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Effect of Selenium- and Glutathione-Enriched Yeast Supplementation on a Combined Atherosclerosis and Diabetes Hamster Model

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Selenium has a central role in antioxidant pathways as a cofactor to glutathione peroxidase. The present study evaluated the effects of four different preparations of inactivated yeast containing various concentrations of selenium and glutathione on a combined atherosclerosis and diabetes hamster model. The hamsters were supplemented with the yeast products for three months. The enriched yeast with the highest selenium and glutathione levels reduced the weight loss induced by diabetes, inhibited an increase in plasma cholesterol and triglyceride caused by a high-cholesterol and high-fat diet, increased the time taken for oxidation of lower density lipoproteins (lag time), and inhibited the formation of atherosclerosis better than low selenium/glutathione yeast supplementation. It was concluded that the yeast prepared to provide high selenium and high glutathione was the best for effecting beneficial changes in glutathione, cholesterol, atherosclerosis, and for demonstrating an antioxidant effect. The high selenium and low glutathione yeast was the best for improving selenium and glucose levels.

KEYWORDS: Atherosclerosis; diabetes; selenium; glutathione

INTRODUCTION

Selenium (Se) is a required micronutrient in mammals, for a well-functioning immune system and for enzymes that contain selenocysteine at their active site (*1*). Selenium also functions as a regulator of glutathione, a major cellular and plasma antioxidant (*1*). Several isoenzymes of glutathione peroxidase (GPx) and thioredoxin reductase contain selenocysteine, and thus the nutritional status of Se in tissues can have a significant impact on the steady-state level of reactive oxygen species (*2*). Selenium as a component of GPx may be beneficial in insulin resistance, hence potentially modifying the risk of diabetes and cardiovascular disease (CVD) (*3*).

GPx is formed in the liver and constitutes part of the body's natural antioxidant defense system. It works with vitamin E to protect cell membranes from damage caused by harmful free radicals and plays a role in the liver to detoxify harmful compounds so that they can be removed from the body. Additionally, some glutathione (GSH) is released into the blood stream, where it helps to maintain the integrity of red blood cells while protecting immune system white blood cells as part of the body's defense (*1*). Se, in an organic form found in yeast (selenomethionine, SeMet), prompts cells in culture to initiate DNA repair, a key mechanism in preventing cancer (*4, 5*).

Selenium deficiency promotes certain disease states. Epidemiological findings have linked a lowered Se status to neurodegenerative and cardiovascular diseases as well as to increased cancer risk. For instance, a recent study of women aged 70–79 found that those with low serum Se had a greater mortality than those with high Se (*6*). Because brain oxidative stress is a cause of cognitive impairment, Se may protect against cognitive decline. A study of elderly subjects in France found that 9-year cognitive decline with aging was associated with a decrease in their plasma Se levels: the greater the decrease in plasma Se, the higher the probability of cognitive decline (*7*). Thus, evidence exists to suggest that additional dietary seleno compounds would be beneficial in some health conditions, but results from future intervention trials are needed to substantiate the argument for increasing Se intake (*8*).

Atherosclerosis is a systemic, multifactorial disease of the arterial intima caused by the retention of modified low-density lipoproteins and by hemodynamic and redox stresses (*9–11*). It is the leading cause of mortality in the industrialized world and

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Table 1. Hamster Groups and Food Composition

parameters	control	low Se/ low GSH	low Se/ high GSH	high Se/ low GSH	high Se/ high GSH
concn Se in yeast (μg/g)	<300	<259	<294	2360	2260
concn GSH in chow (mg/g)	ND[a]	8.2	15.8	4.8	18.2
chow preparation	chow	2.5 g of yeast 1/ kg of chow	2.5 g of yeast 2/ kg of chow	2.5 g of yeast 3/ kg of chow	2.5 g of yeast 4/ -kg of chow

[a] Not determined.

develops at an accelerated rate among persons with diabetes mellitus (*12, 13*).

This study was designed to test the efficacy of different preparations of heat-inactivated yeast containing Se and GSH on a combined atherosclerosis and diabetes model in Syrian Golden hamsters. To avoid any possibility of investigator bias, the trial was blinded and the code was not broken until all the data had been collected and analyzed. The conventional heart disease risk factors were measured, including cholesterol, triglycerides, glucose, and peroxides. Lower density lipoprotein oxidation was also measured since it is hypothesized to be the initiating step in atherogenesis (*14*).

EXPERIMENTAL PROCEDURES

Yeast Analysis. Se- and GSH-enriched yeasts (yeasts 1–4) were prepared by aerobic propagation at Lallemand, Inc. (Montreal, Canada) and sent as coded blind samples to the investigators. Fed-batch propagations were carried out using molasses as substrate, and enrichment of the yeast in selenium, glutathione, or a combination of both was carried out during the molasses feeding phase. Yeast was harvested from the propagator and concentrated to a cream of about 20% (w/w) dry matter. This yeast cream was then inactivated by holding at 70 °C for 30 min prior to spray-drying (Buchi) at 166 °C.

Total Se content in the yeasts was measured through Covance (Madison, WI) using an flame atomic absorption spectroscopy method for selenium, modified from analytical methods for atomic absorption spectrophotometry (*15*). The measurement of Se-Met was done with aid of the Amino Acid Analyzer at the Advanced Protein Technology Centre at the Hospital for Sick Children (Toronto, Canada). The dry yeast samples were hydrolyzed using a Waters PICO-TAG workstation. After neutralization, the amino acids present in the hydrolysate were derivatized using a solution containing phenyl isothiocyanate (PITC) before separation and quantification were carried out in a Waters Alliance 2690 deparation module with a dual-wavelength absorbance detector. The column used for the separation was a Waters PICO-TAG column (3.9 mm × 150 mm) with a modified Waters PICO-TAG gradient at 47.5 °C. Detection was at 254 nm. Data acquisition was achieved using Waters Millenium chromatography software.

GSH content was determined using DTNB (5,5′-dithio-bis(2-nitrobenzoate), Ellman's reagent) in a modified assay following acid hydrolysis. A 0.4 g sample of dry yeast was mixed with 10 mL of 0.1 M HCl in a centrifuge tube (15 mL). The tubes were then vortexed thoroughly to ensure that the sample was completely solubilized. The tubes were then incubated for 1 h at room temperature with occasional vortexing to ensure homogeneity. After hydrolysis, the samples were centrifuged at 700*g* for 20 min or until the supernatant appeared visually clear. A 0.1 mL aliquot of each supernatant was mixed with 4.9 mL of the DTNB reagent and vortexed. The mixture then reacted for 10 min before absorbance was read at 412 nm in a spectrophotometer. The readings of the samples were carried out within 1 h. The absorbances for the samples were compared to a standard curve prepared from known amounts of GSH dissolved in 0.1 M HCl.

Animal Care and Diet. Forty-five weanling male Syrian Golden hamsters were purchased from Charles River and acclimated to laboratory chow and housing, two or three per cage, in the University of Scranton animal suite. The trial was run in accordance with the practice and principles of the institutional ethics review board. The animals were maintained in temperature- and light-controlled conditions.

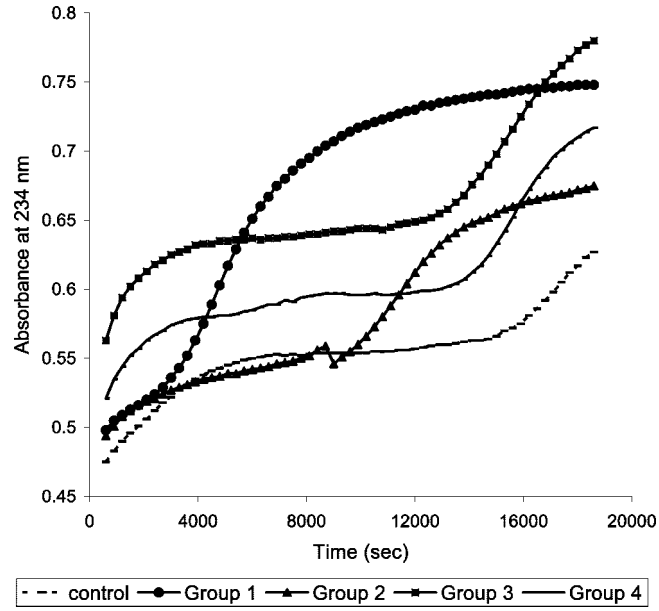

Figure 1. Oxidation of LDL+VLDL from pooled hamster plasmas.

The animals were made hyperglycemic by daily injection (i.p.) for 3 days of 25 mg/kg body weight of freshly prepared streptozotocin (STZ, Sigma) in physiological saline. Animals whose urine tested hyperglycemic by a urine test strip were considered diabetic. The STZ decreases the activity of the Isles of Langerhans in the pancreas, which are responsible for producing insulin. Thus, the model is one of type 1 diabetes in humans. The control animals did not receive the injections. After 2 weeks those animals that were made hyperglycemic were divided into four groups of nine per group and fed 0.2% cholesterol and 10% coconut oil (Sigma and Aldrich, respectively) in powdered Purina rodent chow made into brownies by mixing with 500 mL of water (*16*). The control group was given normal chow only. The supplemented chow was the basic food to which four different yeasts supplied by Lallemand, Inc. with a blind code were added before mixing according to **Table 1**. The four different yeast supplementation codes were broken only at the end of the study, and the group names are listed in **Table 1**.

The weights of the animals were recorded at the beginning and periodically throughout the study. After 3 months, the animals were fasted for 18 h and sacrificed after pentobarbital anesthesia and cardiac puncture (blood collection). Plasma was separated from blood samples and stored at −80 °C.

Selenium and Glutathione Assays. An aliquot of whole blood was stored for Se and GSH analysis. Se was analyzed in blood after digestion in nitric and perchloric acids with heating. This converted the organics in the blood to carbon dioxide and water to minimize interferences in the flameless atomic absorption method. A standard addition methodology with nickel ion as a matrix modifier was used (*17*). GSH was measured colorimetrically in the blood samples after protein precipitation with the use of Ellman's reagent (*18*).

Lipids and Oxidation Indices. Plasma cholesterol, triglyceride, and glucose were measured using Raichem enzymatic kits (Columbia, MD). High-density lipoprotein (HDL) was measured as cholesterol after precipitation of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) using magnesium ion and dextran (Sigma). The

Table 2. Effects of Selenium and Glutathione Supplementation on the Weight, Glucose, Lipid Profile, and Extent of Atherosclerosis of Atherosclerotic/Diabetic Hamsters

parameter	control	low Se/ low GSH	low Se/ high GSH	high Se/ low GSH	high Se/ high GSH
weight gain (g)	17 ± 13	8 ± 16	1 ± 16	7 ± 17	16 ± 17
total cholesterol (mg/dL)	210 ± 26	242 ± 63	252 ± 32	254 ± 33	208 ± 43[b,c]
HDL cholesterol (mg/dL)	80.7 ± 25.4	60.1 ± 15.8	79.5 ± 31.9	75.8 ± 42.6	69.3 ± 40.1
triglycerides (mg/dL)	63.1 ± 28.5	100 ± 64.9	62.4 ± 40.0	23.5 ± 19.4[a,b]	26.2 ± 12.8[a,b]
glucose (mg/dL)	168 ± 16	263 ± 40[a]	244 ± 53[a]	200 ± 56[b]	236 ± 60[b]
Atherosclerosis (%)	0.04 ± 0.09[b]	9.91 ± 10.12	7.82 ± 5.84[b]	11.20 ± 8.55[b]	2.81 ± 3.36[b]

[a] Significantly different from the control group, $p < 0.05$. [b] Significantly different from the low-Se/low-GSH group, $p < 0.05$. [c] Significantly different from the high-Se/low-GSH group, $p < 0.05$.

sum of hydrogen peroxide and lipid hydroperoxides was measured in the plasma by a colorimetric xylenol orange method using hydrogen peroxide as the standard (*19*). Lipid peroxidation was measured with fluorescent thiobarbituric acid reactive substances using malondialdehyde as the standard (*20*).

Atherosclerosis Analysis. The aortal dissection and atherosclerosis assay was carried out as described by Nicolosi (*16*). The heart was perfused with physiological saline, and the aorta was surgically removed, washed, and stained with Oil Red O dye. The *en face* samples were examined using a microscope, and digital photos were taken. The photos were analyzed for foam cells and total aortal area using SigmaScan software, which measures irregular areas. The percentage of aorta covered with foam cells (% atherosclerosis) was then calculated.

A portion of the individual hamster plasmas was pooled, and lower density lipoproteins (LDL+VLDL) were isolated in each group for some analyses. The LDL and VLDL of the pools were separated by our affinity column method. Protein was assayed by Coomasie blue reagent (Sigma) using albumin as the standard. In a glass cuvette, each LDL+VLDL sample was aliquoted to give a final protein concentration with phosphate-buffered saline of 70 μg/mL after addition of 25 μM cupric ion to initiate oxidation at 37 °C using our standard method (*21*). The appearance of conjugated dienes was monitored at 234 nm in a Genesys 5 spectrometer, and the oxidation–time curve was plotted (**Figure 1**).

Statistical Analyses. The biochemical data were analyzed with SigmaStat (Jandel Scientific, San Rafael, CA) using a Student's *t* test for normally distributed data, or a rank sum test for non-normally distributed data. The results are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

The effects of Se and GSH supplementation on weight gain, cholesterol, triglycerides, HDL, glucose, and atherosclerotic profile in the five groups of hamsters are presented in **Table 2**. Weight gain of the experimental groups was abnormally small compared to previous hamster atherosclerosis studies in our laboratory (*22*). This may be due to the mild diabetes induced at the beginning of the study as earlier reported (*23*). Thus, the diabetes induced a reduction in weight, which counters weight gain from the high-cholesterol diet. Only the normal group and the high-Se/high-GSH group had significant weight gain during the study ($p < 0.01$). This weight gain for the diabetic high-Se/high-GSH group indicates it was the healthiest among the experimental groups. Except for high-Se/high-GSH animals, the plasma cholesterol of the experimental groups was higher than that of the control group, as expected since the experimental groups were fed cholesterol and saturated fat. However, due to the variations, there was no significant difference between the control and experimental groups. The cholesterol concentration of high-Se/high-GSH animals was 14% lower than that of low-Se/low-GSH animals, 17% lower than that of low-Se/high-GSH animals, and 18% lower than that of high-Se/low-GSH animals. The high-Se/high-GSH group had significantly lower cholesterol than the other Se/GSH groups. This is in agreement with a previous rat study (*24*). There was no significant difference among the yeast-treated groups with respect to HDL in the present study. Groups 3 and 4 (the high-Se groups) were significantly lower in triglycerides than the low-Se/low-GSH animals ($p < 0.001$ and $p = 0.003$, respectively). The high-Se/high-GSH diet lowered triglycerides 74%, and the high-Se/low-GSH diet lowered triglycerides over 76%, compared to the level observed with the low-Se/low-GSH diet. The low-Se/high-GSH diet lowered triglycerides 38% compared to the level observed with the low-Se/low-GSH diet, but this was not significant due to the large variation in both groups. We also found significantly lower triglyceride levels when a high-Se yeast (uncharacterized for GSH) was given to hamsters with a normal diet for 14 days (*25*).

Table 3. Effects of Selenium and Glutathione Supplementation on Lag Time and Its Indices of Pooled LDL+VLDL of Atherosclerotic/Diabetic Hamsters

parameters	control	low Se/ low GSH	low Se/ high GSH	high Se/ low GSH	high Se/ high GSH
lag time (s)	16227	4321	10200	12658	15487
abs before	0.552	0.503	0.494	0.645	0.645
abs after	0.627	0.748	0.675	0.780	0.717
change abs	0.075	0.245	0.181	0.135	0.115
slope ($\times 10^{-5}$)	2.0	4.7	2.5	3.0	3.5

The experimental groups' animals, but not those of the control group, had type 1 diabetes induced by administration of streptozotocin at the beginning of the study. Our control group was significantly lower in glucose than the low-Se/low-GSH ($p < 0.001$), low-Se/high-GSH ($p = 0.017$), and high-Se/high-GSH ($p = 0.045$) groups. The high-Se/low-GSH group had the lowest plasma glucose, which was not significantly different from that of the control group; i.e., this Se/GSH yeast has essentially normalized the plasma glucose. The high-Se/low-GSH group's diet was 24% lower in glucose than that of the low-Se/low-GSH group, $p = 0.006$. These results indicate that Se in the yeast is the hypoglycemic agent.

The atherosclerosis results represent the clinical end point of the study. It has previously been reported that experimentally induced diabetes enhanced atherosclerosis, with predominantly fatty streaks and intermediate lesions in the aorta and femoral arteries (*12, 26, 27*). In the present study, the control group had no atherosclerosis, as expected. The low-Se/high-GSH, high-Se/low-GSH, and high-Se/high-GSH groups had significantly less atherosclerosis than low-Se/low-GSH group ($p = 0.01$, $p = 0.048$, and $p < 0.001$, respectively), giving a percentage decrease of 61%, 44%, and 87%, respectively. The high-Se/high-GSH group also had significantly less atherosclerosis than the low-Se/high-GSH group ($p = 0.017$) and less than the high-Se/low-GSH group ($p = 0.006$). The development of atherosclerosis was accelerated by the induction of diabetes in

Table 4. Effects of Selenium and Glutathione Supplementation on Oxidative Parameters in Atherosclerotic/Diabetic Hamsters

parameters	control	low Se/ low GSH	low Se/ high GSH	high Se/ low GSH	high Se/ high GSH
plasma hydroperoxides and hydrogen peroxides (μM)	79.8 ± 7.6	78.1 ± 17.2	83.9 ± 14.2	79.8 ± 18.4	89.9 ± 18.2
lipid peroxides (μM)	0.459 ± 0.17	0.673 ± 0.30[a]	0.622 ± 0.28[a]	0.620 ± 0.28[a]	0.942 ± 0.24[a]
plasma glutathione (μM)	925 ± 341	176 ± 29[a]	605 ± 363[a,b]	402 ± 439[a]	735 ± 404[b]
blood Se (μg/L)	856 ± 173	554 ± 156[a]	1448 ± 862[a,b]	1714 ± 592[a,b]	928 ± 301[b]

[a] Significantly different from the control group, $p < 0.05$. [b] Significantly different from the low-Se/low-GSH group, $p < 0.05$.

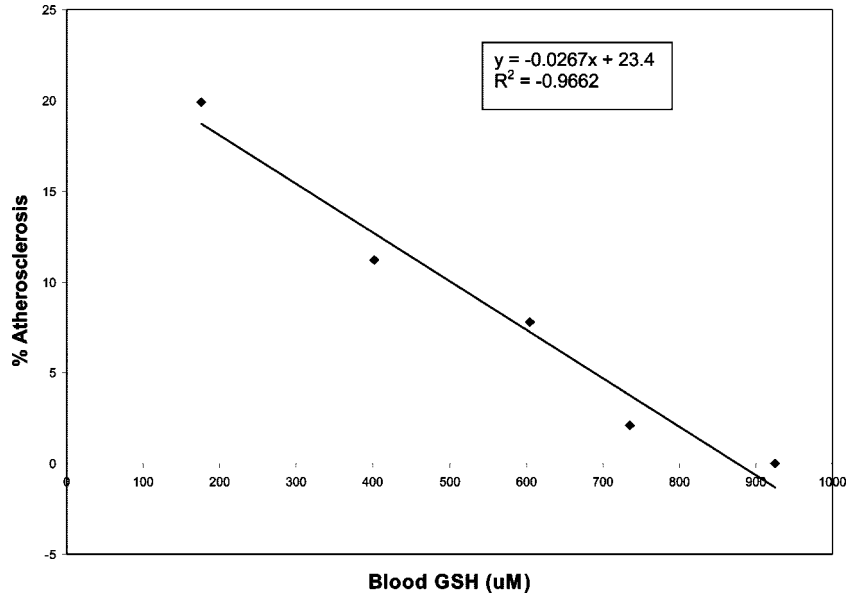

Figure 2. Correlation of blood glutathione and atherosclerosis.

experimental animals as compared to our previous atherosclerosis studies (*28–32*). The high-Se/high-GSH supplementation is the best antiatherosclerotic composition compared to our previous results obtained with chocolate, red wine, grape juice, grape seed extract, berry extract, chromium yeast, beer, and citrus extract (*28–32*).

Oxidation data are shown seen in **Table 3**. **Figure 1** represents the results of the lag time when the slope of the line changes dramatically, indicating a shift from the propagation phase to the rapid oxidation phase. This lag time indicates when the antioxidants present in the LDL+VLDL particles are oxidized and there is no more protection for the oxidizable unsaturated lipids from free radicals. These measurements are relevant to heart disease and atherosclerosis, as the oxidation of lower density lipoproteins (LDL+VLDL) is hypothesized to initiate the atherosclerosis process in the aortic intima. The longest lag time indicates the least oxidative susceptibility of the LDL+VLDL pools. As was observed, the low-Se/low-GSH yeast group has the shortest lag time, indicative of the most oxidizable LDL+VLDL. The other experimental groups show increasing lag times. The lag time of the high-Se/high-GSH group is essentially the same as that of the normal group. The high-Se/high-GSH group has a lag time 3.6 times longer than that of the control group; that of the low-Se/high-GSH group is 2.4 times longer, and that of the high-Se/low-GSH group is 2.9 times longer. Thus, the control group and the high-Se/high-GSH yeast have the least susceptible lipoproteins. The oxidized LDL has potential atherogenic effects on vascular cells in culture, including uncontrolled uptake by the scavenger receptors of macrophages, resulting in the formation of lipid-laden or foam cells, which are the hallmarks of fatty streaks (*33*), associated with increased risk of coronary heart disease (*34*). The change in absorbance indicated the amount of oxidation that had occurred from LDL+VLDL. The low-Se/low-GSH yeast group had the highest absorbance among the experimental groups. The absorbance values of groups with low Se/high GSH and high Se/low GSH were lower than that of low Se/low GSH group, indicating a protective effect of Se and GSH. High-Se/high-GSH group had the lowest amount of oxidation compared to other groups, but it still was 40% more than tht of the normal control pool of LDL+VLDL. The experimental groups have a lower slope of oxidation than the low-Se/low-GSH group, indicating a slower rate of oxidation, which is almost normal in the case of the low-Se/high-GSH group. Thus, Se/GSH yeast supplementation diminishes the oxidizability of lipoproteins by acting as an *in vivo* antioxidant.

The selenoprotein, SelenoP, has been shown to be an excellent *in vitro* antioxidant, protecting human LDL from oxidation (*35*). We have shown that a high-Se yeast was an excellent *in vitro* hamster LDL+VLDL antioxidant compared to selenite, and a yeast with no Se was not active (*25*). It is possible that Se binds to the lipoproteins in place of sulfur in the protein. Selenium is less oxidizable than sulfur in the 2− oxidation state covalently bound in protein. During the oxidation, both unsaturated lipids and proteins are oxidized. *In vivo*, Se supplementation might protect the LDL+VLDL from oxidative modifications and further atherogenic changes (*36, 37*). Our previous study showed that LDL+VLDL in normal hamsters was also protected from oxidation by high-Se yeast supplementation (*25*). In the present study, we obtained a direct relationship between atherosclerosis and lipoprotein oxidizability, and we observed that decreased lipoprotein oxidizability results in less atherosclerosis. The correlation coefficient was -0.8828, $p = 0.018$.

The plasma oxidation reaction products are also relevant to heart disease and are presented in **Table 4**. There was no difference in plasma hydrogen peroxide plus hydroperoxides

in the plasma between the groups. On the other hand, all the cholesterol-fed groups presented significantly more oxidation than the normal group. These results support the reports by earlier researchers, who found a generalized increase in oxidative stress in diabetic patients (*38–42*). Thus, there was a generalized increase of plasma lipoperoxidation in the high-cholesterol-fed and diabetic hamsters that was not alleviated by Se or GSH.

GSH is one of the antioxidants in the yeast, and its concentration in blood is presented in **Table 4**. The control group had the highest GSH, as it was not subjected to oxidative stress. As for the experimental groups, the GSH levels roughly parallel GSH in the yeast, but there is no significant correlation. From the oxidative stress caused by cholesterol and saturated fat consumption, the yeast groups would be expected to have lower GSH levels. All but group 4, with the highest Se and GSH, were significantly lower than the control group. GSH in the lowest Se and GSH group was 81% lower than in the control group ($p = 0.006$), and that in the high-Se/low-GSH group was 57% lower than in the control ($p < 0.05$) but not significantly lower than in the groups with low Se/high GSH and high Se/low GSH. GSH in the low-Se/high-GSH group was 2.5 times higher than in the low-Se/low-GSH group ($p = 0.019$), and in the high-Se/high-GSH group, GSH was 4 times higher ($p = 0.004$). Of importance was the inverse correlation of GSH with atherosclerosis ($p = 0.003$) (**Figure 2**). The correlation coefficient was -0.9662, indicating that GSH can explain 96.6% of the atherosclerosis. This is the first known example of an effect of GSH consumption on atherosclerosis.

Selenium was also measured in the blood by flameless atomic absorption. Low-Se/low-GSH animals had significantly lower Se than the control animals ($p = 0.015$). The normal diet contained 0.3 mg of Se/kg, as stated by the manufacturer. The low-Se/low-GSH animals ate <0.6 mg of Se/kg in the chow; i.e., the Se level had at most doubled. Perhaps the high-cholesterol diet, hyperglycemia, and oxidative stress caused a decrease in blood Se. Groups with higher doses of GSH and Se in the diet had higher blood Se levels. Blood Se concentration after consumption of a low-Se/high-GSH diet is not significantly different from that of the control group due to large variances, although it is higher by 69%. Blood Se concentration of high-Se/low-GSH animals is twice that of the control group and also significantly higher than that of low-Se/low-GSH animals (by over 3 times, $p = 0.035$). Group 4 animals, with the same level of Se in the yeast diet as group 3 animals, were not significantly higher in Se concentration than the control group animals, although blood Se was higher by 68% compared to low Se/low GSH animals. We did not observe any correlation between Se in the blood and atherosclerosis or lipoprotein oxidizability.

Conclusions. The most efficacious yeasts were the two yeasts with high Se levels. The yeast with both high Se and high GSH had the most beneficial effects on GSH levels, cholesterol, atherosclerosis, and lipid oxidation. The high-Se/low-GSH yeast was most effective for increasing Se and decreasing glucose. The importance of GSH for the beneficial effects of the yeasts needs to be further explored. Cellular GSH has been shown to be increased by dietary Se given to apo E-deficient mice (*41*) and humans with oxidative stress (*42*). This may be the link between Se and GSH, as the group with low Se/low GSH, which had lower GSH in the diet than the low Se/low GSH diet, still has much higher blood GSH because there is much higher Se in the yeast. A recent epidemiology study in healthy adults showed an inverse relationship between plasma GSH and early atherosclerosis (*43*), as did our animal study. Human supplementation studies should be done to determine the efficacy of the Se/GSH yeast in normal subjects and subjects with oxidative stress such as diabetics and smokers.

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