

Serum glutathione transferase does not respond to indole-3-carbinol: A pilot study

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Background: Despite the well recognized protective effect of cruciferous vegetables against various cancers, including human colorectal cancers, little is known about how this effect is conferred. It is thought that some phytochemicals found only in these vegetables confer the protection. These compounds include the glucosinolates, of which indole-3-carbinol is one. They are known to induce carcinogen-metabolizing (phase II) enzymes, including the glutathione S-transferase (GST) family. Other effects in humans are not well documented. We wished to assess the effect of indole-3-carbinol on GST enzymes.

Methods: We carried out a placebo-controlled human volunteer study. All patients were given 400 mg daily of indole-3-carbinol for three months, followed by placebo. Serum samples were tested for the GSTM1 genotype by polymerase chain reaction. Serum GST levels were assessed using enzyme-linked immunosorbent assay and Western Blot methodologies.

Results: Forty-nine volunteers completed the study. GSTM1 genotypes were obtained for all but two volunteers. A slightly greater proportion of volunteers were GSTM1-positive, in keeping with the general population. GST was detected in all patients. Total GST level was not affected by indole-3-carbinol dosing compared with placebo. Although not statistically significant, the GSTM1 genotype affected the serum GST level response to indole-3-carbinol.

Conclusion: Indole-3-carbinol does not alter total serum GST levels during prolonged dosing.

Keywords: pilot study, colorectal cancer, glutathione transferase, human, indole-3-carbinol

Introduction

There is strong evidence that diets including a large vegetable component confer protection against various human cancers including colorectal cancer.¹⁻⁶ The underlying mechanism(s) for this protective effect is poorly understood. Vegetables of the brassica genus are known to provide significant protection. Compounds from these vegetables, including the glucosinolates and their metabolites, have been shown in *in vitro* studies to inhibit the proliferation of and induce apoptosis in various human cancer cell lines, act as strong antioxidants, and induce the production of both phase I and phase II carcinogen-metabolizing enzymes.⁷ The glucosinolates are found in several plant groups, but the only human dietary source is from cruciferous vegetables.⁸⁻¹⁰ We wished to quantify the effect of one of these compounds, indole-3-carbinol, on human serum glutathione transferase levels as a logical progression of our *in vitro* work,^{11,12} in a manner similar to that suggested by Kristal and Lippman.¹³

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Materials and methods

Volunteers

Fifty-six volunteers consented to take part in the double-blind pilot study. They were recruited from advertisements in the local press, from family members of patients attending the Hunter Family Cancer Service, and as a result of media interest in our laboratory studies. Because this was a pilot study, no sample size or power calculation was made. Ethics approval was obtained from the Hunter Area Research Ethics Committee.

Dosing and blood collection

A dose of 400 mg of indole-3-carbinol was to be taken each day based on our previous laboratory work and other studies.¹⁴ At the initial visit, volunteers provided a baseline blood sample and were provided with sufficient tablets until the following meeting. Venous blood was obtained in the usual manner, mostly from the antecubital fossa, using the Vacuette® system (Greiner Bio-One, Interpath, West Heidelberg, Australia). Blood suitable for genotyping was retrieved in standard specimen bottles containing ethylenediamine tetra-acetic acid. A total of 50 mL of blood was obtained. A further five meetings occurred, during which additional blood samples were taken and further tablets provided. After the third visit, volunteers changed from indole-3-carbinol to placebo tablets for the following three months. No tablets were given at the final appointment. Volunteers were advised to continue their normal diet throughout the study period.

Genotyping for glutathione transferase Mu

Whole blood was stored at -20°C and the genotype assessed using the polymerase chain reaction (PCR). The following primers were utilized (Life Technologies, Melbourne, Australia):

- GSTM1.16com GCTTCACGTGTTATGAAGGTTCC
- GSTM1.E7B TTGGGAAGGCGTCCAAGCAG
- BGLOBIN.FOR CAACTTCATCCACGTTCCACC
- BGLOBIN.REV GAAGAGCCAAGGACAGTTAC

DNA was extracted from a whole blood sample using reagents specified for the BioRobot M48 (Qiagen, Doncaster, Australia). This was then mixed with 30.55 μL of H_2O , 4 μL dNTPs (2.5 mM) 200 mM (Promega, Annandale, Australia), 5 μL buffer (10 \times) (Promega), 1.25 μL of primers, 0.2 μL

Taq (5 U/mL) (Promega) up to a total volume of 50 μL . An automated PCR was carried out using the Eppendorf Mastercycler (EpGradient S, Sydney, Australia). Initially the samples were denatured for five minutes at 94°C , with a further 14 cycles, each cycle comprising 30 seconds at 94°C , 45 seconds at $60\text{--}54.5^{\circ}\text{C}$ (step-down $0.5^{\circ}\text{C}/\text{two cycles}$), and one minute at 72°C . This was then followed by 30 cycles, each cycle consisting of 94°C for 30 seconds, 53.5°C for 45 seconds, and 72°C for 60 seconds. Once the cycling reaction was complete, a final extension period of 10 minutes at 72°C was performed. The resulting 132bp GSTM1 samples were digested with HaeII enzyme (10 U/mL) at 37.5°C for 3.5 hours. This revealed the homozygous, heterozygous, or null genotype (alleles) of GSTM1. The polymorphism was evaluated by gel electrophoresis (2% agarose), and the DNA levels were checked using a spectrophotometer.

Phenotyping of glutathione transferase Mu

Serum was initially separated from the whole blood and stored at -20°C . The degree of glutathione S-transferase (GST) expression was assessed using both an enzyme-linked immunosorbent assay (ELISA) and Western Blot technique.

ELISA methodology

The wells of 96-well Polysorb ELISA plates (NUNC, Sydney, Australia) were coated with 10 μL of serum from volunteers. These were then incubated at 37°C for 2–3 hours. The plates were then washed and incubated following administration of blocking buffer (10% skim milk, 1% Tween 20) for a further 60 minutes at 37°C . The primary antibody (rabbit anti-human GST, donated by Professor Clancy, University of Newcastle, Australia) was added and incubated for another 120 minutes at 37°C . The plates were washed again and more blocking buffer applied prior to further incubation for 60 minutes at 37°C . Following this, the secondary antibody (anti-rabbit IgG-HRP conjugated, donated by Professor Clancy, University of Newcastle, Australia) was applied and reincubated at 37°C for two hours. The plates were then read in a microplate reader at 405 nm after the horseradish peroxidase (HRP) substrate (ABST) was fixed. A control plate of pure GST was run in a similar fashion for reasons of comparison.

Western Blot methodology

The serum samples were subjected to polyacrylamide gel (12.5%) electrophoresis. The gel was run at 110 V for 75 minutes. Following this, the gel was placed over a sheet

of nitrocellulose and the protein in the gel was transferred to the nitrocellulose electrophoretically in transfer buffer (1× Tris, glycine plus 20% methyl alcohol). Blocking buffer was then prepared by adding 0.2% Tween 20 and 10% skim milk powder to a solution of 10 mM Tris and 100 mM NaCl. The nitrocellulose was then placed in this buffer solution for 120 minutes with shaking. The blocking buffer was removed and a 1/10000 dilution of the first antibody was prepared and added to the membrane. The reaction was incubated for 90 minutes at room temperature and removed. A further application of blocking buffer was applied for 90 minutes. The nitrocellulose was then placed in the secondary antibody (1/5000 or 1/10000 in blocking buffer) and incubated for another 90 minutes at room temperature (shaking). Two milliliters of ECL developing reagent (Amersham Biosciences, Rydalmere, Australia) was prepared and spread onto two glass slides, according to the manufacturer's instructions. The resultant membrane was exposed to Hyperfilm ECL (Amersham Biosciences) for five to 10 minutes, followed by developing and visualization. This was done primarily to validate the results of the ELISA tests.

Statistics

The outcome measure of serum glutathione transferase level was measured for both treatment and placebo. These measures were analysed as continuous variables and the effect of the predictor variables (ie, age, sex, diet) on the mean value for the outcome variables was determined with regression or analysis of variance (ANOVA), (unpaired t-test if the predictor had only two levels). To determine the effect of indole-3-carbinol, the difference between the treatment and placebo groups was analyzed to determine the impact on the mean change. The null hypothesis of no change was assumed, and statistical significance was taken as $P < 0.05$.

Results

Of the 56 volunteers who consented to take part in the study, seven withdrew. The reasons given were inconvenience ($n = 3$), a newly diagnosed medical condition (1), tiredness (1, whilst on the trial preparation), and diarrhea (2, whilst on the trial preparation). The GSTM1 genotype could not be determined in two volunteers, who were excluded from further analysis. Glutathione transferase was detectable in all the remaining volunteers (Figure 2).

Demographics of volunteers

The median age of volunteers who completed the trial was 58 years (range 20–79). Slightly more females took part

(57.1% versus 42.9%) and the majority were “never” smokers (never, 55.1%; no longer, 38.8%; current, 6.1%).

Results of genotyping

Of the 49 volunteers who completed the trial in the two phases, a GSTM1 genotype was obtainable for all but two volunteers (one male and one female). Of the remaining 47 volunteers who completed the study, 25 (53.2%) were positive for GSTM1, which is comparable with the normal population in other studies.^{15–20} Of these, six were 1a/1a or 1a/null-positive and the remainder were 1b/1b or 1b/null. There was no significant difference between GSTM1 genotype for volunteers by age (GSTM1-null: <50 years, 60.0% versus >50 years, 40.6%; $P = 0.21$) nor by gender (GSTM1-null: male, 45.0% versus female 48.1%; $P = 0.82$).

Effects of indole-3-carbinol on serum GST

When all results were considered, there was no significant difference in mean serum GST levels for volunteers receiving indole-3-carbinol or placebo (indole-3-carbinol, 0.168; placebo, 0.168; $P = 0.84$; Figure 1).

The results were then analyzed as matched pairs; that is, the difference in the mean serum GST level at times 1 and 2 for indole-3-carbinol against the mean serum GST level at times 1 and 2 for placebo. There was no statistically significant mean difference (indole-3-carbinol, 0.173; placebo, 0.170; difference, 0.003; $P = 0.67$).

We then considered the matched pairs for all the serum GST levels on indole-3-carbinol against the baseline serum GST levels and the matched time equivalent on placebo. In general, indole-3-carbinol did not affect serum GST levels (Table 1); however, there was nonstatistically significant increase in serum GST level following the three months of treatment with indole-3-carbinol against the baseline.

Effect of indole-3-carbinol on serum GST by GSTM1 genotype

We also compared the effect of indole-3-carbinol on serum GST levels according to GSTM1 genotype. Although not statistically significant, indole-3-carbinol caused a decrease in GST levels in GSTM1-null volunteers and an increase in GST levels in GSTM1-positive volunteers ($P = 0.48$; Table 2).

Discussion

We have successfully completed a double-blind, placebo-controlled, pilot study using indole-3-carbinol, without undue

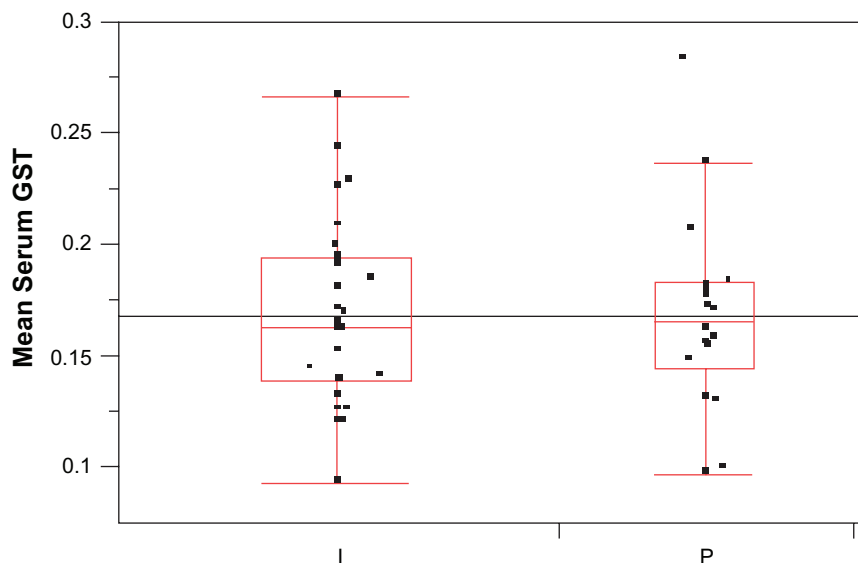


Figure 1 Mean serum GST levels for I3C (I) and placebo (P) – [Units – $\mu\text{g/ml}$].

or intolerable adverse events. In this study, indole-3-carbinol had no effect on serum glutathione transferase levels when compared with placebo. Using the matched-pairs analysis again there was no difference in serum glutathione transferase levels. Against the baseline serum glutathione transferase levels, however, treatment with indole-3-carbinol did increase serum glutathione transferase levels, although not to a level of statistical significance. Such differences were not seen when treatment was compared with the equivalent placebo period. These differences between treatment and placebo periods and treatment and baseline periods are not easily explained, but they may be accounted for by the distribution and function of glutathione transferase and its isoenzymes.

Cytosolic glutathione transferases “mop up” the byproducts of normal oxidative stress, such as lipid peroxidation

as well as xenobiotics.^{21–24} The enzymes do this primarily by attaching a glutathione molecule to electrophiles, making them water-soluble, which facilitates urinary or biliary excretion.^{19,25–27} This is the first step in the formation of mercapturic acids, a pathway which results in the elimination of potentially toxic compounds from the body.^{19,28–30} Within mammalian cells, at least five different isoenzymes of glutathione transferases have been discovered. The most widely studied to date have been named as follows: GST α , GST μ , GST π , GST θ and GST γ .^{20,22} They all have different substrate specificity, but there is significant crossover of function.^{22,28,31} The regulation of cytosolic glutathione transferases is subject to a complex set of endogenous and exogenous parameters including developmental-, gender-,

Table 1 Results of matched pairs t-tests for all samples

Paired test	Mean difference	Standard error	Probability
I3C 1 - Blood 0	0.01037	0.00910	0.26
I3C 2 - Blood 0	0.01393	0.00788	0.08
I3C End - Blood 0	0.00192	0.00978	0.85
I3C 1 - P 1	0.01083	0.01088	0.33
I3C 1 - P 2	0.00145	0.01039	0.89
I3C 2 - P 1	0.00926	0.00930	0.33
I3C 2 - P 2	0.00110	0.00964	0.91

Abbreviations: Blood 0, baseline; I3C 1, indole-3-carbinol after first treatment period; I3C 2, indole-3-carbinol after second treatment period; I3C End, indole-3-carbinol end of treatment period; P 1 after first placebo period; P 2 after second placebo period.

Table 2 The effect of indole-3-carbinol on serum GST levels by GSTM1 genotype

Variable	Least squares mean	Standard error	Difference	Probability
GST null + placebo	0.1835	0.0116	-0.0032	0.48
GST null + indole-3-carbinol	0.1803	0.0113		
GST positive + placebo	0.1582	0.0108	0.0089	
GST positive + indole-3-carbinol	0.1671	0.0108		

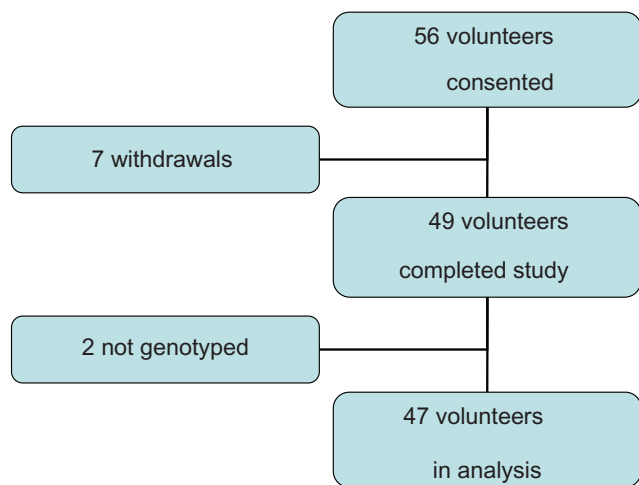


Figure 2 Flow diagram of volunteer selection process.

and tissue-specific factors, as well as a large number of xenobiotic agents, such as polycyclic aromatic hydrocarbons, reactive oxygen species, isothiocyanates, and drugs.³¹ The genetic and sporadic loss of glutathione transferase isoenzymes is known to cause upregulation of the remaining transferases.²² Persons deficient in certain isoenzymes are thought to be more susceptible to cancers of the lung, bladder, prostate, colon, and rectum.^{17,20,32–35}

The vast majority of glutathione transferases in the human body are found in the liver and small intestine. GST μ is less organ-specific than the other glutathione transferases.¹⁹ It is found in relatively low levels in lung and colon tissue^{36,37} and in high levels in the liver.³⁶

The mechanism and site of action for the chemoprotective effects of cruciferous vegetables is not known. It has been postulated that the effects are localized within the gut lumen. This may be as a result of direct inhibition of proliferation/induction of apoptosis in abnormal/cancer cells or the induction of local carcinogen-metabolizing enzymes. This does not explain the regression of carcinoma *in situ* of the cervix when indole-3-carbinol is administered orally.¹⁴

The evidence for an increase in overall GST activity and that within specific tissues is equivocal. Studies in rats fed brussels sprout extract or cabbage-substituted diets showed an overall increase in GST activity.^{38,39} In human studies, no effect on overall GST activity was noted in subjects fed test diets of brussels sprouts^{40,41} or broccoli pills.²⁵ Other studies of rats which were fed brussels sprouts have shown an increase in the glutathione transferase activity of intestinal cells.⁴²

Several studies have looked at cancer risk in GSTM1-null patients in relation to brassica consumption. These studies

would suggest that a greater intake of brassica vegetables or extracts could confer greater protection in GSTM1-null patients. These results have been explained by the slower metabolism of the isothiocyanates in the absence of the GST μ , prolonging their chemoprotective effect.

In our study, indole-3-carbinol was found to cause an increase in serum glutathione transferase levels in those who were GSTM1-positive and, conversely, a decrease in those who were GSTM1-null. One might expect such a pattern because GST μ expression cannot be induced in null individuals. This may also indicate that indole-3-carbinol induces GST μ specifically and has an inhibitory or no effect on the other isoenzymes, despite the observed compensatory mechanism described above. It may be that isoenzyme activity in specific tissues is induced following administration of brassica vegetables, unrefined extracts, or specific glucosinolate metabolites. This induction may be at a level not detectable when overall glutathione transferase activity is considered.⁴³ Glutathione transferases are abundant in the liver and much less so in other tissues,⁴⁴ such that induction of already saturated cells is futile. In extrahepatic tissues, however, a modest induction in specific glutathione transferase isoenzymes is achievable,^{31,41,43,45} and may be of biologic significance.

GSTM1-positive volunteers with a 1a/1a or 1a/null allele status had higher serum levels of GST (not significant, data not shown) than the rest. It may be that the 1a genotype gives rise to greater expression of the GST μ isoenzyme, increasing overall GST levels, or 1a may in fact be relatively inactive so there is greater compensatory expression of the other isoenzymes. Different phenotypes have been found between homozygous and heterozygous GSTM1-positive individuals. In a study by Moore et al, individuals who were GSTM1^{+/-} were found to be at greater risk of developing colorectal adenomas than either GSTM1^{+/-} or ^{-/-} individuals.⁴⁶ Some authors have suggested that these phenotypic differences may account for the lack of evidence for any increased cancer risk attributable to glutathione transferase deficiency, as most studies do not differentiate homozygous from heterozygous positive individuals.²²

These results raise many questions. The relatively small sample size may have reduced the statistical power of some of the observations, and a larger trial looking particularly at specific isoenzymes should answer some of these. The differences observed for indole-3-carbinol against the baseline tests implies that indole-3-carbinol has some effect in the manner to be expected from the previous work by ourselves and others. The lack of any difference against placebo might

suggest that the placebo tablets, which only contained rice flour, may themselves have had some effects on the systems we were investigating. Measurement of individual GST isoenzymes was not available in this study. We intended to measure GST Mu levels using the formerly available MuKit (Biotrin, Ireland). However, this was not available in sufficient quantities to enable testing of all samples. More enzyme- or tissue-specific testing may provide very different results from those we found.

The results also question the perceived role that indole-3-carbinol plays in chemoprevention. It may be that indole-3-carbinol is very specific in terms of which glutathione transferase is induced whereas other components of brassica vegetables, for example, sulforaphane, may have a more global influence on phase II enzymes. Indole-3-carbinol and other isothiocyanates are known to act on cancer cells by mechanisms other than induction of carcinogen-metabolizing enzyme systems.⁴⁷ We plan to carry out a further trial using sulforaphane, and do additional work using human serum and cancer cell lines.

Conclusion

We have conducted a study of indole-3-carbinol in humans with no deleterious or significant side effects. We found glutathione transferase to be present in all volunteers even those who were GSTM1-negative. Dosing with indole-3-carbinol did not appear to alter serum levels of glutathione transferase. This can be explained by several mechanisms. Indole-3-carbinol is very specific and it does globally induce the production of glutathione transferase, but only one of its isoenzymes. Small increases in isoenzymes may not be detectable when global glutathione transferase levels are being measured in the serum. This may indicate that indole-3-carbinol has a role to play in the treatment of colorectal cancer, either in those who are at increased risk or following diagnosis. Whilst some aspects of indole-3-carbinol metabolism have been described,⁴⁸ much of its metabolism and actions remain undiscovered.

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Disclosures

The authors have no conflicts of interest to report in this study.

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