this naturally occurring molecule.

The first human clinical trial to administer exogenously supplied GSH to individuals with PD took place in Italy in 1996, based on the post-mortem evidence of GSH deficiency described above. In this open-label trial of nine individuals with early, untreated PD, 600 mg of intravenous GSH, (iv)GSH, was administered twice daily for 30 days. The authors reported "All patients improved significantly after GSH therapy, with a 42% decline in disability. Once GSH was stopped the therapeutic effect lasted for 2-4 months."[22] (Figure 1)



[22]

Figure 1: Intensity of resting tremor in patients with PD at A) baseline, B) after 30 day of IV glutathione (600 mg twice daily), C) 60 days after glutathione withdrawal, and D) 30 days after Sinemet 25/250 C/L, ½ tablet thrice daily. (Taken from Sechi et al, 1997.) Vertical calibration is 200 microvolts, horizontal scale is 1 second.

The second clinical trial of exogenously administered glutathione in PD was performed by Hauser et al. was published in 2009. This randomized, double-blind, placebo-controlled pilot study was performed to evaluate the safety, tolerability, and preliminary efficacy of (iv)GSH in 21 individuals with PD. After four weeks of 1400 mg three times per week (iv)GSH, the data suggested the GSH group had a mild symptomatic effect over placebo.[23]

Around this time, a private practice neurologist in Naples, FL, USA, and one of the authors on the second (iv)GSH study, posted videos on social media demonstrating almost immediate improvement in PD motor symptoms following (iv)GSH,[24] triggering tremendous patient

demand for (iv)GSH. The therapy, although biologically plausible, is expensive, invasive, and inconvenient. (Figure 2) In an attempt to identify alternative methods of GSH augmentation, I became aware of ear, nose, and throat specialists in Seattle, WA, USA that were administering intranasal GSH, (in)GSH to individuals with chronic sinusitis and environmental medicine practitioners who were using it to treat multiple chemical sensitivity (MCS).[25]

There is an increasing trend in pharmaceutical science to use intranasal delivery to bypass the (in)GSH. The majority of survey respondents reported using (in)GSH to treat multiple chemical sensitivity (MCS) (n=29) and allergy/ sinusitis (n=25); 7 reported using (in)GSH for PD, of which 57% (n=4) reported a positive effect on PD symptoms.[25]

Intranasal Administration

Advantages	Limitations
Non-invasive, rapid, comfortable	Concentration in various brain structures may vary
No modification of drug is required	Delivery is expected to decrease with molecular size
Reduces systemic exposure	Mucosal irritation may occur
Rich vasculature enhances uptake	Nasal congestion may interfere with delivery
Drug degradation is minimized	Ongoing use could lead to mucosal damage
May bypass the blood brain barrier	

IV Administration

Advantages	Limitations
No modification of drug is required	Invasive – risk of complications
Effectively raises serum glutathione	Expensive – medical staff required for IV administration
Minimal drug degradation	Inconvenient – mobility issues plus frequent visits
Method used in preliminary studies	Non-specific access to CNS
'Standard of care' among CAM clinicians	One report of hepatic injury following IV GSH

Oral Administration

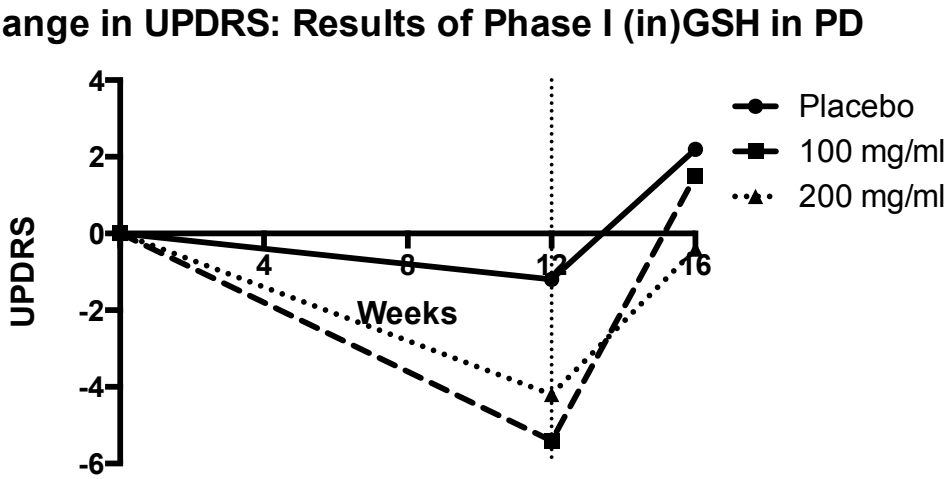
Advantages	Limitations
Convenient	Poor absorption
	Non-specific access to CNS

Nebulized Administration

Advantages	Limitations
Comfortable	Nebulizer is loud and time consuming
	Portion of dose wasted (non-specific dosing)
	Does not reach systemic circulation

Figure 2: Advantages and limitations associated with various methods of GSH administration. (Taken from Mischley, 2011)[26]

Anecdotal reports of symptomatic improvement following (in)GSH, evidenced by clinical chart notes, and reported safety from the survey led to funding of a Phase I Study of (in)GSH in PD, sponsored by the National Institutes of Health (NIH). The goal of the study was to formally evaluate whether the (in)GSH is safe and tolerable enough to use in an efficacy study. In this double-blind, placebo-controlled study, 30 participants with PD were randomized to receive placebo, intranasal saline, (in)saline, 100 mg GSH/ 1 ml saline, or 200 mg GSH/ 1 ml saline thrice daily for 12 weeks, followed by a four week washout. We concluded (in)GSH was safe and tolerable, and in accord with previous trials, these data also demonstrate an improvement in clinical symptom scores, as measured by the gold standard Unified Parkinson's Disease Rating Scale (UPDRS).[27] (Figure 3) It should be noted that for all three clinical GSH intervention studies, a rebound worsening of symptoms was reported. Such exacerbation of symptoms upon withdrawal is further suggestive of a symptomatic effect although none of these trials was appropriately powered to determine a clinical improvement over placebo.



[27]

Figure 3: Change in UPDRS score, by cohort, over three months administration of (in)GSH or placebo, followed by a 4 week washout.

The existing body of data support the notion that exogenously administered GSH results in mild symptomatic improvement in PD. As a non-dopaminergic molecule, GSH may offer new therapeutic strategies. Sufficient data exist to warrant a Phase III multi-center clinical intervention trial, employing the existing formula, dose and delivery of (in)GSH previously described.

1.2 Metabolism of Reduced Glutathione (GSH)

1.2.1 Endogenous synthesis and distribution in the CNS

Glutathione (γ-glutamylcysteineylglycine) is a tripeptide consisting of glycine, glutamic acid, and cysteine. GSH synthesis occurs in the intracellular space; no evidence exists of glutathione transport directly into cells exists. Glutathione is synthesized intracellularly via dependent glutamylcysteine ligase (GCL) and glutathione synthase (GS), with both reactions requiring a single molecule of ATP.

In the CNS, GSH is concentrated in astrocytes and neuronal axons and terminals, but not neuronal cell bodies. In the healthy nervous system, astrocytes serve as the primary reservoir of GSH, providing a first line of defense against endogenously produced metabolic waste and xenobiotics and supplying surrounding neurons with the amino acid substrate for intracellular GSH synthesis, as needed. When the reservoir of glial GSH is depleted, detoxification processes are impaired, further depleting GSH, perpetuating a cycle of impaired

clearance, redox stress, autophagy, whilst surrounding cells suffer from a paucity of substrate no longer being provided by astrocytes (Figure 4).

As the primary circulating thiol, oxidized glutathione, GSSG, is rapidly reduced back to GSH via glutathione reductase and $NADPH^{+}$, so that at any given time 95-99%% of intracellular glutathione is in the reduced state. There is evidence the ratio of GSSG:GSH increases with redox stress in PD.[28, 29]

GSH can undergo transpeptidation to cysteinylglycine and the γ-glutamyl moiety.[30] γ-glutamyl transpeptidase is essential for GSH recycling of glutathione S-conjugates and other γ-glutamyl compounds which will allow for the products of cleavage to be reused in GSH synthesis. The cellular availability of cysteine has been shown to be, in part, reliant on γ-glutamyl transpeptidase activity.

In the periphery, gamma glutamyltranasferase (GGT) is widely distributed in plasma membranes, where the active site is facing the extracellular space, facilitating entry of precursors into the intracellular space. The primary role of GGT is to metabolize extracellular GSH, recycling the component amino acids. Recently, epidemiologic data have suggested that

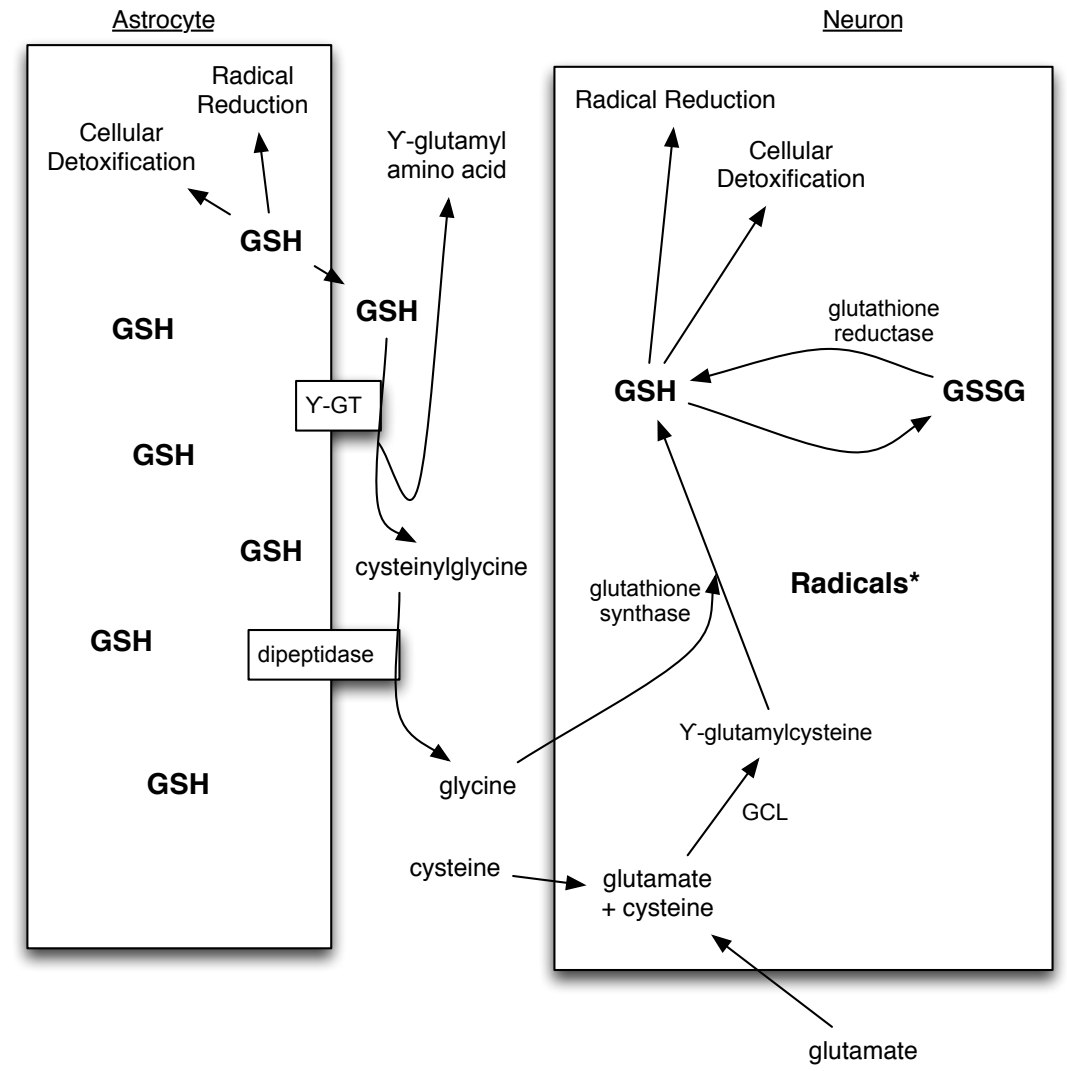


Figure 4: Provision and assembly of GSH in the CNS. GSH: reduced glutathione, GSSG: oxidized glutathione, GGT: γ-glutamyl transpeptidatse, GCL: glutamylcysteine ligase.

serum GGT levels within the normal range may be a biomarker for early oxidative stress, as evidenced by associated increases in C-reactive protein, fibrinogen, and F2-isoprostanes.[31] It has yet to be determined whether the GGT-associated with oxidative stress is relevant in CNS disease.

1.2.2 Regulation

There is evidence that brain concentrations of GSH decline with age in insects, rodents, and humans. Using ^{1}H-MRS, depletion of GSH was observed in healthy aging, when elderly (~67 years old) were compared to young controls (~20 years). Glutathione concentrations were 0.31±0.05 in healthy young and 0.20±0.08 in elderly ($p<0.005$).[32] Measurements were reported as institutional units, which were intended to approximate the number of micromoles per gram.

The age-dependent decrease in GSH is not due to insufficiency of GSH precursors cysteine, glycine, or glutamate. Since the concentrations of amino acid constituents do not decrease with age, the GSH depletion is thought to be due to insufficient biosynthetic enzyme function or diminished coenzyme availability, e.g. magnesium, NADPH, and ATP. Human red blood cells (RBC) also demonstrate an age-related decrease in GST and in rodents, with elderly mice displaying an approximate 60% reduction in RBC GSH. [33]

1.2.3 Role of GSH in the Circadian Rhythm

Across a wide range of species, the activity of glutathione peroxidase, glutathione S-transferase, glutathione reductase, and γ glutamyl cysteine synthetase has been shown to

fluctuate in a rhythmic fashion, although not always on a 24-hour schedule:[34] Increased exposure to ROS occurs during the day, via ultraviolet light, potential toxicants in food and air, and increased metabolic activity with physical exertion. Glutathione peaks in the morning and detoxification activity of glutathione S-transferase (GST) peaks in the late afternoon.[35]

Not only do circadian rhythms impact GSH, GSH status may also impact chronobiological rhymicity. When rodents were chronically fed lipid peroxides, they saw a decrease in intestinal GSH/GSSG and abrogation of the circadian peak ornithine decarboxylase, which regulates cell proliferation.[36]

The role of sleep and the circadian rhythm cannot neglect the role of melatonin, a neurohormone synthesized by the pineal gland. It is strong antioxidant against a variety of radicals and stimulates other antioxidants including glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd).[37, 38] Administration of pharmacological doses of melatonin have been shown to raise GSH-Px in the brain (most significantly), liver, and kidney of rats, and to ameliorate the depression in GSH-Px seen in response to chronic light exposure.[39] It is well known that melatonin decreases with age, a finding which has been proposed to play a role in the age-related loss of glutathione.[40]

There is increasing evidence that the circadian clock plays a role in the regulation of redox homeostasis. Clock-controlled output genes, such as *per* and *tim*, play a role in the regulation of energy balance, DNA repair, and detoxification processes. [35] Following the

nocturnal rise in melatonin, which peaks between 1-3 am, there is an increase in GSH peroxidase (GSH-Px) and GSH reductase (GSH-Rd) activities in the brains of chicks.[41] Studies in pinealectomized rats demonstrate a suppression of GSH-Px circadian rhythm, not an amelioration of it, suggesting factors other than melatonin influence the GSH-Px fluctuations.

Of note, a major role of GSH is in cellular detoxification, which occurs largely through the conjugation of xenobiotics and metabolic byproducts with glutathione S-transferases (GSTs). Previous studies demonstrate susceptibility to pesticide exposure is regulated by circadian rhythm.[42] Recently, a researchers were able to restore GSH homeostasis in a study of individuals with muscular dystrophy via administration of oral melatonin. [43]

1.3 GSH Functions in the Central Nervous System

1.3.1 Cellular Detoxification

As a mechanism of cellular protection, GSH can directly conjugate with electrophilic xenobiotics or may enzymatically catalyze their detoxification via GST, in preparation for cellular excretion. [44, 45]In addition to GST, cellular detoxification also occurs via S-glutathionylation (-SSG), the reduction of protein cysteine residues by the reversible covalent addition of GSH. The nucleophilic thiol group of cysteine allows it to interact with a wide range of molecules, which affects protein stability and function, signaling pathways, and the adaptive cellular response to redox insult.[46] Efforts are underway to understand which proteins are most susceptible to oxidative stress by models employing addition of biotinylated GSH, which mimics an increase in cellular GSSG, triggering a thiol disulfide exchange.[47]

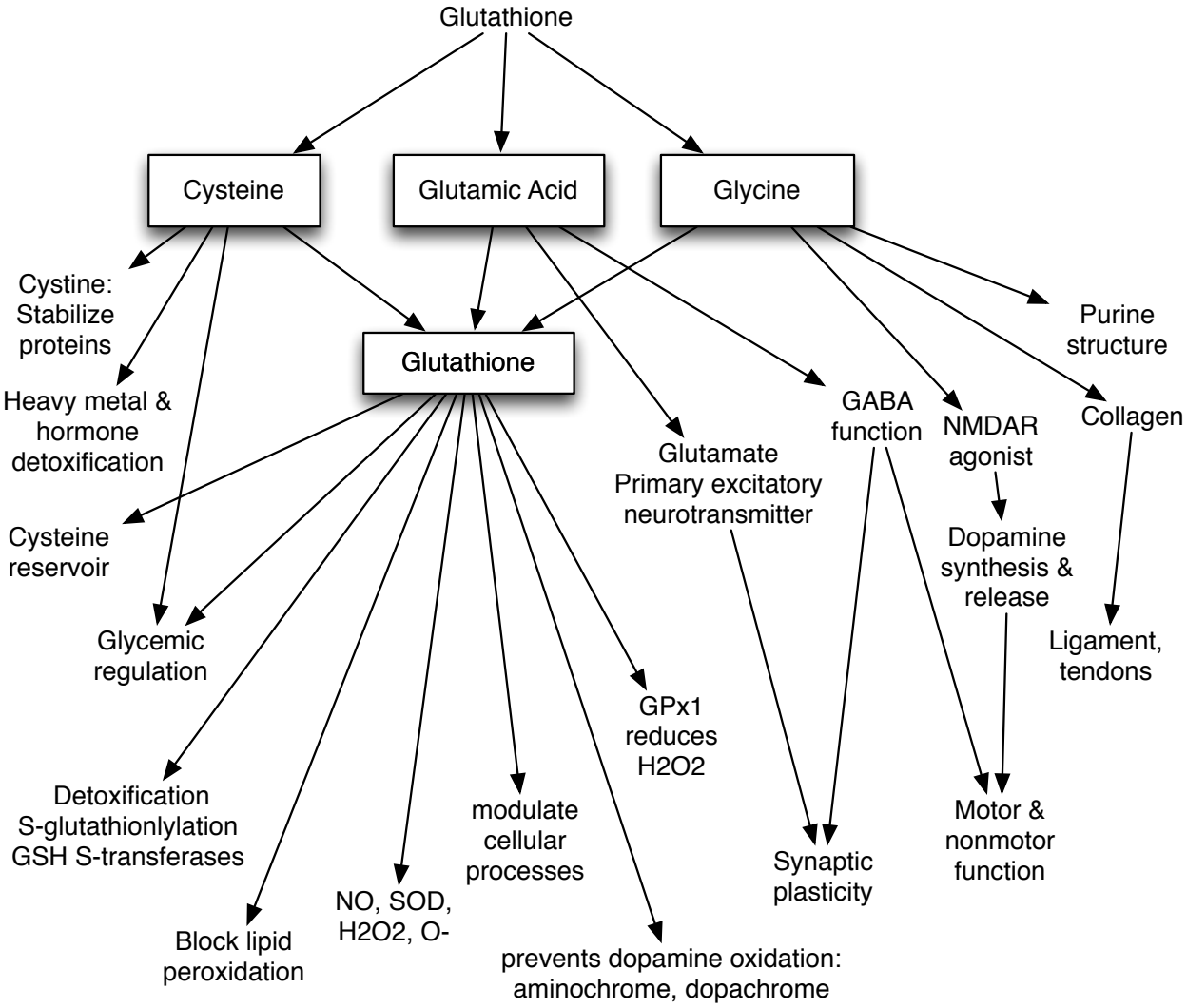


Figure 5: Cellular and Biochemical Mechanisms of Glutathione in PD

(Abbreviations: NO: nitric oxide; SOD: superoxide dismutase, H202: hydrogen peroxide; O-: superoxide; GST: glutathione S-transferase; GABA:gamma-aminobutyric acid; NMDAR: N-methyl-D-aspartate receptor)

1.3.2 Redox regulation

In every cell in the body, homeostastasis between radical exposure, both naturally occurring and environmentally induced, and the antioxidant mechanisms of the body are carefully regulated. When the capacity to reduce radicals is outweighed by the production of radicals, oxidative stress ensues. The high oxygen-dependent metabolic activity naturally makes the brain vulnerable to oxidative damage. Given the delicate environment, it is reasonable that a generalized state of excessive oxidative or nitrosative stress, the brain will be among the organs most severely affected.

The human brain is particularly vulnerable to oxidative stress due to its high metabolic activity. While it represents only about two percent of body weight, it is responsible for approximate 20% of total body metabolic activity. Glucose is the main fuel of the brain, but when glucose is low (i.e. fasting, ketogenic diets, exercise), the brain can use lactate during exercise and ketone bodies for fuel during glucose depletion. The general reliance on glucose means that, per tissue volume, the brain uses disproportionately more oxygen than other tissues. As a course of normal metabolic function, oxidative phosphorylation (occurring in the mitochondria) converts NADH into ATP, producing superoxide radical and hydrogen peroxide as byproduct.

Dopamine, produced in the SN, is especially prone to oxidation. When dopamine is produced (or possibly supplied as l-dopa) in excess of what can be removed from the synaptic clefts by the monoamine transporters, the dopamine molecule may autoxidize, it may be oxidized to dopamine quinones (which can react with intracellular cysteine to cause cellular toxicity), or it

can react with iron, copper, or oxygen resulting in superoxide, or hydrogen peroxide.[48, 49] Figure 4 demonstrates how GSH availability can prevent the formation of the neurotoxic molecule, aminochome, by increasing production of neuromelanin.

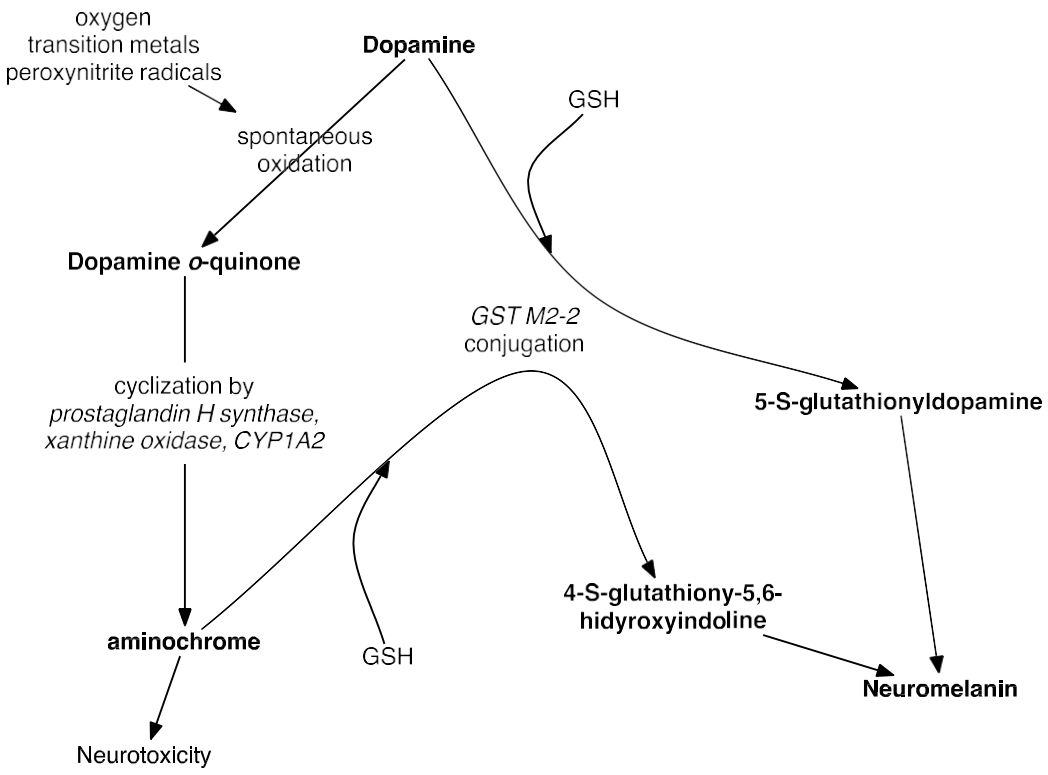


Figure 6: GSH protects against formation of amiochrome.

SN cells have high levels of iron in the CNS, which is essential for the function of the rate-limiting step in dopamine synthesis, tyrosine hydroxylase.[43] Iron is also present as a natural, essential mechanism of supplying oxygen to the highly oxygen-dependent brain tissue. Normally, the ratio of reduced iron (Fe^{2+}) to oxidized iron (Fe^{3+}) in the SN is approximately 1:1, although in the PD SN, Fe^{2+} is elevated up to 3-fold, leading to the Fenton reaction, in

which Fe^{2+} leads to the formation or hydroxyl radicals.[44] Iron chelators, such as green tea, have been shown to reduce the incidence of PD and one of the purported mechanisms for the benefit may be the reduction of Fe^{2+}.[50]

Neuromelanin is responsible for giving the SN its characteristic dark appearance in the brain. The molecule binds easily with iron and other transition metals, as well as lipids, paraquat, etc. In the presence of excessive radical burden, neuromelanin can further stimulate the production of radicals. The higher ratio of microglia:astrocytes in the SN than surrounding brain regions further renders the SN vulnerable to redox stress.[51] Micoglia are highly metabolically active and potent immune system activators; their activation by ROS/ RNS initiates and perpetuates the inflammatory cascade.

The brain is rich in lipids, which are inherently prone to peroxidation, and thus oxidative damage in PD is not specific to dopamine and the SN. The anterior cingulate cortex (ACC) is part of the corpus callosum; it is involved in the regulation of autonomic functions (e.g. heart rate, blood pressure) and susceptible to the accumulation of alpha-synuclein in PD. Using postmortem tissue, the ACC of individuals with PD had a 44% increased susceptibility to lipid peroxidation, compared to controls.[46] Once formed, lipid peroxides degenerate to aldehydes, such as acrolein, which bind covalently with thiol groups of proteins, leading to protein dysfunction and accumulation of DNA adducts.[52] *In vitro*, GSH protects against the formation of DNA adducts. These studies demonstrate cysteine is less able than GSH to protect against formation of DNA adducts, and protection by N-acetyl cysteine (NAC) was not detectable. [48]

Most free GSH reduces H_2O_2, which is itself not a free radical; in the presence of glutathione H_2O_2 is reduced to water via glutathione peroxidase, generating GSSG. Among the reactive species, H_2O_2 has a relatively long half-life and can traverse membranes, eventually catalyzing the formation of more potent radicals such as hydroxyl radical.[53]

When GSSG concentrations accumulate, glutathione reductase is activated in an effort to restore GSH:GSSG ratios. When redox homeostasis cannot be maintained, the high concentrations of GSSG can induce formation of glutathione-protein adducts (PS-SG) that may alter protein activity.[54]

1.3.3 Protein Synthesis

Physiologic adequacy of GSH is required for protein synthesis. With moderately oxidizing conditions, there is an increased demand for protein synthesis, triggering the release of the cysteinyl moiety of GSH.[24] While not described in neurons, in liver cells, cysteine release catalyzes the formation of cysteine-protein mixed disulfides, which regulates protein degradation and synthesis. Elongation factor 2 contains a number of sulfhydryl groups that are essential for the translation step of protein synthesis.[55]

1.3.4 Reservoir for constituents

As a reservoir of cysteine, glycine, and glutamate, GSH functions to supply substrate for connective tissue, purine and porphyrin synthesis. The component amino acids themselves

exhibit neurochemical activity at NMDA, glutamate, and GABA receptors. For instance, the N-methyl-D-aspartate class of glutamate receptors are highly expressed throughout the basal ganglia and the limbic system. NMDR receptor agonsists, such as glycine and serine, have been shown to promote dopamine release and synthesis. Like serine, glycine may be able to improve negative symptoms of PD via allosteric modulation of NMDA receptors.[56]

1.3.5 Nourishment to nasal mucosa cells

While not technically part of the CNS, the nasal mucosa, like hepatocytes, provide an essential role in protection and nutrient provision for olfactory neurons. Glutathione S-transferase concentrations are highest in the nasal epithelium.[57]

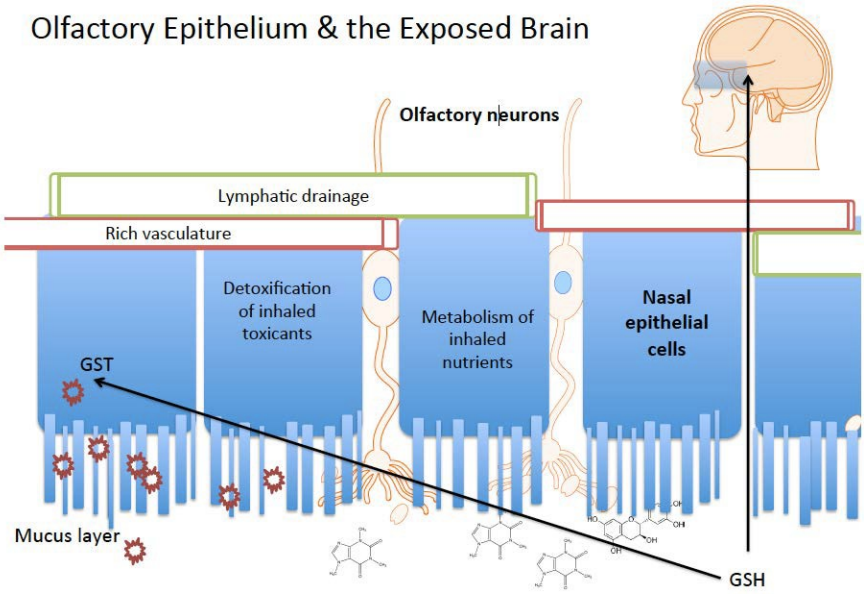


Figure 7. Intranasal GSH may support nasal epithelium GST, olfactory neurons, and may reach CNS target tissue via intracellular space, or the rich nasal vasculature and lymphatic system. Epithelial cells have one of the highest concentrations of GSH in the body.

1.4. Inborn errors of glutathione metabolism

Although rare, hereditary defects have been described in several enzymes in the gamma-glutamyl cycle. All are inherited in an autosomal recessive manner and range in severity, with most patients retaining some enzymatic activity. Because erythrocytes lack gamma-glutamyl transpeptidase and 5-oxoprlinase, leukocytes or fibroblasts are required for accurate analysis.[58]

GSH synthetase deficiency be the most frequently recognized genetic defect in GSH metabolism, occurring in less than one hundred people worldwide.[58] The second enzyme in the synthesis of GSH, it is responsible for adding glycine to gamma-glutamylcysteine. Clinically, GSH synthetase deficiency presents with hemolytic anemia, metabolic acidosis, recurrent bacterial infections and progressive CNS dysfunction.[59, 60] Individuals with a heterozygous mutation have an approximate 45% deficiency in GSH. 5-oxyoproline accumulates due to the GSH synthetase deficiency, causing 5-oxoprolineuria in most patients. [61] A study of 28 individuals demonstrated that fibroblast GSH synthetase activity was not associated with presence of neurological symptoms, which occurred in approximately half of individuals. Supplementation with vitamin C and vitamin E beginning in early in life was associated with survival; regardless of disease severity, fortification for all patients with GSH synthetase deficiency is recommended preventative measures.[60]

Gamma-glutamylcysteine synthetase deficiency incapacitates the synthesis of GSH at its rate-limiting step. Thus far, four different mutations have been identified in the heavy subunit in four distinct families, resulting in low levels of GSH, accumulation of 5-oxoproline, and mild hemolytic anemia.[58] The disease is characterized by hemolytic anemia, and approximately half of those affected have neurological symptoms including learning disability, delayed

psychomotor development, progressive sensory neuropathy, ataxia, hyperreflexia, dysarthria, and spinocerebellar degeneration.[58, 59]

Gamma-glutamyl tranpeptidase deficiency is extremely rare and is associated with increased concentration of GSH in blood and urine. Nucleated cells, such as leukocytes or cultured skin fibroblasts, express reduced activity of gamma-glutamyl tranpeptidase. While cellular levels of GSH are normal, both plasma and urinary GSH are elevated, with urine concentrations up to 10 times higher than controls.[58] In a knock-out mouse model of gamma-glutamyl transpeptidase leads to glutathionionuria, glutathionemia, cataracts, lethargy, growth failure, reduced life span, and infertile. In the model, two weeks of NAC administration to the rodents restored fertility.[62]

5-oxoprolinase deficiency is an extremely rare and heterogeneous disease characterized by 5-oxoprolinuria and low activity of 5-oxoprolinase in nucleated cells. The disease can manifest as mental retardation, kidney stone formation, enterocolitis, microcytic anemia, microcephaly, or neonatal hypoglycemia.[58]

Conclusions

The body of research presented here demonstrates blood GSH decreases as disease severity increases, that (in)GSH is able to augment CNS GSH concentrations, and that (in)GSH is a safe and tolerable therapeutic. Collectively, these data contribute to the hypothesis that GSH depletion is involved in PD pathogenesis and make a compelling case for an efficacy trial designed to evaluate whether (in)GSH results in long-term symptomatic improvement or disease modification in PD. The longest formal exposure to GSH in an intervention trial of patients with PD has been three months of (in)GSH.[27] There is an ongoing internet-based ecological study,

"Complimentary and Alternative Medicine Care in Parkinson's Disease" (CAM Care PD), tracking PD symptoms in individuals who do, and do not, use oral, IV, and (in)GSH.[38] Long-term randomized, placebo- controlled intervention studies will need to be conducted to evaluate whether exogenously administered GSH can slow PD progression or improve symptoms. Now that (in)GSH has been shown to increase CNS concentrations of GSH [63], future efforts in pharmaceutical science can work to increase uptake, prolong duration of augmentation, improve product stability and delivery.

There is no evidence that GSH is capable of regenerating dopaminergic neurons, although GSH has been shown to increase striatal dopamine transport [36] and cysteine has been shown to play a role in dopamine receptor to ligand binding and sensitivity to radical stress.[37] Thus, it is possible that GSH indirectly facilitates the function of the existing dopaminergic pool, rather than contributing to it. Beyond modulation of dopamine, GSH has the capacity to reduce radical stress, facilitate cellular detoxification, regulate protein synthesis, and serve as a reservoir for biologically active amino acids. It is yet to be determined how this biological activity translates to clinical consequence, although early intervention trials are promising.

Collectively, this dissertation support the hypothesis that GSH deficiency occurs early in the course of PD, that cellular GSH depletion exacerbates the degenerative process, and that CNS augmentation is safe and feasible. As the consequences of GSH depletion are elucidated, it will become easier to identify outcome measures sensitive to GSH depletion and repletion in clinical trials. Based on its physiological role, non-motor symptoms and reduced rate of progression would be expected to benefit more from GSH augmentation than the motor symptoms which

result from dopaminergic cell death, and thus should be considered as primary outcome measures in future efficacy trials.

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Conditionally Essential Nutrients: The State of the Science

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"The functioning of the brain is affected by the molecular concentrations of many substances that are normally present in the brain. The optimum concentrations of these substances for a person may differ greatly from the concentrations provided by his normal diet and genetic machinery. Biochemical and genetic arguments support the idea that orthomolecular therapy, the provision for the individual person of the optimum concentrations of important normal constituents of the brain, may be the preferred treatment for many mentally ill patients. Mental symptoms of avitaminosis sometimes are observed long before any physical symptoms appear. It is likely that the brain is more sensitive to changes in concentration of vital substances than are other organs and tissues "[1].

Humans are parasites of the planet. In order to survive, there are minerals and molecules, ultraviolet waves, and other organisms on which we rely. We need some of Earth's resources to function optimally and in some cases, we need them to function at all. These nutrients on which we rely are considered essential nutrients if they are used by most humans most of the time. The mid-twentieth century was a fertile time for nutrition research, during which time a lot of feeding studies were taking place on what are now known as essential nutrients.

Today, the US Department of Agriculture (USDA) Food and Nutrition Information Center (FNIC) maintains an information database of Dietary Reference Intakes (DRI) for vitamins, minerals, and macronutrients developed by the Institute of Medicine (IOM) of the National Academy of Science (NAS). The DRI levels have largely replaced the Recommended Daily Intake or Reference Daily Intake (RDI) system still used for product labeling. The RDI is the intake considered to be sufficient to meet the needs of 97.5% (2 standard deviations below the mean) of the *healthy* population. No set of recommendations has been developed to meet the needs of the unhealthy population. Whether values are set and how they are set has tremendous implications for product labeling, allocation of public health dollars, reimbursement by health insurance companies, etc.

A nutrient is considered essential if it "serves an indispensable physiologic function, but cannot be synthesized endogenously at an adequate rate by healthy subjects."[2] *Conditionally essential nutrients* are those that can usually be synthesized in adequate amounts endogenously, but may require exogenous supplementation during some circumstances. In some cases, these increased requirements can be a result of impaired absorption (e.g. additional fat-soluble vitamins in steattorhea), increased anabolic requirements (e.g. pregnancy, and lactation), increased metabolic demand (e.g. protein in burn, trauma).

The determination of dietary essentiality had traditionally been established through classic feeding studies using purified diets with, or without, the nutrient being studied. Over time, if a nutrient is essential, a deficiency syndrome will emerge as signs and symptoms of impaired growth, function, biochemical alterations, or symptoms of illness become apparent. The quantification of the minimum required dose to prevent deficiency symptoms is determined by incremental re-feeding until the dose resulting in syndrome resolution is reached [2].

Chipponi et al. describe the stages of the deficiency syndrome that results from deprivation studies:

Stage 1 Deficiency: Physiologic function continues normally while stores are being depleted. Adipose tissue, bone, muscle, and circulating storage forms (e.g. ferritin) act to maintain serum concentrations.

Stage 2 Deficiency: The depletion of body stores results in biochemical alterations, although clinical symptoms are not yet apparent. (e.g. C-reactive protein, hemoglobin A1c, homocysteine, altered enzyme activity)

Stage 3 Deficiency: In addition to biological perturbations, clinical symptoms become apparent. (e.g. bleeding gums and easy bruising in scurvy, dementia and dermatitis in pellagra)

Most of the work done to date on nutritional essentiality was conducted at a time when laboratory methodology was more rudimentary and notably less was known about disease pathophysiology. The early work surrounding conditional essentiality was done following the introduction of central ve-

nous access for nutrient delivery in 1969,[2, 3] when uncommon nutritional deficiencies became readily apparent in those patients receiving early TPN formulas. The insufficiencies of the nutrient formula became quickly apparent, and potassium, phosphate, and essential fatty acids were soon added. Long term users were found to need additional zinc, copper, selenium, chromium, etc.[2] and recently rubidium was shown to be a conditionally essential nutrient in dialysis patients, its stage 3 deficiency syndrome manifesting as depression [4].

In the presence of polymorphisms, or under certain physiological (e.g. pregnancy, lactation, aging), or pathological conditions, humans have been shown to have unique nutritional requirements. When disease (e.g. autoimmunity to pancreatic beta cells) or circumstance (e.g. burn victims) results in a metabolic circumstance where the needs of the body cannot be endogenously supplied and the amount recommended during a state of health is insufficient, the substance becomes conditionally essential. This could be an increase in dose for an already recognized essential nutrient (e.g. thiamin in Wernicke's encephalopathy is dosed higher than the RDI), or a condition may render an accessory nutrient essential (e.g. coenzyme Q10 in congestive heart failure.)

The principle of biochemical individuality states that the optimal dose of any nutrient will normally vary between individuals. This is well supported by the science and considered in statistical considerations of biomarkers. The feeding studies that have been done have, in the nutrients studied, demonstrated what occurs in healthy, young individuals who are depleted of a single nutrient.

Carnitine, taurine, arginine, cysteine, glycine, choline, are all generally recognized conditionally essential nutrients. Carnitine, for example, is an FDA-approved prescription drug for carnitine-deficiency syndromes. It is also available as an over-the-counter supplement intended to support athletic performance and weight loss, by facilitating the conversion of fat to fuel. An excellent review on glycine was recently published by Want et al., in which they describe the structural (glutathione, heme, nucleic acid, uric acid synthesis) and functional (immune and metabolic regulation) roles of glycine. The authors provide support for the idea that there are inflammatory disorders (obesity, diabetes, cardiovascular disease, cancer) that require more glycine than the body is capable of synthesizing [5].

Whether 'conditionally essential' should refer only to the nutritional impact caused by a condition is debatable. For instance, the MTHFR mutation is a common SNP that has been associated with depression, miscarriage, and possibly cardiovascular disease and dementia. The SNP can be circumvented with use of the active form of folic acid, 5-MTHF. For those MTHFR homozygotes, the 5-MTHF form should be considered the required form of the nutrient. "MTHFR homozygote" is not a medical condition, although it may increase risk of disease. As discussions about conditional essentiality evolve, efforts should be made focus on prevention. E.g. "Smokers" are not a disease and yet they have unique vitamin C requirements due to the increase in oxidative damage. Similarly, "MTHFR homozygotes" should have unique folic acid recommendations.

It is important to state that conditional essentiality is distinct from the question of causality. Individuals with steatorrhea require additional vitamin A, D, E, and K, but these vitamins are not the cause of their steatorrhea. Similarly, whether a nutrient deficiency predisposes to disease, or whether a disease state induces a deficiency, is irrelevant. Regardless of cause or consequence, the question best serving public health is,

"Would the patient's health be improved if ___ were exogenously supplied?"

This question is not always so easy to answer. Vitamin B12, may cause a deficiency with a striking similarity to MS. Vitamin B12 levels have been shown to be lower in MS and vitamin B12 plays a role in immune system recognition. Since the process of remyelination is upregulated with MS activity, it stands to reason that the nutritional requirements for synthesizing myelin may also have increased requirements. A study has demonstrated that individuals with MS and B12 deficiency are more likely to have diminished neurological capacity [6]. Several trials have attempted B12 supplementation in patients with MS, but no consistent improvement has been demonstrated. This may because the outcome measure with which we are attempting to document benefit is inadequate, or that low B12 itself does not contribute to disease, but low levels may be an indicator for something else. For now, B12 deficiency remains a diagnosis of exclusion in the evaluation of demyelinating disease and B12 deficiency is more common in MS than controls, but additional supplementation does not appear to significantly, or consistently, impact disease outcomes [7].

While an increased nutritional demand in some conditions is biologically plausible, and some diseases are associated with deficiency, until fortification improves a clinically relevant outcome measure, it should not be referred to as a conditionally essential nutrient.

As a solution, one option for nutritional augmentation research efforts is to focus on a particular subset of symptoms within a disease. For instance, approximately 80% of people with PD report constipation. Cassani et al. demonstrated a probiotic supplement improved symptoms of bloat, pain, and improved stool consistency in patients with PD [8]. They did not include PD status or progression as an outcome measure for a probiotic intervention, but rather focused on gastro-intestinal health as an outcome measure.

Clinical epidemiology is evolving and we are beginning to ask the questions differently. Peterson, et al. recently compared vitamin D levels of individuals with PD to determine whether low levels were associated with increased risk of cognitive decline. After correcting for multiple comparisons, they found higher vitamin D levels to be associated with improved outcomes measures of mood, concentration, and verbal memory [9]. The `question has yet to be answered whether vitamin D supplementation can improve mood, concentration, and memory. The importance of preventing vitamin D deficiency in PD patients is only beginning to become clinically relevant, and whether higher-than-normal doses are required, or if in-

dividuals with poor mood and cognitive difficulty should be treated differently.

Another suggested research methodology is to concentrate efforts on the subpopulation of the group who have biological evidence of deficiency. There is a tremendous amount of cell line and animal research suggesting coenzyme Q10 improves mitochondrial function in PD. A pilot trial found a ~40% reduction in rate of progression in those on 1200 mg/day [10]. Beal et al. conducted a multi-center Phase III efficacy trial of 1200mg, 2400mg, or placebo which was stopped early based on calculations that it could not possibly meet clinical endpoints for efficacy. During this same time, data were published showing a statistically significant increase in frequency of deficiency in PD patients over controls. Using the SpectraCell FIA measure, ~35% of individuals with PD were shown to be deficient [11]. Perhaps a better way to ask this question is, "among individuals with PD who exhibit biochemical or clinical signs of Q10 deficiency, does supplementation result is a measurable benefit to biochemical or clinical sign or symptoms? Rather than ignoring the tremendous heterogeneity of these diseases, and trying to overpower them with large sample sizes, we should direct our research efforts toward identifying those most likely to benefit from supplementation.

Table 1: Examples of Nutrients Gaining Acceptance as Conditionally Essential in Certain Disease States

CONDITION	NUTRIENT	Phase 1 Defic	Phase 2 Defic	Phase 3 Defic
Parkinson's	Q10	Synthesized by HMG-CoA reductase (commonly inhibited by statins)	SpectaCell FIA 4-fold risk of Q10 defic [11].	TBD: Myalgia? Fatigue? Weakness? Cardiomyopathy?
Parkinson's	Glutathione	Synthesized on demand, not stored.	40% depletion of nigral GSH at diagnosis; GSH defic leads to inflammation, ROS, mitochondrial dysfunction	GSH depletion associated with aging, GSH progression
Parkinson's See also: *Epilepsy* *Mental illness* *Tics* *Addiction* *ADHD* *Migraine* *Alzheimer's* *MS, ALS, HD*	Lithium	Not stored. Typically obtained	Ecological studies from 1970s show municipal supply assoic with body level. Depletion in rainy regions, highest in desert. *To-do: Repeat in WA	Low Li associated with psychosis, depression, aggressive behavior, and suicide. Calls in the literature for more research and Li the water supply of depleted regions have been ignored. *To-do: Study Li repletion among those deficient. Reverse feeding study.
Parkinson's See also: *IBS, IBD* *Autoimmune disease* *Atopia*	Probiotics		Following probiotic supplementation, improvement in normal stools, bloat, and pain in constipated PD patients [8].	Constipation affects 80% of PD pts. Individuals who have a bowel mov. every other day are 4x as likely to develop PD as those who have 2+ bowel mov./ d. [Abbott 2001]
Parkinosn's See also: *Alzheimer's* *Epilepsy* *MS*	Vitamin D		Levels lower in PD than healthy elderly or AD [11, 7].	PD associated with osteoporosis, balance, weakness, depression- all also assoc with D. Higher vit D assoc with better mood and cognitive function in PD [9].
Metabolic syndrome	Glycine		[5]	
Coronary Heart Disease	DHA + EPA		Immune dysfunction [Calder 2010]	Cognitive decline [Calder 2010]
	Flavonoids		Inflammation	CVD, obesity, neurodegeneration, neuronal hyperexcitability: tics, seizures, ADD, etc.

In complicated, chronic, multi-system diseases like metabolic syndrome, cancer, and neurodenegeration, one cannot expect that replacing one nutrient will shift primary disease outcome measures like BMI or dementia. Physiologically speaking, if an individual had a deficiency of several nutrients,

would benefit be expected if one nutrient were replaced but not the others? Combination protocols have been very effective in HIV+ research and *H.pylori* eradication, but are rarely implemented in chronic disease research related to quality control and regulatory oversight typically being too cumbersome to work within a funding cycle [12].

In table 1, examples are given of conditions and nutrients that meet the criteria for conditional essentiality using the criteria outlines in Chipponi et al., although this concept has not been translated clinically or in federal guidelines.

There is a tremendous disconnect between metabolomics, clinical epidemiology, and public health practices for nutrient provision. Inborn errors of metabolism provide an interesting framework from which to evaluate conditional essentiality. For instance, all newborn babies are screened at birth for inborn errors of metabolism. In the case of PKU, an individual must avoid the amino acid phenylalanine in order to prevent mental retardation. Other inborn errors of metabolism require additional arginine supplementation, to overcome defects of the urea cycle. In these cases, the nutritional products are regulated by the Food and Drug Administration as foods and dietary supplements and often referred to as 'medical foods.' Insurance coverage for these medical foods is reasonably good through childhood, but inconsistently described or regulated in adults [15]. It is as though we, as a culture and a health care system, understand that metabolic differences between individuals can interfere with growth and development in infancy and childhood, but have not come to fully appreciate the degree to which unique metabolic consideration must also be given to adults. In fact, several insurance companies stop paying for specialized amino acid formulas when the individual turns 18.

Physiologists do not contend that dysfunction ensues when levels of lithium, vanadium, and flavonoids are inadequate, and yet DRIs have not been set. Are they essential? What are symptoms of deficiency? What doses are required to not experience the symptoms of deficiency? Who is responsible for educating individuals and healthcare providers? I am hopeful our evolving understanding of human metabolism, nutrigenetics, nutrigenomics, and biochemical individuality as well as improved research methodologies will lead to a revolution in nutritional medicine in the years to come.

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Title page

Glutathione as a Biomarker in Parkinson's Disease: Associations with Aging and Disease Severity

Running Header: GSH Biomarker in PD

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LK Mischley created the PRO-PD outcome measure (freely available).
None of the authors have any other conflicts to disclose.

Glutathione as a Biomarker in Parkinson's Disease: Associations with Aging and Disease Severity

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Abstract

Objectives: Oxidative stress contributes to Parkinson's disease (PD) pathophysiology and progression. The objective was to describe central and peripheral metabolites of redox metabolism and to describe correlations between glutathione status, age, and disease severity.

Methods: 58 otherwise healthy individuals with PD were examined during a single study visit. Descriptive statistics and scatterplots were used to evaluate normality and distribution of this cross-sectional sample. Blood tests and magnetic resonance spectroscopy (MRS) were used to collect biologic data. Spearman's rank-order correlation coefficients were used to evaluate the strength and direction of the association. The Unified PD Rating Scale (UPDRS) and the Patient Reported Outcomes in PD (PRO-PD) were used to rate disease severity using regression analysis.

Results: Blood measures of glutathione decreased with age, although there was no age-related decline in MRS glutathione The lower the blood glutathione concentration, the more severe the UPDRS (P=0.02, 95% CI: -13.96, -1.14) and the PRO-PD (P=0.01, 95% CI: -0.83, -0.11) scores.

Discussion: These data suggest whole blood glutathione may have utility as a biomarker in PD. Future studies should evaluate whether it is a modifiable risk factor for PD progression and whether glutathione fortification improves PD outcomes.

Keywords: Parkinson's disease; parkinsonism; glutathione; deficiency; nutrient; biomarker; nutrition

Introduction

It is well established that redox stress contributes to PD pathophysiology and progression.[1, 2] Comprised of reduced (GSH) and oxidized (GSSG) forms, total glutathione (Glu) is essential for maintaining redox homeostasis, clearing metabolic waste, and serving as a reservoir for amino acids in the central nervous system (CNS). GSH deficiency was first hypothesized to play a causative role in PD in 1982.[3] *Post mortem* analysis of nigral tissue of individuals with PD exhibits deficiency of the antioxidant GSH compared to controls early in the course of disease.[4, 5] GSH deficiency is thought to incapacitate the cell's ability to metabolize cellular waste and impair defense against reactive oxygen species (ROS), reactive nitrogen species (RNS), and H_2O_2.

GSH deficiency has long been implicated in PD degeneration,[6, 7] although little is known about human GSH or Glu concentrations in living humans with PD. It is not known whether peripheral measures of Glu status correlate with CNS Glu status. Recently, magnetic resonance spectroscopy (MRS) technology has evolved to permit the non-invasive estimation of regional CNS glutathione concentrations[8], although ranges have not been described in a PD population. 'Antioxidant Status' blood and urine panels are readily available. The goal of this study was to describe central and peripheral measures of Glu status in individuals with PD and evaluate whether Glu status was associated with PD severity.

PD research is in desperate need of a biomarker that predicts disease progression and can be modified and improved outcomes.[9] Any redox measure suggestive of age-related decline and/ or associated with disease severity may have potential to serve an unmet need in PD research and practice.

Materials and Methods

This is a combined dataset from two separate studies on topics related to PD and redox status. Blood samples for both studies were drawn within a year of one another, were all collected in Seattle, WA, by the same study staff. The demographics and referral sources were similar between the groups. (Figure 1)

CNS Uptake Study of (in)GSH (n=15)

This study was approved by the University of Washington (UW) IRB approved and listed on ClinicalTrials.gov prior to enrolling study participants. All participants were over 18 years of age, spoke English, had at least three of the required positive criteria for PD from Step 3 of the UK Brain Bank Diagnostic Criteria for PD, and had Hoehn and Yahr scores between 2-3. Due to the circadian rhythm of Glu, all urine collected were first morning samples, all MRI scans were scheduled at 8 am, and all blood collection occurred immediately following the scan, at approximately 9:45 am. All subjects were fasting at time of data collection.

Phase IIb Study of (in)GSH (n=45)

This study was approved by the Bastyr University and UW IRBs and registered on Clinicaltrials.gov before the first participant was enrolled. As this was a longitudinal study, for this analysis only baseline measures were used. All participants had Hoehn and Yahr scores ranging from 1-3 and a diagnosis of PD made by a clinical neurologist in the previous 10 years. Due to the circadian rhythm of GSH, all urine samples were first morning. Blood draws were performed when the participant was in a non-fasting state. Participants were asked

to maintain their regular diet but avoid large meals. There were two participants who enrolled in both studies, they were dropped from the second dataset.

For both studies, participants were invited to participate whether or not they were on dopaminergic medications. Both studies excluded individuals with a history of epilepsy, stroke, brain surgery, or structural brain disease, the presence of other serious illnesses, pregnant or at risk of becoming pregnant, a history of sulfur sensitivity, e.g. prior reaction to N-acteylcysteine (N-AC), methylsulfonylmethane (MSM), S-adenosyl methionine (SAMe), a recent history of asthma, supplementation with any form of glutathione or glutathione precursor (i.e. NAC) for six months prior to baseline study visit, current drug or alcohol use or dependence, or diagnosis of mental illness.

Laboratory Methodology

Total whole blood glutathione, enzyme activity of superoxide dismutase, and glutathione peroxidase were measured spectrophotometrically from RBC lysate using the Abbott Diagnostics Architect System (ADAS). Lipid peroxides were measured in serum and urine following hydrolysis and reaction with thiobarbutyric acid. Malondialdehyde was used as the standard for the determination of concentrations of lipid peroxidation products, as previously described.[10]

Serum cysteine and cystine were measured using an adaption of the Gaitonde procedure[11] developed for the detection of amino acids which uses the colorimetric reaction of the amino acids with ninhydrin. Serum sulfate concentrations were determined using a turbidimetric assay which utilizes the chemical properties of sulfate ions to cause the formation of

precipitate that can be measured by the absorbance of light using the ADAS. All tests above were performed by Genova Diagnostics (CLIA: #34D0655571, Asheville, NC, USA) GSH:GSSG ratios were measured at the University of Washington Department of Environmental and Occupational Health Sciences using previously described methods.[10]

MRS Glutathione

A 3 Tesla Philips Achieva magnetic resonance imaging (MRI) scanner (Best, The Netherlands) was used at the University of Washington Integrated Brain Imaging Center to obtain spectra with a 32-channel SENSE phased-array head coil. A cubic volume of interest (VOI), 4 x. 4 x 5 cm, was centered on the left dorsal putamen at the level of the lentiform nucleus. This VOI was selected because of its relatively homogenous composition of neurons and astrocytes and suitable distance from bone and fluid, which could compromise signal quality. All scans were scheduled for 8 am.

Statistical Analyses

Scatterplots were evaluated to observe trends and normality of distributions. Spearman correlation coefficients and regression analyses were determined using Stata (StataCorp, Stata/IC 13.1 for Mac). It is possible that the influence of increasing age on the risk of neurodegenerative disease is mediated in part by diminishing levels of GSH with increasing ago. Therefore, analyses were performed with and without adjustment for age.

Results and Discussion

Two individuals enrolled in both studies, data obtained on that subject in the second study were discarded, reducing the total to N=58. MRS data was discarded due to participant motion in scanner (n=1) or glutathione:creatine concentration in the brain was below the level of detection (n=8).

Most participants resided in the Pacific Northwest, although a few traveled 5+ hours to participate. All but one participant identified as Caucasian. Demographics and baseline disease severity are presented in Table 1.

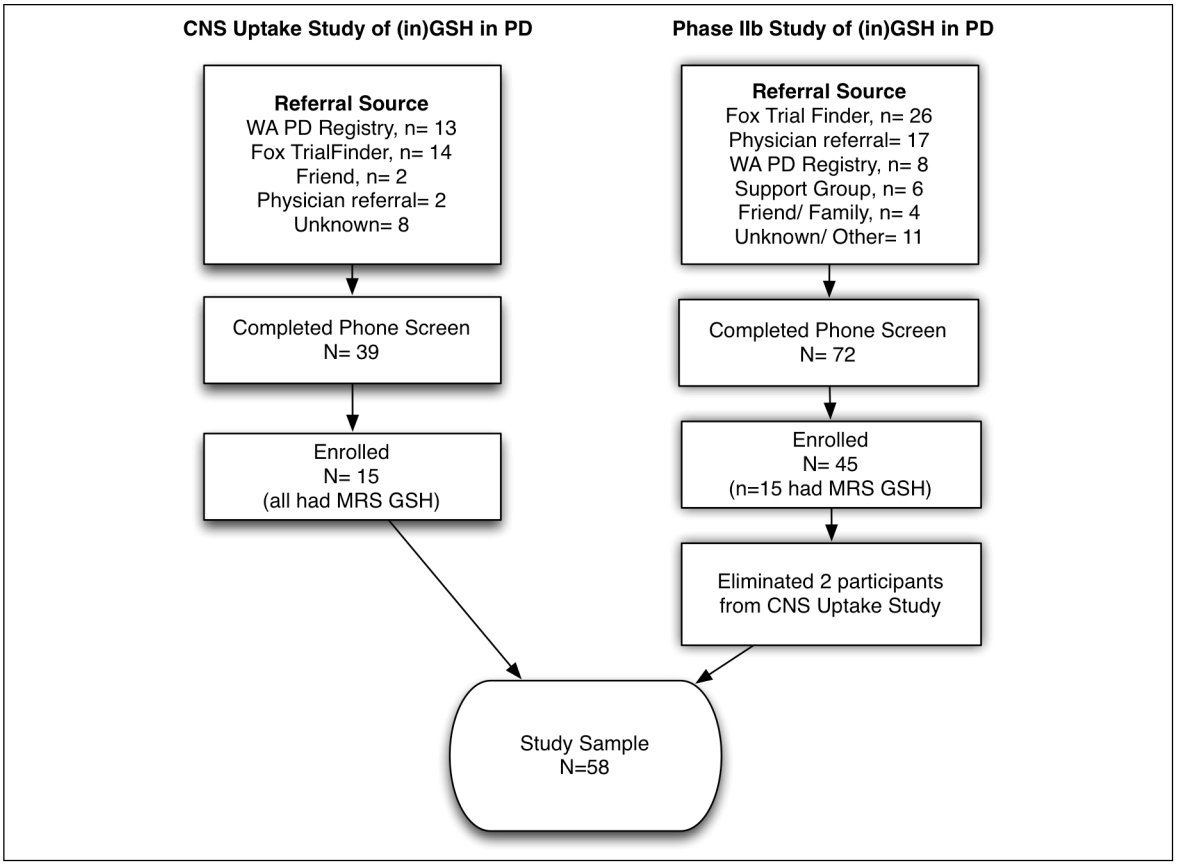

Figure 1: Enrollment Algorithm describing where participants from both studies were recruited and how the databases were merged for this study.

Table 1: Participant Demographics

	CNS Uptake n=15 Mean (SD)	Phase 2b n=43 Mean (SD)	Combined N=58 Mean (SD)
Gender			
Male	11 (73%)	22 (51%)	33 (57%)
Female	4 (27%)	21 (49%)	25 (43%)
Age, years Mean, SD	65.5 (11.2)	62.2 (10.8)	62.2 (10.8)
Disease Severity			
Years since PD diagnosis	6.15 (6.24)	3.54 (2.2)	4.26 (3.9)
Unified PD Rating Scale (UPDRS)	78.9 (15.4)	67.8 (9.5)	70.7 (12.2)
Patient-Reported Outcomes	869 (334)	804 (422)	821 (399)
Hoehn & Yahr			
1		9 (20.9%)	9 (15.5%)
1.5		6 (14.0%)	6 (10.3%)
2	6 (40%)	17 (39.5%)	23 (39.7%)
2.5	4 (27%)	7 (16.3%)	11 (19.0%)
3	5 (33%)	4 (9.3%)	9 (15.5%)

To evaluate whether any of the GSH measures may reflect expected age-related physiological decline, scatterplots were drawn to evaluate GSH measures in a cross-sectional fashion. Whole blood Glu decreased with age (r=-0.2218), although MRS glutathione:creatine and percent GSSG did not (r=-0.01 and r=0.1263, respectively). Based on this sample, there is an estimated six point decrease in micromol/L total GSH for each year of age (p=0.104, 95% CI: -13.34, 1.27). (Figure 2)

Measurements of brain, blood, and urine redox measurements from individuals with PD are described. Data on % GSSG from the Phase 2b were discarded due to high variability between results run in triplicate. (Table 2)

The UPDRS and PRO-PD were used to evaluate disease severity. In both scales higher scores represent more severe disease on both outcome measures used. There was a statistically significant correlation between whole blood total glutathione levels and UPDRS score (P=0.022, 95% CI: -13.96, -1.14). There was a statistically significant association between PRO-PD scores and whole blood total glutathione concentrations, with a 100 point increase in umol/L glutathione being associated with a 47 point decrease in PRO-PD score (P=0.01, 95% CI: -0.826, -0.119). (Figure 3)

Table 2. Measures of brain, blood, and urine redox status in individuals with Parkinson's disease.

`	**CNS Uptake n=15**	**Phase 2b Baseline n=43**	**Combined N=58**
Brain Glutathione Concentrations			
MRS glutathione:creatine ratio (putamen) (n=21)	0.026 (.018)	.030 (.017)	.028 (.017)*
Whole blood			
Total GSH (micromol/L) (n=55)	1027.64 (279.15)	1076.02 (311.85)	1063.71 (302.08)
RBC %GSSG	0.96%	--	--
Serum			
Sulfate (mg/dL) (n=56)	3.83 (.62)	3.60 (.70)	3.66 (.69)
Cysteine (mg/dL) (n=56)	.69 (.12)	.70 (.11)	.70 (.11)
Cystine (mg/dL) (n=56)	2.78 (.34)	2.66 (.43)	2.69 (.41)
Lipid Peroxides (micromol/ L)	6.95 (2.49)	6.52 (1.87)	6.63 (2.03)
Total Antioxidant Capacity(mmol/L)	0.78 (0.07)	0.75 (0.084)	0.76 (0.08)
Urine			
Lipid Peroxides (micromol/g cre)	8.43 (1.92)	8.14 (2.34)	8.22 (2.22)
8-OHdG (microg/g creatinine) (n=55)	9.86 (1.99)	9.92 (9.99)	9.91 (8.60)
Enzyme Activity, Whole blood			
Glutathione Peroxidase (GPX) (U/g Hb)	34.64 (7.94)	31.55 (6.80)	32.34 (7.16)
Superoxide Dismutase (U/g Hb)	11189 (4463)	14622 (4189)	13748 (4481)

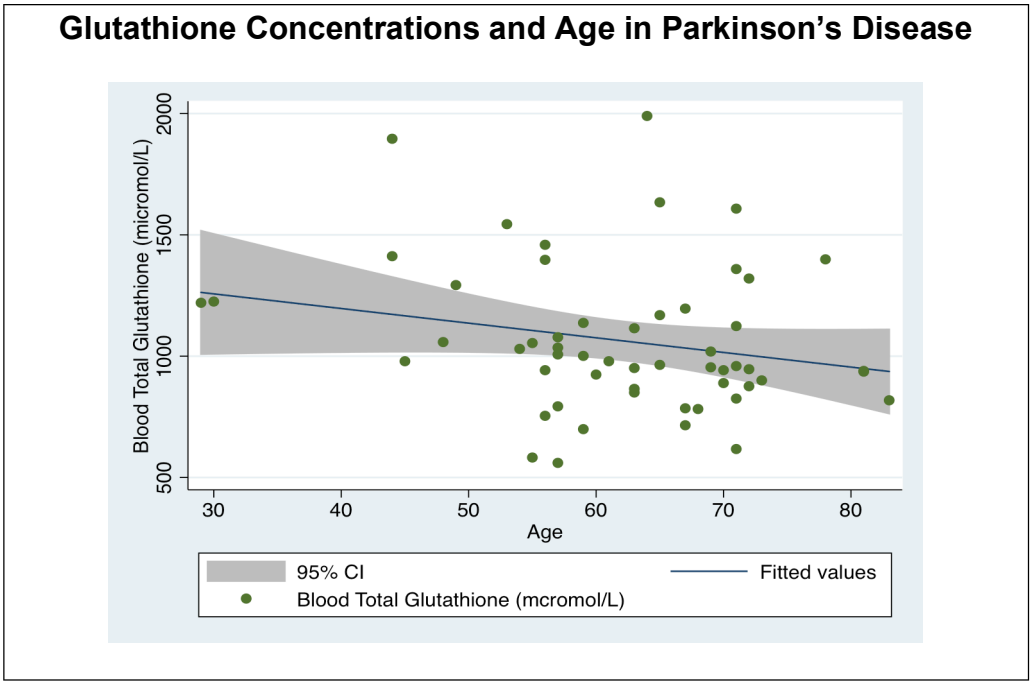

Figure 2: Cross-sectional analysis of whole blood total GSH concentrations

The approximate midpoint for blood glutathione was 1000 umol/L; an analysis was performed to evaluate whether individuals with glutathione concentrations over 1000 umol/L had better PD outcome scores than those with glutathione concentrations < 1000. (Table 3)

Table 3: High vs. Low Blood Glutathione Concentrations in PD. In order to evaluate whether blood glutathione may be associated with rate of progression, regression analysis adjusted for age and years since PD diagnosis.

High vs. Low Blood Glutathione in Parkinson's Disease Severity			
	GSH <1000 umol/L n=28	**GSH ≥1000 umol/L** n=31	**P (95% CI)**
Unified PD Rating Scale (UPDRS)	75.82 (12.19)	65.9 (10.33)	0.087 (-11.90, 0.83)
Patient-Reported Outcomes in PD (PRO-PD)	1013.46 (365.37)	675.53 (381.57)	0.012 (-525.28, -67.49)

Conclusions

Of the panel of redox measurements evaluated here, whole blood Glu was the most sensitive to aging and the only measure that was associated with statistically significant severity of PD, as measured by UPDRS and the PRO-PD. The correlation between Glu concentrations and PRO-PD was stronger than that with the UPDRS, most likely because PRO-PD is more inclusive of non-motor symptoms, does not fluctuate throughout the course of the day, and the slider bar design permits greater sensitivity to change.

The absence of well-established reference ranges, modest sample size (n=58), and lack of control data are all limitations of this study. The strength of consistency between studies bodes well for the reliability of these clinically available biomarkers.

The lack of decline in MRS GSH with age or disease severity may have several explanations. First, eight of 30 participants had brain concentrations below the limit of detection of CNS GSH and were thus deleted from the dataset, which artificially drove up the mean. This could be statistically managed with appropriate *a priori* hypotheses in future studies (e.g. assign mid-point score between zero and lowest detectable).

Although MRS is capable of detecting increases in CNS GSH following (in)GSH administration, these data suggest MRS putamen concentrations cannot be extrapolated to reflect body status. In support, MRS GSH did not correlate with disease severity by either outcome measure; while MRS GSH may be useful for demonstrating target validation for augmentation efforts, MRS GSH does not appear to predict clinical status.

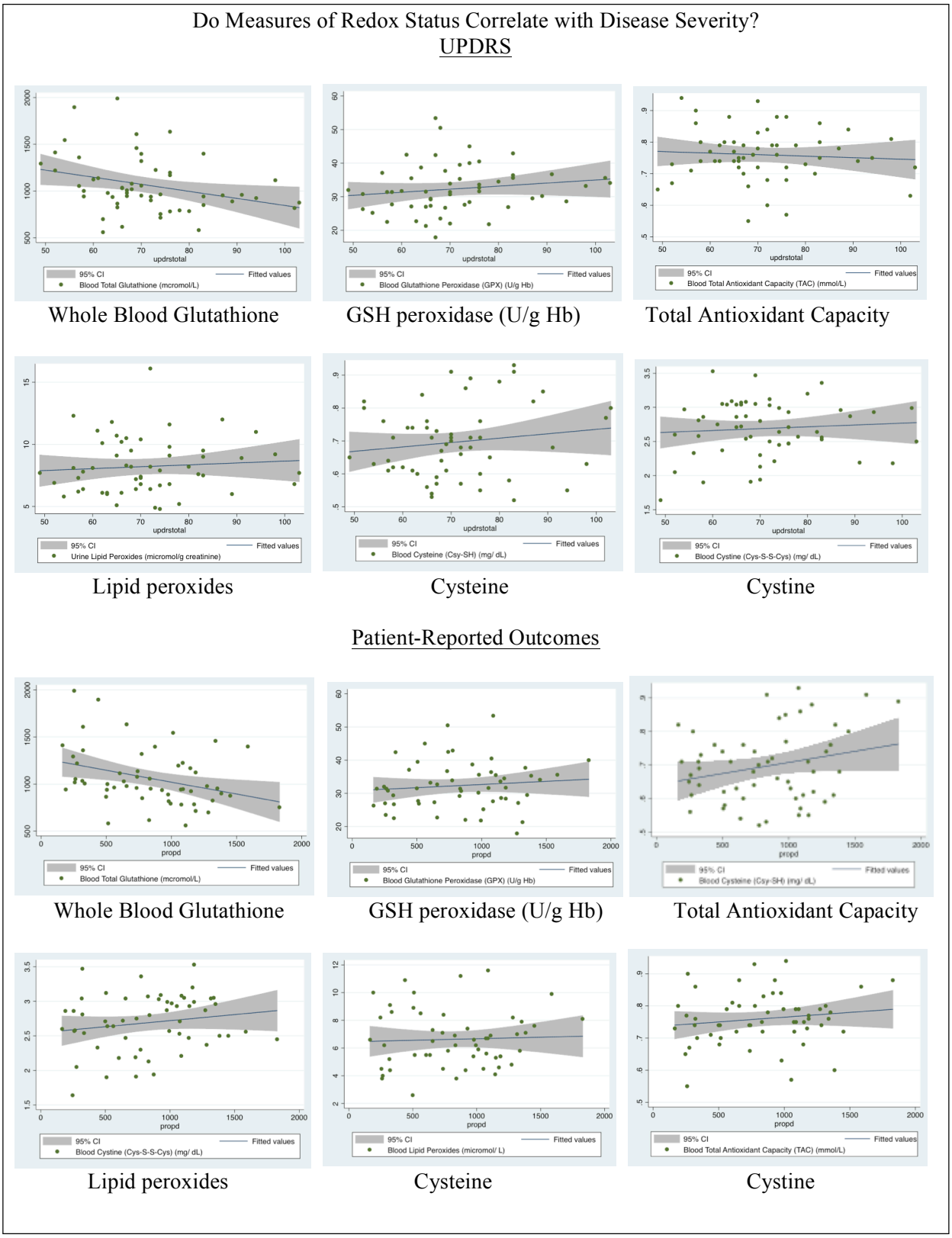

Figure 3: Scatterplot correlations between clinically available redox measurements and Parkinson's disease severity.

Of the assessments evaluated here, whole blood glutathione was the most sensitive to aging and the only measure that was statistically associated with better PD status, as measured by UPDRS and the PRO-PD, suggesting it may have utility as biomarker for PD progression. Whole blood GSH is relatively stable when appropriately stored; banked samples from prior PD studies and ongoing study repositories should consider measuring Glu. The degree to which GSH is a modifiable risk factor in PD progression should be evaluated prospectively in an appropriately controlled study, giving consideration to dietary glutathione intake.

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Original Article

Safety Survey of Intranasal Glutathione

Laurie K. Mischley, ND, Marco F. Vespignani, ND, and John S. Finnell, ND

Abstract

Purpose: Glutathione depletion has been documented in several disease states, and exogenous administration has been hypothesized to have therapeutic potential for some conditions. In an effort to reach target tissues of the sinuses and central nervous system (CNS), glutathione is being prescribed as an intranasal spray, although no literature exists to support this mode of administration. The objective of this study was to describe patient-reported outcomes in a population of individuals who have been prescribed intranasal reduced glutathione, (in)GSH.

Methods: A survey was designed to assess individuals' perception of tolerability, adverse events, and health benefits associated with (in)GSH use. Using a pharmacy database, 300 individuals were randomly selected to receive a survey; any individual who had received one or more prescriptions for (in)GSH between March 2009 and March 2011 was eligible for participation.

Results: Seventy (70) individuals returned the survey (23.3% response rate) from 20 different states. Reported indications for (in)GSH prescriptions were multiple chemical sensitivity (MCS) ($n=29$), allergies/sinusitis ($n=25$), Parkinson disease (PD) ($n=7$), Lyme disease ($n=3$), fatigue ($n=2$), and other ($n=10$). Of the respondents, 78.8% ($n=52$) reported an overall positive experience with (in)GSH, 12.1% ($n=8$) reported having experienced adverse effects, and 62.1% ($n=41$) reported having experienced health benefits attributable to (in)GSH use. Over 86% of respondents considered the nasal spray to be comfortable and easy to administer.

Conclusions: This is the first study to evaluate patient-reported outcomes among individuals across the country who have been prescribed (in)GSH. The majority of survey respondents considered (in)GSH to be effective and without significant adverse effects. (in)GSH should be further evaluated as a method of treating respiratory and CNS diseases where free-radical burden is a suspected contributor to disease progression.

Introduction

GLUTATHIONE IS AN ENDOGENOUSLY synthesized tripeptide consisting of cysteine, glutamate, and glycine. Glutathione is an essential antioxidant, and an imbalance of glutathione homeostasis has been implicated in the pathogenesis of many diseases of the respiratory, immune, and central nervous systems (CNS). While diminished glutathione levels have been described in numerous diseases,[1–4] little attention has been given to exogenous repletion as a therapeutic strategy. This unpatentable molecule is being compounded by pharmacists and used nationwide, and yet no published information has been available regarding safety or efficacy.

Glutathione plays an important role in the detoxification of reactive oxygen species (ROS) in the central nervous system, where it directly quenches radicals in nonenzymatic reactions and reduces peroxides generated by glutathione peroxidase. Antioxidant activity occurs in the cytosol, membranes, and within the mitochondria.[5] In addition, glutathione plays a role in detoxification, the transport of cysteine, cell proliferation, and the regulation of apoptosis.[6]

Intranasal administration of glutathione has been hypothesized to be advantageous over other methods of administration, when the target tissue is the brain or upper respiratory tract.[7] While the rich vasculature of the nasal mucosa might facilitate systemic absorption, intranasal administration provides direct contact with the mucous membranes of the nasal passages and sinuses, which may be advantageous in conditions of the upper respiratory tract. For diseases of the CNS, intranasal administration may be a unique means of bypassing the blood–brain barrier. Absorption into the CNS after intranasal administration has been postulated to occur via the olfactory and trigeminal neuronal pathways (intraneuronal) and diffusion across

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Table 1. Advantages and Limitations of Intranasal Administration of Medications

Advantages	*Limitations*
No modification of therapy is required	Delivery is expected to decrease with increasing molecular size
Reduces systemic exposure	Mucosal irritation or damage may occur
Noninvasive, easy to administer	Nasal congestion may interfere with delivery
Drug degradation and metabolism are minimized	Unknown delivery to various brain tissues
May bypass the blood–brain barrier	Limited data on central nervous system absorption, utilization, and metabolism

mucosal barrier and olfactory plate (extraneuronal), depending on the size, polarity, and odorant nature. Intraneuronal absorption requires axonal transport and thus occurs over hours to days to reach target tissue throughout the brain. Diffusion across the olfactory plate results in immediate delivery and has been shown to occur for small, water-soluble particles less than 1000 daltons (Da) in size.[8] Glutathione is a small, odorant, polar molecule of only 307.33 Da, suggesting that the molecule may be well absorbed without enhancers, but pharmacokinetic studies of intranasally administered glutathione have not been conducted in either healthy or diseased populations (Table 1).

The history of intranasal reduced glutathione [(in)GSH] dates back to 2003, when environmental medicine practitioners began using it to treat MCS. In 2004, one of us (LKM) began using it for PD, Huntington disease, Down syndrome, and autism. Over 2000 individuals have received prescriptions for intranasal glutathione through Key Pharmacy, a compounding pharmacy in Kent, WA, since 2003.[9] Because it is a sterile product, it is regulated in all states by the respective state board of pharmacy according to USP sterile guidelines 797. Only one peer-reviewed publication exists in the literature that mentions (in)GSH, and it is not indexed on PubMed.[7]

Surveys are an inexpensive and efficient instrument for collecting patient-reported outcomes. In response to increasing prescribing and a paucity of data, the authors decided to survey patients who have used (in)GSH about their experiences.

Methods

Key Pharmacy, a pharmacy with 14 years of experience compounding glutathione products, offered to make their database available for this study. This particular pharmacy was chosen because they compound the glutathione using a stabilization formula that results in >97.4% stability of reduced glutathione after 30 days, and 94% stability at 60 days.[10] Institutional Review Board approval was obtained from Bastyr University to send surveys to 300 randomly selected individuals from the Key Pharmacy database. Thousands of prescriptions have been written by hundreds of practitioners[11] across the country. In an effort to maximize the number of patients who returned the survey, only individuals who had received a prescription between March 31, 2009 and March 31, 2011, the 24 months prior to mailing (n=558), were eligible. Pharmacy staff used their database to identify all individuals who received a prescription for (in)GSH, which was exported into Microsoft Excel. The list was sorted by last name, and then a blank column A was added, in which the formula "=RAND()" was added to every cell. This function allocates a random rational number between 0 and 1 in each cell where the function is used. The entire database was then sorted by column A (the random number) in ascending order, and the top 300 patients on the list were selected as our sample group. Key Pharmacy conducted the mailing to protect individuals' privacy, and anonymous surveys were returned to the principal investigator at Bastyr University. Key Pharmacy did not play a role in data collection, analysis, or manuscript preparation.

Data were compiled 2 months after the surveys were mailed. Patients were asked when they began using (in)GSH. When an exact date was provided, that start date was used. So as not to over- or underestimate treatment duration, the midpoint of the date provided was entered (e.g., when only a month and year were provided, the 15th of that month was entered; when only a year was provided, July 15 was entered).

Results

Of the 70 respondents, 66 (94.3%) reported having filled a prescription for (in)GSH. Of those 66 respondents, 51 (77.3%) were female. The age of (in)GSH-treated respondents ranged from 20 to 78 years, with a mean age of 56.8 years (standard deviation [SD], 12.9). The mean age of women treated with (in)GSH was 56.2 (SD 13.1), compared with 58.6 (SD 12.5) for men. Of the 66 respondents, 36 (54.6%) resided in Washington State; the remaining respondents resided in 19 other states. The duration of prescription use ranged from 2 to 178 months, with a mean of 37.4 months (SD 37.3) (Fig. 1) (Tables 2 and 3).

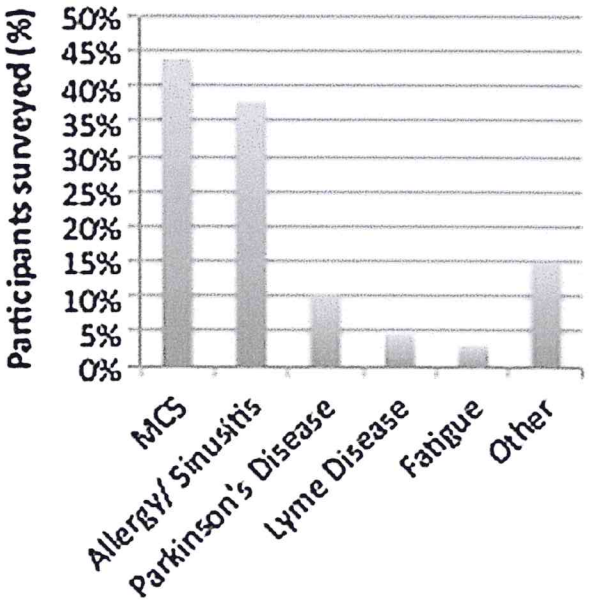

FIG. 1. Reported indications for use of intranasal reduced glutathione [(in)GSH], presented as percent (%) of individuals surveyed. MCS, multiple chemical sensitivity.

TABLE 2. PARTICIPANT-REPORTED EXPERIENCES ATTRIBUTABLE TO INTRANASAL GLUTATHIONE

Diagnosis	*MCS 42.0% (N=29)*	*Allergy/sinusitis 36.2% (N=25)*	*Parkinson 10.1% (N=7)*	*Other 18.2% (N=12)*	*Total 100% (N=66)*
Single diagnosis selected	75.9% (*n*=22)	84.0% (*n*=21)	100.0% (*n*=7)	41.7% (*n*=5)	83.3% (*n*=55)
Multiple diagnoses selected	24.1% (*n*=7)	16.0% (*n*=4)	0.0% (*n*=0)	58.3% (*n*=7)	16.7% (*n*=11)
Demographics					
Female	93.1% (*n*=27)	76.0% (*n*=19)	14.3% (*n*=1)	75.0% (*n*=9)	77.3% (*n*=51)
Mean/median age	54.1/54.0	54.2/53.0	69.6/68.0	55.3.54.0	56.8/58.0
Overall experience					
Positive	72.4% (*n*=21)	88.0% (*n*=22)	57.1% (*n*=4)	66.7% (*n*=8)	78.8% (*n*=52)
Neutral	13.8% (*n*=4)	12.0% (*n*=3)	42.9% (*n*=3)	16.7% (*n*=2)	15.2% (*n*=10)
Negative	6.9% (*n*=2)	–	–	–	3.0% (*n*=2)
Median duration of use: months (p25/p75)	32.5 (16.0/65.0)	22.0 (10.0/52.0)	9.4 (9.0/11.1)	34.0 (22.0/44.5)	24.0 (10.0/56.0)
Frequency of use					
Consistent	55.2% (*n*=16)	44.0% (*n*=11)	42.9% (*n*=3)	50.0% (*n*=6)	50.0% (*n*=33)
Intermittent	31.0% (*n*=9)	44.0% (*n*=11)	42.9% (*n*=3)	25.0% (*n*=3)	36.4% (*n*=24)
Discontinued	10.3% (*n*=3)	12.0% (*n*=3)	14.3% (*n*=1)	16.7% (*n*=2)	12.1% (*n*=8)
Negative effects	20.7% (*n*=6)	–	–	25.0% (*n*=3)	12.1% (*n*=8)
Health benefits	62.1% (*n*=18)	60.0% (*n*=15)	42.9% (*n*=3)	58.3% (*n*=8)	62.1% (*n*=41)
Perceived consequences of (in)GSH use					
Irritation of sinuses or nasal passages	31.0% (*n*=9)	4% (*n*=1)	14.3% (*n*=1)	16.7% (*n*=2)	18.2% (*n*=12)
Headaches	20.7% (*n*=6)	–	–	8.3% (*n*=1)	9.1%
Loss of smell	–	–	–	–	–
Worsening of disease symptoms	3.4% (*n*=1)	–	–	–	1.5%
Bloody nose	13.8% (*n*=4)	4% (*n*=1)	–	–	7.6%
More frequent sinus infections	–	–	–	–	–
More frequent ear infections	–	–	–	–	–
Improved energy	17.2% (*n*=5)	20% (*n*=5)	28.6% (*n*=2)	25.0% (*n*=3)	24.2% (*n*=16)
Improved sense of well-being	31.0% (*n*=9)	16% (*n*=4)	14.3% (*n*=1)	33.3% (*n*=4)	28.8% (*n*=19)
Improvement in sense of smell	10.3% (*n*=3)	12% (*n*=3)	14.3% (*n*=1)	8.3% (*n*=1)	12.1% (*n*=8)
Improvement in disease symptoms	44.8% (*n*=13)	52% (*n*=13)	28.6% (*n*=2)	33.3% (*n*=4)	45.5% (*n*=30)
Reduced frequency of headaches	13.8% (*n*=4)	16% (*n*=4)	–	16.7% (*n*=2)	15.2% (*n*=10)
Less frequent sinus infections	13.8% (*n*=4)	48% (*n*=12)	–	16.7% (*n*=2)	27.3% (*n*=18)
Less frequent ear infections	3.4% (*n*=1)	4% (*n*=1)	–	–	3.0% (*n*=2)

MCS, multiple chemical sensitivities; (in)GSH, intranasal reduced glutathione.

Discussion

This study is the first to utilize a survey to evaluate patient-reported outcomes among users of (in)GSH. From the results of the survey, it appears the therapy is well tolerated with few reported side-effects. That the therapy has been in use for years and that 78% of total survey respondents consider their overall experience with (in)GSH to be positive are among the most notable findings of this survey. For all conditions combined, the most frequently reported benefits include a reported improvement in disease symptoms (45.5%), improved sense of well-being (28.8%), decreased frequency of sinus infections (27.3%), and improved energy (24.2%). The adverse events most commonly reported were

TABLE 3. EXPERIENCE WITH ADMINISTRATION OF INTRANASAL GLUTATHIONE

	Yes	*No*	*Total*
Do you use the spray bottle provided by Key Pharmacy?	93.9% (*n*=62)	4.6% (*n*=3)	98.5% (*n*=65; 1 missing)
	Comfortable	*Uncomfortable*	
Do you consider the administration to be comfortable or uncomfortable?	86.4% (*n*=57)	7.6% (*n*=5)	94.0% (*n*=62; 4 missing)
	Easy	*Difficult*	*Total*
Do you consider the nasal spray to be physically easy or difficult to administer?	87.9% (*n*=58)	9.1% (*n*=6)	97.0% (*n*=64; 2 missing)

irritation to the nasal passages (18.2%), headaches (9.1%), and bloody nose (7.6%). These data suggest that individuals who claim to have a diagnosis of multiple chemical sensitivity (MCS) are approximately twice as likely to have these adverse events than those who identify with other diseases, a finding that warrants greater attention.

The diversity of indications for which (in)GSH is being prescribed warrants separate discussions of clinical significance, so the three primary indications will be discussed separately below.

Multiple chemical sensitivity

MCS is a chronic condition where a diverse array of symptoms are attributed to heightened sensitivity to low-level exposure to chemicals, including solvents, volatile organic compounds, perfumes, etc. Symptoms are nonspecific and include odor intolerance, fatigue, headaches, dizziness, anorexia, and shortness of breath; comorbidities include chronic fatigue syndrome and fibromyalgia.[12] MCS is a poorly understood condition; theories regarding its pathogenesis include genetically determined impairments of detoxification enzymes and CNS sensitization,[13] and an elevation of nitric oxide/peroxynitrite.[14] In all of these proposed pathogenetic mechanisms, excessive ROS are involved. Recently, decreased levels of reduced and oxidized glutathione, as well as of glutathione-metabolizing enzyme activities, were reported in erythrocytes of individuals with MCS.[15] At least one published report exists of an individual being prescribed glutathione as a therapy for MCS,[16] numerous websites encourage the therapy, and this survey suggests improvement in patient-reported outcomes with (in)GSH therapy in people with self-reported MCS.

Chronic sinusitis/allergies

Excessive free-radical formation and depleted antioxidant defenses have been associated with the pathogenesis of several chronic inflammatory disorders of the respiratory tract. While extensive reviews have been published on the therapeutic potential of glutathione in the lower respiratory tract,[2] few have addressed the upper respiratory tract. Decreased levels of reduced glutathione have been observed in patients with chronic sinusitis,[17] providing scientific rationale for repletion as a therapeutic strategy. One study administered 600 mg GSH per day or placebo by nasal aerosol to children with chronic otitis media. GSH levels were dramatically increased in the nasal mucosa in the first hour after treatment and resulted in a statistically significant improvement in nasal obstruction, rhinorrhea, and ear fullness.[18,19]

Parkinson disease

Decreases in glutathione concentrations are the earliest reported biochemical event to occur in the parkinsonian substantia nigra.[20,21] This is supported by the finding that GSH is decreased to almost the same degree in patients with incidental Lewy body disease, considered to be a preclinical form of PD.[3] Depletion in levels of GSH in the substantia nigra precede the loss of complex I activity and subsequent dopaminergic cell death.[20,22] The loss of this primary antioxidant so early in the course of the disease suggests that GSH deficiency may be involved with disease initiation.[23] In 2008, Zeevalk et al. published an extensive review on the role of glutathione deficiency and redox perturbation in the pathophysiology of PD.[24] Two (2) studies have attempted intravenous augmentation of glutathione,[25,26] and both concluded that further research into glutathione supplementation in PD was warranted. A phase I safety and tolerability study of (in)GSH is under way in a population of individuals with PD.[27]

The major limitations of this study are the lack of verifiable diagnoses reported by respondents, lack of objective symptom improvement, and low survey response rate, all of which were anticipated weaknesses given the study design. A limitation unique to this study is the degree to which this population is reflective of the rest of the population with the same diagnosis. It is possible that providers utilizing unconventional, non-U.S. Food and Drug Administration-approved therapies may attract a unique population of patients, and these results may not be generalized to the rest of the population. Future studies should be condition-specific, randomized controlled trials focusing on both objective clinical improvements as well as patient-reported outcomes. Future studies should also seek to determine whether therapeutic efficacy is dependent on endogenous glutathione status.

Conclusions

The three self-reported conditions for which most individuals are using (in)GSH are MCS, chronic sinusitis/allergies, and PD. In these conditions, diminished glutathione has been implicated in the disease pathogenesis, thus providing scientific rationale for glutathione augmentation as a therapeutic strategy. (in)GSH is inexpensive (~$50/month), can be self-administered, and may be a novel method of directly reaching target tissues of the respiratory tract and CNS. This survey of patient-reported experiences suggests (in)GSH is easy and comfortable to administer, with few reported adverse events and results in perceived improvement in health among those returning the survey. Future intervention studies should be conducted in each of these conditions to determine whether the individual's perception of improvement can be objectively verified and whether such benefits are generalizable to a larger population.

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Disclosure Statement

The authors have no partnerships or financial interests to disclose.

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ARTICLE OPEN

Central nervous system uptake of intranasal glutathione in Parkinson's disease

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Glutathione (GSH) is depleted early in the course of Parkinson's disease (PD), and deficiency has been shown to perpetuate oxidative stress, mitochondrial dysfunction, impaired autophagy, and cell death. GSH repletion has been proposed as a therapeutic intervention. The objective of this study was to evaluate whether intranasally administered reduced GSH, (in)GSH, is capable of augmenting central nervous system GSH concentrations, as determined by magnetic resonance spectroscopy in 15 participants with mid-stage PD. After baseline GSH measurement, 200 mg (in)GSH was self-administered inside the scanner without repositioning, then serial GSH levels were obtained over ~1 h. Statistical significance was determined by one-way repeated measures analysis of variance. Overall, (in)GSH increased brain GSH relative to baseline ($P < 0.001$). There was no increase in GSH 8 min after administration, although it was significantly higher than baseline at all of the remaining time points ($P < 0.01$). This study is the first to demonstrate that intranasal administration of GSH elevates brain GSH levels. This increase persists at least 1 h in subjects with PD. Further dose–response and steady-state administration studies will be required to optimize the dosing schedule for future trials to evaluate therapeutic efficacy.

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INTRODUCTION

Glutathione (GSH) deficiency is one of the earliest biochemical perturbations in Parkinson's disease (PD),[1,2] leading to the hypothesis that GSH supplementation may have therapeutic value in alleviating PD symptoms or modifying progression.[3] Reduced GSH (GSH; γ-L-glutamyl-L-cysteinylglycine) is a tripeptide involved in the scavenging of hydroxyl radical (*OH) and singlet oxygen, the reduction of H_2O_2, and for cellular detoxification through GSH-*S*-transferases.[4,5] Deficient GSH synthesis has been associated with oxidative stress in aging,[6] and GSH concentrations decrease with age, a factor thought to explain, in part, why the elderly are at greater risk of neurodegenerative diseases.[7,8]

Two major factors have limited progress toward investigating the utility of GSH supplementation as a therapeutic strategy in PD. First, GSH bioavailability is very low following oral administration. Alternative repletion strategies have focused on oral administration of GSH precursors (e.g., cysteine and glycine supplementation[9]), and intravenous administration of GSH,[10] which although promising, is invasive and inconvenient, and therefore unlikely to be a practical solution. Second, the inability to quantify central nervous system (CNS) GSH concentrations *in vivo* has substantially hindered therapeutic trials targeting CNS augmentation. The current study addressed these limitations by testing a noninvasive nasal GSH repletion strategy, and measuring CNS uptake via proton magnetic resonance spectroscopy (^{1}H-MRS).

^{1}H-MRS is a noninvasive approach that enables the determination of *in vivo* concentrations of specific neurochemicals, including GSH. GSH brain concentrations are not commonly measured using ^{1}H-MRS, because relative to other ^{1}H-MRS-detectable neurochemicals (e.g., creatine (Cr), choline, *N*-acetylasparate), GSH concentrations are substantially lower. In addition, the GSH signal from ^{1}H protons of the cysteinyl β-CH_2, which forms a resonance peak at 2.95 p.p.m., is obscured by nearby spectral peaks from other neurochemicals. The development of editing techniques such as Meshcher-Garwood point resolved spectroscopy (MEGA-PRESS) to effectively suppress nearby resonance peaks of other neurochemicals (e.g., Cr: 3.03 p.p.m.; aspartate: 2.82 p.p.m.; GABA: 3.01 p.p.m.) has provided a practical method for the measurement of GSH concentrations by ^{1}H-MRS.[11] MEGA-PRESS editing has been used to demonstrate alterations in GSH brain concentrations in a handful of conditions, including normal aging,[12] Alzheimer's disease,[13] and schizophrenia.[14]

Little is known about the capacity of exogenously administered GSH, or its precursors, to modify CNS GSH levels. In a 2013 study,[15] the GSH precursor *N*-acetyl cysteine (NAC) was administered as a single 60-min intravenous infusion (150 mg/kg) to individuals with PD and healthy controls. NAC administration increased brain GSH concentrations by 55% in subjects with PD, and 34% in healthy controls ($n = 3$). The authors reported that maximal brain GSH concentrations were measured ~90–110 min after the start of the infusion, and had not returned to baseline levels by 120 min after the start of the infusion.[15] These results support the hypothesis that NAC is capable of crossing the blood–brain barrier and providing cysteine substrate to CNS cells, thus enhancing GSH

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synthesis. Although these findings appear promising, the utility of intravenous NAC repletion as a therapeutic strategy in PD is greatly limited by invasiveness and inconvenience of intravenous delivery.

Recently, a dose-dependent increase in cerebrospinal fluid (CSF) total and reduced NAC concentrations was demonstrated in association with oral NAC administration.[16] Despite increases in NAC concentrations, there was no observed increase in CSF total or reduced GSH concentrations, presumably because the conversion of NAC to GSH occurs intracellularly. Under normal physiologic conditions, intracellular GSH concentrations of neurons are 250- to 500-fold higher than in the CSF,[17] thus limiting the utility of CSF to serve as a biomarker of cellular GSH status. In spite of their limitations, these studies are the first to demonstrate a capacity of exogenously administered NAC to reach the CNS. An oral NAC supplementation trial is underway to assess changes in brain GSH levels using similar ^{1}H-MRS.[18]

Intranasal administration of reduced GSH, (in)GSH, could be an effective approach for delivery of GSH to the CNS. Many studies suggest that small, polar molecules may be able to "bypass" the blood–brain barrier with nasal delivery, as the interface between the nasal cavity and brain is considered a potential point of vulnerability in the blood–brain barrier. On the basis of the biological plausibility and anecdotal case reports of clinical improvement, (in)GSH has been recommended as an off-label therapy for GSH augmentation in PD since 2004.[19–21] Recently, a double-blind, placebo-controlled randomized clinical trial of phase I study of (in)GSH in PD ($n = 30$) demonstrated (in)GSH was safe and tolerable, and both active study arms demonstrated an improvement over placebo in total Unified PD Rating Scale scores, specifically in activities of daily living and motor Unified PD Rating Scale subscores.[22]

The current proof-of-concept study was designed to evaluate whether (in)GSH is capable of augmenting CNS GSH concentrations, as measured by ^{1}H-MRS.

Table 1. Characteristics of study participants

	Total (n = 15)	*Min*	*Max*
Gender			
Male	11 (73%)		
Female	4 (27%)		
Age (years)			
Mean (s.d.)	65.5 (11.2)	44	83
Race: Caucasian	15 (100%)		
Years since PD diagnosis (s.d.)	6.1 (6.2)	0.7	23
UPDRS, total score			
Mean (s.d.)	78.9 (15.4)	56	103
Levodopa dose equivalents (s.d.)	557 (477)	0	1,600

Abbreviations: PD, Parkinson's disease; UPDRS, Unified PD Rating Scale.

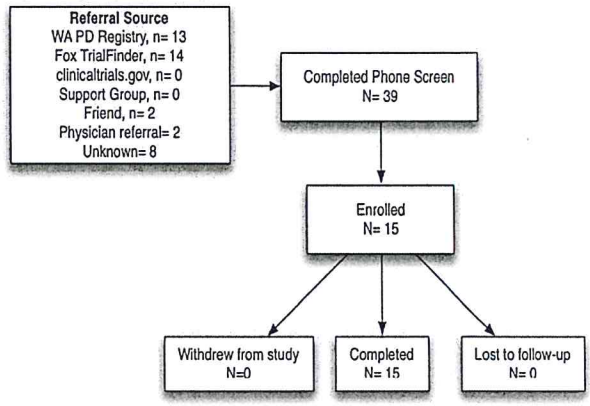

Figure 1. CONSORT enrollment algorithm.

RESULTS

Subject screening and enrollment

In all, 31 individuals were screened in order to identify 15 who qualified. Most study referrals came from the Michael J Fox Foundation Trial Finder (45%) and Washington State PD Registry (42%), with health-care providers and friends contributing to the remaining referrals (13%). The subject population was highly diverse in terms of age, socioeconomic status, education, and geographic neighborhoods throughout the Pacific Northwest, although all participants were Caucasian. The characteristics of study participants are presented in Table 1 and the enrollment algorithm is presented in Figure 1.

Study medication quality and tolerability

Independent analysis of three separate samples compounded to contain 200 mg/ml demonstrated the product potency to be 190 mg/ml (95%) upon receipt, and potency reduced to 144 mg/ml (72%) at 4 weeks in one sample, and 161 mg/ml (80.5%) at 6 weeks in a separate batch.

One participant experienced a single adverse reaction to the study medication, cephalgic paresthesia, which resolved within 1 h.

Changes in brain GSH levels after (in)GSH administration

The duration of post-dose measurements was driven by participant comfort and scheduled time in scanner, up to the duration of time approved by the institutional review board. Six subjects underwent three post-dose measurements, eight subjects underwent four post-dose measurements, and one subject underwent a fifth measurement (Table 2).

GSH-edited spectra were successfully obtained from all 15 subjects (representative spectrum, Figure 2). For one subject, the spectrum obtained at the second post-dose time point was of insufficient quality, and was omitted from analysis. For six subjects, baseline GSH peaks were undetectable. For these six subjects, baseline GSH levels were all assigned the same value (the lowest measured GSH/Cr ratio value across all subjects and scans, divided by 2), in order to calculate the absolute change in GSH/Cr ratio relative to baseline. Thus, of the 70 spectra acquired, a total of 7 spectra were omitted from analysis. For the remaining 63 spectra, mean fit error was 38%. The combined fit error for GSH and Cr varied by less than a factor of 2 over the course of each subject's serial scans. For point-resolved spectroscopy (PRESS) spectra, all scans met the stated quality control criteria. CSF fraction within the voxel ranged from 7 to 25% (mean 17%, ±4.9% s.d.).

Mean GSH levels increased consistently with time relative to baseline (Table 2 and Figure 3a,b), although levels fluctuated somewhat among individual subjects (Figure 3c,d). GSH/Cr (as well as absolute GSH levels) were significantly different from each other (one-way repeated measures analysis of variance, $P < 0.001$). The increase in GSH/Cr or absolute GSH immediately after (in)GSH administration (7.5 min) was not significantly different from baseline, however, GSH levels were significantly higher than baseline at all of the remaining time points ($P < 0.05$; Figure 3a,b). Between the baseline and the 45-min scan, there was a mean 269% increase in GSH/Cr (240% increase in absolute GSH).

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Table 2. Change in brain glutathione levels (as GSH/Cr peak ratios) relative to baseline after 200 mg nasally administered GSH

	Glutathione level (GSH/Cr peak ratio)				
Time post dose (min) as midpoint of scan (s.e.m.)	*Subjects (n = 15)*	*Mean (s.e.m.)*	*Min*	*Max*	*Mean difference relative to baseline (s.e.m.)*
0 (baseline)	15[a]	0.0170 (0.0046)	0.0035	0.0620	
7.5 (0.0)	15	0.0259 (0.0039)	0.0080	0.0580	0.00890 (0.00507)
19.9 (0.17)	14	0.0364 (0.0057)	0.0120	0.0760	0.0201 (0.00585)
32.0 (0.17)	15	0.0385 (0.0053)	0.0130	0.0810	0.0215 (0.00532)
44.7 (0.22)	9	0.0457 (0.012)	0.0130	0.114	0.0340 (0.0135)

Abbreviations: GSH, glutathione; Cr, creatine.
[a]For the baseline scans where the GSH peak was undetectable, a GSH/Cr value (lowest measured value/2) was substituted, as described in the Materials and Methods section.
For six subjects, baseline GSH levels were undetectable, and one post-dose spectrum from one subject was omitted owing to poor data quality.

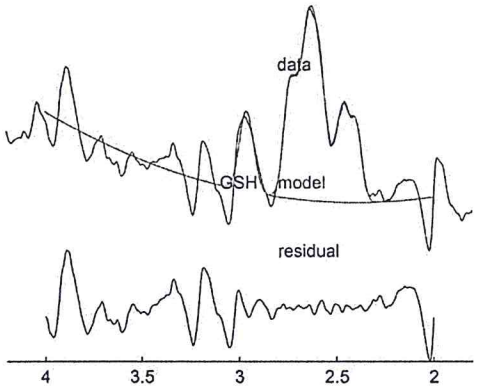

Figure 2. Representative fit to glutathione (GSH) peak. The GSH-edited spectrum is shown in blue. The upper red line illustrates the best fit of a 5-Gaussian model to GSH and co-edited molecules (overall fit), and the lower red line illustrates the fit of a simple Gaussian model to the GSH peak (GSH quantification). Below the plot, the residual between the spectrum and model best fit is shown in black.

DISCUSSION

To our knowledge, this is the first study to demonstrate an increase in CNS GSH levels with a noninvasive GSH augmentation strategy. GSH augmentation as a potential therapeutic strategy in CNS disease has been suggested for decades,[23] although repletion efforts have been hindered by inability to assess human CNS GSH concentrations *in vivo* and poor oral absorption of GSH.[24] Here we demonstrate that both of these obstacles are surmountable by using a ^{1}H-MRS editing method to measure CNS GSH levels, and a noninvasive (in)GSH administration strategy. GSH augmentation deserves investigation as a beneficial therapeutic approach for not only PD but also numerous other CNS disorders for which GSH deficiency and GSH-related enzyme deficits have been documented (multiple sclerosis,[25,26] autism,[27–29] Alzheimer's disease,[30,31] schizophrenia,[32,33] and bipolar disease[34]).

Previously, i.v. NAC was demonstrated by magnetic resonance spectroscopy (MRS) to augment CNS GSH concentrations.[15] Although effective, i.v. therapy requires trained medical personnel for administration, thus raising costs, patient burden for clinic visits, and risk of discomfort and phlebitis. Ours is the first study to demonstrate CNS GSH augmentation using a noninvasive, self-administered therapy in humans. The single dose, short (1 h) observation period, and lack of placebo arm are limitations in this proof-of-concept study and provide direction for follow-up studies.

GSH levels were calculated both relative to Cr (GSH/Cr peak area ratio) and as absolute (water-referenced) GSH concentrations. Although water-referenced neurochemical concentrations are considered by some as the "gold standard" approach for MRS, there are numerous technical challenges and assumptions that can limit the utility of water-referenced measurements.[35] Alternatively, reporting of neurochemical levels relative to a reference neurochemical in the same voxel is a common approach, as measurements are technologically uncomplicated (only a single spectrum must be collected) and no correction for partial-volume effects is required. However, a ratio approach can complicate interpretation of data, if it is unclear whether the reference neurochemical is altered by treatment as well. For this reason, both GSH/Cr ratios and absolute GSH concentrations are reported. We observed good correspondence between the relative and absolute GSH levels (Figure 3), and the statistical significance of GSH level changes with time post dose was the same regardless of the quantitation approach. In addition, although comparison of absolute MRS neurochemical concentrations across different instruments and sites is highly challenging, the baseline absolute GSH levels that we observed (mean 0.109 IU (institutional units); range 0.0183–0.435) are within the ranges reported in the literature for postmortem CNS concentrations of GSH in subjects with PD.[2] Of note, the range of reported brain GSH concentrations is extremely variable, ranging over an order of magnitude.[15,36] The strength of this study lies in the demonstration of a consistent increase in brain GSH levels with time post dose. Absolute Cr concentration in the voxel (mean 6.09 IU) were also comparable to reported values.[37]

It should be noted that this study was not designed to differentiate between GSH in brain tissue versus CSF. However, as reported concentrations of GSH are in the range of 0.2 μmol/l in CSF and 1 mmol/l in brain tissue,[7] GSH in CSF would not appreciably change the MRS determination of GSH in brain tissue. Owing to the velocity of blood movement through the voxel, MRS does not detect GSH signal from blood, thus, the measured GSH values reflect only GSH in brain parenchyma or CSF.

This pilot study was designed to demonstrate that (in)GSH results in an increase in the GSH signal in brain, and not to generate a comprehensive pharmacokinetic profile of the increased brain GSH signal. In light of the data generated in this study, a more complete pharmacokinetic investigation designed to quantify the magnitude and duration of increase in GSH is warranted. Additional studies could be directed toward optimizing delivery techniques, dosing schedules, product stability, and intranasal formulations.

Recently, a small phase I/IIa study of (in)GSH in PD demonstrated a mild symptomatic improvement in PD symptoms as measured by the Unified PD Rating Scale, with return of symptoms upon withdrawal of (in)GSH.[38] Although numerous questions remain about the mechanism by which GSH may ameliorate PD symptoms, this study demonstrates that a single dose of (in)GSH does, in fact, reach the target tissue. In addition to biological activity as an essential intracellular antioxidant, GSH facilitates

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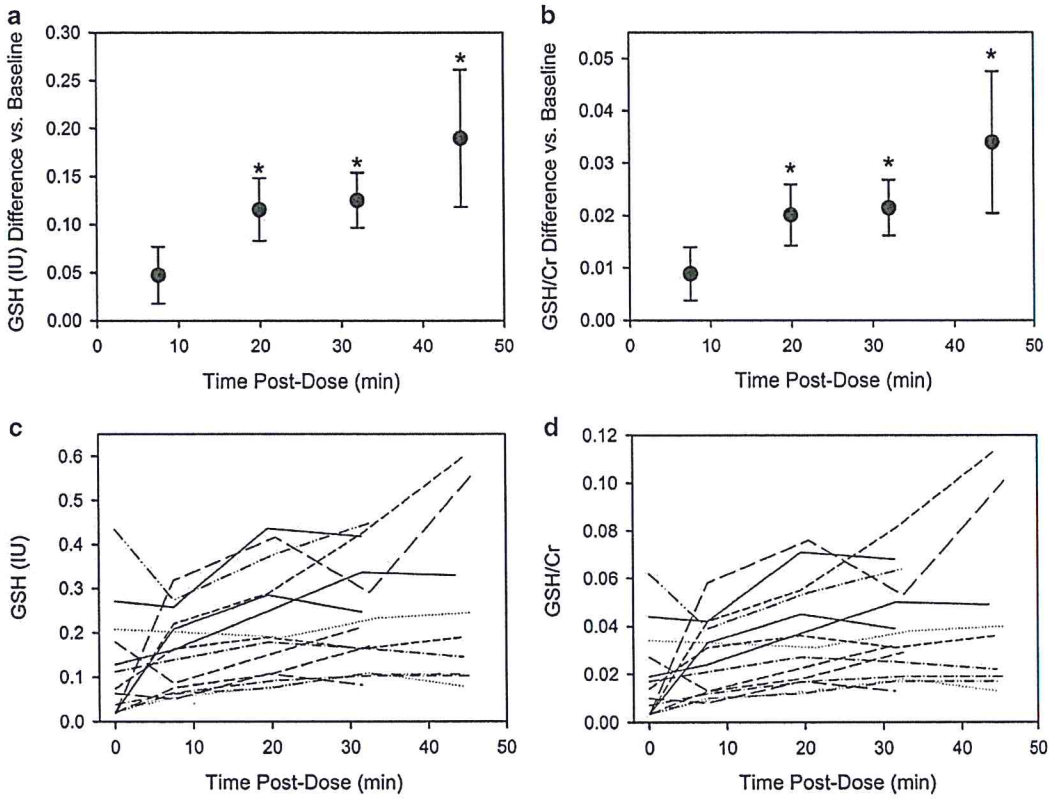

Figure 3. Differences in glutathione (GSH) levels, as absolute GSH (**a**) and GSH/Cr (**b**), relative to baseline versus time after 200 mg intranasally administered GSH. Data are presented as mean ± s.e.m. Asterisks indicate points that are significantly different from baseline (one-way repeated measures analysis of variance comparing each point to baseline, $P < 0.05$ after correction for multiple comparisons). Shown below are time courses of change in absolute GSH (**c**) and GSH/Cr (**d**) in individual subjects over time post dose.

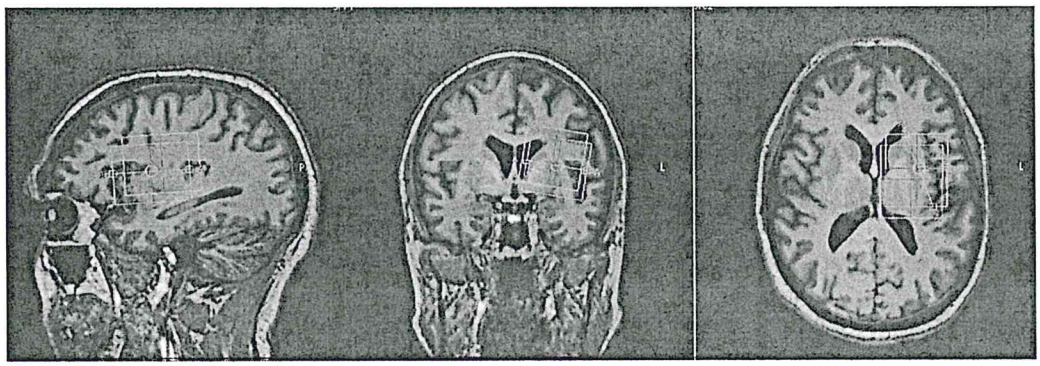

Figure 4. Volume of interest. For all participants, glutathione (GSH) was administered into the left nostril, and a voxel was placed over a 4×4×5-cm region centered on the left dorsal putamen at the level of the lentiform nucleus.

the clearance of metabolic waste via GSH-*S*-transferases and may function as a neuropeptide.[39] In astrocytes, GSH serves as a reservoir for cysteine, glycine, and glutamic acid, each with their own biochemical activities. Glycine, a *N*-methyl-D-aspartate receptor agonist, has been shown to significantly improve negative symptoms in patients with schizophrenia when supplemented.[40] Cysteine availability has been shown to regulate extracellular glutamate concentrations, and thus neuronal excitability, via the cystine–glutamate antiporter.[41] Using 123-IFP-CIT single-photon emission computed tomography, high doses of (iv) GSH significantly influenced putamen dopamine transporter in PD patients.[42] A follow-up study is underway to evaluate the effects of 3 months of (in)GSH on PD symptom status and MRS GSH concentrations. Considering the potential for therapeutic development of (in)GSH, the established safety and tolerability data, biological plausibility, and pilot level clinical evidence of benefit are further supported with this demonstration that (in)GSH is able to augment brain GSH levels.

MATERIALS AND METHODS

Institutional Review Board approval was obtained at the University of Washington for this single-center study of 15 participants with mid-stage PD. Recruitment occurred through the Michael J. Fox Foundation Trial

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Finder,[43] the Washington PD Registry,[44] ClinicalTrials.gov (NCT02324426), and referral from local health-care providers. Inclusion criteria required participants to be over age 18, read and speak English, have a Hoehn & Yahr score between 2 and 3 (bilateral disease, not severely disabled), and have three or more of the required positive criteria for the diagnosis of definite PD from Step 3 of the UK Brain Bank Diagnostic Criteria for PD.[45] Exclusion criteria included any contraindication to MRI, history of epilepsy, stroke, brain surgery, or structural brain disease, pregnancy, history of sulfur sensitivity, ongoing asthma or drug dependence, ongoing chronic diseases, history of mental illness, or acute infection during the prior 30 days. Informed consent was obtained from all participants. In light of evidence from animal models that brain GSH concentrations peak in the morning,[46] the single study visit was scheduled at 0700 hours for each participant in an attempt to control for circadian fluctuations.

Brain GSH assessment

Imaging and voxel selection. Brain GSH levels were determined using a Philips Achieva 3.0-T whole-body scanner (Best, The Netherlands) equipped with a 32-channel SENSE phased-array head coil. From each subject, a detailed brain image was first acquired, using a magnetization-prepared rapid gradient echo[47] high-resolution T1-weighted sequence (repetition time = 6.6 ms, echo time = 3.0 ms, flip angle = 8°, matrix = 256 × 240, slices = 170, and slice thickness = 1 mm). Images were evaluated in real-time to select a cubic volume of interest, 4 × 4 × 5 cm, centered over the left dorsal putamen at the level of the lentiform nucleus. As CNS GSH concentrations are thought to be reduced in PD, a relatively large voxel size was selected in order to maximize signal to noise. The dorsal putamen was selected as the center of the volume of interest due to its relatively homogenous mix of neurons and astrocytes, and suitable distance from bone and other regions that could compromise signal quality. The voxel was positioned to avoid the skull and, to the extent possible, the left lateral ventricle (Figure 4).

^{1}H-magnetic resonance spectroscopy. The cysteinyl β-CH_2 of GSH exhibits a characteristic chemical shift at 2.95 p.p.m., which distinguishes it from other cysteine-based molecules.[48] GSH levels were determined within the volume of interest using MEGA-PRESS double-editing for the cysteinyl β-CH_2 residue of GSH[7] (repetition time = 2,000 ms, echo time = 122 ms, free induction decay points = 2,048, spectral width = 2,000 Hz, number of averages = 8 per phase cycle ON or OFF, 320 acquisitions total requiring just under 11 min). Spectral editing was accomplished by refocusing GSH J-evolution during every other acquisition (ON), using a 43-ms Gaussian pulse centered at the cysteinyl α-CH resonance of GSH at 4.56 p.p.m. During the alternate acquisitions (OFF), the pulse was applied symmetrically about the water peak. The difference-edited GSH spectrum was generated by subtraction of the OFF and ON spectra.

To facilitate quantification of GSH, additional spectra were collected from the same volume of interest (Figure 4) using a short-echo PRESS sequence with vapor water suppression (repetition time = 2,000 ms, echo time = 36 ms; free induction decay points = 2,048, spectral width = 2,000 Hz, number of averages = 64). To account for T_2-weighting differences, PRESS water spectra were also collected using both echo times (echo time = 122 or 68 ms, repetition time = 2,000 ms, free induction decay points = 2,048, spectral width = 2,000 Hz, number of averages = 8).

After baseline MEGA-PRESS and PRESS spectra were acquired, 200 mg GSH was self-administered into the left nostril by each subject inside the scanner without repositioning. Immediately after administration (within 2 min), serial GSH MEGA-PRESS spectra were obtained over the course of up to 62 min post dose (11 min per scan, for a total of three to five measurements post dose). For consistency, the study medication was always administered in the left nostril and spectra were collected ipsilaterally.

Quantification. For each subject, brain GSH levels were quantified from difference-edited spectra using the Gannet 2.0 Toolkit, a Matlab-based automated program for analyzing MEGA-PRESS spectra.[49] Gannet processing steps include 3 Hz exponential line broadening, and frequency- and phase-correction of individual spectra. The edited spectra are fit with Gaussian models, and the GSH signal is expressed relative to the Cr signal; GSH/Cr ratio. Assessment of inter-and intra-subject data quality was accomplished by comparing fit errors (calculated as the s.d. of the residual of the analyte peak, expressed as a percentage of the analyte peak amplitude). In cases where the GSH peak was undetectable, a value (the lowest measured GSH/Cr ratio value across all subjects and scans, divided by 2) was assigned.

To calculate absolute (i.e., water-referenced) GSH levels from GSH/Cr ratios, concentrations of total Cr (Cr plus phosphocreatine) were calculated. Cr concentrations were determined from PRESS spectra using standard model-fitting procedures (LCModel software version 6.2-0T (ref. 50)). A decomposition-fitting algorithm was used to subtract residual water signals. Free induction decays were zero-filled, smoothed with a 1.1-Hz exponential-dampening filter, and then zero- and first-order phase corrected. Quality control criteria included a peak width $\leqslant 0.1$ p.p.m., signal-to-noise ratio $\geqslant 5$, and Cramer–Rao lower bounds $< 20\%$ (as percentage of the estimated concentration). Absolute (water-referenced) Cr concentrations were determined by scaling the spectrum to the unsuppressed water peak, resulting in values with IU that approximate millimolar (mmol/l) concentrations.

To correct for the partial-volume effect, fractions of CSF and brain tissue (gray and white matters) were determined within the voxel using FSL FAST segmentation.[51] As GSH is known to be present in CSF at very low concentrations (~0.2 μmol/l in both healthy and PD subjects),[52] relative to brain tissue concentrations (~1 mmol/l),[7] LCModel-calculated Cr concentrations ($C_{measured}$) were corrected ($C_{corrected}$) using the following formula, which assumes negligible contribution of CSF GSH to the total GSH signal:

$$C_{corrected} = C_{measured} - \left(\frac{1}{\text{fraction CSF in voxel}}\right)$$

Absolute GSH levels (IU) were calculated by multiplying GSH/Cr ratios by CSF-corrected absolute Cr concentrations (IU).

Changes in GSH levels with time post dose were calculated as the difference in GSH/Cr peak ratios for each subject at each time point ($GSH/Cr_{post\ dose} - GSH/Cr_{baseline}$), or similarly, the difference in absolute GSH for each subject at each time point.

Study medication

Powdered GSH was obtained from MEDISCA (Plattsbergh, New York, USA) and compounded by Key Pharmacy (Federal Way, WA, USA). The study medication was stored in a study refrigerator and protected from light until 30 min before administration, when it was allowed to come to room temperature, for participant comfort during administration. All participants were administered an identical intervention ($1 cm^3$ of saline containing 200 mg GSH) using a syringe attached to a Mucosal Atomization Device supplied by Wolfe-Tory Medical (Salt Lake City, UT, USA). This dose is the highest dose meeting tolerability and safety criteria in the phase I study of (in)GSH in PD.[38] As a quality control measure, medication samples were sent for independent potency analysis (Eagle Analytical, Houston, TX, USA) upon receipt, and at 4 and 6 weeks after production.

Statistical analysis

Using data generated from a pilot study and G*Power 3.1 software (Düsseldorf, Germany), it was determined that a sample size of 15 would provide 80% power to detect an increase in CNS GSH concentrations between pre- and post-administration values, with an accepted alpha value of 0.2. Descriptive statistics for study participants are listed in Table 1.

A single brain GSH level was determined from each 11-min MEGA-PRESS acquisition. For the purpose of illustrating changes in GSH level with time, levels were treated as corresponding to the midpoint of each scan. Changes in brain GSH levels over time were determined as the difference between the GSH/Cr ratio (or absolute GSH) at each time point, relative to baseline. Significance was determined by one-way repeated measures analysis of variance, and the Holm–Sidak method for multiple comparisons versus the control group, using SigmaPlot 10.0 software (Systat Software, San Jose, CA, USA). Variance among all groups was not statistically different.

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CONTRIBUTIONS

LKM: conception, design, and execution of clinical trial, and manuscript preparation; TJK, MER, and CCW: methodology/analysis of erythrocyte glutathione concentrations;

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JED: study design and manuscript preparation; EGS, TKW, and KEC: acquisition of MR image; PUDLT: execution of clinical trial, data management, and laboratory glutathione analysis; JMP: study design, MRS analysis, and manuscript preparation.

COMPETING INTERESTS

The authors declare no conflict of interest.

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A Randomized, Double-Blind Phase I/IIa Study of Intranasal Glutathione in Parkinson's Disease

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ABSTRACT

Background: Depletion of reduced glutathione is associated with PD and glutathione augmentation has been proposed as a disease-modifying strategy. The aim of this study was to determine the safety and tolerability of intranasal reduced glutathione in individuals with PD.

Methods: Thirty individuals with PD were randomized to either placebo (saline), 300 mg/day, or 600 mg/day of intranasal glutathione in three divided daily doses. Follow-up visits included side effect screening of PD symptoms and cognition, blood chemistry, sinus irritation, and hyposmia. Tolerability was measured by frequency and severity of reported adverse events, compliance, and withdrawals from the study.

Results: After 3 months, there were no substantial differences between groups in the number of adverse events reported or observed among all safety measures assessed. All groups met tolerability criteria.

Conclusions: These data support the safety and tolerability of intranasal glutathione in this population. Pharmacokinetic and dose-finding studies are warranted.
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Key Words: glutathione, antioxidant, nutrition, neuroprotection, deficiency

Glutathione is a well-established antioxidant, hydrogen peroxide reducing agent, essential for cellular detoxification, as a neuropeptide, and as a reservoir for cysteine, glycine, and glutamic acid.[1,3] Loss of reduced glutathione (GSH) is the most consistently reported alteration in the antioxidant defense system in PD.[7-10] Whereas most individuals can synthesize enough GSH to maintain redox equilibrium, this is not the case in Parkinson's disease (PD) and other neurodegenerative disorders, which have consistently been shown to be associated with GSH depletion,[10] defining GSH as a conditionally essential nutrient in PD.[11]

The value of exogenously administered GSH to patients with PD has been formally studied twice using intravenous GSH, (iv)GSH, based on the understanding that oral GSH is poorly absorbed.[14] Both studies concluded that further research was warranted on GSH as a neuroprotective agent in PD.[15,16] (iv)GSH is limited by invasiveness, expense, and necessary clinic visits, which restrict therapeutic utility.

Intranasal GSH, (in)GSH, has been used as a potential route of central nervous system (CNS) glutathione augmentation since 2004, based on an acceptable safety and tolerability profile,[17,18] the biological plausibility that small, polar molecules may bypass the blood–brain barrier by intranasal administration, and anecdotal case reports of improvement.[17,19]

Patients and Methods

This study was designed to evaluate the safety and tolerability of (in)GSH in a double-blind, placebo-controlled fashion in a cohort of individuals with PD. The study was approved by the Bastyr University Institutional Review Board and conducted in accord with The Code of Ethics of the World Medical Association for experiments involving humans. The U.S. Food and Drug Administration granted Investigational New Drug status. All clinical evaluations were conducted at Bastyr University Clinical Research Center (Kenmore, WA). Only the data monitoring committee, the database manager, and the compounding pharmacy were unblinded. The study was registered on ClinicalTrials.gov (#NCT01398748).

All participants were English-speaking residents of the Pacific Northwest of the United States who reported having been diagnosed with idiopathic PD by a clinical neurologist within the previous 10 years, had a modified H & Y stage ≤ 3, were ≥ 21 years of age, and had been stable on medications, supplements, diet, and exercise

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for 30 days before study entry. Individuals were excluded if they had abnormal liver enzymes or kidney function, cognitive impairment (Montreal Cognitive Assessment [MoCA) score <25), epilepsy, a history of stroke, a history of brain surgery, structural brain disease, diseases with features common to PD (e.g., essential tremor), chronic sinusitis, or a history of intranasal telangiectasia. All individuals agreed to try to maintain stability of medications, diet, lifestyle, and alternative therapies throughout the study trial, although deviation from baseline routine throughout the trial did not disqualify them from continued participation.

Key Pharmacy (Kent, WA) compounded the study medication for each participant enrolled according to a randomized schedule generated by the study statistician. Purity and potency of glutathione, both in powdered and compounded liquid form, was independently validated by Eagle Analytical (Houston, TX) at the beginning of and throughout the study. Liquid glutathione was assessed for potency and purity from both unissued medication and from medication returned by subjects after 30 days of storage. Mucosal Atomization Device (MAD) tips, used to turn the liquid glutathione into a mist for easier administration, were supplied by Wolfe-Tory Medical (Teleflex) SLC, UT and replaced monthly.

Study medication was dispensed as sterile, capped 1-mL syringes in a light-impermeable plastic bag shipped on ice and stored in the refrigerator. GSH has a sulfur smell; to limit risk of unblinding, study clinicians did not participate in dispensation, collection, counting, or disposal of study medication. Participants were instructed to store the study medication in the refrigerator and to rinse MAD tips with warm water and let air dry after each use.

The maximum dose, 4,200 mg/week, was chosen to match the dose used in a 2009 pilot study of (iv)GSH, 1,400 mg three times weekly.[16] Subjects who passed screening were randomized into one of four groups: 600 mg (in)GSH/day; 300 mg (in)GSH/day; placebo (sterile saline); or watchful waiting using simple random allocation with uneven distribution (n = 10, 10, 10, and 4, respectively). In order to evaluate the impact of the saline spray on nasal symptoms, the study sponsor requested that 4 additional individuals be enrolled to a watchful waiting arm, to provide a point of comparison for nasal irritation that could be caused by either the saline placebo or the active glutathione. Because these individuals did not receive placebo, they were excluded from all analyses other than those evaluating nasal irritation. Subjects randomized to intervention arms were instructed to spray one 1-mL syringe full of study medication three times daily for 3 months total. The medication was dispensed one month at a time, with instructions to return both used and unused syringes at the end of each month. Self-reported doses taken were confirmed through counts of returned syringes. Along with the medication, subjects were given a daily log and told to report medication use and any changes in symptoms and well-being.

Subjects returned at weeks 2, 4, 8, 12, and 16 for assessments of complete blood count (CBC), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine, and a urinalysis. Monitoring of Side Effects Scale (MOSES) is a standardized questionnaire designed to assess 83 potential symptoms across 8 body systems, and was used to screen for side effects. The SinoNasal Outcomes Test (SNOT-20), a validated measure of rhinosinusitis,[20] was employed in this study because sinus irritation was anticipated. For our purposes, questions 1 to 10, specific to sinusitis (e.g., runny nose, sneezing, and cough), were used to screen for sinus-specific adverse events (AEs). The UPDRS was used to monitor PD symptoms, and the Sensonics Smell ID Test was used to test olfactory function. AEs were predefined to reflect clinically relevant worsening or occurrence of all outcomes evaluated. According to protocol, study clinicians were blinded and required to have successfully completed the International Parkinson and Movement Disorder Society UPDRS Training Program. Each participant was asked to select a time of day when they were most likely to be *on*; once that time was selected, all subsequent evaluations were scheduled at the same time to minimize the impact of circadian fluctuations of PD symptoms.

All comparisons presented are between the active arms of the study and placebo; data from the no-intervention arm (n = 4) were eliminated from all analyses except sinus irritation, which was anticipated in all arms. Individuals who did not make the 3-month study visit were dropped from the analysis. Descriptive statistics were the primary outcome measure, and thresholds for reporting were determined a priori. Clinical side events were defined as a 2-point change on the MOSES or a rating of 3 or 4 (severe) on the 0 to 4 MOSES scale; laboratory AEs were predefined as a deviation from accepted reference ranges (e.g., ALT >50 IU/L). Tolerability was defined as 80% of the group taking 80% of the prescribed dose of study medication.

Results

Of the 30 participants assigned to a treatment arm, 28 completed the study intervention; 1 participant withdrew because of schedule conflicts and the other withdrew because of an AE attributed to the study medication (Figure 2). The AE necessitating withdrawal from the study was a "ringing in her head" subsequent to the first use of study medication exacerbation of chronic pruritus that had been several months quiescent before the screening visit. The participant reported that the ringing sensation resolved

TABLE 1. Side effects by cohort.

Table of Side Effects	Placebo (n = 9)	300 mg/day (n = 8)	600 mg/day (n = 8)
Number of individuals reporting symptom:			
Negative Side Effects			
Labored breathing	0	0	2
Sore throat/ redness	0	0	2
Flatulence	2	0	1
Increased thirst	0	0	2
Contortions/ neck-back arching	0	2	0
Chills	2	0	0
Positive Side Effects			
Improved blink rate	1	5	0
Improved arm swing	1	1	2
Fewer muscle pains or aches	2	1	2
Reduced edema	0	0	2
Improved incontinence/ Nocturnal enuresis	0	2	0
Reduced urinary frequency	3	0	0
Reduced agitation	0	3	0
Improved drowsiness/ lethargy/ sedation	2	1	2
Improved insomnia	1	0	2
Less crying/ feelings of sadness	2	0	0
Deviation from laboratory normal reference ranges			
Hemoglobin	0	0	2
Hematocrit	0	0	2
Creatine	1	1	2
Uric acid	0	0	2
Change from baseline, mean (SD):			
Sinusitis (SNOT–20 Score 0-1)	0.275	0.185	0.213
Change in PD Symptoms			
UPDRS total (0-199)	−1.1 (4.1)	−5.3 (4.8)	−4.3 (7.5)
UPDRS Part 1: Mentation, behavior, and mood	−0.6 (1.2)	−1.4 (2.0)	−0.8 (1.7)
UPDRS Part 2: Activities of daily living	−1.3 (3.5)	−0.8 (2.3)	−1.3 (3.5)
UPDRS Part 3: Motor score	0.8 (3.7)	−3.1 (2.9)	−1.4 (3.7)
UPRDS Part 4: Complications of dopaminergic therapy	1.0 (1.5)	−0.1 (1.0)	−0.9 (2.4)

The table reports the number of individuals meeting criteria for adverse events and includes only those symptoms reported by two or more participants in any cohort. Sinusitis and UPDRS reported as mean change in absolute score from baseline, by cohort. SD, sdandard deviation

over 4 to 6 hours and the dermal inflammation resolved within 2 weeks. Across study arms, the predominately Caucasian (96%) participants were evenly distributed for gender (50%/50% male/female) and H & Y (median, 2).

Subject compliance with study medication use met criteria for tolerability in all cohorts. GSH retained 89% of its potency after over 30 days of home storage. As expected, individuals in all intervention arms reported an increase in sinus symptoms, and this was approximately equivalent across arms. There were no statistically significant differences in frequency of laboratory events as defined by CBC, white blood count with differential, ALT, AST, creatinine, BUN, uric acid, or urinalysis. UPDRS scores, included as a safety measure, improved in both treatment arms over placebo. In post-hoc analysis, UPDRS trends remained consistent after excluding all individuals (n = 10) who changed medications throughout the study. Side effects, deviations from laboratory reference ranges, and change from baseline clinical scores are listed in Table 1 and Figure 1.

To evaluate whether individuals were unblinded by the smell, participant feedback was evaluated. Qualitative interviews generated 189 total comments; two comments referenced the salty taste, one mentioned the smell of sulfur in nose and stool. Of the 6 participants who expressed confidence in knowing their group assignment, 2 were correct.

Discussion

In this phase I/IIa clinical trial, (in)GSH was well-tolerated. A naturally occurring molecule, exogenously administered GSH has an excellent record of safety. The few studies that have evaluated exogenous administration of GSH to humans with PD have been reassuring.[16,18]

Mild clinical improvement in UPDRS symptoms came as a bit of a surprise for this nondopaminergic therapy, although exogenous GSH has been shown to increase dopamine transporters.[22] The benefit measured may be explained by regression toward the mean, although anecdotal reports suggest at least some individuals do

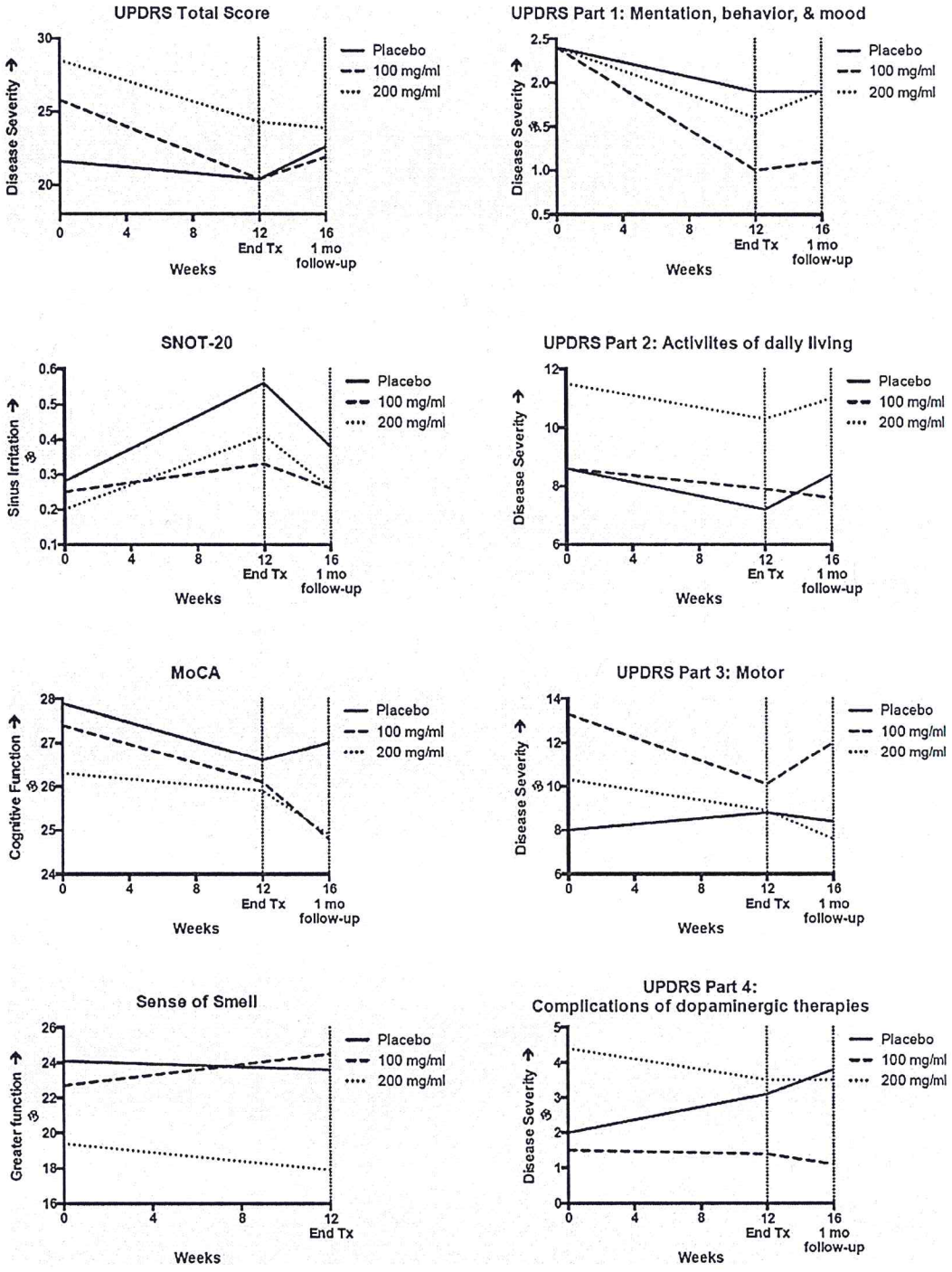

FIG. 1. Outcomes associated with study arms. The mean change, by treatment arm, in clinical outcomes assessed over the course of the 3-month study intervention and after a 1-month wash out period. Sense of smell was determined by Sensonics Smell Identification Test.

experience an acute improvement in clinical symptoms after administration of exogenously supplied gluathione.[19] Whereas the study was double blind with a placebo control, GSH has a distinct smell that unblinded at least 1 participant.

The clinical response, though fortunate for patients, suggests that delayed-start trial (or similar) design should be utilized when attempting to determine the neuroprotective capacity of (in)GSH over time. Symptomatic improvement with (in)GSH should be verified in a larger study powered for detecting differences between groups.

Overall, this study supports the safety and tolerability of (in)GSH in a sample of patients who are within

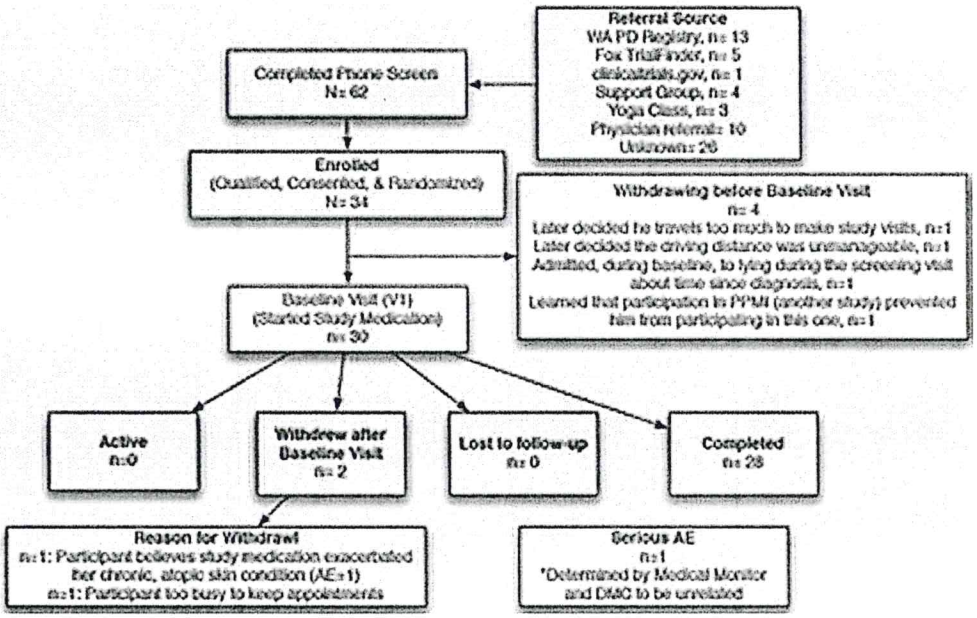

FIG. 2. Enrollment algorithm according to CONSORT guidelines.

10 years of PD diagnosis. The identification of a nondopaminergic strategy capable of improving UPDRS scores may herald a new generation of therapeutics. GSH perturbations have been documented in numerous other disorders of the CNS, such as schizophrenia, dementia, Huntington's disease, and autism, and thus the therapeutic potential of (in)GSH may not be limited to PD. ■

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Cerebellar Transcranial Direct Current Stimulation in Patients With Ataxia: A Double-Blind, Randomized, Sham-Controlled Study

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ABSTRACT

Background and Objective: Numerous studies have highlighted the possibility of modulating the excitability of cerebellar circuits using transcranial direct current stimulation. The present study investigated whether a single session of cerebellar anodal transcranial direct current stimulation could improve symptoms in patients with ataxia.

Methods: Nineteen patients with ataxia underwent a clinical and functional evaluation pre- and post-double-blind, randomized, sham, or anodal transcranial direct current stimulation.

Results: There was a significant interaction between treatment and time on the Scale for the Assessment and Rating of Ataxia, on the International Cooperative Ataxia Rating Scale, on the 9-Hole Peg Test, and on the 8-Meter Walking Time ($P<0.001$). At the end of the sessions, all performance scores were significantly different in the sham trial, compared to the intervention trial.

Conclusions: A single session of anodal cerebellar transcranial direct current stimulation can transiently improve symptoms in patients with ataxia and might represent a promising tool for future rehabilitative approaches.

Key Words: cerebellar ataxia, transcranial direct current stimulation, cerebellar stimulation

Cerebellar ataxias (CAs) represent a heterogeneous group of disabling disorders characterized by ataxia of gait, limb dysmetria, oculomotor deficits, dysarthria, and kinetic tremor.[1]

At the present time, the majority of CAs lack effective therapeutic strategies. Several intervention trials with neuroprotective agents or with intra-arterial infusion of autologous mesenchymal stem cells did not lead to exhaustive conclusions.[2-4] Thus, there is a compelling demand to find novel therapeutic approaches to reverse cerebellar motor deficits or amplify the effects of motor rehabilitation in this group of disorders.

The field of cerebellar stimulation with transcranial magnetic stimulation and transcranial direct current stimulation (tDCS) is recently gaining much attention in the scientific community, in particular, because these stimulation techniques are noninvasive, provide novel information on cerebellar physiology, and promote neural plasticity.[5] In particular, cerebellar tDCS consists in the application of a low-intensity (1-2 mA) steady current through a surface scalp electrode over the cerebellum, which has been demonstrated to elicit changes in cerebellar excitability in a polarity-specific manner.[6] Anecdotal case reports have demonstrated the role of tDCS stimulation over the motor cortex in improving gait symmetry in patients with CA[7] and the effect of cerebellar tDCS in modulating locomotor adaptation in healthy subjects.[8]

All the above observations defined the object of this work, aimed at assessing the effects of a single session of anodal cerebellar tDCS on clinical performance in patients with ataxia.

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Red Blood Cell Glutathione Following Intranasal Reduced Glutathione Administration

Mischley LK, White CC, Rosenfeld M, Szymczak E, Kavanagh T, Standish LJ

Abstract

Background: Glutathione depletion is associated with Parkinson's disease (PD) pathophysiology and glutathione augmentation has been proposed as therapeutic strategy for over three decades. Recently, a Phase I/IIa study of intranasal reduced glutathione, (in)GSH, was shown to be safe and tolerable in individuals with PD. A secondary aim the Phase I study was to evaluate whether 3 months of (in)GSH impacted RBC glutathione concentrations, in order to better understand absorption characteristics of (in)GSH.

Methods: Using stored red blood cell (RBC) from the Phase I study, RBC glutathione concentrations were quantified utilizing fluorometry. The mean total glutathione score and standard deviations (SD) were compared across treatment arms.

Results: The mean baseline concentration of RBC glutathione was 3.76 $\pm$ 0.72 , 4.48 $\pm$1.00, and 4.06 $\pm$1.41 nmol/mg protein in the placebo, 300 mg (in)GSH/ d, and 600 mg (in)GSH/d groups, respectively. After three months of thrice daily dosing, the mean RBC glutathione concentrations were not different between groups: 3.68 $\pm$1.28, 5.00 $\pm$2.30, and 3.45 $\pm$ 0.50 nmol/mg protein in the placebo, 300 mg (in)GSH/ d, and 600 mg (in)GSH/d, respectively.

Discussion: There is no evidence that this dose or form of exogenously administered glutathione raises RBC glutathione concentrations. Our results support data suggesting small polar molecules do not enter peripheral circulation, as lipophilic molecules have been shown to do. Subsequent studies should evaluate whether (in)GSH is capable of reaching the central nervous system at all, and if so, by what mechanism.

Background

GSH is a primary antioxidant for the central nervous system (CNS), where it directly detoxifies radicals in non-enzymatic reactions and enzymatically as a substrate for various peroxidases. It is essential for cellular detoxification, and glutathione depletion has been shown to contribute to alpha-synuclein aggregation, mitochondrial insufficiency, dopamine depletion, and cell death.[1] The loss of reduced glutathione (GSH) is the most consistently reported alteration in the antioxidant defense system in PD.[2-5]

Incidental Lewy body disease (iLBD) is considered a preclinical precursor of PD, in which alpha-synuclein accumulation and dopamine depletion are identified postmortem.[6] PD and iLBD exhibit GSH concentrations that are reduced by approximately 40-50% in the substantia nigra, suggesting glutathione loss precedes clinical pathology. Glutathione levels are similarly altered in other neural degenerative disorders, e.g. progressive supranuclear palsy and multiple system atrophy.[7] These data have led to the suggestion that GSH augmentation has potential as a therapeutic strategy in PD and possibly other neurodegenerative diseases.

In postmortem studies of PD brains versus controls, there is an early depletion of glutathione, as well as an increase in lipid peroxidation, as well as protein and DNA oxidation. (The latter is thought to be due, in part, to the former.) Glutathione serves many roles: an antioxidant capable of reducing superoxide radicals, hydroxyl radicals, and peroxinitites, as a storage form of cysteine, it maintains sulfhydryl proteins in a reduced state, recycles other antioxidants, e.g. vit C, vit E, and serves as a substrate for glutathione peroxidases and glutathione S-transferases.

There is no blood-brain barrier at the olfactory plate. Intranasal administration of therapeutics targeting the CNS are thought to enter CNS via several mechanisms, from direct cellular uptake with active and passive transporters, paracellular uptake, via the rich vasculature of the sinuses into systemic circulation, and swallowed into the gastrointestinal tract. Small, polar molecules, like glutathione, are thought to bypass peripheral circulation, which rapidly take up lipophilic molecules, and enter the CNS directly.[8, 9] Swallowed glutathione is thought to be poorly absorbed.[10] At the time of study initiation, the technology did not exist to non-invasively measure brain concentrations of glutathione, the ideal outcome measure. Thus, the total concentrations of red blood cell (RBC) glutathione were measured over the course of the study to evaluate whether there was a change in RBC glutathione in active arms over placebo, and if so, if the change was dose-dependent. The hypothesis was that GSH, a polar molecule, would bypass peripheral circulation and not substantially raise RBC concentrations.

Methods

In 2013, this team completed a phase I study of intranasal reduced glutathione, (in)GSH, or placebo in 30 indivduals with PD. The study intervention consisted of a 1 cc syringe pre-loaded with study medication compounded fresh monthly by Key Compounding Pharmacy (Federal Way, WA, USA) and administered using a Teleflex Mucosal Atomization Device (MAD) tip (Limerick, PA). Study participants were instructed to spray 1 cc of placebo (sterile saline), 100 mg GSH, or 200 mg GSH intranasally three times daily, for a total daily dose of 300 mg (in)GSH or 600 mg (in)GSH in the two active arms of the study. All cohorts administered more than 80% of their medication. Due to the small sample size, the decision was made *a priori* to exclude from the analysis anyone who did not complete the study (n=2).

The glutathione concentration of red blood cells (RBC) is substantially higher than in serum. Immediately following the draw, the red blood cells were separated and the samples were preserved according to protocol described for serum by Sakhi et al. (2006) [11] and stored in triplicate in 100 mL aliquots at minus 80. Samples were transported on dry ice to the University of Washington Department of Environmental and Occupational Health Sciences for analysis.

Upon thawing, 100 uL 30mM HEPES buffer pH 8 was added to each 100 uL aliquot of RBC sample, and triturated to increase the buffering capacity of the sample and restore it to a liquid form. 400 uL M-per was added to each sample and gently shaken for 15 minutes in an oscillating rocker.

Each well should contain a total volume of 195 mL:
25 µL sample
30 µL NEM/ KOH
10 µL TCEP
100 µL 0.1 NaOH
10 µL NDA
+ 20 µL H20
195 µL

Figure 1: Well composition

Samples were then refrozen in a ethanol/dry ice bath for two minutes, and rethawed on a rocker for 10 minutes. The cells were examined under a microscope and observed to be completely lysed. Samples were then acidified by adding a 1:1 ratio 10% sulfosalicyclic acid to Sample and placed on ice for 10 minutes to enhance protein precipitation. Following protein precipitation the samples were centrifuged at 14000 RPM at 4C for 3 minutes and placed on ice while standards were being prepared.

25 µL sample, or standard, was added to a 96 well plate in triplicate. 30 uL 0.2 NEM/ 0.2 KOH buffer was added for optimal TCEP reduction, bringing the pH to ~ 7.5. To reduce the GSSG to GSH, 10 µL 10mM TCEP was added each well and incubated at room temperature for 15 minutes.

100 µL 0.1N NaOH was then added, bringing the pH to 12 for optimization and stabilization of the NDA/GSH conjugate. 20 µL deionized water and 10 µL 10 mM naphthalene

dicarboxyaldehyde were added. The plate was sealed and incubated in the dark at room temperature for 30 min. The samples were then read at 472 nm ex/ 528 nm emission on a Molecular Devices SpectraMax Gemini XS fluorescence plate reader. Total GSH concentrations in nMoles GSH/mg protein were calculated utilizing SpectraMax and Excel software.

Results

Two subjects withdrew from the study after beginning therapy and were eliminated from the dataset (N=28), one due to a scheduling conflict and one due to an adverse reaction to study medication. The characteristics of the participants who initiated and completed the study are presented in Table 1.

Table 1: Participant Demographics.

	Total (N=29)	**Group1 (n=9)**	**Group2 (n=10)**	**Group3 (n=10)**
Gender				
Male	16 (55%)	7 (78%)	5 (50%)	6 (60%)
Female	13 (45%)	2 (22%)	5 (50%)	4 (40%)
Age, years Median (range)	64.4 (41-83)	65.6 (58-73)	63 (41-83)	64.8 (53-80)
Ethnicity				
Hispanic	2 (7%)	0	2 (20%)	0
Non-Hispanic	27 (93%)	9	8 (80%)	10 (100%)
Race				
White	28 (97%)	8 (89%)	10 (100%)	10 (100%)
Native American	1 (3%)	1 (11%)	0	0
Hoehn & Yahr Stage Median (range)	2.5 (1-3)	2 (1.5-3)	2.5 (1-3)	1.5 (1-3)
MoCA Score	27.1 (25-30)	26.1 (25-29)	27.9 (25-30)	27.1 (25-29)

There was no statistically significant increase in RBC glutathione levels over the course of the three study months. There was no dose-response effect, and no trend toward improvement in the active arms over the placebo arm. (Figure 2, Table 2)

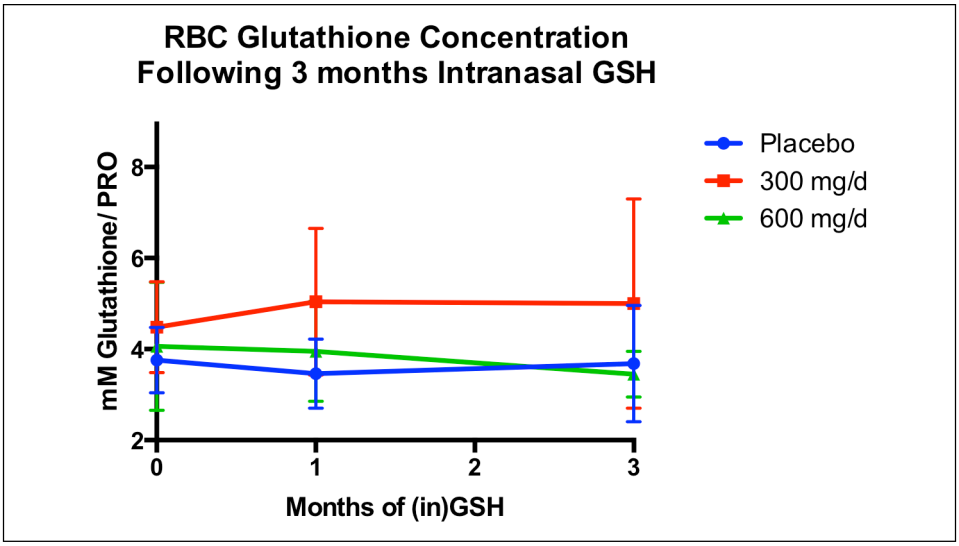

Figure 2. Change in red blood cell glutathione concentrations following administration of (in)GSH for three months in the Phase I Study of (in)GSH.

Table 2: Red blood cell glutathione concentration (nMoles/mg protein) following three months (in)GSH or placebo.

	Baseline	1 mo	3 mo	P-value (95% CI)
Placebo (n=9)	3.76 (0.72)	3.46 (0.76)	3.68 (1.28)	P=0.9553 (-1.296, 1.282)
300 mg/d (n=8)	4.48 (1.00)	5.04 (1.61)	5.00 (2.30)	P=0.4952 (-1.678, 1.969)
600 mg/d (n=8)	4.06 (1.41)	3.96 (1.10)	3.45 (0.50)	P=0.1041 (-0.6501, 0.2301)

Discussion

That RBC glutathione concentrations did not increase over the three month study could be interpreted several ways. First, the concentrations of GSH administered were insufficient to raise RBC levels. A second possibility is that intranasal glutathione, being hydrophilic, bypasses the vasculature and is taken up directly into the CNS. The lack of RBC glutathione augmentation

does not negate the value of (in)GSH as a therapeutic, since there is no evidence that RBC glutathione is necessary for therapeutic efficacy.

It is possible that the glutathione administered acts locally and is utilized by the nasal epithelium or that (in)GSH directly enters the CNS via a route other than circulation. Just as the liver affords protection to oral environmental exposures, the nasal mucosa provides a similar phase I and phase II detoxification system within the respiratory epithelial cells. It is well-established that the detoxification processes of the liver, namely phase II detoxification via glutathione s-transferase (GST), are highly dependent on glutathione; the cells of the nasal epithelium actually contain *more* glutathione than hepatocytes.[ ] As oral glutathione supports hepatic detoxification, so might intranasal glutathione support local nasal epithelial and olfactory cell detoxification.

The rich vasculature of the nasal mucosa has been tremendously useful in the delivery intranasal therapeutics. Lipophilic molecules can easily pass through endothelial cells and into peripheral circulation; as a result, lipid moieties are being added to molecules to enhance intranasal uptake. Small, polar molecules, like glutathione, are unable to pass through the lipid bilayer and are refused entry into the circulatory system. Instead, the fate of hydrophilic molecules less than 1000 Daltons is to pass through the tight junctions of the paracellular space. The smaller the molecule, the more easily this transport mechanism occurs. That glutathione is a small, polar molecule of 307 Da suggests it is likely to easily pass thought the tight junctions.

While biomarkers of glutathione status have the potential improve clinical research, the most important consideration is whether exogenous administration improves clinical outcomes. Future studies should aim to evaluate the ability of (in)GSH, intravenous GSH, and oral glutathione and

N-acetyl cysteine for their ability to reach CNS target tissue and affect clinically meaningful change.

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Glutathione Deficiency in Parkinson's Disease: Future Research

Laurie K Mischley

(in)GSH as a Therapeutic in PD

The FDA has two indications for which they approve PD therapeutics, "Symptomatic" or "Disease-Modifying." There are currently no FDA approved disease-modifying therapies in PD. In the almost 200 years since the disease was first described, we have not identified a single intervention capable of slowing disease progression. There are many FDA-approved interventions that conceal some of the symptoms of PD, but the disease continues to progress as the need for dopamine fortification increases.

The study design of therapies with both symptomatic and disease-modifying potential requires special attention. Study designs such as delayed-start or wash-out have been employed as a way of teasing out symptomatic vs. disease-modifying effects. These studies are more expensive and statistically challenging, and require a substantial amount of effort in the early stages. There have now been four intervention studies of exogenous GSH, all of which have demonstrated a symptomatic improvement.[1-4] Thus, future (in)GSH intervention trials should focus on whether the improvements seen are sustained more than three months and are superior to placebo, a description of the symptoms most improved, and whether the use of (in)GSH is disease modifying.

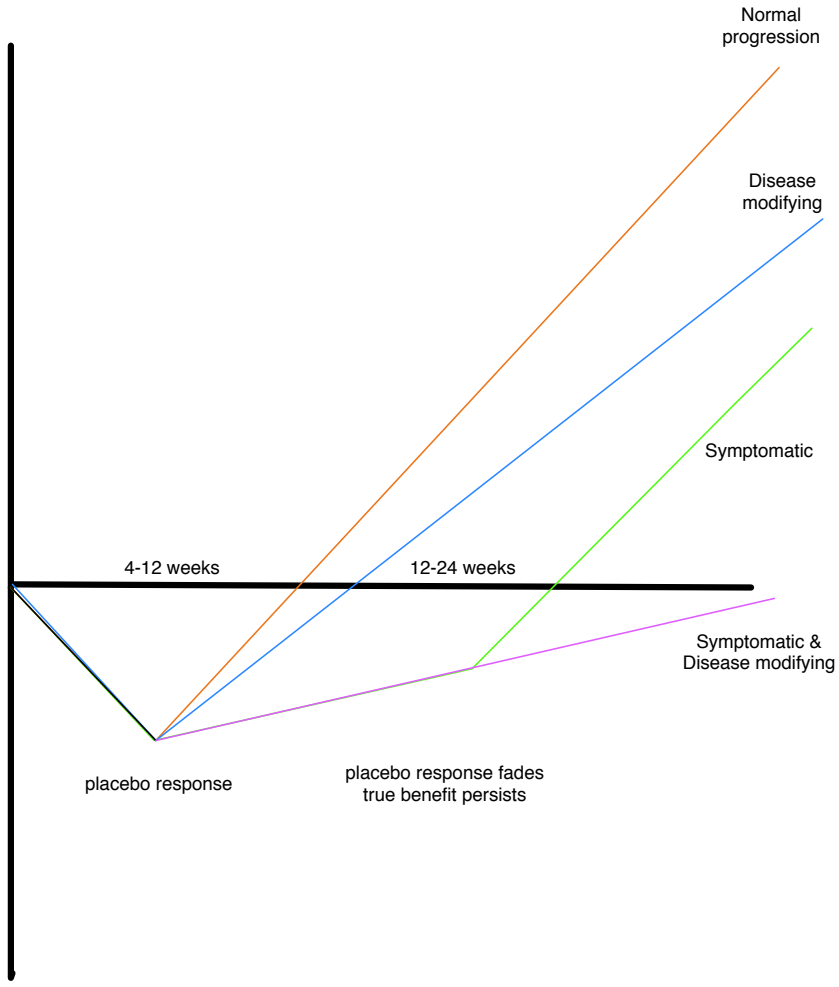

Figure 1: Anticipated response curve of PD therapeutics with disease-modifying and/ or symptomatic effects for any clinical outcome measure.

The novelty of the intervention alone may increase dopamine synthesis and it is notable that our placebo group had a robust response. Dopamine is a key neurotransmitter involved in emotions of 'hope' and 'enthusiasm' and the symptomatic effects of placebo response in PD have been described.[5] Future studies should take into consideration the potential therapeutic effect of (in)saline or some other aspect of study participation. It has been previously described that the more intensive the intervention, the stronger the placebo response.[6] It can be anticipated that the act of administering (in)GSH would elicit a more substantial result than taking an oral medication. Non-pharmacological interventions, such

as yoga, have been shown to improve GSH status. Future studies using MRS as an outcome measure should include a placebo arm to evaluate whether (in)saline boosts MRS glutathione.

In the recent Phase 2b, the highest dose cohort of (in)GSH met *a priori* statistical endpoints for improvements in UPDRS Total and Motor subscore (unpublished data). Future research should evaluate whether more frequent dosing improves outcomes further. Consideration should be given to the MRS glutathione peak following (in)GSH administration. We have demonstrated 200 mg/ ml (in)GSH, administered supine with a Mucosal Atomization Device (MAD) tip, boosted brain GSH by > 200% for up to an hour. The next step will be to describe how long the augmentation persists and what happens with repeated exposures. These data will be used to inform the dosing schedule for the Phase III clinical trial.

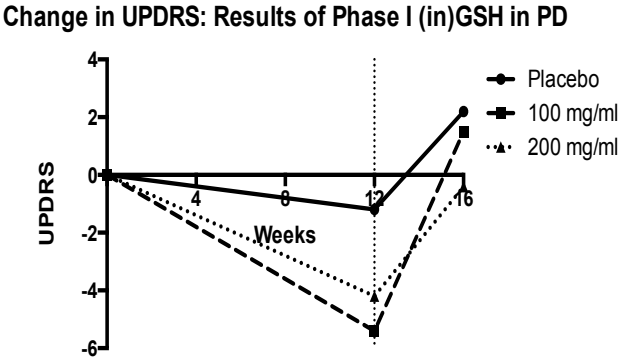

Figure 2: Normalized change in the Unified PD Rating Scale (UPDRS) in response to 3 months of (in)GSH or placebo.

The current formula being used in the ongoing trials has numerous limitations. GSH oxidizes quickly in response to heat, light, and air, in life, patients are unable travel with an item that must be refrigerated. Strategies for improving product stability are important. The

current product does not have a patent; in the public domain there is little incentive to invest in the current formula. The current formula is being produced by a compounding pharmacy, it is unclear whether all compounding pharmacies are capable of generating a comparable product, the impact of GSH supplier, and many other questions will need to be answered in the process of making this therapy available to individuals with PD.

Blood and MRS Glutathione as a Biomarker of PD

Biological assessments are not employed in the routine care of individuals with PD, in the current medical model. Data presented here suggest whole blood total glutathione (Glu) is associated with PD severity. The higher the blood Glu, the lower the Unified PD Rating Scale (UPDRS) and Patient Reported Outcomes (PRO-PD) scores. The next step will be to repeat this analysis in a larger sample of individuals with PD; controls should be included to obtain age-matched reference ranges. Should these data be reproducible, there are implications for the interpretation of future clinical trials, e.g. adjustments for blood GSH, as well as implications for clinical practice. Whole blood Glu is a clinically available measure through specialty laboratories. Future efforts should evaluate the clinical utility of periodic measurements of Glu in patients with PD.

Magnetic resonance spectroscopy (MRS) was able to detect short-term increase in brain Glu following (in)GSH administration. Future studies are in development using MRS to describe the pharmacokinetics of CNS Glu over the course of 6 + hours. Whether the FDA will accept MRS as a measure of equivalence as new versions of GSH augmentation become available is yet to be determined. For instance, if a new product is able to show a similar

MRS spike following administration, can they skip early clinical trials? Because MRS Glu did not decrease with age or disease severity in a cross-sectional analysis of 30 individuals with PD, it is not anticipated that MRS Glu will be an effective screening tool or biomarker for progression.

GSH Content of Food

It has been 25 years since the GSH content of food was comprehensively analyzed.[7] During these past few decades, there has been a substantial shift in farming practices and dietary patterns in the US. Analysis of GSH content of common foods should be repeated and efforts should be make to evaluate the degree to which dietary GSH impacts total blood Glu. A large cross-sectional study is a convenient place to begin, although the question more pertinent to public health is whether increased consumption of GSH-containing foods increases blood Glu content, and thus public health. If dietary GSH and blood GSH are correlated, intervention studies prescribing a 'high-GSH diet' are indicated. Anticipated responses in whole blood Glu or clinical outcomes have yet to be determined.

Defining a Glutathione Deficiency Syndrome (GDS)

Glutathione (GSH) is essential for human health and function, and complete absence is incompatible with life. GSH can be endogenously synthesized and is fortified by diet. Little is known about the GSH content of the modern diet, or whether the GSH content of the diet influences risk of disease. GSH Deficiency Syndrome (GDS) is a metabolic state defined by either a shift in GSH:GSSG or an absolute reduction in tissue or circulating GSH. Clinical signs and symptoms of GDS have yet to be fully described; the tremendous diversity of

roles and requirements for GSH, and variously-functioning compensatory systems, will influence which systems or physical regions are most susceptible to GDS. The risk of GDS increases with age, as endogenous production declines. Certain disease states, e.g. Parkinson's disease, schizophrenia[8], HIV[9], may confer unique demands for exogenous supply. Whole blood and MRS GSH both have potential as a GSH biomarker, although reference ranges have yet to be described. Whether GDS contributes to the cause of PD, or is the results of having PD, is not relevant to the patient-centered question, "Can GSH fortification improve outcomes?"

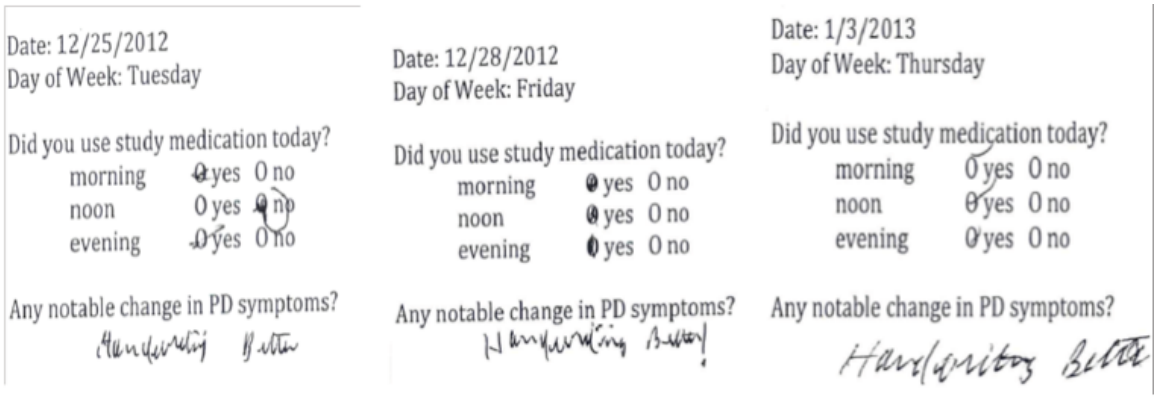

Figure 3: Journal entry reporting improved handwriting from a participant assigned 300 mg/ d (in)GSH in the Phase I/IIa study.

During a Stage I nutritional deficiency, physiologic function continues normally while body stores are depleted. In the brain, it is anticipated the astrocytes, the reservoir for Glu, would be among the earliest cells to be depleted. Peripherally circulating GSH may also serve as a reservoir for GSH status, identifying those with inadequate GSH status. During Stage 2 deficiency, the depletion of body stores results in biochemical perturbations. There is ample evidence in PD models that GSH depletion is associated with increased mitochondrial dysfunction, inflammation, ROS/ RNS stress, and cell death, although no biochemical perturbations associated with GDS have been described in individuals with PD. Future trials

should evaluate whether clinically available measures of inflammation or redox status are associated with reductions in GSH. During State 3 deficiency, clinical symptoms become apparent. If some of the damage due to GSH deficiency in irreversible, it might be anticipated that GSH supplementation will not reverse symptoms entirely. Those symptoms that do improve in response to GSH should be considered symptoms of GSH deficiency, and identifying those particular symptoms should be the focus of subsequent intervention trials.

The United States Department of Agriculture does not consider GSH an essential nutrient. Here, sufficient data are presented to support the hypothesis that individuals with PD are unable to endogenously synthesize GSH in sufficient amounts. The depletion of whole blood GSH is associated with PD severity, and all four studies of exogenously administered GSH in PD have demonstrated symptomatic improvement. Immediate goals include identification of community-based reference ranges for whole blood glutathione, prevalence of deficiency across populations (e.g. elderly, cancer, mentally ill), and whether augmentation improves outcomes in diseases other than PD. Subsequent (in)GSH trials in PD will explore alternative formulas, and focus on distinguishing symptomatic from disease-modifying effects of (in)GSH in a larger, multi-center trial employing motor and non-motor outcome measures. Three decades since GSH deficiency was first hypothesized to contribute to PD progression, there are sufficient data to conclude GSH depletion does play a role in PD pathophysiology. It has yet to be determined whether (in)GSH fortification strategies are able to result in sustainable symptomatic relief and/ or disease modification over time.

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