Effects of a Novel Oral Glutathione Supplement formulated in a Charged Mucosa Binding Liposome Suspension on Serum GSH and Systemic Oxidative Stress Biomarker GSH/GSSG in Humans

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Nov, 2016

Abstract

A novel liquid-based oral glutathione supplement utilizing charged mucosa binding liposomes in a reduced oxygen environment was evaluated for serum GSH (reduced glutathione) and systemic oxidative stress biomarker GSH/GSSG (reduced/oxidized glutathione) levels in four human volunteers. The supplement was taken orally by three of the participants, and applied topically by one participant. Human serum GSH and GSSG levels were sampled initially before ingestion for baseline, and three times over eight hours after ingestion or application of the supplement. The participants continued to take the supplement once every morning at least 30 minutes before meals for four weeks. The participants were sampled once each week during the four week study period to collect data for long-term effects. Every participant experienced a short-term increase in serum GSH of approximately thirty percent, and all participants demonstrated a long-term progressive reduction of oxidized glutathione GSSG of approximately thirty percent. GSH and GSSG results were reported in absolute and ratiometric bar chart form. The ratio of GSH/GSSG known as the redox ratio increased short and long term. This redox ratio is an important and well-understood marker for cellular and systemic oxidative stress. The results indicated that the glutathione delivered by the supplement was immediately bioavailable, unlike other pill and capsule delivery forms, and provided a long-term reduction in systemic cellular oxidative stress.

Introduction

Glutathione (GSH) is often referred to as the body's master antioxidant. Composted of three amino acids - cysteine, glycine, and glutamate - glutathione can be found in virtually every cell of the human body. The highest concentration of glutathione is in the liver, making it critical in the body's detoxification process.

Glutathione is also an essential component to the body's natural defense system. Viruses, bacteria, heavy metal toxicity, radiation, certain medications, and even the normal process of aging can all cause free-radical damage to healthy cells and deplete glutathione. Glutathione depletion has been correlated with lower immune function and increased vulnerability to infection due to the liver's reduced ability to detoxify. As the generation of free radicals exceeds the body's ability to neutralize and eliminate them, oxidative stress occurs. A primary function of glutathione is to alleviate this oxidative stress.

Biochemistry, Metabolism

Reduced glutathione (GSH) is a linear tripeptide of L-glutamine, L-cysteine, and glycine. Technically N-L-gamma-glutamyl-cysteinyl glycine or L-glutathione, the molecule has a sulfhydryl (SH) group on the cysteinyl portion, which accounts for its strong electron-donating character.

As electrons are lost, the molecule becomes oxidized, and two such molecules become linked (dimerized) by a disulfide bridge to form glutathione disulfide or oxidized glutathione (GSSG). This linkage is reversible upon re-reduction.

GSH is under tight homeostatic control both intracellularly and extracellularly. A dynamic balance is maintained between GSH synthesis, it's recycling from GSSG/oxidized glutathione, and its utilization.

GSH synthesis involves two closely linked, enzymatically-controlled reactions that utilize ATP. First, cysteine and glutamate are combined by gamma-glutamyl cysteinyl synthetase. Second, GSH synthetase combines gamma-glutamylcysteine with glycine to generate GSH. As GSH levels rise, they self-limit further GSH synthesis; otherwise, cysteine availability is usually rate-limiting. Fasting, protein-energy malnutrition, or other dietary amino acid deficiencies limit GSH synthesis.

GSH recycling is catalyzed by glutathione disulfide reductase, which uses reducing equivalents from NADPH to reconvert GSSG to 2GSH. The reducing power of ascorbate helps conserve systemic GSH.

GSH is used as a cofactor by (1) multiple peroxidase enzymes, to detoxify peroxides generated from oxygen radical attack on biological molecules; (2) transhydrogenases, to reduce oxidized centers on DNA, proteins, and other biomolecules; and (3) glutathione S-transferases (GST) to conjugate GSH with endogenous substances (e.g., estrogens), exogenous electrophiles (e.g., arene oxides, unsaturated carbonyls, organic halides), and diverse xenobiotics. Low GST activity may increase risk for disease—but paradoxically, some GSH conjugates can also be toxic.

Direct attack by free radicals and other oxidative agents can also deplete GSH. The homeostatic glutathione redox cycle attempts to keep GSH repleted as it is being consumed. Amounts available from foods are limited (less that 150 mg/day), and oxidative depletion can outpace synthesis.

The liver is the largest GSH reservoir. The parenchymal cells synthesize GSH for P450 conjugation and numerous other metabolic requirements—then export GSH as a systemic source of SH-reducing power. GSH is carried in the bile to the intestinal luminal compartment. Epithelial tissues of the kidney tubules, intestinal lining and lung have substantial P450 activity—and modest capacity to export GSH.

GSH equivalents circulate in the blood predominantly as cystine, the oxidized and more stable form of cysteine. Cells import cystine from the blood, reconvert it to cysteine (likely using ascorbate as cofactor), and from it synthesize GSH. Conversely, inside the cell, GSH helps re-reduce oxidized forms of other antioxidants—such as ascorbate and alpha-tocopherol.

Mechanism of Action
GSH is an extremely important cell protectant. It directly quenches reactive hydroxyl free radicals, other oxygen-centered free radicals, and radical centers on DNA and other biomolecules. GSH is a primary protectant of skin, lens, cornea, and retina against radiation damage and other biochemical foundations of P450 detoxification in the liver, kidneys, lungs, intestinal, epithelia and other organs.

GSH is the essential cofactor for many enzymes that require thiol-reducing equivalents, and helps keep redox-sensitive active sites on enzyme in the necessary reduced state. Higher-order thiol cell systems, the metallothioneins, thioredoxins and other redox regulator proteins are ultimately regulated by GSH levels—and the GSH/GSSG redox ratio. GSH/GSSG balance is crucial to homeostasis—stabilizing the cellular biomolecular spectrum, and facilitating cellular performance and survival. GSH and its metabolites also interface with energetics and neurotransmitter syntheses through several prominent metabolic pathways. GSH availability down-regulates the pro-inflammatory potential of leukotrienes and other eicosanoids. Recently discovered S-nitroso metabolites, generated in vivo from GSH and NO (nitric oxide), further diversify GSH's impact on metabolism.

Experimental Procedure

Four persons, one male and three females ranging in age from 23 to 83 years old agreed to participate. Each participant was assigned a number from #1 to #4:

Participant	Age
#1 Female	61 years old
#2 Male	55 years old
#3 Female	23 years old
#4 Female	83 years old

Supplement Formulation The supplement consisted of a 99.9% reduced glutathione (GSH) powder solubilized in a novel deoxygenated water and encapsulated in a charged plant-based phospholipid liposome structure using a proprietary method. The effective dose of each supplementation was 550mg GSH/4g solution. The supplement was packaged in an airless dispenser to protect it from oxygen degradation.

Short Term Evaluation Participants were allowed to eat the morning of first collection, but refrained from taking antioxidant dietary supplements during the course of the four-week study period. The participants' blood was collected for baseline measurements at approximately 8AM by veinous puncture, the serum extracted, processed and frozen for later analysis. After collection, the participants immediately consumed the supplement by dispensing 4ml of the supplement formulation into their mouth and swished for 15-20 seconds and swallowed. They

refrained from drinking for at least 15 minutes afterwards. The participant who used the product topically applied approximately the same amount onto the abdomen and soft areas under the arms. Blood samples were collected again at 2hrs, 6hrs, and 8hrs after first collection.

Long Term Evaluation The participants consumed the supplement one time per day in the morning for a 4 week period. Participants' blood was collected each week at exactly 7 day intervals for 4 weeks at approximately 10 AM. Participants were instructed to consume the supplement 4 hours prior to collection. The serum was extracted, processed and frozen for later analysis. All samples were assayed within 30 days of collection.

Analysis GSH and GSSG serum levels were assayed using the BioVision (Milpitas, Ca) Glutathione Fluorometric Assay Kit (GSH, GSSG, and Total) k264. Wasatch Scientific Laboratories (Murray, Ut) was contracted to perform the assay using the BioVision fluorometric kit and method.

Whole blood samples were collected and immediately centrifuged to separate the serum from the red blood cells and heavier components. Approximately 120µl of serum was added to 40µl of an ice-cold proprietary perchloric acid PCA buffer in a 1ml aliquot, vortexed, and stored on ice for 5 minutes. Then the mixture was centrifuged at 13,000G for 2 minutes, and the supernatant was collected and frozen at -60degC.

The assay for the Short Term Test (first test) and Long-Term Test (second test) utilized their own separate BioVision Glutathione Fluorometric Assay Kit. A standard curve for GSH and GSSG was created, and then the prepared serum samples were tested at two different dilutions to determine the optimal dilution for the best dynamic resolution of the assay. Then samples were processed and assayed in duplicate pairs at the chosen dilution. The results of each pair were reviewed and compared for repeatability and best-fit samples were used.

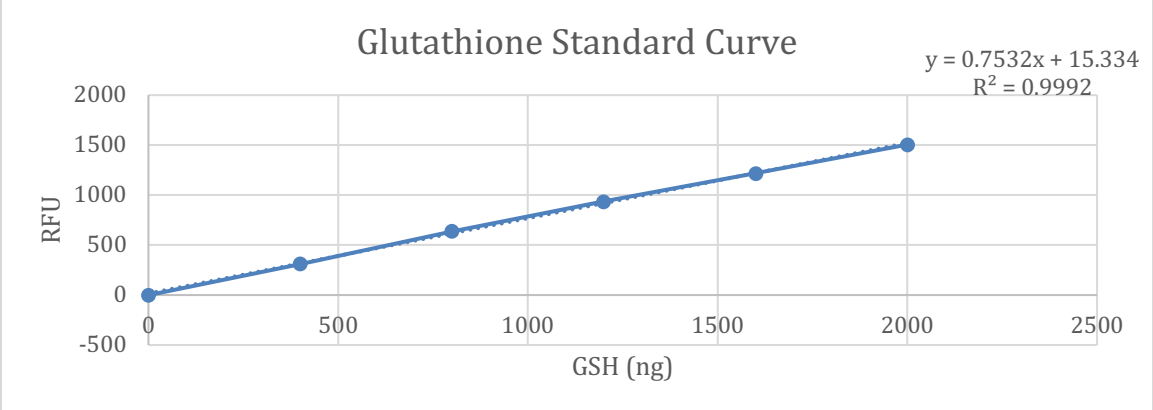

Fig. 1 GSH Standard Curve (Short-Term analysis)

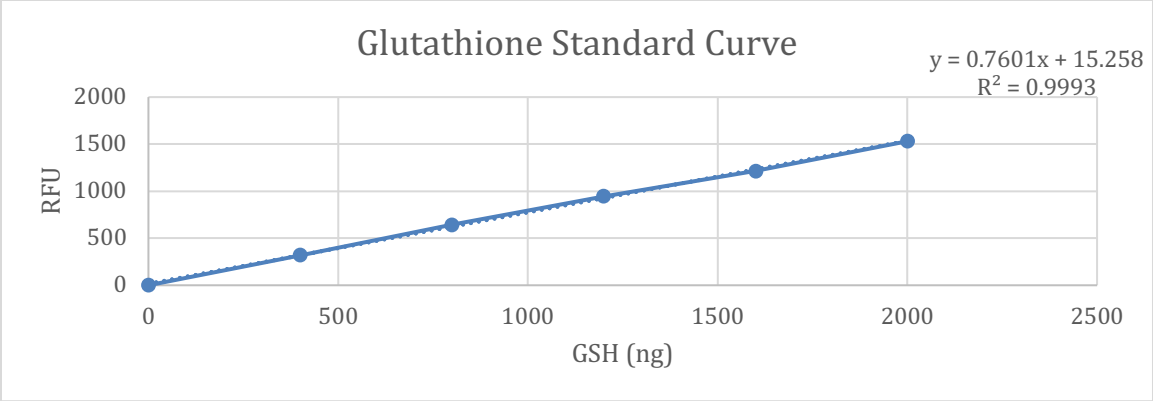

Fig. 2 GSH Standard Curve (Long-Term analysis)

Results

The results of two separate assays, Short-Term Test and Long-Term Test, were compiled and displayed in tabular and graphical form in an Excel spreadsheet. In addition, ratiometric results of GSH/GSSG were created for each sample.

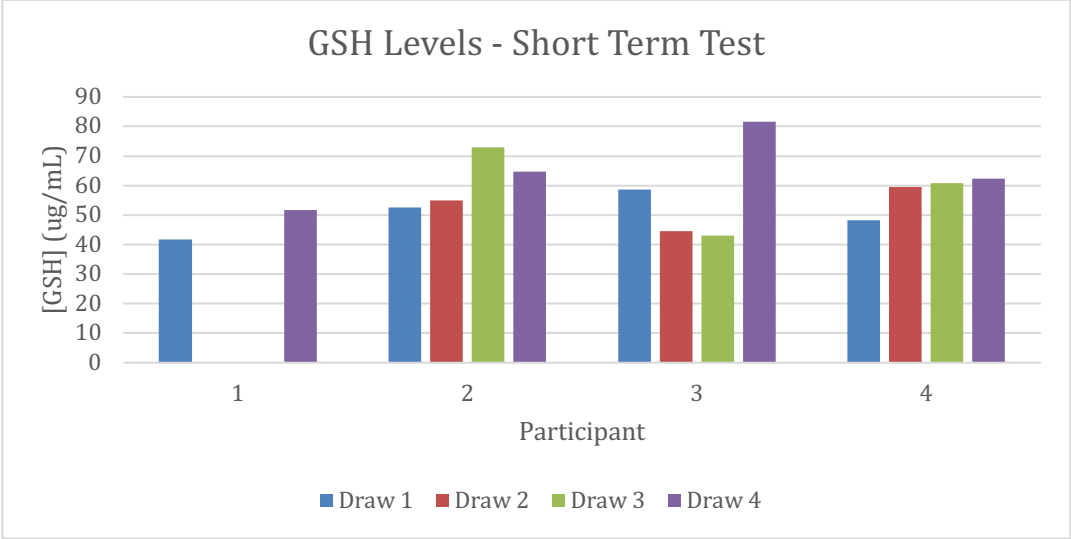

Fig. 3 GSH Levels Short-Term Test

Draw 1 = Baseline (B)
Draw 2 = B+4 hours
Draw 3 = B+6 hours
Draw 4 = B+8 hours

Short-Term Test Results

All participants in the Short-Term Test experienced an increase in serum GSH levels that appeared to peak approximately 6-8 hours after ingestion. The GSH increase was on average 30% above baseline levels. Participant #1 a female, applied the supplement topically and still experienced elevated GSH serum levels. This participant was not assayed at Draw 2 and 3 because of limitations of the number of sample wells in the initial assay, due to first run dilution test requirements.

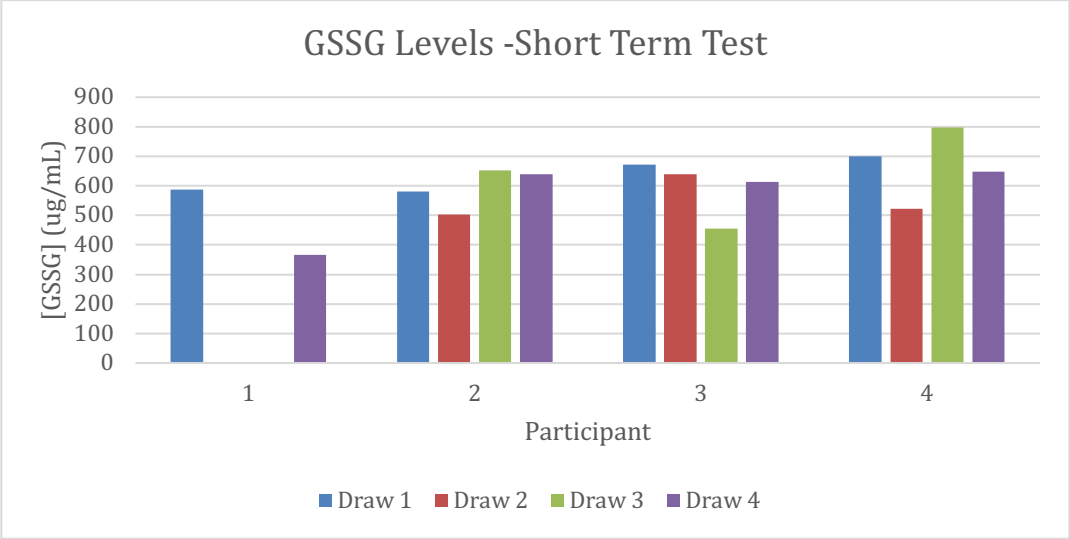

Fig. 4 GSSG Levels Short-Term Test

Note that GSSG levels in the Short-Term Test reported in Fig. 4 above, appeared to respond differently for each participant. The most significant change was an almost 50% reduction in participant #1 who applied the supplement topically. In participants #3 and #4, the GSSG levels on average reduced slightly from baseline.

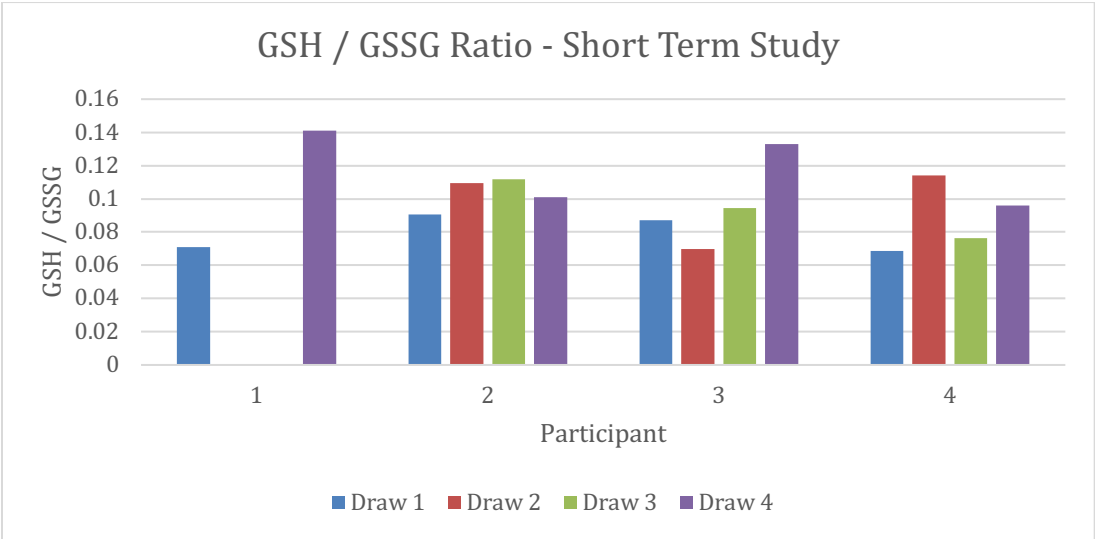

Fig. 5 GSH/GSSG Ratio – Short Term Study

However, Fig. 5 above showing the ratio of GSH/GSSG remarkably shows that the supplement provided a short-term antioxidant benefit. The GSH/GSSG redox ratio is an accepted standard to measure cellular oxidative stress. For this study, the most important feature is the redox ratio trend over time. After ingestion, every participant experienced an increase in the redox ratio compared to baseline, even after more then 8 hours after consuming or applying the supplement.

Long-Term Test Results

The Long-Term Test was designed to determine if a lasting or cumulative benefit of supplementation was evident. Once a week, immediately after the Short-Term Test for 4 weeks the participants were sampled, and human serum GSH and GSSG was assayed at the end of the trial. Samples were collected at the same time of day at 10AM for each blood draw. Note that Draw #1 – Week 1 results were not shown, due to an accidental thaw of the samples causing GSH oxidation.

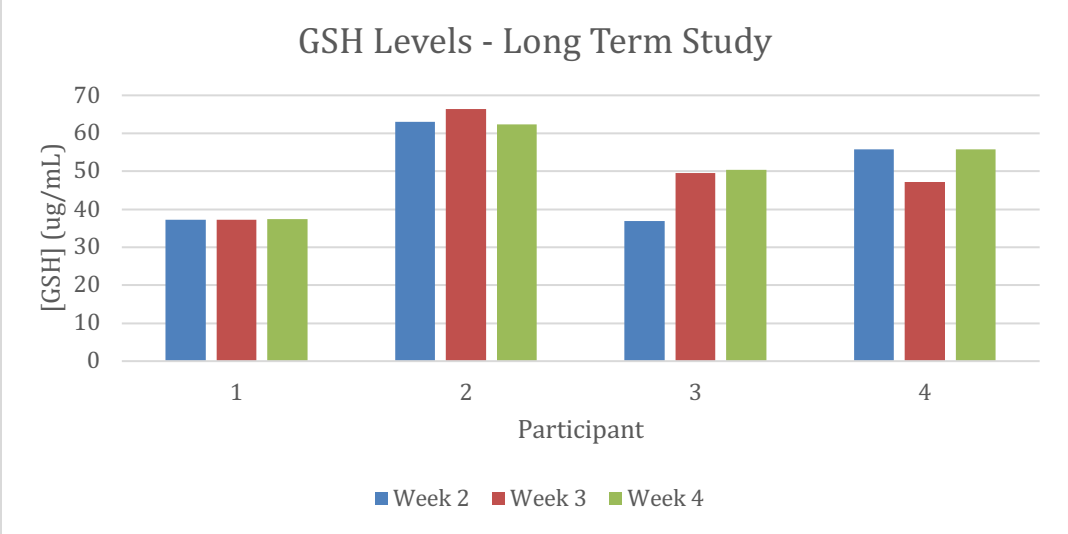

Fig. 6 GSH Levels – Long Term Study

Participants' GSH serum levels which are displayed above show mixed results. Participants #1 and #3 GSH levels are very close to their study baseline levels with no significant increase. Participants #2 and #4 produced a long-term increase of approximately 20-22%. Long-Term serum GSH levels when viewed alone showed positive results in in both of these participants, but unremarkable for participants #1 and #3. However, more importantly their GSSG levels indicated an important trend for all of the participants as demonstrated in figure 7 below.

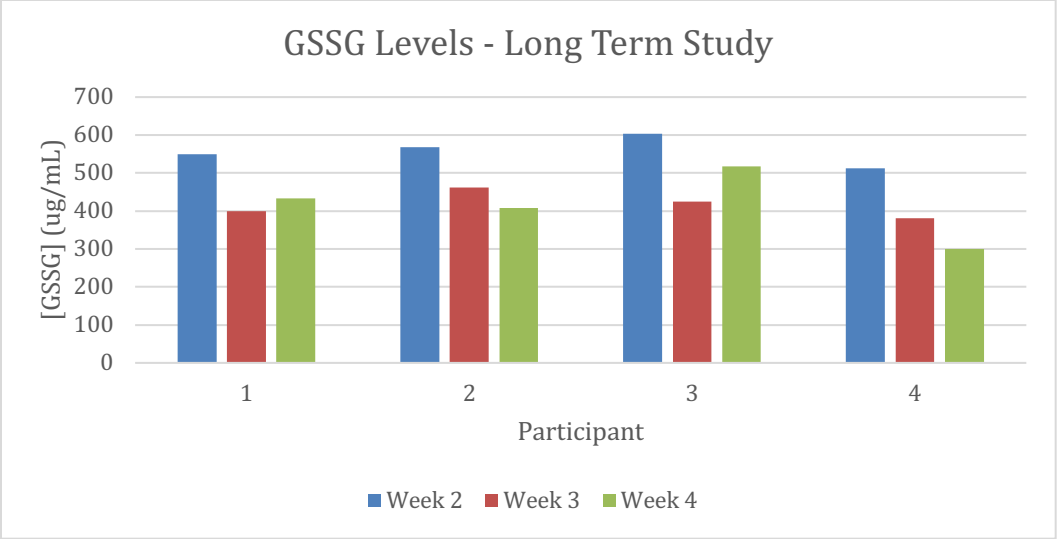

Fig. 7 GSSG Levels – Long Term Study

As shown in the figure above, all participants experienced a decrease in long-term oxidized GSSG serum levels. GSH is oxidized inside cells and converted to GSSG. Then a portion of the GSSG is shuttled out of the cells, into the intracellular space and into the blood. When the GSH/GSSG redox ratio is calculated, a true reduction in cellular oxidation is evident.

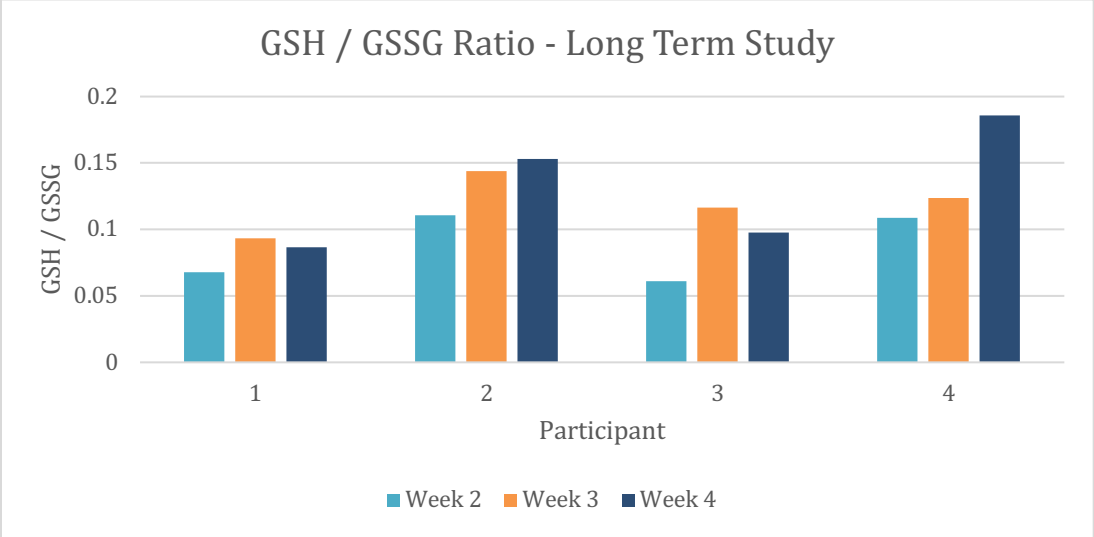

Fig. 8 GSH/GSSG Ratio – Long Term Study

The GSH/GSSG redox ratio increased for all participants indicating a sustained and cumulative decrease in cellular oxidative stress. It is interesting to note that participants #3 and #4 represent a wide age and health gap, female 23 years and healthy vs. female 83 years with health issues due to age respectively. The supplement had a positive long-term effect in both young and old, with the older participants experiencing a greater rise in the redox ratio. Also, the data shows that participant #1 received a redox benefit, even by applying the product topically. It

should be mentioned that this participant only applied about one third of the dose compared to the oral participants, not following specific dosing instructions. The results for all of the participants clearly show the benefits of the novel glutathione supplement as seen in the GSH/GSSG redox ratio.

Figure 9 below displays the GSH/GSSG ratio that has been normalized to the participant's baseline levels. This figure gives a truer comparison of the change in redox over time as a result of supplementation of the test product.

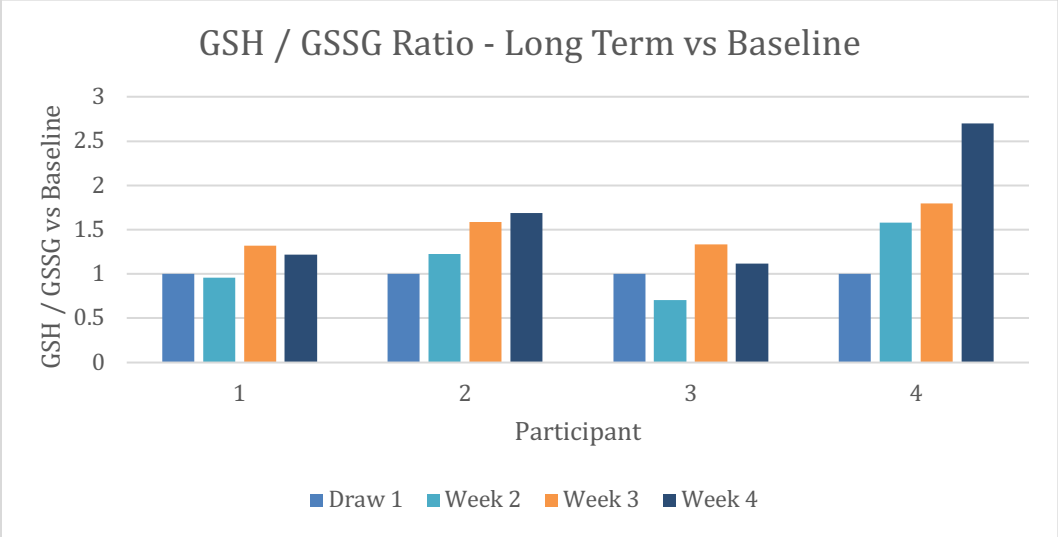

Fig. 9 GSH/GSSG Ratio – Long Term vs. Baseline

This figure reveals some interesting results. All participants had a net average increase above baseline redox ratio, with participants #2 (Male 55 years) and #4 (female 83 years) showing the greatest increase. It is anticipated that participant #1 (female 61 years) would have produced a higher result if the dosage requirements were followed more consistently.

Conclusion

The effects of the novel glutathione supplementation indicated a bioavailability of reduced glutathione and its' short-term and long-term effects as indicated in the serum concentrations of reduced glutathione GSH and oxidized glutathione GSSG. All participants produced a noticeable increase in GSH/GSSG redox ratio indicating a true reduction in cellular oxidative stress, with the older participants (age 55 – 83) producing higher ratios. This may be consistent with an assumption that the older participants may have had greater cellular oxidative stress to be reduced. However, it is interesting to note that the older participants (55 years +, excluding younger participant #3) had higher redox ratios at baseline.

The results of this study are particularly encouraging compared to a more comprehensive oral glutathione study[1] published in the Journal of Alternative and Complementary Medicine In 2011. This randomized, double blind, placebo controlled study administered 500mg 2X per day of reduced glutathione orally in capsule form to human participants. It measured GSH, GSSG, total glutathione, and two other inflammation marker assays. The study reported no antioxidant effects, no increase in GSH or decrease in GSSG, or reduction in measured inflammatory markers. It is interesting that the oral dose of reduced glutathione in that study was almost twice of the current study.

A greater statistical confidence in the results in the current study can be achieved if it is repeated with a greater number of participants and introducing placebo control. Another recommendation is to include other means of measuring oxidative stress and inflammation such as urinary Hydroxydexoyguanosine (8-OHdG) and urinary F2 isoprostanes (F2-isoP) assays.

[1] Allen, Jason and Ryan, Bradley D., *Effects of Oral Glutathione Supplementation on Systemic Oxidative Stress Biomarkers in Human Volunteers*. J Altern Complement Med. 2011 Sep; 17(9): 827–833.