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Research paper

Oral administration of γ-glutamylcysteine increases intracellular glutathione levels above homeostasis in a randomised human trial pilot study☆

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ABSTRACT

Objective: To determine if orally dosed γ-glutamylcysteine (γ-GC) can increase cellular glutathione (GSH) levels above homeostasis. Many chronic and age-related disorders are associated with down-regulation, or impairment, of glutamate cysteine ligase (GCL). This suggests that γ-GC supply may become limiting for the maintenance of cellular GSH at the normal levels required to effectively protect against oxidative stress and any resulting physiological damage.

Methods: GSH levels were measured in lymphocytes of healthy, non-fasting participants before and after single oral doses (2 and 4 g) of γ-GC. Blood samples were immediately processed using high speed fluorescence-activated cell sorting to isolate 10^6 lymphocytes that were then assayed for GSH content.

Results: A single 2 g dose of γ-GC increased lymphocyte GSH content above basal levels (53 ± 47%, $p < 0.01$, n=14) within 90 min of administration. A randomized dosage (2 and 4 g γ-GC) crossover design was used to explore the pharmacokinetics of this GSH increase. In general, for both dose levels (n=9), GSH increased from initial basal levels over 3 h (t_{max}) before reaching maximum GSH concentrations (C_{max}) that were near two (2 g γ-GC) to three (4 g γ-GC) fold basal levels (0.4 nmol/10^6 lymphocytes). Beyond t_{max}, GSH levels progressively declined reaching near basal levels by 5 h. The GSH half-life was between 2 and 3 h with exposure (AUC) to increased GSH levels of 0.7 (2 g γ-GC) and 1.8 (4 g γ-GC) nmol.h/10^6 lymphocytes.

Conclusions: Oral γ-GC is a non-toxic form of cysteine that can be directly taken up by cells and transiently increase lymphocyte GSH above homeostatic levels. Our findings that γ-GC can increase GSH levels in healthy subjects suggests that it may have potential as an adjunct for treating diseases associated with chronic GSH depletion. This trial was registered at anzctr.org.au as ACTRN12612000952842.

1. Introduction

All living organisms have evolved elaborate redox codes that manage metabolic organization and function. These codes dictate and modify the redox chemistry within different cell types, and cellular compartments, during different stages of life cycle and in response to external environmental influences. Disruptions of the redox code that lead to dysregulation of redox steady states will detrimentally impact on cell biochemistry, tissue function and overall health [1]. Glutathione is the most abundant low molecular weight thiol found in cells and plays an important role in the maintenance and regulation of the thiol-redox status of the cell. Thus, the homeostasis of glutathione at optimal concentrations and reduced/oxidised ratios in cell compartments could be argued as being fundamental to a healthy cellular redox [2].

Reduced glutathione (GSH) is a tripeptide (γ-L-glutamyl-L-cysteinylglycine). It is often referred to as the "master antioxidant" and it is produced in the cytosol of all cell types at concentrations up to 10 mM [3]. Beyond its roles as a reducing agent and major antioxidant, GSH is also involved in numerous physiological functions. These include cell cycle regulation, proliferation, apoptosis, xenobiotic metabolism and thiol disulphide exchange. It also serves as a reservoir of cysteine [4].

The intracellular GSH concentration, or homeostasis, is determined

Abbreviations used: FACS, Fluorescence activated cell sorting; γ-GC, gamma-glutamylcysteine; GCL, glutamate cysteine ligase holoenzyme; GCLC, catalytic subunit of glutamate cysteine ligase; GCLM, modifier subunit of glutamate cysteine ligase; GR, glutathione reductase; γ-GT, gamma-glutamyltransferase; GS, glutathione synthetase; GSH, reduced glutathione; NAC, N-acetylcysteine; NADP, nicotinamide adenine dinucleotide phosphate; PBMC, peripheral blood mononuclear cell $\bar{x}$ - mean; s, standard deviation; C_0, initial basal concentration (GSH); C_{max}, maximum concentration (GSH); t_{max}, time to reach C_{max}; AUC, Area under the curve. Overall exposure (to GSH change)

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by a dynamic balance of synthesis, consumption and transport, and in some tissues; the oxidant levels [4,5]. Cytosolic GSH *de novo* synthesis occurs in all mammalian cells by two sequential ATP dependent enzyme catalysed reactions. In the first, glutamate cysteine ligase (GCL) forms the unusual γ-peptide bond between L-glutamic acid and L-cysteine to produce γ-glutamylcysteine (γ-GC). In the second, glutathione synthetase (GS) then adds glycine to γ-GC to generate GSH [6,7]. Cellular GSH homeostasis is controlled by non-allosteric feedback inhibition exerted by GSH on the activity of GCL. There is no such inhibition on GS activity [7].

Indisputable cause and effect links have been demonstrated between changes in GSH levels and/or redox state and chronic diseases, such as Alzheimer's and Parkinson's diseases, diabetes, cystic fibrosis, HIV/AIDS and aging [8–13]. Though it has been observed in healthy epithelial tissues such as the lung, that GSH expression can increase in response to elevated exposure to oxidants [4,5], it is generally accepted that, as we age, our body's capacity to maintain an appropriate homeostatically controlled GSH level progressively declines, leaving us vulnerable to many age-related diseases and disorders [2,14,15]. A deficiency in GSH manifests itself largely through an increase in susceptibility to oxidative stress. The resulting damage is thought to be a determinant of the onset and progression of many chronic disease states [2]. It is widely thought that drugs or supplements able to elevate glutathione (GSH) levels could have therapeutic potential in treating chronic and age related disorders [8–10].

Disease associated cellular GSH depletion is often a result of down regulation of expression or lowering of the specific activities of the first biosynthetic enzyme GCL [16]. Rodent studies have shown GCL levels declining with increasing age, corresponding to a lowering of homeostatic GSH levels [17,18]. The second enzyme involved in GSH synthesis, glutathione synthetase (GS), is a much simpler homodimer composed of two identical catalytic subunits. It generally has a higher specific activity than GCL so that cellular levels of γ-GC are negligible [19]. Many diseases [20–23] have associated impaired GCL activities from genetic or environmental factors that lower GSH homeostasis to levels that may be insufficient to protect against the onset of oxidative stress. This supports the theoretical potential for the use of γ-GC, the immediate precursor to GSH, as a means to increase cellular GSH levels. As cytosolic concentrations of γ-GC are in the order of 7 μM [19], any passive flow of exogenous γ-GC, unlike GSH, would be directed into the cell [24]. Should cellular GSH depletion arise as a result of damaged regulatory control of GCL activity, NAC or other cysteine prodrugs would theoretically not be expected to be effective in elevating GSH levels above the lowered homeostasis [25]. On the other hand, exogenous γ-GC taken up intact, should feed directly into the unregulated GS enzyme and potentially increase GSH levels above homeostatic levels [26].

Early rodent studies demonstrated in mice that intraperitoneal administered γ-GC could restore depleted GSH content within organs [6]. More recently, γ-GC has been demonstrated to ameliorate oxidative injury in neurons and astrocytes *in vitro* and increases brain glutathione *in vivo* [27]. Further *in vivo* studies of neural [28] cardiac [29] and liver [30] tissues have demonstrated that the extracellular addition of γ-GC ethyl ester also increases intracellular GSH concentrations. [31]. *In vitro* studies with isolated mitochondria have shown that γ-GC can directly replace the role of GSH [32]. The same researchers also determined that γ-GC can take over the antioxidant and neuroprotective functions of GSH by acting as glutathione peroxidase-1 cofactor in a mouse model [33].

In this current human study, we investigated the potential systemic bioavailability of orally administered γ-GC. Dipeptides are not expected to be particularly useful as oral therapeutics as they are often readily hydrolysed by digestive or serum proteases; however, the unusual γ-glutamyl bond found in γ-GC is resistant to hydrolysis by most proteases and aminoproteases [34,35].

Animal safety trials have demonstrated γ-GC to be safe at limit acute and repeated doses [36]. Change in GSH content of lymphocytes was chosen as a surrogate measure for cellular γ-GC uptake, and hence its bioavailability. Changes in GSH levels in erythrocytes were not determined as, unlike lymphocytes, their metabolism, physiology and structure are not considered representative of most cell types in the body. In spite of having an efficient productive capacity for GSH *de novo* synthesis, they lack a nucleus and most organelles, and are unable to utilize extracellular GSH due to the absence of the membrane bound ectoenzyme γ-glutamyltranspeptidase (γ-GT) [37–39]. In addition, during the GSH analysis procedure, samples are normally acidified to prevent GSH autoxidation and its hydrolysis by γ-GT. This, however, is ineffective for erythrocytes which release a large amount of iron that reacts with GSH even under acidic conditions [40,41]. Similarly, the monitoring of any changes in plasma GSH or γ-GC levels was not considered relevant to the study objectives since any increase in plasma GSH would require cellular uptake of exogenous γ-GC followed by secretion of synthesised GSH into the plasma. A single oral dose of γ-GC, rather than multiple doses over a period of weeks or months, was investigated. If γ-GC can effectively increase GSH above homeostasis, then the effect should be able to be observed after a single dose.

2. Materials and methods

2.1. Study protocol

The study's focus was to determine whether single doses (2 and 4 g) of orally ingested γ-GC can transiently increase the GSH content of lymphocytes. The study was sponsored by the University of New South Wales (UNSW) and approved by the UNSW Human Research Ethics Committee (HREC Ref#HC12511). It was prospectively registered with the Australian New Zealand Clinical Trials Registry (Trial ID ACTRN12612000952842) and filed under the Clinical Trial Notification (CTN) scheme of the Therapeutics Goods Administration (TGA, Australia; Ref #20120750). The trial was conducted at the UNSW according to the principles of the Declaration of Helsinki. Thirteen healthy adult male and female volunteer subjects (aged 25–70) were recruited by word of mouth from friends, family and work colleagues The γ-GC (CAS No. 636-58-8) administered in the study was provided by Biospecialties International, Mayfield, NSW, Australia as a sodium salt (Glyteine®). The γ-GC and placebo (glucose) were both packaged in identical 500 mg capsules.

A non-fasting requirement for participants was included to eliminate the risk of substrate (in particular cysteine) limitation decreasing GSH levels below homeostasis and thereby confounding the experimental interpretation. If, following overnight fasting, a subject's lymphocyte GSH levels were below homeostasis due to substrate limitation, any observation of increased GSH levels following the γ-GC administration would have been potentially due to a return to homeostasis rather than an increase above homeostasis. No alcohol consumption was allowed on the day of the trial due to its potential to deplete cellular GSH [42]. Throughout the day, subjects were requested to record all food and drink consumption.

The study involved a single dose of 2 g γ-GC (4×500 mg capsules) plus 2 g placebo (4×500 mg capsules) or 4 g γ-GC (8×500 mg capsules) taken with water. For the *before and after* study, subjects (n=13) were administered only the 2 g γ-GC dose, with blood samples being taken before γ-GC administration and at 90 min afterwards. The pharmacokinetic component of the study aimed to investigate the rates of increase in lymphocyte GSH content and the expected subsequent decline to basal homeostatic levels. Six subjects were selected for a randomized double blind crossover 2 and 4 g dose comparison (see Table 1 for subject demographics). Blood samples were taken over periods of up to 7 h after γ-GC administration. The subjects were tested with the opposite dose after a minimum two week washout period, with the dose sequences for individuals selected by block randomization. The dose selection process, coding and capsule management was